Download the CYTO 2015 mobile app via iPhone/iPad and Android native apps or via Blackberry, Windows Phone, and your desktop through the mobile web.

To access the CYTO 2015 mobile app online, visit http://ddut.ch/cyto2015
ISAC gratefully acknowledges the following outstanding sponsors for their generous support of CYTO 2015:

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**ISAC acknowledges Visit Scotland and Glasgow City Marketing Bureau for the generous grants in support of the 30th Congress of the International Society for Advancement of Cytometry.**
Dear Colleagues,

On behalf of the CYTO Organizing Committee and ISAC Council, it is our pleasure to welcome you to CYTO 2015, the 30th Congress of the International Society for Advancement of Cytometry. CYTO 2015 will focus on cutting-edge cytometry opening new horizons in analyzing biology on the level of individual cells. Recent developments in flow cytometry, advanced microscopy, data evaluation and fluorescent reagents enable a new understanding of basic molecular mechanisms and human disease. On the 30th anniversary of ISAC’s first World Congress, we proudly recognize that CYTO is the signature venue for showcasing the state of the art and science of cytometry.

The CYTO 2015 Program includes elements for scientists working in all areas of cytometry and at all career stages. The CYTO 2015 Education Program starts on Friday, June 26, with Scientific Tutorials providing focused updates on a range of research and clinical topics in flow and image cytometry, as well as shared resource lab (SRL) and core facility management. Again this year, the tutorials are included in the CYTO 2015 registration fee and are free to all already registered for CYTO. First-time CYTO attendees are encouraged to attend a welcome and orientation tea late Friday.

The CYTO 2015 Scientific Program opens Friday June 26 with a Special Lecture from Eric Betzig on “Imaging Life at High Spatiotemporal Resolution”. This year’s Hooke Lecture, “Engineered T Cells for Cancer Therapy” will be presented by Carl June on Sunday, June 28. The CYTO 2015 scientific program continues with Frontiers and Plenary Sessions featuring cutting edge cytometry technology and applications, Parallel Sessions with examples of contemporary cytometry from the research lab to the clinic, and Poster Sessions that provide an opportunity for detailed discussions between authors and delegates. A diverse program of interactive workshops provides an opportunity for experts and novices alike to discuss and debate emerging or controversial issues in cytometry.

CYTO 2015 will feature educational tracks on Shared Resource Lab management and another on innovation and entrepreneurship. The SRL track will focus on topics of interest to SRL staff and scientists and includes tutorials, workshops, posters, and a SRL managers’ forum. On Friday afternoon, CYTO Innovation spotlights the challenges and opportunities for the development and commercialization of cell analysis technologies and will focus on cytometry in a world demanding rapid responses to emerging global health issues.

The CYTO Commercial Exhibition features more than 70 companies displaying hardware, software, and reagents for cytometry research. We appreciate the support of all of our exhibitors, and especially our Sponsors, for helping to make CYTO possible. Help us recognize the many contributions our 2015 award winners have made to cytometry, science and the Society during the Awards ceremony Tuesday afternoon. CYTO 2015 will end with the Closing Reception at the Arches on Tuesday, June 30 because all cytometrists appreciate a good party.

We want to express our sincere thanks to the members of the CYTO Organizing Committee and to the 50+ members of the CYTO Program Committee, including the ISAC Scholars and Shared Resource Lab Emerging Leaders, who proposed themes and speakers, and assisted with abstract review. Thanks go also to our Course and Tutorial faculty, Workshop leaders, and Session Chairs for contributing their time and talents. Lastly, we want to thank the FASEB Office of Scientific Meetings and Conventions team, Kanika Pulliam, ISAC education manager, and Michelle Butler, ISAC Executive Director, for their tireless work in ensuring that CYTO 2015 is a success.

We encourage all members to participate in ISAC’s efforts to advance cytometry. Please attend the Business Meeting on Tuesday morning to hear about ISAC’s recent efforts and future plans and share your thoughts on the future of the Society and cytometry with the ISAC Council and management at any time.

Finally, enjoy CYTO and Glasgow!

Alan Waggoner  
CYTO 2015 Program Chair

Andreas Radbruch  
ISAC President
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Efficiency requires that you have the right tool for every job. That is why we created the S3e, an affordable and easy-to-use benchtop cell sorter designed specifically for 1- to 4-color sorts. Software-driven instrument setup and operation allow even a novice to run this instrument while real-time sort monitoring permits true walk-away automation. Reserve your time and expertise for complex sorts by adding the S3e Cell Sorter to your instrument portfolio. Now that's sorting smarter.

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Commercial Tutorial
"Analysis of CD163 mRNA for Monocyte Pathobiology in Systemic Juvenile Idiopathic Arthritis: and Integration of PrimeFlow™ RNA Assay into a Flow Cytometry Core"
Monday, June 29th at 12:45–1:45 PM
Sherry L. Thornton and Monica Dickey, Cincinnati Children’s Hospital

Scientific Tutorial
RNA Flow Cytometry — Tutorial 5
Friday, June 26th at 12:45–2:15 PM
Paul Wallace, Roswell Park Cancer Institute
Chris Grosses, Mediterranean

Visit ebioscience at Booth #301 and discover the power of PrimeFlow™ RNA Assay.

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The Closing Reception will be held on Tuesday, June 30, 2015, from 19:00 until 23:00 at The Arches. The Arches is a unique venue set in Grade A Listed Victorian Vaults in a prime Glasgow City Centre Location, and only a 10 minute walk from the Scottish Exhibition and Conference Centre. You may also take the ScotRail to the Closing Reception by boarding the train at "Exhibition Centre," just 0.3 miles from the Scottish Exhibition and Conference Centre, and getting off the train at "Glasgow Central." Exit "Glasgow Central," leave by Platform 11 onto Argyle Street. The Arches is directly across the street. This venue is renowned for its versatility and daring spirit in hosting spectacular events.

Plan on a fabulous night of fun with your colleagues at this unique venue...there will be music, dancing, whisky tasting, food, drinks, and more! Two complimentary drink tickets will be provided to those attending the reception.

Full registration includes an exchange coupon for the Closing Reception at the Arches. The Closing Reception coupon must be exchanged for an actual ticket on-site at the registration desk beginning Friday, June 26, 2015, until Sunday, June 28, 2015. Coupons will be exchanged on a first come first served basis until maximum capacity is reached. A ticket is required for admittance. Be sure to exchange your coupon early so you don't miss out on the fun!
Raise your career and your lab to the next level by becoming a certified cytometrist!

Register today at cytometrycertification.org

“I was able to obtain an advancement and increased compensation for one of our staff based partially on her obtaining certification.”

– Lab Manager and Certified Cytometrist

“It had a positive impact on a grant application for a study including cytometric analyses. It further helped in discussions during peer-reviewing. I generally would appreciate any study using cytometric data to document its cytometric competence by stating this certificate.”

– Prof. Dr. med. Andreas OH Gerstner, Leitender Oberarzt, Klinik und Poliklinik für HNO-Heilkunde / Chirurgie, Universität Bonn

“I think certification is a great opportunity for cytometrists at all levels. I have been using flow cytometry in my research for over 20 years, but I was one of the first to get certified. It solidifies your knowledge which really helps in developing assays and troubleshooting.”

– Dana Buckman, CCy, Flow Paradigm

“It had a positive impact on a grant application for a study including cytometric analyses. It further helped in discussions during peer-reviewing. I generally would appreciate any study using cytometric data to document its cytometric competence by stating this certificate.”

– Lab Manager and Certified Cytometrist

Featuring Courses, Webinars and Recordings from CYTO

Visit CYTO U Today to Learn More!

http://cytou.peachnewmedia.com
Use FlowRepository to access, review, download, deposit, annotate, share and analyze flow cytometry datasets.

FlowRepository is supported by Cytometry Part A, and is freely available thanks to support from the International Society for Advancement of Cytometry, the Wallace H. Coulter Foundation and from the donation of source code for the underlying technological platform from Cytobank Incorporated.
ISAC Fund for Global Cytometry Education helps further the mission of ISAC and support ISAC’s educational efforts around the world including:

- CYTO University
- ISAC International Cytometry Workshops
- ISAC Scholars Program
- Shared Resource Lab (SRL) Emerging Leaders Program
- CYTO Travel Support for Students and Junior SRL Staff
- FlowRepository
- International Cytometry Certification Exam (ICCE) Program
- Where Support is Most Needed

Individuals, corporations and foundations who share ISAC’s passion for cytometry education are encouraged to donate to ISAC’s Fund for Global Cytometry Education. You may choose to support one of the program areas listed above or designate the donation should go where the support is most needed. Donations of any size are welcome.

DONATE NOW AT WWW.ISAC-NET.ORG

100% of your donation will be applied to the support area you designate. ISAC will absorb all administrative costs. By virtue of ISAC’s 501(c)3 status with the IRS, contributions may be tax-deductible for people who pay US taxes.
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Diether Recktenwald
Desatoya LLC

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Vaccine Research Center, NIAID, NIH

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Shankey Biotechnology Consulting

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Paul Wallace
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NIAID, NIH

Nima Aghaeepour
Stanford University
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<thead>
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<th>Name</th>
<th>Institution</th>
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<tr>
<td>Donat Alpar</td>
<td>Institute of Cancer Research</td>
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<td>Kewal Asosingh</td>
<td>Cleveland Clinic</td>
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<td>David Basiji</td>
<td>Amnis Corporation</td>
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<td>University College Dublin</td>
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<td>Mark Bray</td>
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<td>Anne Carpenter</td>
<td>Broad Institute</td>
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<td>Sungwhan Cho</td>
<td>Nanosort</td>
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<td>Queensland Institute of Medical Research</td>
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<td>University of Modena and Reggio Emilia</td>
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<td>Los Alamos National Laboratory</td>
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<td>Benjamin J. Daniel</td>
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<td>Monica Delay</td>
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<td>Bruce S. Edwards</td>
<td>University of New Mexico</td>
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<td>Cardiff University</td>
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<td>Andrew Filby</td>
<td>Newcastle University</td>
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Visit the Exhibits & Posters

SATURDAY, JUNE 27, 2015

1600 – 2000  Authors Must Place Posters on Boards
1800 – 2000  Commercial Exhibits Open

SUNDAY, JUNE 28, 2015

800 - 1900  Poster Viewing
1130 – 1900  Commercial Exhibits
1530 – 1600  Coffee Break
1730 - 1830  Poster Session 1 (Authors of odd numbered posters present)
1800 – 1900  Happy Hour
1800 – 1900  Exhibitor Showcase

MONDAY, JUNE 29, 2015

800 – 1900  Poster Viewing
1130 – 1900  Commercial Exhibits Open
1530 – 1600  Coffee Break
1730 – 1830  Poster Session 2 (Authors of even numbered posters present)
1800 – 1900  Happy Hour
1800 – 1900  Exhibitor Showcase

TUESDAY, JUNE 30, 2015

800 – 1630  Poster Viewing
1130 – 1630  Commercial Exhibits Open
1530 – 1600  Coffee Break
1530 – 1630  Poster Session 3 consists of Poster Highlights Tour

Posters must be removed by 1630 on Tuesday, June 30, 2015.
# ISAC Special Committees & Task Forces

## Task Forces & Committees 2014 – 2016

### Awards Committee

<table>
<thead>
<tr>
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<td>John Nolan</td>
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<td>Paul Smith</td>
<td>Janos Szollosi</td>
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<td>Paul Wallace</td>
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### Biosafety Committee

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<td>Phil Hogarth</td>
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<td>Simon Monard</td>
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<td>Hank Fletcher</td>
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<th>Vice Chair</th>
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<td>Bruce Greig</td>
<td>Joanne Lannigan</td>
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<td>Teri Oldaker</td>
<td>Elizabeth Stone</td>
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<td>Carina Torres</td>
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### Council of ISAC Associated Societies

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<tr>
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<td>Andrea Cossariza</td>
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<td>Peter Lopez</td>
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<td>Adrian Smith</td>
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<td>Janos Szollosi</td>
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### Cyto Innovation Steering Committee

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<td>Dietner Recktenwald</td>
<td>Allen Poisson</td>
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### Education

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<td>Zosia Maciorowski</td>
<td>John Nolan, Co-Chair</td>
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<td>Awtar Krishan</td>
<td>Peter Lopez</td>
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<td>Jonni S. Moore</td>
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### Elearning Delivery Task Force

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<th>Leader</th>
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<td>Pratip Chattopadhyay</td>
<td>Kewal Asosingh</td>
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<td>Anna Belkina</td>
<td>Alfonso Blanco-Fernandez</td>
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<td>Zosia Maciorowski</td>
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<td>Joseph Tario</td>
<td>Jennifer Wilshire</td>
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### Flow Cytometry Content Task Force

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<td>Jonni Moore</td>
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<td>Joe Trotter</td>
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<td>Paul Wallace</td>
<td>Jennifer Wilshire</td>
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### Flow Cytometry Data Standards Task Force

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<td>Josef Spidlen</td>
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<td>Adam Treister</td>
<td>Jim Wood</td>
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<td>Michael Zordan</td>
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<td>Paul Wallace</td>
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Ryan Brinkman  
Rachel Errington  
Jeannine Holden  
Wayne Moore  
Mario Roederer  
Andreas Radbruch, (ex officio)  
Paul Wallace, (ex officio)

**Finance**

Rachel Errington, Chair  
Nima Aghaeepour  
Tim Bushnell  
Andrea Cossarizza  
Monica Delay  
Andreas Radbruch, (ex officio)  
Paul Wallace, (ex officio)

**Image Cytometry Content Task Force**

Gustavo Rohde, Leader  
Rachel Errington  
Andrew Filby  
Michael Halter  
Silas Leavesley  
Stephen Lockett  
Raluca Niesner  
John Nolan  
Anil Parwani  
Bartek Rawja  
Shantanu Singh  
Karen Thickman  
Gyorgy Vereb

**ISAC Scholars Program Committee**

Alex Nakeff, Chair  
J. Paul Robinson, Co-Chair  
Stephen Lockett  
Susann Mueller  
Mario Roederer  
Andreas Radbruch, (ex officio)  
Paul Wallace, (ex officio)

**Live Education Delivery Task Force**

Awtar Krishan, Leader  
Gulderen Yanikkaya Demirel  
Paresh Jain  
Tomáš Kalina  
H. Krishnamurthy  
Zosia Maciorowski  
Kovit Pattanapanyasat  
Alan Saluk  
Vivek Tanavde  
Bill Telford  
Qianjun Zhang  
Andreas Radbruch, (ex officio)  
Paul Wallace (ex officio)

**Membership Services**

Peter Lopez, Co-Chair  
Rachael Walker, Co-Chair  
David Haviland  
Sung Hwan Cho  
Rui Gardner  
Frank Schildberg  
Adrian Smith  
Andreas Radbruch, (ex officio)  
Paul Wallace, (ex officio)

**Shared Resource Lab (SRL) Content Task Force**

Peter Lopez, Chair  
Bridget McLaughlin  
Patricia Simms  
Adrian Smith  
Andreas Radbruch, (ex officio)  
Paul Wallace (ex officio)

**Shared Resource Lab (SRL) Services Oversight Committee**

Rui Gardner, Chair  
Alfonso Blanco-Fernandez  
Tim Bushnell  
Monica DeLay  
David Haviland  
Joanne Lannigan  
Peter Lopez  
Adrian Smith  
Paul Wallace  
Andreas Radbruch (ex officio)

**Shared Resource Lab (SRL) Task Force**

Joanne Lannigan, Chair  
Lora Barsky  
Matt Chochrane  
Ben Daniel  
Monica DeLay  
Rui Gardner  
Desiree Kunkel  
James Marvin  
Rob Salomon  
Carina Torres  
Rachael Walker

**Scientific Communications Committee**

Jessica Houston, Chair  
Enrico Lugli  
John Nolan  
Bartek Rajwa  
T. Vince Shankey  
Andreas Radbruch, (ex officio)  
Paul Wallace, (ex officio)
General Information

All Congress activities will be held at the Scottish Exhibition and Conference Centre (SECC) located at Exhibition Way, Glasgow, G3 8YW, unless noted otherwise.

Participation in CYTO 2015 is limited to registered delegates. Full congress registration includes admission to all CYTO sessions such as workshops, parallels, plenaries, frontiers, state of the arts, Special Lecture by Eric Betzig, 2014 Nobel Prize Winner Carl June, Robert Hooke Lecture, FSSCA Guest Symposium, CYTO Innovation, scientific tutorials, commercial exhibits, poster sessions, commercial tutorials, refreshment breaks, happy hours, opening reception and the closing reception at The Arches*.

Congress Registration – Hall 4 Lobby
Registration for CYTO 2015 will be open during the following days and hours:
Friday, June 26.......................... 900 – 2000
Saturday, June 27......................... 700 – 1930
Sunday, June 28.......................... 730 – 1830
Monday, June 29.......................... 730 – 1830
Tuesday, June 30.......................... 730 - 1700
Refund Policy: No refunds will be issued after May 24, 2015.

Exhibitor Registration – Hall 4 Lobby
Exhibitor registration provides admittance into the Exhibit Hall only. Exhibitor registration will be open during the following days and hours:
Friday, June 26.......................... 800 – 2000
Saturday, June 27......................... 700 – 1930
Sunday, June 28.......................... 1030 – 1830
Monday, June 29.......................... 1030 – 1830
Tuesday, June 30.......................... 1030 - 1700

Badges
Participation in CYTO 2015 is limited to registered attendees. The official badge is required for admittance to all sessions, social activities and the exhibit hall. A fee may be charged to reissue lost or misplaced badges. Please do not place a business card into the badge holder as identification. If there is an error on a badge, please have it corrected at the registration desk.

Business Centre
The Scottish Exhibition and Conference Centre has a well equipped Information & Business Centre for attendees. Available services include PC Use/Internet Access, Photocopying, Printing, Scanning, Faxing, a Gift Shop and information on the city and restaurants. The Business Centre will open from 930 until 1700 daily. If you have any questions about the services provided, please contact info@secc.co.uk.

Bank
An ATM is located on the main concourse next to the SECC Box Office and at McColls shop in the main SECC.

Cell Phones
Please silence cell phones and pagers prior to entering a session room. If you must leave a session early, please use the rear entrance and exit quietly.

Child Care
Please check with your hotel’s front desk or concierge service for names of babysitters who can provide care in your hotel room. Parents and guardians are required to perform their own reference checks and arrange child care independently. ISAC is not responsible for child care or the quality of care provided.

Closing Reception at the Arches
Transportation will not be provided

The Closing Reception will be held on Tuesday, June 30, 2015, from 19:00 until 23:00 at The Arches. The Arches is a unique venue set in Grade A Listed Victorian Vaults in a prime Glasgow City Centre Location, and only a 10 minute walk from the Scottish Exhibition and Conference Centre. You may also take the ScotRail to the Arches by boarding the train at "Exhibition Centre," just 0.3 miles from the Scottish Exhibition and Conference Centre, and getting off the train at "Glasgow Central." Exit "Glasgow Central", by Platform 11 onto Argyle Street. The Arches is directly across the street. This venue is renowned for its versatility and daring spirit in hosting spectacular events.

Plan on a fabulous night of fun with your colleagues at this unique venue...there will be music, dancing, whisky tasting, food, drinks, and more! Two complimentary drink tickets will be provided to those attending the reception.

*Full registration includes an exchange coupon for the Closing Reception at the Arches. The Closing Reception coupon must be exchanged for an actual ticket on-site at the registration desk beginning Friday, June 26, 2015, until Sunday,
June 28, 2015. Coupons will be exchanged on a first come, first served basis until maximum capacity is reached. A ticket is required for admittance. Be sure to exchange your coupon early so you don’t miss out on the fun!

**COMMERCIAL EXHIBITS – HALL 4**
Visit the commercial exhibits featuring displays by leading suppliers and vendors. A complete directory of exhibiting companies as well as the exhibit hall floor plan is located under the Exhibits tab of this program.

Exhibits will be open during the following days and hours:
- **Saturday, June 27**………………..1800 - 2000
- **Sunday, June 28**………………..1130 - 1900
- **Monday, June 29**………………..1130 - 1900
- **Tuesday, June 30**………………..1130 - 1630

Note: children under the age of 16 are not permitted in the exhibit hall without parent or guardian supervision.

**COMMERCIAL TUTORIALS**
Sixteen (16) commercial tutorial sessions are offered from 1245 – 1345 on Saturday, June 27, through Tuesday, June 30. Please refer to the Commercial Tutorial tab of this program for a complete list of offerings.

**COMPANION/GUEST REGISTRATION**
Registered attendees of CYTO 2015 may sign up a spouse/guest as a Companion for $200 USD. Companion registration allows entrance to the Opening Reception, Happy Hours and the Closing Reception only. The Opening Reception is scheduled to be held Saturday, June 27, 2015 and the Closing Reception on Tuesday, June 30, 2015. Companion registrants are not permitted in the session rooms or the exhibit hall at any other time.

**CYTO U CYBER CAFÉ**
For your convenience, ISAC has set up several computers with free Internet access in the CYTO U Cyber Café. Attendees may use computers to browse the Internet and/or to check email. In consideration of others, please limit your use to 15 minutes. The CYTO U Cyber Café will be open during the Commercial Exhibit hours in the exhibit hall for attendee use beginning Saturday, June 27.

**CMLE**
This continuing medical laboratory education activity is recognized by the American Society for Clinical Pathology as meeting the criteria for 27.5 hours of CMLE credit. ASCP CMLE credit hours are acceptable to meet the continuing education requirement for the ASCP Board of Registry Certification Maintenance Program.

If you’re interested in earning CMLE credits, please follow these steps:
1. Be sure to add your name to the sign in sheet which will be located at the back of each session room (sign in sheets will be present in every session that is eligible to earn CMLE credits);
2. Complete an evaluation form for each session you attend and leave it in the box at the back of the room, immediately following that session;
3. Use the CMLE form available in the session rooms to track the sessions you attend. Please follow the instructions on the form to finalize the process. You will need to drop off your completed form at the Registration Desk or in the back of the session room before leaving the Congress.

CMLE certificates will be issued by the ISAC Executive Office upon request via email to isac@isac-net.org.

**CYTO INNOVATION**
This forum explores the challenges and opportunities for the development and commercialization of cell analysis technologies and will focus on cytometry in a world demanding rapid responses to emerging global health issues. The CYTO Innovation Program will include short talks offering perspectives on current trends in the industry, a panel discussion, and a showcase of short presentations by innovators and entrepreneurs on new technologies and applications for cell analysis. If you are involved in the development, evaluation, or commercialization of new cell analysis technologies, please contact the CYTO Innovation Program Committee to join us for this stimulating program. CYTO Innovation is open to Full Congress Registrants; no additional fees apply. See page 37 for full description.

**DINING OPTIONS**
Drink vending machines are available at various locations throughout the SECC. McColls, situated on the main concourse, sells a range of snacks and other products. Clydebuilt Bar & Kitchen is conveniently located at the East Entrance of the SECC. A food cart serving snacks and sandwiches for purchase will be available in Exhibit Hall 4 during lunch hours.

Glasgow has an amazing and eclectic restaurant and cafe scene. During the summer months, you may enjoy alfresco dining in the city centre’s Royal Exchange Square, Buchanan Square, West End, or Merchant City. Dining options range from traditional Scottish fare to Indian, sushi, whisky to cocktails. For a list of area pubs and great places to dine and to make reservations visit glasgowdinearound.com.
Disabilities and Special Needs
If you have a disability or special need that may have an impact on your participation in the meeting, please contact Meeting Management at info@cytoconference.org or visit the registration desk on site. ISAC cannot ensure the availability of appropriate accommodations without prior notification of need.

Exceptional Student Award
The Exceptional Student Award will be awarded for an outstanding presentation by a pre-doctoral student. All abstract submitters who are enrolled in a pre-doctoral graduate program are eligible. Finalists will be notified prior to the CYTO Congress and will be expected to make a formal presentation of their work in a special session of the Congress. The winner will be recognized at the Award Ceremony and presented with a certificate and a check for $1,000 USD.

Please check the Congress Addendum and Mobile App for the date, time, and location of this event.

CYTO Showcase – Hall 4
CYTO 2015 will feature two Exhibitor Showcases from 1800 – 1900 on Sunday, June 28, and Monday, June 29. Each Exhibitor Showcase includes presentations by several exhibiting companies. The CYTO Showcase area is located in the back of the exhibit hall next to the ISAC booth.

ICCE
ISAC is an approved provider of continuing education for the ICCE certification. Any one hour of ISAC educational programming is worth one credit. CYTO 2015: Cytometry Advancing Science, 30th Congress of the International Society for Advancement of Cytometry, is worth 27.5 ICCE continuing education credits. For more information on the International Cytometry Certification Examination and how to become a certified cytometrist, visit http://cytometrycertification.org.

Internet/Wireless Access
Complimentary WiFi Internet Service is available in meeting rooms and the Exhibit Hall. To connect to the WiFi please log into the “SECC” network with the username and password “cyto2015.”

ISAC Booth – Exhibit Hall 4
The International Society for Advancement of Cytometry (ISAC) serves a multidisciplinary community by leading technological innovation, scholarship, and the exchange of knowledge in the quantitative cell sciences to advance the impact of cytometry in meeting current and emerging challenges in the live, biomedical, and physical sciences. Please visit the ISAC booth in the exhibit hall to learn more about the society and CYTO U, meet society representatives such as the executive director, education manager or the editor-in-chief of Cytometry Part A, and discover how ISAC can help you advance your career.

Medical Centre
The Medical Centre is located on the concourse of the main building, at the East entrance. The highly qualified medical team will be pleased to help, or simply answer any queries you may have. They may be reached by phone at 0141 275 6333.

Mobile App
Download the CYTO 2015 mobile app via iPhone/iPad and Android native apps or via Blackberry, Windows Phone, and your desktop through mobile web. To access the CYTO 2015 mobile site go to ddut.ch/cyto2015.

Poster & Multimedia Presentations – Hall 4
Over 300 poster presentations will be on display in the Exhibit Hall. Please refer to the Poster Board Map in the Congress Addendum for the assigned location of presentations. Please refer to the schedule below for viewing hours.

Saturday, June 27
1600 – 2000 Authors must set up posters on assigned board

Sunday, June 28
800 – 1900 Poster Viewing
1730 – 1830 Poster Session I
(Authors of odd numbered boards must be present at their poster to answer questions and discuss their presentation)

Monday, June 29
800 – 1900 Poster Viewing
1730 – 1830 Poster Session II
(Authors of even numbered boards must be present at their poster to answer questions and discuss their presentation)

Tuesday, June 30
800 – 1630 Poster Viewing
1530 – 1630 Poster Session III
(consists of Poster Highlights Tour)

CYTO 2015 Poster Highlights Tour
One of the highlights of CYTO 2015 is the hundreds of posters spotlighting cutting-edge cytometry, science and SRL management. When there are so many great posters to view, where do you start? You don’t want to miss any of the ones your peers will discuss for years to come. Tuesday afternoon some of ISAC’s most senior and revered
Cytometrists will lead poster highlights tours. Everyone interested in joining these tours should meet in front of the Exhibit Hall at 1515.

**Outstanding Poster Awards**

All poster presenters who are students or postdoctoral researchers (who have received their doctorate within the last five years) are eligible. The posters will be judged at the time they are scheduled to be presented by the author. Those posters not attended at their scheduled time will not be considered. The names of authors selected for this award will be posted on the Announcement Board in the Registration Area on the morning of Tuesday, June 30. Poster winners will be presented a prize and recognized at the Awards Ceremony from 1630 - 1730 on Tuesday, June 30.

**Practical Information for Glasgow**

**Banking and Foreign Exchange**

The official currency of Scotland is the Great British Pound (GBP, Sterling). Foreign currency and travelers checks can be exchanged at banks, post offices, travel agencies, bureau de change kiosks, and some hotels throughout the city. Credit cards are accepted in Glasgow and Automated Teller Machines (ATMs) can be found throughout the city.

ATM bank machine service is available on the main concourse of the Conference Centre.

There are also International Currency Converters:

www.GoCurrency.com

www.Oanda.com

**Climate**

The month of June in Glasgow is characterized by gradually rising daily temperatures, with daily highs around 63°F and lows around 55°F.

**Dialing Codes**

The UK country code is 44, while Glasgow landlines start with a 141 area code, followed by a 7 digit number. To call abroad, dial 00 before the area code.

**Electricity**

The standard voltage in Scotland is 240V AC, 50Hz. North American appliances need a transformer and an adapter; Australian/Asian appliances need only an adapter. Plugs have 3 square pins.

**Language**

The official language of the Congress is English. Translation services will not be provided.

**Presidents’s Award for Excellence**

The President's Award for Excellence will be awarded for an outstanding presentation by a postdoctoral scientist. All abstract submitters who have received a doctoral degree within the last 5 years are eligible. Finalists will be notified prior to the CYTO Congress and will be expected to make a formal presentation of their work in a special session of the Congress. The winner will be recognized at the Award Ceremony and presented with a certificate and a check for $1,000 USD.

Please check the Congress Addendum and Mobile App for the date, time, and location of this event.

**Recording**

Recording any presentation or session (oral or poster) by any means including audio taping or video taping is prohibited, except by an ISAC authorized agent for official purposes or by first authors who want to record their own poster presentations.

**Scientific Tutorials**

The first day of the Congress is dedicated to the scientific tutorial program. Fourteen ninety minute tutorials will be offered. Admission to Scientific Tutorials is included in CYTO 2015 full congress registration.

**Speaker Ready Room – Gallery Bistro**

The Speaker Ready Room is available for speakers who want to review and check the compatibility of their presentation at least 4 hours prior to their session. The speaker ready room is open during the following days/hours:

- **Friday, June 26** .................................. 900 – 1700
- **Saturday, June 27** ............................... 730 – 1700
- **Sunday, June 28** ................................. 730 – 1700
- **Monday, June 29** ............................... 730 – 1700
- **Tuesday, June 30** ............................... 730 - 1700

Speakers must arrive in the session room 30 minutes prior to the scheduled start of their session to allow the operator time to load their presentation onto the computer. The operator will be seated at the table next to the stage. ISAC is not responsible for slides, laptops, or cables left in session rooms.
Speakers are not required to bring a laptop. All session rooms will be equipped with a data projector, screen, a networked PC running Windows XP and Powerpoint XP with dual mouse control, a laser pointer, as well as a Lectern microphone, table microphone and aisle microphone (depending on room size).

TRANSPORTATION

By Air
Glasgow is served by three international airports with direct flights from 130 destinations.

Glasgow International Airport
Glasgow International Airport is Scotland’s most popular airport, with 30 airlines serving 90 destinations worldwide. It is located just eight miles from the heart of the city and serves as the gateway to the West of Scotland. Glasgow Airport links with major scheduled carriers, such as Lufthansa and United Airlines, with daily service from New York to Glasgow.

Glasgow Prestwick Airport
Glasgow Prestwick Airport is south west of the city and is a 45 minute direct train link from Glasgow City Centre.

Edinburgh International Airport
Direct coach from the main terminal to Glasgow City Centre is available every 30 minutes, from 0500 until 2330 daily. The travel time is 55 minutes at a cost of 16.00 pounds/18.00 pounds return.

By Rail
Glasgow is well connected by train from across the UK. Glasgow Central Station links Glasgow by rail to all UK cities. Glasgow Queen Street Station operates routes mainly to Central and Northern Scotland.

<table>
<thead>
<tr>
<th>Average travel time by train from across the UK</th>
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<tbody>
<tr>
<td>Station</td>
</tr>
<tr>
<td>London</td>
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<tr>
<td>Birmingham</td>
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<td>Manchester</td>
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<td>Liverpool</td>
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<tr>
<td>York</td>
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<tr>
<td>Edinburgh</td>
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</table>

Virgin Trains
Virgin Trains offers online booking, route maps, travel information and useful details on chosen destinations.

www.virgintrains.co.uk

National Rail Enquiries
National Rail Enquiries provides a searchable timetable and useful journey planners, as well as details on facilities at major UK stations and points of interest in the local area.

www.nationalrail.co.uk

ScotRail
Scotland’s Rail Operator gives you details on the West Highland Line, the North Highland Line and Caldeonian Sleepers. You will also find timetables, special ticket offers, and other useful links.

www.scotrail.co.uk

By Road
Glasgow is easily accessible via Scotland’s extensive road network. The city’s venues and hotels are comfortably accessed by road and the SECC is located just off the M8 motorway.

AA
The AA provides information about driving in the UK, including maps, route planners, and travel insurance.

www.theaa.com

Traffic Scotland
Traffic Scotland is a website that provides traffic and road work information in real time.

www.trafficscotland.org

By Sea
Traveling to Glasgow by sea is made simple by the ferry connections. Traveling by ferry from the major ports of Belfast and Larne to Cairnryan or Troon in the southwest of Scotland is a popular option for visitors from Ireland. The ferries on these routes are modern and well-equipped and sail several times daily. Crossing times are between 1 and 2 hours.

The port of Troon in Ayrshire is approximately 35 minutes from Glasgow while Loch Ryna Port, Cairnryan in Dumfries & Galloway is around 80 miles away. Both enjoy good road, rail, and bus links with Glasgow.

Getting Around in Glasgow
Compact and built on a grid system, Glasgow is easy to navigate on foot or by public transport.

Subway
Scotland’s only Subway system’s circular route is a fast and efficient way to travel, particularly in the city centre, west end, and south side areas.

ISAC 2015 Program and Abstracts 23
Operating hours are Monday to Saturday, 0630 - 2330; Sunday 1000 - 1800.

Runs every 6-8 minutes

1.60 pounds single; 3.00 pounds return

Day ticket 4.00 pounds

www.sptico.uk/subway

Trains
Glasgow Central Station links Glasgow by rail to the South. Glasgow Queen Street Station operates routes mainly to Edinburgh, the North of the city, and Northern Scotland.

ScotRail offers a Roundabout Ticket for one-day unlimited travel by rail and subway to over 100 stations in the Greater Glasgow area. The cost is 6.30 pounds for adults and 3.15 pounds for children.

www.scotrail.co.uk

Buses
First Bus Glasgow is the largest bus operating company in Scotland, with over 1,000 buses in operation.

www.firstgroup.com/ukbus/Glasgow

Taxis
Glasgow Taxis operate 5-seater “black cabs” in and around the city. If their yellow light is on, they can be flagged down on the street, or alternatively call +44 (0) 141 429 70 70 to book.

Cycle Hire
The nextbike cycle hire scheme is available in Glasgow, with 170 bicycles for hire in 31 locations around the city that provide a quick and easy way to get around.

www.nextbike.co.uk/en/glasgow

City Sightseeing Bus
Hop on/hop off buses include 28 stops along the route and are a great way to see the city of Glasgow. The Scottish Exhibition and Conference Centre, Glasgow Science Centre, Botanic Gardens, Merchant Square, and many more points of interest are among the stops.

www.Citysightseeingglasgow.co.uk

Beyond the City
Glasgow is ideally located for exploring Scotland. More information can be found at www.conference.peoplemakeglasgow.com

David James Chauffer Drive
Enjoy a tailored sightseeing tour of Scotland’s beautiful countryside and vibrant cities with an experienced chauffer acting as your guide.

www.davidjameschauffeur.co.uk

Discover Scotland Tours
Discover Scotland Tours provide a superb selection of one day coach tours to some of the most dramatic and scenic places in Scotland, including visits to Loch Lomond, Loch Ness, and the Highlands.

www.discoverscotlandtours.com

Edinburgh
Edinburgh is the capital of Scotland, where centuries of history meet a vibrant city. It is also the nearest location to Glasgow where castles can be found.

Golf
Scotland is the home of golf and has more than 600 golf courses, including renowned championship courses such as Loch Lomond, Turnberry, and Gleneagles.

Rabbie's Trail Burners
Experience Scotland’s stunning scenery and visit waterfalls, castles, and whisky distilleries, all the way to the Highlands and Islands.

www.rabbies.com

Seaplane
Loch Lomond Seaplanes operate a seaplane service from a new purpose-built terminal at the Glasgow Science Centre on the River Clyde to Oban Bay - a journey time of approximately 24 minutes, flying over areas of outstanding natural beauty.

www.lochlomondseaplanes.com

Timberbush Tours
Travel Scotland in an exciting range of one, two, and three day luxury tours departing daily from Glasgow City Centre all year round.

www.timberbush-tours.co.uk

Waverly
This is the only sea-going paddle steamer in the world, built in Glasgow by A&J Inglis in 1947 for the LNER. It has enchanted millions of passengers who have boarded for a cruise from the heart of Glasgow.

www.waverlyexcursions.co.uk

ISAC 2015 Program and Abstracts

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## Service Locations & Telephone Numbers

### Meeting Management - Hall 4 Lobby

Tel: 0141 5763102

### Congress Registration - Hall 4 Lobby

Tel: 0141 5763102

<table>
<thead>
<tr>
<th>Day</th>
<th>Time</th>
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<tbody>
<tr>
<td>Friday, June 26</td>
<td>900 – 1730</td>
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<tr>
<td>Saturday, June 27</td>
<td>700 – 1930</td>
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<td>Sunday, June 28</td>
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<td>Monday, June 29</td>
<td>730 – 1830</td>
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<td>Tuesday, June 30</td>
<td>730 - 1700</td>
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### Exhibitor Registration - Hall 4 Lobby

Tel: 0141 5763103

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<td>Tuesday, June 30</td>
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### Speaker Ready Room - Gallery Bistro

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<td>730 – 1700</td>
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<tr>
<td>Tuesday, June 30</td>
<td>730 – 1700</td>
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Committee Meetings
All meetings are by invitation only and held in the Scottish Exhibition and Conference Centre unless specified otherwise.

**THURSDAY, JUNE 25, 2015**

ISAC COUNCIL STRATEGIC MEETING
930 – 1800  Leven Room

**FRIDAY, JUNE 26, 2015**

ISAC SCHOLAR PROGRAM COMMITTEE
900 – 1000  Gala 1 & 2 Rooms

SCIENTIFIC COMMUNICATIONS COMMITTEE
1000 – 1100  Gala 1 & 2 Rooms

CYTOMETRY PART A BOARD MEETING
1200 – 1400  The Forth Room

PRE-CYTO FLUORESCENCE CALIBRATION TASK FORCE MEETING
1430 – 1630  Gala 1 & 2 Rooms

FLOW CYTOMETRY TASK FORCE
1600 – 1630  Lomond Auditorium

**SATURDAY, JUNE 27**

IMAGE CYTOMETRY
800 – 900  Gala 1 Room

FLOW REPOSITORY STEERING COMMITTEE
800 – 900  Gala 2 Room

BIOSAFETY COMMITTEE MEETING
1245 – 1345  Gala 2 Room

FLOW CYTOMETRY DATA STANDARDS
1245 – 1345  Gala 1 Room

ASSOCIATED SOCIETIES LUNCHEON
1245 – 1345  The Forth Room

ISAC SCHOLARS SCIENCE SESSION
1930 – 2230  The Forth Room

**SUNDAY, JUNE 28**

FINANCE COMMITTEE
800 – 900  Etive

MEMBERSHIP SERVICES COMMITTEE
800 – 900  Gala 1 Room
<table>
<thead>
<tr>
<th>Event</th>
<th>Time</th>
<th>Room</th>
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<tbody>
<tr>
<td><strong>ISAC Scholars Luncheon</strong></td>
<td>1245 – 1345</td>
<td>The Forth Room</td>
</tr>
<tr>
<td><strong>SRL Oversight Committee</strong></td>
<td>1700 – 1800</td>
<td>Gala 1 Room</td>
</tr>
<tr>
<td><strong>Live Education Delivery Task Force</strong></td>
<td>1700 – 1800</td>
<td>Gala 2 Room</td>
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<tr>
<td><strong>Monday, June 29</strong></td>
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<tr>
<td><strong>Education Committee</strong></td>
<td>700 – 900</td>
<td>Gala 2 Room</td>
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<tr>
<td><strong>SRL Task Force</strong></td>
<td>800 – 900</td>
<td>Gala 1 Room</td>
</tr>
<tr>
<td><strong>Exhibit Advisory Committee</strong></td>
<td>1000 – 1100</td>
<td>Gala 1 Room</td>
</tr>
<tr>
<td><strong>ISAC Emerging Leaders Luncheon</strong></td>
<td>1245 – 1400</td>
<td>The Forth Room</td>
</tr>
<tr>
<td><strong>ISAC Microvesicle Analysis Interest Development Group (IDG) Meeting</strong></td>
<td>1700 – 1800</td>
<td>Gala 2 Room</td>
</tr>
<tr>
<td><strong>E-Learning Delivery Task Force</strong></td>
<td>1730 – 1830</td>
<td>Gala 1 Room</td>
</tr>
<tr>
<td><strong>Tuesday, June 30</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SRL Content Task Force</strong></td>
<td>730 – 830</td>
<td>Gala 1 Room</td>
</tr>
<tr>
<td><strong>ISAC Business Meeting (Open to All)</strong></td>
<td>830 – 900</td>
<td>Clyde Auditorium</td>
</tr>
<tr>
<td><strong>CYTO 2016 Planning Meeting</strong></td>
<td>1200 – 1330</td>
<td>The Forth Room</td>
</tr>
</tbody>
</table>
# Congress Overview

All Congress activities will be held at the Scottish Exhibition Conference Centre unless noted otherwise.

## FRIDAY, JUNE 26, 2015

<table>
<thead>
<tr>
<th>Time</th>
<th>Activity</th>
<th>Location</th>
</tr>
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<tbody>
<tr>
<td>800 – 2000</td>
<td>Exhibitor Registration</td>
<td>Hall 4 Lobby</td>
</tr>
<tr>
<td>900 – 1730</td>
<td>Scientific Registration</td>
<td>Hall 4 Lobby</td>
</tr>
<tr>
<td>1000 – 1130</td>
<td>Scientific Tutorial Sessions 1-4</td>
<td>Lomond Auditorium, Alsh, Boisdale Room, Carron Room</td>
</tr>
<tr>
<td>1000 – 1200</td>
<td>ICCE Exam</td>
<td>Seminar Suite</td>
</tr>
<tr>
<td>1600 – 1630</td>
<td>Coffee Break</td>
<td>Clyde Auditorium Foyer</td>
</tr>
<tr>
<td>1630 – 1730</td>
<td>Special Lecture by Eric Betzig</td>
<td>Clyde Auditorium</td>
</tr>
<tr>
<td>1745 – 1845</td>
<td>CYTO First Time Attendees’ Welcome &amp; Orientation Tea</td>
<td>The Forth Room</td>
</tr>
</tbody>
</table>

## SATURDAY, JUNE 27, 2015

<table>
<thead>
<tr>
<th>Time</th>
<th>Activity</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>700 – 1930</td>
<td>Scientific Registration</td>
<td>Hall 4 Lobby</td>
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<tr>
<td>700 – 1930</td>
<td>Exhibitor Registration</td>
<td>Hall 4 Lobby</td>
</tr>
<tr>
<td>900 – 1030</td>
<td>State of the Art Lectures</td>
<td>Clyde Auditorium</td>
</tr>
<tr>
<td>1030 – 1100</td>
<td>Coffee Break</td>
<td>Clyde Auditorium Foyer</td>
</tr>
<tr>
<td>1100 – 1230</td>
<td>Concurrent Parallel Sessions</td>
<td>Lomond Auditorium, Alsh, Boisdale Room, Carron Room, Seminar Suite</td>
</tr>
<tr>
<td>1245 – 1345</td>
<td>Commercial Tutorials</td>
<td>Alsh, Boisdale Room, Carron Room</td>
</tr>
<tr>
<td>1430 – 1600</td>
<td>Concurrent Workshops</td>
<td>Alsh, Boisdale Room, Carron Room, Seminar Suite</td>
</tr>
<tr>
<td>1430 – 1800</td>
<td>CYTO Innovation</td>
<td>Clyde Auditorium</td>
</tr>
<tr>
<td>1600 – 1630</td>
<td>Coffee Break</td>
<td>Clyde Auditorium Foyer, Lomond Auditorium Foyer, Seminar Suite</td>
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<tr>
<td>1630 – 1800</td>
<td>Concurrent Workshops</td>
<td>Alsh, Boisdale Room, Carron Room</td>
</tr>
<tr>
<td>1800 – 2000</td>
<td>Commercial Exhibits Open</td>
<td>Exhibit Hall 4</td>
</tr>
<tr>
<td>1830 – 1930</td>
<td>Opening Reception</td>
<td>Exhibit Hall 4</td>
</tr>
<tr>
<td>1930 – 2230</td>
<td>ISAC Scholars Science Session</td>
<td>The Forth Room</td>
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## SUNDAY, JUNE 28, 2015

<table>
<thead>
<tr>
<th>Time</th>
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<tbody>
<tr>
<td>730 – 1830</td>
<td>Scientific Registration</td>
<td>Hall 4 Lobby</td>
</tr>
<tr>
<td>800 – 1900</td>
<td>Poster Viewing</td>
<td>Exhibit Hall 4</td>
</tr>
<tr>
<td>900 – 1030</td>
<td>Frontiers Session 1</td>
<td>Clyde Auditorium</td>
</tr>
<tr>
<td>1030 – 1100</td>
<td>Coffee Break</td>
<td>Clyde Auditorium Foyer</td>
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<tr>
<td>1030 – 1300</td>
<td>Exhibitor Registration</td>
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<tr>
<td>1100 – 1230</td>
<td>Concurrent Parallel Sessions</td>
<td>Lomond Auditorium, Alsh, Boisdale Room, Carron Room, Seminar Suite</td>
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<tr>
<td>1130 – 1900</td>
<td>Commercial Exhibits Open</td>
<td>Exhibit Hall 4</td>
</tr>
<tr>
<td>1400 – 1530</td>
<td>Plenary Session 1</td>
<td>Clyde Auditorium</td>
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<tr>
<td>Time</td>
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<td>1530 – 1600</td>
<td>Coffee Break</td>
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<tr>
<td>1600 – 1700</td>
<td>Robert Hooke Lecture</td>
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<td>1730 – 1830</td>
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<tr>
<td>1800 – 1900</td>
<td>Happy Hour</td>
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<td>1800 – 1900</td>
<td>Exhibitor Showcase</td>
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<tr>
<td>1930 – 2230</td>
<td>Shared Resource Lab Forum</td>
<td>Crowne Plaza Hotel</td>
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**MONDAY, JUNE 29, 2015**

<table>
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<tr>
<td>730 – 1830</td>
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<tr>
<td>800 – 1900</td>
<td>Poster Viewing</td>
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<tr>
<td>900 – 1030</td>
<td>Frontiers Session 2</td>
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<td>1030 – 1100</td>
<td>Coffee Break</td>
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<td>1030 – 1830</td>
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<td>1100 – 1230</td>
<td>Concurrent Parallel Sessions</td>
<td>Lomond Auditorium, Alsh, Boisdale Room, Carron Room, Seminar Suite</td>
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<td>1130 – 1900</td>
<td>Commercial Exhibits Open</td>
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<tr>
<td>1400 – 1530</td>
<td>Plenary Session 2</td>
<td>Clyde Auditorium</td>
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<td>1530 – 1600</td>
<td>Coffee Break</td>
<td>Exhibit Hall 4</td>
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<tr>
<td>1600 – 1730</td>
<td>Concurrent Workshops</td>
<td>Lomond Auditorium, Alsh, Boisdale Room, Carron Room, Seminar Suite</td>
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<td>1730 – 1830</td>
<td>Poster Session 2</td>
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<td>1800 – 1900</td>
<td>Happy Hour</td>
<td>Exhibit Hall 4</td>
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<tr>
<td>1800 – 1900</td>
<td>Exhibitor Showcase</td>
<td>Exhibit Hall 4</td>
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**TUESDAY, JUNE 30, 2015**

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<td>730 – 1700</td>
<td>Scientific Registration</td>
<td>Hall 4 Lobby</td>
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<tr>
<td>800 – 1630</td>
<td>Poster Viewing</td>
<td>Exhibit Hall 4</td>
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<tr>
<td>830 – 900</td>
<td>ISAC Business Meeting</td>
<td>Clyde Auditorium</td>
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<td>900 – 1030</td>
<td>Frontiers Session 3</td>
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<td>Coffee Break</td>
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<tr>
<td>1030 – 1700</td>
<td>Exhibitor Registration</td>
<td>Lomond Auditorium, Alsh, Boisdale Room, Carron Room, Seminar Suite</td>
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<tr>
<td>1100 – 1230</td>
<td>Concurrent Parallel Sessions</td>
<td>Exhibit Hall 4</td>
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<tr>
<td>1130 – 1630</td>
<td>Commercial Exhibits Open</td>
<td>Exhibit Hall 4</td>
</tr>
<tr>
<td>1245 – 1345</td>
<td>Commercial Tutorials</td>
<td>Alsh, Boisdale Room</td>
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<tr>
<td>1400 – 1530</td>
<td>ESCCA Guest Symposium</td>
<td>Clyde Auditorium</td>
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<tr>
<td>1530 – 1600</td>
<td>Coffee Break</td>
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<tr>
<td>1530 – 1630</td>
<td>Poster Session 3</td>
<td>Exhibit Hall 4</td>
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<tr>
<td>1630 – 1730</td>
<td>Awards Ceremony</td>
<td>Lomond Auditorium The Arches</td>
</tr>
<tr>
<td>1900 – 2300</td>
<td>Closing Reception</td>
<td>(Must present ticket for admittance)</td>
</tr>
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</table>
Visit the Exhibits & Posters

**SATURDAY, JUNE 27, 2015**
- 1600 – 2000 Authors Must Place Posters on Boards
- 1800 – 2000 Commercial Exhibits Open

**SUNDAY, JUNE 28, 2015**
- 800 - 1900 Poster Viewing
- 1130 – 1900 Commercial Exhibits
- 1530 – 1600 Coffee Break
- 1730 - 1830 Poster Session 1 (Authors of odd numbered posters present)
- 1800 – 1900 Happy Hour
- 1800 – 1900 Exhibitor Showcase

**MONDAY, JUNE 29, 2015**
- 800 – 1900 Poster Viewing
- 1130 – 1900 Commercial Exhibits Open
- 1530 – 1600 Coffee Break
- 1730 – 1830 Poster Session 2 (Authors of even numbered posters present)
- 1800 – 1900 Happy Hour
- 1800 – 1900 Exhibitor Showcase

**TUESDAY, JUNE 30, 2015**
- 800 – 1630 Poster Viewing
- 1130 – 1630 Commercial Exhibits Open
- 1530 – 1600 Coffee Break
- 1530 – 1630 Poster Session 3 consists of Poster Highlights Tour

*Posters must be removed by 1630 on Tuesday, June 30, 2015.*

Don’t forget to exchange your Closing Reception coupon for a ticket to the Tuesday evening Closing Reception at The Arches!

**Coupons will be exchanged on a first come, first served basis until Sunday, June 28, or until maximum capacity is reached.**

*(ticket required for admittance; see page 19 for details)*
Special Lectures

Friday, June 26
1630 – 1730

Imaging Life at High Spatiotemporal Resolution
Eric Betzig, Janelia Farm Research Campus, Howard Hughes Medical Institute, USA

After obtaining a BS in Physics from Caltech, Eric Betzig moved to Cornell, where his thesis involved the development of near-field optics -- the first method to break the diffraction barrier in light microscopy. Betzig became a PI at AT&T Bell Labs in Murray Hill, NJ, where he further refined the technology and explored many applications, including high density data storage, semiconductor spectroscopy, and superresolution fluorescence imaging of cells. In 1993, Betzig was the first to image single fluorescent molecules under ambient conditions, and determine their positions to better than 1/40 of the wavelength of light. Tiring of academia, he served as Vice President of R&D at his father’s machine tool company, developing a high speed motion control technology based on an electrohydraulic hybrid drive with adaptive control algorithms. The commercial failure of the technology left him unemployed and his search for new directions culminated in the invention and demonstration of the superresolution technique PALM by himself and fellow Bell Labs expatriate, Harald Hess. Since 2005, Betzig has been a Group Leader at the Howard Hughes Medical Institute’s Janelia Farm Research Campus, developing new optical imaging technologies for biology. Betzig won the 2014 Nobel Prize in Chemistry for “The development of super-resolved fluorescence microscopy”.

Sunday, June 28
1600 – 1700

Engineered T Cells for Cancer Therapy
Carl June, University of Pennsylvania, USA

Carl June is the Richard W. Vague Professor in Immunotherapy in the Department of Pathology and Laboratory Medicine. He is currently Director of the Center for Cellular Immunotherapies at the University of Pennsylvania, and is an Investigator of the Abramson Family Cancer Research Institute. He is a graduate of the Naval Academy in Annapolis, and Baylor College of Medicine in Houston, 1979. He had graduate training in Immunology and malaria with Dr. Paul-Henri Lambert at the World Health Organization, Geneva, Switzerland from 1978-79, and post-doctoral training in transplantation biology with E. Donnell Thomas and John Hansen at the Fred Hutchinson Cancer Research Center in Seattle from 1983 - 1986. He is board certified in Internal Medicine and Medical Oncology. He founded the Immune Cell Biology Program and was head of the Department of Immunology at the Naval Medical Research Institute from 1990 to 1995 before joining the faculty of the Perelman School of Medicine in 1999. He maintains a research laboratory that studies various mechanisms of lymphocyte activation that relate to immune tolerance and adoptive immunotherapy for cancer and chronic infection. He has published more than 300 manuscripts and is the recipient of numerous prizes and honors, including election to the Institute of Medicine in 2012, the William B Coley Award, and the Richard V Smalley Memorial Award from the Society for Immunotherapy of Cancer in 2013. In 2014, he was elected to the American Academy of Arts and Sciences.
<table>
<thead>
<tr>
<th>Time</th>
<th>Session</th>
<th>Location</th>
<th>Details</th>
</tr>
</thead>
</table>
| 1000    | **Scientific Tutorials**                                                                     | Boisdale Room| 1 Fluorescent Proteins in Flow Cytometry  
| 1000    | 2 Label-Free Imaging: Ptychography-Label-Free Cytometry  
P. O'Toole and M. Humphry. Univ. of York and Phasefocus, Sheffield, U.K. | Lomond Auditorium | 3 Making Resource Technology a Career: Developing, Promoting and Retaining Core Leadership and Staff  
| 1000    | 4 OMERO in Action  
W. Moore and P. Walczysko. GRE Sch. of Life Sci., Univ. of Dundee. | Carron Room  | 5 RNA Flow Cytometry  
S. McClellan, P.K. Wallace and C. Groves. Univ. of South Alabama, Roswell Park Cancer Inst. and MedImmune Inc., Gaithersburg, MD. |
| 1000    | 6 Cellcognition: Image Analysis of Live Cell Imaging Data  
| 1000    | 8 Ask the Experts: Quality Control in Image Cytometry  
M. Halter, S. Leavesley and S. Lockett. Natl. Inst. of Standards and Technol., Gaithersburg, MD, Univ. of South Alabama, Frederick Natl. Lab. for Cancer Res. , NCI. | Boisdale Room | 9 Cell Sorting: Fundamentals, Applications and Troubleshooting  
| 1245    | **TUTORIAL 5 – 9** 1245 - 1415                                                                 | Carron Room  | 10 Forensic Flow Cytometry: Crimes against Cytometry  
P. Chattopadhyay and J. Wilshire. NIAID, NIH and Mem. Sloan-Kettering Cancer Ctr. |
| 1245    | 11 "Super" Resolution: Fluorescence Microscopy  
| 1245    | 13 Processing Images Using the Free and Open-Source Software Icy  
| 1600    | **Coffee Break** 1600 – 1630                                                                  | Clyde Auditorium |  |
| 1630    | **SPECIAL LECTURE: 2014 NOBEL PRIZE WINNER**                                                  | Clyde Auditorium | 15 Imaging Life at High Spatiotemporal Resolution  
E. Betzig. HHMI Janelia Res. Campus, Ashburn, VA. |
## CYTO First Time Attendees’ Welcome & Orientation Tea

1745 - 1845  
The Forth Room  
Is this your first time attending CYTO? ISAC Welcomes you to its 30th International Congress - CYTO 2015!  
All first time attendees as well as nonmember registrants are invited to a Welcome & Orientation. New attendees are welcome to join the CYTO family for afternoon tea and to meet new friends. The session will be informal and friendly with the opportunity to meet with old timers of the CYTO conferences as well as other first time attendees.  

During the event, you will meet ISAC Council members, the Membership Services Committee members (MSC), and other leaders of ISAC. Peter Lopez, ISAC Council member and chair of the MSC, will make opening remarks, and representatives from the ISAC Scholars and Shared Resource Lab Emerging Leaders programs will speak about their first CYTOs and advice they have to share. After the formal presentation, attendees will be encouraged to network with each other and ISAC Leaders, who will be sitting at different tables throughout the room.  
There will be opportunity to ask questions; find out the resources available at the conference; bring up specific topics of interest and request to be put in contact with specific cytometrists who will also be attending CYTO 2015.

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**Saturday, June 27, 2015**

### Opening Remarks & State-of-the-Art Lectures

<table>
<thead>
<tr>
<th>Time</th>
<th>Lecture</th>
</tr>
</thead>
</table>
| 900  | Characterizing Immune Cell Types, Responses and Landscapes Using Mass Cytometry  
*M. Davis, Stanford Univ. Sch. of Med.* |
| 930  | Detection of Extracellular Vesicles by Flow Cytometry: Small, Smaller, Smallest  
*R. Nieuwland, AMC, Amsterdam.* |
| 1000 | Developing Sensors to Probe the Pharmacology and Function of Lipid Sensitive G Protein-Coupled Receptors  
*Graeme Milligan, University of Glasgow, United Kingdom.* |

**Coffee Break**  
1030 – 1100  
Clyde Auditorium Foyer

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### Concurrent Parallel Sessions

#### Parallel 1: Flow Cytometry Instrumentation

<table>
<thead>
<tr>
<th>Time</th>
<th>Lecture</th>
</tr>
</thead>
</table>
| 1100 | Evaluation of Cell Sorting Aerosols and Containment by an Optical Airborne Particle Counter  
*M. Xie and M. Waring, Ragon Inst. of MGH and MIT and Harvard.* |
| 1115 | Measurement of Light Scattering in Backward Hemisphere: A New Way for the Study of Platelet Aggregation with Scanning Flow Cytometry  
| 1130 | Tunable Green Fiber Lasers with Expanded Wavelength Ranges for Flow Cytometry  
*W. Telford, V. Akulov, V. Kapoor, N. Hawk and S. Babin, NCI, NIH, Inversion Fiber Ltd., Novosibirsk and Novosibirsk State Univ., Russia.* |

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ISAC 2015 Program and Abstracts
1145 21 The LED Pulser: A New Device for Instrument Characterization and Panel Development

1200 22 Low-Latency Time-Resolved Fluorescence Lifetime Flow Cytometry and Cell Sorting Using a CMOS Silicon Photomultiplier

PARALLEL 2: MASS SPEC AND FLOW CYTOMETRY DATA ANALYSIS I
1100 - 1230
Alsh
Chair: Nima Aghaeepour
Cochair: Pia Kvistborg

1100 23 The First Multi-center Comparative Study Using a Novel Technology Mass Cytometry Time-of-Flight Mass Spectrometer (CyTOF2) for High-Speed Acquisition of Highly Multi-parametric Single Cell Data: A Status Report

1120 24 Measuring Protein-Protein Interactions by Fluorescence Lifetime Flow Cytometry

1140 25 High Content Dissection of Human Melanoma Tumor Heterogeneity during Treatment Using Mass Cytometry

1200 26 Modeling Human Immune Dynamics in Surgery and at Steady State with Mass Cytometry

PARALLEL 3: IMAGE CYTOMETRY I
1100 - 1230
Boisdale Room
Chair: Silas Leavesley
Cochair: Carolina Wählby

1100 27 Cell-o-pane – An Interactive Environment for the Visualisation and Interrogation of Cellular Lineage Data Derived from Timelapse Microscopy

1120 28 A Multifunction Workstation Underpinning Lanthanide-Based Luminescence Techniques

1140 29 Optimal 3D Segmentation of Cell Nuclei in Tissue and Acinar Structures
S. Lockett, R. Chellappa and K. Nandy. Frederick Natl. Lab., MD and Univ. of Maryland College Park.

1200 30 Precise Pinpointing of Luminescent Targets Empowers Quantitative Scanning Cytometry

PARALLEL 4: IMMUNOLOGY I
1100 - 1230
Caron Room
Chair: Pratip Chattopadhyay
Cochair: Elisa Nemes

1100 31 A Reference Framework for Modeling a Dynamic Immune System

1120 32 Cell Surface Proteomics Reveals Changes Associated with TGFβ-Induced EMT in Lung Cancer

1140 33 Flow Cytometry of the Intestinal Microbiota in Health and Chronic Inflammation
Use of Flow Cytometry and Correlate GFP Fluorescence Lifetimes to pH Shifts in Macrophage Cells Infected by *E. coli*

W. Li and J. Houston. New Mexico State Univ.

**PARALLEL 5: NEW PROBES, ASSAYS, BIOMARKERS I**

1100 - 1230
Seminar Suite

Chair: Marcel Bruchez
CoChair: Rui Gardner

1100 36 Phenotypic Changes on Blood Neutrophils Differentiate Patients with Sepsis from Those with Noninfectious Systemic Inflammation


1120 37 SmartFlare Probes Detect Only the Translationally Available mRNA in Live Cells, but Not the Total Pool of mRNA Quantified by Real-Time RT-PCR: A Novel Limitation of the Method


1140 38 Genomic Cytometry: Flow Analysis and Sorting of Rare Cancer Cells Using qRT-PCR Signatures on a Single Cell Basis


1200 39 DOTS qPCR: A Handheld, Rapid Molecular Diagnostic Tool for Pathogens


**Cyto Innovation**

1430 - 1800
Clyde Auditorium

Chair: Diether Rechtenwald
CoChair: Paul Smith

Open to Exhibitors

This forum explores the challenges and opportunities for the development and commercialization of cell analysis technologies and will focus on cytometry in a world demanding rapid responses to emerging global health issues.

**AGENDA:**

1430 436 Creating Health and Wealth through Cell Therapy

*John Brown, CBE, FRSE, United Kingdom*

1510 40 Cytometry Technology Speeding the Response to Global Health Issues

*Francis Mandy, African Institute for Mathematical Sciences, South Africa*

1540 41 Sound Technology for Infectious Disease Diagnostics

*Julien Rebud, University of Glasgow, United Kingdom*

1610 Break

1640 Accelerating New Technologies into Medical Practice (panel discussion)

Moderators: Allen Poirson, Paul Smith
Panelists: John Brown, Francis Mandy, Julien Rebud, Mario Roederer
Industry Representative: TBD

1720 Technology Showcase Presentations

1800 Session Ends

**Coffee Break**

1600 – 1630
Clyde Auditorium Foyer, Lomond Auditorium Foyer, Seminar Suite Foyer

**CONCURRENT WORKSHOP SESSIONS**

1430 - 1600

**WORKSHOP 1**

Alsh

1430 42 Lost in Translation

*S. Eck and V. Litwin. MedImmune Inc., Gaithersburg, MD and Covance Inc., Indianapolis.*
**Workshop 2**
Boisdale Room
1430 43 Cytometry with Tunable Ultrafast Lasers?

**Workshop 3**
Carron Room
1430 44 Metrics of a Successful SRL: Publications and Acknowledgements

**Workshop 4**
Seminar Suite
1430 45 Foundations of Microvesicle Cytometry
P. Hexley and J. Lannigan. Univ. of Nebraska Med. Ctr. and Univ. of Virginia.

**Workshop 5**
Alsh
1630 46 When Worlds Collide: Research Complexity and Clinical Validity of Flow Cytometric Assays Used for Drug Development
T. McCloskey and V. Litwin. ICON Labs., Farmingdale, NY.

**Workshop 6**
Boisdale Room
1630 47 Mucosal Immunology

**Workshop 7**
Carron Room
1630 48 Conflict Management: Dealing with Difficult Situations

**Commercial Exhibits**
1800 – 2000
Exhibit Hall 4
See pages 78 - 98 for full details.

**Opening Reception**
1830 – 1930
Exhibit Hall 4
**Sunday, June 28, 2015**

**Poster Viewing**
800 – 1900
Exhibit Hall 4

**Frontiers Session 1**
900 - 1030
Clyde Auditorium
Chair: David Hedley
Cochair: Alan Waggoner

900 49 Connecting the Whole and the Parts: Organs on Chips and Cytometry
J. Wikswo, Vanderbilt Univ.

945 50 Accelerating Discovery of Autoimmune Mechanisms
M. Roederer, NIAID, NIH.

**Coffee Break**
1030 – 1100
Clyde Auditorium Foyer

**Concurrent Parallel Sessions**
1100 – 1230

**Parallel 6: Mass Spec and Flow Cytometry Data Analysis II**
1100 - 1230
Lomond Auditorium
Chair: Jonathan Irish
Cochair: Enrico Lugli

1100 51 Connecting the Dots – A Literature-Based Global View of the Immune Intercellular Circuitry

1120 52 Deep Profiling of the Murine Myelopoietic System: Signalling and Cell Cycle Responses to Neurotropic Viral Infection Profiled by Mass Cytometry (CyTOF)
T. Ashhurst, A. Smith and N. King. Univ. of Sydney and Centenary Inst., Sydney.

1140 53 Detection and High Dimensional Phenotyping of HIV-Specific T-Cell Responses by Mass Cytometry (CyTOF)

1200 54 Comparative Exploration of Multi-dimensional Flow Cytometry Software to Analyze the Influence of pMHC-TCR Affinity on T Cell Polymutationality
T. Spear, P. Simms, Y. Wang, L. Hellman, B. Baker, H. Rosen and M. Nishimura. Loyola Univ. Chicago, Univ. of Notre Dame and Univ. of Colorado Denver.

**Parallel 7: Flow Cytometry Data Analysis, Informatics I**
1100 - 1230
Alsh
Chair: Hervé Luche
Cochair: Greg Finak

1100 55 Automated Supervised and Unsupervised Analysis of Big Flow Cytometry Data: Results from the International Mouse Phenotyping Consortium

1120 56 Improving AutoGate’s Automation of Discovery Gating

1140 57 Latent Modeling of Flow Cytometry Cell Populations for Joint Automated Gating

1200 58 Data Confidentiality, Integrity, and Authentication in the Archival Cytometry Standard
A. Irvine and B. Rajwa. Indiana Univ.-Purdue Univ. Indianapolis and Purdue Univ.
**Parallel 8: Cell Cycle, Proliferation and Death**

1100 - 1230

Boisdale Room

*Chair: Peter Lopez*

*Cochair: Xu Huang*

1100  59 Validation of a Whole-Blood In Vitro Assay Immunotoxicity of Chemical Compounds Based on Quantitative Analysis of NF-κB Translocation by Imaging Flow Cytometry


1120  60 Resistance to Intrinsic Apoptosis Stimuli in Lymphocytes: A Role for Mitochondrial Dysfunction

C. Bortner, A. Sculock and J. Cidlowski. NIEHS, NIH, Research Triangle Park.

1140  61 Nuclear Apoptotic Volume Decrease in Individual Cells: Time-Lapse Confocal Microscopy Imaging and Molecular-Kinetic Modeling


1200  62 G2 Transit Times in Pancreatic Cancer, Their Relationship to Genomic Instability, and the Therapeutic Potential of G2 Checkpoint Inhibitors

D. Hedley and S. Chow. Princess Margaret Hosp., Toronto.

**Parallel 9: Other Biological Applications of Cytometry**

1100 - 1230

Carron Room

*Chair: Andy Filby*

*Cochair: Rachael Walker*

1100  63 Characterising the Heterogeneity of Inheritance across Mitosis


1120  64 Subcellular Cytometry – Quantitative 3-Dimensional Analysis of Discrete Subcellular Structures and Events Applied to Studies of DNA Damage and Repair


1140  65 Examining Inheritance during Mitosis, in U-2OS Cells, Using Quantum Dot Labelled Vesicles


1200  66 Acoustically Enhanced High Throughput Flow Cytometry for Remote Plankton Monitoring


**Parallel 10: Immunology II**

1100 - 1230

Seminar Suite

*Chair: Kewal Asosingh*

*Cochair: Anis Larbi*

1100  67 Flow Cytometry In Vivo: Recent Advances


1120  68 Flow Cytometry-Based Assay for Detection of BCR-ABL Fusion Protein in Blood Cells from CML Patients


1140  69 Cytometric Determination of Mobility of Memory Lymphocytes


1200  70 Improved Molecular Genetic Analysis of Clinical FFPE Tissue Cores Using Combined Image-Based Cell Selection with Dielectrophoretic Movement (DEPArray) and NGS


**Commercial Exhibits**

1130 – 1900

Exhibit Hall 4

See pages 78 - 98 for full details
Commercial Tutorials
1245 – 1345

FEATURED COMPANIES:
Beckman Coulter – Lomond Auditorium
BD Biosciences – Alsh
Merck Millipore – Boisdale Room
Milenyi Biotec GmbH – Carron Room
IntelliCyt Corporation – Seminar Suite

PLENARY SESSION 1: FLOW CYTOM BIOMED
1400 - 1530
Clyde Auditorium

Chair: Andreas Radbruch
Co-chair: Andrea Cosarizza

1400 71 *Dynamics in the Microbial Cytome – Unraveling Functions in Natural Systems*

1430 72 *Cytometric Evaluation of Immunological Memory*

1500 73 *What T Cells See on Human Cancer*

Coffee Break
1530 – 1600
Exhibit Hall 4

ROBERT HOOKE LECTURE
1600 - 1700
Clyde Auditorium

Chair: Mario Roederer

1600 74 *Engineered T Cells for Cancer Therapy*
C. June. Univ. of Pennsylvania.

Poster Session 1
1730 – 1830
Exhibit Hall 4
Authors of ODD numbered poster boards present.

Happy Hour
1800 – 1900
Exhibit Hall 4

Exhibitor Showcase
1800 – 1900
Exhibit Hall 4

SHARED RESOURCE LAB FORUM
1930 - 2230
Argyll Suite, Crowne Plaza Hotel

Chairs: Joanne Lannigan and Rui Gardner
1930 Crucial Accountability Workshop
Sharon Rush. Grahame Robb Associated, United Kingdom.

The workshop will introduce crucial diagnostic skills that will support you in holding others accountable for falling short of set expectations. A framework that both motivates and enables others to successfully change their behaviour will also be explored during this session. In the workshop you will:

- Gain an insight into how to hold anyone accountable regardless of position or personality
- Open up a performance discussion in a respectful way avoiding defensiveness and costly argument
- Diagnose the underlying cause of misaligned or poor behavior
- Take away a few key ‘Crucial skills’, which you can start to use and achieve results the very same day

2030 - 2230

Networking and Socializing
Network and socialize with your fellow SRL and Core managers and staff. Appetizers and refreshments will be served.
**MONDAY, JUNE 29, 2015**

**Poster Viewing**
800 – 1900
Exhibit Hall 4

**FRONTIERS SESSION 2**
900 - 1030
Clyde Auditorium

*Chair: Bill Hyun
Cochair: Rachel Errington*

900  75  Fluorogenic Reporters and Modulators in Living Animals  

945  76  Nanoparticle-Based Analysis of Biomolecules, Cells and Tissue  
D. Graham. Univ. of Strathclyde, U.K.

**Coffee Break**
1030 – 1100
Clyde Auditorium Foyer

**Commercial Exhibits**
1130 – 1900
Exhibit Hall 4
See pages 78 - 98 for full details.

**CONCURRENT PARALLEL SESSIONS**
1100 – 1230

**PARALLEL 11: NEW PROBES, ASSAYS, BIOMARKERS II**
1100 - 1230
Lomond Auditorium

*Chair: Katarzyna Piwocka
Cochair: Joseph Tario*

1100  77  Cell-Based Biomarkers of Infection/Sepsis: Critical Assessment from the Perspective of Needing Rapid Time to Result  
B. Davis. Trillium Diagnostics, Bangor, ME.

1120  78  Selection and Optimization of Near-Infrared Fluoromodules for Imaging through Tissue  

1140  79  Identification of Immunosenececence-Associated Gene Signatures in Purified Cells of the Human Innate and Adaptive Immune System  

1200  80  An Extracellular Vesicle Probe Effectiveness Comparison Using Simultaneous Labelling  

**PARALLEL 12: FLOW CYTOMETRY DATA ANALYSIS, INFORMATICS II**
1100 - 1230
Alsh

*Chair: Ryan Brinkman
Cochair: Michael Thomson*

1100  81  Automated Analysis of Flow Cytometry Data to Reduce Inter-Lab Variation in Detection of MHC Multimer Binding T Cells  

1120  82  FlowJo Exchange: A Means of Meeting the Computational Needs of the Flow Community  

1140  83  A Novel Flow Cytometric Cell Cycle ER Stress Model for the Study of Autophagy  
G. Warnes and A. Patel. Queen Mary Univ. of London.

1200  84  immuno Clust – An Automated Pipeline for Population Detection in Flow Cytometry  
PARALLEL 13: IMMUNE MONITORING, THERAPEUTICS, TUMORS, BIOLOGY I

1100 - 1230
Boisdale Room

Chair: Paul Wallace
Cochair: Frank Schildberg

1100 85 Modeling Cell-Surface Proteomic Changes Associated with Neoplastic Transformation and Invasion

1120 86 Multiparametric Flow Cytometric In Vitro Toxicity Assays on Rat Cell Lines for Risk Assessment and Classification of Chemical Compound

1140 87 Cytometry of Anticancer Prodrug OCT1002 Activation and Targeting Using In Vitro and In Vivo Models of Tumour Hypoxia

1200 88 High Throughput and Quantitative Measurements of Integrin Conformational Changes Using Fluorescence Lifetime-Dependent Cytometry
J. Sambrano, Jr., A. Chigaev, Y. Smagley, L. Sklar and J. Houston. New Mexico State Univ. and Univ. of New Mexico Sch. of Med.

PARALLEL 14: STEM CELLS, REGENERATIVE MEDICINE, DIAGNOSTICS

1100 - 1230
Carron Room

Chair: Vera Donnenberg
Cochair: Gergely Toldi

1100 89 The Stem Cell Orchestra – A Non-narrative Approach to Communicate Experimental Processes

1120 90 Barcoding and Tracking Stem Cells

1140 91 Muscle Extracellular Matrix Scaffold Is a Multipotent Environment for Stem Cells Differentiation

1200 92 Lectin-Induced Erythrocyte Agglutination – A Potential New Noninvasive Tool for the Diagnosis of Myocardial Infarction

PARALLEL 15: IMAGE CYTOMETRY II

1100 - 1230
Seminar Suite

Chair: Adrian Smith
Cochair: Stephen Lockett

1100 93 Photoswitchable Flow Cytometry for Analysis of Single Circulating Cells In Vivo

1120 94 Mapping Spectral Signatures of Matrix Components in Decellularized Lungs Using Excitation-Scanning Hyperspectral Imaging

1140 95 Kinetic Measurements of Cellular Parameter Changes Evoked by Nanosecond Pulsed Electrical Field
Commercial Tutorials
1245 – 1345

Featured Companies:
Beckman Coulter – Lomond Auditorium
ThermoFisher Scientific – Alsh
Merck Chemicals Ltd – Boisdale Room
eBioscience, an Affymetrix Company – Carron Room
Cytek Development – Seminar Suite

Plenary Session 2: Imaging
1400 - 1530
Clyde Auditorium

Chair: Bob Murphy
Cochair: Raluca Niesner

1400 97 Imaging-Derived Computational Maps of Local Concentrations to Understand Actin Regulation in T Cells

1430 98 Parameterizing Spatial-Temporal Tissue Simulation Models from Image Information

1500 99 Model-Based Image Analysis: Towards Versatile and Robust Microscopy Image Quantification

Coffee Break
1530 – 1600
Exhibit Hall 4

Concurrent Workshops
1600 – 1730

Workshop 9
Lomond Auditorium
1600 100 Flow Cytometry Trends and Drivers
G. Vacca. Kinetic River Corp., Cupertino, CA.

Workshop 10
Alsh
1600 101 The Devil's in the Details: Implementation of High Complexity Flow Cytometric Methods in Global Multi-center Trials
V. Litwin. Covance Inc., Indianapolis.

Workshop 11
Boisdale Room
1600 102 Multilaser Cytometry – Developments, Opportunities, Challenges, and Pitfalls
A. Smith and W. Telford. Univ. of Sydney and NCI, NIH.

Workshop 12
Carron Room
1600 103 Educating Users in New Advancements in Cytometry
M. Cochran and M. Black. Univ of Rochester and Univ of Washington.

Workshop 13
Seminar Suite
1600 104 Back to the Future: CYTOMICS – Translational System Cytometry

Poster Session 2
1730 – 1830
Exhibit Hall 4
Authors of EVEN numbered boards present.
Tuesday, June 30, 2015

Poster Viewing
800 – 1630
Exhibit Hall 4

ISAC Business Meeting
830 – 900
Clyde Auditorium

Frontiers Session 3
900 - 1030
Clyde Auditorium
Chair: Bob Hoffman
Cochair: Jessica Houston

095 Highly Multiplexed Imaging with Subcellular Resolution by CyTOF Mass Cytometry

096 Microfluidic-Based Cell Sorting
D. Weitz. Harvard Univ.

Coffee Break
1030 – 1100
Clyde Auditorium Foyer

Concurrent Parallel Sessions
1100 – 1230

Parallel 16: Cell Sorting, Chip Cytometry, Fluidics
1100 - 1230
Lomond Auditorium
Chair: David Haviland
Cochair: Steve Graves

107 Inkjet-Printed Cell-Counting Chambers with Integrated On-Chip Sample Preparation

108 Single Cell Isolation Technology Based on Single Cell Self-Seed Microwells

109 Integrated III-V Semiconductor Platform with Capillary Fill Microfluidics for Chip-Based Flow Cytometry

110 Acoustic and Magnetic Methods for Cell Sorting and Single Cell Analysis in a Microfluidic Device

111 Fluorescence Activated Cell Sorter Based on On-Chip Micro Vapor Bubble Jet Flow

Parallel 17: Flow Cytometry Data Analysis, Informatics III
1100 - 1230
Alsh
Chair: Josef Spidlen
Cochair: Qian Jun Zhang

112 Earth Mover’s Distance Approach for Quantitative Analysis of Multivariate Changes in Biomarkers Measured by Flow Cytometry

113 Surface Proteome of Adipose Stromal Vascular Subpopulations

114 The Tenets of Helper T-Cell Classification: Checked and Challenged with 30-Parameter Flow Cytometry
P. Chattopadhyay, S. Perfetto, J. Hill, R. Nguyen, D. Ambrozak and M. Roederer. NIAID, NIH.
1200  115  Automated Analysis of 16-Color Polychromatic Flow Cytometry Data Maps Immune Cell Populations and Reveals a Distinct Inhibitory Receptor Signature in Systemic Sclerosis  

**PARALLEL 18: IMMUNE MONITORING, THERAPEUTICS, TUMORS II**  
1100 - 1230  
Boisdale Room  
Chair: Yolanda Mahnke  
Cochair: Tomas Kalina

1100  116  Patients Pre-operative Immune States Correlate with Recovery from Surgical Trauma  

1100  117  Deep Immunophenotyping by ChipCytometry Reveals CD11c⁺ B Cell Subset in Inflamed Tonsils  

1140  118  Quantification of Germinal Centre T and B Lymphocytes in Lymph Node and Gut Biopsies during HIV Infection  

1200  119  Anti-TNF Therapy Restores Peripheral Blood Cell Subsets and CD40 Expression in Inflammatory Bowel Diseases  

**PARALLEL 19: MICROVESICLES AND PARTICLES**  
1100 - 1230  
Carron Room  
Chair: Joanne Lannigan  
Cochair: Philip Hexley

1100  120  Scanning Flow Cytometry Study of Cell-Derived Microparticles and Their Aggregates in Platelet-Rich Plasma  

1115  121  Standardization of Flow Cytometry-Based Determination of Plasma Microvesicles: Recent Progress  

1130  122  Quantification of Cell-Derived Microvesicles in Blood  
A. Brisson, N. Arraud, C. Gounou and R. Linares. UMR-CBMN, Univ. of Bordeaux.

1145  123  High-Resolution Multiparameter Characterization of Individual Extracellular Vesicles by High Sensitivity Flow Cytometry  
X. Yan, Y. Tian, L. Ma, C. Chen and S. Zhu. Xiamen Univ., China.

1200  124  Functional Sorting of Extracellular Vesicles and Nanoscale Viral Particles with nanoFACS  

**PARALLEL 20: IMAGE CYTOMETRY III**  
1100 - 1230  
Seminar Suite  
Chair: Michael Halter

1100  125  Using Analysis of Cellular Heterogeneity in High Content Screening Data to Guide Compound Prioritization in Drug Discovery  
### Poster Session 3

1330 – 1630
Exhibit Hall 4

Consists of Poster Highlights Tour (See page 21)

Authors must remove their posters from boards by 1630.

**Awards Ceremony**

1630 – 1730

Lomond Auditorium

**Master of Ceremonies:** John Nolan, Awards Committee Chair and Past President

**Recognition of New ISAC Scholars:**
- Greg Finak
- Pia Kvistborg
- Elisa Nemes
- Raluca Aura Niesner
- Josef Spidlen

**Recognition of New Shared Resource Lab (SRL) Emerging Leaders**
- Jessica Black
- Anna Belkina
- Gelo Víctorniano De La Cruz
- Nina Lane
- Michael Thomson

**To Be Announced:**
- Cytometry Part A: 2014 Best Paper Award
- Distinguished Service Award
- Exceptional Student Award Finalists and Winner
- Membership Award
- Outstanding Poster Awards
- President’s Award for Excellence Finalists and Winner
- The Fulwyler Award for Innovation Excellence

**Closing Reception at The Arches**

1900 – 2300

Transportation will not be provided

Ticket required for admittance. See page 19 for full details.
ISAC Scholars 2011-2015

Alfonso Blanco-Fernández
Scientific Director Conway Core Facilities
Director of Flow Cytometry
UCD - Conway Institute of Biomolecular & Biomedical Research
University College Dublin
Dublin, Ireland

Education
Honors Degree in Biology in 1995 from the University of Santiago de Compostela.
M.Sc. Biology from the Universidade de Vigo (Excellent) in 1997.
Ph.D. Biology from the Universidade de Vigo (Excellent cum Laude) in 2003.

Scientific Interest and Avenues for Collaboration
Research, Clinical, Biotechnology/Industry

Participation/Support of ISAC
ISAC Councillor (2012-2016)
CYTO U E-Learning Delivery Task Force member

Donat Alpar
Postdoctoral Fellow at The Institute of Cancer Research, London

Education
MSc in Biology 2003
MSc in Chemistry 2006
PhD in Medical Science 2009

Scientific Interest and Avenues for Collaboration
Cancer genetics, Hematological malignancies, GI cancers

Participation/Support of ISAC
ISAC 2008 and CYTO 2010 participant
CYTO 2011 poster award judge
CYTO 2012-14 Programme Committee member

Brian T. Grimbren
Assistant Professor of International Health, Infectious Disease and Immunology
Center for Global Health and Diseases
Case Western Reserve University School of Medicine
Cleveland, OH, USA

Education
Bachelor of Arts (BA) in Biology and History, University of Rochester, Rochester, NY, USA
Doctor of Philosophy (PhD) in Biology, Wake Forest University, Winston-Salem, NC, USA
Geographic Medicine Fellow, Case Western Reserve University, Cleveland, OH, USA
Hematology and Oncology Fellow, University Hospitals of Cleveland, Cleveland, OH, USA

Scientific Interest and Avenues for Collaboration
His professional interests include: cytometric analysis of malaria parasite species particularly; P. vivax, P. knowlesi, and P. falciparum. He performs high throughput drug screening for novel antimalarial compounds and investigates the induction of parasite apoptosis—like mechanisms for new treatments. Also by focusing on parasite:ligand interactions he searches for new vaccine candidates for P. vivax. He is currently involved in research projects in Thailand, Cambodia, Papua New Guinea, Peru, Kenya, and Madagascar.

Participation/Support of ISAC
Attend ISAC meetings
Cytometry Part A reviewer

Website
case.edu/orgs/cghd/faculty-research/brian-grimbren.htm

Kewal Asosingh
Staff Scientist and Assistant Professor
Cleveland Clinic Lerner College of Medicine of the Case Western Reserve University (CCLCM)

Education

Scientific Interest and Avenues for Collaboration
Our research is focused on proangiogenic hematopoietic progenitor cells in lung vascular disease. We have established mouse models and close collaboration with clinicians facilitates rapid transition of the basic research findings to clinical studies. Flow cytometry interest include: Enumeration and characterization of circulating and bone marrow proangiogenic progenitor cells, mitochondrial organelle function and micro particles.

Participation/Support of ISAC
Member of abstract review committee
Program Committee CYTO meetings
Co-Chair and Member of ISAC eLearning Delivery Task Force

Tomas Kalina
Scientist, Associated Professor
Charles University in Prague
2nd Medical School, Prague, Czech Republic

Education
Medical Doctor, PhD in Immunology

Scientific Interest and Avenues for Collaboration
Polychromatic flow on Primary Immunodeficiency (EuroFlow-PID)
Polychromatic flow on leukemia and minimal residual disease
CMV specific assay using simultaneous IFNg, IL2, CD40L and CD107a detection
Single cell sorting of rare cells
Standard Operating Protocols for 8 color flow in multicenter study (EuroFlow)
Automated flow data analysis
Affinity proteomics by flow (SEC-MAP)

Participation/Support of ISAC
Finance Committee Member
CYTO Congress Program Committee Member
Live Education Delivery Task Force Member
Regular CYTO meeting attendee

Website:http://cllp.l2.cuni.cz/
ISAC Scholars 2012-2016

Pratik K. Chattopadhyay
Staff Scientist, ImmunoTechnology Section, Vaccine Research Center, NIH, Bethesda, Maryland, USA

Education
Bachelors of Arts, Biology: University of Virginia
Ph.D., Molecular Microbiology and Immunology: Johns Hopkins University

University School of Public Health Postdoctoral Training: National Institutes of Health

Scientific interest and Avenues for Collaboration
My work aims to identify the characteristics of T- and B-cells that predict successful immune responses. For this work, I develop and optimize multi-parametric proteomic and transcriptomic technologies, collaborating closely with engineers and bioinformaticians. I then apply these new technologies to better understand basic T-cell immunology and the immune response to vaccines or natural disease, using large cohorts of individuals with well-defined clinical outcomes (particularly in HIV and EBV-associated diseases). I have extensive experience collaborating with groups 1) developing new hardware platforms or reagents, 2) searching for settings to test their data analysis tools, and 3) clinicians or epidemiologists interested in comprehensive characterization of immune responses. I am always eager to work with new collaborators in these areas.

Participation/Support of ISAC
Within ISAC, I have been involved in a number of activities, including developing tutorials and day-long pre-congress courses on mass cytometry, data analysis, and polychromatic cytometry. I am currently a member of the Education Committee, and I lead the Electronic Learning (e-learning) Task Force. As part of this group, I played a key role in the early development of our on-line education portal (CYTO U), and I currently manage the electronic delivery/production of new CYTO U courses. As an ISAC Scholar, my colleague Nima Aghaeepour and I applied for (and won) funding for the first Scholar Collaboration project, and have developed a proposal for more high-profile presentations of Scholar work at the CYTO meeting.

Enrico Lugli
Junior Investigator, Unit of Clinical and Experimental Immunology Humanitas Clinical and Research Institute, Rozzano, Italy

Education
Ph.D. University of Modena and Reggio Emilia, Italy (supervisor: Prof. Andrea Cossarizza)

Scientific interest and Avenues for Collaboration
Mechanisms of memory T cell differentiation and maintenance in humans
Cytokine-based cancer immunotherapy
Immune reconstitution in acquired immunodeficiencies (bone marrow transplantation, HIV infection)

Participation/Support of ISAC
Organization committee of CYTO 2014

ISAC-sponsored flow cytometry workshop @ ESID
(Prague, Nov. 1-2, 2014)
Cytometry Part A Editorial Board

Gergely Toldi
Resident in Pediatrics, First Dept of Obstetrics and Gynecology Semmelweis University, Budapest

Education

Scientific Interest and Avenues for Collaboration
T lymphocyte activation, kinetic flow cytometry, autoimmunity, immunology of pregnancy and neonates

Participation/Support of ISAC
CYTO 2013, 2014 & 2015: Program Committee Member, Session Chair
Outstanding Poster Award, CYTO 2012 Reviewer for Cytometry A

Michael Zordan
Staff Engineer, Sony Biotechnology, Inc.
San Jose, CA

Education
Duke University B.S.E. Biomedical Engineering
Purdue University. Ph.D Biomedical Engineering

Scientific interest and Avenues for Collaboration
Within my field of work, I am interested in collaborating with anyone interested in developing their technology or with researchers to design technology to perform novel measurements they can not currently perform.

Participation/Support of ISAC
Member of ISAC Data Standards Task Force Co-Chaired Parallel Sessions at CYTO 2013 and CYTO 2014
Invited Speaker, GLIFICA 2013 Invited Speaker, Metflow 2014

Qianjun Zhang
Application Scientist FlowJo, USA
General Manager, Emerald Biotech, located in Hangzhou, China

Education
M.S., University of Colorado Health Sciences Center
Scientific Interest and Avenues for Collaboration
I am enthusiastic about the new technology within flow cytometry and also dedicated to enhancing the training and knowledge of flow techniques within resource limited areas such as South East Asia and China. I have helped organize a few international flow cytometry workshops and would like to continue contributing and help the educational committee with my resource and expertise.

Participation/Support of ISAC
ISAC Live Education Delivery Task Force member (2013 – present)
Nima Aghaeepour
Postdoctoral Fellow, Stanford University

**Education**
Ph.D., Bioinformatics, University of British Columbia, 2008-2012
B.Sc., Computer Science, University of Tehran, 2003-2008

**Scientific Interest and Avenues for Collaboration**
Nima Aghaeepour is currently a postdoctoral fellow in Garry Nolan’s laboratory at Stanford University, a Fellow of the Canadian Institute of Health Research, and an Ann Schreiber Mentored Investigator of the Ovarian Cancer Research Fund. His research is focused on computational analysis of single cell proteomics and genomics data to enable a systems level understanding of complex cancers as well as hematologic and immune system malignancies.

**Participation/Support of ISAC**
He currently contributes to ISAC as a member of the scientific communications committee and the CYTO conference’s program committee. He has also chaired several CYTO parallel sessions, has co-organized the FlowCAP parallel sessions, workshops, and tutorials during CYTO 2014, and was an invited faculty in ISAC’s pre-congress advanced data analysis course.

**Website**
http://linkedIn.com/in/naghaeep

Anis Larbi
Principal Investigator, Immunity and Aging Program
Technologist, Head of Flow Cytometry Platform
Singapore Immunology Network (SIgN)
Biopolis, A*STAR, Singapore

**Education**
2003 - PhD Immunology: Research Center on Aging, Laboratory of immunology and signaling, Sherbrooke, Faculty of Medicine, University of Sherbrooke, Canada
2000 - BSc Biology, Claude Bernard University, IUT-A, Lyon 1, France

**Scientific Interest and Avenues for Collaboration**
I am interested in understanding the changes that occur in human aging and how this happens. We are performing various immune phenotyping panels and associated functionality of the cells. We are open to collaboration in immunology, immunosenescence, immunophenotyping, and cohort studies.

**Website**

Sung Hwan Cho
CTO, NanoCellect Biomedical Inc., located in San Diego, CA, USA

**Education**
Ph.D., University of California San Diego (UCSD), USA
Research Engineer at West Wireless Health Institute, San Diego, CA, USA

**Scientific Interest and Avenues for Collaboration**
As an inventor of the core technology of the company (NanoCellect), I am interested in integrated microfluidic systems where fluidics, optics, electronics and acoustics all work together synergistically to develop next generation flow cytometer and medical devices.

**Participation/Support of ISAC**
ISAC Membership Committee member (2013 – present)
Organization committee of CYTO 2013, 2014, 2015
Attended CYTO 2012, 2013, 2014

**Website**
http://www.linkedin.com/pub/sung-hwan-cho/6/140/108

Katarzyna Piwowka
Associate Professor, Nencki Institute of Experimental Biology
Head of the Laboratory of Cytometry
Head of the Department of Biochemistry

**Education**
2013 Habilitation in Biological Sciences, Nencki Institute, Warsaw, Poland
2001 PhD with honours in Biochemistry, Nencki Institute, Warsaw, Poland
1994 Msc in microbiology, Biology Department, Warsaw University, Poland

**Post-graduate training:**
2003-2004 Cell Development and Disease Laboratory, BioSciences Institute, University College Cork, Ireland

**Scientific Interest and Avenues for Collaboration**
My scientific interest concerns the investigation of prosurvival mechanisms promoting leukemia development and resistance to therapy as well as looking for novel potent therapeutic targets to treat leukemia. Especially, my group investigates signaling pathways regulating cellular stress response, chromosomal instability and aneuploidy as well as the exosomes-mediated cross-talk of leukemia cells with the microenvironment. We use flow cytometry mostly to study apoptosis/viability, cell cycle and proliferation, redox state, signal transduction, intracellular and membrane proteins, cell tracking, RNA detection and many others. We are looking for collaboration in the field of cancer biology, signal transduction, microparticles detection and analysis.

**Participation/Support of ISAC**
Member of the Program Committee - CYTO 2014, CYTO 2015
Participation in CYTO 2014 - Organization of the ISAC Scholars Session and Co-Chair of the Parallel Session: Cell Proliferation and Death
2007 ISAC membership for prospective young researchers
Website http://cytometry.nencki.gov.pl/

Bruno Diaz Paredes
Researcher I
Hospital São Rafael – Center of Biotechnology and Cell Therapy (CBTC)
Salvador City, Bahia - Brazil
Education
Graduate of Biology Sciences – Biomedicine Module in Federal University of Rio de Janeiro (UFRJ)
Master’s degree in Physiology and Doctor’s degree in Sciences at Carlos Chagas Filho Biophysics Institute (UFRJ)
Research Technologist in Flow Cytometry sector of National Center of Bioimage (CENABIO)
Assistant Professor in Department of Gastroenterology and Hepatology of Yamaguchi University
Scientific Interest and Avenues for Collaboration

ISAC Scholars 2013-2017

Er Liu
Senior Scientist, Roche Tissue Diagnostics, Tucson, Arizona
Education
Wuhan University of Technology
B.S.2001 CHEMICAL ENGINEERING
Wuhan University of Technology
M.S.2004 BIOMEDICAL

ENGINEERING
Rutgers University Ph.D 2011 BIOMEDICAL ENGINEERING
Scientific Interest and Avenues for Collaboration
Interested in bioanalytical instrumentation development (e.g. portable fluorescence imager, next generation flow/image cytometer, nano-particle analyzer and sequencing instrumentation). Developing multiplex-able screening/single cell analysis platform for cancer profiling and drug screening.
Participation/Support of ISAC
2013 CYTO 2013 conference President’s Award of Excellence.
2013 Program committee, CYTO 2013: ISAC’s XXVIII International Congress.
2014 Program committee, CYTO 2014: ISAC’s XXIX International Congress.
2015 Program committee, CYTO 2015: ISAC’s 30th International Congress.

Frank Schildberg
Investigator - Harvard Medical School, Boston, MA, USA
Education
Ph.D, University of Bonn, Germany
Research Scientist, University of Bonn, Germany
Research Scientist, Dana-Farber Cancer Institute, Boston, MA, USA
Scientific Interest and Avenues for Collaboration
Stem cells (adult, embryonic, iPS) for liver, neural and cardiac diseases; Immunology of stem cells; Innovation on Cell-based therapies; Cell sorting.
Participation/Support of ISAC
Attended 4 CYTO meetings (2011-2014)
Participation as poster’s referee on CYTO meetings
Co-ordinator of 1st ISAC Scholars and SRL Emerging Leaders Business Meeting
Website http://lattes.cnpq.br/0347294341083336

Joseph D. Tario
Post Doc, Roswell Park Cancer Institute
Department of Flow and Image Cytometry
Education
PhD (Pathology)
BS (Biology)
Scientific Interest and Avenues for Collaboration
A liver immunologist by training, I work on different cellular and molecular aspects of how our immune system is regulated and how this knowledge could be translated into novel therapies for infectious diseases, autoimmune diseases, cancer and fibrosis. A major focus of my research is related to stromal cell biology, myeloid derived suppressor cells and co-inhibitory molecules and how these entities shape our immune responses. My research involves advanced genetic in vivo mouse models, functional assays with human tissue samples and cutting-edge cytometric analysis.
Participation/Support of ISAC
ISAC Membership Services Committee member (2014 – present)
Program committee member of CYTO since 2014
CYTO attendee since 2012
Website https://www.linkedin.com/in/frankschildberg
https://www.researchgate.net/profile/Frank_Schildberg

Joseph D. Tario
Post Doc, Roswell Park Cancer Institute
Department of Flow and Image Cytometry
Education
PhD (Pathology)
BS (Biology)
Scientific Interest and Avenues for Collaboration
I study the biology of immunotherapeutic dendritic cell vaccines in the treatment of cancer. I am also interested in the use of flow cytometry as a clinical tool for the diagnosis of hematologic malignancy.
Participation / Support of ISAC
Member of E-Learning Delivery Task Force, CYTO U
Michael Halter
Scientist, National Institute of Standards and Technology
Education
Ph.D. in Bioengineering from the University of Washington at Seattle in 2004
B.S. in Materials Science and Engineering from Purdue University at West Lafayette in 1998
Scientific Interest and Avenues for Collaboration
- Live cell imaging of stem cell populations
- Engineering fluorescent protein gene reporter stem cell lines
- Modeling gene expression dynamics in pluripotent stem cells
- Quality control for quantitative optical microscopy
Participation/Support of ISAC
Image Cytometry Content Task Force
CYTO U Course on Quality Control for Optical Microscopy
Attendance at CYTO conferences since 2008
Website
http://www.nist.gov/mml/bbd/cell_systems/michael_halter.cfm

Yiqing Lu
Macquarie University Research Fellow
Advanced Cytometry Laboratories
ARC Centre of Excellence for Nanoscale BioPhotonics (CNBP)
Macquarie University, Australia
Education
I completed my B.E. degree in Electronic Engineering from Tsinghua University, and PhD degree in the cross-disciplinary area of biophotonics with a joint program between Tsinghua University and Macquarie University.
Scientific Interest and Avenues for Collaboration
My research is focused on the development of analytical methods and instrumentation for biomedical and photonic applications. I am particularly interested in high-throughput techniques for rare-event detection and multiplexed analysis. Currently, I am investigating new nanophotonic approaches toward early-stage diagnosis of cancers and infectious diseases.
Participation/Support of ISAC
Member of the E-Learning Delivery Task Force
ISAC membership since 2011
Attendance at CYTO 2011, CYTO 2012 (received Outstanding Poster Award) and CYTO 2014 (finalist of President’s Award for Excellence)
Website
http://web.science.mq.edu.au/directory/listing/person.htm?id=ylu

ISAC Scholars 2014-2018

Jessica P. Houston
Assistant Professor
Department of Chemical Engineering
New Mexico State University, Las Cruces, NM
Education
M.S. Chemical Engineering Texas A&M University, 2002
Ph.D. in Chemical Engineering, Texas A&M University, 2005
Director’s Postdoctoral Fellow, Los Alamos National Laboratory, 2006
Scientific Interest and Avenues for Collaboration
Flow cytometry instrumentation development; fluorescence lifetime measurements; fluorescence decay kinetics; phase-sensitive flow cytometry
Participation / Support of ISAC
ISAC Councilor Term 2014-2018
Program Committee CYTO 2015
Website
http://dept-wp.nmsu.edu/flowcytometry/

Xu Huang
Lecturer/Principal Investigator of Haematopoietic Stem Cell Immunology/Endothelial Cell Systems Medicine Group
Paul O’Gorman Leukaemia Research Centre
Institute of Cancer Sciences, MVLS University of Glasgow
Glasgow, Scotland
U.K.
Education
2004 Ph.D., University of Peking, Beijing, P.R.China
2004-2008 Postdoctoral Researcher at MRC-Protein Phosphorylation Unit with Prof. Dario Alessi, Dundee, U.K.
2008-2014 Senior Research Associate at CRUK Manchester Institute with Dr. Tim Somervaille, Manchester, U.K.
Scientific Interest and Avenues for Collaboration
My lab has recently identified and validated few potential candidates as therapeutic treatment targets in Acute Myeloid Leukaemia (AML). (Huang et al. Leukaemia 2014, Harris and Huang et al. Cancer Cell 2013). The future research will focus on epigenetic regulation in leukaemia cancer stem cells, and continue to identify novel pathways and targets in leukaemia by using combinations of different systems biology.
approaches and by exploring novel polyomics methods in the study of cancer stem cells.

**Participation/Support of ISAC**
Attended CYTO 2014
Presented short talk at scholar evening session at CYTO 2014

**Website**
http://www.gla.ac.uk/researchinstitutes/cancersciences/research/units/paulogormanleukaemiaresearchcentre/xhuang

**Hervé Luche**
R&D Manager at Center for Immunophenomics (CIPHE), FRANCE

**Education**
Ph.D in Immunology, Medical School of Ulm, GERMANY
Post-doctoral fellow on T cell and dendritic cell development in the laboratory of B&M MALISSEN, FRANCE
Scientist at CIPHE, US-012, INSERM

**Scientific Interest and Avenues for Collaboration**
I am interested in trying to understand how immune cells integrate signals and communicate with their environment to actually change their transcriptional programs, fate or acquire a new function. I use a combination of mouse genetics with high content multi-parameter flow and mass cytometry as well as transcriptomic analysis. I am looking for collaborations in the field of meta-analysis and data integration of multiple datasets.

**Participation/Support of ISAC**
Attended CYTO 2012, 2014
Volunteered for French translation of CYTO-U tutorial material

**Website**
https://www.linkedin.com/profile/view?id=30427463&trk=nav_responsive_tab_profile

**Yolanda Mahnke**
Education
BSc in Biology from Imperial College of Science, Technology and Medicine, University of London, London, UK
PhD in Immunology from The Open University, London, UK; work performed with Prof. V Schirrmacher in the Dept. of Cellular Tumour Immunology, German Cancer Research Center (DKFZ), Heidelberg, Germany
PostDoc with Prof. V Schirrmacher, Dept. of Cellular Tumour Immunology, German Cancer Research Center (DKFZ), Heidelberg, Germany
PostDoc with Prof. P Romero, Div. of Clinical Onco-Immunology, Ludwig Institute for Cancer Research (LICR), Lausanne Branch, Lausanne, Switzerland
PostDoc with M Roederer, ImmunoTechnology Section, Vaccine Research Centre (VRC), National Institutes of Allergies and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD, USA

**Scientific Interest and Avenues for Collaboration**
I have a long standing interest in multi-parameter flow cytometry, using this tool to study the evolution of T-cell responses in the context of tumours, infections, vaccinations, or cellular therapies. Other immune or tumour cell types are also investigated in a project-dependent manner. I assist and train others in building multi-colour reagent panels, as well as in data analysis.

**Participation/Support of ISAC**
CYTO meeting attendance 2010, 2014
Developed a novel publication platform in Cytometry A for the publication of optimized multicolor immunofluorescence panels (OMIP), together with Mario Roederer and Pratip Chattopadhyay
Taught at pre-CYTO Workshop 2010
Lectured at CYTO 2010
Judged posters at CYTO 2014
Co-chairing a parallel session at CYTO 2015

**Carolina Wählby**
Professor in Quantitative Microscopy, Centre for Image Analysis
Dept. of Information Technology, and Science for Life Laboratory
Uppsala University, Uppsala, Sweden
Principal Investigator, Imaging Platform of the Broad Institute of Harvard and MIT
Cambridge, MA, USA.

**Education**
PostDoc at the Dept. of Genetics and Pathology, Uppsala University
Associate Professor in Digital Image Processing 2009.

**Scientific Interest and Avenues for Collaboration**
My research is focused on development of algorithms for image analysis of microscopy data with applications in biomedical research. This includes contributing to Broad’s free and open-source CellProfiler project, and development of advanced methods to quantify and mine information in microscopy images, primarily from high-throughput experiments and tissue samples. I’m interested in collaborations in the exciting intersection between computer science and biomedicine as microscopy is becoming a quantitative measurement tool, and not only a means of visual observation.

**Participation/Support of ISAC**
I’m interested in having a more active part in the ISAC community by attending meetings and networking activities.

**Website**
http://www.cb.uu.se/~carolina/
ISAC Scholars 2015-2019

Greg Finak
Senior Staff Scientist
Vaccine and Infectious Disease Division
Fred Hutchinson Cancer Research Center
Seattle, WA

Education
I have an MSc and PhD from the Biochemistry department at McGill University. My PhD was in Bioinformatics.

I did my post-doctoral work at the IRCM (Institut de Recherches Cliniques de Montreal) and at the Fred Hutchinson Cancer Research Center, where I focused on computational flow cytometry problems including automated gating, quality control, and infrastructure for data sharing and reproducibility.

Scientific Interest and Avenues for Collaboration
I am interested in developing computational tools for the identification and characterization of antigen-specific T-cells by integrating novel high throughput single-cell technologies (e.g. single-cell RNAseq, flow cytometry, Fluidigm Biomark, CyTOF) with applications to immune correlates discovery in vaccine trials. I am always open to collaborations with researchers who have novel data from designed experiments that pose new and interesting analysis problems.

Website

Pia Kvistborg
Head of Experimental Immunomonitoring
Senior Post Doc at the Netherlands Cancer Institute

Education
PhD in Tumor immunology from University of Copenhagen

Scientific Interest and Avenues for Collaboration
Immunomonitoring of cancer patient’s T cells, dissecting what clinically relevant T cells look like in human cancer

Elisa Nemes
Research Scientist
South African Tuberculosis Vaccine Initiative
Cape Town, South Africa

Education
MSc in Medical Biotechnology
PhD (topic: Immunological effects of antiretroviral therapy interruption in HIV infection)

Scientific Interest and Avenues for Collaboration
Clinical trials of novel tuberculosis vaccines
Prospective immunological biomarkers of risk of tuberculosis disease and immune reconstitution inflammatory syndrome
Paediatric immunology
Development and standardization of novel assays to measure immune cell function and phenotype

Participation / Support of ISAC
Attendance at CYTO 2014
Website
http://www.satvi.uct.ac.za/

Raluca Aura Niesner
Group leader, Biophysical Analytics and Leader of JIMI – a network for intravital microscopy
German Arthritis Research Center
Berlin (DRFZ, Berlin), Germany

Education
2009-2011 “Rahel-Hirsch” habilitation fellowship of the Charité – University Hospital, Berlin
Jan. 2005 PhD defense (magna cum laude)
2001 – 2004 PhD thesis at TU, Braunschweig

“New advancements in two-photon fluorescence microscopy – theoretical approaches and bioscientific applications”

Supervisor: Prof. Dr. K.-H. Gericke
May 2001 Diploma at TU Braunschweig
“Single molecule detection with a two-photon laser-scanning microscope”
1999 – 2001 Chemistry study at TU Braunschweig
1996 – 1998 Chemistry study at University Bucharest / Romania

Scientific Interests and Avenue for Collaboration
The development of new techniques which better meet the general requirement in biosciences and biomedicine to monitor vital processes at high spatial and temporal resolution, on a molecular basis, in their genuine environment is of central relevance for biophysics in general, and for microscopy in particular. In this frame, we focus on the improvement and extension of multi-photon laser-scanning microscopy (MPLSM) with application to intravital microscopy (imaging in living anesthetized rodents) to better understand immunological processes in different disease contexts. Thereby, we focus on the improvement of optical performance, e.g. spatial resolution, imaging depth, photobleaching/phototoxicity, imaging time window, and of molecular selectivity to monitor cell and tissue functions by time-resolved fluorescence techniques.

Participation/Support of ISAC
May 2011 “Enhancing the optical performance in dynamic intravital two-photon microscopy”
“Frontiers” talk at CYTO 2011, Baltimore, MD – invited talk
June 2012 “NADPH oxidase detection by fluorescence lifetime imaging in chronic neuroinflammation” poster, CYTO 2012, Leipzig, Germany
Chair of the “Frontiers session” CYTO 2012
Reviewer for abstracts of oral and poster presentations
May 2013 “Intravitral marker-free NAD(P)H FLIM – in vivo selective enzyme detection”
Talk at CYTO 2013, San Diego, CA
May 2014 “Intravitral multi-photon microscopy: an update”
“State-of-the-art” talk at CYTO 2014, Fort Lauderdale, FL – invited talk
“In vivo two-photon microendoscopy in murine bone marrow” – “CYTO Innovation” at CYTO 2014, Fort Lauderdale, FL
Reviewer of poster presentations – student award CYTO 2014
2015 Reviewer for abstracts of oral and poster presentations for CYTO 2015
Involved in the organizing committee at CYTO 2011 and CYTO 2014
Josef Spidlen  
Staff Scientist  
BC Cancer Agency  
Vancouver, BC, Canada  

**Education**  
Postdoctorate Fellow, 2005 – 2008, BC Cancer Agency, BC, Canada (Flow cytometry data standards and analysis tools)  
Ph.D. in Biomedical Informatics, 2005, Charles University in Prague, First Faculty of Medicine, Czech Republic  
M.Sc. in Computer Science, 2002, Charles University in Prague, Faculty of Mathematics and Physics, Czech Republic  

**Scientific Interest and Avenues for Collaboration**  
My scientific interest and research are mainly focused on the development and application of software tools for automated high throughput flow cytometry data analysis. I am the author and maintainer of several R packages, the main author of most ISAC data standards and also the chief developer of FlowRepository.org. I am always excited to engage in collaborations allowing me to apply my computational background to address data analysis challenges or to gain novel biological insights.  

**Participation/Support of ISAC**  
ISAC Data Standards Task Force: first author of most ISAC data standards  
FlowRepository Steering Committee: Chief Developer of FlowRepository  
Website  
http://spidlen.ca
Andrew Filby  
Flow Cytometry Core Facility Manager, Faculty of Medical Sciences  
Newcastle University Centre for Life, UK  

Education  
B.Sc. Hons. Biochemistry  
(University of Huddersfield, UK)  
PhD Molecular and Cellular Immunology (National Institute for Medical Research, UK; affiliated with University College London).  

Scientific Interest and Avenues for Collaboration  
I specialise in imaging (flow) cytometry, conventional flow cytometry (sorting and analysis) as well as molecular and cellular immunology. I am also very involved in the educational and training initiatives within the UK/European and international cytometry communities. I am also an active voice for the shared resource laboratory best practices.  

Participation/Support of ISAC  
I serve on the image cytometry content and e-learning delivery task forces. I have attended four out of the last five Cyto conferences and organised a total of five workshop sessions at these meetings as well as submitting scientific abstracts for talks and posters.  

Websites  
http://scholar.google.co.uk/citations?user=TLlBYYAAAAJ&hl=en  
http://www.researchgate.net/profile/Andrew_Filby  
http://www.london-research-institute.org.uk/technologies/facs

Michael Gregory  
Laboratory Manager, NYU Langone Medical Center, New York, NY  

Education  
Masters, Biology (in progress), New York University  
B.A., Biology (2005), New York University  

Scientific Interest and Avenues for Collaboration  
T-Cell Immunology, Centrifugal Counter-Flow Elutriation  

Participation/Support of ISAC  
I have recently been a member of program committee for CYTO since 2010  
Attended CYTO regularly since 2006  

Website  
http://ocs.med.nyu.edu/cytometry-and-cell-sorting-core

Robert (Rob) Salomon  
Flow Cytometry Manager/ Senior Flow Cytometry Scientist, Garvan Institute of Medical Research, Sydney, NSW, Australia  

Education  
BSc (Anatomy and Physiology)  
Honors 1st class (Animal models of Prostate Cancer)  
MSc (Molecular Profiling of Human Prostate Cancer)  
Dip Eng (electrical)  

Scientific Interest and Avenues for Collaboration  
My interests lie in the accurate application of technology to develop deeper understanding of science, particularly the biological sciences. I have a particular interest in the application of single cell genomics to understanding the underlying process involved in real disease states. I believe that strong, sustainable shared resource laboratories have the capacity to enhance scientific understanding and to participate in the discovery of cures for many diseases.

Participation/Support of ISAC  
Co-chair of Membership Services Committee  
Member of SRL task force  
Secretary of RMS Cytometry Committee and Secretary of FlowcytometryUK (ISAC affiliated societies)  
Lecturer in ISAC Pre-congress courses 2010-11  
Attended CYTO regularly since 2006  
Member of program committee for CYTO since 2010  

Website  
http://www.babraham.ac.uk/science-services/flow-cytometry/rachael-walker

Rachael Walker  
Head of Flow Cytometry  
Babraham Institute, Cambridge, UK  

Education  
PhD in Tissue Engineering, Department of Clinical Engineering, University of Liverpool – 2005  
BMedSc (Honours) – Biomedical Materials Science, University of Birmingham – 2001  

Scientific Interest and Avenues for Collaboration  
I head a very busy and fast growing flow cytometry core facility, working closely with both academic researchers and local biotech companies. I have recently been awarded a government grant for writing and hosting a Flow Cytometry training course for Industry, the first course to be held, Oct 2014.  

Participation/Support of ISAC  
Co-chair of Membership Services Committee  
Member of SRL task force  
Secretary of RMS Cytometry Committee and Secretary of FlowcytometryUK (ISAC affiliated societies)  
Lecturer in ISAC Pre-congress courses 2010-11  
Attended CYTO regularly since 2006  
Member of program committee for CYTO since 2010  

Website  
http://www.babraham.ac.uk/science-services/flow-cytometry/rachael-walker
Jessica B. Back
Associate Director, Microscopy, Imaging, and Cytometry Resources (MICR) Core
Wayne State University and Karmanos Cancer Institute
Detroit, MI
Education
B.S., Biochemistry – Ohio Northern University, Ada, OH
Ph.D., Biochemistry – Wayne State University, Detroit, MI
Post-Doctoral Fellow, Tumor Immunology – Karmanos Cancer Institute, Wayne State University, Detroit, MI
Scientific Interest and Avenues for Collaboration
My goal is to ensure the success of the researchers using MICR by providing expertise in flow cytometry techniques, individualized assay design consultation, and data analysis support. I also strive to push my users out of their comfort zones, expand their use and understanding of flow cytometry, and make their data more functional and reproducible. My personal research interests are in carcinogenesis and tumor immunology.

Participation/Support of ISAC
Meeting Attendance: CYTO 2013, GLIFICA 2013, CYTO 2014
Website
http://micr.med.wayne.edu

Anna Belkina
Research Specialist
Flow Cytometry Core Facility
Boston University School of Medicine
Education
MD (Russian State Medical University) 1997-2003
Ph.D. (Boston University School of Medicine) 2007-2012
Postdoctoral Fellow, Department of Microbiology 2012-now
Boston University School of Medicine
Scientific Interest and Avenues for Collaboration
As a Research Specialist and acting manager of a larger institution Core Facility, I am very focused on engaging our research community in practices matching the highest standards in the field. My current research efforts include a number of collaborative projects focusing on characterizing T cell immunity in chronic inflammatory diseases (Type 1 and Type 2 diabetes, periodontal disease, scleroderma) and cancer.

Participation/Support of ISAC
ISAC E-Learning Delivery Task Force/CytoU
Attended CYTO 2014
Website
https://www.researchgate.net/profile/Anna_Belkina/

Gelo Victoriano B. Dela Cruz
Flow Cytometry Core Facility Manager, The Danish Stem Cell Center
Copenhagen, Denmark
Education
BSc Molecular Biology and Biotechnology (University of the Philippines)
Masters credits (NYU)
Scientific Interest and Avenues for Collaboration
I am interested in cell sorting technologies and how cell sorting affects the survival and viability of different stem cell lines, progenitors and differentiating cells.

Participation/Support of ISAC
Certification Advisory Committee member
Website
http://danstem.ku.dk/research1/core_facilities/flow-cytometry-core-facility/

Nina Lane
Senior Scientific Officer in Flow Cytometry Core Facility, Cambridge Institute, Cancer Research UK,
Education
PhD Immunology (2012)
Nottingham University
Msc Immunology & Allergy (2006)
Nottingham University
Bsc Biomedical Science (2005) Anglia Ruskin University
Scientific Interest and Avenues for Collaboration
T cell immunology, stem cells and single cell technologies.

Participation / Support of ISAC
First time attendee at CYTO
Attend flowcytometryUK meetings
Secretary to Cambridge Cytometry Club

Michael Thomson
Flow Cytometry Coordinator, St Vincent’s Institute of Medical Research
Melbourne, Australia
Education
M.Biomed – Monash University
B.AppSc (Biotechnology/Biochemistry) – Swinburne University
Scientific Interest and Avenues for Collaboration
My interests involve providing a high standard of education to enable researchers utilising the core facility’s Flow and Imaging Cytometry capabilities to expand their scope for scientific discovery. I have assisted groups with a research focus in type 1 and 2 diabetes immunology, cancer, virology, allergy and hematology; thus have an interest in developing and providing appropriate biosafety practices.

Participation/Support of ISAC
Member of organising committee for ACS (ISAC affiliated society) Melbourne Meeting
Website
https://www.svi.edu.au/research_themes/facs
Multimedia and Poster Sessions
Exhibit Hall 4

Author presentation and discussion times:

SATURDAY, JUNE 27
1600 – 2000 Authors must set up posters on assigned board.

SUNDAY, JUNE 28
800 – 1900 Poster Viewing
1730 – 1830 Poster Session 1: Authors of ODD numbered poster boards present.

MONDAY, JUNE 29
800 – 1900 Poster Viewing
1730 – 1830 Poster Session 2: Authors of EVEN numbered poster boards present.

TUESDAY, JUNE 30
800 – 1630 Poster viewing
1530 – 1630 Poster Session 3 (Consists of a Poster Highlights Tour)
1630 All poster material must be removed from the boards.

MULTIMEDIA PRESENTATIONS

AUTOMATED SAMPLE PREPARATION
800 - 1900
B1 132 A Small Device to Expedite and Automate Thawing of Viable Frozen Cells
P. Chattopadhyay, M. Beddall and M. Roederer. NIAID, NIH.

CELL-DERIVED MICROVESICLES
B2 133 Extracellular Vesicle Isolation by Flow Cytometric Sorting and Characterization by Analytical Ultra-Centrifugation and Dynamic Light Scatter
C. Ross, T. Ramin, A. Morales-Kastresana and J.C. Jones. Beckman Coulter Inc., Fort Collins and NCI, NIH.

FLOW CYTOMETRY INSTRUMENTATION
B3 134 High Volumetric Rate Flow Cytometry for Process Monitoring, Large Particle Applications, and Rare Event Analysis
S. Graves, T.A. Woods, A.P. Shreve and M.J. Cumbo. Eta Diagnostics Inc. and Univ. of New Mexico.

B4 135 Build Your Own Flow Cytometers: Teaching Tools for Flow Cytometry Education

HIGH THROUGHPUT INSTRUMENTATION
B5 136 Miniaturization of High Throughput Flow Assays for 384-Well Low Volume and 1536-Well Plates: Detection of Highly Multiplexed Analytes in 6 μL Assays with the iQue Screener and MultiCyt QBeads

IMMUNE MONITORING
B8 139 Advanced Analysis of Human T Cell Subsets by Flow Cytometry Using a 13 Color Tube Based on DuraClone Dry Reagent Technology
A. Boehmler, M. Kapinsky and N. Weit. Beckman Coulter GmbH, Krefeld, Germany and Beckman Coulter Immunotech SAS, Marseille.

NEW SOFTWARE DEVELOPMENT
B9 140 Chromocyte: An Online Resource for Assisting the Flow Cytometry Community
A. Pockley. Nottingham Trent Univ. and Chromocyte Ltd., Sheffield.
**Poster Presentations**

**Antigen-Specific Immune Responses**

B10 141 Development of a Mass Cytometry Platform to Investigate Immunological Correlates of TB Risk in an Endemic Setting  

B11 142 Flow Cytometry Methods Allow the Detection of Low Frequency Proliferative CD4 Memory T Cells in Human Primary Peripheral Blood Mononuclear Cells  

**Automated Microscopy**

B12 143 On the Inaccuracy of Image-Based Cell Counters  

**Automated Sample Preparation**

B13 144 Reagent Release from Hydrogel Coated Cell Counting Chambers for On-Chip Sample Preparation  
X. Zhang, J. van Dalum, D. Wasserberg, L.W.M.M. Terstappen and M. Beck. Univ. of Twente, Netherlands.

**Biomarkers**

B14 145 Ii p10 Neoepitope Assay: A Pharmacodynamic Biomarker Assay for Therapies Targeting MHC Class II-Peptide Complex Formation through Inhibition of Cathepsin S  

B15 146 Quantification of Proteins by Flow Cytometry: Quantification of Human Hepatic Transporter P-gp and OATP1B1 Using Flow Cytometry and Mass Spectrometry  

B16 147 High-Throughput miRNA Profiling on Standard Flow Cytometers  

B17 148 Quantitative Flow Cytometry Measurements in Antibody Bound per Cell Based on CD4 Reference  
L. Wang, H. Degheidey, F. Abbasi, H. Mostowski, G.E. Marti, S. Bauer, R.M. Hoffman and A. Gaigalas. NIST, Gaithersburg, MD, FDA, Silver Spring, MD and Consultant, Livermore, CA.

B18 149 Lessons Learned from a Four-Year Multi-site International Proficiency Testing Program for Luminescence Biomarker Profiling Assays  

B19 150 Monocyte HLA-DR by Flow Cytometry – Assay Development for a Multi-site Clinical Trial  

B20 151 Urinary Endothelial Cell Microparticles as Biomarker of Pulmonary Vascular Disease  

**Biopharmaceutical Applications**

B21 152 Assessment of Receptor Occupancy by Flow Cytometry: A Powerful Tool in Drug Discovery  
A. Lackey, N. Jones, C. Hauther and K. Pennline. Laboratory Corporation of America® Holdings, Brentwood, TN.

B22 153 In-Depth Characterization of Drug Effects Using Multiparametric Plate-Based Cytometry on Guava easyCyte™ Platforms  
J. Clor, K. Gillis and K. Tyagarajan. EMD Millipore, Hayward, CA.

**Cell Proliferation and Death**

B23 154 High-Precise Measuring of Morphological Changes in Mononuclear Cells during Early Stages of Apoptosis Using a Scanning Flow Cytometer  
B24 155 Image-Based Flow Cytometry Method to Assay the Capacity of PBMCs to Resist Apoptotic Stimuli

B25 156 Curcumin Induces a Crosstalk between Autophagy and Apoptosis: A Biochemical Approach Coupling xCELLigence and Flow Cytometry

B26 157 A Novel Image-Based Cytometry Analysis for Measuring Cell Migration in Wound Healing Assay
L. Chan, C. Swingler, O. Dery, S. Cribbes, S. Kessel, D. Kuksin and J. Qiu. Nexcelom Bioscience, Lawrence, MA.

B27 158 A High-Throughput Direct Adherent Cell Cycle Analysis Method for Cell Cycle and Apoptosis Using Celigo Imaging Cytometer
L. Chan, D. Kuksin, S. Kessel, O. Dery, C. Swingler, S. Cribbes and J. Qiu. Nexcelom Bioscience, Lawrence, MA.

B28 159 Development of a Flow Cytometric Assay to Detect Annexin-V Expression in Conjunction with Cleaved PARP, Cleaved Caspase-3, Phospho-histone H2A.X and DNA Content for Screening of Pathway Response in Hematological Malignancies
L. Flye-Blakemore, R. Wilson, T. Jordan, A. Franko and K. Pennline. Lab. Corp. of America, Brentwood, TN.

B29 160 An Annexin V Based Unitized, Room Temperature Stable Assay for Detection of Apoptosis in Small Volume Tube

CELL SORTING AND SELECTION

B30 161 Filtration System for the Detection of Circulating Tumor Cells

B31 162 Comparison of Cell Sorter Performance Using BD™ Accudrop Technology and Rmax on the BD Influx Platform
M. Maj, C. Cossetti and R. Schulte. Univ. of Cambridge.

B32 163 Platelet Isolation by Fluorescence-Activated Cell Sorting

B33 164 Proposal for a Sort-Performance Index

B34 165 New Technique for Sorting Rare Populations of Cells
N. Loof. Univ. of Texas Southwestern Med. Ctr..

B35 166 Practical and Technical Considerations in Sorting C. elegans Larvae on a BD FACS Aria II
L. Tauzin, V. Glutz and M. Garcia. EPFL, Lausanne.

B36 167 FASS and FAST-FIN, Two New FACS Based Methods for the Purification of Brain Tissue Organelles after Subcellular Fractionation

B37 168 Effect of Soluble ST2 on Activation of Type 2 Innate Lymphoid Cells in a Murine Model of Asthma

B38 169 Using Flow Cytometric Cell Sorting to Improve the Inducible Expression of the Rat Serotonin Transporter in a Mammalian Cell Line for Structural Studies of the Protein

B39 170 Enrichment of Live Cells Based on Intracellular Cancer Biomarker Detection Allows for Additional Downstream Functional Testing
D. Weldon, V. Koong, Y. Williams and H. Liu. EMD Millipore, Temecula, CA.

B40 171 Flow Cytometry Analysis and Sorting for In Vitro Selection Applications
V. Romashova, J. Attwater, F. Wachowius, M. Daly and P. Holliger. MRC Lab. of Molec. Biol. and Univ. of Cambridge.
B41 172 Evaluating the Effects of Cell Sorting on Gene Expression  
M. Cochran. Univ. of Rochester.

B42 173 Two Dimensional Acoustic Prefocusing Free Flow Acoustophoresis Enables High Performance Microchip-Based Separation of Leukocyte Subpopulations  

B43 174 Cell Separation by Molecular Computation on the Cell Surface  

B44 175 Evaluation of Novel Cell Sorter Aerosol Containment Methods  
S. Perfetto, H. Fletcher, R. Nguyen, D. Ambrozak, R. Mario and K. Holmes. NIAID, NIH and Univ. of Pennsylvania Cancer Ctr..

B45 176 Pre-sort Debris Removal by Counter-Flow Centrifugal Elutriation Reduces TIL Cell Sorting Times  
M. Gregory, N. Hanson, K. Kobylarz, K. Ryan and P.A. Lopez. NYU Med. Ctr..

B46 177 Enabling Single Cell Sorting of Protozoans on a Beckman Coulter MoFlo ASTRIOS: Application in the Study of Molecular Biology, Epidemiology and Taxonomy  

CELL-DERIVED MICROVESICLES

B47 178 Selection and Optimization of Exosome Labels for nanoFACS  

B48 179 Sorting and Characterization of Functional Viral Particles Using Cytometer with Digital Focusing System  

B49 180 A Rapid Image Cytometric Analysis Method for Phagocytosis Using Celigo Imaging Cytometer  
L. Chan, S. Cribbes, D. Kuksin and T. Smith. Nexcelom Bioscience, Lawrence, MA.


B51 182 Measurement of Microvesicles as a Tool for Pharmaceutical Bioprocess Monitoring  

CLINICAL TRIALS

B52 183 Development of a CLL-MRD Staining Protocol without the Use of Tandem Dyes, and Comparison with the ERIC 6-Color Protocol  
D. Van Bockstaele, M. Lenjou, A. Buys, M. Hansen, N. Jones and K. Pennline. Lab. Corp. of America® Holdings, Clin. Trials, Mechelen, Belgium, Los Angeles, CA and Brentwood, TN.

B53 184 Performance Monitoring of Standardized Flow Cytometry Instruments by Covance Central Laboratory Services  

B54 185 Immunophenotyping the Course of Immune Response following Influenza Vaccination in Aging  

B55 186 Performance Evaluation of the BD FACSCanto™ Flow Cytometer, 4-3-3 Configuration  
Y. Zeng, F. Oreizy, A. Chen, F. Mosqueda, V. Fraser, L. Byrne, A. Lin and K. Judge. BD Biosciences, San Jose.

B56 187 Ex Vivo Immunophenotyping of Adoptively Transferred T Cells Using the SP6800 Spectral Analyzer  
**COMPUTATION AND INFORMATICS**

B57 188 Computation of the Dependence of the Chronolog of Flow Cytometry Data
Univ. of Ghent and Univ. Hosp. Ghent, Belgium.

B58 189 Differential Population Identification in FlowSOM
S. Van Gassen, T. Dhaene and Y. Saeys. 

B59 190 Accelerating Cytometry Analysis via Cell Subset Automatic Labeling and Context Identification

**CYTOMETRY IN RESOURCE POOR SETTINGS**

B60 191 Single-Step On-Chip Differential White Blood Cell Count
Univ. of Twente, Netherlands.

**DIAGNOSTICS**

B61 192 Detection of Donor T and B Cells Specific C4d Fixing Alloantibodies Using Flow Cytometry: A Novel Diagnostic Approach
Medanta - The Midcity, Gurugram, India.

B62 193 NIST High Accuracy Particle Counting of Calibration Beads for ERF Value Assignments in Flow Cytometry
NIST, Gaithersburg, MD.

B63 194 The Increase of the Effective cdB3 Amount in Human Erythrocytes by Magnesium Sulfate Studied with Scanning Flow Cytometry
E. Chernyshova, A. Chernyshev, D. Strokotov and V. Maltsev. 

B64 195 Autoimmune Cellular Immune Response Correlates with Type 1 Diabetes Severity – A Pilot Study
Peking Univ., Shanghai Med. Col., Fudan Univ., China and Harvard Univ.

B65 196 Characterization of Main Physical and Physicochemical Parameters of Polymer Microparticles Using the Scanning Flow Cytometry
A. Polshchitsin, A. Krasulina, V. Kuzin, D. Strokotov and V. Maltsev. 

B66 197 Eyelis™ Software-Assisted Analysis of Stem/Progenitor Cell Types Detected by the SCALPEL™ Assay in a Blood Sample with Relapsed Chronic Myeloid Leukemia

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| **FACILITY MANAGEMENT** | | | |
| B80 | 211 | Year One of CyTOF in a Core Facility Setting: Considerations and Concerns | B. Carter. Stanford Univ. |
| B82 | 213 | Training Tool for Flow Analyser Users in a Cytometry Core Facility | J. Graham, B.L. Ng and C. Kirton. Wellcome Trust Sanger Inst., Cambridge, U.K. |

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| B88  | 219 | Automated Analysis and Sorting of Human iPS Cell Clusters by Large Particle Flow Cytometry  
R. Bongaarts, F. Smet, P. Wanek and M. Zemke. Union Biometrica Inc., Geel, Belgium and RWTH Aachen, Germany. |
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| B89  | 220 | Micro-imaging Cytometry by Focused Flying Laser Spot  
M. Yamamoto and J.P. Robinson. Miftek Corp. and Purdue Univ. |
| B90  | 221 | Near Ultraviolet Laser Diodes for Brilliant Ultraviolet Fluorochrome Excitation  
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| B91  | 222 | Early Adopter Report Regarding the Innovative Sony SP6800 Spectral Analyzer: Advantages, Disadvantages, and Data Comparison with a BD LSR-II  
| B92  | 223 | The Use of Imaging Flow Cytometry as a Support Tool for the Traditional Flow Lab  
S. Mordecai. Massachusetts Gen. Hosp., MD. |
| B93  | 224 | Optimizing Flow Cytometer Sensitivity by Upgrading Hardware Components  
K-E. Witte. UKT Univ. Children's Hosp., Tubingen. |
| B94  | 225 | Implementation of a System for Full Pulse Shape Data Extraction in Flow Cytometry  
| B95  | 226 | Effect of Isotope Transmission on Antigen Detection and Comparison of Different CyTOF Instruments for Improving Standardization  
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| B101 | 232 | Fluidics Modifications to BD Bioscience’s LSRII and Fortessa Flow Cytometers Enable Reduced Maintenance and Downtime  
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**B111 242** A Six Color Flow Cytometry Screening in the Diagnostics of MDS  

**B112 243** Omics’ Approach to Elucidate the Critical Epigenetic Regulatory Machinery Selective for Acute Myeloid Leukaemia Stem Cell Function  
X. Huang. MVLS, Univ. of Glasgow.

**B113 244** Real-Time Measurement of Platelet Shape Distribution with the Scanning Flow Cytometry  

**B114 245** Analysis and Identification of Alkaline Phosphatase-Positive Leukocytes Using Unlysed Whole Blood  

**B115 246** The Predictive Significance of CD20 Expression in Follicular Lymphoma  

**B116 247** Euroflow Quality Assurance Program: Proposal for Structure and Implementation  

**B117 248** Developmental Haemostasis: Difference in Platelet Function between Children and Adults  

**B118 249** B-CLL Response to Bacterial Toxin Listeriolysin O with Monoclonal Antibody Anti-CD20 (Rituximab) in Therapy  
B119  250  BCL-2 Overexpression as a Predictor of B-Cell Lymphomas in Fine Needle Aspiration Biopsy Samples
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B120  251  CD58 Downregulation in Pediatric-B-ALL at Day 15 of ALL IC-BFM 2009 Treatment Protocol and Its Implication on Minimal Residual Disease Detection

B121  252  Telomere Length Measurement in Human Hematopoietic Cells: Comparative Analysis of Q-PCR and Flow-Fish Techniques
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B122  253  BCR-ABL1 and CD66c Exhibit High Concordance in Minimal Residual Disease Detection of Adult B-Acute Lymphoblastic Leukemia

B123  254  Automatic Quantification of Sickled Human RBC by Image Cytometry Combined with Immunophenotyping Provides a Powerful Assay for the Study of Sickle Cell Disease
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B124  255  The Role of CD49d Expression in the Survival of Chronic Lymphocytic Leukemia Cells

B125  256  A Paroxysmal Nocturnal Haemoglobinuria Clone Presenting Type II Monocyte and Type II Granulocyte Clone

B126  257  Quantification of Hematopoietic Progenitors and Stem Cell Subpopulations in Fanconi Anemia Patients

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**B131**  262  A High Throughput Method for Generating Uniform 3D Coculture Tissue Models
J. de Lora, D. Kalb, J. Dorsey, E. Innis, J. Freyer and A. Shreve. Univ. of New Mexico.

**B132**  263  High Throughput Single Cell Gene Expression Analysis Using SH800Z Cell Sorter

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**B133**  264  High Throughput Flow Cytometry Screening 4 Plates and 1536 Wells at a Time
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**B142** 273 Immune-Regulatory Role of Garlic Protein on Entodotoxin Induced Sepsis and Its Associated Multiple Organ Failure 

**B143** 274 A Standardized Platform and Immune Monitoring Methods to Evaluate Immune-Mediated Oncology Biotherapeutics by Flow Cytometry
S. Fuhrmann, K. Mulgrew and C. Groves. MedImmune LLC, Gaithersburg, MD.

**B144** 275 In-Depth Phenotyping of Healthy European Adults by 10-Color Flow Cytometry

**B145** 276 Polynctical Response of Invariant Natural Killer T Cells in Patients Affected by Multiple Sclerosis Displays Th-1 and Th-17 Profiles

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**B136** 267 Reducing Dimensions While Keeping Information in Image-Based Screening

**B137** 268 Label-Free Sperm Morphology Assessment Using Imaging Flow Cytometry
S. Vaidyanathan, B. Didion, A. Li, S. Friend and P. Morrissey. EMD Millipore, Seattle and Mofa Global, Verona, WI.

**B138** 269 New Tool for High Throughput Analysis of Topographic Fluorescence Intensity on Single Cell: Application in Fundamental Immunology

**B139** 270 Automated Analysis of Asymmetric Cell Division Using IDEAS® Image Analysis Software
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**B140** 271 Cytotoxic Populations in American Cutaneous Leishmaniasis Lesion: Simple and Distinct Ways to Analyze
**B148** 279 Changes of Monocyte HLA-DR Expression, IL-6 and LPS Induced TNF Levels in Different Anticoagulated Whole Blood Samples over Time under Various Temperature Conditions

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**B155** 286 Regulatory T Cells Inhibit Efficient Antitumor Immune Responses by Setting Up a Trap for CTLs in Tumor Draining Lymph Node

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**B160** 291 Development of a Polychromatic Flow Cytometry Panel for Analysis of Antigen Presenting Cell Populations in Human Blood and Lymph Nodes
A. Brooks and R. Dunbar. Univ. of Auckland.

**B161** 292 Modulation of Regulatory T Cell by Differential Ratios of Fish Oil and Corn Oil in Experimental Colon Carcinoma
R. Malik, N. Agnihotri and A. Bhattachar. Panjab Univ., India.

**B162** 293 PKCδ Is Involved in Macrophage Polarization Both Directly and by Affecting T Cell/Macrophage Cross-Talk

B164 295 B7 Costimulation and Intracellular Indoleamine-2,3-Dioxygenase Expression in Peripheral Blood of Healthy Pregnant and Non-pregnant Women

B165 296 Assessment of Mast Cell/Basophil Degranulation by Combined Cell Surface Protein Analysis and Intracellular Histamine Measurement

B166 297 Investigating Changes in Monocyte Phenotypes and Functions in Active Visceral Leishmaniasis Patients

B167 298 Flow Cytometric Identification of DP T-Cells Associated with Tumor Regression in Melanoma Pigs

B168 299 A High-Throughput Direct Cell Counting Imaging Cytometry Method for Natural Killer Cell-Mediated Cytotoxicity Detection
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B170 301 Cytotoxic Involvement of NKT Cells in the Immunopathogenesis of Human Cutaneous Leishmaniasis

B171 302 Regulatory Macrophages Derived from Peritoneal Dialysate Reduce Renal Injury in Adriamycin Nephropathy

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B172 303 High Throughput Multiplexed Assays to Facilitate Detection of Shiga Toxin Producing E. coli in the Beef Chain

B173 304 A New Phagocytosis Assay in Whole Blood by Flow Imaging Cytometry with GFP-Expressing Bacteria

B174 305 13-Color Immunophenotypic and Functional Analysis of Whole Blood and Spleen from Human T-Lymphotropic Virus Type-1 (HTLV-1) Infected Humanized BLT Mice

B175 306 iNKT Cells and Their Subpopulations Are Not Restored in HIV+ Patients with Low CD4/CD8 ratio after Prolonged Effective Therapy, and Display a Pronounced Th-1 and Th-17 Pro-inflammatory Profile
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**B180 311 The Use of RiboTagger FISH Probes and Cell Sorting to Isolate and Characterise Novel Microbial Populations in Complex Microbial Communities**

**B181 312 Advanced Volumetric Cytometric Analysis of Marine Phytoplankton Bacteria, and Viral Concentrations Utilizing the Acea NovoCyte**

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**B185 316 Observing Surface Glycosylation Changes on Apoptotic B-Cells Using the Lab in a Trench Platform**

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**B186 317 Use of Mass Cytometry to Identify Circulating T Cell Populations Involved in Pathogenic Responses to Human Rhinovirus**
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**B188 319 Standardizing and Packaging a High-Content Discovery Analysis Workflow with Plug-and-Play Application Architecture**

**B189 320 Real-Time Cytometry Reveals Biochemical Interactions between Fluorescent Probes in the Analysis of Oxidative Stress**

**B190 321 The Hitchhiker’s Guide to CyTOF: Standards, Guidelines and Everything**
B191 322 Multi-dimensional Flow Cytometry Unravels Novel Populations in the Developing Mouse Heart

B192 323 New Fluorescent Dyes for Sensitive Detection of Regulatory T Cells by Flow Cytometry Analysis

B193 324 A Biomarker Discovery Approach for Urinary Cells in Lupus Nephritis

B194 325 NK Cells as Biosensors for Responsiveness to Etanercept in Ankylosing Spondylitis (Morbus Bechterew)

B195 326 Classification of Flow Cytometry Samples with a Dimension Reduction and Binning Approach

B196 327 The Immunophenome of the Mouse: An Integrative Approach to Identify New Key Genes of the Immune System

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B199 330 Phenotypic and Epigenetic Mechanism of Action Determinations of Histone Methylase and Demethylase Inhibitors Using Digital Widefield Microscopy

B200 331 A Rapid 3D Tumor Spheroid Imaging Cytometric Analysis Method for Drug Discovery

B201 332 Multiplex Human Whole Blood Phenotyping with Cell Signaling Assessment Using Imaging Flow Cytometry


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B203 334 Spatial Information on Receptor Clustering in Flow Cytometry via FRET

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B227 358 Light Scattering Simulation for Depolarization Measurement of Birefringent Crystals via Flow Cytometry
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B239 370 Alkaline Phosphatase Live Stain for Stem Cell Research

B240 371 Side Population Stem Cell Identification by Vybrant® DyeCycle™ Violet Stain

B241 372 In Search of Hematopoietic Stem Cells: Functional Heterogeneity of CD34+ Cells by Co-staining with Vybrant® DyeCycle™ Violet
B242 373 Negative Cell Sorting for Removal of Undifferentiated Cells for Regenerative Medicine

B249 380 Calibration of Eleven Flow Cytometers for the Fine Multi-color Analyses of a Large Cohort of Patients during the European IMI Project PRECISESADS

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B244 375 Phenotypic Chemotaxis by the Use of Flow Cytometry

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B246 377 Triterpenoids Alter Mitochondria Function and Cause Cell Death in RKO Human Colon Cancer Cells

B247 378 Polychromatic Flow Cytometry in Cancer Stem Cells Characterization from Lung Cancer Samples

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B257 388 Loss of Trop-2 Expression Is Associated with Mesenchymal Phenotype in Prostate Cancer Cells


B258 389 Tissue-Associated M2 Macrophages Localize to CD90+ Cancer Stem Cells in Barrett’s Esophagus but Not in Normal Esophagus and Invasive Adenocarcinoma


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B266 397 Methyl Green: Old Dye with New Applications


B267 398 Automated High-Throughput Method for Assessing Pathogenic Infectious Dose (TCID50) Using Celigo Imaging Cytometer

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B270 401 Flow Cytometric Study of Melphalan-Induced Cytotoxicity in the Bone Marrow of Rats


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B273  **404** Development and Optimization of an 18-Color Immunophenotyping Panel and Intracellular Cytokine Staining Assay for the Measurement of T Cell Responses from Rhesus Macaques in Pre-clinical Vaccine Studies  
K. Foulds, S-F. Kao, M. Donaldson, V. Letukas, P. Chattopadhyay and M. Roederer. NIAID, NIH.

B274  **405** Induction of Interferon-gamma and Interleukin-17 in T Cells by a Novel Vaccine for Tuberculosis  

**LATE-BREAKING ABSTRACTS**

B275  **406** CD8+/CD28- Naïve T Cells: A Potential Biomarker of Immunosenesence and Active Vaccine Responsiveness  

B276  **407** Effect of Eosinophils on Wnt-5A and TGF-β, Expression by Airway Smooth Muscle Cells in Asthma  

B277  **408** Purification of Replicating Pancreatic β-Cells for Gene Expression Studies  

B278  **409** Correlation of UV-Excited, Blue NADH Autofluorescence with Probes of Plasma Membrane Alterations for Measurement of Apoptosis  
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| B287 | 418 | Violet SSC: An Alternative to FSC PMT of Fluorescence in the Detection of Extracellular Vesicles  
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| B289 | 420 | Sorting and Maintaining Haploid Stem Cells Using a Beckman Coulter MoFlo Legacy and a Bio-Rad S3 by Light Scatter  
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| B290 | 421 | Using a Spectra Physics Tsunami Infrared Pulsed Laser and Second Harmonic Generation on a Beckman Coulter MoFlo Legacy Sorter: A Tunable Light Source  
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| B291 | 422 | Simple Method for Characterizing Cytometer Noise  
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| B294 | 425 | Effect of IL10, IFNγ, and IL4 Receptor Knockouts on Macrophage Response to Stimulation In Vitro and to Peripheral Nerve Injury In Vivo  
| B295 | 426 | Live/Dead Bacteria Discrimination by Styryl Fluorescence Staining and Spectral Intensity Ratio Analysis  
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| B296 | 427 | Optimisation of Polychromatic Flow Cytometry Panels for T Cell Analysis in a Clinical Research Project  
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| B297 | 428 | Demonstration of a 7-Color TBNK Application with Viability Dyes Using Beckman Coulter CytoFLEX Flow Cytometer  
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| B301 | 432 | Analysis of Intracellular Signaling in Different T-Cell Subpopulations Using CytomyFLEX and PerFix EXPOSE  
| B302 | 433 | Genomic Alterations in Invasive Melanoma Cell Lines  
M. Balázs, V. Koroknai, S. Ecsedi, L. Vízkeleti, T. Kiss, I. Szász, A. Lukács, O. Papp and R. Ádány. Univ. of Debrecen, Hungary. |
| B303 | 434 | Optimisation of Cell Counting Using the MACS Quant Flow Cytometer  
Commercial Tutorials

SATURDAY, JUNE 27

**CELL SORTING AND BIOSAFETY - PROTECTING YOU AND YOUR RESEARCH SAMPLES**

*BD Biosciences*
2350 Qume Drive
San Jose, CA 95131
Phone: 877-232-8995
Email: answers@bd.com
Web: www.bdbiosciences.com

**1245 – 1345 – Alsh Room**

*Presenter: Morgan Blaylock and Jens Fleischer*

Biological safety in flow cytometry is a growing issue for core laboratories concerned about the potential incidental exposure of operators to biological samples. Scientific studies have demonstrated that there is an increased health risk from aerosol generation when performing cell sorting experiments on biohazardous material. Therefore a higher biosafety level of containment may be required during sorting than the one recommended for the infectious material alone.

Containment includes the use of a Biosafety Cabinet that can pass international biosafety standards for product, personnel, and environmental protection. In this presentation we will show examples of product, personnel, and environmental safety testing for research use in accordance with the testing criteria defined in the national norms. In this context, research instrument solutions from BD Biosciences will be presented and usability for biosafety applications discussed.

**CHIPCYTOMETRY: A NEW 65-PLEX IMMUNE MONITORING PANEL, EXCITING NEW APPLICATIONS AND INSTRUMENTS**

*Zellkraftwerk*
Bosestrasse 4, Saxonia
Leipzig 04109
Germany
Phone: 49 15152385628
Email: info@zellkraftwerk.com
Web: www.zellkraftwerk.com/cyto.html

**1245 – 1345 – Boisdale Room**

*Presenter: Christine Hennig*

ChipCytometry is a high-content cytometry platform combining the unsurpassed quantitative phenotyping ability of flow cytometry with the unparalleled information depth of microscopy. Besides some features that are quite similar to conventional flow cytometry, three technology features make ChipCytometry an exciting technology for explorative high-content analysis:

1. Long-term sample storage/no sample consumption: ChipCytometry uses microfluidic chips enabling biomarker-preserving long-term storage of samples for a period of at least 20 months. Cell storage is particularly useful for precious samples like patient samples, rare cells and (multicenter) clinical trials because cells are not destroyed during transport, storage and analysis and can be stored for further tests exploring more and more markers on these pre-analyzed samples.


3. Tissue Cytometry: ChipCytometry can work with as sorts of cell specimens as well as with a broad range of tissue types.

At CYTO 2015 Zellkraftwerk will launch the Cytobot™, the first fully automated high-throughput ChipCytometer including fully automated data processing and lab management software. The Cytobot™ is a new instrument designed for core facilities, whereas the ZellScanner ONE (launched in 2014) is a compact benchtop instrument for low to medium sample throughput. Join us for an introduction to the future of “precious samples cytometry”.

Typical German lunch bags will be served on a first come first serve basis.

**MAXIMIZE YOUR FlowJo: APPLYING FlowJo ENTERPRISE TO A CLINICAL RESEARCH STUDY**

*FlowJo, LLC*
385 Williamson Way
Ashland, OR 97520
Phone: 541-201-0022
Email: caitlinf@flowjo.com
Web: www.flowjo.com

**1245 – 1345 – Carron Room**

*Presenter: Jack Panopoulos and John Quinn*

Clinical research studies present unique software challenges to data management, analysis, reports and sharing. Advances in technology have allowed them to grow in complexity and have produced richer data sets to mine. At the same time the rigor required for work related to clinical research has not depreciated. FlowJo Enterprise...
enhances the clinical research environment by providing centralized and consolidated analysis pipelines, facilitating push-button report generation. This centralized server-based system can assist with the compliance aspects of clinical research by generating electronic logs of all data and associated derivatives’ activity from acquisition to analysis and reporting. Server logs can easily be queried by an authorized administrator for various activities, including data upload, workspace manipulation, security access and reports. Restricted access to data, reports and derivatives is regulated by a high-level administrator, further increasing security and compliance with good data management practices.

FlowJo’s workshop tutorial will include a review of federal compliance guidelines and provide an overview of FlowJo Enterprise’s utility as applied to a common clinical research study.

**ACEA NovoCyte™ System - Redefining Benchtop Flow Cytometers with High Performance, Efficient Tools in Customizable Budget Friendly Platforms**

**ACEA Bioscience**
6779 Mesa Ridge Road, # 100
San Diego, CA 92121
Phone: 858-724-0928
Email: info@aceabio.com
Web: www.aceabio.com

**1245 – 1345 – Seminar Suite**

*Presenters:* Derek Davies and Alan Saluk

In this workshop key opinion leaders will present complex multi-parameters studies, comparative studies as well as microparticle applications using the new innovative NovoCyte flow cytometer from ACEA.
SUNDAY, JUNE 28

OPTIMIZING STRATEGIES FOR RELIABLE MULTICOLOR FLOW DATA

BD Biosciences
2350 Qume Drive
San Jose, CA 94087
Phone: 877-232-8995
Email: answers@bd.com
Web: www.bdbiosciences.com

1245 – 1345 – Alsh Room
Presenter: Robert Balderas

Flow cytometry has been a powerful tool for the immunology laboratory and continues to advance from using 3-4 parameters to using >15 parameters and beyond in a single panel. The innovations have made multi-color flow cytometry more accessible to researchers worldwide working on other areas of biology such as marine sciences, microbiology and stem cell biology. In this tutorial we will review various approaches that can be taken to ensure data quality and reproducibility whether we are using 4 or 15 parameters. We will explore how choices in instrument capability, reagent selection and approach to setup strategies can improve our confidence in data and simplify workflows for both cell analysis and cell sorting. We will also look at experimental approaches for studying rare/dim populations, and facilitating transitions from a low to high complexity panel. These multicolor optimization strategies for cell analysis and sorting populations of interest make it easier for researchers to generate good quality data and drive discovery.

MULTICOLOR FLOW CYTOMETRY ON YOUR BENCHTOP: NOW MORE SENSITIVE AND VERSATILE WITH THE GUAVA EASYCYTE™ SYSTEMS

Merck Millipore
Merck Chemicals Ltd.
Boulevard Industrial Park, Padge Road
Nottingham, NG9 2JR
United Kingdom
Phone: 44 115 943 0840
Web: www.merckmillipore.com

1245 – 1345 – Boisdaile Room
Presenters: Katherine Gillis and Jessica Reed

The guava easyCyte™ microcapillary flow cytometry systems are simpler to operate than traditional sheath-fluid based instruments and are far easier to maintain. They utilize small sample volumes, generate minimal waste, and have lower operating costs. In this workshop, attendees will learn how the simplicity and sensitivity of the improved guava easyCyte™ systems enhances data collection and analysis. Please come visit us and learn more!

MICROFLUIDIC DROPLET-FREE SORTING: THE GENTLE WAY TO PURIFY CELLS

Milteny Biotec GmbH
Friedrich-Ebert-StraBe 68
Bergisch Gladbach 51429
Germany
Phone: 49 2204 8306 0
Email: macs@miltenyibiotec.de
Web: www.miltenyibiotec.de

1245 – 1345 – Carron Room
Presenter: John Foster and Christian Peth

Microfluidic sorting offers a number of advantages compared to conventional state of the art jet-in-air cell sorters. High sample throughput, purity, yield and low cell stress can be achieved simultaneously. The benchtop cell sorting instrument MACSQuant® TytoTM uses a sterile disposable cartridge with a microfluidic chip to purify samples in a closed environment. The heart of the microchp is the world’s fastest valve that enables fast and precise flow switching in less than 10µs. Therefore, sort rates up to 50,000 events per second can be achieved. The droplet-free microfluidic approach minimizes cell stress during the sort process resulting in high cell viabilities and functionality. In addition, the absence of aerosols in combination with a closed cartridge ensures operator safety during operation. Simple workflows without complicated setup and alignment procedures allow for sorting with just a few mouse clicks.

NOT YOUR AVERAGE FLOW: CREATING CUSTOMIZED FLOW CYTOMETRY SOLUTIONS USING THE MACSQUANT PLATFORM

Presenter: Jeffrey Carrell

As flow cytometry core lab managers, we are challenged with providing access to methods and technology across global sites, often without full time on-site core staff. To this end, we advocate for standardized instruments and methods enabling the sharing of protocols across a large organization. Our solution was to evaluate an easy-to-operate and highly capable cytometer platform that was well-supported with a line of reagents and assay kits. We chose the Miltenyi Biotec MACSQuant platform as it met all of these criteria. We describe several projects we are
developing in collaboration with Miltenyi, such as: creating a custom barcoded antibody cocktail, incorporating an automated data collection and analysis template for immunophenotyping; building a multi-assay toolkit for the evaluation of biotherapeutics; and producing a series of training videos that we will deploy across global sites. These customized solutions have led to faster data generation and real-time analysis, improved access to flow cytometry methods for non-experts, and more efficient use of core staff time. Most importantly, the platform allows for global harmonization of capabilities, enabling more of our users to conduct sophisticated flow cytometry experiments.

ADVANCES IN HIGH THROUGHPUT FLOW (HTF): THE IDEAL PLATFORM TO ENHANCE PRODUCTIVITY IN IMMUNOLOGY RESEARCH

IntelliCyt Corporation
9620 San Mateo Blvd., NE
Albuquerque, NM 87113
Phone: 505-345-9075
Fax: 866-781-3140
Email: support@intelliCyt.com
Web: www.intelliCyt.com

1245 – 1345 – Seminar Suite
Presenter: Joseph Zock

Key features of the iQue Screener make it uniquely capable to screen both cells and beads, separately and together. By sampling as little as 1 ul from 96, 384 and 1536 well plates, it allows for dramatic reduction in assay volumes to as little as 5 μL. Combined with its ability to analyze an entire 384-well plate in less than 20 minutes, multiplexed screening with precious samples is a reality. ForeCyt Software manages the acquisition and analysis of highly multiplexed data and provides insightful data visualizations at per cell, per well and per plate levels. These features and more place the iQue Screener as the ideal platform for combinatorial immunotherapy characterizations and immunotarget screening. In this tutorial you will see how the Integrated Screening Solution that iQue keystones makes screening of Immunology and Immuno-oncology applications a reality. You will also learn about recent advances to the iQue Screener platform.

CYTOFLEX – HOW NEW TECHNOLOGY TRANSLATES INTO EXCITING APPLICATIONS

Beckman Coulter
22, rue Juste-Olivier
Nyon 1260
Switzerland
Phone: 49 2151 333-723
Web: www.beckmancoulter.com

1245 – 1345 – Lomond Auditorium
Presenter: Martin Adelmann

Beckman Coulter has recently launched a new Cytometry platform called CytoFLEX. Its revolutionary technology leads to unmatched performance and results. Learn how CytoFLEX can bring your research to the next level.

MONDAY, JUNE 29

OVERCOMING CHALLENGES IN CELLULAR ANALYSIS: RARE EVENT DETECTION IN THE INNATE AND ADAPTIVE IMMUNE SYSTEM

Thermo Fisher Scientific
3 Fountain Drive, Inchinnan Business Park
Paisley Renfrewshire, PA4 9RF
United Kingdom
Phone: 44 1418145955
Web: www.lifetechnologies.com

1245 – 1345 – Alsh Room

Presenters: Ryan P. Larson, PhD; Andrea Cossarizza, MD/PhD, University of Modena and Reggio Emilia School fo Medicine, Italy

Analysis of rare cell types in complex biological systems is a major challenge in biomedical research. The multi-parameter capability and high analysis rate make flow cytometry one of the key technologies in identifying and characterizing rare cell types. This seminar will discuss the application of this technology to the analysis of rare cell types in the immune system. Acoustic focusing cytometry allows for high sample collection rates without compromising data integrity, thus allowing for the rapid analysis of rare events in dilute samples or in a no-lyse, no-wash whole blood format. We have developed multiple techniques for no-lyse, no-wash assays for whole blood analysis with minimal sample processing along with assays to characterize phagocyte phenotype and function utilizing pHrodo® BioParticles® phagocytosis/phagosome acidification assay. In addition, we will discuss the identification and functional characterization of innate lymphoid cells, circulating antigen-specific lymphocytes, and innate-like cells (such as invariant natural killer T cells and circulating endothelial cells).
Morphological Analysis, Internalization, Colocalization: What Does Imaging Flow Cytometry Reveal?

Merck Chemicals Ltd
Bolevard Industrial Park, Padge Road
Nottingham, NG9 2JR
United Kingdom
Phone: 44 115 943 0840
Web: www.merckmillipore.com

1245 – 1345 – Boisdale Room
Presenter: Sherree Friend, PhD, Amnis-part of Merck Millipore

Imaging flow cytometry combines the speed, sensitivity, and phenotyping abilities of flow cytometry with the detailed imagery and functional insights of microscopy. In this workshop you will learn how the features and benefits of the Amnis® ImageStream®X Mark II and FlowSight® imaging flow cytometers can be applied to your research. Please come visit us and learn more!

Analysis of CD163 mRNA for Monocyte Pathobiology in Systemic Juvenile Idiopathic Arthritis; and Integration of PrimeFlow RNA Assay into a Flow Cytometry Core

eBioscience, an Affymetrix Company
Campus Vienna Biocenter 2
Vienna 1030
Austria
Phone: 43 1 796 4040 305
Fax: 43 1 796 4040 400
Email: info@ebioscience.com
Web: www.ebioscience.com

1245– 1345 – Carron Room
Presenters: Sherry L. Thornton, PhD and Monica Delay

Integration of Primeflow™ technology into a shared facility will be discussed. Examples of using this technology will focus on examination of macrophage differentiation of monocytes in Systemic Juvenile Idiopathic Arthritis, SJIA. SJIA, is a chronic inflammatory disease of childhood with prominent extra-articular features, including prolonged spiking fevers, rash, lymphadenopathy and serositis. Macrophage activation syndrome occurs in approximately 10% of SJIA patients, and is a potentially fatal episode of overwhelming inflammation with emergence of hemophagocytic macrophages. SJIA monocytes exhibit both classically activated M1 attributes as well as markers associated with alternatively activated M2 macrophages, such as CD163. Primeflow™ technology was used to detect CD163 mRNA in THP-1 monocytes, as protein levels assessed by CD163PE antibody binding show minimal cell surface expression. Upon stimulation of THP-1 cells with IL-10, CD163 mRNA was increased approximately 3-fold. Regulation of CD163 via microRNAs is also under investigation and will be discussed.

Comparison of QbSure and CS&T QC

Cytek Development
4059 Clipper Court
Fremont, CA 94538
Phone: 510-657-0102
Email: cytekdev@cytekdev.com
Web: www.cytekdev.com

1245 – 1345 – Seminar Suite
Presenter: Eric Chase PhD

QbSure and CS&T QC programs use different measurements of instrument performance to determine pass/fail results. QbSure uses the resolution limit (number of MEFs required for full resolution from noise) and the hydrodynamic CV of a bright bead for its pass/fail criteria. CS&T uses a shift in the target PMT voltage and the hydrodynamic CV of a bright bead for its pass/fail criteria. The shift in the target PMT voltage is with respect to a baseline measurement, so is a relative measurement. The resolution limit measurement is an absolute criterion. Both programs track instrument performance over time. However, the QbSure program’s use of a resolution limit to evaluate each parameter allows a direct between-instrument comparison of performance. Without absolute criteria, CS&T cannot provide feedback when instrument performance is poor relative to other instruments. Extensions of QbSure software to DiVa data files will be discussed. This will allow the measurement of the resolution of instruments running DiVa.

Novel Approaches to Extra Cellular Vesicle Detection and Sorting

Beckman Coulter
22, rue Juste-Olivier
Nyon 1260
Switzerland
Phone: 49 2151 333-723
Web: www.beckmancoulter.com

1245 – 1345 – Lomond Auditorium
Presenter: Laura Pajak

Andreas Spittler, M.D. (Medical University of Vienna), Jennifer Jones, Ph.D. (NIH) and Carley D. Ross, Ph.D. (Beckman Coulter) will discuss how to utilize the Astrios EQ for extracellular...
vesicle sorting. Instrument configuration and results obtained will be shown when sorting extracellular vesicles from cells such as HeLa and whole blood.

**Tuesday, June 30**

**Calculation of Sort Recovery and Using Rmax as an Improved Measure of Sorter Performance**

*Bio-Rad Laboratories*

2000 Alfred Nobel Drive

Hercules, CA 94547

Phone: 510-741-4494

Web: www.bio-rad.com

**1245 – 1345 – Alsh Room**

*Presenters:* Dr. Rui Gardner and Carol Oxford

At Bio-Rad, we are committed to measuring the most important parameters to determine optimal sorter performance. A new method of sorter performance has been described to evaluate sorter performance based on the measurement of sort recovery. The method precludes the need for absolute counting and re-sampling the sorted fraction, and requires only determining the ratio of target and non-target populations present in the original pre-sort sample and the non-sorted fraction in the waste stream. The method involves the calculation of Rmax, which is a measure of the maximum recovery expected in the conditions the sort was carried out, i.e., for a given instrument configuration and sort setup. Rmax can also be used to troubleshoot the instrument, to control daily drop-delay determination, and to evaluate and troubleshoot sample preparations by comparing them with an ideal sample. Rmax is a measure of the instrument’s maximum recovery, is dependent on instrument performance, and is independent of sample-related losses. It is calculated based on the cytometric analysis of the original sample, and avoids sampling and absolute counting of the sorted product. Bio-Rad Laboratories patented ProDrop process provides automated calculation of the drop delay based on actual events in the waste stream, providing a recovery based drop delay calculation.

**Spectral Cytometry Session: Introducing the Sony SA3800 High-Throughput Spectral Analyzer and its Unmixing Algorithms**

*Sony Biotechnology Inc.*

1730 North First Street
San Jose, CA 95112

Phone: 800-275-5963

Fax: 408-352-4130

Email: sales@sonybiotechnology.com

Web: www.sonybiotechnology.com

**1245 – 1345 – Bolsdale Room**

*Presenter:* Marsha L. Griffin PhD, MPH

In this talk we will focus on the features of the newest addition to the family of spectral analyzers and the details of Sony's own spectral unmixing algorithms. Spectral flow cytometry is proving to be an exciting technology for cytomics and systems biology. Spectral flow cytometry differs from conventional flow cytometry in that the measured parameters for events are fluorescence spectra taken across all detectors as opposed to being primarily the fluorescence signal measured from one detector. This gives spectral flow cytometry capabilities and flexibility that far exceeds those of conventional flow cytometry. Additional flexibility has been engineered into Sony’s latest analyzer, the SA3800, by extending to users software-driven high-throughput functions that adapt to both plate and well formats permitting walk-away acquisition for the busy scientist. The core functions of a spectral detection scheme facilitate the simultaneous independent measurement of signals and the data processing power to unmix such signals for real time visualization. These fundamental differences enable spectral flow cytometers to perform applications that are not readily possible on conventional flow cytometers. Because a spectral flow cytometer measures the whole fluorescence spectrum for each fluorophore, overlapping fluorophores can be resolved based on spectral shape allowing for set-up of panel combinations and the use of markers that would not be resolvable by conventional flow cytometry. Additional options for analysis include unique spectral graphs for each laser line. These graphs enable the visualization of different cell subsets and data checking.

**Presenters:** Dr. Rui Gardner and Carol Oxford

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**Exhibitor Showcases**

**BD BIOSCIENCES**

**Expanding the Horizons of New Colors: More, Brighter Choices**

**1800 – 1810 – EXHIBIT HALL 4**

To provide more choices for your multi-color panel designs, BD is continuing to utilize our Sirigen polymer technology to develop new and improved fluorochromes for research use in flow cytometry. BD Horizon Brilliant™ Ultraviolet 805 and 661 provide new options off the 355 nm UV laser. BD Horizon Brilliant™ Blue 700 is a new fluorochrome which can be used in the PerCP-Cy5.5 and had improved fluorescence and stability characteristics. Come and see our newest additions and learn how they can be used in your assays.

**VERITY SOFTWARE HOUSE**

**V-Comp™: Automated Compensation System**

**1815 – 1825 – EXHIBIT HALL 4**

Compensation continues to be one of the biggest technical issues facing clinicians and researchers. It has become even more complicated by the ability to collect high dimensional data. Verity Software House is announcing the release of V-Comp™: a fully-automated compensation utility system that is efficient, accurate, and easy to use for any number of parameters. V-Comp only requires identification of appropriate controls; no gating to identify positive or negative populations or other user input is needed. V-Comp detects and eliminates outlier events and areas of drift or instability in control files ensuring the most accurate compensation. Quality control reports are generated with time and user stamps and saved automatically. Compensated files are tagged with a suffix and saved to a user-designated folder, preserving the original data. Compensation set-ups can be reused, edited, shared, or locked. V-Comp can even un-mix Spectral data for further analysis. V-Comp has been rigorously tested and is compatible with major acquisition software programs. Come see how easy compensation can be with V-Comp!

**CELL SIGNALING TECHNOLOGY**

**Analysis of Cellular Signaling Using Activation State-specific Antibodies - Useful Tips to Optimize Imaging and Flow Cytometric Assays**

**1830 – 1840 – EXHIBIT HALL 4**

Accurate detection and quantification of protein expression and post-translational modifications are critical components to our understanding of disease-related signaling. Activation state-specific antibodies allow the quantitative analysis of cellular signaling using imaging and flow cytometric applications, enabling researchers to efficiently assess treatment-induced effects. Join CST for this presentation, explaining how antibodies that specifically detect protein modifications such as phosphorylation, acetylation, ubiquitination, and methylation are used individually to monitor the expression of a specific protein or the activity of a specific pathway. We will also demonstrate the use of these antibodies in multiplex cellular assays. Additionally, we will discuss fixation, permeabilization, and staining protocols that have been optimized for use with signaling antibodies and cell-based fluorescent applications.
LeukoDx

Accellix Automated Flow Cytometry

1800 – 1810 – EXHIBIT HALL 4

LeukoDx’s CE Marked Accellix™ platform is designed to provide 24/7 flow cytometric capability at moderate complexity level labs, the majority of hospital labs, & ultimately at a CLIA waived level. Accellix provides the answers physicians need with a compact, tabletop device operated on location. It’s simple and automated operation requires just a drop of blood in a standardized, disposable cartridge to return results in 10-20 minutes. LeukoDx plans to launch its first product for the rapid diagnosis and monitoring of sepsis, in the EU during 2015, utilizing its clinically validated exclusive licensed CD64 Sepsis Biomarker. The Accellix multicolor (4 color) flow cytometer enables complete automation in 3 steps: biochemical sample preparation in a dedicated disposable cartridge, flow cytometer reading & analytical data processing utilizing a proprietary algorithm & in-process software quality controls.

Assays implemented on Accellix platform include: Sepsis diagnosis: CD64 Index, T cell subsets/HIV monitoring: proportion of CD4 to CD8, Population analysis of cells: T cells, B cells, NK cells & monocytes and Sepsis induced immunosuppression: HLA-DR expression.
Exhibitor Listing

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Exhibit Hours

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<td>Monday, June 29</td>
<td>1130 – 1900</td>
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<td>Tuesday, June 30</td>
<td>1130 – 1630</td>
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</table>
CYTO 2015 Exhibits
Saturday June 27 – Tuesday, June 30
Exhibiting Companies

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Web: www.omicron-laser.de

Since 1989, Omicron has been developing, building and producing innovative lasers and laser combiners. Omicron specialized in customized solutions for applications in the fields of medicine, research, biotechnology, such as microscopy and flow cytometry, digital imaging and optical data storage as well as quality assurance. Product development and production comply with European and US guidelines.
PIC’s WhisperIT diode-based lasers cover the UV, Visible, and IR wavelength range and utilize a patented technology that creates a beam with excellent mode quality, low noise under all conditions, and low speckle, but at a substantially reduced cost, footprint, and power consumption compared to ion or DPSS lasers. PIC customizes its laser products to be quickly and seamlessly integrated into biotechnology instrumentation to ensure optimum performance.

**Phase Holographic Imaging**

Scheelev. 22  
Lund  223 63  
Sweden  
Phone: 46-46-386084  
Email: info@phiab.se  
Web: www.phiab.se

Phase Holographic Imaging (PHI) develops time-lapse cytometry instrumentation and software. The company offers products for long-term quantitative analysis of living cell dynamics without toxic stains or labels. PHI trades through a network of international distributors. Committed to promoting time-lapse cytometry, PHI actively expands the customer base and scientific collaborations in cancer research, inflammatory and autoimmune diseases, stem cell biology, gene therapy, regenerative medicine and toxicological studies.

**Propel Labs**

345 E. Mountain Ave.  
Fort Collins  CO  80524  
Phone: 970-295-4570  
Fax: 970-372-5664  
Email: info@propel-labs.com  
Web: www.propel-labs.com

Propel Labs is a team of flow cytometry experts who specialize in cutting edge technology. Following development of the Avalon Cell Sorter, Propel partnered with Bio-Rad Laboratories who now offers the system as the S3e High Performance Bench-Top Cell Sorter. Propel Labs also offers MoFlo and CyAn upgrades including Co-Lase Towers and NanoView for small particle detection.

**Silicon Biosystems**

Via dei Lapidari 12  
Bologna  
Italy  
Phone: 39 051 4071 300  
Fax: 39 051 7459-550  
Email: admin@siliconbiosystems.com  
Web: www.siliconbiosystems.com

Silicon Biosystems specializes in cutting edge technology. Following the development of the Avalon Cell Sorter, Propel partnered with Bio-Rad Laboratories who now offers the system as the S3e High Performance Bench-Top Cell Sorter. Propel Labs also offers MoFlo and CyAn upgrades including Co-Lase Towers and NanoView for small particle detection.

**Sony Biotechnology Inc.**

1730 North First St.  
San Jose  CA  95112  
Phone: 800-275-5963  
Fax: 408-352-4130  
Email: sales@sonybiotechnology.com  
Web: www.i-cyt.com

Sony Biotechnology analyzes, sorts and imagers incorporate advanced technologies and intuitive functionality to bring support for scientific discovery to a new level. See how spectral technology in our analyzers delivers high sensitivity, as it simplifies application design and workflow. See imaging differently with our 2015 SLAS award winning Cell Motion System. Its real time, cell observations and quantification is built on high speed video microscopy and motion vector analysis.

**Spherotech, Inc.**

27845 Irma Lee Circle, Unit 101  
Lake Forest  IL  60045  
Phone: 847-680-8922  
Fax: 847-680-8927  
Email: service@spherotech.com  
Web: www.spherotech.com

Spherotech manufactures a variety of microparticles for flow cytometers. These particles are used for calibration, alignment, multiplexing, compensation, absolute counting and drop delay determination. Specifically, the calibration, alignment, and drop delay particles are used extensively for QC and long term performance tracking. In addition, Spherotech has particles for confocal microscopy.

**Stratedigm, Inc.**

6541 Via Del Oro  Suite A  
San Jose  CA  95119  
Phone: 408-884-4029  
Fax: 408-351-7700  
Email: info@stratedigm.com  
Web: www.stratedigm.com

Stratedigm manufactures configurable, scalable, and upgradeable flow cytometry solutions that fit every lab and every budget. Users can configure their S1000EXi cytometer up to 4 lasers and 24 parameters. Complimenting any S1000EXi or SE520EXi cytometer, the Stratedigm automation suite provides automated reagent mixing, digital temperature control, sterilization, and 100% automated runs of up to 320 plates. Stratedigm – truly, cytometry without compromise.

**Stratocore**

21 rue Loigny La Bataille  
Chartres  28000  
France
Stratocore is a leader in Resource Management. Our software PPMS, in development since 2003, is used by thousands of clients in over 60 research institutions worldwide. PPMS is scalable and affordable; providing flexible tools that adapt to the changing needs of any facility. It optimizes productivity, increases revenue and provides Resource Managers real time control.

**Sysmex Partec**
Bornbarch 1
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Partec, a Sysmex Company is the collaboration of Sysmex Corporation, a supplier of in vitro diagnostic products and Partec, a pioneering developer and manufacturer of flow cytometry systems. Their products are used in many applications in healthcare, microbiology, cell biology, agrosciences, plant breeding, aquaculture and in pharmaceutical, food and beverage industries. The company also offers solutions for gel electrophoresis, PCR, fluorescence and transmitted light microscopy. Their products are available worldwide.

**Thermo Fisher Scientific**
102 Fountain Crescent
Inchinnan Business Park
Paisley PA4 9RE
United Kingdom
Phone: 44 1418145955
Web: www.lifetechnologies.com

Life Technologies now a Thermo Fisher Scientific brand is the world leader in serving science. Our mission is to enable our customers to make the world healthier, cleaner and safer. Through our Thermo Scientific, Applied Biosystems and Invitrogen brands, we offer an unmatched combination of innovative technologies, purchasing convenience and support.

**Toptica Photonics AG**
Lochhamer Schlag 19
Graefelfing/Munich 82166
Germany
Phone: 49 8985837-0
Fax: 49 8985837-200
Email: sales@toptica.com
Web: www.toptica.com

Toptica Photonics AG develops and produces research grade lasers for scientific applications as well as industry grade lasers for OEM integration. Key technologies comprise tunable single frequency diode lasers from 190 nm to 2880 nm, ps and fs pulsed fiber lasers from 488 nm to 2100 nm, multi-color laser engines, THz systems for frequency-domain or time time-domain spectroscopy, and high power laser diodes & semiconductor optical amplifiers.

**Union Biometrica, Inc.**
84 October Hill Rd
Holliston MA 01746
Phone: 508-893-3115
Email: sales@unionbio.com
Web: www.unionbio.com

Union Biometrica Large Particle Flow Cytometers automate the analysis and sorting of objects too big/fragile for traditional cytometers, e.g., large cells/clusters, cells in/on beads and small model organisms (10-1500 micron diameter). The new LP Sampler module can aspirate these samples from multiwell plates and deliver them intact to the BioSorter. The new VAST Biomager system automates cellular-level imaging of 2-7 dpf zebrafish larvae.

**Verity Software House**
45A Augusta Road P.O. Box 247
Topsham, ME 04086
Phone: 207-729-6767
Fax: 207-729-5443
Email: verity@vsh.com
Web: www.vsh.com

Verity Software House, an industry leader in flow cytometry software development, offers a unique combination of innovative software for flow cytometry and unparalleled technical and customer support. ModFit LT, WinList, and GemStone: an unbeatable combination. Now introducing V-Comp, a fully automated compensation system. Please stop by and see us in booth 501, and learn more about V-Comp at the Exhibitor Showcase on Sunday, June 28, 1815-1825. Without Verity, it’s just software.

**Vortran Laser Technology**
Unit 9, Avro Court Ermine Business Park
Huntingdon Cambridge PE29 6XS
United Kingdom
Phone: 44 1933 461 666
Email: sales@laser2000.co.uk
Web: www.laser2000.co.uk

Vortran Laser Technology is a next-generation manufacturer of laser diode systems ideally suited for applications in bio-life sciences including flow cytometry and fluorescence microscopy. Using
patented technology they are able to offer a new level of functionality, stability and reliability in an otherwise flat technological landscape. Laser 2000 is a world leader in the distribution of photonics and optoelectronics products, offering a broad range of standard and customised solutions.

**VyCAP**

A. Rademakerstraat 41  
Deventer    7425 PG  
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Web: www.vycap.com

VyCAP is a provider of technology for the isolation, identification and analysis of cells and single cells from biological fluid samples. The company designs, and manufactures innovative solutions for both Life Science and Clinical applications. We combine molding technologies with MEMS micromachining and standardized bio-analytical methods. Examples are: · Detection of Circulating Tumor Cells (CTC) by filtration using Silicon Microsieves · Single Cell isolation from Silicon Microwells followed by DNA analysis

**YOKOGAWA ELECTRIC CORPORATION**

323  
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Kanazawa-shi, Ishikawa 920-0177  
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Phone: 81 76 258 7011  
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**ZELLKRAFTWERK**

405  
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Phone: 49 15152385628  
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Email: detmers@zellkraftwerk.com  
Web: www.zellkraftwerk.com

Highly Multiplexed Cytometry Zellkraftwerk manufactures the ZellScanner ONE a revolutionary bench-top instrument for highly multiplex cytometric bio-analysis using transformative Chipcytometry technology. Chipcytometry is comparable to Flow Cytometry, but it has two very exciting technology features: Multiplexing and long-term sample storage. Zellkraftwerk runs an instrument business and a clinical service division.
1 Fluorescent Proteins in Flow Cytometry
Teresa Hawley¹, William Telford², John Nolan³
¹George Washington University, Washington, DC, United States, ²National Cancer Institute, National Institutes of Health, Bethesda, MD, United States, ³Scintillon Institute for Biomedical and Bioenergy Research, San Diego, CA, United States

Course Objectives: In a scientific tutorial in 2011, one of us (W.T.) covered the fluorescent proteins (FPs) available at the time. As a follow-up, we will focus on recent developments in the field, including new FPs, new lasers, and the advances made in detecting FPs using spectral flow cytometry. We will discuss considerations for practical use of FPs and make recommendations for choice of FPs in flow cytometry. We will analyze FP data using fluorescence compensation for conventional flow cytometry and unmixing algorithms for spectral flow cytometry. Attendees are encouraged to bring along their own data.

Course Details or Outline:

1. Basics/Overview
   - Timeline of major developments in FPs
   - List of commonly used FPs
   - Considerations for practical use of FPs
   - Recommendations for choice of FPs in flow cytometry

2. Methods
   - Strategies for simultaneous detection of multiple FPs
   - Matching lasers with FPs
   - Conventional vs spectral flow cytometry of FPs
   - Flow cytometer calibration using FP beads

3. Data Analysis
   - Fluorescence compensation in conventional flow cytometry
   - Unmixing algorithms in spectral flow cytometry
   - Display of properly compensated FP data

4. Discussion

2 Label-Free Imaging: Ptychography-Label-Free Cytometry
Peter O'Toole¹, Martin Humphry²
¹Technology Facility, Department of Biology, University of York, York, United Kingdom, ²Phasefocus, Sheffield, United Kingdom

Course Objectives: This tutorial will cover the emerging area of label free microscopy, focusing on ptychography, but also introducing holography and other quantitative and semi-quantitative label free imaging approaches for cytometric analysis of live cells. Examples of cell cycle, cell proliferation, cell death and live cell tracking will be given.

The student will leave with an insight into what will possibly be the next big area of development in biological imaging. They will be able to understand the basic concepts, need and approaches available and leave enthused about the potential applications that this may open up for themselves and bioimaging in general.

Course Details or Outline:

1. Basics/Overview of the fundamental physics that lie behind these fast developing label free imaging techniques
2. A basic outline of how the ptychographic microscope is set-up will be described.
3. An explanation of how bioimages are acquired, highlighting advantages over labelled techniques and some of its limitations.
4. Discussion of example data sets and their cytometric analysis.
5. Open discussion - how will label free microscopy develop in the future

3 Making Resource Technology a Career: Developing, Promoting and Retaining Core Leadership and Staff
Jonni Moore, Charles "Hank" Pletcher, Jr.
Abramson Cancer Center, Flow Cytometry and Cell Sorting Shared Resource, Department of Pathology and Laboratory Medicine, Perelman School of Medicine, Univ. of Pennsylvania, Philadelphia, PA, United States

Course Objectives: The success of any shared resource lab is primarily dependent upon the quality of the resource scientists in the lab. It is recognized that this is a unique career where experience often plays a more important role than formal education and customer service is as important as technical knowledge. The “human capital” in a SRL is worth way more than the capital equipment, and deserves significant attention to maintain and develop. This tutorial will cover challenges and solutions to recruiting, developing and retaining high quality SRL staff and leadership.

Course Details or Outline:

- Participation in this session should provide the attendee with
- An understanding of how a resource scientist differs from a research scientist
- How to work with institutional human resources to create appropriate job classifications
- How to work with academic leadership to define faculty roles in SRLs
- How to work continuing education and staff development into your workflow and budgeting.
- How to identify and institute best practices to assure a rewarding work environment for SRL staff

4 OMERO in Action
Will Moore, Petr Walczyzko
GRE school of Life Science, University of Dundee, Dundee, United Kingdom

Course Objectives: In this workshop, we will outline and demonstrate the OMERO platform, and show how you can use it to work with your microscopy and/or HCS data. In addition we will demonstrate some of the applications that have been released by OME and some of the integration with 3rd party tools, including:

OMERO.figure - fast figures from your OMERO images
OMERO.webtagging - automatically tag your data and search for tags
ImageJ - improved interaction with OMERO
We will also outline other applications and integration e.g. FLIM image data analysis, object tracking, Matlab Analysis tools, etc. We’ve designed OMERO to be as flexible as possible, and this has enabled its use in a range of imaging domains, including light and electron microscopy, high content screening. Come along to the workshop and bring your favourite data

Course Details or Outline:

- General introduction: What is OMERO?
- Importing data into OMERO
- Image Selection
- Add to container
- Tag at import
- Storage and integrity report
- File in its original format on disk
- Organizing data
- Browsing data
- Metadata access
- Image preview and Rendering settings
- Export as JPEG, OME-TIFF, original
- Viewing images
- Projections
- Split View
- Save
- Movie export
- ROI creation
- Annotations
- Tagging
- File attachments
- Key-value annotation
- OMERO.webtagging
- Batch tagging
- Searching tag
- Searching
- Search by ID
- Search data
- Searching across groups.
- OMERO.figure
- Figure creation
- Multi-T & Z series
- Auto scalebar & labels
- Cropping to ROIs
- Imagej integration
- Import directly from imagej
- ROI reading and writing

5 RNA Flow Cytometry

Steven McClellan¹, Paul K. Wallace², Christopher Groves³

¹Flow Cytometry Core Laboratory, Mitchell Cancer Institute, Univ of South Alabama, Mobile, AL, United States, ²Department of Flow & Image Cytometry, Roswell Park Cancer Institute, Buffalo, NY, United States, ³Dept. of Respiratory Inflammation and Autoimmunity Research, Medimmune Inc, Gaithersburg, MD, United States

Course Objectives: Flow cytometry permits the simultaneous measurements of many biomarkers in individual cells from bulk populations. Until now analysis has been limited, however to primarily analysis of proteins and total DNA or highly abundant DNA sequences. Since most RNA gene transcripts are present at very low quantities our ability to detect these mRNA species by flow cytometry has been limited. In 1993, Patterson et al. (Science, 1993. 260:976) used a PCR driven in situ hybridization technique to detect HIV mRNA in infected cells, however the stringent conditions required by this technique prevented its wide spread application to and development for flow cytometry. Two new techniques, PrimeFlow™ (Affymetrix/Ebioscience) and SmartFlare™ (EMD Millipore) have recently been commercially introduced and are seeing increasing use among research scientists.

This tutorial will discuss:

- The methodology leading up to the current techniques
- The PrimeFlow™ branched DNA methodology and applications
- The SmartFlare™ technique and procedures
- Pitfalls, quality control and analysis strategies for both Techniques
- Methods to simultaneously combine mRNA measurements with labeling of surface and intracellular proteins

The PrimeFlow™ RNA assay system from Affymetrix/Ebioscience allows for the determination of differential RNA expression within a mixed population of cells. The assay improves sensitivity and lowers background of fluorescent in situ hybridization (FISH) through the use of branched DNA signal amplification. Protocols and data will be presented showing the usefulness of this system to identify RNA expression on bone marrow B cells subsets. We will show a direct comparison of this method to a much more time consuming process of sorting cells for microarray analysis. This method provides a sophisticated molecular tool for the characterization of gene expression on rare cell subsets within tissue.

The SmartFlare™ system from EMD Millipore employs a gold nanoparticle that is actively endocytosed into most cell types. In the cytoplasm, interaction with the specific mRNA target causes the generation of a fluorescent signal, which can be detected by flow cytometry or microscopy. Protocols and data will be presented showing the usefulness of this system to sort cells based on mRNA expression, for several applications such as isolation of cancer stem cells from tumors and optimizing shRNA or CRISPR knockdown clone selection. This new technology now enables the non-destructive detection of intracellular epitopes in living cells.

By the end of this tutorial, attendees will have an understanding of which technique is most appropriate for their applications; how to apply them to their research and most importantly gain an understanding of the pitfalls and solutions from individuals who have learned these the hard way.

Course Details or Outline:

A. Introduction
   1. Overview of methods to detect RNA in bulk preparations of cells
   2. Initial flow cytometric methods to detect HIV by in situ PCR
   3. RNA-Seq and Fluidigm methodologies
B. PrimeFlow - branched DNA
   1. Theory
   2. Method and data analysis
   3. Pitfalls
   4. Comparison of flow to microarray based expression profiling
C. SmartFlare
   1. Theory of detecting RNA in live cells
   2. Protocols and data analysis
   3. Pitfalls
   4. Use for isolation of cancer stem cells from tumors
   5. Optimizing shRNA or CRISPR knockdown clone selection

6 Cellcognition: Image Analysis of Live Cell Imaging Data

Christoph Sommer
Institute of Molecular Biotechnology (IMBA), Vienna, Austria

ISAC 2015 Program and Abstracts
Course Objectives: Automated time-lapse microscopy has become an enabling technology to monitor and quantify dynamics of cells and subcellular structures. A typical workflow to analyze time-lapse movies comprises the segmentation of cells, linking of cell objects over time by tracking, and classification of cellular phenotypes. In this tutorial session, the participants will be familiarized with common methods and workflows for the analysis of live cell imaging data.

The tool for this tutorial session will be CellCognition (www.cellcognition.org), an open-source software platform for the analysis of live cell imaging data in the context of High-Content-Screening. The software contains a graphical user interface, which allows also non-experts to parameterize and run complex workflows on time-resolved image data.

Course Details or Outline:
1. Introduction
   a. Live cell imaging data
   b. Data sources and preprocessing
2. Methods and workflows
   a. Cell segmentation
   b. Supervised classification of cell morphologies
   c. Tracking of cells over time
   d. Error correction utilizing temporal context
3. CellCognition walkthrough
4. Summary/Conclusions

7 Bridging Flow Cytometry with New Technologies
Monica DeLay, Sherry Thornton
Research Flow Cytometry Core, Division of Rheumatology, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, United States

Course Objectives: Research projects frequently encompass technologies from more than one Shared Resource Lab (SRL). Novel applications, such as single-cell RNASeq downstream of cell sorting, may require the involvement of several SRLs from sample collection to data analysis. Often little to no communication between the client and the core staff occurs with regards to the experimental goals or the upstream and downstream processes related to a service request. This tutorial will discuss applications for which multiple core technologies are needed to complete a research project and strategies for effective communication, cooperation, and coordination between staff of different cores. Upon conclusion of the tutorial, attendees will have specific tools to aid in the integration of new technologies from other cores into investigator-driven projects that utilize their SRL.

Course Details: Research projects and applications using cytometry techniques that require cross-core collaboration such as cell sorting to single cell RNASeq using the Fluidigm C1. Critical parameters for experimental success and strategies for interaction between cores to support the investigator.

8 Ask the Experts: Quality Control in Image Cytometry
Mike Halter, Silas Leavесs, Stephen Lockett
1 Biosystems and Biomaterials Division, National Institute of Standards and Technology, Gaithersburg, MD, United States
2 Dept. of Chemical and Biomolecular Engineering, University of South Alabama, Mobile, AL, United States
3 Optical Microscopy and Analysis Laboratory, Frederick National Lab for Cancer Research, National Cancer Institute, Frederick, MD, United States

Course Objectives: Quantitative fluorescence microscopy is an important tool for measuring cellular properties (i.e. image cytometry). The reproducibility of these measurements can be significantly improved by using the standard instrument performance criteria, such as the limitation of the detection sensitivity, linear range of spatial resolution (point spread function), and spectral response. Assuring that an instrument’s performance is comparable from day to day can provide evidence that the instrument is working reliably and facilitate the evaluation of quantitative image cytometry data. This tutorial will instruct students on practical and routine methods for evaluating the performance of a fluorescence microscope used for an image cytometry assay. When possible, reference materials for benchmarking the performance of a microscope will also be suggested. Students will also be provided with a checklist that can be used to assure that fluorescence microscopy image data is appropriate for quantitative analysis.

Course Details or Outline:
1. Basics/Overview of fluorescence intensity measurements on a microscope
2. Benchmarking the limit of detection, sensitivity, saturation, and intensity response with fluorescent glass
3. Benchmarking the spectral response of a fluorescence microscope
4. Measuring point spread function (resolution) with sub-diffraction sized beads

9 Cell Sorting: Fundamentals, Applications and Troubleshooting
Simon Monard
Walter and Eliza Hall Institute of Medical Research, Parkville, Australia

Course Objectives:
1. Provide information on the fundamentals of electrostatic cell sorting and an overview of the application of cell sorting in practical situations.
2. Discuss some of the areas where things can go wrong, and what steps can be taken to improve sorting experiments.

Course Details or Outline:
The aim of this session is to provide participants with a solid background in the principles of cell sorting. Ideally the material is presented in a manner that encourages interactions with the participants and stimulates them to think about how cell sorting can be practically applied to solve scientific problems.

The session will discuss how electrostatic cell sorters operate. Starting from basic principles, the hardware that is widely used will be covered following the practical constraints that hardware has on experimental design and the expected outcomes.

The types of sort strategies that can be made, the expected results and some of the reasons things go wrong, and what to do about them, will also be addressed.

10 Forensic Flow Cytometry: Crimes against Cytometry
Pratip Chattopadhyay1, Jennifer Wilshire2
1 ImmunoTechnology Section, Vaccine Research Center, National Institutes of Health, Bethesda, MD, United States
2 Memorial Sloan-Kettering Cancer Center, New York, NY, United States

Course Objectives: Troubleshooting is a critical skill in flow cytometry. This tutorial, we will demonstrate how to troubleshoot flow cytometry data through the use of case studies and real-life examples. We will cover common issues in staining, compensation, and data analysis and discuss practical methods to avoid these issues.

Course Details or Outline:
- Example Topics:
  - Antibody Titration
  - Viability Markers
  - Gating

ISAC 2015 Program and Abstracts 101
11 "Super" Resolution: Fluorescence Microscopy
Rainer Heintzmann

1Leibniz-Institute of Photonic Technology, Jena, Germany
2Institute of Physical Chemistry, Friedrich-Schiller University Jena, Germany, and King’s College London, Randall Division, London, United Kingdom

Course Objectives: This tutorial covers superresolution microscopy from the basic concepts to some recent schemes. It will include methods such as stimulated emission depletion (STED) or photoactivated localization microscopy (PALM), which received the Nobel prize for chemistry in 2014. However, other techniques such as linear and non-linear structured illumination microscopy (SIM) or optical reassignment (OPRA) will also be covered. Finally, an implementation of light-sheet microscopy, which is not really superresolution microscopy, but allows the high-resolution imaging of large 3-dimensional sample volumes is discussed.

The student will gain a good understanding of Abbe’s theory of resolution and a feeling of several ways, how to circumvent this limit in fluorescence microscopy.

Course Details or Outline: (for example)
- Resolution, why do we need it. Abbe’s resolution limit.
- Fourier-space
- Superresolution methods I: STED, PALM/dSTORM
- Short break
- Superresolution methods II: SIM, OPRA
- Large sample volumes at 1µm isotropic resolution:
  - Light-wedge microscopy
  - Summary

12 Flow Cytometry Data Management – Drinking from the Firehose (without Getting Wet)
Wade Rogers

Department of Pathology and Laboratory Medicine,
Perelman School of Medicine, University of Pennsylvania,
Philadelphia, PA, United States

Course Objectives: Flow Cytometry is a high-content technology, and unique in the complexity, not to mention the sheer volume of data that it generates. Typical experiments in the “pre-digital” era averaged in the 1-10 Mbyte range, whereas experiments done on modern digital instruments average over 100 times as much, and some extend well above 1000-fold larger. Techniques that were once adequate to manage data were not scalable, now leaving researchers and core facilities in a quandary: how do they store, organize, transfer, back up, archive and analyze these very large volumes of data? In this workshop we will get into the way-back machine and travel back to the time when storing and distributing data on CDs seemed so much better than floppy disks or ZIP drives. We will then pose the problem of today’s data in the context of yesterday’s technology and finally illustrate with examples some solutions that current computing technology, together with innovative software, have to offer.

After the tutorial, the participant will understand the full dimension of the challenge of data management, and will be conversant in the technologies and strategies that can be utilized to effectively “drink from the firehose”.

Course Details or Outline:
- Historical Perspectives: How Did We Get Here?
- Cytometry Instrumentation and Capabilities
- Evolution of Computing Technologies
- Current State: What Do We Do Now?
- Cytometry Instrumentation and Capabilities

13 Processing Images Using the Free and Open-Source Software Icy
Fabrice de Chaumont

Biolmage Analysis Unit, Institut Pasteur, Paris, France

Course Objectives: This tutorial presents how to investigate an image, by extracting quantitative information. This tutorial is presented as an interactive study which is performed live, using Icy. The audience will participate and will propose interpretation of the problem, and of course, there will be a lot of traps! It covers a large number of topics: understanding the nature of noise in the image, understanding the interest of different representations of the images: the richness of 2D and 3D rendering in different modalities, the use of color maps and the practical use of histories. In a second step, more advanced algorithms such as wavelets for spot detection and MHT for tracking of particles are also covered.

During this presentation, a number of Icy’s functionalities are covered: visualization, use of ROI, histogram, look up table, running and installing plugins, scripting in JavaScript and Python, graphical scripting using protocols, management of data, method reusability and how to reach unknown functionalities.

Students coming to this session will learn in a didactic and ludic way why the noise is so important in the images and what the good practices of the image analysis are. In this interactive session, they will discover that one needs to deeply understand his/her data before performing an analysis. Each step performed during this tutorial is reproducible since the software and the data are free and available for download. At the end of the session, the attendants can perform and extend their analysis directly on their laptops!

Course Details or Outline:
1. Installing Icy
2. Opening an image
3. Understanding the histogram and the look up table (color map)
4. Finding functionalities in seconds without knowing the software!
5. The ImageJ compatibility.
6. Website presentation, browsing script resources and graphical protocols.
7. The screen is lying to us! Representation of 16 bits images.
8. Interpreting an atomic force microscopy output as an image.
9. Viewing background information
10. Viewing data that cannot be displayed in 2D with false color map.
11. Characterization of noise
12. Using a 3D ray-traced volume rendering vs a 3D elevation map rendering
13. Interpretation of the histogram
14. Data are lying to us: understand the defects of the data acquisition
15. Detecting density of spots representing neurons in an image
16. Automatically perform segmentation of cell in an image, and then density detection
17. Interpreting results
18. Interpreting results of several users over the same sample
19. Detection of vesicle and tracking
20. Description of global and individual movements
21. Concluding remarks
14 Flow Cytometry Biosafety

Kevin Holmes¹, Hank Pletcher², Phil Hogarth³, Robert Mundley⁴, Simon Monard⁵, Steve P. Perrietto⁶, Ingrid Schmid⁷
¹NIH, Bethesda, MD, United States, ²University of Pennsylvania, Philadelphia, PA, United States, ³Animal Health and Veterinary Laboratories Agency, Addlestone, United Kingdom, ⁴Mater Medical Research Institute, Brisbane Area, Australia, ⁵WEHI, Parkville Victoria, Australia, ⁶VRC, NIAID, NIH, Bethesda, MD, United States, ⁷UCLA, Los Angeles, CA, United States

Course Objectives: This tutorial will provide a summary of biosafety principles as they apply to flow cytometry and cell sorting, with emphasis on the new ISAC Cell Sorter Biosafety Standards.

This tutorial will also provide a forum in which to discuss with experts in the field, specific scenarios that operators or core facility managers encounter.

After participation in this tutorial the attendee should have a clearer understanding of the principles and practices of biosafety as it pertains to flow cytometry, in particular cell sorting. Additionally, the attendee will have a list of resources to aid in risk assessment and the development of Standard Operating procedures in their own lab.

Course Details or Outline:
- Basics/Overview Biosafety Principles and Practices in Flow Cytometry for sorters and analyzers
- Discussion of scenarios that might be encountered
- Open forum to discuss specific issues
- Summary/Conclusions

15 Imaging Life at High Spatiotemporal Resolution

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As our understanding of biological systems has increased, so has the complexity of our questions and the need for more advanced optical tools to answer them. For example, there is a hundred-fold gap between the resolution of conventional optical microscopy and the scale at which molecules self-assemble in complex cellular structures. Furthermore, as we attempt to peer more closely at the three-dimensional dynamic complexity of living systems, the actinic glare of our microscopes can adversely affect the very cellular structures we are trying to study. As a result, the heterogeneity of living tissue can seriously impede our ability to image at high resolution, due to the resulting warping and scattering of light rays. I will describe three areas focused on high-resolution microscopy for imaging specific proteins within cells at various lengths scales. This is especially valuable in immunology because clinical specimens, especially from young children, are often very limiting. The data is also more easily analyzed because there is little or no overlap to factor. Here I will discuss the value of this technology in three separate contexts—one being the redefinition of virtually every major immune cell type where this has been employed, the second being how it has vastly expanded the number of T cell specificities that can be surveyed using peptide-MHC tetramers, and lastly the way in which we can use this data to define immune landscapes—that is, using this information to create maps of a person’s immune cells such that we can easily compare one individual or group of individuals to another individual or group. Thus, mass cytometry represents an extremely important addition to the single cell analysis toolkit.

17 Detection of Extracellular Vesicles by Flow Cytometry: Small, Smaller, Smallest

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Body fluids contain not only cells, but also smaller cell-derived (extracellular) vesicles (EV). EV are present in high concentrations, and their major functions are protection and transfer of biomolecules between cells. Although EV are present in body fluids under normal and pathological conditions, their concentration, cellular origin, composition and function change in most if not all diseases, including cancer, cardiovascular disease, etc. This knowledge has raised great scientific and clinical interest, because vesicles potentially hold an entirely new level of clinically relevant and hitherto unexplored information, for example for diagnosis, prognosis, monitoring of therapy, etc.

To gain access to this information, ideally every single vesicle present in a body fluid is detected, and the clinically relevant information—e.g. cellular origin—is extracted. Unfortunately, this is not trivial: EV are highly heterogeneous in diameter and the majority is < 100 nm, not all EV are spheres, contaminants in the size range of EV are abundantly present in most samples, the refractive index of EV is low, a 100 nm EV will not expose many identifying proteins, due to their high concentration ‘swarm detection’ may occur, etc.

So far, the instruments capable of extracting information from single EV in suspension are resistive pulse sensing, nanoparticle tracking analysis, and flow cytometry. The first two methods determine concentration and size distribution of vesicles, but the clinical usefulness of such methods is otherwise limited. FCM, however, is also not an ideal tool, because most commercially available instruments detect only EV larger than 500 nm, and thus measure less than 1% of all EV present in body fluids. At present, (i) new flow cytometers have become commercially available capable of detecting single vesicles from 180 nm and larger, (ii) a worldwide standardization of FCM measurements of EV is ongoing, supported by the European Metrology Research Program (METVES; www.metves.eu) and the International Society on Thrombosis and Haemostasis (ISTH; www.isth.org), and (iii) technical improvements are being tested and developed to improve the sensitivity of flow cytometers to detect EV.

A clinically useful instrument for single EV detection is capable of identifying the cellular origin based on the presence of ~10 antigens per vesicle and capable of measuring ~10⁶ EV in 15 minutes, and provide a size estimate of each EV. For the near future, the development of a new generation of flow cytometers will get us closer to this goal. Nevertheless, extracting clinically relevant information from such measurements will remain a challenge for years to come. With the help of ISAC in exploring...
the physical and biochemical limits, we have confidence that such instrumentation will become available in the coming years. Moreover, such instrumentation may also be useful to detect other small particles, such as bacteria, and viruses.

18 Evaluation of Cell Sorting Aerosols and Containment by an Optical Airborne Particle Counter

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Understanding aerosols produced by cell sorting is critical to biosafety risk assessment and validation of containment efficiency. Characterization of the aerosols produced by cell sorters has been performed using an Aerodynamic Particle Sizer, but the device is prohibitively expensive for the widespread, routine evaluation and monitoring of aerosol containment. In this study an Optical Airborne Particle Counter was used to analyze aerosols produced by the BD FACSaria and to assess the effectiveness of its aerosol containment. The suitability of using this device to validate containment was directly compared to the Glo-Germ method put forth by the International Society for Advancement of Cytometry (ISAC) as a standard for testing.

It was found that high concentrations of aerosols ranging from 0.3 µm to 10 µm can be generated in failure mode, with most less than 5 µm. Although the aerosols are effectively contained by the Biosafety Cabinet (BSC), the Aerosol Management System (AMS) operated at the recommended settings fails to contain small aerosols (<0.5 µm) from escaping to the BSC. In most cases, while numerous aerosols smaller than 5 µm were detected by the Optical Particle Counter, no Glo-Germ particles were detected. The results indicate that small aerosols are able to escape AMS containment and are likely underevaluated by the Glo-Germ method.

Our data in this study has clearly illustrated that:

1. BD FACSaria Cell sorters are able to generate aerosols in the range from 0.3 µm to 10 µm and the data strongly suggest that containment of small (<5 µm) aerosol particles needs to be validated for infectious material sorting. This has so far not been clearly addressed in the ISAC guidelines. Our study has advocated that the cell sorting aerosol containment validation protocol developed in the Ragon BL3 core facility using a Fluke 985 airborne particle sizer could practically fulfill this task well.

2. Both the BD AMS at 20% and the integrated AMS of the Bioprotect IV in 'high or low' mode were not able to completely contain aerosols generated in failure mode, especially if the tube holder was not installed. Small aerosols were still detected. This does not present an immediate risk to the operators if the cell sorter is housed inside of a BSC, but it does stress the importance of using a BSC and a need of proper enforcement of additional PPE for arms/hands (such as a sleeve and additional layer of gloves) when reaching into the inside of the BSC if biohazardous cell sorts are conducted.

3. The importance of the tube holder or splash guard in containing aerosols is strongly illustrated in our testing.

4. The Flueke 985 airborne particle counter can be used routinely as an aerosol containment testing tool for cell sorting. It is portable, gives a rapid reading of aerosol sizes and concentrations, and is more sensitive than the Glo-Germ method. It can also be used to periodically check the sort chamber area to test aerosol containment without interrupting the ongoing operation. We believe this device is an invaluable tool for flow cytometry and strongly suggest others to implement its use.

19 Measurement of Light Scattering in Backward Hemisphere: A New Way for the Study of Platelet Aggregation with Scanning Flow Cytometry

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Background: Blood platelets play a central role in hemostasis and are involved in many diseases, including thrombosis, hemorrhage, inflammation, and cancer. Many methods for the evaluation of platelet function are based on platelets aggregation testing. These ‘gold standard’ among those methods is light-transmission aggregometry. However, the alterations are not noticeable on the very first stage of aggregation, the dimerization of platelets, while this process precedes clot formation and is critically important for the hemostasis. Single-particle technique, such as flow cytometry, may help to overcome this limitation. However, the identification of platelet monomers and dimers from flow-cytometric data is impossible due to high variability of cells volume and shape.

Methods: We use the scanning flow cytometry, which is based on the measurement of angle-resolved light-scattering profiles (LSPs) of individual particles. The range of scattering angles where the LSP is measured was 10-70°. We measure LSPs with the Scanning Flow Cytometer fabricated by CytoNova Ltd. (Novosibirsk, Russia, http://cyto.kinetics.nsc.ru) during platelet aggregation. The aggregation was initiated by the addition of adenosine diphosphate (ADP).

For the simulation of LSPs of single platelets and their aggregates we used discrete dipole approximation software ADDA v 1.1. Each platelet were modeled as an oblate spheroid, and platelet aggregates were modeled as spheroids in contact.

Results: LSPs of platelets and their aggregates were measured with the SFC in several time points, i.e. 0, 1, 5, 10 and 15 min after the addition of ADP. The overall intensity of LSPs slightly increased with the increasing aggregation time, while the structure was approximately the same. This fact is non-trivial, because the LSP structure strongly depends on particle shape. We explained this effect by the numerical simulation, which showed that the scattering by platelets in aggregate can be considering single. Also we showed that the interference structure along the azimuthal scattering angle φ which is present only for aggregates, is washed out by the integration over φ. This leads to the additivity of platelet LSPs, which results in the identical structure of monomer and dimer LSPs.

Numerical simulations showed several ways to overcome this problem. The simpler way is probably the measurement of LSPs in backward hemisphere. In this range of scattering angles (110-170°) LSPs of monomer and dimers differ from each other, which is promising for the identification of aggregates. The novel version of SFC with extended angular range is now in the process of manufacturing and testing.

Conclusions: The features of light scattering by platelets aggregates makes it impossible to separate monomers from dimers with existing instruments, including modern flow cytometers. The device capable of measuring light scattering in backward hemisphere is needed for this purpose. The modernized version of the SFC may shed a light on the process of platelet aggregation.

20 Tunable Green Fiber Lasers with Expanded Wavelength Ranges for Flow Cytometry

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Tunable Green Fiber Lasers with Expanded Wavelength Ranges for Flow Cytometry

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The Fluke 985 airborne particle sizer can be used routinely as an aerosol containment testing tool for cell sorting. It is portable, gives a rapid reading of aerosol sizes and concentrations, and is more sensitive than the Glo-Germ method. It can also be used to periodically check the sort chamber area to test aerosol containment without interrupting the ongoing operation. We believe this device is an invaluable tool for flow cytometry and strongly suggest others to implement its use.
Multiple single wavelength lasers are now standard equipment on most flow cytometers. In addition to the common blue-green 488 nm, red 633 to 642 nm and violet 400 to 410 nm wavelengths, a number of other lasers in visible range are often included, either to improve excitation of conventional fluorochromes, or to excite newer ones. Green 532, green-yellow 552 nm, yellow 561 nm and orange 592 nm lasers are only a few of the wavelengths that are now often included on advanced systems. Many modern flow cytometers can now be equipped up to 10 or more laser sources, providing an unprecedented level of excitation flexibility.

Nevertheless, inclusion of many laser wavelengths on a flow cytometer is an engineering challenge, and does not provide total flexibility with regard to excitation wavelength. There will always be excitation gaps that single wavelength lasers do not reach. Tunable laser sources that cover several areas of the visible spectrum are now available that have the potential to provide the same level of excitation flexibility present in spectrophotometry or spectrofluorometry. The user can simply specify the wavelength required, and the laser unit will tune to the desired wavelength. In a previous study, a tunable green laser relying on non-linear fiber optic technology (developed by Inversion Fiber Ltd., formerly Zecotek Photonics) was described that was capable of tuning from 540 to 550 nm in 1 nm increments (a 10 nm tunable range). The spectral bandwidth and power levels (50 to 150 mW) from this source were comparable to single wavelength lasers. This unit was used on a commercial flow cytometer to replace existing green and green-yellow single wavelength lasers.

In this study, an improved version of this tunable green fiber laser was tested with a 21 nm tunable range, from 540 to 561 nm. More than doubling the tunable range of this laser technology allowed the inclusion of both the 552 and 561 nm lines commonly used in flow cytometry. Once again, the wavelength bandwidths, noise levels and power levels were similar to those found with traditional single wavelength lasers. The optimal excitation wavelength for specific green excited fluorochromes (phycoerythrin, rhodamine, sulforhodamine 101) and red fluorescent proteins (DsRed, mCherry, etc.) could be selected. Excitation wavelength for specific green excited fluorochromes - bandwidths, noise levels and power levels were similar to those found with traditional single wavelength lasers. The optimal excitation wavelength for specific green excited fluorochromes (phycoerythrin, rhodamine, sulforhodamine 101) and red fluorescent proteins (DsRed, mCherry, etc.) could be selected.

22 Low-Latency Time-Resolved Fluorescence Lifetime Flow Cytometry and Cell Sorting Using a CMOS Silicon Photomultiplier

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Background: Accurate and fast measurement of fluorescence lifetime in flow cytometry would prompt application to studies of protein-protein interactions by Forster resonance energy transfer (FRET), which are otherwise difficult or impossible to analyse [1]. Time-correlated single photon counting (TCSPC) has the best signal to noise ratio of any fluorescence lifetime technique [2] but cannot readily be implemented within a cytometer due to the requirement of complex software algorithms, severely restricting the throughput and preventing real-time sorting of cells [3].

The SiPM is a single-chip silicon photomultiplier detector employing a CMOS single-photon avalanche diode (SPAD) array light detector and time to digital converters (TDCs) [4]. The sensor’s pile-up resistant embedded centre of mass (CMM) processor accomplishes low-latency measurement and thresholding of fluorescence lifetime. A digital control signal is generated with a maximum 16.6 μs latency for cell sorter actuation.

Methods: The detector was validated in autonomous detection and lifetime-based sorting of fluorescent bursts resembling flow cytometer fluorescent transients. Series of regularly repeating fluorescent bursts were created by laser scanning over a sample of capillaries containing Firefly and FITC-stained beads with 2ns and 4ns lifetimes respectively. Excitation light was provided by a pulsed laser operating at 20MHz frequency and 473nm emission wavelength. Burst-integrated fluorescence lifetime data was collected using the SiPM detector and a commercial fluorescence lifetime detecting system for validation [1]. The processor was subsequently applied to create a real-time electronic actuation signal that matched the validated fluorescence lifetime and intensity data.

Results: The SiPM achieves an exposure sampling rate higher than 1kHz using TCSPC. The embedded CMM algorithm transfers event data to a field-programmable gate array (FPGA) with a

The photomultiplier tubes (PMTs) in flow cytometers capture fluorescence light and convert it into voltage pulses. The precision and resolution of this process are affected by the total background noise (B) and detection efficiency (Q) of the PMT hardware. In particular, B and Q affect how well cellular receptors with low expression can be measured. Staining panels may even be designed to avoid measuring dim markers on PMTs with poor resolution. Unfortunately, current methods to assess PMT resolution only provide rough estimates of Q and B. Thus, staining panel design remains a largely empirical process, requiring time and intimate experience with an instrument’s performance.

Recently, Jim Wood (Wake Forest University) developed a new device, known as an LED Pulser, to measure B and Q directly and accurately. This device delivers consistent and uniform pulses, the amplitude of which can be adjusted with a potentiometer. Our presentation will demonstrate how this device is used to calculate Q and B values. In practice, the LED Pulser, along with calibrated fluorochrome-loaded beads, can determine the relationship between fluorescence channel numbers and the number of photoelectrons a PMT captures (the statistical photoelectron estimate (Spe)). This information, along with estimates of receptor density, can be used to calculate an index value that provides quantitative guidance for panel design. Our presentation will describe this process step-by-step, with examples. Additionally, we will show how the metrics can be used for inter-laboratory comparisons.
5.3 μs latency. The FPGA generates a real-time actuation signal to sort detected particles with a maximum event processing delay of 11.3 μs and an error rate of 0.6%. The 16.6μs latency actuation control enables fast lifetime determination and thresholding, thus increasing cell throughput and eliminating the requirement for external processing in time-resolved flow cytometers to implement sorting.

Conclusions: Sensor architecture and experimental results will be presented validating the detector functions of fluorescence lifetime measurement and fluorescence-activated sorting. Preliminary flow cytometry tests will also be introduced accomplishing the integration of the SiPM detector in a flow cytometer system.

References:

23 The First Multi-center Comparative Study Using a Novel Technology Mass Cytometry Time-of-Flight Mass Spectrometer (CyTOF2) for High-Speed Acquisition of Highly Multi-parametric Single Cell Data: A Status Report

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CyTOF2 is a novel technology for real time analysis of single cells using inductively coupled plasma time-of-flight mass spectrometry. Mass cytometry is a significant advance for studies in medical fields including immunology, hematology, and oncology. Scientists from the Ragon Institute of MGH, MIT and Harvard; Dana-Farber Cancer Institute; Icahn School of Medicine at Mount Sinai; University of Virginia School of Medicine; Stanford University School of Medicine, Fluidigm, and Yale University School of Medicine are pooling CyTOF2 instrumentation data in a cross-center comparison and validation for single-cell analysis. Human Peripheral blood mononuclear cells (PBMCs) were stained with 8 metal-conjugated antibodies and the samples shared with each center. Multiple concentrations of cells (1 μL, 350K, 100K, & 50K/ml) were acquired on the CyTOF2. All samples were run within a 3 day window of being labeled. Prior to acquisition, all systems were calibrated and their performance were checked. Our validation samples will consist of beads with a mixture of four metals. Validation parameters such as mass resolution, limit of quantitation, linearity, precision, accuracy, ruggedness, stability, and system suitability will be evaluated in this report. System suitability tests was based on the concept that the equipment, electronics, analytical operations, and samples formed a uniform system that could be evaluated as a whole. System suitability was used to ensure performance before or during the acquisition of the samples using the reproducibility of replicate injections, single cells numbers and resolution, as the criteria. These parameters will be evaluated throughout the analysis. A computational analysis of the data was performed in CYTOBANK. Analysis of pooled CyTOF2 instrumentation data will be presented. This work is the first of its kind, to cross-examine and validate the capability of CyTOF2 systems at seven different sites across the US and compare the reproducibility of the mass cytometry data produced using these machines.

24 Measuring Protein-Protein Interactions by Fluorescence Lifetime Flow Cytometry
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2University School of Medicine are pooling CyTOF2

Protein binding, dimerization, clustering or conformation changes play important role in cell signaling, drug efficacy or disease development. In intact cells, these functionally important events can be probed by measuring the Förster resonance energy transfer occurring between two suitable fluorophores separated by less than a few nanometers. The fluorophores can be linked to different staining antibodies, constitute expressed fluorescent proteins, organellar specific dyes or their combinations. Currently, FRET detection in conventional flow cytometry is restricted to semi-quantitative ratiometric measurement requiring consistent and well defined fluorophore stoichiometry. It is well understood in the field of microscopy that reduction in donor fluorescence lifetime is the most reliable, quantitative and accurate measure of FRET. It is compatible with the widest range of assays, imposes the least experimental restrictions on the sample and offers the highest dynamic range and sensitivity. Only very recent developments have made fluorescence lifetime measurements in flow cytometry sufficiently accurate and sensitive to detect its minute changes involved in FRET.

We have initially demonstrated FRET measurements on a custom-built fluorescence lifetime flow cytometer in studies on HER1 kinase activity. In this work, a fluorescent HER1-GFP construct served as the energy donor for Cy3 labeled antibodies binding ubiquitous phosphorylated tyrosines. Antibodies binding specifically phosphorylated HER1 were distinguished by FRET-induced GFP lifetime shortening upon stimulation with EGF. In our newest set of experiments, we have focused on overcoming the need for transfection or permeabilization of cells. We examined the dimerization of HER2 and HER3 on SKBR3 cell membrane by measuring FRET from anti-HER2-Alexa546 to anti-HER3-Cy5 antibodies. Cells were stimulated with Neuregulin-1, driving cell signaling through HER2/HER3 dimer formation and thus bringing the fluorescence labels into close proximity such that FRET occurred. HER2/HER3 dimerization-induced FRET was validated both by fluorescence lifetime flow cytometry and fluorescence lifetime imaging.

Experiments demonstrated that fluorescence lifetime flow cytometry can facilitate FRET measurements in studies on protein dimerization or clustering. Direct antibody labeling of extracellular epitopes allowed examination of intact cells. Using combination of these techniques, relevant experiments can be designed for cell signaling, cell-to-cell interactions and drug binding studies or patient screening for drug selection in personalized medicine. Crucially, these experiments could not be directly performed with conventional flow cytometry, as consistent fluorophore stoichiometry could not be ensured. Until recently, fluorescence lifetime imaging microscopy would be the only available acquisition method. However, our comparably sensitive and accurate fluorescence lifetime flow cytometer benefits from vastly enhanced analysis throughput, speed of data acquisition and volume of analyzed cells, enabling rigorous statistical data analysis and rare feature detection.
Background: Tumors are composed of diverse cell types, including cancer cells that can differentially evade therapy and stromal cells that may aid or impede treatment. The emergence of therapy resistant cell subsets is a primary cause of treatment failure in aggressive malignancies, but most high content approaches in cancer research do not achieve single cell resolution and rely on culture-adapted cell lines as model systems. Quantitative fluorescent studies of melanoma can also be confounded by variable intrinsic fluorescence of primary melanoma cells.

Methods: To address these challenges, a 28-antibody mass cytometry (CyTOF) panel including markers of immune cells, endothelial cells, and neural origin melanoma cells was created to characterize melanoma tumor biopsies. A key protein measured in the panel was Nestin, an intermediate filament protein expressed in melanoma cells. In healthy cells, Nestin is expressed embryonically and in rare adult cells, including neural stem cells. Primary tumors from 16 individual patients and 7 melanoma cell lines were characterized by mass cytometry. For 4 patients, the comparison included biopsies obtained before, during, and after treatment as part of a clinical study targeting mutant BRAF and MEK signaling proteins. All specimens were obtained in accordance with the Declaration of Helsinki following protocols approved by the Vanderbilt University Institutional Review Board. Single cell suspensions for mass cytometry analysis were prepared using collagenase-based disaggregation. Data analysis was performed on Cytobank using unsupervised tools (SPADE and viSNE) and traditional directed analysis.

Results: Within primary human tumors, infiltrating leukocytes were distinguished by high CD45, comprising 14.3% ± 23.3% of tumor cells, and were dominated by CD8 T cells expressing CD45RA. Endothelial cells were rare (<5% of CD45 cells) and expressed CD31 and CD61. The remaining CD45 - tumor cells were >98% cancer cells distinguished by hallmark melanoma proteins (e.g., Nestin, MCAM, NGFR). In vivo changes in intra-tumor cellular heterogeneity were quantified following therapy and used to identify phenotypically distinct melanoma cell populations that persisted despite treatment. Lower median Nestin protein expression distinguished melanoma cells that persisted following treatment from those that regressed (p < 0.0001, Bonferroni corrected α = 0.002). Within 6 established melanoma cell lines, cells with this melanoma persister phenotype were present, but rare, and constituted on average 7.0% +/- 3.9% of cells.

Highlights: This study establishes a high content single cell mass cytometry approach for characterizing human melanoma tumors. Loss of Nestin protein expression was a significant feature of melanoma cells that persisted in vivo, despite therapy targeting BRAF and MEK signaling. Clinically significant melanoma cell subsets observed in human tumors were uncommon in established cell lines.

26 Modeling Human Immune Dynamics in Surgery and at Steady State with Mass Cytometry

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Recent advances in single cell technologies have enabled immune monitoring in unprecedented detail. These developments hold significant promise for evaluation, diagnosis, and treatment in cases of immune perturbation and disease. Our recent work used mass cytometry to profile patients undergoing hip replacement surgery (Gaudilliére and Fragiadakis, et al, Sci Transl Med, 2014). Blood was sampled at pre-surgical baseline, one hour, two four hours, 72 hours, and 6 weeks post-surgery. The simultaneous measurement of 21 surface markers and 11 functional proteins enabled the tracking of both the redistribution of major immune cell subsets in the blood as well as changes in signaling activity within each subset over time. When regressed against measures of clinical recovery including fatigue, pain, and hip function, strong correlations were found with pSTAT3, pNFκB, and pCREB signaling in monocyte subsets (R=0.7-0.8, FDR < 0.01). Signaling in these monocyte subsets serves as an early prognostic indicator of surgical recovery to be developed as a diagnostic test using traditional flow cytometry available in the hospital setting.

This study further serves as a proof-of-concept for the ability to use mass cytometry as an immune monitoring tool in a clinically relevant context. We applied these methods to further explore the immune system at steady state, asking to what extent the immune system fluctuates over time in an individual, as compared to immune variation between individuals. Twenty healthy volunteers were serially sampled at four time points over the course of one month, their samples subdivided and stimulated ex vivo with a series of immune perturbagens. Sources of experimental variability were minimized through the development of a 50-antibody lyophilized staining cocktail, sample pre-permeabilization barcoding, and bead-based normalization. A subset of samples was run in triplicate as a metric of experimental variability to use as a threshold for considered immune variability.

Immune features were modeled to create metrics of immune health and to determine the relative stability of immune features within an individual over time. This suggests which aspects are inherent immune traits in an individual versus those that are fluctuating immune states. This data will be compiled as a reference for other mass cytometry studies to compare against clinical samples in both the acute setting such as surgery and infection, and in the context of chronic disease.

27 Cell-o-pane – An Interactive Environment for the Visualisation and Characterisation of Cellular Lineage Data Derived from Timelapse Microscopy

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Studying how tissues (animal or plant) grow, develop and also respond to environmental and genetic influences reveals the fundamental mechanisms of cell cycle function, cell routing and stem-cell behaviour. Deciphering cellular status and response to different perturbing conditions such as a tumour population responding to a drug or a plant root responding to the environment remains a key research emphasis. The analysis of cell origin and the changes in progeny requires a detailed understanding of tissue cell types and their position in the lineage hierarchy. Perturbation of the system causes a series of time-dependent cellular responses, where an understanding of the variation in responses could provide insight into complete lineage behaviour. We propose that in cytometry terms, an encoded cell lineage reflects the relationships between descendants from a common ‘start cell’ exposed to a particular external influence over time. The behaviour of both the progenitor and its progeny reveals the time-integrated response to an influence such as a bioactive drug and may also reveal asymmetry in the evolution of the response within the lineage. The project undertaking was to...
connect a series of tools, creating an integrated pipeline for the analysis of high-content time series image data. Here we focus on the visualization of lineages, and the provision of a simple tool that enables cell biologists to interact and interrogate lineage information in a systematic way. Cell-o-panes was designed to emphasize three important properties. (i) Multi-modality – cell lineages combine spatial, temporal and other properties (ii) Symmetry – which characterizes the lineage branching structure; and (iii) synchrony which is related to temporal alignment of cellular events. Our approach has been applied to a case study for determining the pharmacodynamic action of anti-cancer agents. Thus we have used Cell-o-panes to dissect the impact and operation of asymmetric events and inter-relationships of cells revealing the strategies for lineage expansion, including differential patterns of cytotoxicity across the branching structure. 

### Methods

We engineered a multifunction workstation to facilitate time-resolved luminescence detection under various modes - from microplate reader and scanning cytometry to wide-field and confocal imaging. Modular design was implemented on top of an epifluorescence microscope, providing flexible integration of excitation, scanning and detection modalities to accommodate different down-conversion and upconversion lanthanide materials. The data acquisition and processing algorithms were specifically designed for slow-decaying luminescence, allowing the microsecond-region lifetimes of individual targets to be measured in a precise and rapid fashion.

### Results

After proper optical alignment and electronic synchronization, the detection limit of the system was evaluated to be ~100 photoelectrons. Signal-to-noise ratios over one order of magnitude were easily achieved for molecular assays down to sub-nM concentration. This allowed automated detection for lanthanide-labeled cells that were randomly distributed on a sample slide, conferring unambiguous results in less than 3 minutes. Moreover, taking advantage of open-loop position sensors, we were able to precisely pinpoint every target during the rapid scanning, and retrieve individual's location for true synchronization, the detection limit of the system was evaluated.

### Conclusions

We demonstrated a versatile workstation suitable for long-term lanthanide probes, which provides flexible workflow to efficiently address diverse sample formats. As research on lanthanide materials keeps advancing, we envision this workstation shall support the development of new probes and broaden their applications in biosensing and analytical sciences.

### 29 Optimal 3D Segmentation of Cell Nuclei in Tissue and Acinar Structures

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A major bottleneck preventing widespread analysis of individual cells in tissues is the lack of an efficient method that assures correct 3D segmentation of each cell or cell nucleus. In this study, we present a fully automatic method for segmentation of cell nuclei from 2D or 3D images of tissues and acinar structures, which was inspired in part by a globally optimal surface segmentation method for volumetric medical images (doi: 10.1109/TMI.2014.2304499). Given a pre-defined edge measure of the user's choice calculated at each voxel in the image, the proposed method transformed the image of the edge measures to a geometric volume in spherical space with respect to a point of reference internal to a target nucleus. It then found the globally optimal surface (defined as the surface with the highest possible total edge strength) in that geometric volume that separated the target nucleus from the rest of the volume. The algorithm for obtaining the optimal surface posed the problem as finding a minimal closure in a directed graph and solved it efficiently using the maxflow-mincut algorithm. It was highly efficient and operated in near real time on a standard desktop computer. The method was restricted to each segmented object being point convex, in other words, every straight line from the optimal surface to the reference point was entirely within the object. This, however, is a mild limitation for cell nuclei in 2D and 3D images. For full automation, a reference point had to be found for each cell nucleus. For 2D images, an initial segmentation was performed by a dynamic programming method that equally weighted edge strength, edge direction and edge curvature, from which a reference point approximately near the center of the nucleus was calculated. The method readily extended to full automation of 3D images by utilizing a voting scheme amongst points found in each 2D slice of the 3D image. In an alternative, interactive version of the method, the user manually marked each point of reference, and in addition the method allowed manual correction of apparent segmentation errors by utilizing border points on the surface marked by the user. The method was evaluated using 2D and 3D cell nuclei samples in tissues and a 3D acinar structure labeled with a DNA dye such that 3D fluorescence microscope images showed edges at the interface between bright nuclei and dark background; a 2D image of cultured cells labeled with a plasma membrane marker, and computer-generated 3D images of closely juxtaposed nuclei in tissue so that ground-truth nuclear borders were known. Experiments compared the segmentation obtained by the proposed method to that obtained from a geodesic distance transform based segmentation method published in 2014 in terms of three evaluation metrics: Rand index, overlap ratio and boundary deviation. The proposed method outperformed in all tests.

In conclusion, by utilizing methods based on global optimization, we demonstrated superior robustness and accuracy for cell nucleus segmentation in 2D and 3D images. Given that all the methods extend to higher dimensions, in future work, we will investigate methods to circumvent the restriction to point convex objects thus enabling optimal tracking of cells in 4D.
Precise Pinpointing of Luminescent Targets Empowers Quantitative Scanning Cytometry

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Background: Image cytometry has emerged as a complementary method to flow cytometry when samples are of small volume and ready to examine in solid phase rather than in suspension. It has found diverse applications in cellular and molecular biology, pharmaceutical discovery and clinical diagnostics, where confirmation of the results holds the priority. However, existing techniques, either employing confocal laser scanning or relying on advanced image analysis, require detailed recording for the entire sample area and sophisticated data processing before the targets are recognized and quantified. Moreover, they often find difficulties to address complex samples containing multiple analytes as well as environmental substances, which effectively limits their broad practice for high-throughput and high-content screening.

Method: We explored rapid sample scanning in conjunction with real-time signal processing for detecting and measuring luminescent targets of micrometer size. Two linear encoders and an autofocus unit were implemented to provide synchronous spatial reference in X-Y and Z directions, respectively, coinciding with the photodetector output. This enables precise pinpointing of every individual target, allowing the luminescence signal to be measured under identical conditions. A scanning cytometer was thus developed, capable of statistically analyzing target populations with enhanced quantification at high speed.

Results: We evaluated the scanning cytometer using polymer microspheres incorporating upconversion nanoparticles (UCNP), which were randomly distributed onto glass slide samples. The signal profiles of luminescence vs. displacement recorded during continuous scanning were used to determine the locations of individual microspheres. The spatial precision with respect to the middle of the wide field-of-view (FOV) was found to be ±1.31 μm and ±1.75 μm (in Standard Deviation) for the X and Y directions, respectively, and all targets appear exactly on focus during subsequent imaging confirmation. Three microsphere populations with incremental amounts of encapsulated UCNP were statistically examined, and well-separated intensity histograms were obtained with coefficient of variation (CV) ranging from 6.35% to 17.1%, around 2.5-folds better than previous results using standard image cytometry (CV between 15% and 42%). Moreover, upconversion microrods consisting of sections of upconversion nanoparticles (UCNP) were statistically examined, and well-separated intensity histograms were obtained with coefficient of variation (CV) ranging from 6.35% to 17.1%, around 2.5-folds better than previous results using standard image cytometry (CV between 15% and 42%).

Conclusion: We demonstrated a new scanning cytometry method capable of precise pinpointing and quantifying luminescent targets during rapid scanning. Being easier and faster to operate than conventional image cytometry, it may open new opportunities in biological and clinical applications including rare-event detection, multiplexing sensing and quantitative assays.

A Reference Framework for Modeling a Dynamic Immune System

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Immune cells comprise an interacting hierarchy that coordinates its activities according to the context of the organism. We adapted a force-directed graphical approach to construct an extensible immune reference map from mass cytometry data of different organs, incorporating landmark cell populations as flags on the map to compare distinct samples. Using healthy murine bone marrow as a reference point, the unique organization of immune cells within various organs across the body was revealed. The maps recapitulated canonical cellular phenotypes while revealing reproducible, tissue-specific deviations. The approach revealed the influence of genetic variation and circadian rhythms on immune structure, enabled direct comparisons of murine and human blood cell phenotypes, and even enabled archival fluorescence-based flow cytometry data to be mapped onto the reference framework. The framework additionally enables zooming in on regions of interest to reveal local structure and spatial distributions of subpopulations of interest. This foundational reference framework provides a working definition of systemic immune organization to which new data can be integrated to reveal deviations driven by genetics, environment or pathology.

Cell Surface Proteomics Reveals Changes Associated with TGFβ-Induced EMT in Lung Cancer

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The epithelial to mesenchymal transition (EMT) plays a key role in wound healing and cancer progression. The highly epithelial lung adenocarcinoma cell line A549 can be induced to undergo EMT within 48 hours of exposure to TGF β. We used the Lyoplate scanning panel (BD Biosciences) to investigate changes in expression of 242 antibodies to cell surface markers accompanying TGFβ-induced EMT (48h, 10 ng/mL). EMT was confirmed by morphometric analysis of bright-field image data, assessment of motility in the ‘scratch assay’ and expression of key mesenchymal and epithelial markers by immunofluorescence microscopy and 3D-color flow cytometry. Manual file-level data analysis followed by K-means clustering on percent positive, MFI positive and MFI total were used to classify each marker into one of 4 groups corresponding to negative, weak positive, positive and strong positive. Eight markers were upregulated and twelve were down-regulated with EMT. Among the strongest upregulated markers, four were associated with migration/motility (CD29, CD62e, CD99, CD108), two were immuno-stimulatory (CD15/SSEA-1, CD278), one a known mesenchymal marker (CD90) and one an enzymozyme involved in calcium signaling (CD38). Four down-regulated markers are involved in epithelial differentiation (CD24, CD227/MUC-1, CD321, CD326/EpCAM), four are adhesion molecules (CD104, CD49f, CD47, CD66), three involved in coagulation or complement activation (CD141, CD201, CD46) and one a receptor tyrosine kinase (CD221). CD105, a known mesenchymal marker, was negative and remained unchanged. Eight color-flow cytometry revealed further upregulation of CD73 which was highly expressed in untreated A549 cells and therefore not detected by the clustering algorithm, as well as marked upregulation of intracellular vimentin, both known mesenchymal markers. Immunofluorescence confirmed modulation of vimentin and EpCAM. The ‘scratch’ motility assay, combined with morphometrics and immunofluorescence, confirmed the mesenchymal morphology and phenotype of TGFβ-treated migratory cells. Modulation of many of these markers has not been previously associated with EMT, demonstrating the utility of the cell surface marker proteomic screening system and providing...
multiple leads for markers of progression and targets of therapy in epithelial cancers.

33 Flow Cytometry of the Intestinal Microbiota in Health and Chronic Inflammation

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The intestinal microbiota is a complex and heterogeneous composition of 10\textsuperscript{12} bacteria of several hundreds of bacterial species. In mice, the microbiota was shown to be critically involved in the control of immunity and immune-mediated diseases. In humans, the situation is less clear, largely because of the lack of analytical and preparative methods to dissect the heterogeneity of the microbiota. Currently, most studies of the microbiota rely on qPCR- or pyrosequencing-based analysis of the bacterial metagenome. This is expensive, population-based, restricted to the genome and transcriptome and does not allow to isolate defined bacteria for further analysis.

Here we present a cytometric approach to unravel the complexity of the commensal microbiota. High resolution forward and side scatter detection, together with quantification of DNA content, already allows to distinguish over 35 different bacterial subpopulations from formaldehyde-fixed murine stool samples. Using this method, we tracked the changes of the microbiota in the course of several mouse models for inflammatory bowel disease. We observed a significant loss of microbial complexity upon intestinal inflammation. Intriguingly, changes in the microbiota induced by Th1-mediated intestinal inflammation differed from those brought upon by Th17 cells, highlighting the power of the method to identify disease-specific patterns of changes in the microbiota (dybiosis).

Flow cytometry thus provides unique options for the analysis of the microbiota and its role in disease, at the level of proteome, phenotype and single cells, and permits to isolate defined bacteria, for functional and molecular analysis, but also for therapeutic corrections of the microbiota in dybiosis.

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34 Use of Flow Cytometry and Correlate GFP Fluorescence Lifetimes to pH Shifts in Macrophage Cells Infected by E. coli

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Background: Macrophage cells ingest and degrade foreign substances, such as bacteria through phagocytosis. It is a defense mechanism that involves engulfment, proton-pumps activation, membrane fusion events (e.g. phagosome-lysosome fusion), etc. The evolvement of phagosome maturation ensures the degradation of the engulfed bacterium with low pH, yet some bacteria that survive in the host are capable of manipulating the pathway to the phagosome maturation or escaping from the phagosome to the cytoplasm. Hence, tracking pH shifts during phagocytosis process is rather important in observing bacterial infection. The engulfment of bacteria is heterogeneous, random and a time-dependent process. In order to solve the problem caused by heterogeneity, time-limiting microscopy, spectral overlap issues, and subtle shifts in fluorescence intensity, we propose the use of fluorescence lifetime-based flow cytometry (FLFC).

Methods: FLFC uses digitally-modulated laser excitation at a radio frequency of 25MHz. The subsequent fluorescence and side-scattered light signals are collected and the fluorescence lifetime of each event is computed by the phase difference between the side scatter and fluorescence. This approach not only provides the average intensity of individual infected and fluorescently labeled macrophages, which is related to the number of bacteria engulfed, but also has the ability to measure the average lifetime or multiple lifetimes of individual fluorescently labeled macrophages, which is related to the pH level inside the vacuoles. RAW264.7 cells were infected by GFP expressing E. coli with 10 multiplicity of infection (MOI) for t = 5, 30, 60, 90, 120 or 180 min, respectively.

Results: Our initial results show that differences in the populations of E. coli and RAW264.7 cells are detectable by their fluorescence and side scatter intensities, as well as by average fluorescence lifetime measurements. The fluorescence intensity of the infected RAW264.7 cells gradually increases as infection time increases and the average GFP fluorescence lifetime decreases. At t \textsuperscript{=} 5, 30, 60, 90, 120 or 180 min, the average GFP fluorescence lifetime of the infected cells is 3.0 (+/-0.1) ns, 2.8 (+/-0.06) ns, 2.3 (+/-0.09) ns, 2.1 (+/-0.07) ns, 2.0 (+/-0.06) ns, 2.0 (+/-0.08) ns, respectively. The average GFP fluorescence lifetime, in contrast, does not change 3.1-3.3 (+/-0.1) ns when E. coli is not confined inside macrophage vacuoles.

Conclusions: These results demonstrate that intracellular accumulation of E. coli, which express GFP inside of macrophage cells, is correlated to a decrease in the GFP fluorescence lifetime when compared to control cells. We believe this presents an interesting step toward the use of GFP fluorescence decay kinetics as a pH indicator inside of cells. In this example, phagocytosis is known to involve localized pH changes, and while it is probable that this mechanism be studied at a cytometry throughput with pH-sensor dyes, the process of engulfment itself is heterogeneous. The results presented are a first step toward obtaining quantitative cell-to-cell statistics of subtle, intracellular pH shifts.

36 Phenotypic Changes on Blood Neutrophils Differentiate Patients with Sepsis from Those with Noninfectious Systemic Inflammation

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Introduction: Differentiating patients with the systemic inflammatory response syndrome (SIRS) from those with sepsis (SIRS plus infection) is a major challenge for the clinical management of critically ill patients in intensive care units (ICUs). Neutrophils are the main cellular host response to destroying pathogenic bacteria and these cells also participate in the initiation and perpetuation of inflammation. The aim of this study was to determine whether an alteration in the expression of molecules on the surface of blood neutrophils, particularly those associated with adhesion, could delineate patients with sepsis from those with non-infectious (N-I) SIRS.

Methods: A total of 112 critically ill patients, who satisfied the criteria for SIRS, were enrolled into the study within 48 hours of ICU entry and were retrospectively characterized into the following groups: 38 with definite sepsis (positive microbiology), 33 with clinical sepsis (negative microbiology but a strong clinical suspicion of infection) and 41 with N-I SIRS (negative microbiology and no clinical suspicion of infection). Fourteen healthy volunteers were also included. By means of flow cytometric analysis, neutrophils in whole blood samples were stained with antibodies against CD64, CD49e, CD35, CD29, CD11c, and CD11b. Results were expressed as the median flow intensity (MFI). Neutrophils were identified by their reactivity with anti-CD15 antibodies. Analysis of the flow cytometry data was by using VenturiOne software.
Results: Table 1 shows that the MFIs of CD64, CD49e, CD35, CD29 and CD11c were higher on neutrophils from patients with definite sepsis when compared with patients with N-SIRS. Of the patients with clinical sepsis the only marker that was upregulated on neutrophils was CD64. The expression of CD11b was similar in all 3 patient groups. The table also shows that by using receiver operator characteristic (ROC) curves CD64 had the highest sensitivity and specificity for diagnosing patients with sepsis.

Conclusion: This study demonstrates that several surface molecules are upregulated on blood neutrophils from patients with definite sepsis when compared with N-SIRS. We propose that a combination of these cellular biomarkers could have diagnostic significance for sepsis.

37 SmartFlare Probes Detect Only the Translationally Available mRNA in Live Cells, but Not the Total Pool of mRNA Quantified by Real-Time RT-PCR: A Novel Limitation of the Method

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Background: The possibility to detect mRNA in live cells by flow cytometry demonstrates big potential for research and medicine, including ability to identify and sort cells based on the internal RNA markers as well as the use of mRNA markers for diagnostics. SmartFlare technology was recently introduced as providing possibility for detecting, isolation and characterization of viable cells based on the RNAs. These studies were performed to investigate whether the SmartFlare probes recognize the total pool of mRNA, which is detected by conventional RT-PCR method, or detect only the translationally available pool of mRNA. Especially we were interested, if mRNAs which are present in the cell but are sequestered from translation due to formation of mRNA-protein complexes and storage in stress granules are available for SmartFlare Probes.

Materials and Methods: We used the previously described model, in which we showed that the BRCA1 protein level is decreased in chronic myeloid leukemia cells not because of decreased BRCA1 mRNA level but because of inhibited translation (1). CML cells showed higher BRCA1 mRNA level compared to parental cells, as determined by RT-PCR, although the BRCA1 mRNA was bound to mRNA-binding protein TIAR and protected from translation. BRCA1 mRNA level was estimated either by specific SmartFlare detecting BRCA1 mRNA or by real-time RT-PCR. Simultaneously, scramble control and uptake control SmartFlare probes were used. Translation was estimated by luciferase reporter containing 3'UTR of BRCA1 mRNA. Protein-mRNA complexes were studied by immunoprecipitation followed by real-time RT-PCR. TIAR localization in stress granules was verified by immunofluorescence. To finally verify our hypothesis we used thapsigargin, leading to activation of stress response and TIAR, formation of stress granules and decreased BRCA1 translation.

Results: We found here that the specific SmartFlare probes bind only the translationally available, but not the total pool of mRNA. Inhibition of BRCA1 translation led to decreased detection of BRCA1 mRNA level by SmartFlares. Thus, the mRNA level measured with the SmartFlares differed from those detected by real-time RT-PCR, which quantifies the total pool of mRNA. This results from inability of SmartFlares to recognize and bind mRNAs stored in complexes with mRNA binding proteins and sequesterated from translation.

Conclusion: We described a novel limitation of the SmartFlare technology connected with inability to detect translationally inactive mRNAs. This can be especially important for identification and sorting of cells dependently on the marker mRNAs, such as proposed for stem and other rare populations of cells as well as for the diagnostic applications.


38 Genomic Cytometry: Flow Analysis and Sorting of Rare Cancer Cells Using qRT-PCR Signatures on a Single Cell Basis

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Genomic cytometry is a new field combining the high-throughput capabilities of flow cytometry and cell sorting with single-cell multiplex genomic analysis in microfluidic drops. The genomic cytometry method we will describe is PCR-activated cell sorting (PACS). PACS is analogous to Fluorescence-Activated Cell Sorting (FACS) in throughput and the ability to analyze single cells; however, unlike conventional flow cytometric immunophenotyping, PACS uses extremely sensitive microdroplet TaqMan PCR reactions to identify and, if needed, sort cells of interest for additional downstream molecular characterization. This unique approach enables PACS to identify cells based on the presence of transcripts, genomic DNA sequences, mRNA splice variants, SNPs, non-coding RNAs, and other biomarkers not amenable to antibody-based probe detection. PACS can be used to sort and enrich cells targeting single-copy genomic DNA for the analysis of circulating tumor cells (CTCs), intra-tumor heterogeneity, rare stem cells, viral or pathogen infections and genomic characterization of cancer immune system response and disorder. With PACS, we identified and sorted prostate cancer cells from a heterogeneous population by performing ~132,000 simultaneous single-cell TaqMan RT-PCR reactions targeting vimentin mRNA. Following vimentin-positive droplet sorting and downstream analysis of recovered nucleic acids, we found that cancer-specific genomes and transcripts were significantly enriched. We will report on the ability of PACS to efficiently recover and perform downstream qRT-PCR characterization on rare cancer cells.

39 DOTS qPCR: A Handheld, Rapid Molecular Diagnostic Tool for Pathogens

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As the global human population continues inexorable increase, the likelihood of emergence and transmission of infectious diseases also increases. The recent outbreak of Ebola in West Africa offers a particularly deadly example of this process. Strategies to control and contain infectious diseases require accurate, speedy and cheap detection of the infectious agent, establishment of treatment centers that minimize human-to-human transmission, and development of effective treatments. Ebola virus infection provides considerable current cause for concern due to a lack of effective interventions, its high lethality,
and a lack of rapid and sensitive means to detect viral presence. This is further complicated by disease emergence within highly populated regions of Africa that lack a reasonable health care structure, and by the social activities associated with burial of infected deceased individuals.

Molecular diagnostics, based on detection of the Ebola virus genome based on the Polymerase Chain Reaction (PCR), represents a sensitive approach to screen for infection. However, current PCR machines are large, expensive, and complicated, and do not operate at the speeds required to make clinical decisions at the point-of-care. We have developed a novel PCR device, termed Droplet-On-Thermocouple Silhouette Quantitative PCR (DOTS qPCR), which is fully automated, small and portable, able to provide detection directly from patients, with sample-to-answer times as fast as 5 minutes. The novel DOTS qPCR methodology, which will be described in this presentation, utilizes interfacial effects for droplet actuation, inhibition relief, and sensing, with sub-picogram limits of detection, high specificity, and thermocycling speeds of 28 seconds/cycle in the presence of blood and/or tissue contaminants. This device can potentially be interfaced with standard smartphones for data collection, processing and transmission. It is universally applicable for detection of any biological disease organism of significance, such as other emerging human and agricultural diseases, and even biological weapons.

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cytometry Technology Speeding the Response to Global Health Issues
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During the 1980’s, cytometry was introduced as an analytical instrument to immunologists who were confronted with the expanding threat of AIDS pandemic. The decades that followed saw rapid development in biosciences leading to unprecedented evolution in the field of biotechnology in general and in cytometry specifically. Efficient optical configurations, improvement of light sources, and dramatic enhancement in electronic signal processing continued. Applications including differential diagnostics to support hematopathology became a reality. By the end of the 20th century, flow cytometry was recognized as a biomedical platform. The instrument, once limited to research, continues to expand its global field of applications. Countless scientific disciplines are now on board using cytometry as routine analytical instrumentation. The diverging list of application is extensive: probing ocean plankton distribution patterns, performing rapid epidemiological assessment of children’s immune-profiles, sorting of XY chromosome bearing sperms for animal gender pre-selection, exploring oceans and accommodating astronauts with health status-monitoring platom to support future pursuit of manned deep-space exploration.

We are also facing emerging global health threats such as Ebola from Africa. While there has been stellar progress over the past 35 years in biotechnology, however technology by itself without great insight and expertise can be stagnant. However, with passionate and selfless visionary scientific architects, focused on public health issues, dramatic progress can occur an example of initial failure was how the Ebola outbreak was not contained. Once experts are placed in the field results follow. Three remarkable individuals who are no longer with us with unlimited devotion to improve the human condition pushed our horizon to implement technological advantages such as cytometry and related skills to speed the response to public health challenges years ahead. Our colleagues with social awareness, steadfast vision and determination have managed to set a course that will assist us to navigate the hazardous path beyond the first quarter of the 21st century in terms of better addressing global health issues.

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Sound Technology for Infectious Disease Diagnosis
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Despite important improvements in prevention and treatment over the last decades, infectious diseases still represent a major threat to human health worldwide, with a significant impact on economies globally. To reach the ambitious goals set by WHO towards elimination of diseases such as Malaria or sleeping sickness (Human African Trypanosomiasis - HAT) in the next two decades. To respond to emerging threats, a step change in diagnostic methods is required on at least three fronts.

The tests have to be carried out in a decentralised fashion, at the ‘point-of-care’, to increase coverage and speed.

They have to provide high sensitivities to enable early diagnostics and potentially the discovery of asymptomatic carriers, which in the case of HAT translates into the detection of less than 100 parasites/ml.

Diagnostic assays also need to provide access to more information, for example through genetic typing or access to drug resistance susceptibility.

Here we show how acoustic technologies have provided us with a route towards addressing these challenges. In the form of Surface Acoustic Waves (SAW), the mechanical energy carried by sound has allowed us to enrich specific cells and parasites from blood samples and extract and amplify their DNA for diagnosis. Using the novel technology of phononics, akin to holograms for light, we shape sound waves on low-cost disposable devices, to integrate all necessary diagnostic functions in a ‘point-of-care’ device. This presentation will also discuss our efforts to commercialise this innovation through a start-up company.

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Lost in Translation
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Panel Members

- MS. Cherie Green, Genentech, South San Francisco, CA, USA
- Dr. Wendy White, MedImmune, Gaithersburg, MD, USA
- Dr. Fiona Germaschewski, GlaxoSmithKline, Stevenage, U.K.

The ability of flow cytometry to characterize large numbers of suspended cells by simultaneously assessing multiple markers with a reasonable degree of quantitation makes the technology very attractive for use in clinical trials. The utilization of flow cytometry in clinical drug development generally falls into a few broad categories including assays that help determine subject enrollment, evaluate safety, test hypotheses, or drive hypothesis generation. Enrollment determining assays are intended to establish whether an individual meets the predefined study specifications and are classified as either safety or biomarker assays. Safety assays typically examine a known or suspected health risk, the presence of which could impact the risk/benefit assessment of the drug or warrant clinical intervention for the study subject. Hypothesis testing assays generally have a pre-specified analysis sufficient to test a specific hypothesis and typically include pharmacodynamics (PD) and biomarker assays. The last category tend to be more purely discovery endeavors looking to identify a potential biomarker and/or PD markers associated with response to treatment or mechanism of action and typically involve tissue (most commonly blood) profiling to look for a pattern of response. Examples of the different types of
assays and considerations around their implementation in translational studies will be discussed.

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Cytometry with Tunable Ultrafast Lasers

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Ultrasound lasers with picosecond and femtosecond pulse duration and a repetition rate of 80 MHz or more are a well proven light source for microscopy applications. They deliver broadly tunable and very low-noise light at virtually any wavelength between 355 nm and 1600 nm. The high peak power allows for efficient driving of nonlinear optical effects utilized in several new imaging techniques such as two photon microscopy.

Intravital two-photon laser-scanning microscopy (TPLSM) proved to be the most versatile tool to study cell migration and cell-cell interactions in health and disease. Its power to enable investigations in genuine environment - the living organism - in a dynamic way, with high resolution is its hallmark among current high-performance technologies used in cell biology and biomedicine. The use of Ti:Sa lasers, fs-pulsed (100 fs – 140 fs), high-repetition (80 – 90 MHz) lasers, tunable between 690 nm and 1080 nm, made possible the excitation of various dyes as well as blue to orange fluorescent proteins. The development of red fluorescent proteins and their usage to generate reporter mice exceeded the excitation capacities of Ti:Sa lasers. Among other tunable sources adequate for this purpose, e.g. Cr:forsterite, Cr:BeAl2O4 lasers, actinid or lantanid fiber-lasers, parametric oscillators (OPO), and parametric amplifiers (OPA). The optical parametric oscillators (OPO) in particular has been proven to be the most versatile and reliable solution for TPLSM, delivering similar power levels and the same short pulses (sub-200 fs), but with an extended tuning range from 700 nm to 1900 nm. The latter feature is especially useful since many more and relevant fluorescent proteins can be investigated.

Recently, the reliability of such light sources has also been greatly improved, and the introduction of fiber lasers enabled very compact, no-maintenance systems, which are directly integrated into instruments. However, in imaging cytometry and in flow cytometry these light sources are currently seeing only limited use, although first experiments with ultrafast lasers have shown promising results. Here we report on the latest generation of light sources extending the tuning range of classical lasers and providing multiple wavelength output. An overview of their use in advanced microscopy methods such as fluorescence lifetime based methods, CARS, and SRS is presented.

Based on the reported methods an open discussion will be held with an expert panel and the audience to investigate the challenges and advantages of cytometry with ultrafast lasers.

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Metrics of a Successful SRL: Publications and Acknowledgements

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There are many ways in which a Shared Resource Laboratory (SRL) can justify its existence. These include such metrics as cumulative hours of use, number of users, income generated and so on. All of these can be modified by fiscal approaches that are designed to maximise income and for the usage per resource. Although these metrics will satisfy many financial departments, for an SRL to move forward they may be insufficient. From a career progression perspective, for staff satisfaction and retention, and for staff recruitment, the fact that an SRL is seen to contribute to the science of an institutional setting is important. But how can this be done? There are basically three ways; the SRL can publish independently, it can publish in collaboration with users or it can be acknowledged on papers. However, in the real world, all three approaches can be fraught with difficulty and many SRLs are all too often seen as part of a service industry that is ‘just doing its job’. How can this be changed? We will look at ways that SRLs can disseminate technical advances, how collaborations can be established and, at the minimum, how users of a facility can be encouraged to show proper acknowledgement. This workshop will concentrate on the ways that metrics of publication can be generated and look at ways of facilitating publication within an SRL. There will be some short presentations to allow maximum time for audience discussion.

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Foundations of Microvesicle Cytometry

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In recent years the cytometric analysis of microvesicles has increased dramatically, however there still remains much controversy in the literature due in part to lacking standardization and calibration. This workshop will focus on key foundations aimed to move the field forward. These are, guidelines for reviewers and editors in manuscript submission of cytometric microvesicles, and the current status of standardization and calibration.

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When Worlds Collide: Research Complexity and Clinical Validity of Flow Cytometric Assays Used for Drug Development

Thomas McCloskey1, Virginia Litwin2

1ICON Labs, Farmingdale, NY, United States, 2Covance Inc.

Panel Members

Dr. David Lanham, Eurofins, Milton Park Abingdon, United Kingdom  
Dr. Christele Gonneau, Covance, Geneva Switzerland  
Dr. Maxime Moulard, BioCytex, Marseille, France

Clinical flow cytometric methods used guide patient diagnosis and treatment decisions must be validated according to CAP/CLIA requirements. The innovative, high complexity methods used to support basic research do not require formal validation. The application of high complexity methods during the drug development process presents several challenges surrounding the validation approach. This workshop will address the numerous factors which must be considered when developing a validation study plan using the Fit-for-Purpose approach and challenges of implementing novel technology in a regulated environment.

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Mucosal Immunology

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Innate immunity serves as the front line of defense from invading pathogens in the mucosal tissues. With our growing understanding of the mucosal tissues, so is our realization of the complexity of the cell types and processes. Multi-parameter cytometry has influenced our understanding of these complex sites by allowing separation and visualization of cellular lineages, origins, plasticity and trafficking. We encourage discussion about new methods for tracking and visualizing innate immune cells in the mucosal tissues.
This workshop will highlight some of the advances in understanding mucosal immunity with novel cytometry approaches.

48  Conflict Management: Dealing with Difficult Situations

Joanne Lannigan1, Rui Gardner1

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Conflict occurs in every day life in one form or another. Working in a ‘Shared’ environment such as a Shared Resource Lab (SRL) can have its fair share of conflicts. Dealing with these conflicts is of critical importance in managing a SRL. Often we neglect to address situations when they seem minor because we are uncomfortable or unsure of the best way to address the situation, until the situation becomes a major one and even more difficult to resolve without relationship damage. Also, dealing with these conflicts incorrectly may lead to the same outcome.

This workshop will address:
- Recognizing when there is a conflict that requires intervention
- Understanding the sources (causes) of the conflict from multiple perspectives
- Finding creative ways to address the conflict with a win/win outcome
- Identifying process changes that can prevent future conflicts

This will be a very interactive workshop so bring your smartphones and come prepared to participate!

49  Connecting the Whole and the Parts: Organs on Chips and Cytometry

John Wikswo

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The reductionist quest in biology began 2000 years ago as physicians and physiologists dissected animals and cadavers and studied organ relationships. Beginning ~130 years ago, isolated organs and tissues helped explain much of physiology. We now have detailed descriptions of many constituent biological parts, i.e., individual cells, organelles, and biomolecules. Fluorescence and mass cytometry and other single-cell assays provide detailed information on gene expression and protein translation in individual cells and cell populations, and enable characterization of gene regulatory networks. But reductionism alone cannot explain all of biology. Many physiological functions involve dynamic, multiscale, spatiotemporal interactions between numerous components that span molecules, cells, tissue, and organs. It is difficult to study these dynamics in detail with readouts that either dissociate or destroy the system. Now that we can engineer the genome, proteins, cells, and tissues for particular functions or properties, including remote sensing and control, we can turn to a constructionist approach and begin to assemble the individual parts into a functional whole. This could be done with complete, natural biological systems, traversing computationally or experimentally back over the well-trodden path of biology toward the instrumented and controlled animal that can be interrogated in real time and provide dynamic readouts of its state. Alternatively, one could follow a more synthetic path, wherein engineered cells, tissues, pumps, valves, and sensors are used to create organs-on-chips (OoCs) that can be connected together to create a new microphysiological model system - a milliHuman or a microHuman. Such homuncular models offer a new means to characterize the signaling and metabolic dynamics within and between organs and answer questions in systems biology, drug development, toxicology, and biodefense. Real-time non-destructive readouts already used to assay OoC performance and interactions include sensing molecules that record calcium, pH, voltage, etc.; genetically encoded, fluorescently labeled biomolecules that report cellular phenotype; electrochemical metabolic activity sensors (glucose, lactate, oxygen, pH, etc.); micromechanical strain gauges; multiplexed ELISA; and UPLC-nESI-IM-MS characterization of the dynamic exometabolome. With low enough cost and cell numbers, and high enough chip-to-chip reproducibility, many parallel OoC experiments can be stopped at different times after a drug or toxin challenge to allow destructive readouts such as flow cytometry, mass cytometry, and imaging mass spectrometry to reconstruct dynamic signaling events. A combination of sensors and actuators will enable closed-loop control. With these techniques, microphysiological homunculi will close the hermeneutic circle of biology, wherein we cannot understand the whole until we understand the parts, and cannot understand the parts until we understand the whole.

50  Accelerating Discovery of Autoimmune Mechanisms

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Background: The human body carefully regulates the number of cells in the blood of many different levels. For example, in healthy adults CD4:CD8 ratio is approximately 2:1; within CD4 or CD8 T cells, the ratio of naive to memory cells is typically 1:1. Mechanisms that regulate these balances are poorly, if it all, known. Furthermore, dysregulation in the homeostasis of these cells often accompanies or leads to disease. We hypothesized that insight into homeostasis mechanisms could be revealed by quantifying the heritability of the representation of leukocyte subsets in healthy related adults, and performing GWAS to identify the associated genetic loci.

Methods: We developed and optimized seven 14-color immunophenotyping panels to assess the representation of a broad range of leukocytes. Cryopreserved PBMC from 668 healthy twins were analyzed in a discovery phase (with complete statistical analysis) followed by an independent validation phase. Over 78,000 gates (subset representation) and nearly 700 MFI values (subset phenotype) were computed on each individual. Standard twin based heritability analysis was used to rank all immunological traits: the top 150 independent traits were subjected to GWAS analysis.

Results: From this initial analysis, we identified 297 SNP associations at 11 genetic loci that explain up to 36% of the variation of 19 traits. We found multiple associations with canonical traits of all major immune cell subsets, and uncovered insights into the genetic control for regulatory T cells. Importantly, the data also revealed immune traits associated with loci known to confer autoimmune susceptibility, providing novel mechanistic hypotheses that link the traits to the etiology of diseases.

Conclusion: Our data establish a biosource linking genetic control elements associated with normal immune traits to common autoimmune and infectious diseases. This provides a shortcut to identify potential mechanisms immune related diseases. In addition, our study illustrates the value of detailed immunophenotyping of healthy adults to identify potential disease mechanisms.

51  Connecting the Dots – A Literature-Based Global View of the Immune Intercellular Circuity

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Background: Protective immunity is not the end outcome of any single cell, but rather drawn on functionality elicited by many cell types communicating between one another. To date, using
reductionist techniques, immunologists have elucidated many of the basic principles of individual cell type behavior at a given condition. Complex inter-cellular circuitry and whole system effects, however, are difficult to capture or understand.

**Methods:** Here, we describe immuneXpresso, an information extraction system, tailored for parsing the primary literature of immunology using Natural Language Processing techniques and ontologies at its core. More than 13 million research abstracts are examined to automatically extract semantically related cells and inter-cellular signaling factors as well as identify their interactional context.

**Results:** We used immuneXpresso to mine all of PubMed and compile a global high-resolution directed cell-cytokine interaction network together with the dimensions of diseases, drug treatments, tissues and biological functions. The beta release of immuneXpresso is available online through ImmPort. We demonstrate how the literature-reported evidence can be further computationally analyzed to build disease-specific interaction networks, unveil disease/drug similarities, interpret experimental data, predict novel cell-cytokine interactions and even suggest cross-species immune system differences.

**Conclusions:** immuneXpresso facilitates ‘connecting the dots’ between very different areas of immunology establishing a framework for knowledge refinement as well as systematic de novo hypotheses generation.

52 Deep Profiling of the Murine Myelopoietic System: Signalling and Cell Cycle Responses to Neurotropic Viral Infection Profiled by Mass Cytometry (CyTOF)

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Viral infection of the central nervous system (CNS) results in a rapid influx of bone marrow (BM)-derived monocytes/macrophages, that ultimately induce fatal pathology in the mouse. Whilst these cells are derived from the bone marrow, little is known about the kinetic and migratory events that mobilise BM monocytes and their progenitors in response to CNS infection. The BM is home to a complex system of haematopoietic cells, where cell profiles represent the differentiation of long-term haematopoietic stem cells through various lineage-committed progenitors into mature forms. This differentiation occurs in accordance with a genetically encoded rise and fall of various overlapping cell surface markers. The complexity of this progression requires high-dimensional single cell technologies to fully unravel, which has so far been unavailable to the discipline of stem and progenitor cell research. However, the success of both multiparametric fluorescence and mass cytometry (CyTOF) technologies and novel data analysis software has enabled the study of single cells with more than 40 parameters with minimal overlap of reporter signals. Here we report the analysis of complex BM systems and kinetic changes that occurred in monopoiesis, a process that is not well characterized, during neurotropic viral encephalitis. To do this, we compared fluorescent flow cytometry assays developed in our laboratory to characterize bone-marrow stem and progenitor kinetics, with novel mass cytometry assays run on Australia’s first CyTOF II, acquired by the Ramaciotti Facility for Human Systems Biology. Using our first panels, we were able to characterise cell cycle and transcription factor changes in BM progenitor populations, and associated cellular kinetic changes induced by viral encephalitis.

53 Detection and High Dimensional Phenotyping of HIV-Specific T-Cell Responses by Mass Cytometry (CyTOF)

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HIV is a chronic viral infection with a progressive prognosis if not treated with antiviral drugs. However, some individuals control the infection in absence of therapy (so-called elite controllers) and CD8 T cells are thought to play an important role in this process. CD8 T cells from elite controllers are more effective at killing HIV-infected targets than those from progressors. The underlying mechanism is not completely understood, but is affected by HLA type, T cell receptor clonotype, viral epitopes targeted, and likely also by a complex regulatory network of activation and inhibition. During the course of chronic infections in general, CD8 T cells acquire a state of exhaustion characterized by reduced proliferation and cytokine expression as well as upregulation of co-inhibitory molecules like PD-1, CD160 and CD244. However, the particular phenotype of activation and exhaustion markers associated with the increased killing of HIV-specific CD8 T cells from elite controllers has remained unclear.

To elucidate these regulatory networks, we performed high-dimensional phenotyping of HIV-specific CD8 T cells using mass cytometry (or CyTOF-Cytometry by Time of Flight), a cutting edge technology combining flow cytometry and mass spectrometry that enables analysis of up to 40 markers by metal-labeled antibodies with minimal signal overlap. We established and validated a CyTOF panel of 33 markers to identify up to six HIV-specific CD8 T-cell subsets using tetramer technology and characterized memory, activation, and exhaustion status of those cells. We compared different methods to analyze and display the data derived from these experiments (i.e. conventional manual gating, SPADE, and viSNE) to determine the best tool for detection of rare cell events, like HIV-specific T cells. We show that our panel enables in-depth characterization of HIV-specific CD8 T cells at a level of unprecedented granularity and may reveal signatures of activation and exhaustion networks that underlie the enhanced lytic capacity of these cells in HIV elite controllers.

54 Comparative Exploration of Multi-dimensional Flow Cytometry Software to Analyze the Influence of pMHC-TCR Affinity on T Cell Polyfunctionality

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Advancement in flow cytometry instrumentation and reagent development has lifted the ceiling for simultaneous analysis of large numbers of cellular parameters. These improvements have allowed for synchronized identification of numerous immune system components including lineage and functional markers. The use of polychromatic flow cytometry requires, however, proper analytical software to answer the intricate questions that result from the generation of highly complex datasets, and different questions may require different optimal tools to analyze the same dataset.

We are interested in deciphering how an HLA-A2-restricted, CD8-independent, hepatitis C virus (HCV) NS3:1406-1415-reactive T cell receptor (TCR) mediates recognition of a wide variety of naturally occurring mutant epitopes. Stimulating with peptide-loaded T2 cells or an HLA-A2 human hepatocellular
Using multi-dimensional flow cytometry, we measured the presence of 6 intracellular cytokines (IFNγ, TNFa, IL-2, IL-4, IL-17A, IL-22) and lytic marker CD107a in CD4+ or CD8+ TCR-transduced lymphocytes and compared polyclonality potential across wildtype and mutant peptide and tumor stimulation conditions. The analysis of these 7 parameters after gating on our lymphocyte population markers resulted in 128 possible combinations of negativity/positivity for these functional markers. Further, 10 different peptide or tumor stimulation conditions yields a grossly complex dataset that is difficult to analyze with basic flow cytometry software and requires additional tools to answer the various complex questions from such a large amount of data. Here, we comparatively assess various multi-dimensional flow cytometry software to answer the questions posed by the accumulation of this data by outlining advantages and disadvantages and displaying graphical output each can offer. Of the various tools investigated, such as GemStone, VISON, and R-based tools including SPADE and RchyOptimyx, coupled-analysis in Pestle and SPICE provided the most user-friendly manipulations and graphically pleasing output to answer effects of differential antigen stimulation on T cell polyclonal activity. This exploration of other multi-dimensional analysis software may prove to better assess the feasibility of answering these difficult questions.

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Improving AutoGate’s Automation of Discovery Gating

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Background: Automated high-dimensional clustering methods for flow cytometry data are starting to replace traditional manual gating, which is widely criticized as too time-consuming and subjective. Given the complexities, challenges and slowness of full automation based on concurrent clustering with 3 or more parameters, however, we created an alternate, semi-automated approach named ‘AutoGate’ (AG) and released it at CYTO 2014 as a free software application distributed via the web. To gate data, the user first creates a gating model on one sample using a semi-automated, 2-dimensional projection pursuit approach that requires user input to select the projection parameters and identify subsets. Once constructed, his model can then be applied automatically to any sample with compatible parameters.

AG alleviates the manual task of gate drawing by doing clustering via density-based merging (DBM) and allowing the user to pick one or more clusters as a subset. AG assists the manual task of selecting the parameter set by providing a parameter pair-finder feature that ranks and visual orders 2-D plots according to entropy metrics for biological informativeness.

After a year of use, feedback identified opportunities to improve. First, the clustering process required users to tune a “% outlier” setting in order to increase sensitivity to smaller subsets. This user input requirement was judged as too manual. Second, because of the way the smoothing parameter was being chosen, a non-locality effect existed that caused the clustering of a subset to be influenced by the presence or absence of events that were clearly not related. Clustering of a small population could change depending on the size of distant unrelated populations. Finally, our parameter pair-finder feature was deemed visually confusing with too many colours; moreover, it did not always order best using Shannon entropy or differential entropy as a metric for biological informativeness.

Methods: Using MatLab, we were able to rapidly evolve AG and get ‘same day’ feedback from expert gaters thanks to a new instant web update-upon-launch capability that we created for any compiled MatLab application. We focused on improving DBM’s calculation of density bandwidth wherein we set a predefined minimum on the kernel width and allow it to be greater than that for small samples. We implemented the silhouette coefficient (SC) which investigates biological informativeness in terms of the distinctive differences between certain cell-populations in both groups using RchyOptimyx, a computational tool which illustrates cell populations in a hierarchy based on their scores by using dynamic programming and optimization tools. Our automated analysis identified ten populations of interest that were compared to the results of obtained by independent, comprehensive manual analysis. Additional manual analysis validated each of the phenotypes as true positives. Two were artifacts (i.e., the signal was real but due to technological or biological reasons), three were known populations also identified by the manual analysis and six were novel populations missed by the manual analysis. Several of the novel populations would not have been identified even by a comprehensive and extremely time consuming manual analysis due to their unexpected marker combinations. Our results comprehensively demonstrate that automated analysis can not only robustly replicate manual analysis, but can also be used as a tool for biomarker discovery in massive datasets.
57 Latent Modeling of Flow Cytometry Cell Populations for Joint Automated Gating

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Background: The lack of absolute measurements of markers in flow cytometry means that data samples only can be interpreted in relation to each other. At the same time, pooling samples is suboptimal since technical and biological variation gives the cell populations different sizes, locations (mean fluorescence intensities) and shapes. Despite this, traditionally automated gating algorithms either treat the samples individually or ignore the variation in location or shape by for example pooling the data samples. We have developed an algorithm for joint automated gating of a collection of samples, using a latent model for the cell population parameters describing location and shape so that variation in these is taken into account.

Methods: We use Gaussian Mixture Models (GMM), which are often used for automated gating of single flow cytometry samples, as models for each sample in a collection together with a latent layer modeling the parameters in each GMM. Hyper parameters describe how closely connected cell population locations and shapes are across samples. The model can furthermore accommodate for that different cell populations might be present in different samples, by including variables indicating presence or absence of populations. We infer the model using Markov Chain Monte Carlo (MCMC) sampling, with a reversible jump step for detecting the absence of cell populations in a sample. As a post processing step, we merge mixture component with sufficient overlap, enabling modeling of cell populations with non-Gaussian distributions.

Results: We have applied our algorithm to a data set with 30 samples of hematopoetic stem cells for which four markers were measured. This dataset was part of the FlowCAP I challenge, in which state-of-the-art algorithms for automated gating were applied to it and compared to manual gating. For the 30 samples, our algorithm achieved an average F-measure of 0.98, meaning that the result was very similar to the manual gating (a prefect score of 1 would signal complete concordance). This clearly outperforms the automated gating methods participating in FlowCAP I that did not use additional information such as the true number of populations or the true labels for some of the events. The top performers had F-measures of 0.96 and 0.93 when manual tuning was allowed and 0.94 and 0.93 when not. With our model, we get in addition automatically a systematic overview of cell population characteristics.

Conclusion: To achieve high performance for automated flow cytometry gating, we believe that it is necessary to do the gating jointly for many samples. We have developed a Bayesian hierarchical model enabling this, taking biological and technical variation of location and shape of cell populations into account. We have shown on a dataset previously used for evaluation of a wide range automated gating methods that our approach can indeed improve performance.

58 Data Confidentiality, Integrity, and Authentication in the Archival Cytometry Standard

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Background: The Archival Cytometry Standard (ACS), developed as the next-generation data storage format for flow cytometry (FC), is designed to include components such as metadata and analysis results in addition to FC measurements. Although FC is commonly employed in unregulated research environments, its use in clinical settings is subject to HIPAA compliance rules, which require confidentiality, integrity, and availability of all protected information. Confidentiality can be achieved by encryption, and data integrity can be ensured by the use of digital signatures. However, the current ACS specification does not provide concrete recommendations and leaves open the question of implementations. The XML format does not offer encryption of other documents unless a slow base-64 transformation is applied. W3C XML signatures may be used in ACS files, but unencrypted signatures on uncrypted data are vulnerable to forgery. The ZIP specification also lacks sufficient security measures. The presented research investigates remedies to the security and integrity issues in FC.

Methods: To guard against security vulnerabilities, the developed system signs separate components of the ACS, encrypts the signature appended to the data, and finally signs the encrypted file using a digital signature algorithm (DSA) or elliptic curve DSA (ECDSA). Our implementation is built using the Java cryptography architecture (ICA), which is interfaced to R via the jfca package for prototyping.

Results: Generating a signature using ECDSA requires a smaller public key than does DSA. The AES, Serpent, and Twofish encryption algorithms are comparably secure and efficient in the FC context. However, AES slows down as the key length becomes longer. Despite this disadvantage, our implementation favors AES because of its efficiency on hardware of varying complexity. The process of encrypting/signing components of the ACS file is opposed to encrypting the entire container permits varying levels of access. Our implementation allows for key hierarchy scheme in which a practitioner at a medical facility can retain the ability to decrypt clinical metadata, whereas a collaborating researcher at an academic institution can access only the FCS data but not the patient information.

Conclusions: The presented implementation demonstrates the concept of FC data security and integrity within the ACS standard. The study shows the need for an extension to the standard, as well as modifications in the data-acquisition process in order to provide asymmetric-key digital-signature capability. For high-security environments, this requires the use of hardware modules performing on-line encryption, thereby protecting files from the risk of data being altered after collection. Our research also demonstrates the need for a key hierarchy to allow a facility that generated the data to grant varying levels of access to other institutions via derivation of lower-level keys.

59 Validation of a Whole-Blood In Vitro Assay Immunotoxicity of Chemical Compounds Based on Quantitative Analysis of NF-κB Translocation by Imaging Flow Cytometry

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Background: Detection of human immunotoxicity is relevant to predictive and regulatory toxicology but is usually complex, while nodal steps may provide simple and suitable endpoints. NF-κB activation is both a promising therapeutic target and an important immunotoxicity endpoint. Conventional methods employed to determine nuclear translocation of NF-κB lack statistical robustness (microscopy) or the ability to discern heterogeneity within the sampled populations (Western blotting and gel shift assays). Previously, we developed an in vitro assay of immunotoxicity based on quantitative measurement by Multispectral Image-in-Flow Cytometry (MsIFC) of NF-κB translocation in a monocyctic cell line. MsIFC combines the high image content information of microscopy with the high throughput and multiparametric analysis of cytometry which overcomes the aforementioned limitations of conventional assays. In this study, we have adapted and validated this assay for human peripheral whole-blood samples. Materials and Methods: The degree of NF-κB nuclear translocation was quantified in U937 cells and in peripheral blood samples after treating samples with different compounds affecting in vitro NF-κB-dependent immune functions, including activators (LPS and PMA), inhibitors (PDTC and Wedelolactone) and six test immunotoxicants. Negative controls were cell cultures treated with appropriate solution vehicles. To analyze the effects of NF-κB inhibitors, samples were pre-incubated with or without them for 1h and then treated with activators or pro-oxidant agents. After treatment, surface staining was performed for 100 pl whole blood samples using PC5 anti-CD14, erythrocytes were lysed by addition of 2 ml BD FACS Lysing Solution 1X (Becton Dickinson). Then cells were fixed and permeabilized with 0.1% Triton-4% PFA and stained with anti-NF-κB p50 conjugated to Alexa Fluor 488 (Biolegend). Afterwards, cells were washed and counterstained with 7-AAD (Molecular Probes). MsIFC data were obtained for at least 15,000 events per sample using an ImageStream100 system (Amnis). Results: LPS- or PMA-induced NF-κB nuclear translocation was quantified in U937 cells and peripheral blood samples after 2 and 24 h of treatment with different compounds affecting in vitro NF-κB-dependent immune functions, including test immunotoxicants such as lindane, diazepam, hexachlorobenzene, 1-butylhydroperoxide, verapamil and mercury chloride. Our results show that MsIFC allows to quantify the effect of xenobiotics and biological regulators on NFκB nuclear translocation in peripheral whole blood samples. Automated image processing algorithms allowed to calculate the percentage of lymphocytes and monocytes showing NF-κB nuclear translocation in each condition. IC50 values could be derived, thus classifying the immunotoxicity based on quantitative measurement by Multispectral Image-in-Flow Cytometry.
condensation in single cells. This allowed us to quantitatively characterize the process by lag-phase duration, AAVR rate, nuclear volume shrinkage degree, and degree of chromatin condensation.

**Conclusion:** We present a method based on time-resolved contour microscopic measurements and a mathematical model, describing measured nuclear AAVR dynamics accompanied by chromatin condensation and formation of apoptotic ring, for quantitative characterization of an early nuclear morphological changes during apoptosis.

62 **G2 Transit Times in Pancreatic Cancer, Their Relationship to Genomic Instability, and the Therapeutic Potential of G2 Checkpoint Inhibitors**

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Genomic instability is a hallmark feature of cancer. Pancreatic cancers are highly malignant, and show early onset of metastases and drug resistance that are likely due in part to genomic instability and high mutation rates. The G2 phase of the cell cycle allows proof reading for errors, and the repair of DNA damage prior to entry into mitosis. We hypothesize that pancreatic cancers showing high genomic instability require an extended G2 to resolve post-replication errors, and that agents that disrupt the G2 checkpoint might show selective toxicity towards these aggressive cancers.

The cytokinetics of solid tumours are of fundamental importance to cancer treatment, but in recent years this subject has been largely ignored by the flow cytometry community and the field has not kept pace with developments in basic science. Our experimental approach uses a large series of early passage xenografts derived from pancreatic cancer patients and maintained in the panareas of immune-deficient mice. These tumours closely recapitulate the features of their corresponding patient donor, and their availability substantially overcomes the difficulty obtaining patient samples for flow cytometry-based experiments. Following disaggregation into single cells, combined staining of DNA content, Cyclin A2, and serine 10 phosphorylation of histone H3 allows assignment of cells in the G2/M peak to the sequence G2 → prophase → metaphase/anaphase → late mitosis. Applying this technique to pancreatic cancers we have successfully tracked the kinetics of G2 arrest in response to a single treatment of tumour-bearing mice with 8Gy radiation, and abrogation of the G2 checkpoint by treatment with a wee-1 inhibitor. Interestingly, in the absence of radiation wee-1 inhibition results in rapid exit from G2 into the metaphase/anaphase population, which is consistent with the idea that an extended G2 protects genomically unstable cancers from the effects of unresolved post-replication damage.

Our ongoing work involves the addition of EdU pulse/chase to allow estimation of the actual G2 transit times in vivo, and then to measure these in a series of pancreatic cancers selected based on cytogenetic complexity. This will allow testing of the hypothesis that increasing genomic instability results in prolongation of G2. We plan to use this information and our hypothesis that increasing genomic instability results in replication damage and repair, sexoyosis, endodysis as well as oxidative phosphorylation, proteolytic degradation, etc. Such small foci can be imaged and analysed using concepts and principles similar to the ones that have been developed and are now applied successfully in the realm of flow cytometry and laser scanning cytometry. New optical microscopy methods, including super-resolution imaging techniques, open even more innovative possibilities in cytometric studies of various subcellular structures and processes.

We have developed new analytical and mathematical approaches aimed at measuring and analysing the numbers and volumes of subcellular microfoci, their fluorescence intensities, distances between barycentres of nearest neighbours of microfoci of various types in 3-D space, and spatial correlations and interactions between them. Such analyses were supplemented by the complementary methods of multicolor flow- and laser scanning-cytometry to provide information covering the range from a molecular- to a cellular- and a cell population level. We applied these techniques in studies of the processes of replication and endogenous DNA damage in human cells, as well as damage induced by oxidative stress and by several DNA damaging drugs including DNA topoisomerase I and II inhibitors. DNA damage sites were visualised by detection of phosphorylated histone.
The complexities of the microtubule dynamics, chromosome assembly and chromosome segregation are critical aspects during mitosis. Recent findings suggest that an understanding of the relationship between the temporal segregation of intracellular compartments (endosomes, lysosomes) and links to asymmetric cell division, and cancer, could be crucial to unravel the cell biological basis of tumourigenesis.

The present research involved designing an analysis methodology to quantify the morphological and polarised relationships between the chromatin, mitotic spindle and the quantum dots, during the distinct phases of cell division. The simple question being posed - at which point during mitosis is the inheritance of the quantum dot (Qdot) labelled vesicles determined?

Human osteosarcoma (U-2 OS) cells were seeded onto, coverslip bottomed, Greiner Bio-one, 24 well, plates. The next day they were pulse labelled (1 hr) with 705nm quantum dots, allowing for intra cellular processing into the cells through endocytosis. After a further 24 hours they were fixed and further immunolabelled for anti-β-tubulin and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) to highlight the nuclear compartment. 3D, x, y, z data stacks were collected using In Cell 6000, a high-throughput, laser-based confocal imaging system.

The cells were categorised according to the cell cycle phases, based on the intensity levels and morphological features of the nucleus and microtubules. In total, 109 mitotic cells were identified, and these were split into subsets defined by the different phases of mitosis. Subsequently, the relationships between the nuclear intensity and the spatial positions of the Qdots, and also more specifically the relationship between the microtubule features and the radial intensity of the quantum dots, were examined.

As cells enter mitosis, and the nuclear envelope is disassembled, Qdot localisation changes. Clusters are rapidly formed so that when the cell is entering metaphase, the distribution of the Qdot clusters remains.

H2AX, and 53BP1, RPA, Rad51 and XRCC1 repair factors. DNA replication foci were detected by visualising incorporation of the DNA precursor EdU or imaging PCNA. DNA damage signalling foci were correlated with the DNA replication and repair sites. New aspects of cytotoxicity mechanisms of topoisomerase inhibitors and oxidative DNA damage revealed by these studies will be discussed.

These newly developed analytical approaches are expected to further expand analytical capabilities of cytometry in the direction that will reveal various parameters, features and spatial relationships between sub-resolution structures in cells, including protein aggregates or even individual macromolecules, and make it possible to study and analyse quantitatively the activities and interactions between them.

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Examine Inheritance during Mitosis, in U-2OS Cells, Using Quantum Dot Labelled Vesicles
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Background: DNA damage induces the formation of foci with various factors including histone proteins, replication factors, DNA repair complexes and chromatin modifica-
tions. The foci are responsible for DNA repair and the accurate segregation of chromosomes to daughter cells during cell division.

Methods: The DNA damage response was induced using ultraviolet (UV) irradiation of Hela cells. The distribution of DNA damage foci was examined using immunohistochemical staining. The distribution of foci was quantified with the CellProfiler Cell Imaging Pipeline.

Results: The DNA damage response is characterized by the formation of foci associated with DNA repair proteins. The distribution of DNA damage foci is affected by the presence of different DNA repair pathways. The analysis of the distribution of DNA damage foci indicates that the repair pathways are not equally efficient in all cases.

Conclusions: The DNA damage response is a complex and dynamic process that requires the coordinated activity of various DNA repair proteins. The analysis of the distribution of DNA damage foci can provide insights into the efficiency of DNA repair pathways and the mechanisms that regulate their activity.
from these fully integrated oceanic tests within the submersible cytometer will be summarized.

Conclusion: We have fully integrated the acoustic focusing system into the fully submersible flow cytometer and demonstrated its performance through extended field testing in the ocean. Our optimization of the acoustic system allows us to operate at flow rates that are an order of magnitude faster than those obtainable through the use of hydrodynamic focusing alone.

67 Flow Cytometry In Vivo: Recent Advances
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Background: The sensitivity of conventional flow cytometry is limited by the small volume of blood collected, in which no less than one disease-specific marker can be detected. It leads to missing many thousands of abnormal cells in the whole blood volume (~5 L in adults), which can be sufficient for disease progression to barely treatable or incurable stage. For example, despite enormous efforts to detect circulating tumor cells (CTCs) responsible for 90% of all cancer deaths as a result of the small volume of blood collected, in which no less specific markers can be detected. It

Methods: To solve this problem we introduced in vivo flow cytometry for detection of rare circulating biomarkers directly in the bloodstream using blood vessels as natural tubes with native cell flow. In this technology, laser irradiates vessels of interests followed by the detection of photoacoustic (PA) waves from circulating objects using ultrasound transducer attached to the skin. The PA waves are generated either using intrinsic contrast agents (e.g., melanin) or absorbing low toxic plasmonic nanoparticles functionalized to specific markers.

Results: We summarize recent advances of new generation of in vivo multicolor flow cytometry platform using multispectral lasers, fiber-based transducers, label-free and/or multiplex molecular targeting, plasmonic probes with ultrasharp PA spectral resonances, in vivo magnetic capturing of CTCs, and combination of PA diagnosis with photothermal (PT) elimination of abnormal cells. The capacity of this new technology was first demonstrated in preclinical models for real-time detection of bulk and stem CTCs, S. aureus, and sicle cells. Then, the clinical prototype of in vivo PA flow cytometry (PAFC) provided the assessment of ~1 L of patient blood during 30-60 minutes. The first clinical studies focused on detection of CTCs and cancer-associated clots in melanoma patients. The PAFC data in vivo were validated in vitro with conventional flow cytometry, immunohistochemical staining, and magnetic kits. The obtained results demonstrate that PAFC is more sensitivity (≥100-fold), accurate and rapid (≤1 hour) that conventional technique.

Conclusion: Unlike typical blood sampling involving extraction of a volume of blood ranging from 10 µL (drop) to a few mL (CTCs), in vivo examination involves nearly the entire volume of blood passing through 1–2-mm-diameter peripheral vessels over 0.5–1 h and thus will enable a dramatic increase in diagnostic sensitivity, ultimately up to 104-105 times, reflecting the ratio of the volume of blood sampled in vivo to that in vitro. According to pilot clinical results, the portable personal flow cytometer can provide a breakthrough in early diagnosis of cancer, infection and cardiovascular disorders with a potential to inhibit, if not prevent, metastasis, sepsis, and strokes or heart attack through the use of well-timed personalized therapy.

68 Flow Cytometry-Based Assay for Detection of BCR-ABL Fusion Protein in Blood Cells from CML Patients
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Chronic myeloid leukemia (CML) is currently diagnosed using RT-PCR and/or FISH to reveal the presence of the fusion mRNA transcripts for BCR-ABL, or of the characteristic Philadelphia chromosome. RT-PCR is also used to monitor the effects of treatment by sensitively measuring transcripts representing minimal residual disease (MRD). It has not been possible to use flow cytometry to identify the neoplastic cells but such a method would be helpful in the workflow of a hematopathology lab. We have now developed a method that successfully detects and enumerates cells harboring the fusion protein BCR-ABL by flow cytometry in CML patients. The method uses the in situ proximity ligation assay (PLA) (Söderberg et al 2006, Leuchowius et al 2009), where two antibodies target the BCR and the ABL part, respectively, of the fusion protein. The antibodies are equipped with DNA oligonucleotides that – when brought in proximity – guide the formation of a circular DNA molecule as a template for localized DNA amplification through rolling circle amplification (RCA). Each RCA-product is then labeled with around 1,500 fluorophore-coupled DNA oligonucleotides, allowing cells to be detected by flow cytometry. The method has proven very sensitive, able to detect very low number of cells in patient samples, and it allows identification of patients in relapse at a very early state.

Since the method is developed for flow cytometry all the advantages with multiwavelength fluorescence analysis can be achieved simultaneously and the method allows for standard cytological markers to further characterize the malignant cells.


69 Cytometric Determination of Mobility of Memory Lymphocytes
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We have originally developed the concept that memory lymphocytes rest in dedicated survival niches of the bone marrow, initially for memory plasma cells (1), then also for memory T helper (2) and cytotoxic memory T lymphocytes (3). We found that these cells were resting in terms of proliferation and gene expression, however, when we wanted to determine if these cells were also resting in terms of migration or whether they recirculated between bone marrow and blood, we realized the fundamental technological limitations in the conventional ways of determining mobility of cells. We have thus developed a novel cytometric approach to obtain a ‘snap-shot’ of cellular mobility.

We used three cytometric correlates of mobility: the expression of (a) KLFR, S1PR1, CD62L and CCR7, as an indication of the...
ability of cells to translocate from bone marrow into blood and secondary lymphoid organs, (b) the expression of Tiam1, which is essential for the activation of Rac1 (Rho-GTPase), and thus for the navigation of the cells in chemokine fields, and (c) the cytometric determination of filamentous actin (F-actin) and its intracellular, polarized expression, defining the front end and required for forward movement of the cells. Using these cytometric parameters, we found that memory T lymphocytes of the bone marrow expressed little or no CD62L, CCR7, KLF2 and ST1a1, less Tiam1 than memory lymphocytes of the periphery, and less F-actin, in particular, no polarized F-actin expression. This cytometric diagnosis of ‘immobility’ was confirmed in transwell migration assays of cells isolated ex vivo confirming that memory T lymphocytes of the bone marrow migrated less than memory T cells of the periphery in response to chemokine gradients.

The cytometric determination of these correlates of mobility have thus confirmed the novel concept that immunological short-term memory is maintained by memory cells circulating between blood and secondary lymphoid organs, while immunological long-term memory is maintained by immobile cells, resting in dedicated survival niches of the bone marrow, and probably also other organs, as tissue-resident memory cells. This new understanding of immunological memory impacts tremendously on the development of vaccination strategies, and on the development of therapies against immune-mediated diseases, like chronic inflammations and allergies.


70 Improved Molecular Genetic Analysis of Clinical FFPE Tissue Cores Using Combined Image-Based Cell Selection with Dielectrophoretic Movement (DEPArray) and NGS
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For several cancer types preoperative chemotherapy improves the overall survival of the patients. Massive cell death can be the result of such treatments. This impacts molecular DNA and RNA tissue analysis for primary or compehdent diagnostics. Precision medicine should then be carried out on the preoperative formalin fixed paraffin embedded biopsies. Furthermore, carcinomas are heterogeneous of cellular composition with different amounts of stromal and inﬂammatory cells which further complicates the analysis.

As a model for clinical biopsies we dissociated 0.6 µm diameter tissue cores taken from FFPE lung and colon carcinoma tissue blocks into cell suspensions. Cells were stained for vimentin (A647), keratin (A488) and DNA (DAPI). Stromal cells and carcinoma cells in exact numbers (mean = 107, median 58, range 5 - 600) by combining image-based cell selection with dielectrophoretic movement (DEPArray™). Next DNA was extracted from pure tumour subpopulations. Using IonTorrent AmpliSeq CHPv2, we generated sequencing libraries, after lysis of the pure cells recovered by DEPArray™ (n = 54), or unsorted samples (either QiAmp DNA columns or disaggregated cells). Libraries were sequenced with IonTorrent PGM (mean depth > 2,000x), and analysed using IonTorrent software.

Results: On several loci, we detected somatic mutations with 100% variant frequency, only observable as heterozygous in the unsorted samples and as wild-type in stromal cells of same patient, confirming 100% purity of sorted cells. Frequently, for loci harbouring germ-line heterozygous SNPs with variant frequency around 50% for pure stromal cells, we readily detected loss-of-heterozygosity in tumour cells subpopulations as binary (0% / 100%) variants. Quantitative traits such as copy number gains and losses were also reproducibly identiﬁed in tumour cell replicates as deviations from the 50% variant frequency of germ line SNPs of pure stromal cells.

Conclusions: DEPArray™ technology signiﬁcantly improved molecular genetic analysis of small tissue cores from FFPE carcinoma tissue blocks. Mutations in cancer driver genes were identiﬁed which were left unnoticed in total DNA extracts of the same tissues. The technology described might be considered for molecular genetic analysis of clinical FFPE biopsies taken prior to preoperative therapy.

71 Dynamics in the Microbial Cytome – Unraveling Functions in Natural Systems
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Introduction: Microorganisms are everywhere. They drive the biogeochemical cycles in soil and water and comprise a major part of biomass on earth. As communities they are an integral component of the human body and directly contribute to the well-being of human society e.g. in wastewater treatment and resource management, energy production, or synthesis of chemical building blocks. High complexity in taxonomy usually ensures high functionality as well as stability and resilience due to replaceable functions if microorganisms disintegrate, are inhibited, or destroyed. Though microbial communities are first hand companions for humans in many aspects there is only scarce knowledge how to manage them.

Methods: Microbial ﬂow cytometry is capable of following community dynamics in a fast and inexpensive way. The approach relies on analysis of a very low number of discriminating cellular parameters and high numbers of cells. In addition, abiotic parameters from micro-environmental surroundings are recorded. Newly developed bioinformatic tools are used to enable quantification of single cell dynamics and to make nearly online evaluation of community attributes possible. Thereby, community trends are followed and possible constraints are revealed that inﬂuence community structure and function.

Results: Communities from different ecosystems are characterized coming from wastewater and biogas facilities as well as the intestine of the mouse. Microbial communities show cell-based ﬁngerprint like structures mirroring their speciﬁc micro-environment and keep this structure if remained undisturbed. Perturbations reveal sub-communities that encounter constraints while others fail to survive or do not participate in a restructuration process. A rating of the impact of environmental variables on sub-community behavior is proposed thereby allowing e.g. nutritional bottlenecks, stress factors, or impact of reactor design and management on microbial function to be detected. Thus, the degree of variation in the microbial cytombe allows conclusions on systems stability but also on microbial functions that emerge in response to changing environments. The approach is applicable to managed or natural environments but also to the human microbiome.

Conclusions: Natural microbial systems are highly dynamic due to the short generation time of the comprised organisms and their rapid and distinct reactions to changing environments. Microbial
flow cytometry can make the microbial cytochrome accessible for ambitious ecosystem studies. Specific functions of cells within the cytochrome can be determined either by cell sorting in combination with other omics-approaches of choice or by simple correlation analyses.

72 Cytometric Evaluation of Immunological Memory
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A key property of the vertebrate immune system is its ability to establish long-term immunity against previously encountered pathogens. The cellular correlates of such immunological memory are the memory T, B and plasma cells. However, the analysis of such cells has long time been limited to the blood and secondary lymphoid organs and has given us a very restricted view on the organisation of immunological memory. Thus, the prevalent view was that memory cells are circulating in constant search for their antigen. In contrast, we could demonstrate that many memory cells of mice and humans are maintained in the bone marrow. In the bone marrow, memory T and memory plasma cells reside in specialized niches organized by dedicated VCAM-1+ stroma cells expressing IL-7 and CXCL12, respectively. There, the memory cells are quiescent, resting in terms of cell cycle activity and mobility. For human CD4+ memory T cells, we have analysed their antigen-specificity of in matched blood and bone marrow samples of humans undergoing hip replacement surgery in more detail. The repertoire of memory T cell in the bone marrow comprises specificities to recent as well as past antigenic experiences. In particular for historic antigens they outnumber memory T cells of the blood. Thus, our results show that the bone marrow is a major site for the long-term maintenance of immunological memory calling for a novel concept for the organization of immunological memory.

73 What T Cells See on Human Cancer
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Human tumors contain large numbers of mutations, of which many hundreds can be present within expressed genes. As the resulting altered protein sequences are foreign to the immune system, immune recognition of such ‘neo-antigens’ is likely to be of significant importance to the activity of clinically used immunotherapeutics such as anti-CTLA-4 and anti-PD-1 in melanoma. However, the vast majority of the mutations in human cancers are unique to individual patients and, because of this, broadly applicable approaches to link the consequences of DNA damage in human tumor to tumor-specific T cell activity have long been lacking.

Using in-house developed technologies for monitoring of T cell activity, we have recently demonstrated the feasibility of cancer exome-driven analysis of both tumor-specific CD8+ T cell reactivity and CD4+ T cell reactivity in human melanoma. The data obtained demonstrate that T cell recognition of the consequences of DNA damage is a common feature in human melanoma. Furthermore, based on the distribution of mutation loads in other major human cancer types, we propose that also in many other human tumors, the repertoire of mutant antigens provided by DNA damage (the ‘neo-antigen space’) will suffice to allow T cell recognition.

Collectively, these data indicate that mutational load may form a biomarker in cancer immunotherapy, and that the development of ‘personalized immunotherapies’ that exploit cancer genome information to target patient-specific mutant antigens should be explored.

74 Engineered T Cells for Cancer Therapy
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Relapsed hematologic malignancies pose a substantial unmet therapeutic challenge despite aggressive therapies. Engineered T cells have shown promise for a variety of malignancies and chronic infections. In this session we will review the current status of trials testing chimeric antigen receptor (CAR)-modified T cells for acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), and for myeloma. As of February 2015, 151 patients have been treated with CTL019 for various B cell malignancies, including CLL, ALL, lymphoma, and plasma cell myeloma. 41 pediatric cases of ALL have been treated (28 relapsed post-allo HSCT), and 35 of 39 evaluable patients achieved CR. More than 50 patients with advanced refractory chronic lymphocytic leukemia have been treated with CTL019. Two of the first three patients treated remain in complete remission more than 4 years after infusion. An ongoing randomized phase II trial testing a high and a low dose of CTL019 has not shown a difference in the response rate. The lack of an obvious dose response rate is consistent with the high level proliferation of CAR T cells in vivo and suggests that once a threshold dose is reached, further dose escalation does not increase efficacy. Persistent B cell aplasia is observed in responding patients (more than 4 years in CLL patients and more than 2 years in pediatric ALL patients). In CLL, no patient with persistent B cell aplasia has relapsed with CD19+ tumor. A major issue in the field is whether it is necessary to have ongoing immunosurveillance by CAR T cells or whether they induce ‘sterile remissions’ where every last tumor cell is eradicated.

CAR T cells can target very low levels of targets expressed at the cell surface. Because some myeloma cells express low levels of CD19, and because B cells may be a precursor of malignant myeloma cells, an ongoing pilot trial is testing whether CTL019 cells might recognize and eliminate malignant cells in patients with advanced plasma cell myeloma. Taken together, our findings support the advancement of CAR-modified T cell therapy into multicenter phase 2 clinical trials for leukemia and lymphoma.

75 Fluorogenic Reporters and Modulators in Living Animals
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The interaction between a non fluorescent dye and a cognate molecular recognition protein can be exploited to activate fluorescence or other photochemical properties of the dye. Using analogs of the malachite green chromophore, a triarylmethane dye, we have generated a series of fluorogenic dyes that are bound and activated by a common high-affinity fluorogen activating protein, derived from a human VI domain. Crystallographic analysis of the protein-dye complex revealed strategies to tune the affinity of the protein-dye complex, resulting in a range of distinct, specific fluoromodules with well-defined equilibrium binding properties and spectral properties tuned from blue to infrared wavelengths. This protein can be expressed and labeled effectively in a wide variety of sub-cellular locations, and on a variety of molecular targets. Addition of fluorogenic dyes can be used to assess the abundance of proteins in living cells and animals, including transgenic model organisms like zebrafish and mouse xenograft tumor models. By combining these dyes with physiological indicators, the internalization of targeted proteins can be quantified in real-time using a common detection window in the near-IR spectral range, allowing deep-tissue imaging through the skin.
Nanoparticle-Based Analysis of Biomolecules, Cells and Tissue

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Metallic nanoparticles offer many opportunities in terms of detection including light scattering, surface plasmon resonance and surface enhanced Raman scattering (SERS). We are interested in the optical properties of metal nanoparticles and their potential application in a range of different biological studies. We can make use of the optical properties of nanoparticles in two ways.

1. The nanoparticle can act as an extrinsic label for a specific biomolecular target in the same way as a fluorescent label is used. The advantage of using the nanoparticle is its optical brightness (typically several orders of magnitude more than fluorophores) and the lack of background vibrational signals. Functionalization of the nanoparticle with a specific targeting species such as an antibody or peptide aptamer allows this approach to be used in a wide range of studies including cell, tissue and in vivo analysis.

2. Nanoparticles can be designed to contain a specific recognition probe designed to cause a change in the aggregation status of the nanoparticles resulting in a discernible optical change when it interacts with its biomolecular target. This allows separate free analysis of specific biomolecular interactions and can be applied to a range of different probe/target interactions such as DNA-DNA, peptide-protein and sugar-protein.

We have been making use of nanoparticles in both of these approaches in conjunction with SERS which is an advanced vibrational spectroscopy. To demonstrate the applicability of the two different approaches examples will be given on the use of nanoparticles for cell imaging in two and three-dimensions, imaging of nanoparticles at centimetre depths through tissue and also their ability to report on biological molecules in vitro and in vivo.

Cell-Based Biomarkers of Infection/Sepsis: Critical Assessment from the Perspective of Needing Rapid Time to Result

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Background: Cell diagnostics can provide robust assays of neutrophil and monocyte activation and deactivation, which can be reliably offered for real time patient management. The markers that provide rapid immunophenotypic evidence of neutrophil activation is best exemplified by CD64, but additional markers including CD10, CD11b, CD16, CD15, and various degunulation assays. Monocyte markers include CD16, CD64, CD163, HLA-DR and others. Soluble levels of cell associated biomarkers might also be considered markers of immune cell activation, which might include IL-6, TNF-a, sCD163, sCD206, cell free DNA (cfDNA) and others that can be measured by a number of immunoassays or fluorescence based assays.

Methods: Clinical samples were obtained with local IRB approval to provide data in correlation with clinical outcomes. These samples were used to study the biomarkers developed by Trillium Diagnostics, including the Leuko64, Macro163, and cfDNA Quant assays. Comparative performances of assays were extracted from in-house data and peer-reviewed literature.

Results: Studies with neonates and adults demonstrate the incremental diagnostic information afforded by neutrophil CD64 (ROC analysis typically >0.85), but also lesser and yet incremental diagnostic information is afforded by the addition of other cellular parameters of monocyte CD163 (along with sCD163 levels), monocyte CD64, monocyte HLA-DR, neutrophil CD35, sCD206, TREM-1, cfDNA and others, beyond what is afforded by procollactin, C reactive protein and other "standard of practice" parameters of immune activation associated with diagnosis of infection/sepsis. Findings are discussed in the context of the real clinical need for definitive results available within 2 hours of collection on a 24 hour basis, which is at odds with the current instrumentation and technologist training expectations for most North American and European clinical flow cytometry laboratory practice, specifically specialty lab.

Conclusions: Cell based assay have potential to make a significant impact on health care, both as IVD assays and quantitative QC assays to monitor cell therapies and in vitro cellular product manipulations. However, in order for IVD cell based assays to be implemented into areas of critical patient management, such as severe infection/sepsis, immune compromised states and screening assays, a two hour time to result is necessary. Outside of automated CD4 counting, which is not required for patient management in real time, the level of instrumentation remains a barrier.

Selection and Optimization of Near-Infrared Fluoromodules for Imaging through Tissue

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Optical imaging of live animals allows investigation of pathology and therapy within a richly informative biological context. Increasing use of fluorescence in animal studies drives continued development of labels that provide high specificity, contrast, and sensitivity through tissue. The specificity and contrast provided by fluorescent proteins has proven valuable in samples such as cultured cells, but tissue is highly opaque to the wavelengths at which most fluorescent proteins absorb and emit. Tissue opacity and autofluorescence diminish at near-infrared wavelengths, but engineering natural proteins to fluoresce in this wavelength range is challenging. We have produced bright, near-infrared excited and emitting fluoromodule labels that, like fluorescent proteins, can be used as genetically encoded fusion tags. Fluoromodules consist of non-fluorescent dyes that becomes highly fluorescent upon binding to cognate fluorogen activating proteins (FAPs). The synthetic chromophores in this system impart unique advantages in terms of brightness, wavelength, and versatility.

A near-infrared absorbing analog of malachite green, SC1, was tested against malachite green-binding FAPs. The brightest candidate was optimized by two rounds of PCR-based mutagenesis and cell sorting for intensity to obtain Mars1. An scFv expression library hosted in S. cerevisiae was screened for fluorescence activators of a membrane impermeant SC1 analog called SC1. Enrichment of the brightest clones over several rounds of sorting yielded clones with emission maxima above 750 nm. Mutagenized libraries were constructed using these clones to screen for fluoromodules with increased emission wavelength, using a 780 long pass emission filter. This process yielded mMars2.

The Mars1 FAP possesses fluorescence quantum yields of 0.17 and 0.21 when bound to SC1 and SC1, respectively, with excitation and emission peaks near 700 and 730 nm. We have expressed this FAP as a fusion tag in multiple subcellular contexts, and FAP-expressing cells can be imaged in mice after systemic fluorogen delivery. The mMars2 FAP excites and emits maximally with SC1 at 744 and 764 nm, respectively, and optimization efforts for this clone continue.
The near-infrared fluoromodules described here are brighter and operate at longer wavelengths than existing genetically encoded labels, making them well-suited for in vivo imaging. The applied screening strategy may allow discovery of additional labels for animal imaging, since promising dyes can be quickly screened against existing peptide expression libraries. Dyes may also be engineered with added functionality. This approach represents a promising avenue through which to expand both the palette, and versatility of fluorescent labels for in vivo optical imaging.

79 Identification of Immunosenescence-Associated Gene Signatures in Purified Cells of the Human Innate and Adaptive Immune System

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The phenomenon of immunosenescence is discussed to be responsible for compromised immunity in elderly individuals. In consequence, increased susceptibility and sensitivity to infectious diseases and poor responses to vaccination in the elderly can be ascertained. Although several indications for low-grade chronic inflammatory processes referred to as inflamm-aging exist, detailed knowledge about cell-specific gene signatures for cells of the innate and adaptive immune system in young and elderly are missing. Therefore, we compared genome-wide transcriptional profiles of purified monocytes, granulocytes, naive as well as effector/memory T helper cells in 11 young and 16 elderly healthy donors. Since it is known that naive T cells from elderly individuals exhibit impaired responses to antigen, we segregated the naive T cell population into recent thymic emigrants (RETs) defined by expression of CD31 and central naive T cells, defined by absence of surface expression of CD31. Comparing gene expression between the young and the elderly we identify cell-specific, immunosenescence-associated gene signatures, which revealed substantial qualitative and quantitative differences in their composition and magnitude of expression. Hierarchical clustering of these genes allowed an almost perfect classification of young and elderly leucocyte populations, whereby innate immune cells showed markedly less complex gene signatures when compared to T helper lymphocyte subsets. The RETs, whose numerical decline with age in consequence of thymus involution, showed only 821 differentially expressed transcripts, while 4,839 transcripts were identified in central naive T cells and 6,071 in effector/memory T cells. These numbers impressively demonstrate for differentiation-dependent aging processes in the T cell compartment. Differentially expressed genes were subjected to gene ontology enrichment analysis followed by network analysis of closely connected ontologies. This analysis revealed that age-related gene ontologies of cell types investigated show substantial differences indicating for lineage-specific transcriptional ageing programs in innate and adaptive immune cells. In conclusion, this is the first study giving a comprehensive view on immunosenescence-related transcriptional changes in a cell type-specific manner, which is also of central importance for a better understanding of premature aging processes of the immune system discussed in the pathogenesis of chronic autoimmune diseases.

80 An Extracellular Vesicle Probe Effectiveness Comparison Using Simultaneous Labelling

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Background: Extracellular vesicles range from 30 to >1000nm. Currently microvesicles (100-1000nm) are most often analysed using flow cytometry. As newer generations of custom built flow cytometers are beginning to appear in the microvesicle community, allowing unprecedented resolution, a new generation of probes are required for identifying microvesicles. The most common probe of choice is annexin V, labelling phosphatidylserine in a Ca2+ dependant manner. Accumulating studies are emerging however that suggest other probes have a higher binding affinity to phosphatidylserine and/or are capable of more clearly separating microvesicles from endogenous background noise. Here we compare the labelling of microvesicles simultaneously using phosphatidylserine labelling (annexin V), intracellular Ca2+ concentration (BAPTA-1) and a new generation of membrane loading dye (VRD)

Aim: To directly compare three methods of extracellular vesicle identification using simultaneous labelling. Intracellular Ca2+ labelling, membrane loading and external membrane labelling will be compared using platelet-derived microvesicles.

Method: 6ml of blood was drawn in to citrated tubes, following a 2ml discard. Tubes were spun at 140g for 20min with platelet-rich plasma (PRP) isolated. 5µl of PRP was added to 200µl HBS + 2mM CaCl2. Samples were incubated at 37°C before being centrifuged at 1500g x 2. Supernatants were drawn off and stored at -80°C. Supernatants were thawed at 37°C. 100µl of supernatant was added to 2µl 500µM BAPTA-1 and spun at 20,000g x 30mins. Supernatants were discarded and 100µl HBS + 2mM CaCl2 was used to resuspend the microvesicle pellet. 5µl 25µg/mL Annexin V PerCP-Cy5.5 was added and incubated on ice for 20min. 2µl of 200µM VRD was added and incubated for 5min.

Results: Preliminary data shows annexin V to be the least effective at identifying microvesicles from noise, with a stain index of ~1.31. The VRD and BAPTA however had a stain index of ~1.69 and ~1.75, respectively. Whilst BAPTA-1 outperformed annexin V, the VRD is the only stain that was capable of separating all microvesicles from background noise.

Discussion: From the results above, both BAPTA-1 and VRD appear to be superior labels for identifying extracellular vesicles. Whilst BAPTA-1 is a superior label to annexin V, the wash step required to reduce background noise is a limitation for large studies, both in terms of time and microvesicle concentration lost from pelleting. VRD however is non-fluorescent until integrated into the membrane, therefore having minimal background fluorescence, incubation times are short and staining appears to be more effective than annexin V and BAPTA-1. Furthermore, due to VRD being a ratiometric dye, fluorescence at 585nm for negatively charged extracellular membrane and 530nm for net neutrally charged external membranes, a new avenue of phenotypic information can be gained from microvesicle populations.

81 Automated Analysis of Flow Cytometry Data to Reduce Inter-Lab Variation in Detection of MHC Multimer Binding T Cells

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An Extracellular Vesicle Probe Effectiveness Comparison Using Simultaneous Labelling

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In an effort to improve quality in assessment of antigen responsive T cells, the organizations CMT and CIC has conducted numbers of proficiency panels to enhance quality and promote assay harmonization. This has successfully improved the ability to uniformly identify MHC multimer binding T-cell populations following flow cytometry analysis and the most recent proficiency panel, organized by Immunex on behalf of the CMT, immunoguiding group showed on average high performance with 76.5% of participating laboratories (n=51) within a range of 1.5x the median value for a detected T-cell response. Yet, the gating strategies and subjective gate-border decisions taken by the individual labs may provide variation to the assessment of antigen specific T-cells when comparing data across laboratories, but also over time-courses in individual labs. Therefore strategies to provide automated analysis of MHC multimer binding T-cells form an ideal solution to decrease subjectivity and variation. The challenge of automatic analysis of MHC multimer responsive T-cell populations is that these are often of low frequency and low fluorescence intensity.

We set out to assess, using a recent MHC multimer proficiency panel dataset, if MHC multimer binding T-cells could be analyzed with the computational solution currently available and if such analyses would reduce the variation across different laboratories. We have at first instance used FLOCK, SWIFT and ReFlow (ongoing). The MHC multimer proficiency panel included 51 labs, from which 31 had provided datasets suitable for our analyses. Each lab received the same donor material and same MHC multimers, but used their own antibodies, staining protocols and gating strategies, which varied significantly from lab to lab. The two donors used held T-cell responses against Epstein-Barr virus (EBV)- and Influenza virus (FLU)-derived T-cell epitopes in the range of 0.04 - 5.33% of CD8 T-cells and additionally MHC multimers with an irrelevant peptide were included as a negative control.

Preliminary data shows the feasibility for detection of MHC multimer reactive T-cell populations using FLOCK (n=31) and SWIFT (n=10). Especially when attempting to identify the large and intermediate populations, both software tools performed quite well and correlated with what was found by manual gating (r=0.92 for SWIFT, r=0.88 for FLOCK). When it came to the low frequency populations, both software tools - FLOCK a bit more than SWIFT - did however have some difficulties as there were several cases where no cells were assigned to the specific populations. A disadvantage of both solutions was that they both required extensive manual pre-filtering and/or gating of the data alongside with the computational analysis. Thus to allow a fully automated approach, we did an automated pre-filtering step using a not yet publicly available algorithm. Comparison of the automatically pre-filtered data with the manually pre-gated data is currently ongoing. Furthermore, a central manual gating of all labs is also being conducted in order to evaluate whether this in itself would reduce variance between different labs.

82 FlowJo Exchange: A Means of Meeting the Computational Needs of the Flow Community

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Cyometric analyses continue to grow in both complexity and nuance. Much of the data produced outstrip the ability of a team of human analysts and require analysis automation and/or machine-generated analysis. However, the breadth of unique analyses and growth in applications make it less likely that a software tool has been created that meets the specific analysis and visualization needs and to accommodate the nuances of a study. Building custom analysis tools or learning a new software package for every application is time-consuming and expensive.

Herein, we describe the development and use of the Script Editor for rapid application development. This extensible editor is integrated into the open architecture of FlowJo, the most cited third-party cytometric analysis software package, and gives real-time access to all statistics, gates, groups, and metadata for use in creating custom analysis tools within FlowJo.

The Script Editor allows a user to write code that will be executed within the program using Java script, a straight-forward and widely-used language. As it is integrated into commercial software, the needed libraries, dependencies, Java objects, and references are already included, leaving the user only to code what is unique to their work. The coding can be as simple as including some common FlowJo action within a loop to automate a relatively simple set of actions, or as complex as using the built in math library to create a statistics based analysis germane to a particular application. To demonstrate how this tool might be used to address analysis needs, we created a script that reads the keyword information from files annotated with index sort information and automatically creates gates on all of the single cells with their well position used as the gate name. Moreover, we show that data-dependent custom analysis pipelines may be created using template analysis. FlowJo templates contain an analysis which may be applied to new data sets. We show that template application can be automated along with script execution, and automation decision steps occur conditionally based on criteria derived from the data.

This is an imperfect stand-alone solution as any programming may be too much for a scientist whose focus is the experiment. The FlowJo Exchange addresses this issue. Scripts produced in the Script editor can be saved as text files, shared, and run by anyone in three clicks by loading them into a FlowJo workspace and clicking ‘Run’. The FlowJo Exchange is a repository that allows users to upload a script with annotation or download a shared script. It brings crowd-sourcing to the computational requirements of the Flow Community and provides an open-source hosting platform supporting this tight-knit community. In the Flow community, ideas pass very quickly to the mainstream, making an automatable, extensible, shared resource the ideal way to map the missing spaces of data analysis.

83 A Novel Flow Cytometric Cell Cycle ER Stress Model for the Study of Autophagy

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Thapsigargin (Tg) and Chloroquine (CQ) are known to cause cell cycle arrest, but the role of mitosis in the apoptotic process is less studied & the distribution of the autophagic marker LC3B across the cell cycle phases is not well characterised. Tg is also known to cause ER stress & autophagy which can result in ER phagy. Here we employ a novel ER stress flow cytometric assay which measures the physical and products of ER stress. The ER mass & the levels of misfolded proteins were measured in a cell cycle dependent manner to generate an ER stress index evaluation.

KS62 cells were treated with Tg (0.1, 0.5, 1μM & CQ (25, 50 and 75μM) for 72 h. Cells were loaded with, Hs33542 and EM Tracker Red (ERTR) & analysed flow cytometrically. Cells were then fixed, permeabilised and labelled with anti-LC3B-AB-647, protein aggregate Proteostat (PS) probe (Enzo) & DAPI. The ER Stress Index (ERSI) was determined by comparing changes in ER mass from the Median Fluorescence Intensity (MFI) of ERTR to controls to calculate a percentage change in ER mass for each phase of the cell cycle. This reduction was then multiplied by the level of protein aggregates (PA) as calculated from the MFI of PS. Thus data with lowest ER Stress Index values show the most ER Stress.
KS62 cells showed significant Tg & CQ induced cell cycle arrest, G1 77±2% (P<0.05), S phase 14% (P<0.01), G2/M 8.6±5%; G1 69±2%, S phase 6.5±2.3%, G2/M 14.3±1.2% (P<0.05) respectively after 48h compared to controls. Tg & CQ induced up-regulation of LC3B MFI in a cell cycle dependent manner at 48h with 0.5µM Tg, 401±364, 614±557 & G2/M 985±882; 25µM CQ LC3B MFI, 667±41, 1009±33 (P<0.05) & G2/M 1079±115. Tg & CQ up-regulated LC3B in S phase by 18% more than observed in control S phase cells. While Tg up-regulated LC3B in G2/M by 40% more than observed in control G2/M cells.

Tg & CQ caused equivalent ER phagy in a cell cycle dependent manner at 48h with more in G1 36%±8%, than S 26%±4% or G2/M 25%±1%, P<0.05; G1 32%, than S 21% or G2/M 24%, P<0.01, respectively. Both Tg & CQ induced protein aggregate formation only in a dose dependent manner at 48h 49±5 PA, P<0.01. Tg produced an ESRI after 48h which was cell cycle dependent with more ER stress in G1, -172±190, than S -1261±83 or G2/M -1270±26. While CQ gave an ESRI 11% <Tg in a cell cycle dependent manner with more ER Stress in G1, -1542±247, than S -1033±124 or G2/M -1171±234.

ER stress inducing drugs not only caused G1 cell cycle arrest but up-regulated LC3B in S & G2/M phases above the normal distribution of LC3B. Tg & CQ G1 arrested cells showed more ER phagy than other phases of the cell cycle with low amounts of LC3B, although PA content was constant. The ESRI gave a useful summation of the end physical products of ER stress within a cell, with other doses of drug and times of analysis resulting in differing outcomes of ER mass changes, which included ER elongation & changes in protein aggregate levels, resulting in different levels of ESRI.

84 **immunoClust** – An Automated Pipeline for Population Detection in Flow Cytometry

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Multiparametric fluorescence and mass cytometry offers new perspectives to disclose the diversity of cell populations in the peripheral blood and to provide new approaches in biomarker research. To analyze the complexity of data from high-end cytometric devices that may detect up to 120 individual parameters, adequate software tools are needed.

We developed an automated analysis pipeline, **immunoClust**, for uncompensated fluorescence and mass cytometry data. In a first step, cell events of each sample are clustered. Subsequently, a classification algorithm sorts these cell event clusters into populations, which can be compared between different samples. The clustering of cell events is performed in a global unsupervised method. It is designed to identify rare cell types even when next to large populations. Both parts use model-based clustering with an iterative Expectation Maximization (EM) algorithm and the Integrated Classification Likelihood (ICL) to obtain the clusters.

To test immunoClust for detection and classification of large populations, depletion experiments were performed. Comparison with manual analysis revealed an excellent correlation. Identification of small populations was investigated based on FlowCAP III data sets. Populations with more than 200 events were recognized with a high f-measure. While sensitivity for detection slowly decreased for smaller populations, this decrease was not related to an absolute number of events but to the characteristics of the distribution of events. The algorithm was applicable also to mass cytometry data with up to 28 parameters. It is applicable to compare blood samples from different diseases with controls as well as to compare between blood cells and cells from effusions like synovial fluid. While compensation is no precondition, normalization of data may help to optimize meta-clustering.

In summary, immunoClust is a promising tool to standardize and automate the analysis of high-dimensional cytometric datasets. It supports our efforts in developing biomarkers for monitoring of chronic inflammatory disorders and the development of therapy recommendations in personalized medicine. immunoClust is implemented as an R-package and is provided as source code on the Bioconductor web site.
86 Multiparametric Flow Cytometric In Vitro Toxicity Assays on Rat Cell Lines for Risk Assessment and Classification of Chemical Compound

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Background: European laws demand chemical risk assessment and classification of industrial compound based on their toxicity. This extensive toxicity testing requires in vitro methods to replace, reduce or refine (3R) testing in animals. In previous studies we have shown that multiparametric flow cytometry (MFCM) tests based on human cell lines predicted reasonably acute human in vivo toxicity. However, such assays were less reliable to classify chemicals according to the current UNO/EC Global Harmonisation System (GHS), based on rat in vivo toxicity. To overcome this limitation, we have initiated a series of in vitro toxicity assays performed by MFCM on several rat cell lines.

Methods: The following rat established cell lines were used: B50 neuroblastoma, NRK-52E transformed renal tubular cells (both adherent) and N13 hepatoma (growing in suspension). Cytotoxicity of 57 test compounds (IC50) was determined in range-finding experiments at 24 hour-incubation. Cells were then treated with several concentrations up to IC50 and the following biomarkers of cytotoxicity estimated: viability, plasma membrane potential, mitochondrial membrane potential, cytosolic calcium, mitotic arrest, and tubulin polymerization. All biomarkers were determined by MFCM assays run on a high-throughput system (Accuri C6-HyperCyt). IC50 or EC50 were calculated and correlated to reported human and rat toxicity, and used for compound classification in GHS classes by hyperclustering with rat LD50 values. The correlation with human in vitro toxicity was established on the basis of human toxicity data from the AcutoxBase database.

Results: For all cell lines and most compounds tested, several parameters resulted in better in vitro/in vivo correlation than reported by existent bibliography. N13 cell line gave the better correlations to rat and human in vivo toxicity. In most cases, MFCM assays classified correctly compounds according to the GHS scale. Exceptions were compounds with very low toxicity for which no IC50 or EC50 could be calculated.

Conclusions: Although the GHS-based classification by MFCM is not perfect, the strategy of using rat cell lines improved the results over our previous studies with human cell lines and, the method could be useful for toxicity testing.

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87 Cytometry of Anticancer Prodrug OCT1002 Activation and Targeting Using In Vitro and In Vivo Models of Tumour Hypoxia

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Background: Tumour microenvironment hypoxia (TMH) facilitates disease progression and therapeutic resistance, presenting challenges for the treatment of prostate and pancreatic cancer. The non-toxic anthraquinone prodrug OCT1002 (OncoTherics, UK) is designed to distribute widely in tissues, become irreversibly activated to OCT1001, a potent DNA topoisomerase II inhibitor, thereby acting as unidirectional hypoxia activated prodrug (uHAP). We have investigated the relationship between intracellular generation and persistence of TMH-generated OCT1001 and biological responses both in vitro and in vivo.

Methods: Confocal imaging and FCM exploited the intrinsic far-red fluorescence of the uHAP. Cancer cell lines were exposed to clinically relevant prodrug levels at 1-100 nM under hypoxia (4%; 1-3% oxygen); studied for cell proliferation, OCT1002 bioreduction and cell cycle arrest. Human prostate cancer xenografts expressing a luciferase reporter (LNCaP-luc) in Balb/c SCID mice were used to monitor growth delay, lung metastasis and vascular changes in relation to OCT1002 targeting in dorsal skin-flap/window chambers. pO2 of tumours was recorded using an Oxylite 2000 system and assessed in tissues by Glut1 staining. Xenograft induced hypoxia was achieved by exposure to bicalutamide (Casodex™); a non-steroidal anti-androgen. Pancreatic cancer human BX-PCS/SCID mouse xenografts, presenting intrinsic hypoxia, were used to assess uHAP monotherapy.

Results: Hypoxia-dependent growth arrest induced by OCT1002 was found in a wide range cancer cell lines irrespective of p53 status. Cytostasis and sustained G2 cell cycle arrest was achieved within 1 population doubling at 100 nM [% cells arrested at 1%-3%>>air]. OCT1002 and hypoxia co-exposure generated persistent intracellular fluorescence attributable to the metabolite OCT1001 while the prodrug OCT1012 was not retained. OCT1001 located within LNCaP-luc tumours at regions distant from blood vessels, persisted for >7 days, slowed tumour growth and reduced lung metastasis. A pilot study showed that a single dose OCT1012 induced a growth delay in pancreatic cancer BX-PCS/SCID mice xenografts, which are hypoxic, comparable with that achieved by fractionated Gemcitabine treatment.

Conclusion: Cytometric analyses have shown effective OCT1002 activation in vitro. A quantitative relationship was found between the degree of hypoxia, extent of uHAP bioreduction, persistence of OCT1001, growth delay and first cycle G2 arrest. In vivo imaging, in a prostate cancer model that recapitulates TMH induced by anti-androgen treatment, showed that uHAP activation at TMH locations. The slowing of tumour growth and reduction in metastatic potential in prostate cancer together with monotherapy efficacy in a pancreatic cancer model suggest that OCT1002 has potential for tumour indications and in combined modalities where TMH is considered to be a barrier to effective treatment (Prostate Cancer UK Grant funded).

88 High Throughput and Quantitative Measurements of Integrin Conformational Changes Using Fluorescence Lifetime-Dependent Cytometry

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Background: Integrins are adhesion receptors that mediate cell-to-cell adhesion, cell-to-matrix and cell-to-pathogen interactions. They are expressed on virtually every cell type. An Integrin’s ability to bind to its ligand is modulated through intracellular signaling that regulates conformation. Depending on the integrin conformation, cells can roll or firmly adhere to ligands. This conformational regulation includes rapid molecular affinity modulation, vertical changes in the position of the ligand binding site (bent or extended state) and the lateral distribution of the molecules on the cell membrane. Understanding these conformational changes will assist in understanding the pathogenesis of multiple diseases including blood diseases and
cancer. We use Forster Resonance Energy Transfer (FRET) with intensity-based measurements to study the structural transformations of integrins. FRET with fluorescence lifetime-dependent cytometry can be used to observe these structural transformations and illustrate the heterogeneity of integrin conformational states across the cell population. We expect bent conformations that are engaged in FRET to have shorter lifetimes compared to the extended state, where longer lifetimes are expected.

**Methods:** To test our hypothesis, a human cell line (L937), stably transfected with formyl peptide receptor has been cultured using standard tissue culture techniques. First, cells were treated with a fluorescein analog bound to a Leu-Asp-Val-peptide derivative (LDV-FITC). This compound specifically binds to the headgroup of the VLA-4 integrin and serves as fluoresence donor. Next, a red fluorescent lipid dye, PKH-26, was added to the cell suspension. PKH-26 serves as FRET acceptor, which quenches the LDV-FITC in the resting bent conformation due to close proximity between the headgroup and membrane-incorporated PKH-26. The cells are subsequently treated with n-formyl-Met-Leu-Phε-Phe (fMLPP) to initiate activation of the integrins into the extended conformation. This results in the immediate de-quenching of the FITC donor fluorescence. At each stage of cell treatment, flow cytometry measurements were made using standard cytometry and lifetime-dependent cytometry.

**Results:** With ten repeated measurements, the average fluorescence lifetime of LDV-FITC in a quenched state was 4.00 +/- 0.03 ms. When engaged in FRET, the average fluorescence lifetime of LDV-FITC is 3.1 -/- 0.35. After activation (de-quenching of the LDV-FITC), the fluorescence lifetimes are 3.4 +/- 0.55 ms. In any given cell, a distribution of integrin conformations exist and is reflected in standard cytometry data.

**Conclusion:** The results suggest that FRET between two molecules can be detected using lifetime-based flow cytometry. Current data suggests the occurrence of significant heterogeneity in integrin conformational states in the cell population. Incomplete restoration of the average lifetime after activation may be associated with this heterogeneity. The ability to measure fluorescence lifetimes is promising for future cell signaling studies, because it can lead to the development of quantitative approaches to analyze the population statistics of activated molecules. More work is necessary to estimate the fraction of integrins in various states for each individual cell. We are refining flow cytometers to enable this through a technique called 'multi-frequency flow cytometry.'

**89 The Stem Cell Orchestra – A Non-narrative Approach to Communicate Experimental Processes**

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Translation of basic stem-cell research into therapeutic demands reproducibility and identification of subsequent experimental processes variations, in order to assure cellular quality and efficacy. However, many methodologies of stem cell research remain highly idiosyncratic, and the traditional narrative (textual, verbal or video) approaches still lack the ability to identify or assess process variations. As a result, both the reproducibility and standardisation of the processes remain an elusive goal.

Addressing this reality, here, we introduce a non-narrative audio-visual approach to assess process variations that utilise provenance information. Provenance embodies the history of an object or data with chronological description of the SWS - who, where, when, what and why. Provenance has been extensively used in the manufacturing industry to determine the quality of a product, by providing better interpretation, assessment, attribution and authentication via these SWS. For this project, provenance information was acquired from our open source software ProtocolNavigator, which is a virtual laboratory environment where researchers can emulate their laboratory activities, and the software automatically annotates the provenance information of the process and depicts a time-integrated interactive experimental map, that includes action patterns, manipulations and data acquisition icons. Interlinking and traversing through this map, a provenance trail for samples becomes apparent and the underlying process emerges visualised across a timeline.

Sonification, the use of non-speech audio to convey or contextualize complex spatiotemporal information, has been used as a kinaesthetic learning approach to identify the sources of process loss, determination of critical steps, and most importantly assessment method for process improvement – fundamental perquisite for reproducibility. Using a parameter mapping sonification technique we have transformed multi-dimensional provenance information (eg. activity, location) into auditory dimensions (eg. pitch and rhythm) to facilitate the auditory perception for metadata interpretation. The goal has been to generate a language independent approach so that researchers from any discipline can abstract knowledge and discover relationships between process variation and sample quality. Parallel to this qualitative approach, we are also developing a quantitative approach based around provenance data model PROV-DM and associated algorithms. Together this comprehensive approach will enable us to scope the depth and breadth of process variability in stem cell research and lead to the establishment of “best practice” in qualitative as well as quantifiable terms. This will not only facilitate safe therapeutics development, but provide a ‘trust calculator’ for data derived from cellular studies.

**90 Barcoding and Tracking Stem Cells**

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**Background and Aims:** Understanding how mesenchymal progenitor cells (MPCs) behave as part of a micro-community is fundamental to our knowledge of endogenous tissue repair processes. We have derived populations of MPCs from the dental pulp (DPPCs). Clonally isolated and heterogeneous cell populations have been characterised by expression of MPC markers and their ability to proliferate and differentiate in 2D. Qtracker705QD (Qdot) are far red fluorescent nanoparticles that can label and track single cells according to the innate proliferative capacity. As cells divide, Qdots re-segregate between resulting daughter cells such that the total fluorescent signal of both daughter cells is equal to the originating cell. This approach provides a method to select and separate different cell populations based on the simple biophotonic readout. The overall aim of this study is to develop a barcoding method that will enable the identification (by flow and image cytometry) of specific cell populations within both heterogeneous 2D and 3D environments.

**Methods:** DPPCs were cultured in α-MEM (10% Fetal bovine serum, 100µM L-ascorbate) and labelled with 4nM Qdots. Two defined windows were sorted based on width and amplitude of fluorescence signal to obtain a narrow sort window in order to mathematically de-convolve the subsequent signal attenuation. Cells were then seeded for onward culture and Qdot signal re-distribution was measured again at 48, 72 and 144 hours. The resultant Qdot profiles were further interrogated with a second sort at 72 hours to reveal specific populations based on unique regions across the fluorescence spectrum. RNA was extracted
from these re-sorted cells. Biomarker analysis was carried out for MPC, pluripotency, neural crest and early neural markers.

**Results:** The Qdot approach enables us to label (encode) cells so their cell division and position can be tracked using time series analysis. This simple concept allowed us to extract by signal processing, features of proliferation, cell-cell inheritance and cellular location. Clonal and heterogeneous DPPCs demonstrate variation in their initial Qdot loading and subsequent signal loss over time. Some clonal populations demonstrate similar loading and signal loss to heterogeneous DPPC whereas other clonal populations demonstrate signal maintenance over 72 hours but catastrophic loss of signal after 144 hours. Parallel variation existed in the expression of MPC, pluripotency and neural markers between the specific sorted cell populations based on their evolved Qdot signal.

**Conclusion:** The single barcode approach led to phenotypic descriptors of DPPCs linked to their original and emergent lineage behaviour. The double sorting and enrichment of cells in this way allowed us to track and identify unique sub-populations of differentiated cells within a micro-community based on simple biophysics principles. This is a unified approach where the signal readout is embedded into the biological system - a systems approach, provides the scope for translation to 3D and in vivo tooth repair models.

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**Muscle Extracellular Matrix Scaffold Is a Multipotential Environment for Stem Cells Differentiation**

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**Background:** The multidifferential ability of the native-extracellular-matrix-scaffold (ECMS) is a novel concept and never investigated as yet. Analogously to the multipotency of stem cells, this scaffold has a certain degree of multiplicity in a sense that may be able to influence cell lineage differentiation. ECMS is an emerging tool in tissue engineering for the reconstruction of three-dimensional tissues and organs, respecting their structural and functional features. Indeed, the ECMSs from decellularized organs possess the characteristics of the ideal tissue-engineering scaffold (i.e., histocompatibility, porosity, degradability, non-toxicity). We recently demonstrated that ECMS from skeletal muscles promote myogenesis when transplanted in animal models. The clonal and heterogeneous DPPCs demonstrate variation in their initial Qdot loading and subsequent signal loss over time. Some clonal populations demonstrate similar loading and signal loss to heterogeneous DPPCs whereas other clonal populations demonstrate signal maintenance over 72 hours but catastrophic loss of signal after 144 hours. Parallel variation existed in the expression of MPC, pluripotency and neural markers between the specific sorted cell populations based on their evolved Qdot signal.

**Conclusion:** The single barcode approach led to phenotypic descriptors of DPPCs linked to their original and emergent lineage behaviour. The double sorting and enrichment of cells in this way allowed us to track and identify unique sub-populations of differentiated cells within a micro-community based on simple biophysics principles. This is a unified approach where the signal readout is embedded into the biological system - a systems approach, provides the scope for translation to 3D and in vivo tooth repair models.

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**Lectin-Induced Erythrocyte Agglutination – A Potential New Noninvasive Tool for the Diagnosis of Myocardial Infarction**

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**Introduction:** Myocardial infarction (MI) is an acute life-threatening disease with a high incidence worldwide. As MI also affects blood composition, we tested lectin-carbohydrate binding-induced red blood cell (RBC) agglutination as a potential innovative tool for fast, precise and cost effective diagnosis of MI.

**Methods:** Five lectins (Ricinus communis agglutinin (RCA120), Phytohaemagglutinin for erythrocyte agglutination (PHA-E), Datura stramonium agglutinin (DSA), Triticum vulgaris agglutinin (WGA) and jacalin) were tested for their ability to differentiate RBC agglutination characteristics of patients with MI (MI, n = 101), angiina pectoris without MI (AP, n = 34), and healthy volunteers (HV, n =68). RBC agglutination kinetics was analyzed by light absorbance of a stirred RBC suspension in the green to red light spectrum in an agglutimeter (amtec, Leipzig, Germany) for 15 min after lectin addition. Mean cell count in aggregates was estimated from light absorbance by a mathematical model.

**Results:** Each lectin induced RBC agglutination. RCA led to the strongest RBC agglutination (~500 RBCs/aggregate), while the others induced substantially slower agglutination and lead to smaller aggregate sizes (~150 RBCs/aggregate). For all analyzed lectins the lectin-induced RBC agglutination of MI or AP patients was generally higher than for HV. However, only PHA induced agglutination that clearly distinguished MI from HV. Variance analysis showed that aggregate size after 15 min of agglutination induced by PHA was significantly higher in the MI group (143 RBCs/aggregate) than in the HV (29 RBC/aggregate, p < 0.001).

**Discussion:** We hypothesize that pathological changes during MI induce modification of the carbohydrate composition on the RBC...
membrane and thus alter lectin-induced RBC agglutination. Hence, occurrence of carbohydrate-lectin binding sites on RBC membranes provides evidence about MI. Due to the significant difference in the rate of agglutination between both groups with MI > HV, the crucial differentiation to identify individuals with MI is possible based on PHA-induced RBC agglutination. This novel assay could serve as a rapid, cost effective valuable new tool for the diagnosis of MI.

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93 Photoswitchable Flow Cytometry for Analysis of Single Circulating Cells In Vivo
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Healthy states are impacted and many diseases (e.g., cancer, sepsis) are spread by mobile cells that circulate in the organism through the blood. A big challenge, however, has been tracking single circulating cells and determining their health-hazard status within a highly heterogeneous blood environment. Here we introduce the new generation of in vivo flow cytometry (photoswitchable flow cytometry) for noninvasive single-circulating-cells analysis in vivo that integrates photolabelling of controlled number of cells directly in the bloodstream and tracking them within the whole organism. Photoswitchable flow cytometry (PFC) is based on new principle of ultrafast photoswitch of photoswitchable fluorescent proteins (PSFPs) that change their fluorescent color (e.g., from green to red for Dendra2 PSFP) in response to light. We focused our study on photoswitchable flow cytometry (PFC) is based on new principle of ultrafast photoswitch of photoswitchable fluorescent proteins (PSFPs) in response to light. We focused our study on capability for studying recirculation, migration, and distribution of CTCs during metastasis progression. For the first time, we monitored real-time dynamics of CTCs released from primary tumor and discovered the capability of CTCs to colonize existing metastasis (reseedion phenomenon). It also demonstrated capability for identifying dormant cells, and imaging of CTCs colonizing a primary tumor (self-seeding). Integration of genetically encoded PSFPs, fast photoswitching, flow cytometry, and imaging makes in vivo single cell analysis in the circulation feasible to provide insights into the behavior of CTCs and potentially any circulating cells of interest (e.g., bacteria, immune-related cells) which may inspire advanced strategies for disease therapeutics.

94 Mapping Spectral Signatures of Matrix Components in Decellularized Lungs Using Excitation-Scanning Hyperspectral Imaging
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Background: Hyperspectral imaging is an emerging technology adopted from the remote-sensing field that discriminates multiple fluorescent species by sampling a contiguous spectral range. Our laboratory has developed a novel method for hyperspectral imaging that provides 10-100x higher sensitivity by sampling the fluorescence excitation (excitation scanning) instead of the fluorescence emission spectrum. Traditional spectral imaging approaches have sought to remove autofluorescence within an image while neglecting potential information contained within the autofluorescence signal. However, prior spectroscopic studies have indicated that autofluorescence may be a valuable indicator of tissue composition, viability, and pathology. Unfortunately, assessing tissue composition using autofluorescence is a non-trivial task, as signals arise from both extracellular matrix (ECM) components and cells. We have begun by measuring the spectral properties of matrix components from decellularized rat lungs. Here, we report a preliminary assessment of collagen autofluorescence in the extracellular scaffold of decellularized rat lungs. Our novel approach may be applicable as a biomarker for the pathogenesis of diseases affecting the extracellular matrix such as fibrosis and cancer, among others.

Methods: Lungs were excised from adult rats and decellularized by serial detergent perfusion and immersion. Decellularized lungs were prepared for microscopy and treated with various concentrations of a racemic collagenase mixture. Hyperspectral imaging was performed with an inverted fluorescence microscope (TE-2000, Nikon Instruments). Excitation scanning was accomplished using a thin-film tunable filter system (VersaChrome® filters from Semrock, Inc.) placed in collimated space. The filter system was incorporated after the arc lamp to filter excitation light from 340 nm to 480 nm, in 5 nm increments. Images were acquired using an electron-multiplying charge-coupled device camera (Rohera EM-C2, Q-Imag). Exogenous collagen I (Rat tail, BD Biosciences) was used to obtain a spectral library of collagen. ENVI software (ENVI 5, Exelix Visual Information Solutions) was utilized to isolate collagen I and tissue spectra from lung samples.

Results and Conclusions: Multiple spectrophotometer scans confirmed collagen with excitation peaks of 340 and 360 nm, consistent with previously reported observations. Excitation scans of decellularized lung samples treated with collagenase show multiple excitation peaks at 400 nm, 450 nm, and 520 nm. Our future work will broaden the spectral filter range to 340 nm to coincide with the reported excitation peak of collagen, and to incorporate spectra from components such as elastin and NADH. This work represents a novel approach for identifying autofluorescence of molecular components in the matrix scaffold, and the potential translation to identifying fluorescent biomarkers in multiple diseases, including pro-fibrotic conditions and cancer.

95 Kinetic Measurements of Cellular Parameter Changes Evoked by Nanosecond Pulsed Electrical Field
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Introduction: Nanosecond pulsed electrical field proved to be an efficient method to increase cell proliferation in many cell lines in their normal culture medium without the application of growth factors; hence this method is regarded as an economical way to maintain cell cultures. During the treatment, some of the cells might die during the process, but the surviving ones grow faster than controls. The electroporation of the cell membrane induced by the field produces calcium influx, which has crucial role in the activation process of many cell lines. Although there are many data on the proliferation and apoptosis rate of the treated cells following electroporation, no data is available on alterations of intracellular parameters and short-term cellular consequences evoked by the process. We aimed to develop an instrumental
setup which allows the observation of the effects of electrical field treatment using kinetic flow cytometry.

**Materials and methods:** C6 glioma and HEK 293 transformed cell lines were cultured for testing, and were harvested at 70% confluency. The 50 Hz electrical field was generated by Mitoplicator™ device. Flow cytometry measurements were performed on a BD Accuri C6 cytometer equipped with 488 nm and 640 nm lasers. The instrumental setup was developed to transfer the cell suspension directly from the treatment chamber of the Mitoplicator instrument to the sample input of the cytometer using a 40-cm long silicon tube. The starting pressure for the suspension flow was facilitated by a peristaltic pump. For the kinetic measurement of intracellular calcium concentration Fluo-4 dye was used, while for the viability assay DRAQ7 probe was applied. Measurements were performed in the presence or absence of the electrical field for 600 s following the recording of a 120 s baseline (without electrical field). Ionomycin was used as a positive control at the end of the measurements, and cells were recorded for another 300 s following its addition. Data analysis was executed by the FACSkim software.

**Results:** Intracellular calcium concentration, as well as the rate of calcium influx was lower upon the addition of ionomycin following Mitoplicator treatment in HEK cells, however this alteration was not present in C6 glioma cells. Mitoplicator treatment had no biologically significant effect on viability (measured by DRAQ7 staining) of the investigated cell lines.

**Conclusions:** The applied instrumental set-up allowed us to detect the intracellular changes within a short time frame following different experimental interventions. The flow cytometer could handle the initial cell stream flush sent by the peristaltic pump, and a continuous, consistent flow was maintained during measurements. Our results indicate an enhanced calcium response upon Mitoplicator treatment in HEK, but not in C6 glioma cells. Our system enables further real-time flow cytometry analysis of the effects of nanosecond pulsed electrical field.

**96 Single Cell BH3-Profiling: Fingerprinting through Image Cytometry**

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**Introduction:** Even though deep insights into the molecular pathways that regulate cell death have been gained, it is clear that molecular profiling of key pro-and anti-apoptotic players alone does not provide the predictive capability needed to assess chemo-responsiveness. Thus, there is a role for single cell functional analysis and fingerprinting to extract patterns of cell death within cancer cell populations.

We have developed and implemented novel nano-tools to aid our understanding of apoptotic responses using flow and imaging cytometry of the B-cell lymphoma cell lines Daudi and SU-DHL-4. The aim was to establish and validate a BH3-profiling pipeline suitable for sample stratification, using BH3 pathway inducers and sensitizers.

**Methods:** We used two key readouts to quantify the apoptotic response. Depletion of cytochrome c from the mitochondria and nuclear condensation/fragmentation were analysed using flow cytometry, imaging, and morphometric image analysis to investigate pattering and heterogeneity of the apoptotic response on a single cell basis.

**Results:** Treatment of SU-DHL-4 cells or DoHH2 cells with ABT-737 (a BH3 mimetic) resulted in strikingly different cytochrome c release levels and signal distribution between the two cell types. DoHH2 cells displayed a discrete two-population distribution where cells had either fully released their cytochrome c from the mitochondrial compartment, or had fully retained their cytochrome c. In contrast, SU-DHL-4 cells exhibited a continuous or smeared cytochrome c signal decrease, and at the same time the signal was upheld even at high drug doses.

BH3-profiling with stapled Bak or Bid peptides was used to measure the differential capacity of the two cell types to respond to a pathway sensitizer or inducer respectively. Quantitative image analysis revealed that ABT-737 treated SU-DHL-4 cells had high levels of nuclear fragmentation but that this was not always associated with loss of cytochrome c. This was not evident in DoHH2 cells where a classical loss of cytochrome c was accompanied by reductions in cellular and nuclear volume. This suggests that cytochrome c is not a definitive apoptotic marker for the effectiveness of a drug/peptide. Further analysis of the two cell lines using BAM7 (a direct BAX activator), indicated that SU-DHL-4 cells lacked the ability to respond to BAX activation.

**Conclusions:** We have demonstrated quantifiable heterogeneity of the apoptotic response following targeting of the BH3 pathway. Distinct patterns of cytochrome c release and nuclear fragmentation appear to be cell-type and stimulus-specific. Disparate apoptotic responses not only emphasize the need for multiple readouts of apoptosis but also demonstrate that the key apoptotic events succeeding mitochondrial outer membrane permeabilization (MOMP) do not always occur in a pre-determined, sequential manner. This has implications for the analysis of the potential of drug and peptide treatments for patient stratification.

**97 Imaging-Derived Computational Maps of Local Concentrations to Understand Actin Regulation in T Cells**

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Here we demonstrate how unbiased computational analysis of extensive imaging data allows efficient investigation of complex regulatory processes. Actin dynamics are a general regulator of cell morphology and function. When primary T cells activate in the interaction with antigen presenting cells they accumulate actin at the cellular interface. Genetic and pharmacological data suggest that actin dynamics regulate the efficiency of T cell activation. As the costimulatory receptor CD28 also controls activation efficiency, we wanted to understand how costimulation regulates actin dynamics. This is a problem of substantial complexity as actin turnover is controlled by the integrated interaction of a core group of actin regulators downstream of multiple receptors. As an unbiased strategy to identify components of this interaction network under control of costimulation, we reasoned that loss of enrichment of an actin regulator from a core area of actin turnover would be indicative of diminished function. We therefore imaged actin and eight core regulators under full stimulus and costimulation-blocked conditions, yielding 1836 time lapse movies, and developed computational methods to extract voxel-by-voxel aligned maps of the actin regulator subcellular concentrations. Analyzing these maps for actin regulator localization we found significantly diminished recruitment of WAVE2 and Collin to the area of actin accumulation upon costimulation blockade. This identified WAVE2 and Collin as key targets of costimulation-dependent T cell actin dynamics as experimentally confirmed.
98 Parameterizing Spatial-Temporal Tissue Simulation Models from Image Information

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We will study in how far spatial-temporal tissue models can be parameterized from image information. The examples envisaged are liver regeneration after drug-induced damage, and growing multi-cellular spheroids.

For regeneration in liver we will show how the iterative application of a pipeline consisting of confocal scanning microscopy, image analysis and modeling can be used to set up a quantitative spatial-temporal agent-based model of liver regeneration (Drasdo et. al., 2014), that by simulated predictions successfully guided experiments on spatial tissue organization processes (Hoehme et. al, 2010), liver metabolism (Schliess et. al., 2014), and the molecular control of cell proliferation towards new biological insight. We use our image modeling software TiQuant (Tissue Quantiﬁer), integrated in an analysis pipeline of standardized imaging protocols for confocal laser scanning microscopy and image processing to reconstruct 3D volume data sets from optical serial sections (Hammad et. al., 2014), and subsequently quantify and thereby objectify the image information (Friebel, in rev.).

Spatial temporal simulations are either performed directly in the reconstructed 3D images, or in representative tissue samples obtained by sampling from statistical distributions over the parameters chosen to quantify the image information. Hypotheses on the mechanisms underlying the observed tissue regeneration processes are generated and implemented in our simulation software TiSim (Tissue Simulator). TiSim can import images directly from TiQuant. A simulated sensitivity analysis is performed varying each parameter in its physiological range to identify the best agreement between model simulation and observed data. If no sufficient agreement is obtained, the model is modiﬁed and the same cycle is repeated. If more than one model mechanism permits to explain the data quantitatively, the model is used to search for an experimental situation where these mechanisms would yield a different result. For liver regeneration, in this way an order mechanism during regeneration of liver after toxic damage, the lack of a ammonia-detoxifying reaction and the timing and spacing of HGF-sources have been predicted. For growing multicellular spheroids of SK-MES-1 cells, a small lung cancer cell line, mechanisms explaining their proliferation, extra-cellular matrix and death pattern in space and time for various oxygen and glucose medium concentration have been inferred.

Using the image information permits implementation of a model-guided experimental strategy to identify mechanisms underlying spatial-temporal tissue organization processes.

References:
sites, training of sites; stabilization, freezing, shipment of samples; within study controls; outsourcing of flow cytometry assays.

102 Multilaser Cytometry – Developments, Opportunities, Challenges, and Pitfalls
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The ongoing development of high-dimensional flow cytometry has required advances in both the palette of reagents and in highly-optimised instrumentation. While much of the focus of this development has been aimed at maximising the number of parameters (the “emission” side of the technology), there has also been concurrent development on the “excitation” side as well. In this workshop we will concentrate on the excitation side, in particular, the lasers that drive the emission from fluorophores, and consider best practice in developing high-performance and optimised multilaser cytometry platforms.

Laser technology has advanced significantly in the past ten to fifteen years and it is now possible to purchase lasers of almost any wavelength and outfit (and potentially retro-fit) commercial flow cytometers with up to ten different high-powered laser lines. This great flexibility significantly enhances our cytometric capabilities by allowing us to optimise our excitation characteristics to maximise the signal from individual reagents and/or facilitate the combining of reagents into multiparametric panels. However, this flexibility can come at the cost of significantly increased complexity that limits our ability to obtain maximum benefit from the technology.

In this workshop, through a combination of short presentations and panel discussion, we aim to: review current laser technologies and options to consider when purchasing new (or upgrading existing) instruments discuss the advantages and disadvantages of different wavelengths in the context of different experimental approaches, examine issues around laser power optimisation, discuss some of the pitfalls and cautions in multi-laser cytometry, provide an update on new and forthcoming technologies, including the latest trends in single wavelength lasers and the emergence of tunable laser systems, and identify areas where the further investigation/experimentation is required.

Attendees are encouraged to submit questions and issues to the facilitators prior to the workshop for inclusion in the discussion.

103 Educating Users in New Advancements in Cytometry
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This workshop explores ways Shared Resource Laboratories can provide educational opportunities to their users on advancements in this technology. One of the challenges in cytometry is staying informed of rapidly evolving technologies and reagents. As an SRL, providing services to researchers, it is vital that the lab keeps current and can act as a conduit for information to the researchers with advancements that can directly impact their work. The aim is to find ways an SRL can stay up to date on technology and provide ideas on how best to disseminate information to the users. The workshop will include discussion of two ongoing initiatives at handling this challenge as well as group discussions of the SRL communities topics of interest. A brief survey will be disseminated prior to the meeting to solicit topics that are of importance.

104 Back to the Future: CYTOMICS – Translational System Cytometry
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CYTOMICS —> Translational System Cytometry from Bench to Bedside —relates to the future of our shared passion for cellular systems.

Cytometry is growing as a diagnostic and monitoring field in the research and clinical laboratory and is useful in establishing a forum to exchange information, to present research data and to explore new applications of the rapidly expanding technologies in image and flow cytometry. To better understand and further explore the cellular systems, the merging and bundling of technologies is necessary. Single technology can’t provide the ultimate solution.

Cytomics can be defined as multifunctional study of cellular system, from the genes to the entire cellular diversity in tissue, up to the entire dynamic functionality of organs in the body.

Therefore CYTOMICS, which can be defined as multifunctional analysis of cellular system, from genes to the entire cellular diversity in tissue and body, will give a comprehensive view of a disease. The analysis of already existing data from scientific (omics)- studies or routine diagnostic procedures makes CYTOMICS a valuable platform for studying diseases, that will be of immediate value in translational medicine. This ‘CYTOMICS – Workshop’ will expand the -omics-capacities by adding a further overview of the next generation of cellular technologies to study the cells in their physiological environment in vivo, within the system of an organism, namely ‘in vivo Flow Cytometry’ and ‘In vivo Imaging’. Adapting our technical procedures and applying this to the natural environment of systems will improve our understanding of the dynamics of cellular processes in the nature.

We hope this CYTOMICS workshop will be the platform in future within the Cytometry Society to discuss cutting-edge technologies that bridge the gap of the ‘Translational System Cytometry from Bench to Bedside’.

105 Highly Multiplexed Imaging with Subcellular Resolution by CyTOF Mass Cytometry
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Introduction: Tissues are complicated assemblies of multiple interacting cell types that communicate with each other to achieve physiological states. In cancer, malignant cells and normal cells of the tumor microenvironment (TME) facilitate tumor progression and drug resistance. For an understanding of the underlying processes it is important to comprehensively investigate the components and their relationship within the TME. This necessitates imaging approaches that can simultaneously measure dozens of biomarkers in a targeted and reproducible manner to define cell types, their functional and signaling states, and spatial relationships.

Methods: For highly multiplexed tissue imaging at subcellular resolution, we have coupled immunohistochemical (IHC) methods with high-resolution laser ablation and mass cytometry (1). In mass cytometry, rare earth metals are used as reporters on antibodies. Analysis of metal abundances using the mass cytometer – an inductively coupled plasma mass spectrometer – allows determination of biomarker expression. In the approach presented here, tissue sections were prepared for antibody labeling using IHC protocols. Rare-earth-metal isotope tagged
antibodies were selected to target proteins and protein modifications relevant to breast cancer. After antibody staining, the sample was positioned in a laser ablation chamber developed by Wang et al. (2) to minimize aerosol dispersion for high-resolution, high-throughput and highly sensitive analyses. The tissue was then ablated spot by spot, and the ablated material was transported to the CyTOF mass cytometer using a gas stream. After data preprocessing, the 32 transient, isoantibodies were plotted using the coordinates of each single laser shot, and a high-dimensional image of the sample was generated. Single-cell features, high-dimensional data, and single-cell marker expression data were extracted for downstream bioinformatics analyses.

Result: Imaging mass cytometry provides targeted, high-dimensional analysis of cell type and state at subcellular resolution to study tissues and adherent cells. The novel imaging approach enabled the simultaneous visualization of 32 proteins and protein modifications, with the potential to map up to 120 markers on a single tissue section with the availability of additional isotopes. Application of imaging mass cytometry to breast cancer samples allowed delineation of cell subpopulations and cell-to-cell interactions, highlighting tumour heterogeneity and enabling new routes to patient classification. As such it has the potential to yield novel insights of the TME by exploiting existing large collections of FFPE tumor samples and associated clinical information.


106 Microfluidic-Based Cell Sorting
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This talk will describe the use of microfluidics to create a new class of cell sorter, that both competes with more traditional FACS and that extends its utility by a significant amount. This talk will describe a traditional cell sorter that is fully self-contained within a microfluidic chip, eliminating all aerosol production and using much smaller quantities of sheath fluid. Optical alignment is greatly simplified through the use of the microfluidic chip. Cells are probed optically, using fluorescence or scattering, and are sorted using a surface acoustic wave device which produces a traveling acoustic wave that changes the direction of fluid flow, thereby sorting the cells. This device can operate at rates close to those achieved with a more traditional FACS instrument, while using much smaller volumes of fluid. The flexibility of the device is greatly enhanced by modifying the device to encapsulate the cells in aqueous drops suspended in an inert carrier oil. Because a microfluidic chip is used, there is no problem with the use of an oil as the carrier fluid. Moreover, because the cell is encapsulated in a drop, many of the limitations of FACS are removed. In particular, the detection assay does not have to be chemically fixed to the surface or interior of the cell, but can instead be a secreted fluid that is contained within the drop. This greatly enhances the nature of assays that can be probed. For example the cell can excrete proteins and these can be detected or the cell can be lysed and the lysate can be probed. It is even possible to test multiple cells within the same drop, enabling growth or killing assays to be used. The drops themselves can be easily sorted in the same fashion as the cells, and can even be transferred to wells of a multiwall plate, one drop per well, for further analysis. Examples of the use of this drop-based cell sorting will be described.

107 Inkjet-Printed Cell-Counting Chambers with Integrated On-Chip Sample Preparation
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Current cell counting techniques often require expensive lab equipment or labor-intensive procedures, e.g. for sample preparation or manual counting. Our concept for simple low-cost cell counting uses dried reagent films inside cell counting chambers in combination with large-area quantitative fluorescence imaging. Using this concept, we have demonstrated a CD4 count which can meet the requirements for resource-poor settings [1]. Delayed release of fluorophore-conjugated antibodies or nuclear stains from the reagent layer after filling of the chamber with whole blood allows for very uniform cell staining. Training requirements are minimal, as the entire sample preparation is integrated on the chip, and precision pipetting is not required, since the sample volume per image (~ 1 µl) is determined by the chamber height (~ 25 µm) and the image area (~ 40 mm²).

Recently, we have made major progress with regard to automated fabrication of chambers by inkjet printing, studies of the release kinetics of cell staining reagents from sub-µm films of different materials, as well as new assays based on this principle. We have developed printing processes to replace the manual fabrication of our CD4 counting chambers. Both the reagent layer and the spacer material defining the shape and height of the chambers are deposited onto a flat PMMA slide using printing techniques, which will allow for extremely low cost production. The ink for the reagent layer of the CD4 count consists of an aqueous gelatin solution containing two fluorescently labeled antibodies (CD3-APC, CD4-PerCP) for immunostaining, deposited by a picoliter inkjet dispenser. The chamber walls function as both seal and spacer and consist of a suspension of 20 µm polystyrene beads in a UV-curable glue that is deposited by a nanoliter dispenser. After attaching a standard glass microscope slide as a cover the chambers are immediately ready to use or can be stored for at least 3 months at room temperature. CD4 counts using ‘all-printed’ chambers compare well with reference values obtained by flow cytometry.

Furthermore, our approach for real-time monitoring of the release kinetics of antibodies or nuclear stains from sub-µm thin films of various materials has resulted in better understanding of the release process, and has allowed us to optimize the printing procedures and, to fine-tune the release kinetics of our assays. The measured antibody release kinetics from gelatin films comply with diffusion controlled release. Release times between ~20 s and ~840 s are obtained from dry layers with the thicknesses between 0.25 µm and 1.5 µm. This is consistent with an antibody diffusion coefficient of 0.4 µm²/s in swollen gelatin layers.

The versatility of the cell counting platform was demonstrated by the realization of a differential white blood cell count combined with a CD4/CD4% count.


108 Single Cell Isolation Technology Based on Single Cell Self-Seeding Microwells
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Introduction: The heterogeneity of tumor cells dictates the need for analysis at the single cell level. In addition, gene expression can be altered during the course of the disease and is accelerated under the influence of therapy. This imposes the need for a tumor biopsy each time therapeutic intervention is required. Circulating Tumor Cells (CTC) represent a unique opportunity for a ‘real time liquid biopsy’. Current available CTC counting technologies are hampered by inefficiency to isolate individual CTC for further molecular characterization to unveil the best treatment strategy.
Here we introduce a simple solution to obtain and analyze the genetic make-up of individual cells and CTC.

**Methods:** A Self Seeding Microwell Chip comprises of 6400 microwells in an effective area of 808 mm². Each well has a diameter of 70 μm and a depth of 360 μm resulting in a well volume of 1.4 nL. The bottom of the well is a thin, optical transparent, silicon nitride (Si3N4) membrane with a thickness of 1 μm, that contains a single pore with a diameter of 5 μm in the center. Fluid and cells enter the microwells from the top and exits through the pore at the bottom. A cell with a diameter larger than the pore, will close the pore hereby stopping the flow through this well. The remaining sample will have to find other wells to pass. This results in a fast and efficient distribution of single cells in individual microwells. After identification by fluorescence microscopy, the bottoms and the cells of the microwells that contain cells of interest were punched into the cups of 384-well PCR plate that is placed below the microwells, using a thin needle.

A mixture of differentially stained LNCAp, PC-3 and SKBR3 cells were prepared, seeded onto the microwells and punched into cups of a 386 well PCR plate. Whole Genome Amplification (WGA) was performed on the punched cells using WGA kits of GE Healthcare, New England Biolabs and Silicon Biosystems. Specificity of the system was demonstrated by detection of two specific mutations in the ROBO-1 and PTEN genes using Sanger sequencing.

**Results:** The efficiency for transferring single cells from the microwell to the PCR plate was > 80%, with a single cell isolation frequency of 1 cell / second. Three different WGA kits were used to amplify the DNA of the punched cells. Depending on the WGA kit that was used Whole Genome Amplified DNA was found to be produced in 60% of the wells for the NEB kit, 77% for the GE kit and 62% for the SB kit. The identity of punched single LNCAp, PC-3 and SKBR3 cells, was confirmed by Sanger sequencing on the WGA DNA by detection of the mutations in the Robo-1 and the PTEN genes in the specific cell lines.

**Conclusions:** We introduced a self-sorting nanowell plate in combination with an efficient method for the isolation of single cells. On average 68% of the single isolated cells can be amplified with different WGA kits.

109 Integrated III-V Semiconductor Platform with Capillary Fill Micro-fluidics for Chip-Based Flow Cytometry

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The advent of bench top flow cytometry has reduced some of the cost and complexity associated with cell analysis. However, the potential of bench top systems for low cost point-of-care and resource poor settings is limited. Micro-electro-mechanical systems (MEMS) and micro-fluidic technologies on the other hand, offer a potential route toward truly low cost, portable/disposable flow cytometers [1] but current dependence on external light sources, detectors and fluid pumping equipment undermines true portability and adds significant cost. Emitters and detectors can be incorporated on-chip using 'pick-and-place' style fabrication [2], but, alignment constraints and cost, make this approach impractical for anything but the simplest systems. The issue of alignment of large, multicomponent systems can be addressed with monolithic integration of photonic elements: lasers and photo-detectors are defined on a common substrate using high resolution lithographic techniques [3]. Even so, as long as the device relies on pressure driven fluidics for cell delivery, the chip based flow cytometer remains tethered to large scale and expensive pumping equipment.

Here we present a prototype of an integrated III-V semiconductor platform that combines a coupled array of 10 μm wide ridge laser/detectors with an on-chip capillary fill fluid delivery system, figure 1(a). We demonstrate the potential of this device through the results of a micro-bead counting experiment. A 0.5 μL sample of 10 μm diameter polymer beads, suspended in water, is deposited into an on-chip inlet reservoir from where it flows, under capillary action, through a buried micro-channel. Lasers on one side of the channel are forward biased in pulsed mode and the opposing lasers are operated as photodiodes. Operation of light emitting and detecting sections in a pulsed mode provides sub-μs time resolution of measurement. Figure 1(b) shows a plot of the time-resolved photo-voltage signal measured from a detector as a bead passes through the interrogation region. The sample volume used here sustains a continuous fluid flow of over 30 s and the 30 mV noise floor affords a large dynamic range of well over 1.2 V. The prototype demonstrates the potential for multiple interrogation events per cell and true scalability in a microscale system format.

Figure 1(a) Image of the integrated device showing an array of coupled 1mm long lasers with a 50 μm wide fluid channel. (b) Time-resolved photo-voltage recording the transit of a bead through the lasers.


110 Acoustic and Magnetic Methods for Cell Sorting and Single Cell Analysis in a Microfluidic Device

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**Introduction:** Accurate and high throughput cell sorting is a critical “first step” in isolating target cells from complex, biological fluids. The current gold standard for sorting cells, fluorescence-activated cell sorting (FACS), is capable of multiplexed analyses and can sort up to 50,000 cells/sec. However, FACS systems suffer from high costs, bulky designs and a reliance on purified buffers, all of which make routine clinical use suboptimal.

We propose a microfluidic system as a prototype for a new generation of cell sorting and analysis that is a potentially viable alternative to FACS. This system is comprised of three independently verified modules that operate to: (1) acoustically confine cells bound to elastomeric particles, (2) magnetically separate acoustically pre-focused cells in a non-uniform magentic field (B) and (3) magnetically template those cells into a spatially periodic microwell array for on-chip staining and single cell analysis.

**Methods & Results:** Module 1: Acoustophoresis enables gentle cell separation capabilities based on the acoustic properties of cells and the carrier fluid. 2 A transducer excites the device to resonance whereupon ultrasonic standing waves form across the flow cavity. Cells bound to elastomeric particles are driven to the pressure antinodes (i.e., the walls of the microchannel) and unbound cells are driven to the pressure node (i.e., the center of the microchannel). We show that elastomeric particles, synthesized from the nucleation and growth of alkoxysilane monomers can be used to immunospecifically capture KG-1a cells for their rapid confinement in an acoustic standing wave.
Module 2: Magnetophoresis enables the separation of cells bound to magnetic nanoparticles from cells without magnetic labels. Using forces from an acoustic standing wave (as in Module 1), we show that pre-focused cells can magnetically separate in a non-uniform magnetic field.

Module 3: We show that a magnetographic array, comprised of an overlaid pattern of micromagnets and microwells, is capable of capturing magnetically labeled cells into well-defined compartments with accuracies >95%. As a proof of principle, we show this device can localize CD3+ lymphocytes into microwells on a single chip for on-chip staining to accurately assess their CD4:CD8 ratio.

Conclusion: We have developed a versatile cell-sample processing system in which interchangeable, chip-based modules are used for precise cell manipulation and analysis. These modules are designed to integrate into a holistic instrument capable of comprehensive cell isolation, multi-marker staining and single cell analysis that could enable rapid prognostic and diagnostic evaluations on a microchip.

Conclusions: A µFACS based on integrated jet flow generators is reported. The flow speed, event duration and purity were comparable to commercial FACS, but in an aerosol-free closed fluidic system. The rapid sorting of human cells is demonstrated with no indication of significant cell viability losses. The complete device is produced in a silicon fabrication pilot line. Ultra high throughput sorting with parallel microfluidic channel is being developed.

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112 Earth Mover’s Distance Approach for Quantitative Analysis of Multivariate Changes in Biomarkers Measured by Flow Cytometry

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Background: Changes in frequency and/or marker expression in subsets of cells can serve as important biomarkers of drug response, disease susceptibility, etc. High-resolution characterization of these cellular subsets is possible through improvements in flow cytometry instrumentation, in which measurements are taken for dozens of characteristics on hundreds of thousands of cells, resulting in complex non-parametric multivariate distributions of data. These distributions can be compared between disease and control cell subsets, samples that undergone stimulations, etc. to provide information about the model system. While flow instrumentation has advanced to meet these needs, methodologies for clinically useful quantification of differences between subsets in routine and high-throughput analyses have lagged sorely behind and are limited to hypothesis testing, which detect the presence or absence of differences between subsets without quantitative characterization. Here we present a new quantitative methodology that facilitates flow data analyses and provides reliable, multivariate indices of change in subset representation and marker expression. We introduce this new paradigm by applying the Earth Mover’s Distance (EMD) approach from computer vision literature, in combination with a density-based clustering method (DBM), to provide a suitable metric for high-dimensional (Hi-D) flow data treatment.

Methods: We used DBM to perform clustering of Hi-D flow cytometry data to define cell subsets. Then given defined populations of cells before and after stimulation or treatment, we used the EMD approach to quantify dissimilarity between the samples. Classification analysis was performed using support vector machine method.

Results: We have developed a new methodology based on the EMD approach for quantitative evaluation of multivariate flow cytometry data in a biologically meaningful way. The practical utility of such methodology was demonstrated through three proof-of-concept studies. First, utilizing data from an allergy study, we demonstrated clinically-relevant shifts in the expression of two markers on basophils in response to stimulation with an offending allergen. Second, we used the EMD to quantify changes in the murine colon cancer tumor immune cell microenvironment in response to ablative tumor radiation. Also, we applied the EMD approach on samples of mouse peritoneal cavity cells to rank the immunological difference between three commonly used mouse strains. All experiments involving animals were conducted...
in conformance with approved principles of the care and use of animals in research.

**Conclusion:** The EMD approach can be used with any method that enables identification and isolation of cellular subsets on which markers are expressed, i.e. flow cytometry, mass cytometry, etc. We plan to make this technology available in ways that will allow clinicians and researchers to readily apply it to commonly encountered problems in which recognizing quantitative differences between samples is key. We have implemented this method in a provisional software package, available for free to .edu and .gov users, that provides Hi-D flow data analysis tools.

**113 Surface Proteome of Adipose Stromal Vascular Subpopulations**

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We used the BDV FACSAP 3-color Lyplate system to investigate the cell surface proteome of freshly isolated adipose stromal vascular cells (SVF, n = 4). The Lyplate system includes directly labeled antibodies (FITC, PE, APC or equivalents) in a 96well plate, dispensed with 3 different specificities per well. Coverage consists of 242 surface markers including control antibodies. Isolated cells were stained in bulk with a 7-color backbone panel consisting of anti:- CD19-ECD, CD3-PS, CD45-PS, CD31-PC, CD34-APC, CD14-A700, CD14-A707, CD146-BV711, aliquoted and stained **in situ** in the Lyplate. Stained cells were fixed, permeabilized and stained with DAPI for DNA content analysis prior to acquisition on a BD LSR II Fortessa cytometer equipped with a high throughput sampler. File-level analysis was performed using VenturiOne software. The backbone panel was used to identify a total of 9 non-overlapping subpopulations. Five were previously identified SVF populations: pericytes (P), supraadventitial adipose stromal cells (SA-ASC), endothelial progenitor (EP) and mature cells (EM), and a transitional population (TR) between pericytes and SA-ASC. An additional previously uncharacterized high light scatter CD45dim/CD34+ population was also identified, as were T cells, B cells and macrophages. For each subpopulation, data (percent positive, MFI positive, MFI total) were exported to Microsoft Excel, organized and exported to SYSTAT statistical software for classification of each marker by K-means clustering (4 clusters corresponding to negative, weak positive, positive, strong positive). Starting with the hypothesis that the pericyte is the progenitor of all nonhematopoietic adipose subpopulations, we determined the global differences in marker expression between each subset and pericytes. Compared to pericytes, CD58 (LFA-3), CD45RA, CD49a (integrin α1), CD102 (ICAM-2), CD105 (endoglycan), CD141 (thrombomodulin), CD147 (Eminprin) and HLADR were markedly upregulated during endothelial specification. Upregulation of CD201 and CD271 accompanied adipose differentiation. CD340 (Her-2) was upregulated during endothelial as well as adipose differentiation. Based on similarity of marker expression, the CD45dim/CD34+ may represent an intermediate between the pericyte and the endothelial progenitor cell. Whether this SVF subset, which co-expresses the leukocyte antigen CD45 and CD31, represents an adult equivalent of the hemangioblast requires further investigation.

**114 The Tenets of Helper T-cell Classification: Checked and Challenged with 30-Parameter Flow Cytometry**

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CD4+ (‘helper’) T-cells play a central role in coordinating immune responses to vaccination and natural infection, through the expression and secretion of a myriad of proteins. To better understand T-cell immunity, and find correlates of protective T-cell responses, CD4+ T-cells have historically been classified into subsets, using surface marker expression as a surrogate for a cell’s functional activity.

For example, subsets of CD4+ (helper) T-cells may exhibit different functions depending on the cytokines they express. In turn, it has been postulated that differences in cytokine expression are linked to particular (cell surface) chemokine receptors.

However, because of technical limitations, the overlap between cell types defined by cytokine and chemokine receptor combinations has never been comprehensively examined. With our new 30-parameter flow cytometry technology, we simultaneously tested whether: 1) CXCR3 expression was a surrogate for TH1 cells (that make IFNγ, TNF, or IL2), 2) CRTH2 or CCR3 were surrogates for TH2 cells (making IL4, IL5, and IL13), and 3) CCR4 or CCR6 were surrogates for TH17 expression (TH17 cells), as previously reported based on limited flow cytometry panels or in vitro expansion experiments. We found that, after **ex vivo** stimulation (with SEB or PMA/ionomycin), CCR3-expressing cells did not make IL4 or IL13 (cytokines common in TH2 type cells), and that CRTH2 expressing cells made IFNγ and TNF, which are not typically expressed with TH2-type cells. We also found that many IL17+ cells lacked expression of either CCR4 or CCR6, suggesting that these chemokine receptors are not complete surrogates of a TH17-type T-cell response. We used this data to calculate a ‘polarization index’, which indicates the degree to which a chemokine receptor reliably identifies the cytokine-producing cells it is thought to mark, and have developed a new and straightforward method of graphically representing the cytokine polarization of cell populations.

We have performed similar studies with panels that analyze regulatory T-cells (which are variously thought to be identified by CD25, CD39, CD73, CD127, and FoxP3, along with a number of cytokines) and cytolytic CD4+ T-cells. In summary, the data collected demonstrate the incredible complexity of T-cell responses, and support the need for higher-parameter technology in immune monitoring. Importantly, since the core of this technology involves coupling bright new dyes with tried and true flow cytometry: 1) signals for dim markers are easily detected over background, 2) reagents for replication and extension of this work are readily available, and 3) cell sorting (for downstream cellular assays) will be possible in the near future.

**115 Automated Analysis of 16-Color Polychromatic Flow Cytometry Data Maps Immune Cell Populations and Reveals a Distinct Inhibitory Receptor Signature in Systemic Sclerosis**

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**Background.** The phenotypic profiles of both peripheral blood and tissue-resident immune cells have been linked to the health status of individuals with infectious and autoimmune diseases, as well as cancer. In light of the promising clinical trial results of agents that block the Inhibitory Receptor (IR) Programmed Death 1 (PD-1) axis, novel flow cytometric panels that simultaneously measure multiple IRs on several immune cell subsets could provide the distinct IR signatures to target in combinational therapies for many disease states. Also, due to the paucity of...
Healthy com be applied to polychromatic FCM datasets and provide robust developed for processing multi-dimensional single cell analysis that allow objective and comprehensive population characterization are severely underutilized on data generated from large polychromatic panels.

Methods. A 16-color flow cytometry (FCM) panel was developed and optimized for the simultaneous characterization and purification of multiple human immune cell populations on a 4-laser BD FACSARIA II cell sorter. FCM data of samples obtained from healthy subjects and individuals with systemic sclerosis (SSc) were loaded into Cytobank cloud, then compensated and analyzed with SPADE clustering algorithm. The viSNE algorithm was also employed to compress the data into a 2D map of phenotypic space that was subsequently clustered using SPADE. For comparison, the FCM data were also analyzed manually using FlowJo software.

Results. Our novel 16-color panel recognizes CD3, CD4, CD8, CD45RO, CD25, CD127, CD16, CD56, 6TCR, ve24, PD-1, LAG-3, CTLA-4, and TIM-3; it also contains a CD1d-tetramer and a live-dead dye (with CD19 and CD14 included as a combined dump channel). This panel allows combinational IR signatures to be determined from CD4+ T, CD8+ T, Natural Killer (NK), invariant Natural Killer (iNK), and gamma delta (γδ) immune cell subsets within one sample. We have successfully identified all subsets of interest using automatic SPADE and viSNE algorithms integrated into Cytobank services, and demonstrated a distinctive phenotypic type of IR distribution on healthy versus systemic sclerosis subject groups.

Conclusions. Methods of automatic analysis that were originally developed for processing multi-dimensional mass cytometry can be applied to polychromatic FCM datasets and provide robust results, including subset identification and distinct IR signatures in healthy compared to diseased subject groups.

116 Patients Pre-operative Immune States Correlate with Recovery from Surgical Trauma

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Introduction: Over 60 million surgeries are performed annually in the US alone. Recovery after surgery is highly variable, and protracted recovery causes significant societal and economic costs. Protracted recovery is a widely recognized health care problem; however, the biology that drives recovery remains poorly understood. Furthermore, we lack strategies to predict recovery in individual patients.

We have recently applied mass cytometry at the bedside to survey alterations of the human immune system in patients undergoing major surgery (hip arthroplasty)1. Analysis of the resulting high-dimensional, single-cell dataset revealed patient-specific responses that contained strong correlates of clinical recovery. Specifically, changes in STAT3, NF-kB, and CREB signaling shortly after surgery correlated with recovery from fatigue, pain, and functional impairment of the hip.

The identification of strong immune correlates shortly after surgery is an important advancement in understanding the biology that drives recovery. However, a blood test interrogating a patient's immune system before surgery and accurately predicting clinical recovery after surgery would truly transform perioperative patient care. In this study, an in vitro assay was developed to determine whether differences in patients' pre-surgical immune states containing immune correlates of recovery from surgery.

Methods: 26 patients scheduled for primary hip replacement were enrolled (20 males, 12 females, ASA 1-2, median age 59). Clinical recovery profiles were collected as previously described. Pre-surgical blood samples were obtained and stimulated in vitro with a panel of extracellular ligands (LPS, IL-2, IL-6, IL-10, GM-CSF). Using mass cytometry, 21 phenotypic markers and 10 evoked intracellular responses were simultaneously quantified per single cell contained in each blood sample. Evoked immune responses that correlated significantly with indices of clinical recovery were identified using a False Discovery Rate < 0.01.

Results: Activation of canonical intracellular signaling pathways downstream of selected extracellular ligands was observed in all patient samples. However, the magnitude of this response varied significantly across patients, thereby defining pre-surgical and patient-specific 'immune states'. Among these responses, signaling downstream of the TLR4 receptor in CD14+ monocytes (namely pCREB, ppp38s and pMAPKAP2) strongly correlated with recovery from functional impairment of the hip (R=0.45-0.69, FDR<0.01). The pre-surgical immune responses reported here mirrored post-surgical immune correlates of functional recovery.

Conclusion: Outlined approach provides proof-of-concept as well as the mechanistic basis to develop a pre-surgical diagnostic 'immune stress test' to predict the course of clinical recovery. While mass cytometry is a relatively complex and novel technology, reported immune correlates are readily identifiable with a total of six markers that are adaptable to a traditional fluorescence-based flow cytometry test.

References:

117 Deep Immunophenotyping by ChipCytometry Reveals CD11c+ B Cell Subset in Inflamed Tonsils

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Introduction: Image-based ChipCytometry is an emerging analytical cytometric methodology enabling destruction-free analysis of cells and tissues. Cells or tissue are immobilized on microfluidic chips and are analyzed by biomarkers by iterative immunostaining-imaging cycles. ChipCytometry is highly complementary to classic flow cytometry as immobilized specimens can be stored for <1 year (biorepository) which makes this technology attractive for consecutive studies such as desired inter alia during clinical trials.

Method: To test the feasibility of this technology for patient samples we aimed to study the cytokine of tissue B cells of inflamed human tonsils. We first generated a biorepository with immobilized tonsil tissue section and isolated tonsil leukocytes from 6 donors. Then we conducted deep immunophenotyping on the single cell samples using 27 different antigens as analytes.

Results: ChipCytometry data revealed the classic major T and B cell subsets, confirming the suitability of this technology for immunophenotyping. Unexpectedly, we found in all samples a subset of tonsil B cells which stained positively for CD11c1, a marker generally used to identify antigen-presenting myeloid cells such as dendritic cells 8.8 +/-1.7 % of B cells in the IgD-CD27+ cluster were found CD11c positive while 1.5 +/- 0.9 % CD11c+ cells were found in the IgD+CD27- cluster. In addition, both IgM+ or class-switched IgG+ cells were detected within CD11c+ B cell fraction. In contrast, CD11c+ B cells lacked the plasmablast marker CD318 and the myeloid cell marker CD11b. The expression of CD11c+ was confirmed by PCR and standard
flow cytometry on FACS-sorted tonsil B cells. A 10-plex ChipCytometry marker study on donor-matched tonsil tissue sections demonstrated the localization of CD11c+ B cells in the germinal centers of the tonsil architecture. This suggests an active contribution of these cells to humoral immunity. While the scarce literature on CD11c+B cells relate their existence to autoimmunity and autoantibody formation, functional- or organ-specific features of this interesting cell subset remain to be elucidated.

In conclusion, our study clearly demonstrates the suitability of ChipCytometry for high-dimensional immunophenotyping and underlines the attractiveness to develop and employ this methodology for the assessment of patient samples to address translational and clinical questions.

118 Quantitation of Germinal Centre T and B Lymphocytes in Lymph Node and Gut Biopsies during HIV Infection

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Background: Measurement of HIV-1 reservoirs is usually studied in peripheral blood (PB). However, 98% of CD4 T cells are in lymphoid tissues and other organs, particularly gut-associated lymphoid tissue (GALT). CD4 T cells in these tissues may be greatly depleted early in HIV-1 infection, but histological studies showed characteristic increases in germinal centres (GC) in lymphoid tissue. Recently, increases in the T follicular helper cell (TfH) subset of CD4 T cells, an integral cell type within GC, have been reported, and there is now recognition that GC and TfH are an important reservoir of HIV-1. Ultrasound-guided fine needle biopsies (FNB) from peripheral lymph nodes (LN) may offer a minimally invasive means of longitudinally accessing HIV-1 reservoirs in GC in lymphoid tissue.

Methods: FNB of inguinal LN were performed on 10 healthy adult controls (HC). 11 HIV+ ARV-naive and 10 HIV+ on ART subjects, without adverse events. FNB were analyzed for CD3+CD4+PD-1hiCXCR5+ICOS+CD127dimCD45RA- Tfh cells and CD19+CD20hiCD38hiKi67+IgA+ B cells in intact tonsil tissue. CD4 T cells from FNB and PB were sorted from 19 patients, and HIV DNA and cell-associated (CA) HIV RNA were quantified by real time PCR. Separately, 15 HC and 13 HIV+ subjects on ART provided ten gut pinch biopsies from each of 3 sites: left colon (LC), right colon (RC) and terminal ileum (TI), via endoscopy and colonoscopy. Single cell suspensions were prepared by collagenase digestion, and CD45+ve/EpCam-ve, IgA+CD19+CD27+ B cells and TIh cells were counted.

Results: Median Thh cell number in FNB from ART-naive HIV+ subjects was 46,300 cells, significantly higher than in HC (5,600; p<0.04), but not HIV+ on ART (15,800). Median GC B cell number in FNB from ART-naive HIV+ was 153,493 cells, significantly higher than HC (1,506; p<0.01), but not HIV+ on ART was 30,749 cells, so that 4/10 were above HC normal range. HIV+ FNB yielded a median 518,938 CD4+ T-cells, and HIV DNA was quantified in 19 out of 19, and CA HIV RNA in 13 out of 17, samples. In gut biopsies, median TIh cell counts from HC were 2743, 4526 and 9714 from LC, RC and TI biopsies respectively, not significantly different to corresponding median Thh cell counts from HIV+ subjects (6840, 5236, and 3703, respectively). Median IgA+ B cell counts from HC were 29704, 67780 and 30367 from LC, RC and TI, respectively, not significantly different to HIV+ subject cell counts (4533, 43641 and 35266, respectively). Counts for CD38hiIgA+ plasmablasts were also not significantly different between the two subject groups.

Conclusions: Ultrasound guided FNB of unenlarged LN was safe and well-tolerated, so that high-dimensional flow cytometry and PCR allowed assessment of HIV persistence in LN, and access to Thh and GC B cells for pathogenesis, therapy and vaccine studies. HIV+ subjects on ART appeared to have normalized GALT Thh and IgA+ B cell levels, but 4/10 still had elevated Thh and GC B cells in peripheral lymph nodes.

119 Anti-TNF Therapy Restores Peripheral Blood Cell Subsets and CD40 Expression in Inflammatory Bowel Diseases

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Background & Aims: The role of B lymphocytes in inflammatory bowel diseases (IBD) is largely unexplored. Anti-TNF therapy has become a standard therapy for severe IBD, but its effect on B cells is unknown. IFX also up-regulates blood total memory and pre-switched memory B cells in rheumatoid arthritis. In IBD, IgM (+) memory B cells are decreased. CD19+ B cells in the inflamed intestinal mucosa predicts long lasting remission to IFX in CD. We investigated peripheral blood B cells, B cell subsets and CD40 expression (as a maker of B cell activity) in patients with IBD before and during anti-TNF therapy with infliximab (IFX).

Methods: Blood was taken from healthy controls (HC, n=52) and patients with active IBD before (n=46) and/or during anti-TNF therapy (n=55). B cell markers were identified by immunofluorescent staining and FACS analysis.

Results: We found a numerical deficiency of circulating CD19+B cells, a lower activity state (CD40 expression) and lower proportions of CD5+ B cells and IgM+CD27+pre-switched memory B cells in active IBD patients before therapy, compared to healthy controls. IFX treatment increased CD19+B cell number as well as the proportions of named subsets in responders (RS) but not in non-responders (NRS). IFX more effectively up-regulated CD40 expression in RS than in NRS. Restoration of B cells correlated with the biological response (CRP, Trough levels of IFX correlated with the number of B cells during therapy.

Conclusions: A lower number of circulating B cells, a low CD40 expression, and a decrease in the proportion of CD5+ and of the pre-switched memory subset characterize active IBD. Restoration of these abnormalities by anti-TNF therapy correlates with the clinical response to anti-TNF therapy.

Reference
120 Scanning Flow Cytometry Study of Cell-Derived Microparticles and Their Aggregates in Platelet-Rich Plasma

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Introduction: Reliable detection and analysis of single cell-derived microparticles (MPs) is complicated by their small sizes and low refractive indices (RIs), that motivates development of new techniques to identify MPs in biological fluids, distinguishing them from other constituents, and to characterize MPs by their shape, size, and RI.

Methods: We developed a flow-cytometry-based method for identification and characterization of MPs in platelet-rich plasma from light scattering. Using a scanning flow cytometer we measure angle-resolved light-scattering profiles (LSPs) and side scattering for individual particles in plasma. These data is used to deduce particle size, shape, and RI from the solution of the inverse light-scattering (ILS) problem given the adequate optical model of the particle. We utilized the following light-scattering simulation methods: discrete-dipole approximation, Mie-theory, and T-matrix method for platelets, sphere-like and bisphere-like MPs respectively. These particles were separated from other detected events, including larger MP aggregates and other non-spherical plasma constituents, based on agreement between with measured and calculated LSPs. Thus, we not only identify individual MPs among other particles, but also characterize them by their size and RI, including errors of these estimates.

Results: We analyzed a sample of platelet-rich plasma. All detected events were separated into platelet and MP events, including sphere-like, bisphere-like and not-identified particles (none of proposed models is suitable). Identified MPs were characterized by their distributions over size and RI. Polystyrene beads of 0.4 and 1 µm were also measured. The developed method and current setup of the SFC allowed us to reliably characterize the fraction of single spherical MPs falling in the range of 450–600 nm for size and 1.48–1.52 for RI, the median uncertainties of single measurements were 6 nm and 0.003, respectively. RI of beads is 1.617–1.618, which agrees with the literature data for polystyrene at 405 nm.

Conclusion: Presented light-scattering method allows one to identify single spherical MPs and biosphere-like MP aggregates in platelet-rich plasma and characterize them by their size and RI with high precision.

121 Standardization of Flow Cytometry-Based Determination of Plasma Microvesicles: Recent Progress

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Background: Cell-derived microvesicles (MV) counts may provide useful diagnostic/prognostic information, assessment of their pathophysiological relevance in multicentre studies is hampered by methodological concerns and a lack of standardization. Aiming to define the usefulness of MV as true biomarkers in clinical practice, the Scientific Sub-Committee on Vascular Biology (VB-SSC) of the International Society of Thrombosis and Haemostasis (ISTH) has set up a network aimed at mastering pre-analytical and analytical variables (mainly FCM-related) for MV analysis. First, a pre-analytical protocol significantly reducing the variability of MV measurement was validated in a multicentre study (1). Second, a collaborative workshop using standard Flow Cytometry (sd-FCM) in 2009 defined the inter-laboratory variability of PMV counts (2). Although a specific bead-based calibration system proved to be useful for instrument qualification and monitoring, differential behavior among flow cytometer (FCM) sub-types impeded a universal standard set-up. Third, a modified strategy has been recently proposed to provide optimized scatter-based reference levels for either FSC- or SSC-optimized FCMs (3) and to cope with higher sensitivity FCM (hs-FCM, 4).

Aim: Based on this new approach, a 2nd collaborative workshop was initiated to evaluate inter-instrument reproducibility of MV counts among different platforms.

Methods: The 1st phase (2013) aimed at selecting FCMr based on scatter resolution and background level. In the 2nd phase (2014), selected labs received frozen aliquots of plasma featuring various platelet MV (PMV) counts as well as common reagents. In additon to the bead-based Q.C. tools (Megamix-Plus FSC or Megamix-Plus SSC, depending on instrument), these included PMV staining reagents and 3 µm counting beads compatible with hs-FCM.

Results: From Phase 1, ~85% of n=59 candidate FCMr featuring 14 types from 44 labs passed the MV-oriented Q.C., most of which confirmed qualification a year after. After Phase 2 of this multi-center exercise, the mean MV counts were gained from n=29 FCMr and illustrated that comparable PMV counts can be achieved among different labs, even using different platforms. Indeed, the mean PMP counts measured in each group of FCMr (FSC- or SSC-optimized) were not statistically different from one group to the other nor from those determined by the core-lab with CVs across all labs of 31%, 40% and 51%, depending on the sample. These CV contrast with previously observed differences in orders of magnitude.

Conclusion: The results of this international exercise for the standardized counting of PMVs with currently available FCMr open the door for future multi-center studies. Although it should not stop ISAC’s technological search for higher sensitivity, this standardization effort may already help taking the best from actual FCMr.


122 Quantification of Cell-Derived Microvesicles in Blood

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Background: Cell-derived microvesicles (MV) play major roles in health and disease. Elevated MV levels have been found in plasma and other body fluids in many pathological disorders, leading to the hypothesis that MVs may serve as disease biomarkers. However, the small size of MVs renders difficult their characterization, principally their quantification, as attested by major discrepancies in MV concentrations reported in the literature. It is therefore critical and urgent to design reliable and sensitive methods of MV quantification. We have recently shown that a simple flow cytometry (FCM) method based on fluorescence triggering enabled to detect 50 x more Annexin-5-positive (Ann5+) MVs than conventional FCM methods based on light scatter triggering (1).

Here, we will present the extension of this approach to the enumeration of the main MV populations in blood, namely MVs derived from platelets and erythrocytes, as well as their subpopulations that bind or do not bind Annex5.

**Methods:** Three types of blood samples were analyzed: 1) whole blood (WB); 2) platelet rich plasma (PRP) obtained by centrifuging WB at 200 g for 10 min; 3) platelet free plasma (PFP) samples, obtained by two cycles of centrifugation at 2,500 g for 15 min. Blood samples were single- or double-labeled with Annex5-Cy5, anti-CD41-PE or anti-CD235a-PE mAb. MV concentrations were determined by triggering detection either on a fluorescence signal or on the forward scatter intensity (FS), using a Gallios flow cytometer. In addition, MVs were enumerated by a quantitative method of electron microscopy (EM) in which samples were labeled with gold particles conjugated with Annex5, anti-CD41 or CD235a and sedimented on EM grids (3).

**Results:** In PFP, significantly higher MV concentrations were detected by fluorescence as compared to FS triggering, namely 40 x for Annex5+ MVs, 75 x for CD41+ MVs and 15 x for CD235a+ MVs. We found that about 20% of Annex5+ MVs were of platelet origin, and only 3% of erythrocyte origin. MV concentrations determined by on-grid sedimentation EM were close to those determined by fluorescence triggering. In addition, we were able to determine the concentrations of MVs in WB and PRP, for the first time to our knowledge. We found that: i) by FS triggering, a larger number of MVs was detected in WB or PRP than in PFP; the surplus consisting of MVs of large size; ii) by FL triggering, the total concentration of MVs detected in WB or PRP was about 4 x higher than those measured in PFP.

**Conclusions:** The method of MV detection and phenotyping by fluorescence triggering in FCM is simple, sensitive and reliable. The catalogue of MVs in normal plasma constitutes an important step towards the development of a standardized approach for quantifying MVs and a baseline for future studies of MVs in diseases.


### 123 High-Resolution Multiparameter Characterization of Individual Extracellular Vesicles by High Sensitivity Flow Cytometry

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Extracellular vesicles (EVs) are cell-derived nanometer-sized vesicles that have been increasingly recognized as biomarkers of disease and mediators of intercellular communication. As determined by their biogenesis, the two main classes of EVs are exosomes (30-100 nm) and microvesicles or microparticles (MPs, 100-1,000 nm). The large intrinsic heterogeneity in particle size and molecules enclosed or embedded in the lipid bilayer calls for advanced analytical tools that can probe EVs at the single-particle level. However, the small particle size, low refractive index, and low content of cargo molecules render conventional flow cytometry incompetent to analyze all types of individual EVs due to limited sensitivity. Moreover, background signals generated by impurity particles in the sheath fluid also hamper the detection of the smallest EVs. Adopting strategies for single-molecule fluorescence detection in a sheathed flow, our laboratory has developed high-sensitivity flow cytometry (HSFCM) that allows light scattering detection of low refractive index particles as small as 24 nm in diameter. Particularly, the reduced sheath flow rate and detection volume make the interference of impurity particles drastically diminished on the HSFCM. In this report, we will demonstrate how the HSFCM can be applied to the analysis of single EVs.

Exosomes and MPs were extracted separately from human platelet free plasma (PFP) using appropriate protocols of differential centrifugation. To better approach the refractive index of EVs, monodisperse silica nanoparticles with a range of diameters of 40 to 500 nm were synthesized and employed as the size reference standards. Fluorescent polystyrene microparticles with sizes optimized for conventional FCM (Megamix-Plus beads, BioCyteM, Marseille, France) were additionally used to establish the fluorescence detection. Specific cargo contents such as phosphatidylserine, CD41, CD63, and RNA of single MPs or exosomes can be accurately measured using single particle enumeration. Furthermore, concurrent assessment of particle size with biochemical properties of EVs was carried out on the HSFCM via sensitive fluorescence detection. Specific cargo contents such as Annexin5, CD41 or CD235a were measured via single particle enumeration. Functional Sorting of Extracellular Vesicles and Nanoscale Viral Particles with nanoFACS


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**Introduction:** Cytometric sorting of individual biological submicron particles is limited by two major factors: 1) the small and overlapping sizes of these biological particles and 2) the overlap of particle-associated signals with background instrument noise. In order to identify, sort, and study distinct subsets of Extracellular Vesicles (EVs) and ~100 nm viral particles, we developed nanoscale Fluorescence Activated Cell Sorting (nanoFACS) methods that do not rely on coupling EVs or beads, use a high sensitivity flow cytometric image analysis. NanoFACS uses a high sensitivity scatter threshold parameter and “reference noise” in the trigger channel to allow an extended range of detection of nanoscale particles, including parallel substratum signals detected on independent scatter and fluorescence detectors.

**Methods:** We configured an AstrocytoFACS with two side scatter detectors, to use one as the trigger and the other as a detector. With this configuration, we defined two informative regions of interest in the data- the ‘parallel sub-threshold’ events, and the ‘reference noise’ events, so that side scatter and fluorescent labels were sufficient to discriminate and sort distinct EV and viral preparations. We used two HIV (~100nm) viral preparations (CCR5-tropic BaL and CXCR4-tropic NL4.3) with distinct co-receptor tropism and matched U373-MAGI reporter cell lines to quantify preservation of biological activity (infection and tropism) and specificity among the sorted viral particles.

**Results:** NanoFACS detected EVs, liposomes, and other nanoparticles as small as 40nm, and sorted particles at least as small as 80-100nm. With suitable sort speeds and sample dilution, ‘swarming’ (coincidence) did not occur. We sorted 10-100 million particles per hour, in a two-way sort with this nanoFACS configuration and achieved purity (~96%, based on flow cytometric reanalysis) and preserved biological activity (infectivity and specificity) of sorted HIV particles. Crossover testing of sorted viruses with reporter cell lines demonstrated...
preservation of co-receptor tropism and clean separation of the two populations with nanoFACS. Preservation of receptor-specific tropism and titers of the sorted viruses confirmed that single virions, not doublets were sorted.

**Summary/Conclusion:** NanoFACS can extend the range of high-speed particle sorting to an order-of-magnitude smaller than standard flow cytometry. Our ultimate goal is to use nanoFACS to identify, sort, and study relevant subsets of EVs arising from primary tumors, tumor cell lines as well as in response to tumor treatment (e.g., radiation), and to isolate EVs produced in response to viral infection. NanoFACS is the first method, that we are aware of, that can be used to analyze and isolate discrete EV and viral populations in sufficient numbers and with adequate preservation of EV/viral integrity for functional biological assays.

**125**

**Using Analysis of Cellular Heterogeneity in High Content Screening Data to Guide Compound Prioritization in Drug Discovery**

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One of the greatest challenges in biomedical research, drug discovery and diagnostics is understanding how seemingly identical cells can respond differently to perturbagens including drugs for disease treatment. Although heterogeneity is an accepted and common characteristic of a population of cells, it is rarely evaluated or reported. The standard practice for cell-based high content screens, as well as other cell analysis applications, has been to assume a normal distribution and to report a well-to-well average and the standard deviation. To address this important gap we sought to define a method that could readily be used to identify, quantify and characterize population heterogeneity in assays of both cells and small organisms, and furthermore, to guide decisions in drug discovery, experimental cell/tissue profiling and development of diagnostics. Our study suggests that heterogeneity can be effectively identified and quantified with three indices that indicate diversity, non-normality and outliers. The performance of these indices will be presented using the induction and inhibition of STAT3 activation by interleukin-6 (IL-6) and oncostatin M (OSM) in five cell lines, where the systems response was well characterized and controlled. Results indicate that the heterogeneity in the activation of STAT3 varies by cell line and pathway of activation. Cal33 cells stimulated with IL-6 exhibit a distinctly bimodal distribution while OSM induces a more normal distribution of STAT3 activity. In contrast, 686LN and MCF10A cells show nearly identical profiles of heterogeneity when activated by IL-6 and OSM, in both cases exhibiting a single normally distributed population.

The same analysis was further applied to High Content Screening data from several compound series in an SAR designed to optimize inhibitors of STAT3 activation. We will first present an approach to validating assay data for heterogeneity analysis that is essential to ensuring the consistency of cellular response distributions plate-to-plate and day-to-day. Using validated data we show that heterogeneity in the response to inhibitors varies between series and within series providing key insights into the relative efficacy of the compounds. Our conclusion is that for phenotypic assays, heterogeneity may be as important as potency in guiding an SAR. Understanding heterogeneity in the response to perturbagens, as well as in cancer tissues and organ models, will become a critical factor in designing strategies for the development of therapeutics including targeted polypharmacology.

**126**

**Multifocal Multiphoton Fluorescence Lifetime Imaging for High Speed Protein-Protein Interaction Monitoring by FRET in Live Cells and Tissues**

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Fluorescence lifetime imaging microscopy (FLIM) is a powerful technique for high resolution imaging of the functional spatio-temporal dynamics in situ. Förster resonance energy transfer (FRET) is, by far, the most extensively studied technique for observation of protein-protein homo- and hetero-dimer interactions in intact cells(1,2). For intermolecular FRET, a key benefit of performing donor FLIM (when compared to intensity based ratiometric techniques), is that fluorescence-lifetime measurements of donor emission are independent of acceptor concentration and is thus suited to imaging studies in intact cells(3,4). Multiphoton microscopy confers additional advantages in terms of inherent three dimensional sectioning and enhanced depth penetration for in vivo imaging(5-8). However, the data acquisition rate for FLIM is a significant limitation in current implementations of laser scanning microscopy.

In this paper we demonstrate diffraction limited multiphoton imaging in a massively parallel, fully addressable time-resolved multi-beam multiphoton microscope capable of producing fluorescence lifetime images with sub-50ps temporal resolution. This imaging platform offers a significant improvement in acquisition speed over single-beam laser scanning FLIM by a factor of 64 without compromising in either the temporal or spatial resolutions of the system. We demonstrate FLIM acquisition at unparallelled imaging rates with live cells expressing green fluorescent protein. The applicability of the technique to imaging protein-protein interactions in live cells is exemplified by observation of time-dependent FRET between the epidermal growth factor receptor (EGFR) and the adapter protein Grb2 following stimulation with the receptor ligand. Furthermore, ligand-dependent association of HER2-HER3 receptor tyrosine kinases was observed on a similar timescale and involved the internalisation and accumulation or receptor heterodimers within endosomes. Finally we demonstrate the applicability of this new technique to in vivo imaging in zebrafish. These data demonstrate the broad applicability of this novel FLIM technique to the spatio-temporal dynamics of protein-protein interaction.


**127**

**Preparation of Large and Uniform Cell Monolayers for Imaging Applications**

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Background: A long-standing goal of imaging-based cytometry is the preparation of large, uniform cell monolayers with tunable cell-packing fraction. Such a method would facilitate the inspection of all cells in the sample in a high throughput manner, particularly, for clinical applications that require the detection of rare cells. Traditional approaches to create cell monolayers, such as cyto-spinning and blood smearing, are not scalable over areas larger than a microscopy slide. Furthermore, controlling the density and homogeneity of the cell-packing fraction with these techniques is cumbersome, often requiring modification of initial cell suspension. Lastly, cyto-spinning techniques waste a large portion of the sample, which may be a restriction incompatible with clinical applications investigating highly rare cells or utilizing small sample volumes. Recent approaches to organizing cells on a surface have focused on the use of patterned surfaces, cartridges, or printing-based techniques. However, these methods can be costly to fabricate and do not allow the cell-packing fraction to be tuned during deposition. Here, we describe a novel approach to producing uniform cell monolayers over large substrates with tunable cell-packing fraction, independent of the cell suspension concentration.

Methods: Cell monolayers were deposited using a low-cost, custom robotic system that aerosolizes the cell suspension over a circular substrate that was simultaneously rotated and translated. The physical characteristics of the cell monolayer were controlled by adapting the substrate velocity, fluid flow rate, and sample deposition time. Two different samples were used, swine blood (both whole and diluted) and antibody-stained CD-Chev Plus (Streck) stabilized cell suspensions. Images of the monolayers were acquired with a standard 20x microscope to quantify cell-packing fraction and uniformity. A line-scan of seven images, one centimeter apart, were obtained per substrate and segmented with standard image processing techniques.

Results: We produced 25 separate cell monolayers in Petri dishes covering an area larger than 50 square centimeters. The cell-packing fraction was tunable from 10% to 65%, with good uniformity (<5% deviations in cell concentrations along the line scan area). The overall process was reproducible, with differences between individual samples lower than 3%.

Conclusions: These results show that our approach to preparing large cell monolayers is reliable, scalable, and tunable. This technology is being developed in combination with a high-throughput imaging platform for the clinical analysis of blood samples.

129 Analysis of Subcellular Second Messenger Signaling Events Using Spectral FRET Microscopy and Image Cytometry Approaches

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Background: FRET techniques are exquisitely sensitive to changes in intermolecular distance, and have allowed a broad range of biological measurements. Unfortunately, FRET microscopy data is typically characterized by high noise, resulting in the need for many pixels to be averaged to yield sufficiently reliable data. We have previously developed spectral imaging approaches for measurement of FRET-based CAMP probes. While spectral FRET provided lower coefficients of variation (CVs), the data still presented high noise contributions, precluding analysis of small, subcellular regions. These limitations have made it difficult to assess the role that compartmentation may play in determining cAMP signaling specificity. In this current work, we describe an approach to mitigate noise limitations in FRET data by combining spectral FRET with non-linear image analysis algorithms and cell segmentation approaches.

Methods: Rat pulmonary microvascular endothelial cells were grown on 35 mm round glass coverslips. Cells were transfected with a 3:1 ratio of TransIT-X2:CDN encoding a Turquoise-EPAC-Venus CAMP biosensor for 48 hours and were then stained with Hoechst 33342 (nuclear label), Mitotracker red (mitochondrial label), and wheat germ agglutinin (WGA) - TRITC (membrane label). Cells were imaged using a Nikon A1R spectral confocal microscope with 405 and 562.7 nm excitation. Spectral emission was detected from 414 to 724 nm, in 10 nm increments. Time-lapse images were acquired every 20 seconds for 25 minutes. Cells were treated with 50 μM Forskolin (adenylate cyclase stimulator) at 5 minutes and 50 μM Rolipram + 500 μM BMX (phosphodiesterase inhibitors) at 15 minutes. Spectral image data were processed using a combination of custom MATLAB scripts and Cell Profiler (Broad Institute) analysis. Nuclear identification and cell segmentation was first performed. Subcellular regions were next identified using the Hoechst, Mitotracker, and WGA-TRITC images. FRET efficiencies were calculated for each region. A Perona-Malik anisotropic diffusion algorithm was applied to further reduce noise effects in FRET image data.

Results: Treatment groups displayed expected increases in cAMP (decreased FRET efficiency). Subcellular segmentation allowed FRET traces for subcellular regions. Using raw FRET data, subcellular traces were not statistically significant due to high noise contributions. However, processing with the anisotropic diffusion algorithm greatly decreased noise and produced statistically-significant traces. cAMP response rates were delayed in the para-nuclear region, compared to the near-membrane region.

Conclusions: The combination of spectral imaging and anisotropic diffusion processing provided FRET traces with reduced noise, allowing analysis of cAMP concentrations in subcellular regions. These data show promise for developing new models for compartmentalized second messenger signaling.

129 Endothelial Progenitor Cells and Cancer

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Endothelial progenitor cells (EPCs) play a central role in blood vessel formation in physiologic and pathologic conditions. In cancer, EPCs contribute actively to tumor neoangiogenesis. In response to tumor-derived soluble mediators EPCs, mainly residing in the bone marrow, mobilize to the peripheral blood and home to the tumor site. Endowed with high proliferative potential ad the ability to differentiate into mature endothelial cells, EPCs are incorporated into tumor vessels. They also contribute to tumor vascularization by producing proangiogenic factors that in turn promote local angiogenesis. By these processes, EPCs contribute to the angiogenic switch, the mechanism by which dormant tumors are turned into highly vascularized tumors and favor the dissemination of cancer cells towards surrounding tissues or distant sites.

Indeed, our understanding of the actual contribution of EPCs in tumor vascularization in different types of cancer is still hampered by the lack of standardized methods employed for EPC definition, quantification and analysis. In fact, EPCs are a heterogeneous cell population comprising hematopoietic and non-hematopoietic subsets that collaborate closely to promote endothelial repair and vessel formation by angiogenetic and vasculogenetic processes. In the peripheral blood, these different subsets of EPCs can be studied by means of two main different approaches. One approach consists in identifying and selecting EPCs by cell surface phenotype using fluorescently labeled antibodies and flow cytometry directly performed on blood samples. The second
approach consists in isolating and expanding EPCs in vitro, allowing further functional characterization of cultured cells.

Evidences accumulated so far demonstrating the involvement of EPCs in human cancer will be illustrated and discussed highlighting advantages, limits and critical aspects of the methods used to investigate the role of these cells in the malignant transformation of human tumors.

130 Real-Time Cytometry
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A key point in biochemical analysis is the study of enzyme kinetics, as a necessary step for the characterization of a given enzyme or pathway, as well as for monitoring the action of modulators. It is common to monitor kinetically biochemical processes by following the specific and stoichiometric transformation of fluorogenic substrates into fluorescent products or, conversely, the extinction of fluorescent probes. Most currently available flow cytometers incorporate real time as a parameter, which makes possible to follow the kinetics of fluorescence variations upon specific transformation of adequate substrates. Since sample runs continuously, and a high rate (up to thousands of cells per second) along any flow cytometric analysis, collected data are by definition time-resolved at sub-second resolution. This kinetic approach is not new in flow cytometry and different descriptive terms have been coined, such as ‘flow-cytoenzymology’, ‘in-fluxo analysis’ and, more recently, ‘real-time flow cytometry’ or RT-FCM. Kinetic plots are routinely employed as an aid in quality control procedures in flow cytometry. However, in spite of its potential for biochemical analysis, RT-FCM is not a frequent application of flow cytometry. In this communication, we present the concept of RT-FCM and we illustrate its relevance with examples from our laboratory. In our experience, RT-FCM has been applied to determine the activity of single enzymes or to integrate complex metabolic pathways. Also, novel no-wash, no-lyse RT-FCM assays allow to detect and to follow up specific processes of oxidative stres and find striking differences in nitric oxide- and superoxide-driven generation of peroxynitrite among subpopulations of peripheral blood leukocytes. Moreover, the application of polychromatic RT-FCM assays has led us to detect and characterize biochemical interactions among fluorescent probes commonly used for the analysis of oxidative stress. Finally, some of our RT-FCM assays have been adapted to the use of the imaging flow cytometer (Amini ImageStream), allowing to correlate our previous observations to dynamic processes of intracellular distribution of fluorescent probes and morphological alterations of cell compartments. Part of the data presented in this communication have been obtained in the course of studies supported by grants from the Spanish Ministerio de Ciencia e Innovación (BIO2010-19870) and the Conselleria de Educación de la Generalitat Valenciana (ACOMP2013/102).

131 Overview of European Contribution to Cytometry
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Flow cytometry is a relatively new scientific field and many of the people who contributed to flow cytometry evolution are still alive and active all over the world, including Europe.

If we recall the founders of the various technologies from which modern flow cytometry arose, several Europeans have contributed to the staining techniques, the evolution of modern Microscopy, the blood counting and the modern cytolgy techniques.

Wolfgang Gohde introduced fluorescence into flow cytometry and developed the first flow cytometer in Europe around 1960, named the Phye Impulscyphotometor (ICP)11 instrument. In the 1970s this instrument was mainly used by Europeans studying DNA content, the main cytometry application for many years. In parallel to the development of various instruments in USA, many groups in Europe, developed their own instruments using a variety of light sources, fluidics and electronics.

G.J.F. Kohler and C. Milstein (Nobel prize in Physiology or Medicine, 1984), who invented the technology of monoclonal antibodies production in Cambridge in 1974, made also a major contribution to the development of flow cytometry applications, although monoclonal antibodies would become commercially available many years later.

The contribution to flow cytometry of several significant Europeans should be mentioned, such as the ones who introduced and run the first quality control of cellular phenotyping by flow cytometry around 1990, the ones who founded the European Cytometry Network, or the European Working Group on Clinical Cell Analysis (EWGCCA) or the ones who founded and run Euroflow as well as many basic cytometry researchers.

ISAC and its predecessors, namely the Engineering Foundation and the Society of Analytical Cytometry have been holding many of their meetings in Europe. Many of ISAC presidents have been Europeans. For many years ISAC has been the main society offering training courses in cytometry all over the world.

The educational role of the European National Cytometry Societies should be also underlined. Many European commercial companies specialized on cytometry reagents as well as main distributors of flow cytometers played an important role in the education of flow cytometry users all over Europe.

Last but not least the European Society of Clinical Cell Analysis (ESCCA) and its predecessor, EWGCCA has played and continues to play a very important role in bringing together the European flow cytometrists, especially the ones working in clinical labs, without disregarding basic research in flow and image cytometry. ESCCA Euroconferences and Eurocourses, as well as the international and national schools together with the new web education tools introduced in 2015 form a strong educational network for flow cytometry in Europe.

436 Creating Health and Wealth through Cell Therapy
John Brown
Guy’s Hospital, London, United Kingdom

The Cell Therapy Catapult was established by Innovate UK in 2012 to advance the growth of the UK cell and gene therapy industry, by bridging the gap between scientific research and full-scale commercialisation. Focusing on overcoming the barriers to innovation, the Cell Therapy Catapult has built significant resources with more than 100 employees in its new state of the art facility located in Guy’s Hospital in London and a new large scale manufacturing centre devoted to manufacturing for late stage clinical trials for Advanced Therapies. The Cell therapy Catapult has also built significant resources such as health economics, reimbursement and clinical and regulatory expertise to assist companies to develop and succeed. Working on clinical products and enabling, Dr Brown will discuss progress with the growth of the Cell Therapy Catapult, the project portfolio and its impact so far on accelerating innovation in cell and gene therapies in the UK.
Automated Sample Preparation (B1)

132/B1
A Small Device to Expedite and Automate Thawing of Viable Frozen Cells
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*This abstract describes a novel device, patent pending.*

Cryopreserved cell specimens are fundamental to biological research; increasingly, research and clinical assays depend on viable frozen cells. Indeed, daily sample throughput for batched flow cytometry studies will often be limited by the number of cryovials that can be processed at one time. There is a manifest need for rapid and automated thawing methods.

Many aspects of the common cell thawing method are variable and prone to error. Even with standard operating procedures (SOPs), the method is very subjective: for example, cell vials are ‘partially submerged’ in a 37°C water bath, and then ‘swirled’ so that heat is evenly distributed, until only a ‘pea-sized’ ice crystal remains. The thawed cells are then ‘quickly’ transferred into a vial of warm media to reduce the toxicity of the cryopreservative. This is a laborious and intricate process; practically, it is limited to four samples at a time per technician. The subjective nature of multiple steps also introduces variation, e.g. the time from initial thaw to dilution can vary dramatically between users and thawing. Rigorous QC studies in our laboratory have shown that these steps are critical for viability and cell yield, and the most likely source of performance variation between individuals.

To overcome these limitations, we designed, tested, and optimized a small adaptor that sustains an inverted cryovial of frozen cells over a tube of medium. The combination is loaded immediately into a centrifuge; during the spinning, the sample thaws and falls into the medium, immediately diluting the cryopreservation agent, while washing and pelleting the cells. While simple, this plastic adaptor makes a dramatic impact on the process of recovering viable cells from frozen specimens. It reduces labor time from several minutes to a few seconds, allowing a single technician to simultaneously thaw as many vials as a centrifuge will hold.

The device presented here provides a simple solution to thawing cryopreserved cells. Importantly, our device achieves equivalent viability and yield as current SOPs, while simultaneously eliminating subjective steps, reducing opportunities for technical error, and dramatically increasing throughput.

Cell-Derived Microvesicles (B2)

133/B3
Extracellular Vesicle Isolation by Flow Cytometric Sorting and Characterization by Analytical Ultra-Centrifugation and Dynamic Light Scatter
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Background: The extracellular vesicle (EV) research field has dramatically increased in the last five years. Using a high-speed flow cytometric sorter, EVs may be isolated at high rates such that researchers can differentially separate, isolate and characterize the EVs for downstream analysis. EVs contaminated with proteins, dye or antibody aggregates of the same size, but different mass, can be characterized based on these physical properties. This poster focuses on characterizing the EVs on an analytical ultra-centrifuge XL-A1 (AUC) and dynamic light scatter after isolation by flow cytometric sorting.

Methods: Extracellular vesicles were isolated from a HeLa cell line following serial ultracentrifugation and compared with exosomes were purchased (SBI - H196, MCF7, Serum). HeLa EVs were measured for total protein content (BCA assay, ThermoScientific), and confirmed using CD63 Dyna-beads (Life Technology). Additional stained HeLa EVs were treated with PKH26 and 67 (Sigma Aldrich) in cell culture and EVs were collected. EVs were stained with CM-DiI (Life Technology) and sorted on the MoFlo Astrios EQ (Beckman Coulter) using side scatter and fluorescence. Sorted EVs were concentrated with a centrifugal concentrator. Instrument performance was tested with polystyrene latex beads (Magnabeads) ranging from 22–104 nm, and 100 nm liposomes (FormuMax) for minimum detection limits. Absorbance and interference were used on AUC to measure the EV mass. Control liposomes (100 nm), DiI-stained liposomes, BSA and purchase exosomes were used as AUC control particles. DeltaMax and DeltaMax Core, dynamic light scatter (DLS) instruments, were used to measure the particle sizes of EV samples.

Results: The Astrios EQ was able to distinguish and sort the SBI and HeLa exosomes with some overlap on SSC noise. 100 nm liposomes and 81 nm beads were visible above noise; 58 nm bead population was split above and below the noise. EVs stained with PKH 26 and 67, were mixed and sorted on SSC and fluorescence with populations above and in the noise. Sorted fractions were analyzed on the AUC and DeltaMax for unstained, stained and aggregate mass distributions. AUC identified EV populations with varied sizes. The size distributions of EVs and dye aggregates were measured by the DeltaMax with DLS.

Conclusions: Sorting stained EV populations with the Astrios EQ provided a useful tool for their visualization, separation and characterization. AUC effectively separated particles, based on their mass distribution and clarified issues with dye aggregation vs EV staining. Additionally, the DeltaMax allowed for quick analysis of post-sorted populations. Taken together, the Astrios EQ was able to sort EVs and AUC provided additional analysis for exosome purity.

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Flow Cytometry Instrumentation (B3)

134/(B3-B4)
High Volumetric Rate Flow Cytometry for Process Monitoring, Large Particle Applications, and Rare Event Analysis
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Introduction: Current individual flow cytometers achieve particle analysis rates as high as 70,000 events per second, while delivering sample at up to 1 mL/minute. To increase analysis rates, several groups have used relatively complex combinations of parallel implementations of individual cytometer analysis heads that each interrogate multiple discreet individual channels. These parallel systems were initially carried out to address specific regional application areas requiring detection of very rare events (e.g. circulating tumor cells). However, there are also other application areas such as large particle analysis (e.g. small organism analysis),
and process monitoring that would benefit from dramatically higher throughput, in terms of geometric dimensions of the flow cell, volumetric delivery, and particle analysis rates. Here we are developing a flow cytometer that will enable massively parallel analysis in a single channel using a robust and simple combination of multi-node acoustofluidics and line focused laser interrogation. Importantly, this flow cytometer will be simple to implement and support both high volumetric flow rates and high analysis rates, which will make it useful for numerous applications.

Methods: Specifically, we are developing large aspect ratio acoustic flow cells that can support high volumetric flow rates, particles several hundred microns in diameter, and also provide parallel flow streams to support high analysis rates. Our flow cells use a rectangular aspect ratio and multi-node acoustic standing waves to create a wide channel that has up to 64 parallel flowing sample streams. To accompany these large flow cells, we have developed a highly parallel detection system that uses a line-focused laser in combination with a high-resolution sCMOS camera operating at 25,000 frames per second to detect fluorescence and scatter from up to 50 streams simultaneously.

Results: Our current system uses flow cells with 32 parallel flow streams and has demonstrated focused flow rates of 25 mL/min in a single 8 mm wide by 400 µm high capillary. Combined with our optical detection approaches, we can currently analyze up to 100,000 cells/s and samples at 25 mL/min. Furthermore, we have demonstrated parallel detection of calibration particles with sensitivities as low as 1000 fluorescent microspheres and with sufficient resolution to resolve the lower populations of the Ultra Rainbow microspheres (URCP-100-2, Spherotech).

Conclusions: This work has created a compact affordable parallel flow cytometer prototype that retains the optical properties of conventional flow cytometry. Our current system will be great value to both large particle analysis and process monitoring applications. It also has a realistic path to analysis rates of $>1\times10^6$ events/s and sampling rates of 50 mL/min. Notably, it will retain the sensitivity and resolution that flow cytometry is known for while achieving these rates. As such, this system represents an important technological advance in parallel flow cytometry that will be important for both high-volume, large particle, and very rare event applications in flow cytometry.

135/B4
Build Your Own Flow Cytometers: Teaching Tools for Flow Cytometry Education
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John Martin and James Jett presented the first ‘Build Your Own’ flow cytometry laboratory in 1990 at the National Flow Cytometry Resource flow cytometry course at Bowdoin College. The ‘Build Your Own’ cytometer was a fully functional open platform teaching cytometer with all the components of a conventional instrument. Students could assemble a working flow cytometer from the ground up, giving them a valuable hands-on experience in how flow cytometers really work. The Build Your Own Cytometer laboratory, now taught and managed by John Martin, Mark Wilder and James Jett has since become a regular event and the most popular laboratory at the Bowdoin College Los Alamos / University of New Mexico annual flow cytometry courses, where hundreds of students over the last 25 years have had the unique experience of building a working flow cytometer with their own hands. Over the years the system had been updated and modernized, and has been presented at a variety of university events in addition to the yearly national courses.

The original Build Your Own laboratory relied specialized and often unique components, electronics and software developed at Los Alamos National Laboratories. Recent advances in optical, electronics and software technology are now converging to make the development of such teaching systems accessible to far more investigators and institutions. The recent explosion of ‘maker’ culture has also provided very useful tools for building such systems, including, off-the-shelf optical components suitable for flow cytometers, open-source electronics, operating systems and programming languages like Linux and Python, and fabrication techniques like 3D printing. The National Cancer Institute has recently developed their own Build Your Own system (the Flow Cytometer Maker Laboratory) designed specifically for teaching at international flow cytometry courses and workshops; it is smaller than the traditional Build Lab and constructed of extremely lightweight components, allowing it to be transported to international courses as checked luggage. This system and the original Build Your Own benefit both from modern optical components, materials and electronics/software, including the advanced digital electronics and software Azurite/Kyoto system built by Mark Naivar of DarklingX, LLC. In line with the ‘maker’ philosophy, we are working to make the Build Your Own concept accessible to any scientist with a desire to build a teaching flow cytometer as an educational tool.

High Throughput Instrumentation [B5]

136/B5
Miniaturization of High Throughput Flow Assays for 384-Well Low Volume and 1536-Well Plates: Detection of Highly Multiplexed Analytes in 6 µl Assays with the iQue Screener and MultiCyt QBeads
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Profiling of secreted proteins is ideally achieved by the detection of multiple proteins simultaneously, maximizing contextual and correlative value of the data. However, bead-based multiplex technologies are often slow, complicated and exceed the price per data point goals for screening. IntelliCyt’s Multicyt QBeads assays for soluble proteins run on the iQue Screener enable an affordable, no-wash, miniaturized, multiplex detection of up to 30 simultaneous analytes, and rapid read times. Based on IntelliCyt’s patented high throughput sampling technology, we are able to sample from 96, 384, and 1536 well plate formats with zero dead volume. Here we demonstrate the miniaturization of our standard QBeads assay for cytokine detection for high throughput screening from a standard 30 µL down to 6 µL total assay volumes in both 384 well low volume plates and 1536 well plates. As a biological model, Jurkat cells were stimulated with a combination of PHA and PMA for 24 hours in batch. A triplex QBeads assay was performed in triplicate 384 well plates for the detection of IL-2, IL-3 and IL-6. Bead counts per sample averaged ~400 beads (130 beads per plex) with a 7.3% CV across 3 plates, demonstrating high reproducibility in sampling. Quantification of secreted proteins was likewise robust across all 3 plates, with IL-2 being secreted at high levels (2,311 pg/mL, 10.1% CV), IL-6 secreted at moderate levels (853 pg/mL, 8.1% CV) and IL-3 secreted at moderate levels (485 pg/mL, 11.6% CV). In 1536 plate format, average bead count CV was 8.6% for a 3-plex assay, and all other performance metrics were comparable to data seen in 384-well format. Critical factors in the successful execution of QBeads assays in low volume formats were identified and implemented, including methods for control of sample evaporation and edge effects. In summary, the bead input per well represents a greater than 8-fold reduction in reagent consumption compared to other commercially available multiplexed bead products, greatly reducing the price per data point for HTS without any sacrifice to data quality.
**Immune Monitoring (B8)**

**Advanced Analysis of Human T Cell Subsets by Flow Cytometry Using a 13 Color Tube Based on DuraClone Dry Reagent Technology**

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T cell analysis requires a multitude of markers to capture the various populations that have been described. Accordingly, informative T cell phenotyping can only be realized by the use of a broad menu of fluorochromes including multiple tandem dyes that are known for their susceptibility to photo oxidation and related changes in emission characteristics (‘tandem dye aging’). Simultaneous alteration of various tandem emission spectra within a high content panel is a considerable challenge even for experienced operators, renders pre-mixing of conjugates a questionable approach and antagonizes reproducibility of staining patterns and intensities.

To overcome the described issues, the DuraClone IM brand of dry multicolor reagents for the analysis of human immune cells has been developed. In close cooperation between industry and international study groups, marker combinations were designed by scientific experts and formulated as dry DuraClone tubes that evade aging effects seen for liquid preparations of tandem dyes. Advanced instrumentation with more than 10 fluorescent parameters allows for customizing panels based on the robust DuraClone formulation.

Using the 10 color DuraClone IM T Cell Subset dry reagent kit (CD45RA-PE, CD197-PE, CD28-ECD, CD279-PC5.5, CD27-PC7, CD4-APC, CD8-AlexaFluor 700, CD3-APC-AF750, CD57-Pacific Blue, CD45-Krome Orange) plus 3 additional liquid antibodies for the violet laser (Brilliant Violet 605VB, anti-human CD95, Brilliant Violet 650VB anti-human CD25, and Brilliant Violet 768HB anti-human CD127 antibodies) we defined a 13-color tube that allows for the identification of major peripheral T cell subtypes according to classical and more recent characterization criteria. We demonstrate the identification of naive, memory and effector T cells at various stages of differentiation, but also the detection of rare populations such as stem cell like memory T cells and different Treg subpopulations by applying no more than 3 reagent pipetting steps.

The dry reagent formulation of the DuraClone IM backbone panel guarantees for long term fluorochrome stability at room temperature, reducing changes to the compensation matrix and increasing reproducibility of results. The tube is suitable for all flow cytometers with a 5-3-5 (488 nm & 561 nm / 638 nm / 405 nm) optical layout.

**New Software Development (B9)**

**Chromocyte: An Online Resource for Assisting the Flow Cytometry Community**

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The increasing complexity of flow cytometry instrumentation and the move towards customizable and ‘non-standard’ configurations is making it progressively more time-consuming and difficult for users to identify combinations of fluorochromes that are appropriate for their specific instrument and identify the suppliers of these. Furthermore, many Principal Investigators with no previous experience in multi-parameter flow cytometry are now realising the benefits and strengths of the technique. These issues have prompted the need for resources that allow flow cytométrists to search for antibodies that are suitable for their own particular instrument, identify the suppliers of these and provide a comprehensive listing of flow cytometry-related material and resources. Chromocyte (www.chromocyte.com) has been developed as such a resource and its aim is to improve flow cytometry knowledge and practice globally. Chromocyte consists of four principle environments: **CALCULATE, LOCATE, EDUCATE and COMMUNICATE**.

Using a series of dropdown menus in **CALCULATE**, users can select and configure their instrument, and registered users can save these configurations. Instrument configurations that have been provided by Core Facility / Shared Resource / Laboratory Managers can be made available to other Users via Chromocyte’s ‘Facility Management’ interface. Once configured, the specificity of antibodies required can be defined using a search tool which returns the maximum number of antibody-fluorochrome conjugates that are consistent with the configured instrument. Antibodies can be selected from this panel and the suppliers of these provided. Results can be downloaded as a spreadsheet. **LOCATE** is possibly the quickest way to search for multiple antibodies simultaneously. **EDUCATE** provides links to training resources, tips, as well as to products and services relating to flow cytometry and cell-based analyses. Users can interact with the flow cytometry community via our **COMMUNICATE** online facility.

Our latest **Fluorophore Poster Generation Facility** enables users to generate a colour coded poster which summarises the fluorophores that are consistent with a particular instrument in a few simple steps. For more information, visit www.chromocyte.com/poster.

**Acknowledgment:** Chromocyte is free to use due to the invaluable financial support of our Corporate Sponsors.
**Poster Abstracts**

**Antigen-Specific Immune Responses (B10/B11)**

141/B10
Development of a Mass Cytometry Platform to Investigate Immunological Correlates of TB Risk in an Endemic Setting

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Single cell analysis by flow cytometry has significantly enhanced the field of immunology. An important limitation to this technique, however, is the number of markers that can simultaneously be analyzed. Cytometry by time-of-flight (CyTOF) or mass cytometry, permits analysis of more than 40 parameters on a single cell level in a single panel, facilitating unparalleled multi-dimensional analysis of complex cellular subsets.

We performed duplicate intracellular cytokine staining assays, analyzed in parallel by conventional multiparameter flow cytometry and mass cytometry to directly compare these technologies. Whole blood from healthy donors, stimulated with the i style="font-size: 12pt;"/>Mycobacterium tuberculosis antigens ESAT-6/CFP-10 and Ag85A/Ag85B, PHA, or left unstimulated, was stained with fluorochrome or heavy-metal-conjugated monoclonal antibodies specific for cell subset, phenotypic and functional markers. Boolean analyses of all parameters were performed in FlowJo and analyzed in SPICE (NIAID, NIH) for each method used in order to assess and compare the capabilities and sensitivity of Mass Cytometry in our hands.

Cell subsets (CD4, CD8 and memory subsets based on CD45RA and CCR7 expression) and cytokine-expressing cell frequencies detected by CyTOF were highly correlated with those detected by flow cytometry. CyTOF and flow cytometry yielded equivalent quantification of cytokine-expressing CD4 T-cells by ICS. No significant differences between methods were observed in the functional profiles analyzed (all the possible combinations of IFN-γ, IL-2, TNF-α and IL-17). We have assessed that CyTOF is a powerful tool allowing unbiased characterization of the complex immune response to Mycobacterium tuberculosis. In our hands ICS by mass cytometry performed as well as flow cytometry, a gold standard for many years, while allowing analysis of significantly more markers (innate and adaptive subset of cells as well as additional functional markers), providing novel and more comprehensive immunological insight.

Our aim is to optimize mass cytometry assays to integrate this technology into a broad systems biology approach to identify correlates of risk of tuberculosis disease in adolescents from a TB-endemic area.

142/B11
Flow Cytometry Methods Allow the Detection of Low Frequency Proliferative CD4 Memory T Cells in Human Primary Peripheral Blood Mononuclear Cells

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**Background:** During vaccine or therapeutic clinical trials, we measure the specific activity of effector CD4+ T cells (low frequencies), in particular performing tritium-labeled thymidine (TH3) incorporation test. This method is commonly used for its high sensitivity but it generates radioactive waste. In this study, we chose to compare the sensitivity, time and cost of this radioactive method with two flow cytometry techniques: Cell Trace Violet (CTV, Life Technologies) described as sensitive and allowing the observation of division peaks, and Click-iT ® Edu (Life Technologies), close to TH3 labelling.

**Methods:** For Click-iT ® Edu and TH3, PBMCs of 20 healthy donors were stimulated with tetanus toxoid (TT) during 6 days, before adding the thymidine analog. The day after, the cells labelled with TH3 were lysed and the radioactivity was measured with a beta counter. For Click-iT ® Edu, a CD3-CD4 phenotype was performed before revelation in accordance with manufacturer’s guidelines. For CTV test, the PBMCs were previously incubated with the dye and then stimulated as described above. At day 7, the CD3-CD4 phenotype was performed just before flow cytometry analysis (LSRII, BD Biosciences or Novocyt, ACEA).

**Results:** A proliferative response with TT was detected in 8/14 tested donors (57 %) using CTV method, in 14/19 tested donors using Click-iT ® Edu method (73 %) and in 15/20 of the donors using T-H3 (75 %). With the Click-iT ® Edu method, we were able to detect very low frequencies of proliferative CD4 T cells (0.2%), in contrast to CTV, where the background was higher. The time required to measure T-H3 was about 2 hours (transfer on filter, counting), while the flow cytometry methods require more time (3.5 and 5 hours) but present the advantage of characterizing the population which proliferates thanks to the T CD4 phenotyping.

Concerning the cost, the Click-iT ® Edu method is more expensive (8 euros / condition) compared with two others (2-4 euros).

**Conclusion:** In conclusion, health risks and safety concerns incite us to eliminate the radioactive exposure. We found that the T-H3 method may be replaced by the Click-iT ® Edu technique, which is as efficient at detecting the proliferative responses of primary human CD4 memory T cells.

**Automated Microscopy (B12)**

143/B12
On the Inaccuracy of Image-Based Cell Counters

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**Introduction:** In the last few years, several automated cell counters have been introduced with the goal of replacing manual cell counting with hemacytometer or flow cytometry. These systems have the advantages of being easy to use, having a small size and an attractive price when compared to flow cytometry solutions. However, given the smaller sample size that these systems analyze, its accuracy is often challenged. In order to obtain a reliable measure of several commercial systems accuracy, we have performed a two-fold analysis. On one side, we have calculated the theoretical margin of error introduced by each system given its technical specifications at different cell concentrations. On the other hand, we have performed a comparative practical analysis by performing several cell counts with the different systems from the same original sample.

**Methods:** Flasks of growing HepG2 cell cultures were analyzed alternatively with 4 different instruments: i) Neubauer chamber manual counting ii) flow cytometry (Cytofcs FC500, Beckman-Coulter) iii) Countess (Life Technologies), iv) Scepter portable cell counter (Millipore), v) Micro Counter (Celeromics Technologies).
Each measurement was repeated 20 times shaking the original sample before sample extractions.

**Results:** Both theoretical and practical results indicate that the higher the amount of sample analyzed the lower the error introduced. For concentrations higher than 1 million cells/mL most systems behave correctly, with standard errors below 5%. However, image-based systems that analyze sample sizes below 4 mL behave very poorly below 500,000 cells/mL, introducing errors higher than 40% in their working range.

**Conclusions:** We have determined that most image based cell counting systems are not suitable for research work with sample concentration below 500,000 cells/mL. In order to obtain reliable measurements in that range, the only valid systems are those that analyze samples larger than 4 mL.

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**Automated Sample Preparation (B13)**

**144/B13 Reagent Release from Hydrogel Coated Cell Counting Chambers for On-Chip Sample Preparation**

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Sample preparation for point-of-care diagnostics requires minimum dependence on lab equipment and trained personnel. We developed a cell counting chamber coated with cell staining reagents embedded in hydrogel layers to be used in quantitative fluorescence imaging. In this concept, the chamber is filled with a drop of finger prick blood by capillary force. Subsequent release of fluorescently labeled antibodies or DNA stains from the hydrogel layer ensures a homogeneous distribution of reagent in the chamber and uniform cell staining. In order to achieve such homogeneous staining throughout the image area, hydrogel layers are required to retain the majority of antibodies during sampling. Inflow (~5 s) and release them within the incubation period (~30 min). Thus, the release time of antibody should be of the order of magnitude of hundreds of seconds.

We have developed a real-time and in situ fluorescence imaging method to monitor the release process of fluorescent reagents from sub-μm thick hydrogel layers. Using this technique, we have studied the thickness dependence of the release kinetics of antibody release from gelatin layers and found that the initial antibody release rate complies with a diffusion controlled mechanism and determined an antibody diffusion coefficient in swollen gelatin of 0.4 μm²/s. Desired release time can therefore be tuned by changing the initial layer thickness. For example, 0.5 μm thick gelatin layer results in the release time of ~100 s and yields homogeneous antibody distribution and fast cell staining.

To automate hydrogel layer deposition for low-cost production, inkjet printing is used to replace manual pipetting of hydrogels. We have found that the material properties of gelatin are significantly influenced by the jetting process and drying conditions. The release time of 0.5 μm thick printed layer is ~30 s, resulting in significant antibody wash-off during sample inflow. Our results indicate that shear stresses change the conformation of the gelatin macromolecules causing reduced physical crosslinking and, thus, decreased release time in printed release layers.

In conclusion, we have developed tools to study the release kinetics of cell staining reagents from sub-μm thick hydrogel layers, which improves our understanding of the release process and allows for the optimization of the release time. Using this information we have tuned the release time for the optimization of on-chip sample preparation in our previously demonstrated CD4 count.


**Biomarkers (B14 - B20)**

**145/B14**

**li p10 Neoepitope Assay: A Pharmacodynamic Biomarker Assay for Therapies Targeting MHC Class II-Peptide Complex Formation through Inhibition of Cathepsin S**

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Major histocompatibility complex (MHC) class II molecules are central to adaptive immune responses and MHC class II-restricted autoantigen priming of CD4+ T cells is thought to play a role in the pathogenesis of a number of autoimmune diseases, including systemic lupus erythematosus, psoriasis, lupus nephritis and rheumatoid arthritis.

MHC class II molecules are initially associated to the invariant chain, a protein that blocks peptide binding and needs to be degraded before (auto)antigenic peptides can be acquired. li p10, a 10kDa fragment, is the last intermediate form in the proteolytic cleavage of the invariant chain into CLIP. This last enzymatic step required before peptide binding is mediated by cathepsin S, and inhibition or deficiency in this cysteine protease is known to result in the intracellular accumulation of li p10 in mice.

Here we show that li p10 can be used as a pharmacodynamic biomarker in a flow cytometry assay to evaluate potential therapeutic strategies targeting the antigen presenting machinery through the disruption of cysteine protease activities. The robust assay described allows for the detection of cell type-specific, intracellular accumulation of li p10 in a dose-dependent manner, from a limited blood sample volume and independently of cell frequencies.

In healthy human volunteers, CD19+ B cell specific accumulation of li p10 could be detected after in vitro cathepsin S inhibition in PBMC, simultaneously revealing a reproducible heterogeneity among volunteers in terms of maximum level of accumulation. When applied to safety assessment studies in an in vivo animal model, the assay was successfully used to detect li p10 accumulation in peripheral B cells as early as 2 hours post oral administration of a cathepsin S inhibitor, and could help monitor the pharmacodynamic effect of the inhibitor over time.

The specificity and robustness of the li p10 neoepitope assay, as well as the limited on-site sample processing requirements and the stability of the biomarker in frozen samples make this assay a valuable tool for clinical studies.

**146/B15**

**Quantification of Proteins by Flow Cytometry: Quantification of Human Hepatic Transporter P-gp and OATP1B1 Using Flow Cytometry and Mass Spectrometry**

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Flow cytometry is a powerful tool for the quantitation of fluorescence and is proven to be able to correlate the fluorescence intensity to the number of protein on cells surface. Mass spectrometry can also be used to determine the number of proteins per cell. Here we have developed two methods, using flow cytometry and mass spectrometry to quantitate number of transporters in human cells. These two approaches were then used to analyse the same samples so that a direct comparison could be made.

Transporters have a major impact on the behaviour of a diverse number of drugs in human systems. While active uptake studies by transmembrane protein transporters using model substrates are routinely undertaken in human cell lines and hepatocytes as part of drug discovery and development, the interpretation of these results is currently limited by the inability to quantify the number of transporters present in the test samples.

Here we provide a flow cytometric method for accurate quantification of transporter levels both on the cell surface and within the cell, and compare this to a quantitative mass spectrometric approach. Two transporters were selected for the study: OATP1B1 due to its important role in hepatic drug uptake and elimination; P-gp as a well characterised system and due to its potential impact on oral bioavailability, biliary and renal clearance, and brain penetration of drugs that are substrates for this transporter. In all cases the mass spectrometric method gave higher levels than the flow cytometry method. However, the two methods showed very similar trends in the relative ratios of both transporters in the hepatocyte samples investigated. The P-gp antibody allowed quantitative discrimination between externally facing transporters located in the cytoplasmic membrane and the total number of transporters on and in the cell. The proportion of externally facing transporter varied considerably in the four hepatocyte samples analysed, ranging from only 6% to 35% of intact and viable cells. The sample with only 6% externally facing transporter was further analysed by confocal microscopy which qualitatively confirmed the low level of transporter in the membrane and the large internal population.

Here we prove that flow cytometry is an important tool for future protein analysis as it can not only quantify the number of proteins that a cell express but also identify the number of proteins on the surface and it is easy to apply for routine assays.

**147/B16**

**High-Throughput miRNA Profiling on Standard Flow Cytometers**

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MicroRNAs are short, non-coding nucleic acids that regulate more than half of the genes in our bodies. These molecules have shown enormous potential for understanding, diagnosing, and treating prevalent diseases including cancer, heart disease, Alzheimer’s, and diabetes. Despite their promise, the clinical utility of miRNAs has yet to be realized due to the lack of simple technology to accurately measure miRNAs directly from biofluid or tissue specimens.

Here, we introduce a mid-plex, high-throughput, highly sensitive approach for profiling miRNAs with readout on standard flow cytometers. Using this system, we demonstrate the quantitation of up to 68 targets directly from plasma or serum with no RNA purification. Furthermore, while researchers have typically analyzed populations of cells and tissue to obtain sufficient starting material for analysis, we show miRNA profiling from single cells. The high Pearson correlation between these low-input samples compares very favorably to RT-PCR and sequencing methodologies, but with a significantly faster and streamlined workflow. In addition, our approach does not require investment in dedicated equipment, but utilizes standard flow cytometers for assay readout.

Our assays are built on highly-structured hydrogel microparticles. To enable scanning on standard flow cytometers, each microparticle contains multiple distinct regions, resembling a series of closely-spaced cells that are recorded as multiple events. We configured the microparticles with two regions to encode the particle, and a third region for target quantitation, each region being spatially separated by non-detected spacers. Unlike conventional beads used in multiplex assays, which typically have polystyrene surfaces, the microparticles are composed of a porous, bio-inert poly(ethylene glycol) hydrogel that enables the binding of analyte throughout a 3-dimensional structure. We exploit this structure to effectively hybridize and manipulate miRNAs for efficient fluorometric detection.

Using our platform with a single-laser flow cytometer, we have demonstrated a high level of multiplexing with more than 4 logs of dynamic range. We also compared assay performance across several cytometers from various manufacturers, with nearly-identical results despite differences in lasers, detectors, and fluids. Beyond miRNAs, we are exploring the utility of this platform for the detection of proteins.

In conclusion, we have developed a robust platform that enables high-throughput biomolecule analysis with readout on standard flow cytometers, with the potential to be scaleable by multicolour detectors. This platform can be used as a universal platform for the detection of proteins.

**148/B17**

**Quantitative Flow Cytometry Measurements in Antibody Bound per Cell Based on CD4 Reference**

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Multicolor flow cytometry assays are routinely used with fluorescently labeled antibodies in clinical laboratories to measure the cell number of specific immunophenotypes and to estimate expression levels of specific receptors/antigens either on the cell surface or intracellularly. The cell number and specific receptors/antigens serve as biomarkers for pathological conditions at various stages of a disease. Existing methods and cell reference material for quantitative expression measurements have not yet produced results which are of neither clinical interest nor are the results instrument independent across all fluorescence channels of cytometers. This presentation details a procedure for quantifying surface and intracellular biomarkers by calibrating the output of a multicolor flow cytometry in unit of antibody bound per cell (ABC). The procedure includes the following critical steps (a) quality control (QC) of the flow cytometer, (b) fluorescence intensity calibration using hard dyed microspheres assigned with fluorescence intensity values, (c) compensation for fluorescence spillover between adjacent fluorescence channels, and (d) application of a biological reference calibrator to establish an ABC scale. A few examples will be provided for implementing this procedure for quantifying the expression levels of clinical relevant biomarkers. Additionally, current efforts for quantifying biomarkers in a manner which is independent of instrument platforms and reagent differences will be discussed.

**149/B18**

**Lessons Learned from a Four-Year Multi-site International Proficiency Testing Program for Luminex Biomarker Profiling Assays**

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**Background:** Multiplex Luminex assays are widely used for biomarker quantification due to their sample-sparing capability. There has been, however, no comprehensive analysis of variables impacting assay performance, nor development of a proficiency testing program for labs performing these assays for clinical
**Methods:** Luminex assay proficiency of ~25 US and international sites is monitored semi-annually by EQAPOL through a process that is A2LA accredited in accordance with ISO/IEC 17043:2010 (CerfT 3614:01). Each proficiency test includes a de-identified commercial assay kit to quantify human IFNγ, TNFα, IL-6, IL-10 and IL-2, and a panel of recombinant cytokine-spiked human serum samples. All aspects of test development and shipping are performed under GCLP. A model-based proficiency score comprised of timeliness, protocol adherence, accuracy and precision has been implemented. Site proficiency, bead platform (polystyrene/magnetic) and assay kit source (common/site choice), were evaluated in all, or a subset of, sites over the past 4 years.

**Results:** Overall mean proficiency score across six rounds of testing has remained in the range of 75-90 (100 max). A detailed analysis of site-reported results however indicated an improvement of within and between site variation, suggesting that training/remediation for poor performing sites may have a positive impact on proficiency. Two studies were performed to evaluate site accuracy and precision when quantifying 5 cytokines in 7 samples on a polystyrene (Polyl) or a magnetic (Mag) Luminex assay. Analysis of the Polyl/Mag ratios for each reported analyte (range 0.95-1.19) suggested no biologically significant differences in cytokine pg/mL between sites was observed when a common kit was used, except for TNF which was significantly higher at a polystyrene site. In both studies a significantly lower %CV of reported cytokines in samples using a common EQAPOL kit and a site selected kit. In both studies a significantly lower %CV of reported pg/mL between sites was observed when a common kit was used, suggesting improved accuracy. Significantly tightened precision was detected when sites used a common kit (p=0.026; Wilcoxon Signed-Rank Test).

**Conclusions:** Continued proficiency testing to identify variables affecting Luminex assay outcomes, and investigation of ways to minimize the negative effect of these variables, will strengthen ongoing efforts to bring standardization to multiplex biomarker profiling for multi-site clinical trials/studies.

**150/B19 Monocyte HLA-DR by Flow Cytometry – Assay Development for a Multi-site Clinical Trial**

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The increased development of biologic therapeutics in the pharmaceutical industry has resulted in a need for sensitive flow cytometry assays to assess changes in immune status during clinical trial testing. This in turn presents significant challenges for accurate biomarker analysis and requires the incorporation of novel methods ensuring marker stability during the transit of clinical samples from multiple sites to a central lab for standardized analysis.

Using BD Quantibrite® Monocyte HLA-DR Kit and Quantibrite® Calibration Beads, measurement of HLA-DR expression on monocytes (mHLA-DR) to assess immuno-suppression in acute illness is documented. However, to date, it has been necessary to perform the assay within four hours at the site of sample collection due to the poor stability of this biomarker. The instability of this marker, coupled with the wide range of differences in personnel and instrumentation at multiple sites, make the measurement of mHLA-DR highly impractical for use in a multi-site clinical trial.

Here, using blood from healthy donor volunteers, we compared EDTA, sodium heparin, acid-citrate dextrose, and CytoChex® BCT (Streck) sample collection tubes to demonstrate that sample stability of mHLA-DR can be extended up to 48 hours simply by using CytoChex® BCT tubes for whole blood collection prior to analysis. To confirm the utility of this collection methodology for mHLA-DR anlaysis, we compared mHLA-DR levels in blood from healthy donor volunteers collected into CytoChex BCT tubes and the previously used standard, EDTA tubes. While the absolute mHLA-DR levels measured in BCT tubes were consistently lower than those measured in EDTA tubes, the results demonstrated high correlation (n=13, R²=0.9822). Additionally, with samples collected from healthy donors and acutely ill patients, we compared the lyse/no wash method to a lyse/wash method to further characterize the assay using BCT Tubes. The two methods demonstrated high correlation (n=16, R²=0.9919), with the absolute mHLA-DR levels from the lyse/wash method consistently lower than lyse/no wash results. These results suggest that the mHLA-DR assay can be conducted in multi-site clinical trials by employing CytoChex® BCT tubes. Our experiments characterized assay performance to be comparable to standard of practice. Use of this alternative approach enables the collection of samples in multi-site clinical trials and shipment to a CLIA certified central lab for subsequent measurement of mHLA-DR.

**151/B20 Urinary Endothelial Cell Microparticles as Biomarker of Pulmonary Vascular Disease**

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Pulmonary Arterial Hypertension (PAH) is a lung-heart disorder characterized by severe remodeling of the pulmonary artery and right ventricle. Invasive or costly techniques, such as right ventricular catheterization and heart sonography, are utilized to monitor the disease progression and therapy response.

Microparticles are bioactive molecules carrying cytoplasmic vesicles generated by exocytic budding. They are smaller than 1.0 micronmeter and display surface antigens of the cell of origin. Microparticles can be detected in biological fluids that are easily accessible in all diseases such as blood, urine and saliva. The discovery that microparticles provide biological information about cell activation, cell injury or apoptosis has marked a new era in medicine. Enumeration of microparticles is a technically challenging task. Since recently, micro flow cytometers, such as the Apogee Micro, have become available, allowing quantification and phenotyping of microparticles in an unparalleled method. Early studies using conventional flow cytometers showed that increased numbers of circulating endothelial cell-derived microparticles (EC-MPs) correlated to disease severity in PAH. Here we report optimization of a method to quantify EC-MPs in urine samples. Healthy volunteers and PAH patients were recruited for this study. Heart sonography was performed on PAH subjects. Microparticles were stained for annexin-V combined with CD45 and CD31 or vascular-endothelial cadherin (CD144). Samples were analyzed on Apogee A50-micro equipped with 488nm laser and has a detection limit of <100nm and <10nm resolution. EC-MPs were detected as annexin-V+ CD45-CD31+ or annexin-V+ CD144+. Pilot experiments showed low CD31 expression on EC-MPs in urine samples. In contrast, CD144 exhibited bright expression resulting in clear separation between negative and positive MP's. Based on these findings samples were analyzed by staining for annexin-V and CD144. EC-MPs were increased in PAH urine samples compared to healthy controls. Furthermore, there was a strong correlation between EC-MPs and parameters of right ventricle function measured by sonography. Overall, the findings demonstrate that the detection of EC-MPs is feasible in urine samples and may serve as a biomarker for disease severity in PAH.
**Biopharmaceutical Applications (B21 – B22)**

152/B21

Assessment of Receptor Occupancy by Flow Cytometry: A Powerful Tool in Drug Discovery

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Flow cytometry can be a powerful tool in drug discovery. One of the areas proven to benefit from this technology is the assessment of receptor occupancy. Receptor occupancy assays describe the qualitative and quantitative assessment of binding of a drug to its target receptor. Flow cytometry is an excellent tool for the investigation of monoclonal-based therapeutics, immunomodulators, and small molecules due to the ability to interrogate drug binding, as well as free-receptor simultaneously on multiple cellular targets. Assessment of receptor occupancy by flow cytometry can take several approaches.

Free receptor expression, which defines those receptors not occupied by drug, can be assessed by detection with either an antibody that competitively binds the drug's target epitope or by drug that has been fluorescently labeled.

Total receptor expression, representing the total receptor available for drug binding, can be assessed using a non-competitive antibody that binds to a different epitope than that targeted by the drug or competitive antibody used in free receptor assessment.

Bound receptor expression allows for the assessment of drug that is actively bound to its target epitope. This is performed by using a fluorescently-labeled antibody directed to the drug and ideally to the non-binding site region of the drug to avoid competing of drug away from its receptor.

Receptor modulation assays, when properly designed, can allow for the assessment of the functional effect drug binding may have on receptor expression. An example of this type of assessment may involve the monitoring of inhibition of receptor internalization in response to external modulation challenge as a result of drug-receptor interaction. The degree of this modulation inhibition may be directly proportional to the amount of drug-receptor binding present at the time of assessment.

These assays, when properly designed and implemented, can serve as powerful tools in the pharmacodynamic assessment of drug binding. The authors will present examples of how these assays can and have been used in drug discovery and development programs, and address important aspects of the validation and implementation process required for global, multisite performance of these assays in clinical trials.

153/B22

In-Depth Characterization of Drug Effects Using Multiparametric Plate-Based Cytometry on Guava easyCyte™ Platforms

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Dissection of mode of compound action requires the study and understanding of multiple impacts a compound has on cell stress, survival and function. Traditional approaches have typically investigated 1-2 parameters in parallel and focused mainly on apoptosis and cell death indicators. Recent studies have however shown the importance of studying mitochondrial depolarization in context of apoptosis and cell death in particular since it is common to multiple modes of cell death. Time and sequence of impacts can also provide insights into possible localization or site of major impact for a compound. The guava easyCyte™ systems have greatly facilitated plate-based compound screening due to the use of small amounts of cells per well and the capability to compare impact of several different parameters simultaneously or in parallel using the powerful InCyte™ software. In this study, we applied a powerful multiplexed approach using caspase activation, mitochondrial potential changes, annexin V based response and cell death in a single assay followed by analysis with the guava easycyte 12 instrument and Incyte™ software. The approach was applied the study of dose and time responses of compounds such as niclosamide and thimerosal to elucidate detailed mechanism of action. Our results demonstrate that at shorter times of incubation, thimerosal rapidly causes almost complete depolarization and a significant level of annexin V response with little caspase activity and cellular death at time points up to 6 h. Changes in proliferation were also observed with the treatments. Interestingly, niclosamide treatment results in complete mitochondrial membrane depolarization response but no apoptosis or cellular death is observed for up to 6 h but much longer times treatments eventually leads to apoptosis and death. The data thus demonstrates that the major mode of action of niclosamide proceeds through its impact on mitochondrial function. Powerful multiplexed assay approaches on the guava easycyte platforms combined with Incyte software allows for maximum information to be obtained from fewer cells in drug discovery and mechanism based studies. Such studies provide great depth into the understanding of the mechanism and sequence by which compounds/drugs modulate cellular stress, death and proliferative processes.

**Cell Proliferation and Death (B23 – B29)**

154/B23

High-Precise Measuring of Morphological Changes in Mononuclear Cells during Early Stages of Apoptosis Using a Scanning Flow Cytometer

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Background: Apoptosis is the process of programmed cell death implicated in biological processes ranging from embryogenesis to ageing, from normal tissue homeostasis to many human diseases. Therefore researches of this process are very important for medicine and open new perspectives in the cell biology and immunology. Apoptosis is characterized by distinct morphological and biochemical changes. Usually the early stages of apoptosis are indentified by biochemical or immunological methods. However such methods may have an undesirable effect on the object studied. Therefore this work is devoted to present a new approach to the kinetic study of the early stages of apoptosis and fluorescence-free technique for the multiparameter assay of morphological changes in the mononuclear cells population during this process using a scanning flow cytometer.

Methods: Experimental basis of this work is a scanning flow cytometry technique. In this study we used lymphocyte samples obtained with a density-gradient separation procedure from the whole human blood. Solving the inverse light scattering problem one can obtain some characteristics of cell. In our previous work we applied a bilayer sphere as an optical model of a lymphocyte. In this work we used a bilayer sphere with the eccentric inner sphere that allows us to take into account the lymphocyte nucleus heterogeneity. Lymphocytes were analyzed before induction of apoptosis and during 3 hours after induction.

Results: The use of a new optical model enables us to take into account the lymphocyte nucleus heterogeneity and the chromatin condensation at the nuclear periphery during apoptosis by that increase the accuracy of determining cells characteristics such as the volume and refractive index of the nucleus. The dynamics of nuclear cells volume distribution functions has been received during early stages of apoptosis. Applying a new approach to the
kinetic study of the early stages of apoptosis to process the experimental kinetic data we calculated next parameters: the fraction of apoptotic cells, the characteristic time of the apoptosis lag-phase, and the cell population synchronicity to go into apoptosis. Lymphocytes nucleus volume changes during the process of apoptosis were ~20%.

**Conclusions:** We presented a new approach to the kinetic study of the early stages of apoptosis and experimentally demonstrated the possibility of the fluorescence-free method for the multiparameter assay of morphological changes in mononuclear cells during this process. This method allows one to determine such mononuclear cells characteristics with high precision as the volume of cells and cell nuclei before and after the initiation of apoptosis, refractive index of the nucleus, the fraction of apoptotic cells, the characteristic time of the apoptosis lag-phase and the cell population synchronicity to go into apoptosis. This information may also be useful in the assessment of individual sensitivity of tumors to chemotherapy drugs.

**155/B24**

**Image-Based Flow Cytometry Method to Assay the Capacity of PBMCs to Resist Apoptotic Stimuli**

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**Introduction:** The apoptosis pathway consists of a series of physiological events within the cell that culminate in cell death. Blood monocytes and peripheral tissue macrophages play a role in the healing of muscle following injury. When these cells enter peripheral tissue they are exposed to intramuscular contents that is considered toxic to the cells. The percentage of cells that undergo apoptosis directly influences muscle recovery, thus establishing an assay to assess the ability of peripheral blood mononuclear cells (PBMC) to resist apoptotic stimuli is relevant. The purpose of this study was to determine develop an assay to assess the ability of PBMCs to resist apoptosis as classified into the various early (phosphatidylserine) and mid (caspase events) markers.

**Methods:** With consent, subject venous blood was collected into EDTA treated blood tubes. Peripheral Blood Mononuclear cells (PBMCs) were isolated using a density gradient centrifugation method (final concentration=5.0 x 10^6/mL). Cells were held on ice (control) or incubated with staurosporine (5, 10, or 20 nM) in an incubator (37°C and 5% CO2). Incubations of 8, 16, 24, and 48 h were compared to generate a time course of change in apoptosis resistance. Staining panel allowed for the measurement of mitochondrial dysfunction (CellROX-green), phosphatidylserine ( Annexin-V-eFluor530), caspase cleavage (Guava MultiCaspase), and nuclear fragmentation (7AAD staining). Responses were classified according to classic (CD4+16+) and pro-inflammatory (CD4++16+) monocytes using image-based flow cytometry (Aminis FlowSight; EMD Millipore). Acquired raw data was compensated and analyzed for apoptosis indices using IDEAS software (v.6; EMD Millipore). A two factor ANOVA was used to examine the effect of staurosporine dose vs. incubation time. Significance was set at P<0.05.

**Results:** The quickest induction of apoptosis was achieved with a staurosporine dose of 20 nM and an incubation time of 16-h. Using a dose of 10 nM and incubation times of 16 and 24-h provided the greatest capacity to differentiate changes in apoptosis. We also observed sequential passage of PBMC through, early, middle, and late apoptosis.

**Conclusion:** In this report we demonstrate the development and validation of a new assay system to evaluate the capacity of PBMCs to resist apoptotic stimuli. Specifically, within this assay system we found that the combination of multiple staurosporine doses and incubation times provides the best ability to differentiate changes in PBMC resistance. The next logical step is to apply a bioactive treatment known to improve resistance to apoptotic stimuli.

**156/B25**

**Curcumin Induces a Crosstalk between Autophagy and Apoptosis: A Biochemical Approach Coupling xCELLigence and Flow Cytometry**

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Curcumin, a major active component of turmeric (Curcuma longa), has inhibitory effects on cancers. In vitro studies suggest that curcumin inhibits cancer cell growth by activating apoptosis but the mechanism underlying the anticancer effects of curcumin is unclear. We investigated the mechanism of destabilization of the endoplasmic reticulum (ER) and lysosome that is involved in mitochondrially associated apoptosis in HuH7 cells.

It has been suggested that it may be possible to exploit autophagy for cancer therapy. Indeed, there is a complex interplay involving early autophagy, as soon as mitochondria produce superoxide anions and hydrogen peroxide. We examined the induction of autophagy, marked by the formation of autophagosomes, by staining with acidine orange after exposure to 10 microM curcumin. At this concentration of curcumin, only early events of apoptosis are detectable. The conversion of LC3-I to LC3-II, a marker of active autophagosome formation, was followed by Western blotting. The initial induction of autophagy has been efficiently depicted by the simultaneous use of xCELLigence (ACEA, Invitrogen) and flow cytometry (BD Biosciences). Cells isolated from the culture wells of the xCELLigence system at different times were trypsinized and immediately reanalyzed by flow cytometry for metabolic assays, i.e. mitochondrial membrane potential [DiOC6(3)], superoxide anions production (MitoSOX), calcium levels Flu-JAM1, autophagy with acidine orange (AO) or LC3-II staining, cell cycle analysis (BrdU) or electron microscopy for mitochondrial swelling and autophagosomes formation.

We found that, following curcumin treatment, the production of reactive oxygen species and the formation of autophagosomes was almost completely blocked by all of the following compounds N-acetylcyctein, the mitochondrial specific antioxidants MitoQ10 and SKQ1, Curcumin-induced autophagy failed to rescue all cells and a majority of cells underwent a type II cell death following the initial autophagic processes. However, 20-30% of cells, which where blocked in the cell cycle, escape and give rise to a novel proliferative phase which depends on the curcumin concentration.

This work has been founded by a grant from the 'Association Francaise contre les Myopathies' (AFM 15137 & 15661) to PXP.

**157/B26**

**A Novel Image-Based Cytometry Analysis for Measuring Cell Migration in Wound Healing Assay**

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Cell migration is a multi-stepped, highly complex process that is involved in normal processes of cell proliferation and homeostasis, but also is exaggerated in the pathologies of metastasis and tumour invasion. The coordination of events has been studied at the molecular, biochemical and biophysical level for nearly 50 years. One assay which has been used throughout is the wound-healing or scratch assay. Simply defined, a monolayer of cells are grown and a border is introduced either by scratching through this monolayer to create a wound or by removing a physical barrier. The movement of cells over the margin and into the newly created space is measured. Additional
information that can be gleaned from this type of assay may also be cell morphology and polarity. This biological process can substantially differ depending on the origin of the cells, the matrix they are grown on, the composition of the media and any compounds/nucleic acids that may be added as part of a screen, therefore it is important to set up a robust assay that will allow for many modifications. In this work, the Celigo Imaging Cytometer was used to measure direct in-situ cell migration for label-free or fluorescently labeled cells for cell migration analysis. We used the Oris\textsuperscript{TM} Cell Migration Assay (Platypus Technologies, LLC), cultured the cells with increasing concentrations of Cytochalasin-D to inhibit actin polymerization and cell migration, and measured cell migration in 2-hour increments for 48 hours. Confluency and cell counts were obtained at each time-point and growth curves for each well were created. Data was exported and a Cytochalasin-D dose-response curve was generated. The Celigo Imaging Cytometer generated highly reproducible cell migration results, as well as consistent data using either bright field or fluorescent imaging. The Celigo software allows different plate selection and analysis methods for the researchers to optimize their analysis parameters, which can generate more accurate cell migration results. The Celigo Imaging Cytometer can be an essential tool for researchers to greatly improve accuracy of results and efficiency.

158/B27
A High-Throughput Direct Adherent Cell Analysis Method for Cell Cycle and Apoptosis Using Celigo Imaging Cytometer
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Apoptosis and cell cycle play an important role in various aspects of preclinical pharmaceutical drug discovery and validation. The ability to quickly determine the cytotoxic effect of chemical compounds on cancer cells allows researchers to efficiently identify potential drug candidates for further development in the pharmaceutical discovery pipeline. Recently, a plate-based imaging cytometry system, Celigo Imaging Cytometer, has been used for to high-throughput fluorescence cell cycle and apoptosis analysis. In this study, we demonstrate the use of Celigo imaging cytometry for apoptosis and cell cycle detection by studying the dose response effect of nocodazole on cell cycle and staurosporine on apoptosis. For cell cycle analysis, the cells are treated with 150 nM and 50 μM of nocodazole for 18 hours, and then fluorescently labeled with DAPI and BrdU to measure DNA content. For apoptosis analysis, the cells are treated with staurosporine from 0.5 to 5000 nM for 18 hours, and then fluorescently labeled with Annexin V-PE and Hoechst 33342 to measure phosphorylation. The Celigo imaging cytometric analysis showed showed a clear dose response effect of the nocodazole to the cell cycle arrest as well as the increase of phosphatidylserine externalization as concentration of staurosporine increased. The experimental results were evaluated to validate the imaging cytometric capabilities of the Imaging Cytometry system. The plate-based imaging cytometer utilizes bright-field and three fluorescence channels (Blue, Green, and Red) for multi-channel analysis. By utilizing the F theta lens technology, uniform bright-field image is captured for more accurate cell counting and analysis of the entire well. In addition, Celigo analysis software is used to report numerous parameters allowing detailed fluorescence-based cell population characterization. The ability of Celigo to rapidly and cost-effectively perform plate-based fluorescent assays has the potential of improving research efficiency, especially for adherent cells where plate-based cytometer does not require trypsinization for cell population analysis.

159/B28
Development of a Flow Cytometric Assay to Detect Annexin-V Expression in Conjunction with Cleaved PARP, Cleaved Caspase-3, Phospho-histone H2A.X and DNA Content for Screening of Pathway Response in Hematological Malignancies
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Historically, the detection of Annexin-V was only possible on fresh, unfixed cells, necessitating rapid acquisition of stained samples. Laboratory Corporation of America\textsuperscript{®} Holdings has developed a technique that allows cells to be fixed and permeabilized after Annexin-V staining allowing full assessment of the apoptotic pathway engagement by measuring the expression levels of cleaved PARP, cleaved caspase-3, and phospho-Histone H2A.X in conjunction with DNA dye to discern the cell cycle stage at which expression levels have changed. Time course studies were conducted on multiple malignant cell lines, treated with various chemotherapeutic agents, to assess the effects of treatment on cells of interest. Traditional Annexin-V with Propidium Iodine staining was performed and compared to the new method described. Significant correlation was observed between the traditional Annexin-V method and the fixation/permeabilization method. This method can also be utilized with additional surface markers and additional apoptosis pathway markers to fully elucidate intrinsic and extrinsic apoptosis pathway engagement in hematopoietic malignancies. Examples of these other surface and apoptosis pathway markers will also be shown, displaying the utility of the new method in multiple applications.

160/B29
An Annexin V Based Unitized, Room Temperature Stable Assay for Detection of Apoptosis in Small Volume Tube
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Apoptosis is a sequential process of programmed cell death that occurs in multi-cellular organisms. The Annexin V binding assay has been a valuable indicator for the detection of the early stages of apoptosis. The apoptosis assay kits that are currently available can be cumbersome to use, involving multiple pipetting and washing steps. Reducing the work flow and bringing in a standardization of this assay will prove to be very beneficial to the pharmaceutical industry, which routinely uses apoptosis-based drug screening assays. We have now developed a stable Annexin V-FITC/Propidium Iodide (PI) assay based on the DuraClone technology of dry reagents that enables long-term storage of antibody-dye-combinations at room temperature. Jurkat cells were submitted to cold stress conditions, and apoptosis was quantified with a cocktail of dry DuraClone Annexin V-FITC/PI reagents, allowing for a distinct separation between the apoptotic, necrotic and live-cells.

The DuraClone Annexin V-FITC/PI reagent is dispensed in a unitized format in micro centrifuge tubes; samples can be stained by adding the sample material directly to the tube. These samples can be acquired from the same tubes, through flow cytometers designed for sample acquisition from low volumes of samples in micro centrifuge tubes. A dry, room temperature stable reagent obviates the need for cold storage. The unitized format increases
the sensitivity of this assay by reducing pipetting errors and provides a simple workflow.

This assay was demonstrated in micro centrifuge tubes, using smaller sample volumes. Thus, the DuraClone format has the potential to be suitably modified to 96 well microstate plates or to Micronic™ tubes which can cater to a high throughput screenig assay requirement of the pharmaceutical industry.

**CELL SORTING AND SELECTION (B30 – B46)**

**161/B30**

**Filtration System for the Detection of Circulating Tumor Cells**

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**Introduction:** Circulating tumor cells (CTC) measured with the CellSearch system in patients with metastatic carcinomas are associated with poor survival. Since the introduction of the CellSearch system for the detection of CTC from whole blood, alternative systems have been developed that are not using immuno-magnetic isolation of tumor cells based on their EpCAM expression but rather use physical characteristics of the a CTC, e.g. size, elasticity and charge. Here we present a simple filtration instrument that is based on the use of silicon microsieves together with a tool to label the collected the events.

**Methods:** A silicon microsieve comprises of a thin silicon nitride membrane with a thickness of 1 micron that contains 112,000 precisely dimensioned pores with a diameter of 5 microns in an area of 8 x 8 mm². The membrane is rigid, atomically flat and has no auto fluorescence. The silicon microsieve is placed in a slide that is part of disposable filtration unit, which is connected to a pump unit that applies a negative pressure across the sieve membrane. After completion of the filtration process the slide with microsieve is removed and transferred to a staining holder. Next fluorescent labels (15 microlitre) are added on top of the membrane. After incubation the bottom of the microsieve is pushed onto the absorber of the staining holder and excess labels / buffers flow through the membrane towards the absorber. Images of the collected cells were acquired using a fluorescence microscope. The filtration system was evaluated using four pre-stained cell lines, Colo320 (size 11 µm), SW480 (size 11 µm), T24 (size 16 µm) and SKBR3 (size 16 µm). These cells are spiked in PBS and blood from healthy volunteers. Used filtration pressures ranged from 10 – 150 mbar.

**Results:** To obtain a flow rate of 1 ml/min, a pressure of 15 mbar was needed for PBS, 30 mbar for EDTA blood and 100 mbar for CellSave fixed blood. The cells were homogeneously distributed over the surface of the microsieve and uniformly stained. The recovery percentages of the filtration system was depending on the size of the used cell lines, as well as the pressure and total time required to filter the whole sample. For a negative pressure of 15 mbar, the recovery percentages ranged from 20% for the Colo320 and 80% for the SKBR3 cell line.

**Conclusions:** The results demonstrate that the designed filtration system is easy to use for collecting and labeling of cells present at low frequency. Next step is to derive a filtration model for the recovery in which the physical parameters of the cells are combined with pressure and total filtration time to optimize the filtration settings and recovery.

**162/B31**

**Comparison of Cell Sorter Performance Using BD™ Accudrop Technology and Rmax on the BD Influx Platform**

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Most cell sorters on the market allow for cell sorting with different sorting modes. Depending on demands of each sort, these modes can be applied to focus either on optimization of recovery at the expense of purity or vice versa. To generate good sorting results the drop delay (DD) has to be determined on all systems. Some sorter software allows running a DD assay automatically; other systems need the operator to set the delay manually. To establish the correct drop delay two technologies are mainly used. Either fluorescent beads are deflected to one side and DD adjustments are made to maximise the number of deflected beads (Accudrop™ technology) or instruments analyse the waste stream to check for remaining beads (Central Stream Catch (CSC) or ProDrop™ technology). The CSC technology has the benefit of checking the frequency of the target population that is not sorted and therefore left in the central stream. By combining this value with target-population frequency taken from the original sample it is possible to establish the point of maximal recovery (Rmax). The BD Influx cell sorter platform uses the Accudrop technology. In this study we included four BD Influx cell sorters located in three different flow core facilities to compare the results of instrument setup using the Accudrop and the CSC technology. To this end we used two nozzle sizes on each instrument, performed instrument set up using Accudrop technology and ran the Rmax analysis with both nozzle sizes. Our results show that the Accudrop technology allows for a reliable setup of the cell sorter as the maximal recovery on all instruments lay between the Accudrop setup values plus/minus 2/16 of a drop. The results also highlight that the quality of the Accudrop results rely heavily on the optimal focus of the Accudrop camera on the BD Influx platform.

163/B32

**Platelet Isolation by Fluorescence-Activated Cell Sorting**

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The involvement of human platelets in various physiopathological processes has triggered the study in several areas of basic and clinical research. However, removing platelets from their physiological environment may be difficult due to the lack of suitable isolation methods. Obtaining samples with a high degree of purity without contamination of erythrocytes and leukocytes as well as the artefactual activation of platelets poses a real challenge, not completely addressed by centrifugation or gel filtration, the preferential methods used for platelet isolation [1, 2]. This is particularly evident in biomarker research and proteomic studies where the goal is to compare platelets obtained from patients and controls. To obtain the high degrees of purity required in these types of studies, fluorescence-activated cell sorting (FACS) could be used. However, the stress associated to FACS needs to be avoided in order to prevent any damage on platelets and maintain their functionality and response capacity to different agonists. Therefore, the aim of the present study is to optimize the methodology of FACS making it applicable to isolate functional platelets.

From whole blood samples, platelet were identified by CD61 and sorted in a FACS™ at low pressure, before and after treatment with Pluronics®-F68, a non-ionic surfactant which protects...
different types of cells from shear forces. To characterize platelet activation responses to thrombin (0.5 U/ml) before and after sorting, the expression of P-selectin (CD62P) was monitored. After sorting, platelet capacity to respond to thrombin was significantly impaired as noticed by the decrease in median fluorescence intensities of CD62P comparatively to non-sorted platelets. Contrary to what happens with other cell types, for the tested concentrations, Pluronic®-F68 did not offer protection to platelets during the sorting process. Currently we are trying to understand the platelet activation pathways that are affected during the sorting process and target those specifically to protect platelets during high-speed cell sorting.

In the future this optimized methodology could lead to a better understanding of biomarkers and therapeutic targets, allowing an effective diagnosis and consequently a better treatment of cardiovascular diseases.

References:

164/B33
Proposal for a Sort-Performance Index
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In many research fields, optical cell sorting is utilized to isolate rare cell populations. While target cell purity generally is an obvious metric to score the sorting performance, other aspects can also be mission critical: The yield of target cells can often be important, the number of sorted target cells or last but not least the time it took to process the sample material.

Unfortunately, within the limits of a given technology these are conflicting interests: In order to obtain a highly purified fraction without losing too material much due to coincidence related aborts, the sort process has to be carried out with diluted sample material. As a consequence, the time to sort goes up. Or alternatively, if yield and time to sort are the most important aspects, purity will be compromised.

With regard to the above it is desirable to have a metric to be quickly able to compare experimental setups, different sorting instruments or even different sorting technologies.

We have analyzed different published methods to compare sorting results, have looked at different ways to visualize the results in a comprehensive way and propose a sort-performance index (SPI) in order to have a quick way compare results generated under varying experimental conditions.

This SPI now has enabled us to compare different sorting technologies and allows us to demonstrate the utility of this in the process of planning a complex experiment that could be carried out in single or sequential runs, on a single or on multiple platforms: By applying this methodology it is straight-forward ‘cookbook-recipe’ process to find the optimal experimental setup.

165/B34
New Technique for Sorting Rare Populations of Cells
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New Technique for sorting rare populations of cells

Many abundant cell populations from a variety of tissues have been well characterized by flow cytometry. However, our ability to detect and study rare cell populations is still limited. Many of these cell populations are so rare that it can take over 4 hours of sorting time to recover enough cells for further experiments. Cost and availability of the sorters can make these long sorts impossible to do in regular flow facilities.

Traditionally these rare populations have been enriched for by 1) the classic double sort (yield mode followed by purity mode) however, this does not significantly decrease sorting time or 2) depleting unwanted cells by either lysis (e.g. red blood cells) or by column enrichment using magnetic beads; although these methods are effective, they increase the number of steps in sample preparation which may cause loss of cells of interest.

A novel technique, which utilizes double sorting, has been developed. However, compared to the regular method, the first sort quickly enriches the sample by fooling the instrument by applying high thresholds on multiple parameters. This allows us to run hundreds of thousand of cells per second and decrease the time of the enrichment sort fivefold. The pre-sorted population can then be re-sorted to increase the purity of the population of interest.

The advantage of this new sorting method is that it doesn’t add any sample preparation steps, increasing cell viability, decreases the time on the instrument and increases the yield of the population of interest compared to classical methods of sorting.

166/B35
Practical and Technical Considerations in Sorting C. elegans Larvae on a BD FACS Aria II
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Isolating subpopulations of Caenorhabditis elegans, a nematode now established as a major model and tool in biomedical research, has required the use of low-throughput methods or the use of very specialized equipment. A method for sorting C. elegans L1 larvae on a BD FACS Aria II enabling the use of traditional cell sorters for accurate detection and selection of pure C. elegans subsets has been described, overcoming some limitations for applications requiring high amounts of pure populations of that nematode.

However, that method lacked of statistical analysis for the assessment of cell sorting performance, which can be usually characterized by the following five parameters: purity, efficiency, recovery, yield and post-sort survival. Due to its oblong shape and its multicellular nature, C. elegans L1 larvae represent atypical particles for a classical cell sorter, designed to sort single cells in suspension. In addition, dealing with a live organism able to curl and move adds a level of complexity. We therefore investigated how influential these morphological and physiological features were on sorting performance.

We first questioned the validity of drop delay calculation based on microbeads to worms sorting. C. elegans larvae being particles of a very different size and shape compared to these microbeads. We then noticed the influence of C. elegans movements and their ability to curl on the measurement of optical signals and sorting performance. Within this scope, we also investigated the impact of sample flow rate on larvae curling. Finally, sample preparation and especially larvae concentration was optimized in order to find the right balance between sorting performance and sort duration. This study allowed to fine-tune the method to sort C. elegans L1 larvae on a BD FACS Aria II and to provide a statistical evaluation in terms of sorting performance as well as practical considerations for sample preparation.

167/B36
FASS and FAST-FIN, Two New FACS Based Methods for the Purification of Brain Tissue Organelles after Subcellular Fractionation
Effect of Soluble ST2 on Activation of Type 2 Innate Lymphoid Cells in a Murine Model of Asthma

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Background: Soluble ST2 is a member of the interleukin (IL-1) receptor family. Actually, the ST2 gene produces a soluble form (sST2) and a transmembrane form (ST2L) by alternative splicing. sST2 corresponds to the extracellular portion of ST2L, which constitutes IL-33 receptor with the aid of IL-1 receptor accessory protein. We demonstrated in the previous study that binding of sST2 to IL-33 blocked signal transduction of IL-33 through ST2L in vitro. Type 2 innate lymphoid cells (ILC2) were recently discovered as a novel target of IL-33. IL-33 activated ILC2 and induced production of IL-5 and IL-13, thus caused airway inflammation in asthma. Therefore, ILC2 is a possible target of treatment against airway inflammation. The aim of this study is to investigate whether sST2 suppresses ILC2 activation with IL-33 both in vitro and in vivo.

Methods: Murine sST2 was purified as a tagged protein from culture media of HEK293T cells. The effect of sST2 in vitro was examined using murine ILC2, isolated from lungs of naive BALB/c mice by cell sorting. ILC2 were cultured with or without IL-33 in the presence or absence of sST2. The effect of sST2 in vivo was examined using an ovalbumin (OVA)-induced asthma model of BALB/c mice. Airway inflammation was induced by intraperitoneal sensitization twice, and subsequent intranasal challenge for three consecutive days with OVA. sST2 or PBS was intranasally administrated before OVA challenge once a day. Characteristics of lung ILC2 in cultured cells and asthma model were assessed by flow cytometry. Inflammatory cells and cytokine production were evaluated by microscopy and enzyme-linked immunosorbent assays.

Results: Isolated lung ILC2 clearly expressed ST2L on the cell surface. The stimulation of ILC2 with IL-33 induced cell proliferation and significant production of IL-5 and IL-13. The expression of CD25, Sca-1, and ST2L were also increased on the cell surface. Treatment with sST2 prior to IL-33 stimulation effectively suppressed IL-33-mediated responses of ILC2 in vitro. Furthermore, lung ILC2 in sST2-administered mice in asthma model showed less expression of CD25, Sca-1, and ST2L compared to those of PBS-administered mice. Administration of sST2 also reduced OVA-induced eosinophil infiltration and production of IL-5 and IL-13.

Conclusions: These results indicate that sST2 suppresses the IL-33-mediated activation of ILC2 and the development of airway inflammation in OVA-induced asthma model. Therefore, sST2 may be a candidate for therapeutic agent for ILC2-mediated diseases.
Result: Cells sorted from highly fluorescent tetracycline-induced populations, did not reproduce parental levels of GFP fluorescence. However, when tetracycline was not added, sorting the un-induced population that gave the highest resting levels of GFP expression was successful. Sorting these at low cell numbers into 96well plates gave a selection of cell populations, which when screened gave rise to populations of cells which produce milligram quantities of rSERT on a 10L scale.

Conclusion: Mammalian expression systems are an under-used resource in structural biology and represent an effective way to produce fully functional membrane proteins for crystallisation and future structure determination. The strategy we have used may be successfully applied to improve the expression of other mammalian membrane protein targets.

170/B39
Enrichment of Live Cells Based on Intracellular Cancer Biomarker Detection Allows for Additional Downstream Functional Testing
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Enrichment of cancer cells within a mixed population has traditionally been confined to fixed cells or in the case of live cell sorting to a limited set of cell surface markers which are typically not specific to the target cells. Cell surface antibodies may also activate surface receptors altering the state of the cells such that they are no longer representative of the original population. Intracellular staining requires fixation and permeabilization thus rendering the cells unusable in downstream functional assays. GFP reporters while amenable to live cell detection may alter the function of the protein ultimately affecting the cell which can be the case for many of the tagged fusion proteins.

Using a novel live cell biomarker detection technology we have shown the ability to label specific live cells within a population by fluorescently detecting RNA. This method does not affect or alter the cells and allows further testing of the target population following enrichment through fluorescence-activated cell sorting. We have successfully enriched cell populations based on mRNA and microRNA targets. The introduction of RNA as a new parameter to sort cells greatly expands the available markers that can be used to isolate live cell subpopulations.

The data presented here shows live cell enrichment of a target cell population within a heterogeneous mixture of cells based on their RNA expression levels. We used two independent microRNA probes targeting miR-221 and miR-222, which have been shown to be upregulated during epithelial to mesenchymal transition. miR-221 and miR-222 were used to identify specific cancer cells within a mixed population. Additionally and more interestingly the enriched cells were then further characterized through a functional migration assay. The miR-221 and miR-222 positive (or high) populations showed highly invasive properties while the miR-221 and miR-222 negative (or low) expressing populations did not exhibit the ability to migrate through the collagen matrix. In addition we have shown the ability to enrich for ERBB2 positive cells from heterogeneous populations utilizing a probe specific to ERBB2 mRNA.

The ability to sort live cells based on RNA levels of cancer biomarkers in a non-destructive manner enables researchers the ability to study the expression of rare events and labile species using a diverse group of targets beyond the traditional surface markers with the added benefit of utilizing the enriched cells for downstream functional assays.

171/B40
Flow Cytometry Analysis and Sorting for In Vitro Selection Applications
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Background: The advent of in-vitro selection methods for DNA and RNA facilitated the emergence of new nucleic acid binding motifs (aptamers) and new catalytic functions such as ribozymes and deoxyribozymes. Of a particular interest amongst in-vitro selected ribozymes is the RNA polymerase ribozyme (RPR). The latest version of this ribozyme is capable of synthesizing RNAs up to 200 nucleotides from nucleoside triphosphates (NTPs). Nevertheless, the RPR is still not capable of synthesizing its own sequence, which would be the cornerstone of the RNA world theory; e.g. a RNA replicase.

In general, in-vitro selection techniques for ribozymes are based on the acquisition or loss of a capture tag, which restricts the selection for multi-turnover reactions such as RNA polymerisation.

Methods: We developed a selection method called CBT (Compartmentalised bead tagging) that uses water-in-oil emulsion technology to link ribozyme genes (genotype) to thousands of the corresponding ribozymes (phenotype) via microbead (MyOne Streptavidin C1 Dynabeads, Invitrogen) display. The ribozyme-catalysed primer extension reactions on beads are amplified by rolling circle amplification (RCA). Through labeling with SYBR® Gold Nucleic Acid Stain we were able to detect the primer extension performance of the ribozyme attached to each bead by quantifying the amount of its fluorescent signal. The beads were sorted by SYS200 Cell Sorter (Sony) isolating two populations based on their intensity of fluorescence. Beads with highest intensity of fluorescence (top 1% and 5%) were separated and mixed in various ratios. Genes extracted from the mixture and amplified by PCR (polymerase chain reaction) encoded more active ribozymes, and were used in the next round of selection.

Results and Conclusions: The new CBT selection method allows us to use flow cytometry analysis and sorting as a sensitive tool for the fast separation of the improved bead bound ribozyme variants from a population of 10⁸ different clonal ribozymes. We anticipate our described procedure to be useful for a diverse range of in-vitro selections of functional nucleic acids. Implementing multicolour staining might help to perform parallel selection on different templates and allow direct observation of the progress of selection.

172/B41
Evaluating the Effects of Cell Sorting on Gene Expression
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The ABRF Flow Cytometry Research Group has continued with the goal to establish best practice guidelines for cell sorting conditions that minimize cell stress, perturbation, or injury to the sorted cell populations. In past FCRG studies, gene expression changes in sorted Jurkat cells, a human lymphoblastic T cell line, were correlated to nozzle size and sort pressure. The current study examined the effect sorting has on primary cells (C57Bl/6 mouse splenic B lymphocytes). B lymphocytes were isolated using multiple flow sorters under gentle (100 micron nozzle size/20 psi pressure) and stressful (70 micron nozzle size and 70 psi pressure) sort conditions. The sorts were performed using several instrument types to compare the differences in instrument designs (cuvette hybrid and jet-in-air) in addition to differences in sort conditions. Gene expression was assessed using Affymetrix Mouse Gene ST 2.0 microarrays using targets prepared from the NuGEN Pico reagents and Qiagen Micro minelute columns. Details of the study and results will be presented along with future plans.
173/B42
Two Dimensional Acoustic Prefocusing Free Flow Acoustophoresis Enables High Performance Microchip-Based Separation of Leukocyte Subpopulations

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We report that microchip-based free flow acoustophoresis (FFA) in combination with two-dimensional acoustophoretic cell prefocusing enables concurrent multiple target outlet fractionation of leukocytes from a lysed blood sample into subpopulations (lymphocytes, monocytes and granulocytes).

The basic principle for the separation relies on the fact that different cell types display cell specific acoustophoretic mobilities in an acoustic standing wave field. By designing microfluidic channels to support the formation of a half wavelength standing wave in a micro channel, cells that enter the channel along its sidewalls will migrate to the channel center (acoustic standing wave pressure node) at a velocity that is based on its intrinsic acoustophysical properties while being perfused along the channel. A flow splitter at the channel outlet extracts a centre fraction, i.e. the cells that migrate fastest to the centre will be collected here. In the current study we implemented an acoustophoresis channel with a flow splitter that had 5 outlets to enable the collection of multiple fractions that represent cells having a certain acoustophoretic mobility.

In order to ensure a high performance in the acoustophoretic separation it is of utmost importance that all cells that enter the separation zone are travelling along the channel at the same velocity, else the separation resolution will be heavily dispersed by the fact that cells spend different time in the acoustic force field and hence their final lateral location in the channel is as much dependent of their initial spatial location in the parabolic flow profile. By employing a step of 2-dimensional acoustophoretic prefocusing of the cells prior to entering the separation zone we ensure that all cells travel at a uniform velocity through the separation system. The implementation of the 2-D prefocusing vastly improved the separation outcome. The system performance was calibrated using 3, 5 and 7 micrometer polystyrene beads yielding that 87.5% of the 3 um beads were collected in its targeted outlet and the corresponding data for 5 and 7 micrometer beads were 96.2 and 98.5 %. The slightly lower performance for the 3 micrometer beads reside in the fact that their size is approaching the lower limit for where acoustophoresis separation can be well performed at resonance frequencies around 2 MHz.

The corresponding data for separation of leukocyte subpopulations from a lysed blood sample showed similar performance. Flow cytometry data for lymphocytes demonstrated a high purity (>95.1%) and high recovery (>86.5%) and for granulocytes the corresponding data gave a purity of 98.5 % and a recovery of 68.4%. No subpopulation bias was observed. These data demonstrate an unprecedented separation of leukocyte subpopulations at flow rates of ~100 µl/min and ~1M cells/ml sample concentrations, not previously reported in acoustofluidic systems. The method is a viable alternative to current methods for particle sorting and cell isolation, requiring a minimum of sample preparation, and with potential to lower analysis time and cost.

174/B43
Cell Separation by Molecular Computation on the Cell Surface

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With an aim to separate closely related cells using a single tag in a single step, we report our latest developments using autonomous oligonucleotide-based computing cascades to evaluate cell surfaces. Our method has advantages of minimizing the impact on cell viability by separating in a single step and minimizing time, including directly from whole blood.

Oligonucleotide-based YES-logic gates can be combined linearly to form molecular computing cascades, where the output of the first YES-gate in the cascade sequence becomes the input of the next YES-gate, and so on (thus building-up AND-gate logic). Each logic gate in the cascade can be conjugated to an antibody against a cell surface marker of interest. As long as targeted cell surface markers are mobile, all logic gates assembled on the cell surface can come in contact with one another, thus enabling exchange of inputs and outputs in a sequential manner as programmed. Importantly, the absence of a step in the program will result in failure of the cascade to reach its conclusion, which is the key to achieving a high purity cell separation of closely related cells. Our molecular computing cascade components (i.e. antibody-oligonucleotide conjugates) only rely on choosing a combination of antibodies that define the cell to be selected; there is no need for judicious choosing of multiple fluorescent tags because only the oligonucleotide output in the final step in the cascade will display a tag, which can then be used for separation. We will discuss results of YES/AND-gates; NOT-gate functions for negative selection; OR-gate functions; and incorporation of amplification for low abundant cell surface markers, in magnetic cell separation.

175/B44
Evaluation of Novel Cell Sorter Aerosol Containment Methods

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Cell Sorters are increasingly used for infectious cell sorting procedures, but have the potential to produce aerosols putting the operator at risk. Instrument manufacturers have designed aerosol containment systems to mitigate this risk. However, as recommended in the latest ISAC Cell Sorter Biosafety Standards (Holmes, et al., 2014), containment testing of these systems must be performed at intervals determined by risk assessment. The current published method for containment testing utilizes Glo-Germ beads and the Aerotech impactor (Perfetto, et al., 2003). However, as we reported at CYTO 2012, this method has several drawbacks which led us to investigate the use of the Cylex D non-viable impactor and more standardized fluorescent beads, Polysciences YG beads. Tests of this impactor found that 1um Dragon Green (DG) beads (Bangs Laboratories) were brighter and more hydrophilic (facilitating instrument clean-up), and were used for subsequent tests.

Although the Cylex impactor proved advantageous, bead-based assays for aerosol detection are in general problematic for these reasons: Low frequency of bead-occupied aerosols (<0.1%) in fail mode; inability to accurately control bead event rate, especially at high flow rates; and difficulty in visual identification of beads and fluorescence quenching. Therefore, an alternative non-bead based method was developed and compared with the Cylex D/DG bead assay.

Data will be presented showing a method in which aerosols generated from a cell sorter in fail mode are collected on a Cylex D cassette and are subsequently quantitated with a spectrophotometric assay for phosphate (a constituent of the Phosphate Buffered saline in the sheath fluid). Failure of
containment was simulated by a reduction of the airflow rate of the Aerosol Management System, which also permitted a more consistent aerosol concentration for measurement in the Cycles/Phosphate Assay. Sensitivity of the assay was determined by correlating spectrophotometer absorbance readings with aerosol concentration as measured with a UV-APS aerodynamic particle sizer (TSI, Inc.). Measurement of aerosols by the Cycles D/Phosphate assay was also directly compared with the Cycles D/DG assay by measuring both phosphate and DG capture on the same Cycles coverslip.

The aim of these tests is to facilitate the required aerosol containment testing by providing the most sensitive, reproducible assay that is also simple to perform and does not require a large investment in equipment or supplies.

176/B45
Pre-sort Debris Removal by Counter-Flow Centrifugal Elutriation Reduces TIL Cell Sorting Times

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Isolation of purified tumor-infiltrating lymphocytes (TILs) from solid tumors by flow cytometric cell sorting can often be hindered by the large amount of debris generated during tumor preparation. Since the effective upper speed limit of cell sorting is governed by the amount of total events an instrument can process and successfully sort per second, removal of cellular debris before sorting can greatly reduce cell sorting times, improving efficiency and downstream results.

We show that counter-flow centrifugal elutriation (CCE) can be used to deplete the debris from cells in suspension. The cell suspension is pumped into a rotating flow cell with a set centrifugal force at a flow rate calculated to allow only the smaller debris to overcome the centrifugal force and be washed from the sample. The larger target cells are then collected by decreasing the rotor speed. This removal can be completed without a gradient or other added reagent, without damaging the cells, and in a relatively short time. Other enrichment techniques including positive magnetic bead selection or the use of density gradients can be mitogenic or risk the potential of cell loss.

We present data showing that sorting material pre-processed by CCE can shorten the sorting time needed to isolate high purity TILs from heavily debris-laden tumor preparations, cutting sort time up to 50%, with better recovery of target cells. This technique can easily be adapted to other sample types that include the isolation of small percentage target cells from a sample containing large amounts of debris.

177/B46
Enabling Single Cell Sorting of Protozoans on a Beckman Coulter MoFlo ASTRIOS: Application in the Study of Molecular Biology, Epidemiology and Taxonomy

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Background: Flow cytometry (FCM) has been extensively used to study many biological and molecular phenomena in various parasitic diseases such as Leishmaniasis and Chagas’ Disease. Quantification of the distribution of cell populations (immunophenotyping) and the determination of its functional roles has made flow cytometry, the methodology of choice for studies concerning the immunopathogenesis of these diseases in both ex vivo and in vitro assays from humans as well as experimental models. The sorting by FCM was complementary to these studies allowing the isolation of pure cell populations for use in subsequent experiments of cellular and molecular biology, providing single parasite gene expression analysis. In the case of leishmaniasis and Chagas’ disease, our FCM core has been developing the sorting of parasites in order to get answers to some questions observed in queries related to the molecular characteristics (DNA) and behavioral, providing subsidies for evaluations of entomology, taxonomy and epidemiology of these diseases. Thus, non-color single cell sorting has been used to obtain parasite clones for further identification of species; behavioral and epidemiological analyzes of vertebrate hosts and vectors, by biomolecular and enzymatic techniques, as well as in vitro tests for interaction between protozoan and host cell.

Methods and Results: Single cell sorting was performed in MoFlo ASTRIOS (Beckman Coulter) with a BSL-2, type A2 Biosafety Cabinet. First of all, the fluid system is completely sterilized, as well as the sort chamber. To setup the flow cytometer and demonstrate the accuracy of one-parasite sorting (cloning) in MoFlo ASTRIOS, one, two, five or fifteen flow check fluorescent beads (Beckman Coulter) were sorted onto a slide and counted in UV-immunofluorescence microscopy before and after sample experiment. Any issue in the certification of sorted beads, others fine adjustments should be performed, particularly in frequency and amplitude parameters. Next, a small amount of parasites was acquired to establish the gate strategy and sort decisions in FSC vs SSC density plot. Low concentration of parasites is important to avoid clumps and clogs during the process and using 100 µm nozzle and low sheath pressure ensured successful sort in gentle conditions resulting in one-cell purity. The perform the single cell sorting (cloning), one parasite was sorted into a 96 round-bottom well plate with 200 µL of Schneider medium, cultivated in BOD incubator at 27°C for 5 days. After, each well with parasite growing were transferred to culture tubes to parasite expansion. Parasites were then submitted to PCR multiplex; Real-time PCR or isoenzyme electrophoresis assays.

Conclusion: This method improves the use of cell sorters for accurate one-cell detection of pure protozoan populations, allowing the achievement of parasites clones to be expanded and evaluated in subsequent protocols, enabling the comprehension about the parasitology in these diseases.

CELL- DERIVED MICROTUBULES (B47 – B51)

178/B47
Selection and Optimization of Exosome Labels for nanoFACS

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Introduction: Extracellular vesicles (EVs) are heterogeneous populations of nano-sized vesicles with important regulatory roles. EVs produced by different cells (and under different conditions) have functional differences that we hypothesize are due to and identifiable by the production of distinctive subsets of EVs. In order to determine whether EV subsets are biologically relevant, we must be able to isolate EV subsets and perform functional studies. For this purpose, we developed the nanoFACS method for analysis and sorting of EVs and other nanoscale (50-150nm) particles. In this study we present the use of high sensitivity proteomics to identify surface markers for further analysis and sorting of EV subsets, and the use of nanoFACS with Nanoparticle Tracking Analysis (NTA) to test and titrate EV-specific labels.

Methods: EVs isolated by serial ultracentrifugation and quantified by NanoSight NTA. 104 EVs were used per staining condition. NanoFACS was performed with an AstraEQ flow cytometer, triggered with a high sensitivity SSC detector, and detection on a secondary high sensitivity SSC detector to monitor the parallel
subthreshold noise, in addition to standard FSC, FL parameters. Appropriate methods to prevent particle coincidence and instrument ‘swarm’ were employed. Membrane dyes included PKH26, PKH2, and CM-DiI. Cytoplasmic dyes included Qtracker and CFSE. Proteomic analysis was performed using a Thermo Orbitrap Fusion Mass Spectrometer.

**Results:** Large (>200 nm) aggregates were detected with all membrane or cytoplasmic dyes. We detected especially abundant aggregates in EV samples stained directly with PKH. EV’s generated from PKH-stained cells were relatively free of excess aggregates. nanoFACS delineated specific EV populations in unstained and CM-DiI preparations that are distinct from non-specific dye aggregates. For subset specific EV-labeling, we compared EVs isolated from irradiated and unirradiated cells. We found that among 160 proteins uniquely identified in EVs from irradiated cells, 41 were plasma membrane associated, and 3 (Mcam, Plexin A1, and Stom) were highly represented. For EV samples from both irradiated and unirradiated cells, among 204 proteins overrepresented at least two-fold, 82 were plasma membrane proteins. Thus, few of the candidate proteins that discriminate EV subsets induced by irradiation are surface receptors amenable to standard staining methods for flow cytometry.

Summary/Conclusion: To leverage the tremendous potential of EVs as biomarkers and regulators of disease, we need to identify relevant EV (sub)populations. In these studies, we refined pan-EV staining methods and identified candidate radiation-specific EV markers for further testing. Our results emphasize that when testing labels for nanoparticle subset discrimination, it is important to use methods such as nanoFACS, to discriminate between properly labeled individual particles and unwanted aggregates by analysis and sorting, and NTA to verify the sizes and concentrations of nanoparticles.

179/B48

**Sorting and Characterization of Functional Viral Particles Using Cytometer with Digital Focusing System**

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**Background:** Arenaviruses are highly heterogenous in the size (40-250 nm). However, heterogeneity of virus particles composition has not been addressed, mainly due to the absence of reliable and sensitive methods and instrumentation for their characterization. This study presents a powerful tool to analyze and sort nanosized vesicles using as a model functional JUNV particles.

**Methods:** The JUNV(Candid1) was obtained from the Bavarian laboratory (USAMRIID). JUNV-Alexa647 preparations infected Vero cells or macrophages were stained with the anti-GPC and anti-nuclear-protein antibody to detect infected cells. Viral particles were labelled with YOYO-1, CD9-PE antibody or FITC-CTB. The TR-PCR Quantifast kit also from Qiagen was used for reverse transcription. For FACS we used customized FACSARIA II equipped with 300 mW 488 nm laser (Coherent, FSC-PMT and digital focusing option (DFS). DFS included a tunable picomotor PMT and digital focusing option can be tremendously helpful not only for analysis and sorting of functional viral particles, but also for exosomal and microparticles fractions of EVs, opening new avenues to study mechanisms of extracellular vesicular production.

180/B49

**A Rapid Image Cytometric Analysis Method for Phagocytosis Using Celigo Imaging Cytometer**

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Phagocytosis is an essential process of the immune system to eliminate cellular debris and pathogens. It is a specific form of endocytosis involving vesicular internalization. Phagocytic cells such as macrophages can be attracted to pathogens or cellular debris and engulf the material to be trapped in an internal vesicle called phagosome. The phagosome would fuse with the lysosome to form phagolysosome, where enzymes and toxic peroxides can digest the pathogen or cellular debris at a low pH value. Traditionally, phagocytosis can be qualitatively examined via standard optical microscopy to observe the engulfment of bacteria or cellular debris. In addition, phagocytosis can also be quantitatively measured by incubating phagocytes with particles bound with serum or IgG. After incubation, the excess particles are washed away, leaving only phagocytosed with engulfed particles. These cells are lysed to release the engulfed material, which can be examined using microplate reader or western blot can also be utilized to quantify the level of phagocytosis. However, these methods are time-consuming and require experienced researchers to obtain meaningful results. By using the Celigo Imaging Cytometer, phagocytosis can be measured using a pH sensitive label called pHodo. The labels will only fluoresce when they are trapped in the phagolysosome where the pH value is lower. Therefore, by measuring the amount of fluorescence or percentage of cells fluorescing, one can use the Celigo to determine the fluorescent intensities of the target cell populations and measure the level of phagocytosis. In this work, THP-1 cells were stimulated with LPS into macrophages and incubated for 24 hours. After incubation, the cells were mixed with particles labeled with pHodo, and with 2 different drug treatments (with and without bortezomib). The cells were allowed to incubate with the 2 drugs and particles for 24 hours. The results showed clear difference between the 2 drug treatments, where the difference in fluorescent intensities were measured. The ability of Celigo to rapidly perform plate-based phagocytosis assay has the potential of improving research efficiency, especially for adherent cells such as macrophages.
where plate-based cytometer does not require trypsinization for cell population analysis.

181/B50
Imaging Nanomaterials Secreted from Cells in Microvesicles with an Amnis ImageStreamX MkII
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Background: Microvesicles (MVs) are sub-micron lipid-membrane enclosed particles released from mammalian cells. A growing body of research suggests that MVs are involved in cell-to-cell communication and contain non-random collections of both membrane surface proteins and internal cargo. MV are strong candidate biomarkers for disease screening and monitoring treatment efficacy or disease progression. Single microvesicle phenotypic analysis has been accomplished using both standard flow cytometers and imaging flow cytometers, though recent work has made clear that the Amnis ImageStreamX has less background noise, improved sensitivity and suffers less from the swarm detection than standard flow cytometers. Multiple publications describe immunophenotypic analysis of MV via surface antibody staining. Here we describe imaging peptide-coated internalized silver nanoparticle (AgNP) payload within single microvesicles by imaging flow cytometry.

Methods: PPC1 cells in the presence of peptide-coated AgNP fluorescently labeled with CF647 were cultured in serum-free media with or without amino acids added. Cell culture media supernatant was collected and concentrated by a regular 10,000 X g centrifugation. The resulting fraction was stained with the lipophilic dye PKH26 to identify MV. For some samples AgNP external to MV were dissolved by a chemical etch. Samples were imaged on an ImageStreamX MkII (ISX2) imaging flow cytometer (Amnis, Seattle) with a 60X objective at low speed. PKH26 fluorescence was read in channel 3 and CF647 labeled AgNP was detected in channel 11. Channel 6 was used for Side Scatter from the 784nm laser. The ISX2 injects one micron ‘Speedbead’ calibration particles into the core stream so that real-time automated adjustments can be made to keep the focal plane in line with the core. By default, the INSPIRE acquisition software is configured to exclude Speedbeads from the resulting data file. Since Speedbeads are close in size (pixel area) to MV, and we were concerned that MV would be inadvertently excluded from the data sets, INSPIRE was configured to include Speedbeads. The concentration of AgNP bearing MV was determined by gating on small PKH+ CF647–AgNP+ particles with a high degree of colocalization (bright detail similarity) between the PKH4 and AgNP signal. The gating scheme was validated against data from etched samples where the AgNP fluorescence was deactivated by chemical etching.

Results: Leveraging the ability of the ISX2 to localize fluorescence signals in X and Y enables discrimination between events where MV and AgNP fluorescence is cospatial versus events in which MV coincidently appear in the same image as AgNP but are not occupying the same pixels. We demonstrate that it is possible to image not just surface features of MV, but also internal payloads. Careful gating for colocalized fluorescence between payload and lipophilic dye (PKH26) can discriminate coincident from colocalized MV fluorescence.

Conclusion: We have established an imaging flow cytometry assay and gating strategy to monitor cellular release of AgNP through MV. The ISX is well suited to this task as image analysis can be employed to discard coincidence events and identify events where MV and NP fluorescence occur with similar spatial distribution

182/B51
Measurement of Microvesicles as a Tool for Pharmaceutical Bioprocess Monitoring
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Introduction: In this work we present microvesicles (MVs) as a new factor for optimizing pharmaceutical bioprocess control. Microvesicles (MVs) are membrane vesicles released from the cells into their microenvironment. They have fundamental role in regulating biological processes. Recognition of their role in a variety of diseases mostly stimulates their investigation for clinical purposes. In this work we present MVs from a different point of view, as a tool for the bioprocess monitoring. The release of MVs depends on cell activation, cell stress, cell properties, and dying of cells; therefore, the amount of released MVs could be used for the identification of cell state and cell condition during the bioprocess.

Methods: Microvesicles were quantified by flow cytometry. MVs were measured in direct samples from the bioreactor without any treatment or cells were centrifuged off and MVs were concentrated.

Results: The amount of MVs increases during the bioprocess and indicates deteriorating conditions in bioreactor. The absolute amount of MVs in the bioreactor increased enormously during the bioprocess. Besides, the amount of MVs in comparison to cells increases obviously during the bioprocess: during the early stages of the bioprocess, the amount of MVs regarding to cells only slightly increased; but the amount of MVs in comparison to cells increased significantly at the end of the bioprocess. Results have shown that increasing MVs quantity in bioreactor is the consequence of two processes, cell activation and cell dying; however, the formation of MVs from activated cells has much weaker impact on MVs quantity in bioreactor then cell dying. The most accurate results were obtained with direct untreated samples, making monitoring of MVs quantity very quick, easy, and cheap.

Conclusions: MVs concentration reflects conditions in the bioreactor and provides new information of the bioprocess state. Therefore, we suggest MVs concentration as a new important parameter for assessing the bioprocesses.

CLINICAL TRIALS (B52 – B56)

183/B52
Development of a CLL-MRD Staining Protocol without the Use of Tandem Dyes, and Comparison with the ERIC 6-Color Protocol
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The 6-color ERIC (European Research Initiative in CLL, Rawstron et al, Leukemia (2013) 27, 142-149) protocol has recently received widespread recognition for evaluation of MRD in B-CLL patients.

As a global central laboratory facility with large experience in flow cytometry to follow up on a variety of pathologies in
longitudinal long term clinical trials studies we have some concerns that were further explored and remedied.

A first concern is the use of tandem dyes in this ERIC consensus staining protocol (especially PE-Cy7). Whereas these tandem dyes are certainly valuable in short term research studies it is our experience that the spectral properties and stability of these conjugates is inferior to pure dye conjugates making their use in long term multi-site studies unwarranted. Our alternative staining protocol using pure dye conjugates CD81FITC, CD43FITC, CD22PE, CD79BPE, CD21PerCP-Cy5.5, CD3APC, CD45A700 and CD19V450 proves to be equivalent and provides the same information.

A second concern is this 6-color ERIC protocol is based on the assumption that the markers CDS/CD20/CD43/CD81/CD79b and CD22 suffice to always make a clear distinction between normal B-cells and B-CLL B-cells. It is clear that this is NOT always the case. In our experience about 17% (53 out of 311 screened patients) of the CLL patients present with a non-compliant B-CLL immunophenotype e.g., a normal expression of CD20 or CD79b, absence of CD43, presence of CD81 (or partial) absence of CD5. The screen sample will be decisive to conclude if a given patient can be followed up for CLL MRD using this 6-color MRD protocol or not.

The authors of this report suggest looking into more robust assays where additional markers are being evaluated, especially CD23 and FMC7 are valuable additional markers that already were used decades ago (see Matutes et al, Leukemia (1994) 10, 1640 and FMC7 are valuable additional markers that already were used decades ago (see Matutes et al, Leukemia (1994) 10, 1640). One additional marker in the incubation panel can give rise to multiple additional combinations that can help in distinguishing between normal B-cells and B-CLL B-cells: adding CD23-BV421 to the ERIC panel or CD23-BV605 to our pure dye panel for instance results in 6 additional potential valid B-CLL phenotypes, CD23+CD81, CD23+CD22-, CD23+CD5+, CD23+CD43+, CD23+CD79b+, CD23+CD20dim that can help in MRD assessment in the cases where the 6-color panel fails.

The 185/B54

**Immunophenotyping the Course of Immune Response following Influenza Vaccination in Aging**

Anis Larbi, Crystal Tan, Xavier Camous
Singapore Immunology Network (Sign), Singapore, Singapore

Studies aiming at understanding the age-related immune erosion (immunosenescence) have failed to identify which component(s) of the immune system is/are showing defects. The lack of systematic analysis of the immune response’s kinetics is partly responsible for this gap. In a Phase-IV Clinical Trial using vaccination against Influenza as a readout we tested the hypothesis that several immune populations (subpopulations) are showing alterations during the course of the response and that certain cell populations may predict the later outcome (antibody generation). We have vaccinated 210 elderly individuals and collected blood samples before vaccination and 2, 7 and 28 days after vaccination. We targeted immune cells form the innate and adaptive immunity. We used multi-parametric panels to cover the array of subpopulations identified in T cells, B cells, monocyte/dendritic cells, innate lymphoid cells and progenitors. During my talk I will present data relative to the distribution of these populations in elderly individual with different health conditions and show whether immune cell distribution at baseline may be related to the immune response and how these populations are modulated during the course of the response. The extensive clinical information we obtained from the elderly group enabled us to correlate clinical status with immunological status.

186/B55

**Performance Evaluation of the BD FACScanto™ Flow Cytometer, 4-3-3 Configuration**

Yang Zeng, Farzad Oreizy, Angela Chen, Fred Mosqueda, Veronica Fraser, Laurie Byrne, Anna Lin, Kevin Judge
BD Biosciences, San Jose, CA, United States

**Background:** Multicolor flow cytometry has become an increasingly important tool in the clinical laboratory for the diagnosis and monitoring of a variety of disease states. To address market and medical needs to simultaneously detect multiple cell markers using flow cytometry, BD developed the BD FACScanto™ 4-3-3 configuration (BD FACScanto 4-3-3) to expand the capability of the BD FACScanto™ clinical instrument platform. This new cytometer is equipped with three lasers (blue, red and violet) and ten fluorescence detectors. The new BD FACScanto™ instrument provides the potential to perform 10-color biological assays (6 colors for clinical assays and 4 colors for research assays). BD clinical assays such as 4-color and 6-color TBNK with and without BD Trucount™ tubes have been released with the BD FACScanto 4-3-3 for clinical customers. To promote standardization of BD FACScanto platform instruments for biological applications, especially clinical assays, evaluation of the BD FACScanto 4-3-3 within the BD FACScanto platform is important and necessary.

**Method:** The performance of the BD FACScanto 4-3-3 was compared with other BD FACScanto platform instruments (using the BD FACScanto™II instrument as the predicate). Prospective procured whole blood specimens in BD Vacutainer® tubes were used in the study. The BD Multitest 6-color TBNK clinical reagent which determines the number of T, B, and NK cells for the normal and HIV patient populations, was evaluated for absolute cell counts and percentage relative to total lymphocytes. A total of 112 specimens were enrolled in the study, with results from 111 evaluable specimens used in the accuracy analysis. BD clinical assays such as T-, B-, and NK-subpopulations, the 95% confidence interval (CI) of the mean long term multi-site clinical trials studies we have some concerns that were further explored and remedied.

A first concern is the use of tandem dyes in this ERIC consensus staining protocol (especially PE-Cy7). Whereas these tandem dyes are certainly valuable in short term research studies it is our experience that the spectral properties and stability of these conjugates is inferior to pure dye conjugates making their use in long term multi-site studies unwarranted. Our alternative staining protocol using pure dye conjugates CD81FITC, CD43FITC, CD22PE, CD79BPE, CD21PerCP-Cy5.5, CD3APC, CD45A700 and CD19V450 proves to be equivalent and provides the same information.

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186/B55

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bias was within ±2%. For the CD16+CD56+ subset, the 95% CI of the mean bias was within ±4%. For the percent positive of CD4+, CD19+, and CD16+CD56+ relative to total lymphocytes, the 95% CI of the mean bias was within ±0.2%. For the percent positive of CD8+ and CD3+ relative to total lymphocytes, the 95% CI of the mean bias was within relative ±1.4%.

Conclusion: This study demonstrates that the bias of cell absolute count and percentage is very small when measured within the BD FACSCanto instrument platform. This is significant for clinical labs that quantitatively analyze cellular samples on different instruments in the FACSCanto platform. The BD FACSCanto 4-3-3 enables accurate clinical assay results as well as design flexibility with the potential of performing multicolor biological assays.

187/B56
Ex Vivo Immunophenotyping of Adoptively Transferred T Cells Using the SP6800 Spectral Analyzer
Laila-Aicha Hanafi¹, Andrew Berger¹, Barbara Pender¹, Abir Bhattacharyya¹, Katherine Melville¹, Tanya M. Budiaro¹, Emily Robinson¹, Brian Raden¹, Gregory Veltri², Stanley R. Riddell¹, David G. Maloney¹, Cameron J. Turtle¹
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Background: Genetic modification of T cells to express a CD19-specific chimeric antigen receptor (CAR) enables redirection of T cell specificity to CD19 expressed on B cell malignancies. We are conducting a phase III clinical trial to treat patients with CD19+ B cell malignancies with autologous CD19-specific CAR-modified T cells (CAR-T cells). Data obtained from ex vivo immunophenotyping of adoptively transferred CAR-T cells present in limited quantities of blood and other tissues will improve our understanding of the mechanisms associated with effective therapy and will be key to guiding future immunotherapeutic strategies.

Methods: The lentiviral CD19-specific CAR construct incorporates a non-functional truncated human epidermal growth factor receptor (tEGFR) that serves as a marker of transgene expression in the engineered and transferred T cells. By labeling PBMC with an anti-human EGFR antibody, cetuximab, CAR-T cells can be identified, counted and immunophenotyped by flow cytometry.

Results: Patients with ALL, NHL or CLL have been treated on the trial. We have developed multiplexed flow cytometric assays to immunophenotype adoptively transferred tEGFR+ CAR-T cells in blood, marrow and other tissues, and have determined the kinetics of in vivo expansion and persistence of transferred tEGFR+ CAR-T cells in the CD4+ and CD8+ T cell subsets. By incorporating extensive flow cytometry panels using the Sony Spectral Analyzer we are able to characterize transferred CAR-T cells in distinct memory T cell subsets and establish their activation and proliferation state, expression of chemokine receptors, and expression of regulatory and costimulatory molecules, using limited quantities of tissue. Distinct subsets of viable tEGFR+ CAR-T cells were flow sorted for gene expression and functional studies.

Conclusion: Adoptive immunotherapy with CD19 CAR-T cells of defined subset composition is a promising approach for patients with refractory B cell malignancies. The incorporation of tEGFR to identify transferred CAR-T cells and high resolution multiplexed immunophenotyping allows extensive ex vivo characterization of adoptively transferred CAR-T cells.

Computation and Informatics (B57 – B59)
188/B57
Computational Inference of Developmental Chronology Using Flow Cytometry Data
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In the bone marrow, stem cells mature into different precursor cells that further develop into different terminal cell types, and this developmental process is proceeding in a coordinated fashion. Ideally, when all intermediate cell types would be known, it would be possible to write down the sequence a cell follows from the most immature to the most mature stage according to its developmental chronology, further referred to as a trajectory. As there is still a lot of uncertainty about the developmental trajectories that cells follow, it would be interesting to construct such trajectories automatically using computational methods, thereby providing novel hypotheses about cell differentiation, and possibly revealing new intermediate cell stages.

A recent computational trajectory detection algorithm is the Wanderlust algorithm [1]. Wanderlust is a graph-based, one-dimensional trajectory detection algorithm that uses high-dimensional cell data and a user-defined early cell as input. The most restrictive assumption of this algorithm is that all cells follow a linear differentiation pathway without any branching. In this work, we present TraCy (Trajectory inference from flow Cytometry data), a new computational method to identify trajectories from high-dimensional flow cytometry data, that overcomes the major limitation of the Wanderlust algorithm, and effectively allows for trajectories to branch into d different cell types. This occurs e.g. in the lineage of hematopoietic stem cells, where the current view is a hierarchical one where early stem cells differentiate into various progenitor cells, and these again differentiate into multiple immune cell types.

TraCy allows to find trajectories with multiple endpoints in raw cell data, thereby extending the Wanderlust algorithm, and making it much more applicable to real data where branching mostly occurs. As input, TraCy expects a number of user-defined mature cell states, one for each branch in a d-branched trajectory. These cells will be used to assign all other cells to one of the d trajectory branches. TraCy will then reconstruct the developmental chronology, allowing to identify intermediate cell states.

We used both synthetic as well as real data to evaluate our algorithm. Synthetic data was created by using Bezier curves representing an artificial branching structure in a three-dimensional space. Noise was added by including seven additional dimensions randomly sampled from a Gaussian distribution. TraCy was shown to successfully reconstruct the three-branched trajectory, even in the presence of noise. In addition real flow cytometry data was used from hematopoietic stem cells differentiating into common myeloid and lymphoid progenitors. TraCy was able to detect this two-branched trajectory, thus offering great potential to extend trajectory modeling to d-branched trajectories.


189/B58
Differential Population Identification Using FlowSOM
Sofie Van Gassen¹, Tom Dhæne¹, Yvan Saey²,³
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FlowSOM is a web-based software tool for the analysis of high-dimensional cytometry data. FlowSOM allows to visualize, explore and analyze complex data sets in a guided and interactive way.

The FlowSOM algorithm is based on the single linkage agglomerative clustering algorithm. FlowSOM offers different clustering algorithms (average linkage, single linkage, complete linkage) and uses Ward’s method for the initial k-means clustering. FlowSOM provides different visualization tools for the initial and final clustering results, such as heatmaps, scatter plots and boxplots.

FlowSOM allows to visualize and explore high-dimensional cytometry data in a guided and interactive way. FlowSOM also provides different visualization tools for the initial and final clustering results, such as heatmaps, scatter plots and boxplots.
Two-dimensional scatter plots, which are traditionally used in flow cytometry analysis, offer a very limited way to explore high-dimensional datasets. New techniques such as SPADE and viSNE offer promising alternative visualizations, as they are able to show all cell populations and are thereby reducing the probability of missing interesting effects. However, these techniques suffer from some drawbacks, such as downsampling of the original dataset to keep computations feasible, and the need to compare many plots when analyzing several markers. This motivates research on new visualization methods that overcome these limitations.

Here we describe a new version of FlowSOM, a visualization tool based on Self-Organizing Maps [1], that overcomes the limitations of SPADE and viSNE and is able to directly visualize differential populations between groups of samples in one plot. Opposed to SPADE and viSNE, FlowSOM computations are orders of magnitude faster, allowing to eliminate the downsampling step and thus to visualize the whole original sample. In addition, FlowSOM makes use of star charts, which allows us to visualize multiple marker values in one plot, presenting a clear overview of all cell properties in one figure. The main feature of presenting cell populations in FlowSOM are nodes of star charts, and by adapting visual features of these nodes (e.g. size, background color, positioning) a lot of information can be conveyed in one plot, avoiding the need to scroll through many figures as is currently the case in SPADE and viSNE. In our original paper, we applied two layouts to the FlowSOM nodes: the original SOM grid and a minimal spanning tree (such as used by SPADE). We now also include a t-SNE mapping of the nodes (such as used by viSNE). Using this mapping, clusters of nodes, which typically correspond with cell populations, can be distinguished more easily. The key novelty of the extended FlowSOM algorithm is its intuitive use to find differential populations across sets of samples, allowing to detect cell populations that are strongly increased or decreased in size. Furthermore, we will also present a case study where novel changes in populations, such as shifts in marker intensities for specific subpopulations can be easily detected using FlowSOM. We will show that such shifts would probably not have been detected using the traditional analysis, and thus could give rise to unexpected, new findings.


190/B59

Accelerating Cytometry Analysis via Cell Subset Automatic Labeling and Context Identification

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Background: Recent technological innovations in single cell technologies now allow us to quantify hundreds of thousands of single cells at high dimension from a sample. This yields phenotypically rich datasets that enable a more accurate identification of cellular sub-populations. Clustering and visualization methodologies have been developed to identify meaningful cell subsets in the data. However, none provide a systematic and automatic method for labeling the clusters using all of the markers that distinguish them from all others. This is providing for each cluster a combination of markers levels that describes the cellular sub-population represented by this cluster. Instead, the identification of relevant cellular sub-populations through manual annotation remains standard practice, whereby a user needs to manually interpret and integrate the many separated plots of the different markers. This is made more difficult as single cells assigned to a cluster may not be a homogenous subpopulation in a particular dimension. The scalability and subjectivity inherent in this process slows both analysis and progress and prevents the exploitation of higher dimensionally measurements enabled by the new technologies such as mass cytometry (CyTOF) and single cell RNA-seq.

Methods: To fill this gap, we developed CAL (Cyto-Auto Labeling). CAL takes clustering results as an input and converts them to discrete representation. Then, for each cluster CAL calculates the inter-cluster distributions of each marker. Based on these calculation it automatically identifies for each cluster the combination of markers values that best describe the cellular sub-population represented by this cluster, as well as suggest removing non-homogenous clusters from consequent analyses. By using standardized terms, CAL allows to easily map cell measurements from a given experiment to literature derived information on those cells.

Results: We used CAL to automatically label intra-cellular cytokine experiments in which cells were measured by CyTOF and clustered by Citrus, a dedicated high dimensional cytometry clustering algorithm. Annotated cell subset were then automatically mapped and checked against published knowledge with regards to their ability to express or respond to a variety of cytokines.

Conclusions: Through automated annotation of cell subsets clusters and their subsequent cross-reference against the published literature, CAL enables accelerated discovery in high dimensional cytometry data.

CYTOMETRY IN RESOURCE POOR SETTINGS (B60)

191/B60

Single-Step On-Chip Differential White Blood Cell Count

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One of the most requested blood tests worldwide is the complete blood cell count, providing important clinical information to healthcare professionals. Currently, complete blood counts are performed using automated hematology analyzers in hospital laboratories, which are often not accessible in resource limited settings. To realize a simple, low-cost CD4 count we have demonstrated cell counting chambers with on-chip sample preparation, containing cell staining reagents for subsequent use in quantitative fluorescence imaging.[1] In this concept, the chamber is filled with a drop of finger prick blood by capillary force. Subsequent release of fluorescently labeled antibodies from a hydrogel ensures a homogeneous distribution of reagent in the chamber and hence, uniform cell staining. A simple large-area fluorescence imager using LED excitation and automated image analysis was used to identify and count the cells. In order to extend this concept to a portable robust, easy-to-operate, versatile complete blood cell count platform, we made the first step by demonstrating the feasibility of an on-chip differential white blood cell count.

The combined results of two counting chambers on one diagnostic chip are used to identify lymphocytes, NK cells, monocytes, neutrophils, eosinophils, and basophils. In the first chamber, lymphocytes and monocytes are counted in the image by the use of a combination of antiCD3-APC, antiCD20-APC and antiCD14-PerCP released from the hydrogel. In the second chamber, neutrophils, NK cells, eosinophils and basophils are counted using antiCD15-APC antiCD193-APC antiE-APC and antiCD16-PerCP. Differential white blood cell counts obtained using this chip format are in good agreement with flow cytometry. For confirmation, whole blood was stained with the same reagents.
and the cell populations were sorted using FACS. The sorted cell types were stained with Hemacolor and positively identified as lymphocytes, NK cells (large granular lymphocytes), monocytes, neutrophils, eosinophils, and basophils by brightfield microscopy. 


**DIAGNOSTICS (B61 – B70)**

192/B61 Detection of Donor T and B Cells Specific C4d Fixing Alloantibodies Using Flow Cytometry: A Novel Diagnostic Approach

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**Background:** Donor-specific complement-fixing alloantibody identification, specially, the deposition of C4d in the peritubular capillaries in a kidney biopsy is a valuable marker of antibody-mediated rejection (AMR). Various methods have been reported for the detection of antibodies in recipient serum, which can differentiate the all-antibodies to be HLA or non-HLA, complement-fixing or non-complement fixing, donor T and/or B cell specific. The C4d Flow-PRA is one of the screening methods to identify the HLA specific complement fixing antibodies. However, the results are limited by the lack of donor specificity in this method.

**Method:** We hereby report a novel method christened Donor-specific Flow-C4d (DFC) of identifying donor-specific (T and/or B cell), C4d-fixing allo-antibodies. Inter and intra-assay reproducibility was confirmed by repeating the tests with the stored serum of the cases. With every run, commercial and pooled positive controls were used. The tests sera were run in duplicates. The Cut-off was calculated based on a set of normal non-sensitized control samples with the value calculated as three standard deviation of the mean. The results were compared with the NIH-CDC method and C4d staining on renal biopsies. DSA were confirmed by running the recipient serum for single antigen bead based assay on luminex and subsequently performing the virtual crossmatch with the donor’s HLA antigens.

**Results:** We present a series of cases representing a variety of cases in renal transplants wherein the DFC was beneficial. First two cases were of AMR, where initially was a dilemma of AMR versus Acute cellular rejection, as result of histo-pathological finding of allograft biopsy and FCXM were not sufficient to confirm it as complement mediated rejections. The positivity on DFC method confirmed the diagnosis of AMR due to donor B cell specific, complement fixing, allo-antibodies for both of these cases. Third case was of a deferred pre-transplant donor, wherein the recipient serum was confirmed to be positive for donor T and B cell specific; complement fixing, HLA alloantibodies without using an invasive procedure of allograft biopsy. This was helpful in pre-transplant prediction of a possibility of an AMR. The last case was a clear indication of correlation of the DFC and histo-pathological findings. The patient was managed with the therapeutic protocols used for ACR.

**Conclusion:** With the added advantage of being non-invasive and Donor T and B cell specificity, this newer method provides information pre-transplant; whereas kidney biopsy based C4d evaluation can only be done post-transplant. This method also emphasize the optimal use of Flow cytometry specially in developing countries wherein Flow-cytometers are only being used for hematological disorders and unavailability of advanced technologies like Luminex. We postulate this method incorporates the best features of all the available modalities (i.e. FCXM, C4d-FlowPRA and NIH-CDC).

193/B62 NIST High Accuracy Particle Counting of Calibration Beads for ERF Value Assignments in Flow Cytometry

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**Background:** The accurate determination of antibodies bound per cell (ABC) is still a great challenge in quantitative flow cytometry (QFC). The comparability and accuracy of quantitative cytometry assays often depend on the accuracy and consistency of the ABC values obtained. In QFC, the fluorescence channels being used in an assay must be calibrated, typically using reference beads with known fluorescence intensities, before ABC values of samples can be determined. Fluorophore solutions of known concentration are used to assign fluorescence intensities to these calibration beads in units of equivalent reference fluorophores (ERF). To make this assignment, the bead concentrations of the reference bead suspensions need to be known with high accuracy.

**Methods:** Light obscuration, Coulter counter and flow cytometer measurements were used to determine the concentration of calibration bead suspensions. SRM 1934, comprised of 4 fluorescent dye solutions with known concentrations, was used to compare the fluorescence intensities of dyes in solution to those in hard-dyed calibration beads in the same spectral regions. The NIST High Accuracy Fluorescence Spectrometer was used for this comparison to measure fluorescence intensities.

**Results:** The bead concentrations in suspension, determined by three independent methods, were compared and systematic errors related with each of the three techniques were explored. The concentrations of the beads were determined with an uncertainty of less than 5%. Calibration curves of fluorescence intensity versus free-dye concentration enabled ERF assignments of calibration bead suspensions with uncertainties of about 5% or less.

**Conclusions:** NIST recently certified the concentration of four dye solutions (Coumarin 3D, APC, Fluorescein and Nile Red), which will soon be released as SRM 1934. In order to assign ERF values to bead suspensions, not only do the fluorescence intensities of free-dye solutions with known concentrations (SRM 1934) have to be measured, but the concentrations of calibration bead suspensions also have to be known with high accuracy. The highly accurate particle counting measurements reported here will improve the accuracy of ERF-based fluorescence intensity assignments of reference beads for the calibration of the FC channels centered from 425 nm to 800 nm. We foresee that this improvement in calibration accuracy of flow cytometers will lead to improved comparability of different flow cytometer platforms and accuracy in ultimate ABC determinations.

194/B63 The Increase of the Effective cdB3 Amount in Human Erythrocytes by Magnesium Sulfate Studied with Scanning Flow Cytometry

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**Background:** Currently, in prenatal diagnosis there is a problem to detect and monitor (during tocolitic treatment with magnesium sulfate) the risk of fetal hypoxia in the early stages of pregnancy. It is known that the following erythrocytes characteristics are responsible for CO2/O2 physiological exchange and, therefore, should be monitored to detect the risk of the hypoxia: the concentration, the shape, the anion permeability and the elasticity of the membrane. The determination of these characteristics with high accuracy is possible by using the scanning flow cytometer. In this work, we applied the scanning flow cytometer for the study of the effect of magnesium sulfate on the anion permeability in terms of the amount of the effective
cdB3 (i.e. band 3 proteins) anion exchangers on individual erythrocytes. In order to explain the observed increment we developed a corresponding molecular-kinetic model.

Methods: Scanning Flow Cytometer (fabricated by CytoNova Ltd., Novosibirsk, Russia, http://cyto.kinetics.nsc.ru/) was applied to determine individual characteristics of human erythrocytes. Whole blood was taken from donors (volunteers) on 30-36 week of pregnancy by venepuncture and collected in a vacuum tube containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant (9:1 blood:EDTA). Then the cells were used in experiments at room temperature (22°C) within 3 h. The protocol was approved by the institutional review board of the participating hospital. For in vivo experiments, the blood was taken once a day (at the same morning time) during 4 days of magnesium sulfate therapy (tocolytic treatment). For in vitro experiments, the blood was taken from the donors, which were not under the tocolytic treatment, and a certain (in the range MgSO₄:blood from 1:100 to 10:1) amount of MgSO₄ was added to the sample 5 minutes before the cdB3 measurements. Totally, there were 5 donors with the pathology, such as fetoplacental and placental insufficiency, which lead to the development of hypoxia. The mean effective number of ‘effective cdB3’ per erythrocyte was obtained from the rate of hemolysis in isotonic water solution of ammonium chloride.

Results: It was found that magnesium sulfate change the anion permeability of erythrocytes that was described in the developed molecular-kinetic model through the change of the effective cdB3 amount on the erythrocyte membrane. The parameters of the model, - the membrane permeability of the magnesium ion and the affinity of the magnesium ion to cdB3 inside the erythrocyte, - were evaluated from the comparison of the model with the obtained experimental data. Known literature data support the developed model.

Conclusion: We hope that our investigation will help to reduce the problems associated with preterm birth and fetal hypoxia in the prenatal diagnosis through early detection of pathologies.

195/B64 Autoimmune Cellular Immune Response Correlates with Type 1 Diabetes Severity – A Pilot Study
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Type 1 Diabetes (T1D) is an incurable autoimmune disease against pancreatic β cells, causing insufficient insulin secretion to control normal blood glucose level. Current diagnosis of T1D is rooting on insulin C-peptide release and autoantibodies against pancreatic autoantigens (such as β cell, insulin and GAD, etc.), which is sufficient to identify T1D, but poor at predicting disease severity course. Cellular immunity, cyto-toxic lytic cells (CTLs) to be specific, directly lysing and killing pancreatic cells, here is hypothesized as a straightforward tool to evaluate disease course. Through multi-functional flow cytometric analysis, in this 5-patient pilot study we monitored cytokine profiles against autoantigen epitopes secreted by CTLs in peripheral blood samples from patients with T1D, type 2 diabetes (T2D) or Latent autoimmune diabetes of adults (LADA). We found that the profile of two cytokine molecules expressed by CTLs discriminated out autoimmune patients (both T1D and LADA) from T2D controls, and more importantly, the cytokine profile showed a discrimination of T1D patients from 1 LADA patient, who is presumably in early stage of autoimmune. Therefore, we reason that our data support the notion that cellular immune response correlates with type 1 diabetes severity, and further validation in blinded trials will unveil the diagnostic value of this approach in bedside. Once validated, the test may also be practically used to screen LADA patients in periodic physical examinations.

196/B65 Characterization of Main Physical and Physicochemical Parameters of Polymer Microparticles Using the Scanning Flow Cytometry
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Background: Polymer particles of different composition are widely used in many areas of medical, biological, chemical and physical studies both as carriers of biological molecules and as independent objects of observation. The most promising applications areas for particles are in theranostics, in targeted drug delivery, in vivo optical imaging, and in bioanalysis and diagnostics. Some of the applications (e.g., immuno-diagnostics, targeted drug delivery, or specific labels in electron microscopy) require the ability of particles to bind specifically to the protein or polysaccharide ligands on the cell surface. The key physical and physico-chemical characteristics of the polymer particles, which determine the possibility of their use in various applications, are the size, the refractive index and the surface charge density. This work is devoted to the construction method for estimating the basic parameters of polymer microparticles using the Scanning Flow Cytometer (SFC).

Methods: We studied angle-resolved intensity light scattering patterns (LSPs) of individual particles of different composition with the Scanning Flow Cytometer fabricated by CytoNova Ltd. (Novosibirsk, Russia, http://cyto.kinetics.nsc.ru/). With the use of Mie theory we solved the inverse light scattering problem by approximating the experimental LSPs with the theoretical ones using the DiRect method of global optimization. During this procedure particle sizes and the refractive indices were determined with high precision.

The initial stages of aggregation of polymer microparticles in solutions with different sodium chloride concentrations were studied in the series of the experiments by means of the scanning flow cytometry. Using the LSPs we measured the relative fractions of aggregates composed of different number of monomers.

Results: Based on the Smoluchowski equation we theoretically predicted and experimentally demonstrated the linear increase in the ratio of the total number of aggregates to the number of monomers in time in a wide range of the monomers conversion degree and the fractal dimension of particle clusters. Using the slope of the ratio in time we determined the dimerization rate constants for all experimental sets. Using the Fuchs formula in solving the inverse problem we estimated the surface charge densities of the particles. The values obtained were in good agreement with data in literature.

Conclusions: We present a new method of quantitative determination of main physical and physicochemical parameters of polymer microparticles using the scanning flow cytometry. The proposed technique allows us to evaluate the size, the refractive index and the surface charge density of beads of different composition and surface modifications.

197/B66 Eyelis™ Software-Assisted Analysis of Stem/Progenitor Cell Types Detected by the SCALPEL™ Assay in a Blood Sample with Relapsed Chronic Myeloid Leukemia
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Cell subpopulations in heterogeneous cellular mixtures are typically defined by cell surface antigen expression profile. In flow cytometry (FCM)-based diagnostics, leukocyte subtyping is achieved by labeling the sample with the appropriate set of antibodies to distinguish cell types by their lineage and maturation status. Neoplastic populations are identified by clonal
expansion of cells defined by antigenic aberrances when compared to their normal counterparts. Despite multi-parameter capabilities of FCM, a general lack of the ability to measure functional attributes precludes identification of cells based on their oncogenic potential and deregulated proliferative pathways. Inclusion of markers of signaling activities in a multiplexed cytometry assay has the potential to more precisely identify neoplastic subsets, particularly when differences in the static antigens compared to normal cells are minimal for the markers tested. We demonstrated lymphoid progenitor cells (PCs) in the peripheral blood of a patient with early chronic myeloid leukemia (CML) relapse and a rising BCR-ABL1 transcript (0.285), due to treatment non-adherence. Our novel SCALPEL™ (Single Cell Analysis of Limited Protein Expression Levels) assay using a panel of surface and IC markers identified 3 proliferative stem/progenitor cell types defined by correlatively high expression level of functional markers in activated signaling cascades. However, classification of the cells by lineage and maturation markers was confounded by rarity of these cell types (altogether comprising <1% of analyzed leukocytes) and cells in transition expressing mixed phenotype. Visual displays using high dimensional clustering algorithms lacked the ability to identify and describe pure subpopulations. Thus, Eyelis™ algorithms were developed applying Gaussian Mixture Model and non-parametric Bayesian statistics, rendering an automated method for cell classification. The automated classification results corroborated with manual sequential gating results. Differential expression analysis of lineage, maturation and signaling markers was performed to precisely assess the degree of phenotypic overlap between the lymphoid PCs. Elevated signaling activity levels of markers downstream of BCR-ABL1 kinase suggested the 3 phospho-STAT5a cell types were part of the CML clone. Clonal relatedness of the CD19+ and/or CD3+ PCs and a CD34+/CD117+ subset (0.06%) was postulated, in part due to the presence of transitional cells and resemblance in the signaling activity profiles. Differentiating aspects of the CD34+ subpopulation compared to the lymphoid PCs included lower (-0.5X) phospho-p38 MAPK level. It is important to distinguish the cell types that contribute to the BCR-ABL1 molecular transcript level, given that the CD3+ PCs lack oncogenic potential. The CML SCALPEL™ assay as a novel cell-based diagnostic test for minimal residual disease assessment in conjunction with molecular monitoring will be discussed.

198/B67 Fast Antimicrobial Susceptibility Testing Using Flow Cytometry
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Introduction: Antimicrobial resistance is considered a global threat by the World Health Organization. Worldwide alerts and restrictive antimicrobial policies have been implemented in order to preserve antibiotic efficacy. In case of a severe infection, currently empiric therapy is immediately started once a susceptibility profile takes at least 48 hours. Most antimicrobial susceptibility tests (ASTs) are based on the study of growth ability in the presence of several antimicrobial drugs, i.e. resistance assays. However, even fast growth microorganisms takes time to replicate. Molecular methods and more recently mass spectrometry represented a revolution on microbial identification which differs for different microbe types.

Methods: EDTA, citrate as well as heparin anti-coagulated whole blood was drawn from healthy individuals (n=7 for each anti-coagulant) and was processed immediately or stored for 4h, 8h, 24h and 48h at 4°C and room temperature (RT). Whole blood was stained with three different pre-cocktailed DuraClone dry reagents. In influence of Storage Time, Temperature and Anticoagulants on Leukocyte Antigen-Expression and Survival from Human Peripheral Whole Blood Using DuraClone Dry Reagents
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Background: Immune phenotyping in whole blood samples is a crucial diagnostic factor in the daily work routine. Due to various logistic and organizational issues blood samples are often stored for up to two days or even longer under different temperature conditions. The aim of the present study was to investigate the influence of storage time, temperature and anticoagulants on leukocyte antigen expression from human peripheral whole blood samples utilizing flow cytometry. For this purpose we here performed a standardized staining procedure with various panels of pre-cocktailed DuraClone dry reagents.

Results: At 4°C Zombie™ cells significantly increased in heparinized blood from 4 hours to 48 hours, whereas samples anticoagulated with citrate and EDTA remained stable up to 24 hours. In contrast at 21°C dead cells in EDTA blood significantly increased compared to 4°C. CD3+/Zombie™ cells increased significantly over time for EDTA (p=0.004) as well as for citrate (p=0.018) and heparin (p=0.006). Using DuraClone at 4°C and at RT the percentage of NK cells increased in citrate > EDTA > heparin blood; in citrate blood the percentage of CD3+/CD4+ decreased (p=0.003) but the percentage of CD3+/CD8- cells increased (p=0.034). Under both

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temperature conditions there was a significant increase of CD14+CD16+ monocytes in heparin samples compared to EDTA and citrate samples. Interestingly no significant changes could be observed in B-cell distribution between anticoagulants.

**Conclusions:** Our data provide changes of leucocyte antigen expression which might be a crucial factor during daily work routine when blood is stored over longer time periods and under different temperature conditions. Minimal changes were detected when heparinized blood was stored at 21°C or when anticoagulated EDTA and citrate blood was stored at 4°C.

200/B69

**Changes of Microvesicle Distribution in Whole Blood in Dependence of Storage Time, Temperature and Anticoagulants**

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**Background:** Microvesicles (MV) are cell-derived vesicles in the size of 100nm up to 1000nm. They are produced by a variety of cells into body fluids to interfere in coagulation, inflammation, communication and transport. It is known that the storage and preparation of plasma samples significantly influence the analysis of MVs. Thus, this study evaluated the microparticle release in whole blood samples under the influence of different anticoagulants, storage time and various temperature conditions.

**Methods:** Samples were collected from healthy probands (n=7 for each anti-coagulant) and subsequently stored for 0, 4, 8, 24 and 48 hours at room temperature (RT) or 4°C. Samples were centrifuged in two steps to harvest platelet-free plasma (PFP) which was immediately stored at -80°C until analysis. To identify MVs, PFP was stained with Annexin V (Pacific Blue) and calcein (FITC channel). To detect MV subpopulations, PFP was stained for MVs from red blood cell origin with an anti-CD235a (APC Alexa Fluor 750), for platelet derived MVs with an anti-CD41 (APC) and for myelomonocytic cell (MMV) source with an anti-CD15 (Krome Orange). Measurement was performed on a CytoFLEX (Beckman Coulter) flow cytometer. In addition MVs were quantified with TruCount beads to determine their absolute number. To show the procoagulatory effect of the PFP, a tissue factor activity (TF-MAV) assay was performed.

**Results:** Without prior storage, sodium citrate showed the lowest MV count compared to heparin and EDTA. Interestingly, EDTA showed a significant release of MMVs compared to sodium citrate. In addition, sodium citrate and heparin showed a stable MV count at RT or 4°C in the first 8 hours after blood collection. Overall, the MV count massively increased after 24 hours of storage independent of storage condition or anticoagulant which was related to all subpopulations. In addition, the procoagulatory (TF-MV) potential increased significantly after 8 hour storage.

**Conclusion:** Based on both, this work and literature data, sodium citrate seems to be the best working anticoagulants to analyze MV count and function. Motions less storage did not significantly influence MV count for 8 hours indicating a potential longer storage time prior to analysis. Large-scale studies are needed to evaluate the precise influence of storage time on MV count and, even more interesting, on MV subpopulations.

201/B70

**CD26 Expression in Patients with Mycosis Fungoides**

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**Background:** Mycosis fungoides (MF) is a primary cutaneous lymphoma presenting with typical pruritic erythematous lesions and characterized by the neoplastic T-lymphocytes. As were reported earlier, the phenotypically abnormal T-lymphocytes populations were identified as CD26- or CD7- T-lymphocytes with dim CD3+ and CD4+ populations (Hristov et al., 2011). CD26 is a surface enzyme that is expressed in the majority of lymphocytes in peripheral blood. (Jones et al., 2001). Absent or dim CD26 expression is a feature of almost all cases of MF and can be used to allow more sensitive flow cytometric detection and quantitation of tumor cells, than the use of CD7. We observed the CD26 expression on CD3+ lymphocytes in patients with MF diagnosis and healthy controls in our study.

**Methods:** By using flow cytometry we analyzed the blood samples of 15 patients with Mycosis fungoides. Cell surface was stained with fluorescence labeled monoclonal antibodies (anti-CD7, CD26, CD4, CD8, CD3 and CD45). Flow cytometric acquisition was performed on a FACS Canto II flow cytometer (Becton Dickinson, NJ, USA).

**Results:** Significant differences were found in expression of CD26 and CD7 on T-lymphocytes (p<0.05) as compared to healthy controls. The CD4+/CD8+ ratio (Immunoregulation index) was observed. However, only one patient had the IRI under the physiological level.

**Conclusions:** The aim of this study was to analyze absence of the marker CD26 on the T-lymphocytes in patients with Mycosis fungoides diagnosis. So far, the specific criteria for the diagnosis of Mycosis fungoides haven’t been defined. Despite this fact, some markers as well CD26 can be useful in the diagnostics of this disease. Nevertheless, is necessary to find a specific marker for an accurate diagnostics of the Mycosis fungoides.

**Disease Progression Monitoring (B71 – B77)**

202/B71

**Comparative Study of Bcl2 and CD105 in Radicular Cysts, Dentigerous Cysts and Odontogenic Keratocysts, Using Immunohistochemistry**

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**Background:** Angiogenesis and apoptosis are two main processes controlling lesions formation. The aim of this study was to compare the expression of Bcl2 as a marker involved in apoptosis and CD105 as a marker for MVC evaluation, in radicular cysts (RCs), dentigerous cysts (DCs) and odontogenic keratocysts (OKCs) by using immunohistochemical methods (IHC).

**Methods and Material:** This cross-sectional study was done in 2014. A total number of 45 biopsy samples, embedded in paraffin consisting of 15 cases of OKC, DC and RC and were collected from the archive of the Oral and Maxillofacial Pathology department of Dental school, Babol University of Medical Sciences from 2004 to 2014. All specimens had previously been fixed in 10% neutral buffer formalin solution, dehydrated by graded alcohol, and embedded in paraffin. Hematoxylin and Eosin staining were performed to confirm the diagnosis, followed by IHC using bcl-2 and CD-105 antibodies. A labeling index (LI),
which expresses the percentage of bcl2-stained cells in the basal layer, was calculated for the analysis of bcl2 expression. CD105 immunostaining was analyzed by counting the number of positive blood vessels with luminal space or a single endothelial cell.

Findings: Bcl-2 expression in odontogenic keratocysts was significantly higher than radicular and dentigerous cysts (p<0.05). There was no significant differences between radicular cysts and dentigerous cysts (p=0.694). The highest percentage of CD105-positive vessels was seen in radicular cysts. There were significant differences in MVC between groups (P<0.05).

Conclusions: According to the findings of this study, it may be possible to correlate the different biologic behavior of OKC to bcl-2 expression. This may explain its aggressive behavior compared to radicular and dentigerous cysts. The same rule was not found for CD 105.

Key words: Bcl2, CD105, Radicular cyst, Dentigerous cyst, Odontogenic keratocyst

203/B72
Flow Cytometry Analysis of Superoxide Production in Leucocytes from Patients with Advanced Hepatocellular Carcinoma

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Background: Hepatocellular carcinoma (HCC) is one of the most common lethal malignancies, accounting approximately for 626,000 new cases per year worldwide. Nowadays, one of the challenges in the HCC is to identify biomarkers capable of predicting prognosis and response to treatment. The aim of our study was to evaluate possible variations in intracellular and mitochondrial superoxide production in leucocytes from advanced HCC patients.

Methods: Venous blood samples from 8 untreated patients with advanced HCC and liver cirrhosis (Child-Pugh A) and 8 patients with liver cirrhosis (Child-Pugh A) were collected to determine intracellular and mitochondrial superoxide levels. Leucocytes were isolated from freshly obtained blood by FICOLL density gradient and incubated with hydroethidine (0.5 μg/ml for 20 min), an intracellular superoxide-specific probe, or MitoSOX (1.25 μM for 20 min), a red mitochondrial superoxide indicator. The leucocytes were analyzed by a FACSVerse flow cytometer using the FACS Suite flow cytometry software (Becton Dickinson, San Jose, CA, USA). For each sample about 60,000 events were acquired and the fluorescence was measured on FL2-A channel. The mean fluorescence intensity indicates superoxide levels. Student’s t-tests were performed to determine differences between samples. A value of P<0.05 was considered statistically significant.

Results: A decreased intracellular superoxide production was observed in leucocytes from advanced HCC patients compared to patients without HCC (1,739±105 versus 3,145±443 fluorescence arbitrary units (FAU), P<0.05, respectively). The decreased production of intracellular superoxide was observed in monocytes, neutrophils and lymphocytes. Analysis of mitochondrial superoxide formation with MitoSOX showed an increased (P<0.05) production of mitochondrial superoxide in leucocytes from HCC patients that was measured in neutrophils (2,667±407 vs 1,266±84 FAU, P<0.05) and monocytes (3,807±514 vs 2,762±195 FAU, P<0.05) but not in lymphocytes (1,827±231 vs 1,977±160 FAU, P>0.05).

Conclusions: The results demonstrate a reverse effect of HCC on intracellular and mitochondrial superoxide levels. Leucocytes from patients with advanced HCC showed a decrease in intracellular superoxide and an increase in mitochondrial superoxide. These changes in superoxide production in HCC patients could modulate immune responses through effects on signal transduction cascades. The determination of superoxide levels in leucocytes with FACS analysis might have value in the prediction of response to treatment in HCC patients.

204/B73
Tumor-Associated Macrophages (TAMS) Are Associated with the Epithelial-to-Mesenchymal Transition in Primary Breast Cancer

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Transition in Primary Breast Cancer

The current understanding of cancer invasive potential is that epithelial cancer cells must change their shape and lose adherence to gain motility. This process, termed the epithelial-mesenchymal transition (EMT), confers on cancer cells a series of mesenchymal traits and the ability to enter into the cancer stem cell (CSC) state, with self-renewal and self-protection as the key characteristics. We have shown that tumor stem-like EMT cells, physically located at the invasive front of primary breast cancer, have been linked to tumor metastasis. Further, we demonstrated that these cells interact with tumor-associated macrophages (TAM) to create a CSC-niche, amplify EMT and promote an invasive tumor type. Here we demonstrate that the CSC-TAM interaction can be detected in primary breast cancer at the time of diagnosis and that TAMs are exclusively of M2 polarization and we corroborate our findings in that TAM associate with CD90+ CSCs.

The expression of Cytokeratin, EpCAM, E-cadherin, Vimentin, CD73, CD44 and CD90 was determined by multiparameter flow cytometry on single cell digests from primary and metastatic tumors. Immunofluorescent staining (CD68, CD163, HLA-DR, Cytokeratin, Vimentin, E-cadherin, CD90) was performed on serial paraffin embedded sections from normal (n=19) and adenocarcinomatous tissues (n=25) and was used to identify, to enumerate and to localize CD68+TAMs, CD90+cytokeratin+stromal cells and CD90+cytokeratin+ tumor subpopulations.

In adjacent normal breast ductal tissue, CD90+/CTK+ cells were absent or rare and tissue macrophages were solitary and localized near microvasculature. In ductal carcinoma in situ (DCIS), a non-invasive carcinoma, CD90+/CTK+/Vimentin+ cells were rare, TAMs were present but did not invade tumor cell clusters. In invasive adenocarcinoma, TAMs exhibited an M2 polarization (CD68+HLADR-CD163+), localized to the interface between CD90+Vimentin+CTK+dTNM stem like tumor cells and CD90+stromal cells. The proportion of TAMs positively correlated with the abundance of CD90+CTK+ cells, further supporting the role of TAM in EMT. The juxtaposition of TAMs with luminal space or a single endothelial cell.
205/B74
Mitochondrial Destabilization and Abrupt ROS Production Linked to TAZZ Gene Mutation in the Barth Syndrome Enhance Autophagic Fluxes
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Mutations in the gene that encodes the monolysom-transaclyase, TAZ, leads to Barth syndrome. Individuals affected by this X-linked multi-system disorder present with cardiomyopathy, skeletal muscle weakness, neutropenia, growth retardation and methyl glutaconic aciduria (3-MGA). The previous research on various species models or cells derived from the Barth syndrome have established deep roots laying on top of biochemical characterization of the cellular defects associated to TAZ mutations. Biopsies from the heart, liver and skeletal muscle of patients exhibited malformed mitochondria and dysfunctional mitochondria.

As previously described with patient-derived lymphoblasts, we showed in patient derived fibroblasts that the production of abnormal cardiolipin led to mitochondrial alterations. Indeed, the lack of normal cardiolipin led to changes in electron transport chain stability, resulting in cellular defects. We found a destabilization of the supercomplex (respirasome) 1 + II + IIV and also decreased amounts of individual complexes I and IV and supercomplexes I + III and IV. No changes were observed in the amounts of individual complex III and complex II. We also found decreased levels of complex V. However, these alterations were compensated by an increase in mitochondrial mass, as demonstrated by electron microscopy and measurements of citrate synthase activity. We suggest that this compensatory increase in mitochondrial content prevents a decrease in mitochondrial respiration and ATP synthesis in the cells.

Remarkably, and curiously also, basal levels of superoxide anion production were slightly higher in patients’ cells than in control cells as previously evidenced via an increased protein carbonylation in the taz1delta mutant in the yeast. This ROS aberrant ROS production leads to an increase in autophagic fluxes which primarily discarbed unwanted mitochondria. This may be deleterious to cells in the long term. This results are discussed in light of recent results linking enhanced ROS production and sarcomeric dysorganization in BTHS-iPSCs generated from dermal fibroblasts or BTHS iPSC-CMs where ROS production is linked to sparse and irregular sarcomeres, and in which engineered BTHS “heart-on-chip” tissues contracted weakly. The consequences of mitochondrial dysfunction, enhanced ROS production, dysregulation of autophagy and alteration of apoptotic signal transduction are considered in light of the potential for the development of future treatments.


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206/B75
FlowScript™ Platform Detects Genotypic and Phenotypic Markers Associated with HPV Infection and Cancer Progression
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Introduction: Enzo’s newest flow cytometry platform, FlowScript™, acts as “a snapshot cell query” by allowing the analysis of mRNA transcript expression in individual cells in a mixed cell population. By studying whether a gene or a set of genes is turned on or off, it is possible to obtain clinically relevant information at the single cell level. The first product developed for use with this technology is the FlowScript™ HPV E6/E7 Assay. Integration and ultimately overexpression of HPV oncoproteins, E6 and E7, promotes the growth of malignant cells through the inhibition of tumor suppressors and has been linked with increased likelihood of cervical cancer progression.

Methods: FlowScript™ HPV E6/E7 Assay Kit (ENZ-GEN300) is a flow cytometry-based, molecular detection system which allows for the multiplex analysis of cell function and identity. The assay employs a novel in situ hybridization technique utilizing a cocktail of oligonucleotide probes specific to multiple targets within high risk HPV E6/E7 genes, ensuring detection of the most prevalent HPV species. The results are expressed as the percentage of cells in the analysis gate that over-express mRNA for the two oncoproteins. FlowScript HPV Positive and Negative Control Cells are concurrently processed and analyzed during the assay workflow and act as an external control, confirming E6/E7 probe functionality. To assess the clinical application of the FlowScript HPV assay, a validation study of over 200 ThinPrep and SurePath clinical specimens was performed by Enzo Clinical Labs.

Results: The analytical sensitivity was verified by a dilution series of positive and negative control cells and was detectable down to 0.45% positivity. Precision of the assay was equally notable with an overall reproducibility of 100% and inter-assay CV of 98.2-27.7%. Among the 202 cases analyzed by the FlowScript assay, 46 biopsy cases were also evaluated. Findings support the claim that E6/E7 testing on cytology samples is a useful discriminating test as there was a strong negative predictive value for ASCUS and LSIL cytology results when correlated to biopsies.

Conclusion: The validation study from Enzo Clinical Labs suggests this assay can be used to help guide providers in assessing the risk of progression in cervical cancer. E6/E7 results may help to guide whether colposcopy or follow-up screening can be the preferred course of action. In addition to HPV, the FlowScript™ platform is anticipated to be further utilized for applications related to cancer, immune-mediated disorders, patient monitoring and drug development. With novel features including: a homogeneous assay design, internal and external controls and ability to be multiplexed with specific dyes for analysis of cell function, the FlowScript™ assay is an user-friendly, simple-to-implement method for gathering information on genotypic and phenotypic changes occurring at the single cell level.

207/B76
Minimal Residual Disease Detection in Patients with Mantle Cell Lymphoma: A Comparative Analysis of Flow Cytometry and RQ-PCR Approach
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Background: The monitoring of minimal residual disease (MRD) represents an essential tool for therapy response assessment in patients with hematologic malignancy, including mantle cell lymphoma (MCL). Real-time quantitative PCR (RQ-PCR) is the most sensitive and broadly applied method for MRD assessment in MCL reaching the sensitivity up to \( \leq 10^{-4} \). The aim of our study was to compare molecular analysis of MRD detection in MCL to 8-colour flow cytometry single tube (8-FC) approach.

Methods: Peripheral blood (PB) and bone marrow (BM) samples of continuously observed cohort of 63 patients after or during therapy were included in the study. MRD levels were analyzed employing 8-FC protocol combining anti-CD3, 5, 19, 20, 23, 45, 62L and 200 monoclonal antibody. The 8-FC MRD protocol was previously designed according to mean fluorescence intensity (MFI) of CD surface markers that were able to separate MCL cells
from normal B lymphocytes (Chovancova et al., 2015). Molecular monitoring of MRD by RQ-PCR was performed on PB and BM DNA samples employing bcl-1/IgH translocation or clonal rearrangement of IgH genes.

**Results:** Concordant results were obtained in 77.5% of the samples; 21.6% of the samples were found RQ-PCR positive but 8-FC negative. Moreover, simultaneous observation showed a good correlation (r=0.71, p<0.01) between both approaches.

**Conclusions:** Despite lower sensitivity, flow cytometry technique appears to be an adequate method for MRD detection compared to ordinarily applied molecular biology approaches. Noticeable advantage of 8-FC consists in rapid sample processing and lower financial demands.

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**208/B77 Automated Discovery of Rare Cell Subsets: Identification of Leukemic Cells as Minimal Residual Disease**

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**Background:** Flow cytometry is an excellent tool in the study of cancers of the blood and related tissues. Clinical analysis methods exist for use in diagnosis, efficacy monitoring during treatment, and remission/relapse monitoring. These methods have acceptance but also have shortcomings, one of which is a reliance on expert manual analysis, which can have researcher-specific bias resulting in misidentified or unidentified cells. In addition, manual analysis is difficult to scale and standardize for clinical tests. Even subtle shortcomings in analysis can affect a clinical outcome, especially in the case of monitoring for minimal residual disease (MRD), where very small numbers of cells can be significant (e.g., < 1%). For these reasons, computational methods to assist or automate the detection and classification of aberrant cells in the blood and other tissues will be beneficial to clinical applications of flow and mass cytometry.

**Methods:** Using publicly available 35-parameter CyTOF-based clinical data from Amir 2013 (Nature Biotechnology), we computationally combine a sample of leukemic bone marrow at 0.2% to a sample of otherwise healthy marrow to mimic an MRD condition. We then seek to capture the aberrant cells from the healthy background without prior knowledge of significant biomarkers or type of cancer, and then characterize the significant biomarkers of the cancer cells. Three computational workflows of varying degrees of automation are used in order to reach this goal: 1) dimensionality reduction using vSNE, 2) vSNE followed by clustering via SPADE, and 3) automatic identification of differential biomarkers that separate cancer cells from healthy, using a logit LASSO-based classifier.

**Results:** Using vSNE alone we reproducibly identify the rare subset of leukemic blasts with complete separation from the healthy background. Extending this analysis to clustering by SPADE resulted in output of a targeted cluster of cells of interest that was confirmed to match the known aberrant cells, shortcircuiting their path to discovery. Using the LASSO-based classifier with no prior knowledge of biomarker profile, we were able to automatically identify CD10 and CD34 as the strongest markers that distinguish ALL blasts from healthy cells. 100% of the expected aberrant cells were recovered and no cells were found to be misidentified when their location was unblinded after analysis.

**Conclusion:** We showed that automated methods can be leveraged to reliably identify rare aberrant cells from healthy marrow without previous knowledge of phenotype, a technique of interest for analysis of MRD. In addition, we present an approach for automated identification of biomarkers associated with the aberrant cell type. The benefits of adopting more automated methods into standard workflows are discussed in order to mitigate bias and bring more objectivity to clinical cytometry analysis.

**FACILITY MANAGEMENT (B78 -- B82)**

**209/B78 Human Immunophenotyping Service in the BRC Cell Phenotyping Hub**

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The Cambridge Biomedical Research Centre Cell Phenotyping Hub is a shared resource laboratory (SRL) providing analytical, educational and technical services in the broad range of cytomics (e.g. cytometry, confocal imaging, cell separation and purification, analysis). It is equipped with state-of-the-art technology, including three high-end sorters enclosed in Class 2 cabinets, one high-speed cell sorter for processing of Class 1 samples, five analysers, two confocal and one fluorescent microscope, AutoMACS and other accessory kits.

Historically, the services offered by the Hub focused on training researchers to equip them with all the skills required for self-operation of the equipment and processing of their samples. In that context, the Hub did not provide a tailored service for those groups who would like to use cytomics as a research tool but were not able to invest the large amounts of time, manpower and resources required to realise research projects heavily dependent on flow cytometry (e.g. human phenotyping).

Therefore this year the Hub has established a new phenotyping service, officially launched in August 2014. We have created and optimised 6 standardised panels, including standard T cell, regulatory T cell, Th17, Th, B cell and myeloid panels, which are compatible with all our major equipment ( analysers, sorters and AutoMACS ) and can be offered to groups and individuals requiring a phenotyping service. These panels also offer a modest potential for expansion in terms of ‘free channels’ for specific markers required for additional research questions. The phenotyping service covers initial research advice, further panel modification and optimisation, sample processing, sample staining, MACS separation and/or FACS purification. We operate a flexible approach depending on the various needs of our collaborators: one can either choose one of our specialised ‘all inclusive’ standard immunophenotyping packages with required individual add-ons, or go for just one or several of the customised services we offer.

Despite the fact that the Immunophenotyping service was launched only half a year ago, it has already resulted in several very successful collaborations, involving three clinical trials along with one-off phenotyping inputs into entry-level pilot projects run by researchers with little or no experience in Flow Cytometry.

We believe that implementing new ideas in the supportive collaborative culture of the Phenotyping Hub will allow us to evolve a new range of services to meet current demands of modern research.

**210/B79 ISAC Shared Resource Laboratory Emerging Leaders Program**

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1Flow Cytometry, Garvan Institute of Medical Research, Darlinghurst, Australia, 2Newcastle University Centre for Life, London Research Institute, Newcastle-upon-Tyne, United Kingdom, 3OCS Cytometry Core, NYULMC, New York, NY, United States, 4Flow Cytometry, Babraham Institute, Cambridge, United Kingdom

**Stanford University, Stanford, CA, United States**

Using viSNE alone we reproducibly identify the rare aberrant cells, and that was confirmed to match the known aberrant cells, shortcircuiting their path to discovery. Using the LASSO-based classifier, we were able to automatically identify CD10 and CD34 as the strongest markers that distinguish ALL blasts from healthy cells. 100% of the expected aberrant cells were recovered and no cells were found to be misidentified when their location was unblinded after analysis.
In 2014 the International Society for the Advancement of Cytometry appointed the inaugural Shared Resource Laboratory (SRL) Emerging Leaders to three of its younger members. The program is designed to enhance and develop the next generation of emerging leaders in SRL operations as well as furthering the field of cytometry.

This five-year program provides ISAC SRL Emerging Leaders with a complimentary membership in the Society, subscription to Cytometry Part A, and complimentary registration and partial travel funds to attend the Society's annual conference CYTO as well as eligibility for one-time funding of mentored training. In addition, ISAC SRL Emerging Leaders are given the opportunity to be an integral part of the Society through committee memberships, advisory boards, and creating educational material to further support the goals of ISAC.

The SRL Emerging Leaders Program is an initiative of the ISAC council and the awards for 2014-2018 SRL Emerging Leaders were given to Andy Filby (UK), Michael Gregory (USA) and Robert Salomon (AUS). We would like to take this opportunity to introduce the current ISAC SRL Emerging Leaders, which include Rachael Walker (UK) who transferred from the ISAC Scholars Program, and to outline the projects that will be undertaken during the program.

The SRL Emerging Leader program is running again in 2015 and we hope for the foreseeable future, for more information please contact any member of SRL Emerging Leader program.

211/B80
Year One of CyTOF in a Core Facility Setting: Considerations and Concerns
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Introduction: In 2014, the Stanford Shared FACS Facility (SSFF) at Stanford University (California, USA) added CyTOF to its suite of common core instruments. CyTOF is a new generation of cell analyzer that combines single-cell interrogation common to traditional flow cytometry with the ability to resolve metal probes with minimal signal overlap using atomic mass spectroscopy. Its addition proved to be an excellent complement to existing FACS (Fluorescence Assisted Cell Sorting)-based technology available to current SSFF clientele offering researchers already familiar with flow cytometric-based experimental design an extended range of targets (upwards of 30-40 probes) for single cell analysis. Still, making CyTOF a convenient, cost-effective option for a core facility is not an easy task.

Method and Results: Here, we’d like to share our experiences from our first year of CyTOF operations with an eye on 1) physical and abstract logistics, 2) core expectations and outcomes, 3) breadth and depth of use, and, ultimately, 4) time and cost considerations.

1. Preparing a facility for CyTOF raises a variety of logistical questions quite different from those commonly encountered with FACS instrumentation. These questions range from prepping and planning physical space for mass spectroscopy to deciding how far responsibilities for CyTOF related experimental components (sample prep, instrument operations, etc.) extend to a core facility.

2. Adapting CyTOF to a core community requires the development of an operations scheme to address a vast range of laboratory disciplines, objectives and, even, levels of etiquette. The development of robust SOPs and strict training regimens can help alleviate many of these concerns.

3. Encouraging use of CyTOF from the broad scientific community requires a concerted effort to educate and assist new labs in its implementation. Especially in a setting previously without CyTOF, one can expect a long, steep learning curve for researchers and operators alike. Leaning heavily on other institutes for ‘lesson learned’ scenarios can ease this process significantly. Thus far, we’ve extended use beyond CyTOFs traditional clientele making inroads into the environmental sciences and Bioengineering.

4. Investing in CyTOF is an expensive proposition both in terms of time commitment and general maintenance and expenses. The addition of CyTOF to an existing core can provide a financial buffer for its implementation especially when building a clientele. Dedicating personnel to its maintenance and oversight is crucial to instilling confidence in the technology and keeping CyTOF in working order.

Conclusion: This presentation will review how we at the Stanford Shared FACS facility tackled these hurdles offering useful insights for the addition of CyTOF to your core capabilities.

212/B81
Pharma Flow: Organized Global Core Harmonization Efforts Improve Access to Technology and Improve Data Generation across a Large Organization

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Flow Cytometry is a dynamic and continually evolving technology. Cores are often formed to support the education and training of researchers to new methods and instrumentation, while also providing instrument troubleshooting and maintenance, scheduling, billing, and operation services. In our Biopharmaceutical flow cytometry core we are challenged with providing basic services and support for the use of the technology not only to our site, but also across global sites where often there are no cytometry Core support staff. To do this we have established a global core strategy. We believe the keystone to such a global core begins with a universal and robust website. A unified and single site for our company is essential as a primary hub for sharing instrumentation capabilities, protocols, training, scheduling, and core-staff activities. The training resources and modules enable and ensure on-demand support for users across time zones. Furthermore, advocacy for the use of standard instrumentation promotes the co-development and sharing of optimized multi-parametric protocols, which can be employed universally. Those standard protocols will help ensure the generation of uniform high quality data. Furthermore, consistent data analysis and management tools need to be built and supported which make the utility of large data sets possible. These basic business support tasks are not unique to our core, but something each core must provide to its users and institution. We suggest that an ISAC-sponsored website could be useful in promoting universal basic laboratory management and technology links that would help facilitate the advancement of Cytometry.

213/B82
Training Tool for Flow Analyser Users in a Cytometry Core Facility

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Background: As part of the core facility effort to educate our users in the use of the flow cytometer, the facility has developed a training tool for the flow analysers. The training tool comprises information about how a flow cytometer works, the optics configuration of the flow analysers and most importantly an easy to use compensation chart. The compensation chart is drawn out based on the optics configuration and the laser power used on the
flow analysers. This compensation chart has formed part of a useful tool for our in-house flow analyser training programme.

Methods: In this experiment, Anti-mouse Ig, K/Negative Control BD compensation particles set of 7-7.5 pm in size (cat no: 560497) is used. The particles are labelled with antibody conjugated to a particular fluorochrome as described in the supplier reagents technical data sheet. A total of 17 different fluorochromes each conjugated to an antibody and a DNA chelating dye; DAPI stained on a cell line, are chosen based on the facility flow analyser optics configuration (Table 1). The spectral information for each of the fluorochromes used in this experiment was obtained from the spectral viewer available on the website. The data was acquired on a BD LSR Fortessa flow analyser and analysed using FACS DIVA v.8.0 software. A total of 5000 events was collected and displayed on a series of bivariate plot of one fluorescence channel versus another fluorescence channel. The number of possible two-colour combination plot was calculated using the formula \[ N(N-1)/2 \].

Results: The fluorescence spillover between each detection channel is investigated and corrected using the auto-compensation function of BD FACSDIVA software. The corrected compensated values are tabulated as shown in the compensation chart (Table 2).

Conclusions: We have found that the information gathered from the compensation chart offered a very useful guide to our researchers who work with polychromatic assays as it provides an immediate insight about a particular fluorochrome spectral behaviour with respect to the instrument optics configuration. The compensation chart experiment is simple to perform and can be carried out in any lab who owns a multi-lasers flow cytometer. The information gained has not only helped our researchers in the design of their experiments but most importantly has formed part of a useful tool for our in-house flow analyser training programme.

Table 1: Flow Analyser Optical Configuration and Lasers Power

Table 2: Compensation Chart

The 153 possible two-color combinations that can be generated using eighteen different fluorochromes are shown with the compensated value highlighted. The figure is adapted from Hadrup et al. References:

1. BD Fluorescence Spectrum Viewer a multicolor tool http://www.bdsbiosciences.com/in/research/multicolor/spectrum viewer/index.jsp
2. Biolegend Fluorescence spectra analyzer http://www.biolegend.com/spectraanalyzer

FLOW CYTOMETRY INSTRUMENTATION (B83 – B109)

214/B83

Fibromyalgia Induces Oxidative Stress and Cytochrome C Release in Isolated Rat Muscle Mitochondria by Flow Cytometry

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Fibromyalgia syndrome (FS) is a common rheumatologic syndrome which is manifested as chronic, diffuse musculoskeletal aching and soreness accompanied by poor sleep, fatigue, morning stiffness and, frequently, affective dysfunction. Although FS patients primarily have muscular pain, they may also report localized articular pain, subjective swelling of the hands or knees, numbness or coldness. In this study the muscle toxicity mechanisms of Fibromyalgia syndrome were investigated in the isolated mitochondria obtained from rat muscle. Rat muscle mitochondria were damaged by differential centrifugation and then mitochondrial toxicity endpoints as well as mitochondrial sources of ROS formation were determined in vitro using specific substrates and inhibitors. Our results with isolated mitochondria showed that disorders of Fibromyalgia could induce significant (p <0.05) rise in mitochondrial ROS formation, GSH oxidation, mitochondrial potential collapse, mitochondrial swelling and cytochrome c release before the mitochondrial outer membrane rupture ensued. We also showed that the respiratory complex III is the major source of induced ROS formation. In general, our data strongly supported that Fibromyalgia syndrome induced muscle toxicity is mitochondrial respiratory complexes II and III which are the obvious causes induced ROS formation in muscle cells which leads to cell death signaling via MPT pore opening and cytochrome c release. These events progress rapidly in individual cells but are observed as bi-physic peaks in flow cytometry assay because cell death generally occurs asynchronously in a population. This allows researchers to use flow cytometry to easily distinguish healthy cells with intact mitochondria from those with permeabilized mitochondria.

215/B84

High Throughput Imaging and Parallel Flow Cytometry Using Radiofrequency Multiplexing

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Borrowing concepts and techniques from the field of radiofrequency communications, we present the development of both an imaging flow cytometer and a parallel flow cytometer. These high throughput instruments represent order-of-magnitude increases in throughput for imaging flow cytometry and conventional flow cytometry, respectively. The ability to multiplex fluorescence and light scatter signals in the radiofrequency domain using an optical analog of orthogonal frequency domain multiplexing (OFDM) provides a combination of data throughput and sensitivity unmatched by competitive technologies. Multicolor fluorescence imaging of cells flowing at meter per second velocities is demonstrated, with cellular throughput in the range of 50,000 events per second. Further, we demonstrate conventional flow cytometry operating in parallel using a single optical and electronic platform, which offers an order of magnitude improvement in cellular throughput versus conventional single-channel instruments. This parallel approach to high-throughput flow cytometry offers greatly improved sample throughput without sacrificing the number of cells per sample analyzed. Applications of these two technologies in drug discovery and high content screening will be highlighted.

216/B85

Violet SSC: An Alternative to FSC PMT or Fluorescence in the Detection of Extracellular Vesicles

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Over the past decade, there has been a rapid growth in studies of secreted membrane vesicles, collectively called extracellular vesicles (EVs). (1) Although advances in various fields, including microscopy, have addressed some of the preliminary hindrances, flow cytometry remains the dominant approach for the characterization of submicron cell derived particles. In this independent study, several of those technologies are evaluated and compared. As most of the hardware adjustments are accomplished by enhancements to the FSC parameter, the study will also evaluate the use of Violet SSC on Beckman Coulter’s Cytolsax as a novel approach to small particle detection.

For this flow cytometric assay, particles were chosen at specific sizes for later cell tracking. By using beads of differing sizes and fluorescent intensities, one can optimize the flow cytometer for cellular analysis. Bangs Labs’ Dragon Green Beads (DG) were acquired on Beckman Coulter’s Astrios EQ, MoFlo XDP with...
Propel Labs NanoView attachment, Gallios and Cytoflex flow cytometers. All instruments were peaked to maximize resolution and separation of populations (192nm, 520nm, and 780nm). To further investigate Violet SSC as an alternative to the conventional mechanics. Employing RT-DC, the mechanical phenotype has the potential to become a standard parameter in cytometric assays, with many applications in biology and medicine.

218/B87
AcCellix-Automated Flow Cytometry
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Background: Medical flow cytometry (FC) provides diagnostic answers by detecting the presence and concentration of cell populations, and/or by measuring concentrations of cell surface markers expressed on cells. Currently, FC is limited to high complexity labs by time consuming pre-analytical steps, requiring highly trained technologists. Inter-instrument and inter-operator variability limit broad acceptance of IVD FC. Finally, interpretation of FC results requires highly trained professionals typically available only during business hours.

The AcCellix compact table top multicolor flow cytometer automates the 3 step process required for population identification and/or cell surface marker measurement. Sample preparation and reading are performed in a dedicated disposable cartridge. Analytical data processing utilizing proprietary algorithms provides answers directly to the user.

Acellix Cartridge: This disposable cartridge-based platform provides 24/7 availability in a moderate complexity lab - ultimately CLIA waved setting - by implementing sample preparation using three reagent blisters. With different reagents in the blisters the same cartridge structure can be used for multiple applications. The 3 Acellix CD64 cartridge blisters contain staining cocktail of conjugated monoclonal antibodies, lysis buffer, and reference beads respectively. Once sample processing is complete, the sample flows through a dedicated reading channel where data is acquired.

Applications Implemented on Acellix: 1) Sepsis diagnosis and monitoring based on upregulated CD64 expression on neutrophils. 2) HIV monitoring based on determining T cell subsets: proportion of T helper cells (CD4) to cytotoxic T cells (CD8) compared with total T cells (CD3). 3) Population analysis of cells: differentiating T cells, B cells, NK cells and monocytes based on cell surface marker expression. 4) Measuring sepsis induced immunosuppression via HLA-DR expression on circulating monocytes.

Results and Conclusions: In a demonstration of cell surface marker quantitation a comparison study of 53 blood samples showed a correlation coefficient of 0.91 for Acellix determined neutrophil CD64 compared to those determined using a FACs. In a study to identify lymphocyte subsets a comparison study of 5 samples (run in triplicates) showed a correlation coefficient of 0.99 for Acellix determined T cell differentiation based on CD4/CD8 ratio, and correlation coefficients of 0.94 and 0.99 for lymphocyte population analysis to determine B cells (CD19/CD45) and NK cells (CD56/CD3/CD45) compared to FACs. These initial studies show that the cartridge-based Acellix system can determine the presence and concentration of cell populations as well as determine the concentration of cell surface markers. Thus, implementation of a wide range of fully automatic IVD assays with results in 30 minutes or less is possible using Acellix.

219/B88
Automated Analysis and Sorting of Human iPS Cell Clusters by Large Particle Flow Cytometry
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Biomedical methods based on stem cells are currently the most dynamic areas of life science especially when it comes to cellular test systems for pharmacological and toxicological screenings. Short term goal is establishing new test systems to increase prediction of screenings in the preclinical phase. This will lead to cost reduction and higher efficiency. The development of
reprogramming somatic cells into human iPS cells opens unique perspectives for producing human cell products in a tissue disease and patient specific manner. To fully take advantage of the potential of this technology there is a need to produce a high number of iPS cell lines using high throughput techniques, to standardize the available protocols and to deliver fully characterized cells.

Currently the standard procedure of handling iPS cell production and culture is based on the processing of these cells as clumps. This includes the selection of the primary iPS cell colonies and the passaging during expansion. At the moment these steps require skilled personnel and are highly time consuming.

The availability of a high throughput technique, which is fully automatatable and is capable of selecting and sorting cell clusters is Union Biometra’s large particle flow cytometry technology (COPAS and BioSorter). Here we describe the COPAS technology for the automated cell culture of human iPS cells.

220/B89
Micro-imaging Cytometry by Focused Flying Laser Spot
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Flow cytometry has become a critical tool for quantitative cellular analysis over the past 40 years. Current systems detect scatter and fluorescence signal by illuminating a cell with laser spot significantly larger than the cell itself. In this mode, the laser spot is so-called ‘Top-Hat’ shape for uniform illumination which brings a wider tolerance by reducing the spatial resolution. On the other hand, Gaussian laser beam has intrinsically higher spatial resolution and peak intensity. The motivation for this work was to provide a new method for analyzing every cell features by using a smaller laser spot than the cells being analyzed.

The use of the flying-spot concept is well known in areas such as SEM, CRT, confocal microscopy and the optical disk. But in order to apply this concept to cytometry, the most advanced devices are mandatory: 405nm laser, acoustical optical (AO) device & driver, planar-type flow chamber design, custom objective lens, high speed detectors and a huge data acquisition system. Based upon cell size and optical tolerance, 2μm FWHM laser spot, 0.1–1.0μm flow velocity, 100k–1,000kHz beam deflection were targeted as the proof-of-concept. Further, using 405 nm as the shortest visible wavelength has the advantage of reduced spot size and deflection RF power. In addition 405nm excitation can provide a broadband wavelength range for fluorescence detection. We present a system using wavelength stabilized 405nm laser with a plane wave front & good Gaussian profile. Good beam deflection is the key for micro imaging applications combined with TeO2 crystal for acoustic optical high-speed beam deflection. Finite optics were applied to minimize wave front distortion and satisfy fill conditions. The AO deflection frequency is 250MHz ±50MHz. Combined with an NA=0.16 objective lens, we have confirmed deflection scanning at 1MHz. We have also evaluated 2.1μmFWHM laser spot size on focused plane. Because of the planar flow chamber, the use of the small flying spot allows for the simultaneous detection of multiple cells on the focused plane. To achieve this concept, a unique flow chamber with hydrodynamic focused plane was developed. The prototype shows in-focus (hydrodynamic) flow at 0.1–1.0 m/s mean velocity using a flying spot velocity of up to 400μm/s. In order to detect sufficient spatial resolution, we have developed 70MHz bandwidth detector modules. Si PIN diodes were used for 405nm signal detection and pMPTs were applied for high speed fluorescence detection. A new data acquisition system requiring 16bit/160MHz/4CH A/D converter was developed to capture high speed data. Using this data system, it is possible to record raw data on 1.5 TB solid state memory with 10.24Gbps.
We have developed a prototype data analysis software allowing initial analysis with more extensive system under development. We have used beads and stained cell mounted on slides and flow chamber for live cell analysis is under evaluation for system performance and biological application.

The first proof of Micro Imaging Cytometry is now demonstrated. Future development include migration of laser spatial resolution to 0.25 microns, extending into the UV region and development of unique analysis methods. Transforming from a larger to a smaller spot size than the cell may open new frontiers of cellular analysis for smaller particle detection, polarization analysis, photon fluorescence, time sequential analysis and 3D cell imaging.

221/B90
Near Ultraviolet Laser Diodes for Brilliant Ultraviolet Fluorochrome Excitation
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Although multiple lasers are now standard equipment on most modern flow cytometers, ultraviolet (UV) lasers (325 to 365 nm) remain an uncommon excitation source for cytometry. Nd:YVO4 frequency tripled diode pumped solid state (DPSS) lasers emitting at 355 nm are now the primary means of providing UV excitation on multilaser flow cytometers. Although a number of UV excited fluorochromes are available for flow cytometry, the cost of solid state UV lasers remains prohibitively high, limiting their use to all but the most sophisticated multilaser instruments. The recent introduction of the Brilliant Ultraviolet (BUV) series of fluorochromes for cell surface marker detection and their importance in increasing the number of simultaneous parameters for high-dimensional analysis has increased the urgency of including UV sources in cytometer designs; however, these lasers remain expensive. Near UV laser diodes (NUVLDs), a direct diode laser source emitting in the 370 to 380 nm range, have been previously validated for flow cytometric analysis of most UV-excited probes, including quantum nanocrystals, the Hoechst dyes and DAPI. However, they remain a little-used laser source for cytometry, despite their significantly lower cost. In this study, the ability of NUVLDs to excite the BUV dyes was assessed, along with their compatibility with simultaneous Brilliant Violet (BV) labeling. A NUVLD emitting at 375 nm was found to excite most of the available BUV dyes at least as well as a UV 355 nm source. This slightly longer wavelength did produce some unwanted excitation of BV dyes, but at sufficiently low levels to require minimal additional compensation. NUVLDs are compact, relatively inexpensive lasers that have higher power levels than the newest generation of small 355 nm lasers. They can therefore make a useful, cost-effective substitute for traditional UV lasers in multicolor analysis involving the BUV and BV dyes.

222/B91
Early Adopter Report Regarding the Innovative Sony SP6800 Spectral Analyzer: Advantages, Disadvantages, and Data Comparison with a BD LSR-II
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The Sony SP6800 Spectral Analyzer pioneers a new design for flow cytometry (FC). Conventional FC emerged when computer throughput was a limiting factor, so the technology that was established employed filters to reduce signals to fluorophore emission peaks, to reduce computation. This design has proved very workable, but becomes ever-more challenging as additional fluorophores are added to the same spectral space. The alternate Sony design, which leverages modern computer power to unmix aggregate emissions, employs algorithms to determine each fluorophore’s contribution to a particle’s total signal. This innovative technology allows the simultaneous use of fluorophores whose peaks occupy the same bandwidth spaces,
allows the use of fluorophores with broad or multiple peaks, and makes possible the capture and analysis of broad-spectrum autofluorescence, which can vary from sample to sample. Because full spectra are collected, the technology can use virtual filters to mimic a conventional cytometer. Because the technology does not require fluorophore-specific filter arrangements, it easily adapts to changes in fluorophore preferences. And because spectral overlap is less an issue than with conventional FC, more fluorophores can be used in the same spectral space. Notably, these technological differences require a different logic for panel creation. In conventional FC, the preferred fluorophores have narrow and simple emission peaks, spaced apart from neighbors. But for the SP6800, there are benefits to using broad or multipeak emissions, so fluorophores that have been difficult to use (e.g., Cy2, KromeOrange, BV570, PE-Cy5.5) can be useful additions to panels. Our immunology lab has obtained one of the first SP6800 Spectrum Analyzers (adding a violet laser upgrade to the standard blue/red laser configuration). This instrument has joined two BD LSR-II analysers (V/BR) in our lab. We will discuss issues associated with adoption of this new technology, present qualitative and quantitative data obtained from identical samples run on the SP6800 and the LSR-II, and comment on the advantages and disadvantages, challenges and promise of this design.

223/B92
The Use of Imaging Flow Cytometry as a Support Tool for the Traditional Flow Lab
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Imaging Flow Cytometry (IFC), couples high acquisition rates of hydrodynamically focused cells in suspension and accurate object segmentation, with brightfield and fluorescence microscopy enabling the ability to statistically discriminate objects based on differences in appearance and spatial distribution of signal.

Beyond the many high-end quantitative imaging statistical microscopy applications for Imaging Flow Cytometry, it has also become an established platform for the accurate quantification of singlets, doublets, aggregates, particles and debris in suspended cell preparations. This makes Imaging Flow Cytometry an ideal tool to support standard flow cytometry and droplet sorting applications in the areas of sample assessment staining quality, specificity, artifact rejection and post-analysis of sample populations.

Topics covered will include:
- Brief overview of the technology and a few representative imaging applications
- What’s different from standard flow practices
- What’s different from traditional fluorescence microscopy

The use of Imaging Flow cytometry for the Traditional Flow Lab:
- cellularity assessment
- staining quality and specificity
- artifact rejection
- post-sort analysis
- Rogues Gallery of artifacts

Discussion
- The audience is encouraged to ask questions

224/B93
Optimizing Flow Cytometer Sensitivity by Upgrading Hardware Components
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We are currently upgrading a LSR II and a Fortessa (both from Becton Dickinson) for simultaneously detecting up to 18 fluorochromes (plus SSC and FSC parameters). We have been exploring the effects of installing improved hardware components (laser, improved hardcoated filters and dichroic mirrors, PMTs and PMT sockets) on the measurement sensitivity of fluorochromes available conjugated to antibodies, especially the Brilliant series of dyes developed by Sirigen and other novel dyes/tandems.

While the influence of laser wavelength and laser power is relatively straightforward, the optimization of filters can be quite tricky: While filters can obtain quite similar stain indices from test samples stained with only a single fluorochrome, the spillover from other channels can result in much more pronounced filter preferences for polychromatic FC panels. Dichroic mirrors can exhibit quite pronounced gaps for shorter wavelengths and we have tried to minimize deleterious effects for detecting shorter wavelength fluorochromes off violet and UV lasers.

Standard detectors for both instruments are 1/8” PMTs from Hamamatsu: R9220 while R3896 is slightly superior. A new generation of PMTs shows improved performance: we utilize R10699 as a general replacement with the (affordable) R11540 and (more expensive) R12896 offering additional advantages for 370-490nm and NIR, respectively. While the sensitivity of the new generation of PMTs has improved it is important to note that PMTs have widely differing characteristics (within the limits guaranteed by the manufacturer) and the best placement of individual PMTs within a cytometer should be verified. As sockets for PMTs, we compare the commonly used HPMS-1N–L2BH/ L2BSM (Matsusada) with the C12597 (Hamamatsu).

225/B94
Implementation of a System for Full Pulse Shape Data Extraction in Flow Cytometry
Martin Buescher, Juergen Krieg, Markus Nagel, Andreas Froemming
Miltenyi Biotec, Bergisch Gladbach, Germany

In many research fields, flow cytometry is utilized to identify multiple complex cell populations. With few exceptions the information density has been increased by increasing the number of fluorochromes detected. Information about spatial distribution of dyes is mostly ignored.

However with recent developments in electronic signal processing and increasing storage capacities on computer systems, extracting and exploring information about dye-distribution on- and within cells is becoming possible with commonly available technology.

Herewith we are presenting an approach where we use fast analog-to-digital conversion in combination with appropriate laser shaping optics and digital deconvolution algorithms in order to extract 1-dimensional-dye-distribution data from multiple fluorescence channels simultaneously. We derive multiple scalar values from the data obtained similar to methods used in the imaging area such as cell-complexity, symmetry and polarity.

We demonstrate the usefulness of this 1d data extraction in the field of apoptosis research by showing the ability to quantify the chromatin-condensation. The benefit of implementing this method in the field of flow cytometry is, that this is opening up the available parameter space significantly, without increased system complexity.

226/B95
Effect of Isotope Transmission on Antigen Detection and Comparison of Different CyTOF Instruments for Improving Standardization
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The recent introduction of mass cytometry has greatly increased the number of parameters that can be measured per single cell. As with all new technology, there is a need for dissemination of standardization and quality control procedures. Here, we characterize variations in sensitivity observed across the mass range of a mass cytometer, using different lanthanide tags. To this aim we used a multi-element solution containing isotopes with atomic masses comprised between 139 and 176 Daltons, four element calibration beads and cells stained with antibody-metal conjugates to simulate experimental conditions. The different test were performed on four different CyTOF instruments located in four different core-facilities.

We observed a 5-fold difference in lanthanide detection over the mass range and demonstrated that each instrument has its own sensitivity pattern. Therefore, the selection of lanthanide combinations is a key step in the establishment of a staining panel for mass cytometry-based experiments, particularly for multicenter studies. An important information arise from this data: while in conventional flow cytometry the main differences in sensitivities are related to the used fluorochrome, in CyTOF technology the sensitivity pattern is determined by the instrument. We propose here the sensitivity pattern as the basis for panel design, instrument standardization and future implementation of normalization algorithms.

\textbf{227/B96}

Use of the Separation Index of Flow Cytometer to Evaluate the Effectiveness of Light-Path Design

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\textbf{Background:} Lasers in a flow cytometer excite fluorescent particles to emit fluorescence photons, which are eventually converted to detected electrical signals. Light converting efficiency of this process is proportional to the performance of a flow cytometer. As a key component of the light-path, cuvette-system design is crucial to the efficiency. In the jet-in-cuvette flow cytometer, lasers pass a flat plane before reaching the core stream. Less laser power loss ensures more fluorescence emission. Secondly, the effective cuvette-system design should maximize fluorescence photons collection to get the maximum resolution and sensitivity. Since the photons are emitted in all directions, the cuvette in flow cytometer should be coupled with optical-gel of high N.A. value to collect more. Another way to improve performance is to use reflecting mirrors to compensate light collection. Here, we demonstrate the effectiveness of light-path design in a flow cytometer can be evaluated by separation index.

\textbf{Methods:} Four flow cytometers* with different light-path design were tested. All of them are fixed with 488nm laser power: FCM1=20mW, FCM2=20mW, FCM3=15mW, FCM4=20mW for FITC and PE excitation. Filter for FITC channel: FCM1=530/42, FCM2=533/30, FCM3=530/30, FCM4=530/30. Filter for PE channel: FCM1=585/40, FCM2=585/40, FCM3=585/42, FCM4=585/42. FCM3 and FCM4 fix with gel-coupled cuvettes. FCM1 and FCM2 have cuvettes without gel between the cuvette and the optical-lens. FCM2 has reflecting mirrors.

BD Calibrite beads (BD Biosciences, San Jose, US) were used to evaluate the FITC and PE detecting channel. BD Calibrite beads (BD Biosciences, San Jose, US) were used to calculate the FITC and PE detecting channel. BD Calibrite beads (BD Biosciences, San Jose, US) were used to calculate the FITC and PE detecting channel. BD Calibrite beads (BD Biosciences, San Jose, US) were used to calculate the FITC and PE detecting channel. BD Calibrite beads (BD Biosciences, San Jose, US) were used to calculate the FITC and PE detecting channel.

We calculate Separation index according to the Formula 1\textsuperscript{1-2}.

Some flow cytometer statistics have no SD value, we use the Formula 2 to calculate the SD.

\begin{align*}
\text{Formula 1} & \quad \text{MEAN}_{\text{positive}} - \text{MEAN}_{\text{negative}} / (2 \times \text{SD}_{\text{negative}}) \\
\text{Formula 2} & \quad \text{SD}_{\text{negative}} = \text{Median}_{\text{negative}} \times \text{CV}_{\text{negative}}
\end{align*}

\textbf{Results:} Separation index (SI) of FITC channel on each flow cytometer is: FCM1\textsubscript{FITC} = 140.428, FCM2\textsubscript{FITC} = 202.696, FCM3\textsubscript{FITC} = 172.338, FCM4\textsubscript{FITC} = 225.405. Separation index (SI) of PE channel on each flow cytometer is: FCM1\textsubscript{PE} = 118.600, FCM2\textsubscript{PE} = 171.014, FCM3\textsubscript{PE} = 194.457, FCM4\textsubscript{PE} = 178.237.

\textbf{Conclusions:} Separation index, which shows how well the flow cytometer separates stained from unstained populations, presents the performance (resolution and sensitivity) of a flow cytometer in a biologically important manner. It also can be used to evaluate the effectiveness of light-path design. Build flow cytometer with gel-coupled cuvette system is an efficient method to get better resolution and sensitivity.

*FCM1=NovoCyte, FCM2=Accuri C6, FCM3=FACSCalibur, FCM4= FACSComp

\textbf{References:}

\textbf{228/B97}

Comparison of AQUIOS Tetra and Navios Tetra System Performance

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\textbf{Background:} The AQUIOS CL* is a fully automated flow cytometer with integrated sample loading, preparation and analysis. In this study, we demonstrate that the AQUIOS Tetra algorithm provides accurate results for enumeration of lymphocyte subsets in samples tested up to 24 hours post venipuncture. The recovery of the T, B and NK cell lymphocyte subsets using AQUIOS Tetra method was compared to the Navios Tetra system.

\textbf{Methods:} Sixty seven (67) specimens, comprising HIV+ subjects were tested with tetra reagents on the AQUIOS and Navios systems. Specimens for the comparator method were processed manually and lysed on TQ-Prep system. AQUIOS sample handling and preparation is integrated in the system. Collected samples covered the clinical and normal range for the CD4 positive cells. The distribution included CD4 expression levels at 32 cells/µL – 1300 cells/µL, with the majority (63%) of samples representing the clinical decision points under 500 CD4+ cells/µL.

The statistical analysis demonstrated a negative bias (lower 95% limit of bias for CD3+/CD4+ count at 100, 200 and 500 cells/µL was -17, –20 and -37 cells, respectively) between the two methods. A positive bias (upper 95% limit of bias for CD3-/CD56+ count at 29th, 50th and 75th percentile cell/µL was 29, 38 and 57 cells, respectively) between the methods for enumeration of NK cells.

\textbf{Conclusions:} The AQUIOS Tetra method demonstrated comparable results to the Navios Tetra application for measuring recovery of the T, B and NK cell lymphocyte subsets. The greater difference seen in the enumeration of NK cells was expected given that this population was identified only by the CD56.
**229/B98**

**Enumeration of T, B and NK Subpopulations in Aged Whole Blood Samples by Novel Flow Cytometry AQUIOS-Tetra Method**

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The AQUIOS CL* is a fully automated flow cytometer with integrated sample loading, preparation and analysis. In this study, we demonstrate that AQUIOS-Tetra algorithm provides accurate results for enumeration of lymphocyte subsets in samples stored for 24 hours post venipuncture. A total of 73 specimens including normal and clinical (HIV+) were prepared as fresh (<8hrs), at 24 and 32 hours and analyzed immediately and after 3min incubation as prepared samples. The purity of lymphocyte population was assessed by evaluation of the monocyte and neutrophil contamination** in total of 48 specimens, clinical and normal, tested fresh (≤8hrs) and aged (24hrs).

**Results:** The drift in absolute count and percent positive for T, B and NK lymphocyte subsets was assessed between the reference 0-0 and 24-0 time points at 95% confidence level. The results showed insignificant drift within clinical limits bias when compared to fresh sample recovery. The maximum measured monocyte and neutrophil contamination in the lymphocyte gate was 4.02% and 0.75% for aged samples, respectively.

**Conclusions:** The AQUIOS-Tetra method provides accurate results for recovery of lymphocyte subsets and supports >95% purity of lymphocyte population for analysis of aged blood specimens.

*Pending clearance by the US FDA; not yet available for in vitro diagnostic use within the U.S.A.

**230/B99**

**Evaluation Results of the SA3800: Introducing a Novel Spectral Analyzer for High-Throughput Single-Cell Analysis**

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**Background:** Single cell analysis has proven to be an integral part of life science research. Cell heterogeneity, the significance of rare populations, and the complexity of existing biological variances have each contributed to an increased demand for high-throughput single-cell analysis tools. Spectral analyzers are such a tool. They offer a revolutionary and innovative approach to flow cytometry. Sony spectral analyzers are unlike conventional flow cytometers because their approach to the detection of fluorescence utilizes a linear array of 34 photomultiplier tubes (PMTs) and a custom prism array. This engineering design enables a maximum amount of information to be captured from each interrogated cell because it negates the use of interference filters and therefore a loss of collected photons. At Sony, we have worked to engineer and develop a high-throughput spectral analyzer, the SA3800. This 4 laser analyzer is built on the DNA of other Sony instruments for the biotechnology marketplace (the SH4800 sorter and the SP6800 spectral cell analyzer). This presentation reports the result of instrument evaluation which includes long time stability evaluation and multi-color analysis.

**Methods:** We have implemented commonly used methods for QC and instrument sensitivity determination and tested the instrument accordingly. AlignCheck and 8 peak beads were used for this purpose. Multi-color analysis has been carried out with commonly used cells and probes.

**Results:** We present here daily QC results and data from some representative immuno-phenotyping experiments. The on-board high-speed automatic cleaning system limits carry-over and allow for very fast well-to-well times.

**Conclusion:** The results presented here show that we successfully engineered a new instrument for spectral based, high throughput flow cytometry. The quality and flexibility of the design and the ease-of-use will allow scientists to integrate this instrument in routine multi parameter experiments. Such innovations and walk-away capabilities are requested by scientists in diverse areas of life science research and advance many fields of discovery.

**231/B100**

**Microfluidic Sensors for Simultaneous Impedance and Optical Label-Free Single Cell Analysis of Whole Blood Samples**

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Developments of highly integrated microfluidic components are necessary for building compact and robust single cell analysis systems and are essential for in-vitro point-of-care diagnostics. On the other hand, combination of well established measurement protocols with the methods being still in the stage of development, provides the researchers with the new tools. Integration of the electrical vector impedance analysis into the laser flow cytometer is a good example. To this end, we developed microfluidic devices for combined electrical and optical flow cytometric analysis of blood cell suspensions. Our sensors allow analysis of single particles by simultaneous detection of fluorescence, light scatter and electrical impedance in the frequency range from 300 kHz up to about 100 MHz. As follows, the blood cells can be differentiated based on their size, membrane capacity and resistance of the intracellular contents whereas the identification is validated by standard protocols used in laser flow cytometry. The microfluidic sensors incorporate hydrodynamic shear flow focusing to confine particles in a defined volume. Chips were manufactured applying double-etched glass structures with integrated sputtered Pt electrodes in facing configuration. Stability of hydrodynamic focusing, measurement sensitivity and resolution were benchmarked by measuring the coefficients of variations (CV) of calibration microparticles with specified size and fluorescence intensities. Differential measurement, based on phase-sensitive detection technique assures high sensitivity of...
impedance measurement of particles moving with velocities of up to 6 m s\(^{-1}\). Applying the developed microdevices, we demonstrate the method for simultaneous label-free differentiation of blood platelets, erythrocytes, granulocytes, monocytes and lymphocytes in human whole blood. The method is based on combined impedance and optical analysis without splitting the sample. Immunostaining protocols including CD45, CD235a and CD66b antibodies were applied to verify the assignment of the targeted cell populations. The only sample treatment step involved a necessary sample dilution. To facilitate the demonstration, the whole blood suspension was enriched with white blood cells from the same sample. Additional tests were carried out on the lysed blood samples to verify the identification of the leukocytes.

### 232/B101

**Fluidics Modifications to BD Bioscience’s LSRII and Fortessa Flow Cytometers Enable Reduced Maintenance and Downtime**

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Current fluidics solutions supplied with the BD Bioscience LSRII and Fortessa flow cytometers have been problematic, particularly when utilizing plate sampling systems (HTS) in a busy Core lab. These systems are complicated with a variety of sensors, fittings, tubing, and consumable plastic parts, that when failing, take significant core-staff time to diagnose and repair. In addition, the HTS sampling systems rely on syringe pumps and valves for consistent fluidics operation. Both of these HTS components can be adversely affected by shear fluid salt build-up and result in erroneous measurements and potentially useless data. Our custom built fluidics solutions are comprised simply of stainless steel tanks and fittings, which are more durable and require less maintenance than the vendor-supplied components. We show rigorous quality control measurements that demonstrate a once per day each morning sheath filling provides consistent performance throughout the day. Furthermore, our custom fluidic system supplies distilled water directly to the HTS system pumps and valves eliminating salt buildup. These simple improvements can lead to reduced maintenance and downtime while allowing for better longitudinal performance of these instruments.

### 233/B102

**A Custom, Automated Solution for Immunophenotyping Applications**

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Many investigators in our R&D organization routinely use isolated human leukocyte subsets for their studies. However, most scientists do not perform post-separation purity testing to analyze the contaminating fraction subset composition. We co-developed a solution with Miltenyi Biotec for verifying the purity of commonly isolated subsets. To facilitate this, we developed a custom-designed and barcoded antibody staining cocktail, a no-wash labelling protocol, automated analysis, and real-time reporting. The platform utilizes the MACSQuant VYB flow cytometer, which we brought into our core operations as an easy-to-operate, flexible, and reliable alternative to our other heavily used cytometry instruments. The VYB platform has advantages over our other instruments in that: it can be quickly learned by laboratory technicians with little flow cytometry experience, and the integrated no-wash staining, analysis, and data reporting protocols are entirely automated and can be driven by barcoded reagents. We compared the purities reported via traditional non-automated analysis versus the automated platform for total CD3 T cells, CD4 T cells, CD8 T cells, NK cells, B cells, and CD14 monocytes. Our fit-for-purpose approach allows for a highly standard method for purity testing to be simple to employ for most everyone. This customized solution has led to faster data generation and real-time analysis, as well as improved access to flow cytometry methods for non-experts.

**234/B103**

**Measuring Birefringent Samples Using a Two FSC-PMT Setup**

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Birefringent molecules are known to change the angle of light polarization and can be used to identify particular types of cells such as malaria-infected RBCs containing Haemoglobin, Erythrocytes, or other particles like clay sediment samples. In flow cytometers, lasers are typically polarized at a ratio >100:1 and changes in light polarization can be measured using normal SSC against depolarized SSC by simply replacing a bandpass filter in one of the fluorescence detectors by a polarizer placed perpendicular to the angle of light polarization. Yet currently, the optical path of light detection in most instruments is being replaced by fiber optics. Although highly convenient to reduce the instrument’s footprint, these optical fibers do not maintain the light polarization angle and therefore are unsuitable to measure changes in light polarization caused by birefringent molecules.

Last year at CYTO2014 we showed as a proof of principle that as long as new instruments have one extra FSC detector it may be possible to measure changes in light polarization by comparing normal SSC with depolarized FSC. Expanding on our initial results we show that for different types of birefringent samples regardless of the wavelength used, changes in light polarization can be captured using a two FSC-PMT setup. For this purpose we used a MoFlo Astrios EQ with two FSC-PMTs that could be easily aligned to measure FSC at 405 nm, 488 nm, 561 nm, 594 nm, and 645 nm wavelengths. Furthermore, taking advantage of the different FSC masks available for the HTS system pumps and valves eliminating salt build up. These simple improvements can lead to reduced maintenance and downtime while allowing for better longitudinal performance of these instruments.

It remains to be seen whether changes in light polarization can be detected in instruments with a FSC photodiode vs a FSC-PMT since previous attempts to measure birefringent samples with this setup have failed. Our results indicate that this may be related to the fact that the FSC-PMTs in these instruments are optimized for small-particle detection, though it could still be related to the reduced sensitivity of the photodiodes. In the future, we plan to address this issue by applying different types of obscuration bars before the FSC detectors to evaluate whether measuring light depolarization can be universally applied to instruments containing two FSC detectors.
235/B104
Step by Step Instrument Characterization and Optimization of Optical Configuration: Impact on Overall Fluorochrome Resolution and Multicolor Panel Design

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Instrument configuration has a very strong impact on the overall resolution of multiparametric flow cytometry panels. In order to rationalize the panel design step at CIPHE, we first wanted to optimize our instrument performance. We went through a systematic round of testing of the optical filters on our 5-laser LSRII Fortessa SORP. By using 8 pic bead particles, we optimized the combination of filter sets on our instrument in order to increase resolution of dim fluorescent beads. By this methodology, we could visualize the impact of laser order and highlight the effect of cross-laser contribution to the optical background seen in some PMTs of our instrument. We then verified the impact of this sensitivity optimization on cells by establishing a brightness index of 24 fluorescent dyes coupled to the same anti-mouse CD8a antibody (Clone 53-6.7) on murine splenocyte. Finally, the overall resolution of multiple cell populations using a 12 colors panel was compared to exemplify the impact of instrument configuration on multi-parameter flow cytometry.

By presenting this work, we wish to share our observations with the community of cytometrists and help in identifying key parameters on C&ST baseline reports which could be further improved by filter optimization in order to maximize resolution of multicolor flow cytometry panels.

236/B105
New Ways of Exploring Fluorescence Relaxation Dynamics in Flow

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Introduction: Research on the timing of excited state processes in flow cytometry requires sophisticated technologies and rapid response by each hardware component. For example, photodetectors, lasers, and data systems must meet high-frequency specifications to enable the measurement of the fluorescence lifetime. Therefore revealing intricate fluorescence decay behavior is quite challenging for flow cytometry and not quite routine. Despite this, we have studied a number of different applications where fluorescence excited state times are important. Some of these scenarios include metabolic mapping through measurement of NADH fluorescence decay, Förster resonance energy transfer, fluorescent protein localization in cells, and fluorescence lifetime as a discriminator for spectrally overlapping fluorophores.

Methods: In this contribution we present a handful of new, state-of-the-art fluorescence lifetime techniques to respond to the growing list of applications. The fluorescence lifetime is the average time a fluorescent molecule spends in its excited state prior to relaxation to the ground state. It is a unique photophysical trait that, with time-resolved detection systems, can provide quantitative information for cellular assays. We have developed techniques called: non-modulated flow cytometry, polar-plotted flow cytometry, and NIR fluorescence lifetime flow cytometry. A variety of cytometric hardware were modified including the laser excitation sources, photodetectors, and ways in which we plot and analyze the data.

Results and Conclusions: Overall we demonstrate the ability to measure fluorescence lifetimes without laser modulation for fluorophores whose lifetimes vary from 3 to 20 nanoseconds. We also show the ability to develop polar plots for multi-lifetime data from a single cell that is dual-labeled as well as cells expressing different fluorescent protein variants (e.g. tetrascuor fluorescent protein and a dark-state tetrasucor fluorescent protein). Finally, we have measured the fluorescence lifetimes of E. Coli: expressing near-infrared proteins and NIR fluorescence microspheres which range from 0.5 to 2 nanoseconds. Cytometers can be modified in many ways such that the fluorescence lifetime can be captured. Herein we strive to simplify the ways in which a flow system is modified and at the same time increase the content of the data collected.

237/B106

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The Sony SP6800 Spectral Analyzer pioneers a new design for flow cytometry (FC).

In contrast to conventional FC, which detects light intensity coming through discrete, separated bandpass filters, the Sony design collects entire spectra emitted from laser-excited particles. This difference in design allows the SP6800 to detect broad autofluorescent signals and treat them as a unique channel. This identified signature can either be subtracted out or analysed itself.

The Picker Lab has been using flow cytometry since 2000 in the development of prototype HIV vaccines. A standard assay used in this research takes white blood cells from vaccinees, and exposes those cells in culture to antigens of interest. Cells reactive to those antigens will synthesize cytokines, which can be trapped inside the cells and quantified by the Intracellular Cytokine Staining (ICS) technique. Using ICS, we measure vaccine recall responses induced in HIV antigens engineered into our prototype vaccines.

A longstanding problem has bedeviled this method in our work: The HIV targeting receptor includes peptide sequences that induce death to cells exposed in culture. When FC is used to measure ENV-encoded peptide mixes, it is common to see a death spike run through the data, confounding attempts to accurately measure memory to this viral receptor.

Although there are various ways to reduce this data contamination (including gating methods and live/dead dyes), the death induced by these toxic peptides is associated with an autofluorescent signature. Since our immunology lab has one of the first commercial installations of the SP6800, we were interested to learn whether this cytometer’s unique ability to detect and manipulate autofluorescence would allow it to subtract this signal from our datasets.

We have found this to be the case, and will present data indicating that this capability may have use in the study of memory responses to toxic peptides.

238/B107
Attune NxT: Modular Optics

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For Attune NxT development, extensive voice of customer was collected. From that data, it was clear that customers highly value instrumentation that is designed to be upgradeable, flexible,
and stable. For many platforms, modular design is the key to a robust upgrade path. Modularity allows for a customer to purchase at a price point they can afford and upgrade at a later time. A single base unit can deliver four different product tiers, which allows the sales force to ‘walk the customer up the ladder.’ To deliver on the benefit of modularity and product robustness, a novel optical platform for stable beam delivery and emission collection had to be developed for Attune Nxt. The core of the advancement is in the modular and mechanically-decoupled optical components that allow one to four flat-top laser lines as well as up to four fiber coupled detector arrays with customizable changeable fluorophore filters. For ease of manufacturing and upgrading, all lasers have identical form factors and focal lengths, and can be switched out if a different wavelength is required for excitation. A major hurdle was overcome by the use of patent-pending beam stacking cubes to mitigate aberrations induced by conventional beam combining in the converging flat-top profile. The optical design has demonstrated equivalent or better performance compared to market leading flow cytometers in regards to population resolution, sensitivity, and throughput.

239/B108
Short Wavelength, Low Coherence, Low Noise Lasers for Improved Resolution of Submicron Particles

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Background: A major barrier to single submicron particle analysis and sorting is the detection of small particles above the noise level of the cytometer system. Although cytometer ‘noise’ is due to many factors, the laser source can be a significant contributor to the overall system noise. To improve analyses of submicron particles, picocell extractable virus and viruses in the 50–500 nm size range, we sought to identify laser attributes that could improve signal-to-noise ratio for nanoparticle detection by flow cytometry.

Methods: We employed laser sources with several unique attributes. First, the lasers tested in this study emitted at wavelengths shorter than the traditional 488 nm sources normally used for forward and side scatter measurement. Lower wavelength laser light would be theoretically expected to improve small particle resolution. Blue 450 nm, blue-violet 420 nm and violet 405 nm laser diode sources were compared to traditional blue-green 488 nm laser sources. Second, these lasers (manufactured by Pavilion Integration Corporation, San Jose, CA). Second, these lasers emitted at lower coherence levels than most single wavelength lasers used in flow cytometry, with flat-top spectra. Active mode phase diversification of the laser emission produced lower levels of ‘speckle’ than lasers with higher coherence levels. Laser speckle is a potential source of noise for flow cytometry. These modules also minimized laser speckle by other mechanisms, including internal combining of two beams with differing angles of polarization and generation of multimode beam geometries with sum frequency mixing of the resulting longitudinal modes. For the blue 450 nm module, the active mode phase diversification could be switched on and off, allowing comparison of small particle resolution for both operating modes. Third, these laser sources used extremely low noise control electronics and power supplies to minimize laser noise to an RMS level of less than 0.05% for the 20 kHz to 20 MHz range. These specialized laser modules were compared to conventional laser diodes at the same wavelengths, and to a blue-green 488 nm PIC laser. All lasers were tested on a BD Influx cell sorter (BD Biosciences, San Jose, CA). equipped with small particle detection optics. Laser beam shapes, which did differ somewhat between lasers, were circularized using anamorphic prism pairs and measured using a beam profiling camera. Each laser installation was tested using 100 and 200 nm microparticles (Life Technologies, Carlsbad, CA).

Results: The short wavelength, low coherence, low noise laser sources gave improved forward scatter resolution above noise compared to conventional laser diodes at comparable wavelengths when analyzing 100 and 200 nm particles. The 450, 420 and 405 nm lasers also gave somewhat improved small particle forward scatter resolution when compared to a 488 nm source with the same active wavelength modulation and low noise. Running the lasers in active mode phase diversification lowered both noise and signal level, with no clear improvement in small particle resolution.

Conclusions: Lower wavelengths did somewhat improve small particle resolution compared to operationally comparable blue-green 488 nm sources. A stronger degree of improvement was seen when comparing short wavelength, low coherence, low noise lasers to conventional lasers at the same wavelength. These studies suggest that improving the quality and specifications laser excitation is an important component of instrument design for submicron particle analysis.

240/B109
Rapid and Powerful Cellular Analysis Using Microcapillary-Based Guava easyCyte™ Platforms

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The guava easycyte™ line of flow cytometers are based on microcapillary cytometry and are accessible and easily adaptable platforms for users spanning a range of expertise levels. The mode of usage of these cytometers have undergone a transformation over the years with usage ranging from routine cell counting and apoptosis or antibody screening applications to more complex immunophenotyping, or multiplexed assays for drug-screening, as well as the study of micromasages such as yeast and bacteria. Many of these studies leverage on the fact that these cytomter enable determination of absolute cells counts, utilizes low sample volumes, and are easier to operate and maintain than traditional flow cytometers. Recent updates to the easycyte instrument platforms include more powerful lasers along with enhanced features of Incyte™ software. The combinations thus provides users choice of systems ranging from 1-3 lasers and up to 12 detection channels. Here we present data from our evaluation of the updated systems. Evaluation of 8-peak rainbow beads on all systems showed excellent resolution of peaks; data obtained with a range of sizing beads clearly depicts enhanced detection and resolution in small particle ranges while sensitivity studies indicates improved sensitivity from these platforms. Applications of multi-parametric assays at a cellular level for drug screening/immunophenotyping will also be presented demonstrating clear detection of lower abundance populations. The new performance features of the guava easycyte™ platforms along with powerful Incyte™ software provides users with expanded and enhanced detection capability and increased analytical power without sacrificing ease of use.

HEMATOLOGICAL DISORDERS (B110 – B126)

241/B110
Flow Cytometry as Rapid Diagnostic Tool for Molecular Screening of Chronic Granulomatous Disease

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Abstract: Chronic Granulomatous Disease (CGD) is a primary immunodeficiency disorder with recurrent pyogenic infections and granulomatous inflammation resulting from loss of phagocyte superoxide production. Mutations in the 3 structural genes of the NADPH oxidase complex viz. CYBB and CYBA encoding for membrane bound gp91phox and p22phox; and NCF1, NCF2, and NCF4 encoding for cytosolic components p47phox, p67phox, and p40phox respectively, have been found to cause CGD. The CYBB gene encoding the enzymatic center of the NADPH oxidase, gp91phox, is on the X-chromosome and autosomal recessive forms occur from mutations in CYBA, NCF1, NCF2, and NCF4 gene. Clinical manifestations in CGD and currently used diagnostic tests viz. Nitroblue tetrazolium test (NBT) and Dihydrorhodamine (DHR) assay do not discriminate between underlying genetic defects. Identification of molecular defect is important for patient management as well as for prenatal diagnosis in the affected families which is currently done by laborious and time-consuming method of western blotting. However this technique requires large volume of blood is technically difficult.

The present study was aimed at studying the pattern of genetic defects in a cohort of CGD patients with the help of rapid six color flow cytometric evaluation of protein expression of NADPH oxidase components in the index case and parents.

Method: Expression of NADPH oxidase components was standardized on healthy control peripheral blood neutrophils. Membrane-bound subunits, gp91phox and p22phox expression were analyzed by surface staining with fluorescently tagged 7D5 monoclonal antibody whereas cytosolic components p47phox and p67phox required permeabilization were studied using specific monoclonal antibodies against each component. In the peripheral blood these components normally expressed both on phagocytes as well as on B cells whereas T lymphocytes did not show expression. Sixty patients with abnormal NBT and DHR were further evaluated for expression of NADPH components.

Results: 7D5 (gp91phox) and p22phox expression is defective in both XL-CGD and AR-CGD CYBA gene defect; however mosaic pattern is seen in mother of XL-CGD. 18% patients had XL-CGD and 72% AR-CGD. Absent or reduced expression of p47phox and p67phox suggesting defect in respective genes were further molecularly analyzed. Homozygous Del GT mutation in NCF1 gene was identified in 24% patients. 24% showed abnormal p22phox expression suggesting deficit in CYBA gene.

Conclusion: AR-CGD is more common in this cohort of Indian patients as compared to XL-CGD which is distinct from the western data. Surface staining with 7D5 antibody along with cytoplasmic p47phox and p67phox expression by flow cytometry in index case and mother can be used to discriminate between different subtypes of CGD and it helps in their molecular characterization. Flow cytometry provides rapid and sensitive tool for detecting subgroups of CGD and is valid when CGD genes. Hence this method is helpful in screening and guiding further for molecular analysis of the disease.

242/B111
A Six Color Flow Cytometry Screening in the Diagnostics of MDS
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Background: A number of studies have shown that flow cytometry (FC) is a valuable approach to define immunophenotypic abnormalities in patients with myelodysplastic syndromes (MDS). Therefore, this technique has been proposed as a part of the diagnostic approach together with cyto-morphology (CM), histo-pathology (HP) and cytogenetics (CG). In particular, percentage of myeloid progenitors and CD34+ B cell precursors, CD45 expression on both myeloid progenitors and lymphocytes and the ratio of the side scatter (SSC) of granulocytes and lymphocytes, parameters which constitute the ‘Ogata score’ (Os), have been usually evaluated in suspected MDS cases. In this study we retrospectively evaluated if the single six color tube implemented in our setting for the first FC screening of patient’s bone marrow would be useful for the Os evaluation in MDS diagnosis with a more accurate definition of progenitors.

Methods: 76 consecutive patients with a MDS suspicion and 35 patients without cytopenia (controls) were evaluated with the following single screening tube: CD14-FLTC, CD19-PE, CD3-PeC, CD34-PECy7, CD117-APC and CD45-APC-H7. We analyzed the Os parameters with the only difference on the definition of myeloid precursors as CD34+CD117+ and CD34+ B cell precursors as CD34+/CD19+. A positive Os score was then assigned in the presence of 2 or more alterations in the above mentioned parameters.

Results: Based on clinical parameters, CM, HP and CG, 72 cases were diagnosed as MDS. Four cases remained as inconclusive because samples resulted to be not adequate. Considering a Os cut-off value of ≥2% for myeloid progenitors and of ≤5% for CD34+ B cell precursors we obtained a positive score only in 31/72 (43%) MDS cases. No controls had a positive Os score (24 Os=1, 11 Os=0). Interestingly, in 3 MDS cases with silent CM, HP and CG, the FC analysis showed an Os ≥2. As expected, the percentage of myeloid progenitors among MDS patients was significantly increased as compared to controls [0.9 % (0.04-6) vs 0.5 % (0.01-1.6); median and range; p=0.0003], although it was lower than previously described [Della Porta et al; 1.6 % (0-74.1)]. This could be due to a more accurate but also restrictive definition of myeloid progenitors in our analysis. Analysis of CD34+ B cell progenitors showed a decreased percentage of those cells in MDS cases as compared to controls [2.4 % (0-78) vs 6.7 %, (0-35.2); p=0.03] with values comparable to those from literature [Della Porta et al; 1.6 % (0-74.1)]

Conclusion: Our single six color screening tube is a simple and useful tool for the Os evaluation. In consideration of our more accurate progenitors definition in FC analysis, a revision of the previously defined cut-off values, in particular for myeloid lineage, could ameliorate the sensitivity of the Os in our setting.

243/B112
Omics1 Approach to Elucidate the Critical Epigenetic Regulatory Machinery Selective for Acute Myeloid Leukaemia Stem Cell Function
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Acute myeloid leukaemia (AML) is the most common acute leukaemia. Within AML malignant clones a few cells defined as leukaemia-initiating cells or leukaemia stem cells (LICs or LSCs) are considered the critical cellular component responsible for the initiation and maintenance of the disease. Novel treatments to be used in combination with existing first line chemotherapeutics are required to enhance the elimination of LSCs and prevent disease relapse in the long term.

We have adopted a global comparative approach and established a human haematopoietic hierarchy gene transcriptional regulation control fingerprints between normal haemapoietic stem cells (HSCs) and LSCs. Our data suggests that aberrant epigenetic regulation plays an important role in AML oncogenesis. Consequently we performed a targeted knockdown (KD) screen of chromatin regulatory genes in human AML cells. KD of EP1C or EPC2 induced apoptosis of primary human AML cells but spared the functional potential of normal HSCs.
Therefore we identified EPC1 and EPC2 as critical and selective oncogenic co-factors in AML.

Utilising a combination of genomic, proteomic (High-throughput FACS, co-IP-MS and ChIP-seq/RNA-seq) and bioinformatic approaches, we have obtained preliminary results to characterize differential EPC interactomes and their downstream targets in AML and normal bone marrow cells. Our data favour the model whereby EPC complex serves as an inhibitory element docking at the key LSC gene promoter region, blocking Tip60 and HAT1 acetylase activity towards H4K5 site; the association of HDAC2 enhances this inhibition-induced hypoacetylation. As such, we are currently investigating on the inhibition of HDAC2 as an avenue for specific targeting of LSCs in ML-AML via directly reversing this hypoacetylation leukaemic feature back to ‘normal’.

This finding has a significant implication in current clinical trials: Histone deacetylase pan inhibitors (HDACis), such as suberoylanilide hydroxamic acid (SAHA), are used for various types of cancer, including haemopoietic malignancies, but the outcome for treatment of myeloid leukaemia is still uncertain. This is likely due to the fact that these pan-HDACis target multiple pathways simultaneously, thus compromising their anti-cancer effects. A more specific HDACi is required to target the cancer-specific HDACs more than those required in normal cells. Our current results elucidate the unique role of the EPC epigenetic regulatory machinery in AML stem cell function, via recruiting specific HDAC2 activities at LSC target gene leucemic loci, such as PRC2 module and MYC module genes’ promoter regions. Epigenetic therapies using small molecule inhibitors targeting EPC complex components could represent an novel and efficient way to ‘reverse’ aberrant epigenetic profiles, in turn ‘converting’ LSC signatures back to normal in patients, thereby contributing clinical benefit for cancer treatment in AML and maybe other cancers.

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244/B113
Real-Time Measurement of Platelet Shape Distribution with the Scanning Flow Cytometry
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Background: Blood platelets play one of the most important role in hemostasis. Different diseases are connected with pathology of platelets, including pathology with platelets activation. Many methods have been developed for the evaluation of platelet function. These methods are usually based on platelets aggregation and blood coagulation. Clinical methods for the assessment of platelet activation are not widely spread.

Method: We used the scanning flow cytometry, the method based on the measurement of angle-resolved light-scattering patterns (LSPs) of individual cells with subsequent solution of the inverse light-scattering (ILS) problem. We measured LSPs with the Scanning Flow Cytometer fabricated by CytoNova Ltd. (Novosibirsk, Russia, http://cyto.kinetics.nsc.ru/). The solution of the ILS problem allows one to obtain distribution of platelets over shape. This distribution was used to characterize the shape change during activation and various external influences.

Results: The study of platelets with the scanning flow cytometry was performed for different patients. Platelet shape change led to the shift of the obtained distribution to the region of spherical cells. The amount of activated platelets was evaluated after stimulation of cells by several agonists in different concentrations, showing a monotonic dependence of the latter. Real-time measurement of platelet shape immediately after the start of activation was achieved with the rapid pneumatic sample delivery system. We also studied platelet shape under the influence of EDTA, heparin and the microtubule stabilizing agent taxol.

Conclusions: Together with the ability to detect activated platelets based on their shape, the presented approach allows one to study shape change in live platelets during platelets activation. This novel data may help in understanding of the process and lead to new diagnostic or therapeutic techniques.

245/B114
Analysis and Identification of Alkaline Phosphatase-Positive Leukocytes Using Unlysed Whole Blood
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Background: Many of the “normal” ranges of blood chemistry are quite large. The normal range for alkaline phosphatase, for example, is from 30-120. While a clinician might look at these blood chemistry panels and find nothing abnormal, we have validated a new protocol for alkaline phosphatase live staining (APLS) using unlysed whole blood that could help to provide new evidences for a kind of sequential immune disruption in human malignancies.

Materials and Methods: Unlysed whole blood cells were incubated with Alkaline Phosphatase Live Stain (APLS) in presence of Vybrant® DyeCycle®116 Violet Stain (DCV) for 20 minutes at 37°C. APLS and DCV were obtained from Life Technologies®. Briefly, for APLS loading, anti-coagulated blood samples containing 5 x 10¹¹ cells in HBSS (final volume = 500µL) were prepared. DCV was used for nucleated cell discrimination. Data were collected using the Attune® Acoustic Focusing Cytometer (Life Technologies® equipped with two lasers operating at 405 and 488 nm. Filter combination for APLS analysis was: 555 DLP, BP 530/30 (green). DCV signal was measured using logarithmic scale at 50 mW. APLS was measured using logarithmic scale at 20 mW.

Results: Alkaline phosphatase positive cells were easily detected after 20 minutes incubation at 37°C, with increased levels of human neutrophil alkaline phosphatase in peripheral blood cells, when compared with monocytes and lymphocytes.

Conclusions: Alkaline Phosphatase Live Stain can be easily applied for whole blood immunophenotyping of different leukocyte subtypes. Since alkaline phosphatase is increased in inflammatory disease, AP live staining using un-lysed whole blood could help to provide new evidences for a kind of sequential immune disruption in hematological malignancies.

246/B115
The Predictive Significance of CD20 Expression in Follicular Lymphoma
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Background: The anti-CD20 monoclonal antibody rituximab has improved the overall response rate (ORR), as well as the response duration and the overall survival (OS) of patients with B-cell lymphomas (BCL). There are few reports in the literature examining whether higher CD20 expression parallels with better treatment outcomes. In our previous study, we determined the cut-off value of CD20 expression at the level of 25000 molecules of equivalent soluble fluorochrome (MESF) to be the predictor of response to rituximab containing treatment in patients with BCL. However, in specific histologic types of BCL we could not confirm the predictive significance of CD20 expression because the number of patients was too small. Therefore we were aimed...
to assess if the cut-off value of CD20 expression at 25000 MESF has predictive significance in patients with primary follicular lymphoma (FL), and if this cut-off value is a predictor of OS, disease free survival (DFS) along with the ORR.

Methods: Forty-seven patients with primary FL treated with rituximab and chemotherapy at the Institute of Oncology Ljubljana, Slovenia between 2005 and 2011 were enrolled in the study. In all patients the CD20 expression was assessed prior to the beginning of treatment. The level of CD20 expression was determined by quantitative flow-cytometric measurements using Spherogram Rainbow Calibration Particles, which were measured on the same day as the CD20 expression was assessed. Clinical data were collected from patients records, and the DFS and OS were evaluated by means of Kaplan-Meier survival curves. The study was approved by Republic Slovenia National Medical Ethic Committee (7/006/14).

Results: The cut-off value of CD20 expression, which predicts a better response to rituximab in patients with primary FL was determined at 30000 MESF. Our data show that patients who achieved complete response after rituximab therapy had a significantly higher expression of CD20 antigen (p<0.0001) than those whose disease only stabilized after rituximab therapy. No significant difference was observed in DFS between the patients with CD20 antigen expression above the cut-off value and those expressing CD20 antigen below the cut-off value (HR=0.0; 95% CI=0.003-36.930, p=0.646). Even though, we have proved that patients whose CD20 expression was above the cut-off value and were treated with rituximab had a significantly longer OS (HR=3.922; 95% CI=1.122-66.731, p=0.041) than patients with the level of CD20 expression below the cut-off value. According to our study population, 12.8% had the CD20 expression below the cut-off value.

Conclusions: These results confirmed our previous findings that a higher level of CD20 expression translates into an improved OS and ORR in patients treated with rituximab. The cut-off value of CD20 expression at 30000 MESF suggested to have the predictive significance of better outcome in FL was slightly higher than the one observed in our previous study (25000 MESF).

247/B116

EuroFlow Quality Assurance Program: Proposal for Structure and Implementation

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Flow cytometric immunophenotyping has become essential for accurate diagnosis, classification and disease monitoring in hemato-oncology. The EuroFlow Consortium has established a fully standardized ‘all-in-one’ pipeline consisting of standardized instrument settings, reagent panels, and sample preparation protocols as well as software for data analysis and disease classification. For its reproducible implementation parallel development of a quality assurance (QA) program was required to provide feedback on proper execution of standardized EuroFlow flow cytometry protocols.

The novel QA scheme aimed at monitoring the whole flow cytometric analysis process (cytometer setting, sample preparation, acquisition and analysis) by reading the median fluorescence intensities (MedFI) of defined lymphocytes’ subsets. Each QA participant applied the pre-defined reagents’ panel on blood cells of local healthy donors. An uniform gating strategy was applied to define lymphocyte subsets and to read MedFI values per marker. The MedFI values were compared to reference data and deviations from reference values were quantified using performance score metrics.

We performed four consecutive annual rounds of the novel external QA EuroFlow program. In 4 annual QA rounds, we analyzed 123 blood samples from local healthy donors on 14 different instruments in 11 laboratories from 9 European countries. The immunophenotype of defined cellular subsets appeared sufficiently standardized to permit unified (software) data analysis. The coefficient of variation of MedFI for 7 out of 11 markers performed repeatedly below 30%, average MedFI in each QA round ranged from 86% to 125% from overall median. Calculation of performance scores was instrumental to pinpoint standardization failures and their causes. Overall, the new EuroFlow QA system for the first time allowed to quantify the technical variation that is introduced in the measurement of fluorescence intensities in a multicentric setting over an extended period of time. We found only a mild improvement over the QA years, presumably due to gain in expertise of participants. Likewise, only moderate improvement of variation was observed with usage of lyophilized reagent mixture in evaluated labs (however, eight out of nine participating labs had over 4 years of experience with EuroFlow protocols). EuroFlow QA is a technical proficiency test specific for laboratories that use standardized EuroFlow protocols. It will be implemented as a performance control and open to any laboratory that follows the EuroFlow protocols. Since we proved that the intensity of expression of tested markers is well conserved among healthy donors, the QA test does not require any complicated logistics involved in stabilized sample sending. In practice, each laboratory will perform the QA test and report the MedFI values into dedicated QA result website, central analysis will provide feedback to erroneous results and periodic bi-annual Educational QA meetings will aim at continuous quality improvement.

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248/B117

Developmental Haemostasis: Difference in Platelet Function between Children and Adults

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Aims: Platelets play a protective role in haemostasis at sites of vascular injury. Despite the integral role of platelets in many disease processes, there is very little information available on platelet function and response to agonists in healthy children. We recently reported that circulating monococyte-platelet aggregates (MPA), a sensitive marker of the thrombo-inflammatory process, are increased in healthy children compared to adults. Here we aimed to investigate and compare platelet activation, granule exocytosis and heterotypic aggregation in response to physiologically relevant platelet agonists in healthy children compared to healthy adults.
Methods: Whole blood flow cytometry was used to measure platelet function. Platelets were identified by characteristic light scatter and expression of CD42b. Platelet activation was measured by binding of PAC-1, which recognises an activation dependent conformational change in the GPIb-IIIa receptor. Granule exocytosis was measured by translocation of CD62P to the platelet membrane. Heterotypic aggregates were measured by CD14+/CD42b+ events.

Results: We demonstrated significant age-specific differences in agonist-stimulated platelet activation, granule exocytosis and formation of heterotypic aggregates in response to sub-maximal concentrations of PAC-1 and P2Y12 agonists. Pre-existent MPA was found to be directly correlated to agonist-stimulated MPA in children. An inverse correlation between age and agonist-stimulated MPA was observed in children.

Discussion: These results challenge the general assumption that platelet reactivity in children is similar to adults, and may be important in the administration and monitoring of antiplatelet agents to children.

249/B119
B-CLL Response to Bacterial Toxin Listeriolysin O with Monoclonal Antibody Anti-CD20 (Rituximab) in Therapy

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Background: Listeriolysin O (LLO), the main virulence factor of Listeria monocytogenes belongs to the family of Cholesterol Dependent Cytolysins. It is characterized by cytolytic activity to biological membranes containing cholesterol. LLO molecules in contact with the membrane undergo oligomerization forming transmembrane pores. Physiological effect depends on the concentration of the toxin. At low concentrations it creates pores in the cell membrane facilitating Ca²⁺ influx into the cell which stimulates many cellular pathways and may induce apoptosis. At higher concentrations LLO produces numerous pores causing necrosis.

Rituximab (RTX) is a chimeric monoclonal antibody specific for an epitope located at the large loop of CD 20 antigen. This antibody is used in treatment of patients with B-leukemia and B cell lymphomas, with cell surface antigen CD 20. The activity mechanism of the antibody is associated with the direct induction of apoptosis, complement-dependent cytotoxicity and antibody dependent cellular cytotoxicity (ADCC).

Similar effects of LLO and RTX on eukaryotic cells raise the possibility of combined use of these two factors in the treatment of B-cell lymphocytic lymphoma / chronic lymphocytic leukemia. The aim of our experiment was testing joint the effect of bacteria toxin LLO and RTX on a human B-cells from patient with diagnosed B-CLL.

Methods: In the in vitro experiment, we used a modified Listeriolysin O. Peripheral blood mononuclear cells were pretreated with RTX and then washed, resuspended in RPMI 1640 medium and incubated at 37 °C, 5% CO₂, 1% O₂ at various concentrations of LLO, RTX, and a mixture of these compounds. The effect of the tested agents was measured by the PI population changes of cells were analyzed by standard protocol of BD Simultest.

Results: In vitro experiment involving complement cytotoxicity observed additive growth of cytotoxicity of rituximab and bacterial toxin listeriolysin O. The experiments were performed on human cell leukemia lines Raji and Jurkat as a control. The effect of rituximab and LLO toxin mixture on B-CLL leukemia cells was dose dependent.

Conclusions: Comparing the mechanisms of action and the results of our observations in vitro we assume that the combined effect of bacterial toxin listeriolysin O and antibody rituximab may have therapeutic effect. However requires further study and may in the future result in a more efficient removal of the tumor cells.

250/B119
BCL-2 Overexpression as a Predictor of B-Cell Lymphomas in Fine Needle Aspiration Biopsy Samples

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Introduction: In the histology immunohistochecmical determination of Bcl-2 is commonly used in lymphoma diagnosis mostly for differentiation between follicular hyperplasia and follicular lymphoma and also for classifying of lymphomas. In cytology the role of Bcl-2 in lymphoma diagnosis is not well defined. There are some reports that flow cytometric determination of Bcl-2 can be useful for differentiation between reactive lymphocytic proliferations (RLP) and B-cell lymphomas (BCL) especially in cases, when clonality of B-cells cannot be determined by the surface light chain staining.

The aim of the present study was to evaluate the diagnostic value of flow cytometric determination of Bcl-2 expression in fine needle aspiration biopsy samples when BCL is suspected.

Materials and Methods: The study was approved by The National Medical Ethics Committee of the Republic of Slovenia (109/02/14). In the study, we investigated 215 FNAB lymph node samples where BCL was suspected clinically or microscopically. In all cases expression of Bcl-2, CD45, and CD19 antigens were determined by three-color flow cytometric measurements. Bcl-2 expression was assessed in populations of B-cells and T-cells. When Bcl-2 expression of B-cells was lower or similar to expression in T-cells, the Bcl-2 test was considered negative. On the contrary, the test was considered positive when B-cells showed higher level of Bcl-2 expression (overexpression) than in T-cells. Final diagnosis of BCL and RLP for studied samples was based on histological examination or clinical follow-up.

Results: Among 215 of our samples, 108 (50 %) of them had the final diagnosis of BCL and 107 (50 %) RLP. Among lymphomas 39 (36 %) diffuse large B-cell lymphomas (DLBCL), 36 (33 %) follicular lymphomas (FL), 25 (23 %) marginal zone lymphomas (MZL), 3 (3%) chronic lymphocytic leukemia, 3 (3%) Burkitt lymphomas, and 2 (2 %) Hodgkin lymphomas were found. The Bcl-2 test was positive in 71 (33 %) and negative in 144 (67 %) of 215 cases. The test was positive in 71 of 108 (66 %) BCL and in 1 of 107 RLP (1 %). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 66 %, 99 %, 99 % and 74 %, respectively. Comparison of sensitivity for FL, DLBCL, and MZL based on Bcl-2 expression showed the highest value (78 %) in samples with FL, and the lowest (62 %) in samples with DLBCL. The specificity was 100 % for all three lymphoma types.

Conclusions: Bcl-2 overexpression is a good predictor of BCL in FNAB samples and it is diagnostic for BCL. However, BCL cannot be excluded in cases where Bcl-2 is not overexpressed, because
of the low NPV (74%) of the test. In these cases another test or repeated FNAB in 4 to 6 weeks must be recommended.

251/B120

CD58 Downregulation in Pediatric-B-ALL at Day 15 of ALL IC-BFM 2009 Treatment Protocol and Its Implication on Minimal Residual Disease Detection

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Minimal residual disease (MRD) detection by flow cytometric immunophenotyping in pediatric B-cell acute lymphoblastic leukemia (p-B-ALL) became one of the most important prognostic factors according to current treatment protocols. Detection of leukemic cells during treatment relies on specific immunophenotype of the tumor cells. Among other useful markers CD58 was introduced in the ALL IC-BFM 2009 protocol. Previous studies showed that CD58 is overexpressed in p-B-ALL cells and the expression remains stable during the treatment. Since large-scale studies have not confirmed the stable expression of CD58 yet, we tested the CD58 expression of bone marrow leukemic cells at diagnosis (day 0) and at day 15 in p-B-ALL patients. Furthermore we compared the CD58 expression of the leukemic samples to the expression of normal precursor B-cells in bone marrow samples of patients with normal hematopoiesis as controls.

Twenty-one diagnostic and 17 day 15 bone marrow aspirate samples of p-B-ALL patients were studied along with 22 control bone marrow samples. The CD58 expression was defined in MEFL (Molecules of Equivalent Fluorescein) units using CD58 FITC antibody (EXBIO, clone MEM-63). The immunophenotype of the leukemic cells were identified at diagnosis by 5-color panels. The MRD detection at day 15 was carried out according to the ALL IC-BFM 2009 flow cytometric MRD protocol. In the control bone marrow the precursor B-cells were gated by their CD19, CD45, CD10, FSC and SSC parameters. Using the median CD58 MEFL values of the leukemic population the 3 groups of samples were compared by Mann-Whitney U test and ROC analysis was performed.

In agreement with previous studies we found significant difference (in average 6.3 times overexpression) between diagnostic samples (mean MEFL=7109) and control bone marrow samples (mean MEFL=1127). However in the day 15 samples we found a decrease compared to the diagnostic values in most (16 of 17) cases (mean MEFL=3494). The relative intensity decreased in 11 of 17 cases below 50% of the original value. We tried to define cut-off values to separate CD58 overexpressed samples. The best cut-off value for the day 15 samples was 1367 but with significantly lower specificity (81.8%) and sensitivity (70.6%) compared to the excellent cut-off value (MEFL=2741, specificity: 100% and sensitivity: 95.2%) of the diagnostic samples. When applying the higher original cut-off to the day 15 samples the results show no overexpression of CD58 in more than half of the patients.

In contrast with previous studies we found a remarkable decrease in the CD58 expression during the induction phase of the ALL IC-BFM 2009 treatment protocol at day 15. In conclusion, due to its downregulation, CD58 should be treated with caution as an MRD marker to avoid underestimation of residual leukemic cells in day 15 p-B-ALL bone marrow samples.

252/B121

Telomere Length Measurement in Human Hematopoietic Cells: Comparative Analysis of Q-PCR and Flow-Fish Techniques

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Abnormal telomere lengths have been linked to cancer and other hematologic disorders. Determination of telomere length is traditionally performed by Southern blotting and densitometry, giving a mean telomere restriction fragment (TRF) value for the total cell population studied. Here, we compared a quantitative PCR approach, Q-PCR, and a flow cytometric approach, Flow-FISH (FF), to evaluate telomere length distribution in total PBMC or specific cells population based on situ hybridization using a fluorescein-labeled peptide nucleic acid (PNA) (CCCTAA)1 probe and DNA staining with propidium iodide.

We showed that both Q-PCR and FF provide a robust measurement, with FF measuring a relative length longer than Q-PCR. Both methods showed comparable telomere length reduction with age, and the rate of relative telomere length loss was similar.

253/B122

BCR-ABL1 and CD66c Exhibit High Concordance in Minimal Residual Disease Detection of Adult B-Acute Lymphoblastic Leukemia

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Background: Many lineage specific markers are currently in use to diagnose B-acute lymphoblastic leukemia (B-ALL) and to monitor for minimal residual disease (MRD) by flow cytometry during and following treatment. However, as the status of these markers changes under treatment, the quest for markers that exhibit stable expression even subsequent to treatment remains. Recent reports have illuminated carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6; CD66c) as one such potential marker. The myeloid antigen CD66c expression is always limited to granulocytes and their precursors within the normal hematopoietic system. CD66c has been shown to be aberrantly expressed in a considerable proportion of pediatric B-ALL case and also been correlated to some specific genetic changes in B-ALL, such as Ph/BCR/ABL1, hyperdiploidy, and TEL-AML1 fusion gene negativity. The status of CD66c expression in adult B-ALL, however, has not yet been well characterized. In the present study, for the first time, we investigated the relationship between surface expression of CD66c and the breakpoint cluster region-Abl (BCR-ABL1) fusion gene in adult B-acute lymphoblastic leukemia (B-ALL) at primary diagnosis, and their concordance during minimal residual disease (MRD) monitoring.

Methods: Bone marrow biopsies were collected from newly diagnosed B-ALL patients (n=43) between September 2011 and September 2014. Karyotyping was used to detect Philadelphia chromosome (Ph), and fluorescence in situ hybridization (FISH) and reverse transcription-polymerase chain reaction (RT-PCR) were used to detect BCR-ABL1 fusion gene. Immunophenotyping was performed by flow cytometry for leukemia. Patients with both CD66c expression and BCR-ABL1 were further assessed for MRD during treatment.

Results: Overall, 26/43 (60.5%) B-ALL patients were positive for BCR-ABL1 fusion gene expression, and all Ph positive cases (17/43; 39.5%) expressed BCR-ABL1 and CD66c. CD66c was expressed at significantly higher levels in BCR-ABL1 positive than negative patients (24/26, 92.3% vs. 11/17, 64.7%; P=0.042), and furthermore, in all Ph positive cases (17/17, 100% vs. 18/26, 69.2%; P=0.014). When BCR-ABL1 was set as the gold standard for the presence or absence of MRD after treatment, both CD66c alone and the MRD panel including CD66c demonstrated high diagnostic performance for the detection of MRD, with values of area under the receptor operation curve (ROC) of 0.881 vs. 0.891 respectively.

Conclusions: The stable expression pattern of CD66c has noteworthy clinical value in B-ALL not only in the recognition of abnormal leukemia cells at primary diagnosis but also in monitoring of MRD during the treatment, especially in patients without definitely cytogenetic or molecular abnormal, and thus, warrants further investigation as a routine clinical marker for MRD detection by flow cytometry.
254/B123
Automatic Quantification of Sickled Human RBC by Image Cytometry Combined with Immunophenotyping Provides a Powerful Assay for the Study of Sickle Cell Disease

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Polymerization of deoxygenated hemoglobin and the subsequent formation of sickled red blood cell morphology remain central to the pathophysiology of sickle cell disease (SCD). Image flow cytometry provides researchers a powerful tool to study sickle cell morphology and the potential effects of therapeutic interventions.

Human peripheral blood samples from patients with sickle cell disease were collected via the sickle cell biorespository under an IRB approved protocol. Freshly collected blood was stored for a maximum of five hours before use. In vitro O2, equilibrium curves were generated on a Hemox instrument. While generating the O2 equilibrium curve, consecutive aliquots of 50 ul of sample were collected at known pO2 values. The cell suspensions were immediately transferred into 4% deoxygenated paraformaldehyde in hemox buffer for 15 to 30 minutes, followed by 0.05% deoxygenated glutaraldehyde for additional 3 min. The cells were rinsed with 2.5 mM sodium borohydride solution, permeabilized with 0.1% TX-100 for 10 min, and incubated with pre-conjugated antibodies. Labeled cells were analyzed by image flow cytometry (Amnis ImageStream® MkII). Due to the variation of sickled RBC morphology, there isn’t a single built-in or customer-made feature that can separate sickled cells from normal cells. To automate the quantification of percent sickling, we used sequential approaches that gradually pull out normal RBCs from sickled cells. Circularity and shape Ratio are the two strongest morphometric features that separated of normal and sickle cells. However 50% of cells remained in mixed populations. Therefore we used a sequential approach to further separate normal cells from sickle cells until all cells were distributed to sickle or normal cell populations. We verified the algorithm by hand counting in multiple in vitro sickling assays. To further validate the assay and investigate the functional effects fetal hemoglobin on sickling we applied the algorithm on cells labeled with fluorescent antibodies to gamma globin. The results showed 28% of the RBCs with negative HbF sickled at pO2 30 mmHg. 59% at 20 mmHg and the sickled cells reached over 70% at pO2 10 mmHg. RBCs with high HbF intensity (HbF++) showed a strong anti-sickling effect in the range pO2 30 to 1.8 mmHg. The degree of sickling inhibition is 65% at pO2 20 mmHg and 60% at pO2 10 mmHg. The results suggest that F-cells with HbF++ protected RBCs from hypoxia-induced sickling.

We have developed a new algorithm that can automatically quantify the percentage sickle cells in peripheral blood samples and that this can be combined with immunolabeling to further investigate RBC biology and the process of sickling.

255/B124
The Role of CD49d Expression in the Survival of Chronic Lymphocytic Leukemia Cells

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Chronic lymphocytic leukemia (CLL) is the most common B-cell leukemia of adults in Western countries. The disease is characterized by variable clinical courses, which can be predicted by various prognostic factors. Among them is the recently discovered CD49d, which predicts poor outcome. The CD49d, also known as the α4 integrin subunit, is associated with the CD29 (β1) molecule. The ligands of the CD49d/CD29 complex are VCAM-1 and fibronectin. It is well known that CLL cells resist apoptosis and show increased proliferation as a result of their interaction with the microenvironment. Several studies investigated the role of CD49d in the survival of CLL cells. Some of them showed that CD49d-VCAM-1 interaction inhibited the apoptosis of CLL cells, others suggested that this interaction just helped the binding of CLL cells to bone marrow stromal cells (BMSC).

In our study we investigated whether the VCAM-1 or fibronectin stimulation alone or the bone marrow stromal cells (BMSCs) by themselves can induce the survival and proliferation of CLL cells. We examined the change in the expression of adhesion antigens, cytokine receptors of CLL cells under different culturing conditions. We determined the conformation of CD29 on the surface of CLL cells.

Peripheral blood mononuclear cells from 30 CLL patients with different CD49d expression were cultured on VCAM-1 or fibronectin coated plates as well as in a co-culture with BMSCs for 3-7 days. After 3-7 days culturing we measured the apoptosis of CLL cells by flow cytometry (Annexin V/PI staining, detection of subG1 population), the proliferation by Ki-67 staining and cell counting. We examined the expression of CD80, CD86, CD184, CD5, CD38 and CD49d on the surface of CLL cells after 3-7 days culturing. We determined the conformation of CD29 with a conformation specific antibody.

BMSCs reduced the spontaneous apoptosis of CLL cells after 7 days, while VCAM-1 and fibronectin did not. The apoptosis rate was independent of the CD49d expression of CLL cells. The proliferation rate of CLL cells was not altered in the different culture conditions. The CLL cells co-cultured with BMSCs have increased expression of CD5, CD19, and decreased the expression of CD184; the VCAM-1 and fibronectin did not change the surface antigen levels. We detected only the low affinity conformation of CD29 on the CLL cell surface.

Our results suggest that the interaction of CD49d and its ligands do not, but BMSCs are able to prevent apoptosis of CLL cells.

256/B125
A Paroxysmal Nocturnal Haemoglobinuria Clone Presenting Type II Monocyte and Type II Granulocyte Clone

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Background: Paroxysmal nocturnal hemoglobinuria (PNH) is a chronic, bone marrow failure disorder associated with significant morbidity and mortality. It is a rare hematologic disorder and the cause of the disease is due to the absence of two glycosylphosphatidylinositol (GPI)-anchored proteins, which leads to uncontrolled complement activation. PNH, still remains a clinical situation that should be confirmed with peripheral blood flow cytometry to detect the absence or severe deficiency of GPI-APs on white blood cell (WBC) and red blood cells (RBC). A fluorescein-labeled proaerolysin (FLAER), is increasingly being used to diagnose PNH on WBC. RBC are a notable exception; this may be because both normal and PNH red cells express large amounts of glycophrin, a protein shown to bind aerolysin weakly. Guidelines for the diagnosis and monitoring of PNH have recently been published that recommend the cocktails of monoclonal antibodies and how to report. The report must include the clone sizes of WBCs and RBCs. RBC and WBCs are classified as PNH types I, II, and III according to the mean fluorescence intensities (MFI) of membrane proteins. RBC clones size is also important for the monitoring the efficiency of Eculizumab therapy.
Methods: The case was 44 years old male patient with PNH diagnosis who was treating with Eculizumab since 2010. Total blood cell count present WBC 4.87x10^9/L, hemoglobin (Hb) 12.8 g/dL, MCV 113.7fL and platelet count was 136x10^9/L. Increased Lactate Dehydrogenase (LDH 389 U/L), direct bilirubin: 0.38mg/dL, Erythrocyte sedimentation rate (ESR 40mm/hour) and C-reactive protein (CRP 4.1 mg/dL) were also performed within other laboratory results. Flow cytometric analysis of the peripheral blood was performed according to the guidelines with 4 color by BD instrument. The patient clone sizes were monitoring every 4 month since 2014 in our laboratory.

Results: Analysis of peripheral blood PNH clone size for RBC type II (41-55%) was greater than the type III RBC clones (13-17%) with the efficiency of the Eculizumab therapy. Whereas the granulocyte and monocyte clone size were over 98% and 92% respectively. The patients WBC clones were also showed type II and type III clones. The size of type III granulocyte and monocyte were greater than the type II WBCs.

Discussion: The ability to rapidly diagnose and providing the clone size of PNH by flowcytometry has led to improved patient management and prognosis. Although Type II granulocytes and monocytes can occasionally be recognized RBCs are typically better for the monitoring of the patients with Eculizumab therapy.

257/B126 Quantification of Hematopoietic Progenitors and Stem Cell Subpopulations in Fanconi Anemia Patients

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Fanconi anemia (FA) is a rare disease mainly characterized by bone marrow failure, congenic abnormalities and cancer predisposition, mainly acute myeloid leukemia. The follow-up of the hematopoietic stem and progenitor cells (HSPCs) in the bone marrow of these patients is now routinely evaluated in the Spanish FA patients. Here we describe the flow cytometry technique that is routinely used for the characterization of the HSPCs in different situations: 1) Patients follow up, 2) Recruitment of FA patients for a clinical trial aiming to evaluate the safety and efficacy of a new HSPC mobilization approach in FA patients, and 3) Evaluation of the leukapheresis products obtained from this trial. The expression of the CD34 antigen is used as a primary marker to evaluate the HSPC content in all these samples. Also, the use of multiple surface markers has allowed us to characterize different HSPC subsets within the CD34+ population. In this respect, the CD34+ CD45RA population should be studied to understand the genetic defect in FA patients and the very primitive HSC subpopulation. As an additional study, we have defined a multicolor single flow panel to investigate more deeply the HSPC hierarchy in CD34+ cells. This panel includes the analysis of CD10, CD7, CD135, CD90, CD45RA and CD49f. Understanding the defects at the HSPC compartment in FA patients would improve our understanding of the disease and also the development of advanced therapies for this rare disease.

High Content Analysis (B127 - B130)

258/B127 Cell-Based Assay with High-Throughput and High Content Manners

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Cell-based assay is an essential component of drug development or medicinal studies; they normally follow experiments performed in vitro and precede investigations on animals to check drug effects from the points of view of cellular responses to the treatment. Although diverged assays can indicate additional information about cells, there are some problems for standardizing and integrating these because, depending on the system, they can provide data that is static or dynamic, statistical or individual, or comprehensive or focused for a range of measurements and observation targets. The flow cytometer is used for high-throughput screening (HTP) assays with heterogeneous cell populations. Specific detection relies on labeling objects of cells with fluorophores. Static and multidex evaluation systems has been established. Alternatively, high-content screening (HCS) for cell-based assay with imaging cytometers facilitates cellular-imaging analysis with high spatial resolution, where designed sensing molecules are used.

We have so far tried to generate a functionally merged custom cell-based assay by combining the advantages of HTP and those of HCS. Our system is grounded on the apparatus and fluorescence resonance energy transfer (FRET)-based sensing molecules. The apparatus is a flow cytometer designed to measure the average fluorescence lifetimes of cell population. In some FRET-based measurements, it is necessary to calibrate the apparent changes in fluorescence intensities allow to estimate the net changes in the fluorescent properties of all the components. Fluorescence lifetimes of donor components of FRET pairs reflect the occurrence or disappearance of FRET. A system for measuring equivalent performances by FRET-based analysis can be moderated directly. We have already fabricated chimeric FRET bioprobes consisting of a fluorescent protein as a donor and a fluorescent dye as an acceptor. Such FRET bioprobes can be tuned by appropriate choices of donor and acceptor molecules enable us to use some bioprobes simultaneously.


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Background: The development of new technologies for high-parameter data has resulted in a critical challenge for identification of immune cell subsets discovery and annotation. Flow cytometry has been classically focused on manual analysis, despite computational tools being developed for high-parameter and cross-sample comparisons. Sharing well-annotated data improves transparency and facilitates vital reproduction of results by external groups. Adoption of these new tools for immune subset discovery requires thorough collaborative investigation and validation of identified cell populations against manual results.

Methods: In this study we compare manual vs automated population finding tools by comparing gating against a combination of viSNE and SPADE on PBMCs labeled with 26 different surface markers using Mass Cytometry. We also visualize the gating hierarchy by leveraging the zoomable sunburst, a visual and interactive representation of traditional manual gating. viSNE allows interaction with high-parameter data in the context of two-dimensional space where gating can be accomplished,
whereas in this case we run SPADE to provide automated clustering and visualization for identification of cell subsets.

**Results:** We have demonstrated the ability to automatically elucidate many immune subsets using Cytobank via an iterative analytic approach, combining computational tools (viSNE and SPADE) to recapitulate manually derived cell subsets, with applications to publicly available human mass cytometry data sets. We also show that we are able to automatically find small cell subsets that are relatively difficult to gate by hand (e.g. Basophils) as well as recapitulate canonical cell populations.

**Conclusions:** The combination of automated tools are leading the way towards augmenting and extending traditional single-cell analysis methods, creating global views of high-parameter analysis. In allowing for data to be viewed side by side between manually gated 2D plots and automated analysis, direct comparisons between the two methodologies as well as trust in discovery of novel subsets can be established.

**260/B129**

**Extending Computational Methods for Single-Cell Flow Cytometry Data Analysis: Applications to High-Parameter Single-Cell Data Types and High-Throughput Analysis**

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**Background:** Over the last 10 years, the development of new single-cell technologies has been increasing — in flow cytometry, mass cytometry and imaging, the number of simultaneous proteins measured has grown significantly as has the throughput for number of cells in single-cell RNA sequencing. Pre-processing pipelines exist for flow and mass cytometry, imaging and sequencing. With these new single-cell technologies come new challenges: cell subset characterization and discovery for higher parameters (e.g. RNA-seq data) is necessary. Existing computational analysis tools for single-cell data need to be extended and adapted to these major challenges.

**Methods:** To determine if Cytobank algorithms can retrieve known populations and features in high-parameter data, we ran dimensionality reduction using viSNE on single-cell RNA-seq data (Fan 2015, Science) imported into Cytobank. Twelve genes measured in the Ramos and K562 cell lines were used for dimensionality reduction, followed by SPADE on the tSNE dimensions. viSNE was also applied to 94 genes from two donors pre and post-stimulation with cytomegalovirus pp65 peptide pool. SPADE was applied to imaging data to identify distinct imaging features specific to regions in the image.

**Results:** viSNE is able to automatically separate both the Ramos and K562 cell lines using only a limited set of markers. Exported statistics from SPADE clusters generated from these cell subsets showed statistically significant changes between the two cell types. viSNE is also used to discover rare IFNG cell subsets specific to CD8+ T cells. For high-parameter imaging data, we found distinct imaging features using SPADE which aligned with known cell regions.

**Conclusions:** Characterization of single cell data is the first step prior to integrative data analysis. Dimensionality reduction tools such as viSNE and clustering algorithms such as SPADE can be used to automatically identify different cell subsets in other data types in addition to flow and mass cytometry.

**261/B130**

**High Content Cytometry: Proof of Concept and Practical Considerations with the Fortessa X50**


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**Background:** With the recent integration of the new Fortessa platform at University of Nebraska Medical Center, we are working towards optimizing a 31 parameter analysis. The ability to combine multiple fluorochromes has traditionally been constrained by hardware capabilities and reagent availability. At UNMC we have made progress towards this 31 parameter analysis due to developments in, and access to, new hardware and novel reagent technology.

**Methods:** To achieve this there are several accomplishments that have been made. First, the instrument itself; electronics and interface to acquire ~30 simultaneous parameters. Second being access to novel reagents. The instrument development coupled with the reagent technology has allowed acquisition of more than 18 simultaneous fluorochromes. We generated bead data to show proof-of-concept for high content cytometry using conventional flow cytometry (cFCM) technology. We present our progress with cellular acquisition of high-content cytometry.

**Results:** We have successfully generated a 27 parameter analysis using beads, demonstrating proof-of-concept for high content cytometry using cFCM technology. In addition, we reveal differences with LSRII data that have led to practical considerations in experimental design, data acquisition, and analysis.

**Conclusion:** The new Fortessa X50 platform and reagent technology holds much promise for 1) flexibility in panel design, 2) achieving high content cytometry data using cFCM technology, and 3) because of the conventional approach, a future vision for this technology is the potential for cell isolation. To date, the instrument and reagent technology is in early stage development; very few specialized and well-supported labs are capable of generating biologically relevant, high content cytometry data using cFCM technology. Here we demonstrate the feasibility and promise the Fortessa X50 platform holds, and highlight some practical concerns from a shared resources facility perspective.

**HIGH THROUGHPUT INSTRUMENTATION (B131 – B132)**

**262/B131**

**A High Throughput Method for Generating Uniform 3D Coculture Tissue Models**

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Adaptation of cancer cells to an evolving microenvironment is a crucial aspect of malignant progression. Cells depend on vascular nutrient and waste transport to maintain metabolic and physiological requirements. However, increasing tumor mass creates multiaxial gradients in extracellular pH (pHₑₓ), extracellular oxygen (O₂), and the transcription factor hypoxia inducible factor 1α (HIF-1α). Exactly how these gradients are regulated and their effects on cellular proliferation, metabolism and viability are currently open questions. Producing uniform and controllable in vitro 3D perfusion models of tumors with integrated chemical nanosensing capabilities is critical for discovering how interacting microenvironmental parameters (pHₑₓ, O₂) influence cellular pathophysiology (HIF-1α). Improved methods are needed for generating such 3D models containing multiple cell types and multiplex chemical nanosensors for controllable in situ assays of chemical, metabolic and physiological microenvironments. We are developing new instrumentation that will provide spatially correlated measurements of these gradients, allowing mechanistic investigation of the relationships between pHₑₓ, O₂, and HIF-1α.

As a first step in this design process, we have developed a high-throughput instrument that builds upon the concepts of cell sorting to produce uniform, cell-encapsulating microspheres with integrated optical nanosensors. The instrument uses microfluidic
devices with defined orifice diameters (70-200 μm), pressure driven fluid flow of viscous solutions, and high frequency acoustics tuned to produce uniform droplets in air. The cell-encapsulating biomaterial consists of sodium alginate (ALG) and polyethylene glycol (PEG) in buffer. The ALG/PEG droplets crosslink by introduction of calcium ions in a solution that contains interface stabilizing chemicals, dextran (DEX) and Pluronic F-127. Varying the orifice diameter, the flow velocity (up to 10 mL/min) and piezoelectric frequency (1-40 kHz) controls the size of the droplets in air (70-300 μm diameters). In order to determine initial cell-encapsulation concentrations for droplets initiated without internal microenvironmental gradients, we measured the doubling times of HIF-1α wild type and knockout cells in monolayer coculture using green (WT) or red (KO) membrane dyes. Using flow cytometry to identify the two cell types in a mixture provides the ability to measure growth in coculture by both dye dilution and differential counting. In uniform culture conditions, the cells have doubling times of 11-13 hours when measured by cell counts or dye dilution. These results are informing the design and implementation of a droplet-packed perfusion cell culture system that will produce a controllable and quantitative model of the chemical, metabolic and physiological gradients produced by cellular metabolism in a tumor.

263/B132
High Throughput Single Cell Gene Expression Analysis Using SH800Z Cell Sorter
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Background: Historically, flow cytometry of multivariate populations has involved analysis of cellular phenotype at the population level rather than at the single cell level. Therefore, genetic analysis of populations sorted by flow cytometry used mean values from a heterogeneous population. The recent emphasis on developing methods for studying cells at a single cell level underlines the importance of single cell analysis. High throughput methods that offer the ability to mine data on a larger scale are especially important for single cell analysis.

In this study we demonstrate the utility of sorting and single cell gene expression analysis of single cells in a 384 well plate format using SH800Z cell sorter.

Methods: In the single cell analysis method, to avoid loss by DNA purification sample volume should be reduced the minimum possible as less as possible. As a result sorting target becomes smaller and accuracy of sorting position is more important for achieving accurate single cell gene expression analysis downstream of cell sorting. To realize the accurate sorting position SH800Z utilizes an angled sort angle plate holder and high precision plate stage control unit. Additionally automatic plate calibrating function and user friendly manual sort position adjustment function in SH800Z software can realize more precise positioning. Another element that is indispensable for high throughput analysis is high speed measurement system. The time required to sort into 384 wells is not dominated by sorting speed but by speed of plate stage movement and communication with the analysis software. SH800Z realizes high speed plate sorting with intelligent control software and high speed stage.

We demonstrate an amplification-free single-cell qPCR (ref 1) result as high throughput single cell analysis example by using SH800Z 384 well plate sorting function. Single cells of mouse ES cell were sorted to 192 wells of 384 well plate and one drop of sheath liquid without cells was sorted into 92 wells between the wells in which single cells were sorted. Gnb gene expression of these single cells and sheath drops were analyzed with negative controls and standard controls by qPCR analysis.

Results: In the wells where single cells were sorted, we observed that 96.9% (186/192) scored positive for Gnb gene expression. Moreover, none of the wells containing empty sheath drops scored positive for Gnb gene expression (0/96). This result shows that the 384 well plate sorting function of SH800Z sorter and amplification-free single-cell qPCR analysis are together a good high throughput tool for accurate single cell analysis.

Keywords: Single cell analysis, SH800Z cell sorter, High throughput, 384 well plate, Amplification-free single-cell qPCR

Reference:

High Throughput Screening (B133 – B134)

264/B133
High Throughput Flow Cytometry Screening 4 Plates and 1536 Wells at a Time
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Background: In 2012, we documented performance of the Cluster Cytometer platform, 4 networked flow cytometers used for high throughput HyperCyt analysis of samples displayed in high-density, 1536-well plate format (Cytometry A 81: 419, 2012). The automated platform consisted of 4 BD Accuri C6 flow cytometers integrated with an autosampler that sampled 4 contiguous 384-well segments of a 1536 well plate in parallel. Although 1536-well plates are suitable for assay volumes of <10 μL/well, some high throughput screening (HTS) assays are more optimally performed in larger volumes for which 384-well plates are more compatible. A case in point was a novel high throughput flow cytometry assay for assessing non homologous end joining (NHEJ) mediated repair of DNA breaks, and its use to detect selective repair pathway inhibitors.

Methods: To speed 384-well plate HTS we enabled the Cluster Cytometer platform for parallel processing of 4 separate plates. A 3-D printer was used to custom fabricate 1) 4 plate mounting stations on the autosampler deck amenable to access by an Agilent plate handling robot and 2) an autosampler arm assembly for positioning sample uptake probes in matching well locations in a quartet of plates. Custom software was also developed to enable Agilent automation software to communicate with and control the Cluster Cytometry platform. For HTS, a cell based NHEJ repair assay was performed in 20 μL/well volumes in which GFP expression signaled repair of DNA breaks induced in a chromosomally integrated reporter construct. Cell viability was independent of DNA repair status and assessed by cell count shifts between live and dead cell light scatter populations. Hit compounds produced > 60% decrease in GFP fluorescence intensity with > 60% cells remaining viable.

Results: In HTS of the NCI NoXt Diversity Set (83, 536 compounds) 10-12 minutes was required for parallel analysis of each 4-plate set (~128-154 wells/min, 1536 wells/set). Robot transport of plates between autosampler deck and CO2 incubator required ~5 additional minutes during which plates were unsealed, vortexed and barcode-registered in transit to the autosampler and rescaled on the return trip. Screening statistics for each 4-plate set (Z' values, fluorescence compound anomalies, etc.) were calculated within 15 min post-analysis to immediately identify aberrant plates requiring remedial reanalysis. In HTS of 262 plates, control well cell viabilities were 87 ± 10% and Z' values were 0.69 ± 0.10 (mean ± SD) with a range of 0.50 to
0.90. HTS identified 726 active compounds (hit frequency = 0.87%) that are currently undergoing confirmation testing.

**Conclusions:** A fully integrated platform is now operational for flow cytometry HTS in 1536-well plates or 4 at a time in 384-well plates with robustness in quality and repeatability critical for the HTS environment.

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**265/B134**

A Novel Approach for In Vitro Characterization of Anticancer Effects of Experimentally Selected Mixtures of Amino Acids and Other Small Molecules

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The high-throughput, automated, microscope based imaging systems can be increasingly useful to examine the biological activity in single cells, cultures or (even) whole organisms following treatments with even thousands of the (pharmaceutical) agents.

Culevit™ is a mixture of amino acids, vitamins and other small molecules which was shown earlier to improve the quality of life of cancer patients. Moreover, it had some toxic effect in vitro on many tumorous cell lines and could induce apoptosis of the cancer model cells applied. The in vivo antitumor effect of Culevit™ was also demonstrated earlier in numerous tumor models.

In this study we aimed to show that Culevit™ has no major toxic effects on non-tumorous cells. Therefore, we analyzed the survival of a green fluorescence protein (GFP) expressing A549 lung carcinoma cell line co-cultured with primary cells isolated from different origin using our fully motorized fluorescence microscope platform (Olympus IX83-ZDC2 equipped with scanR™ software system). That instrument works as a high content screening (HCS), image based flow cytometer.

Objects cultured in 96-well plates were exposed to different formulations and/or doses of active (AM) or control (CM) mixture(s). That selection of the components and the related parameters of exposure applied were determined earlier. Following the treatment the living cells with or without cell nuclei staining were subjected to HCS platform.

Using scanR™ the recognized cells/objects could be classified and gated according to both their morphology such as the size, shape or circularity and/or the related fluorescence intensity as well. Gates or the classified group of objects, a reference gallery of objects of the selected area can be easily viewed and manually reviewed for accuracy of the gate settings. Data analyses revealed that AMs had no negative, cytotoxic effects on normal primary cells, but significantly led to the decrease of GFP expression on those cells.

The HCS microscopy results were simultaneously confirmed by similar, conventional flow cytometric measurements on the enzymatically detached objects.

That HCS method may be used for the large-scale screening of the anticancer drugs on intact, living primary cells and/or the related co-cultures. Contemporarily, our results may strengthen the proofs about the promising contribution of Culevit™ in the effective treatment of cancer in the near future.

**Image Processing and Analysis (B135 – B140)**

**266/B135**

Infrared Multi-beam Multi-photon Striped-Illumination Microscopy Based on a Blind-Illumination-Like Algorithm Allows for Deep-Tissue In Vivo Super-Resolution Imaging

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Adult mammals are the only realistic and useful models of us, humans, to study pathogenesis, to develop innovative therapeutic strategies, investigate drug effects and finally to heal diseases. Fluorescence microscopy proved for almost a century now to retain the potential of decisively helping us on this quest: investigating the motion of life. With the development of multi-photon microscopy new insight regarding cellular motility and interaction in the living organism could be gained, in health and disease. However, the major challenge to find the trade off between spatial and temporal resolution still remains to be overcome (Chen et al., Science, 2014). Super-resolution and nanoscopy technologies revolutionized our view on fluorescence imaging by enhancing our vision down to single molecule level even in living cells. On the other side, adaptive optics and technologies based on light-sound interaction have done the same by expanding our insight into tissue beyond the limits of visible or even near-infrared radiation. Still, bringing together the needs of biomedical research to visualize life in adult mammals and the capacities of fluorescence microscopy requires further technological development. We propose here a blind-illumination-like approach for multi-beam multi-photon striped-illumination microscopy, initialized by the previously acquired raw striped-illuminated images and by the image calculated by a Min-Max algorithm, as previously discussed by us (Andresen et al, PLoS ONE, 2012; Cseresnyes et al, JoVE, 2014). Our new approach retrieves super-resolution 3D images in deep-tissue by combining a genetic algorithm with weighted contributions of wave-front distortions and a gradient algorithm for elastic high-frequency scattering. We compare our results obtained with the Min-Max algorithm (20% better lateral and 2.7x better axial resolution) with those achieved with the new approach (1.7x better lateral and 2.7x better axial resolution) on different tissue samples, e.g. agarose or spleen tissue containing randomly distributed 100 nm red fluorescing beads. The power of our new approach becomes striking especially in deep-tissue, in which the pattern illumination is strongly modified by scattering and wave-front distortions as we demonstrated by imaging the whole heart of zebra fish embryos, brain slices and, finally, highly scattering spleen tissue. We additionally employed longer excitation wavelengths (1020 nm or 1050 nm as compared to 850 nm) to multi-beam multi-photon striped-illumination microscopy to further reduce the effect of high-frequency scattering on spatial resolution and, thus, on optical performance in general. Hence, we achieve improvement of lateral and axial resolution beyond the diffraction limit in deep tissue layers (beyond 100 µm depth) and a higher penetration depth (over 300 µm depth in spleen tissue) as compared to conventional multi-photon microscopy.

**267/B136**

Reducing Dimensions While Keeping Information in Image-Based Screening

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Background: Compared to flow cytometry, multi-well image based experiments makes it possible to observe the effects of many different treatments in parallel using very small volumes of drugs. The acquired image data holds a wealth of information on how individual cells were affected by the presence of a specific drug, and a number of digital image processing algorithms have been developed to quantitatively extract this information. Yet, once the data has been collected, the problem is far from solved, as the richness in information mixed with noise and artifacts provide a great data-mining challenge. We present a high-throughput screening, high-content analysis project where the goal is to find therapeutically relevant regulatory differences between cell lines.

Methods: Cancer stem cells from glioblastoma patients are continuously collected and established cell lines are characterized by a number of different molecular assays. Each cell line is exposed to 250 different drugs at 11 doses, stained with a nuclear and a cytoplasmic marker, and imaged with fluorescence and bright-field microscopy, generating tens of thousands of images of millions of cells. We extract morphological descriptors of individual cell nuclei and their surrounding cytoplasmus using established as well as novel approaches. Rather than reducing per-cell measurements to per-well averages, we compare per-well population dynamics by approaches similar to those used in flow cytometry.

High doses of drugs kill all or most cells in a well and provide little information useful in cell biology. On the contrary, doses slightly lower than the killing dose are more likely to provide morphological clues on mechanisms of action. Using the same reasoning, very low doses of drugs will likely not induce relevant changes in cell morphology. It is often difficult to predict which dose may produce useful information and the quick solutions is to test a range of doses. Deep analysis of all doses is however cumbersome. We have therefore developed an approach to reduce the collected dataset by automatically selecting the drug-specific doses that are most likely to convey morphological differences that occur as cells are affected by drugs.

Results: We show that cell cycle dynamics are clearly observable based on per-cell measurements of DNA content. Moreover, reproducible differences are observed between untreated cells from different cell lines. We also show that our approach for selecting drug doses efficiently reduced the data set to one tenth of its original size, while maintaining sufficient information to identify drugs that are known to alter cell cycle behaviour.

Conclusion: Image-based screening experiments have the potential to convey novel clues of mechanisms of action. Our presented methods is a first step towards mining large scale experiments for relevant regulatory differences between patient derived cell lines.

268/B137
Label-Free Sperm Morphology Assessment Using Imaging Flow Cytometry
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Assessment of sperm morphology is one of the most important steps in the evaluation of the health of the spermatozoa. A higher percentage of morphologically abnormal sperm is strongly correlated to lower fertility. Morphological image analysis can be used to segment sperm morphology, extract associated quantitative features, and classify normal and abnormal sperm in large quantity. In the vast majority of fertility clinics and laboratories, brightfield microscopy is used to evaluate sperm morphology. However, since sperm morphology contains a variety of shapes, sizes, positions, and orientations, the accuracy of the analysis can be less than optimal as it is based on a limited number of cells (typically 100–400 cells per sample). To overcome this challenge, we used ImageStream imaging flow cytometer to acquire brightfield and side-scatter images of porcine sperm samples at 60X magnification. We developed novel image algorithms to perform image segmentation in order to detect abnormal sperm cells using salient shape descriptors (invariant of scale, position, and orientation), such as diameter, circularity, elongation, corners, and negative curvatures. Taking advantage of the ability of the imaging flow cytometer to acquire images at a high resolution and speed, we demonstrate the validity of using image based parameters that can be adapted to each spectral image and features to assess sperm morphology in an objective and precise manner.

269/B138
New Tool for High Throughput Analysis of Topographic Fluorescence Intensity on Single Cell: Application in Fundamental Immunology
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Introduction: B cell activation is initiated by the binding of cognate antigen to B cell receptors (BCR). Antigen-BCR complexes accumulate in the center of the immunological synapse, from which they are extracted and targeted to endosomes. During cell cycle, activated B cells segregate its antigen (Ag) content asymmetrically, giving rise to two daughter cells with different interaction capabilities with CD4+ T “helper” lymphocytes, which could determine their fate.

The degree of asymmetry seems to depend on how the mother cell antigen content is distributed in its endosomal compartment. However, the study of this parameter is currently based on the Ag fluorescent quantification by microscopy, which don't allow for a rapid analysis on a large number of cells. This technological limitation currently limits our understanding of this phenomenon.

Objective: To evaluate ImageStream markII (ISX, Amnis-EMD millipore), an imaging flow cytometer that combines the speed of flow cytometry with the detailed imagery of microscopy analysis on single cell, to quantify the distribution of intra cytoplasmic fluorescence intensity.

Methods and Results: Purified B cells were incubated for 30 minutes at 37°C with Ag-coated fluorescent beads (400 nm diameter) at a ratio of 1/100. Beads bound to the surface but not internalized were identified by a staining at 4°C with an antibody against the Ag.

The resolution of the ISX (0.3 μm / pixels) is sufficient to determine, for each cell, the number of endosomal compartments loaded with Ag: i.e. intra-cytoplasmic fluorescent spots. A customized tool was designed using image J to measure the fluorescence of each endosome.

By combining the measurement of the fluorescence quantum for a bead and analysis of APC raw fluorescence data, we were able to accurately quantify the number of beads per endosome for each B cells.

Conclusion: ISX technology allow for high-throughput topographic analysis of single cell fluorescence intensity, a promising tool for a wide variety of scientific projects.

270/B139
Automated Analysis of Asymmetric Cell Division Using IDEAS® Image Analysis Software
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Asymmetric cell division occurs when macromolecules such as proteins are unevenly proportioned into daughter cells during
Cytotoxic Populations in American Cutaneous Leishmaniasis: Simple and Distinct Ways to Analyze

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Background: Flow cytometry is a scientific tool extensively used in the study of American cutaneous leishmaniasis (ATL). Because of lesions are the defining characteristics of the clinical course of the disease, it is important to determine the role of cytotoxic activity in lesions of patients with AML. We know that CD8+ lymphocytes play a key role in immunopathogenesis of this disease, although there is still controversy regarding the beneficial or deleterious cytotoxic effect of these cells. Moreover, other cell populations can play cytotoxic activity such as NK and NKT cells. In the majority of functional studies, researches analyze the data based on percentage or MFI of specific functional markers such granzyme, CD107a or cytokines. ThNKT cells. In the majority of functional studies, researches evaluate the role of cytotoxic populations in active ATL lesions. Methods: After processing the tissue sample, we stained the cells with anti-CD3, anti-CD8, anti-CD4, anti-CD56, anti-CD107a monoclonal antibodies, and with 7-AAD. We acquired the samples on MoFlo Astrios Flow Cytometer (Beckman Coulter) and analyzed on Kaluza 1.2 software. This staining panel allowed us to perform analyzes based on two different points of view. The first one was based on the frequency of six different populations: CD8+ and CD4+ T lymphocytes, CD4-CD8- double-negative T lymphocytes (DN) CD4+CD8+ double-positive (DP) NK cells and NKT and then we analyze the cytotoxic activity of them. The other one is based on the total frequency of cytotoxic cells and then the distribution of these six population based on CD107a+ gate.

Results and Conclusion: These distinct ways to perform cytotoxicity analyze allowed us to evaluate which cell population show the higher frequency of cytotoxic cells, and which population is more committed with cytotoxic activity. By analyzing the distribution of CD107a+ cells, we showed higher percentages of DN cells that are cytotoxic, especially if compared to NK and CD8+ T cells, which usually be considered the main cytotoxic cells. On the other hand, NKT cells are the most committed population to cytotoxic activity in ATL lesion. Here we designed a specific flow cytometric protocol in order to determine cytotoxic populations in lesion environment of ATL patients, which can be applicable to other diseases. We think that this careful and simple exploration of data allowed us to guide the answer of a variety of immunological enquires. Therefore, in order to optimize flow cytometric approach, we encourage flow cytometers and immunologists to explore multiparametric flow cytometry analysis to go farther than the expected results.

IMMUNE MONITORING (B141 – B153)

272/B141
Restoration of Foxp3+ Regulatory T Cell Subsets and Foxp3+ Type 1 Regulatory-Like T Cells in Inflammatory Bowel Diseases by Anti-TNF Therapy

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Background & Aims: A defect in regulatory T cells (Treg) is probably involved in the pathogenesis of inflammatory bowel diseases (IBD). Infliximab (IFX) is a chimeric anti-TNF monoclonal antibody. IFX therapy increases circulating Foxp3+ regulatory T cells (Treg) in patients with IBD rheumatoid arthritis, psoriasis and Behcet’s disease. Several subsets of Treg cells have now been identified. Resting and activated Foxp3+ regulatory T cells (Treg) can be differentiated from Foxp3+ effector T cells (Foxp3+ T eff) by the expression of Foxp3 and CD45RA. An IL-10 and IFN-γ double producing Foxp3+/− regulatory-like T cell (Tr1L) has also been identified in human blood. We have quantified these subsets of Treg cells in patients with active IBD as well as during anti-TNF therapy with IFX.

Methods: Blood samples were obtained from healthy controls (HC, n=54) and patients with active IBD, either before (n=62) and/or during IFX therapy (n=75). Treg cells were identified by immunofluorescent staining and FACS analysis.

Results: A numerical deficiency of circulating rTreg, aTreg and Tr1L in active IBD patients was documented. Clinical responses to IFX could be predicted by baseline levels of these Treg subsets. We documented an up-regulation of all 3 subsets during IFX therapy. Moreover, significant differences in Treg subsets were seen between responders and non-responders to IFX. Restoration of Tr1L correlated with the clinical and biological response(CRP) to IFX therapy. Trough serum level of IFX positively correlated with the proportion of aTreg and Tr1L during therapy. But, Foxp3+ Treg cells were not associated with the therapeutic effects of AZA or S-ASA.

Conclusions: A deficiency of circulating rTreg, aTreg and Tr1L is involved in pathogenesis. Successful IFX therapy differentially up-modulates the different types of Treg cells in blood of IBD patients. This effect might be relevant for understanding the mechanism of action of anti-TNF agents.

Keywords: Anti-TNF therapy; Foxp3+ Treg subsets; Type 1 regulatory-like T cells


273/B142

Immune-Regulatory Role of Garlic Protein on Endotoxin Induced Sepsis and Its Associated Multiple Organ Failure

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Sepsis is the reflection of systemic immune response that manifests in the sequential immune regulatory process with systemic infection and excessive production of inflammatory mediators that cause severe tissue injuries. Garlic is very well known immune modulator and immune regulatory role of garlic protein was observed in this new venture. This garlic protein also seems to be bio-active to moderate various cellular processes. Thus, this finding encourages us to evaluate immune regulatory response of garlic protein in the septic condition.

In this study, we have used bacterial endotoxin stimulated macrophages to evaluate the efficacy of garlic protein (Gp) towards the status of different Th 1 and 2 mediators (IFN-γ, TNF-α, IL-1β, IL-6, IL-4, IL-17, and IL-10). Gp also regulates transcriptional level of signal activation in inflammatory response with the associated of TLR4-CD14 expression. In vivo system, endotoxin induced murine sepsis model was used in focusing on multiple organ injury (Lungs, Liver, and Kidney) and status of different cytokine-chemokine(s). Due to the effect of Gp, endotoxin induced inflammatory cytokine-chemokine(s) levels were significantly altered with the involvement of functional organs. To test whether Gp affects activity of other T lymphocytes, we measured CD8+ T cell populations. The changes in vascular permeability factors affected the expression of cell adhesion molecules in septic mice that was evident by immunocytochemical approaches with the presence of Gp.

These findings indicate that Gp may be proven to be a potential immune regulatory agent against bacterial sepsis with associated organ injury.

274/B143

A Standardized Platform and Immune Monitoring Methods to Evaluate Immune-Mediated Oncology Biotherapeutics by Flow Cytometry

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Immune-mediated therapies have become a main focus for cancer research for a wide range of disease indications. Because of this, understanding the tumor microenvironment and the cells that play a role in the generation of anti-tumor responses has become a key aspect to Biopharmaceutical oncology research and clinical evaluation. Flow cytometry has been used for decades to aid in the diagnosis and measurement of patient responses in a variety of malignancies. New developments in Flow Cytometry instrumentation have enabled customized assays, methods, and acquisition and analysis templates which provide a standardized platform for testing the activity of immune-mediated oncology biotherapeutics. Researchers routinely use in vivo syngeneic mouse models to test biotherapeutics as a means to guide predictive biomarker development and test anti-tumor immune responses in humans. We utilized the CT-26 syngeneic colon carcinoma mouse model and an antagonistic antibody to drive a potent anti-tumor immune response leading to tumor regression. We show how a researcher can easily employ a toolkit of flow based immune monitoring assays aimed at measuring changes in cell phenotype, function, cytokine production and miRNA levels. This approach has allowed us to observe the effects on a single cell basis of an antagonistic monoclonal antibody on the immune infiltrate within the tumor microenvironment as well as the periphery. We observed changes in effector T cells, which correlate with the immunologic events involved in immune-mediated tumor killing. Our flow cytometry-based platform and method facilitates testing, not only a variety of therapeutics but also in characterizing many cell subsets which aid in the anti-tumor response.

275/B144

In-Depth Phenotyping of Healthy European Adults by 10-Color Flow Cytometry

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Background: LIFE (http://life.uni-leipzig.de) is a prospective population-based epidemiological study in Leipzig, where subjects undergo comprehensive medical examinations and biomaterial sampling to find new molecular and cellular markers associated with frequent civilization diseases, health status and lifestyle. LIFE is the first study for long-term immunophenotyping of healthy European subjects on the large scale. For identification of risk phenotypes, characterization of disease states and individualized therapy by cytomtery age- and gender matched reference intervals for leukocytes from blood are essential.

Methods: Immunophenotyping of 608 healthy German adults (age: 40 to 79 yrs; male:female 44%:56%) was performed by 10-color flow cytometry. Gender and age dependent reference values (male 40-59 yrs: 118; 60-79 yrs: 151 and female 40-59 y: 144; 60-79 y: 195) for 15 different leukocyte subsets were determined. Experimental setup was detailed elsewhere (OMIP-023: Cytometry A. 2014;85:781). Reference intervals (90% CI for lower and upper limits) were calculated based on the recommendations of the International Federation of Clinical Chemistry for males and females. Gender differences of the cell counts were analyzed using the Mann-Whitney U test, and age dependent differences using log-linear regression analysis (R2: 0.3-0.7), with p-values < 0.05 considered as significant.

Results: Gender related differences were found for monocytes, T-cells, NK cells and B-cells: Males had significantly higher non-classical (p<.001) and classical (p<.005) monocyte counts, as well as higher NK cells counts, whereas women had higher counts for B cells (p<.001) and CD4+ T-cells (p<.001), as well as for CD4+ regulatory T cells (p<.008). The strongest age-related changes were found for T-cell subsets. CD4+ CD8+ T cells (p=.002) and CD4+ regulatory T cells (p=.009) significantly decreased with age in males, whereas these trends were p=.191 and p=.038 only marginally in females. Other age effects, such as a decrease of neutrophils with advanced ages in females (p=.022) and B cells in males (p=.089) were only weak. But, for both genders with increasing age a decrease in CD8+ T-cells (8-9% within five years; p=.001) and an increase in NK cells (males
4 % within five years; p=0.014 and females 3 %; p=0.061) were observed.

Conclusion and Outlook: The significantly higher absolute NK cell counts in healthy elderly of the LIFE study is in accordance with the literature. Especially the differences between both genders might indicate different abilities to respond to infections. In the LIFE Follow-up these ageing effects (T cell drop and NK cell increase) will be investigated and confirmed in longitudinal studies.

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276/B145
Polyfunctional Response of Invariant Natural Killer T Cells in Patients Affected by Multiple Sclerosis Displays Th-1 and Th-17 Profiles
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Introduction: Multiple Sclerosis (MS), an autoimmune disease with neurodegeneration and inflammation, is characterized by several alterations of different T cell subsets, including iNKT. In this study we investigated the phenotype and polyfunctional response of iNKT cells in different forms and treatments of MS.

Methods: We studied a total of 123 patients followed by the MS Center (NOCSAE, Modena, Italy), 91 with a Relapsing Remitting form (RR), 20 with treated with interferon 1-b (IFN), 20 with natalizumab (Nat), 19 with glatiramer acetate (Gla), 32 without treatment. Thirty-two had a Progressive MS: 16 Primary Progressive (PP), 16 Secondary Progressive (SP), Thirty-five age- and sex-matched subjects were used as healthy controls (CTR). CD3+ T cells were volumetrically counted using a CyFlow Counter (Sysmex, Italy). Isolated peripheral blood mononuclear cells (PBMC) were stained with anti-VA24-18 TCR, CD4, CD8, CD161, CD3, CD19 and CD14 mAbs. We then analyzed the capacity of iNKT cells to produce up to 4 cytokines simultaneously in 18 RR (11 treated with IFN and 7 with Nat and 7 treated with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with simultaneous in 18 RR (11 treated with IFN and 7 with Nat and 7 treated with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with IFN), 4 PP, 6 SP and 16 CTR. For this, PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with T-helper cells expressing CD4+ produced the highest level of TNF-a. In these patients, mainly showed Th1 and Th17 cytokine production. This could suggest that the progressive phase of the disease is characterized by a permanent iNKT activation. RR patients treated with IFN-1b produce higher levels of TNF-a probably as a compensatory mechanism. Thus, immune regulatory effects of IFN-1b could be, at least in part, mediated by a modulation of the activity of iNKT cells.

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277/B146
Real-Time Cytometric Assay of the Interaction between Nitric Oxide and Superoxide Anion in Peripheral Blood Leucocytes: A No-Wash, No-Lyse Kinetic Method
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Background: Nitric oxide (NO) and its related reactive nitrogen (RNS) and oxygen (ROS) species are crucial in monocyte responses against pathogens, but also in inflammatory conditions. Central to both processes is the generation of the strong oxidant peroxynitrite (ONOO) by a fast reaction between NO and superoxide anion. ONOO is a biochemical reaction for ROS- and NOS-cytotoxicity and causes protein nitrosylation. Circulating by-products of protein nitrosylation are early biomarkers of inflammation-based conditions, including minimal hepatic encephalopathy in cirrhotic patients. In this context, we have designed a novel no-wash, no-lyse real-time flow cytometry assay to detect and follow up the NO- and superoxide-driven generation of ONOO in peripheral blood monocytes of normal donors and cirrhotic patients.

Methods: Whole blood samples were stained with CD45 and CD14 antibodies plus each of a series of fluorescent probes sensitive to ROS, NO, glutathione (GSH), namely 5-Amino-5-Methylamino-2',7'-difuoro fluorescein diacetate (DAF-FM DA), dihydrodihydramine 123 (DHR), MitoSOX Red, Dihydroethidium (DHE) and 5-chloromethylfluorescein diacetate (CMFDA) plus a viability marker. Samples were exposed sequentially to NO1, a NO donor, and three different superoxide donors, while analyzed in real time by kinetic flow cytometry. Relevant kinetic descriptors, such as the rate of fluorescence change were calculated from the kinetic plot in the different leucocyte populations gated on the basis of light scatter and CD45/CD14 expression.

Results: The real-time generation of ONOO, which consumes both NO and superoxide, led to a decrease in the intensity of the cellular fluorescence of the probes sensitive to these molecules in all leucocytes from samples preincubated with superoxide donors and then treated with NOR-1. In addition, the NO generation in samples treated with NOR-1 was rapidly reversed by the addition of superoxide donors. Further analysis with fluorescent probes sensitive to ROS and GSH showed striking differences among lymphocytes, monocytes and granulocytes regarding the response to NO and superoxide, indicating a different regulation of oxidative pathways in those cells.

Conclusion: This is a fast and simple assay that may be used to monitor the intracellular generation of ONOO and to explore ROS and NOS metabolism in blood cells.

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278/B147
Role of Naive-Derived T Memory Stem Cells in T Cell Reconstitution following Allogeneic Transplantation
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Early T cell reconstitution following allogeneic transplantation depends on the persistence and function of T cells that are adoptively transferred with the graft. Post-transplant cyclophosphamide (pt-Cy) effectively prevents alloreactive responses from unmanipulated grafts, but its effect on subsequent immune reconstitution remains undetermined. Here we show that T memory stem cells (TSCM), which demonstrated superior reconstitution capacity in preclinical models, are the most abundant circulating T cell population in the early days following haploidentical transplantation combined with pt-Cy, and precede the expansion of effector cells. Transferred naive, but not TSCM or conventional memory cells preferentially survive cyclophosphamide, thus suggesting that post-transplant TSCM cells originate from naive precursors. Moreover, donor naive T cells specific for exogenous self/tumor antigens persist in the host and contribute to peripheral reconstitution by differentiating into effectors. Similarly, pathogen-specific memory T cells generated detectable recall responses but only in the presence of the cognized antigen. We thus define the cellular basis of T cell reconstitution following pt-Cy at the antigen-specific level and propose to explore naïve-derived TSCM cells in the clinical setting to overcome immunodeficiency.

279/B148
Changes of Monocyte HLA-DR Expression, IL-6 and LPS Induced TNF Levels in Different Anticoagulated Whole Blood Samples over Time under Various Temperature Conditions
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Background: LPS induced monocyte TNF-α secretion, constitutive Interleukin 6 (IL-6) expression and monocyte HLA-DR expression are important monitoring parameters in sepsis. Reduced HLA-DR expression, diminished TNF-α production and high IL-6 levels are indicative for a bad prognosis. The time of the analysis of these parameters after patients blood sampling potentially varies. Therefore the aim of the present study was to investigate whether the specified parameters will change over time in stored whole blood samples using different anti-coagulants and different temperature conditions.

Methods: EDTA, citrate as well as heparin anti-coagulated whole blood was drawn from healthy individuals (n=7 for each anti-coagulant) and was processed immediately or stored for 4h, 8h, 24h and 48h at 4°C and 21°C. After the indicated time points blood was stimulated with LPS (0.5μg/ml) and TNF-α and high IL-6 levels are indicative for a bad prognosis. The time of the analysis of these parameters after patients blood sampling potentially varies. Therefore the aim of the present study was to investigate whether the specified parameters will change over time in stored whole blood samples using different anti-coagulants and different temperature conditions.

Results: EDTA, citrate as well as heparin anti-coagulated whole blood was drawn from healthy individuals (n=7 for each anti-coagulant) and was processed immediately or stored for 4h, 8h, 24h and 48h at 4°C and 21°C. After the indicated time points blood was stimulated with LPS (0.5μg/ml) and TNF-α and high IL-6 levels are indicative for a bad prognosis. The time of the analysis of these parameters after patients blood sampling potentially varies. Therefore the aim of the present study was to investigate whether the specified parameters will change over time in stored whole blood samples using different anti-coagulants and different temperature conditions.

280/B149
Case Study Report: Severe Deficiency of Memory B Cells and Expansion of CD3+CD8+CD16bright T Cells Detected by Polychromatic Flow Cytometry
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Background: Common variable immunodeficiency (CVID) is defined as a heterogeneous group of disorders characterized by low levels of serum immunoglobulins and increased susceptibility to infections. It is considered to be the most frequent clinically symptomatic primary antibody disorder, induced by intrinsic B cell defect, T cell defect, mutations in TNF receptors or without a known genetic defect. Diagnosis should be suspected in cases of recurrent sinopulmonary infections exceeding an age-specific frequency and is based on exclusion of other causes of hypogammaglobulinemia. Due to recurrent sinopulmonary infections and previous diagnosis of immunodeficiency disorder (CVID D83.0), 17-year old boy was admitted to our hospital. Family history showed no abnormalities. He showed frequent urinary and respiratory infections from infancy and was hospitalized at the age of 9 months due to agranulocytosis, hypo IgG (1.5 g/l) and sepsis caused by Pseudomonas. From the age of 12 months he is repeatedly receiving immunoglobulin substitution therapy.

Methods: Comprehensive immunophenotyping of whole blood was performed using polychromatic flow cytometry. Peripheral whole blood samples were obtained from patients with the antibodies for cell surface markers CD45, CD3, CD4, CD8, CD19, CD56 and CD16 and for the two activation markers HLA-DR and CD25. Samples were lysed and fixed with 1x FACSLysing solution (BD Biosciences, USA). Upon centrifugation, leukocyte pellets were resuspended in 1% paraformaldehyde/PBS. Acquisition of samples was performed using the Navios flow cytometer (Beckman Coulter, USA). Data was analyzed using the FlowLogic™ software package (Innava Technologies, Australia).

Results: Severe B cell and memory B cell deficiency was detected in four consecutive immunophenotypizations. In addition, noticeable increase in relative number of CD3+CD16+ T cells was detected with expanded CD3+CD8+CD16bright subpopulation which represents approximately 80% of total CD3+CD16+ T cells. In the two latest immunophenotypizations the onset of NK deficiency was also observed.

Conclusion: Suspected deficiency of the memory B cells was successfully confirmed for patient with CVID diagnosis using polychromatic flow cytometry. Expanded immune monitoring panel enabled detection of typical CVID immunophenotype and revealed accompanied NK deficiency and expansion of CD3+CD8+CD16bright cells. Since there are no data in literature about relationship between CVID and expansion of...
CD3+CD8–CD16hi/hi cells, further studies on CVID patients should include described approach in combination with assessment of switched memory B cells in order to get new insights into this complex immunodeficiency syndrome.

### 281/B150

**Polychromatic Flow Cytometry in Routine Immunophenotyping: More Colors May Reveal Hidden Immunophenotypes**

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**Introduction:** Monitoring changes in human immune cell populations such as lymphocytes, monocytes etc. in different clinical phenotypes is crucial. The distributed nature of the hematopoietic system makes it amenable to flow cytometric analysis. Today, both research and clinical laboratories addressing various immunological aspects heavily rely on flow cytometry for phenotypical and functional analyses of immune components in different disease settings, such as primary immunodeficiencies, as well as in the healthy immune system.

Flow cytometry is currently the platform of choice in analyzing the complex components of the immune system—i.e. to separately characterize many phenotypically and functionally distinct subsets of leukocytes, any of which might be clinically relevant. Multi-colour flow cytometry assays, therefore, need to be developed with great care in order to ensure reliability of data generated therewith.

**Aims and objectives:** The aim of this research was to analyze the phenotype of human peripheral blood mononuclear cell subsets (lymphocyte and monocyte) in the diagnostics and monitoring of primary immunodeficiencies (PID) using polychromatic flow cytometry assays.

**Methods:** Comprehensive immunophenotyping of whole blood was performed using polychromatic flow cytometry. Peripheral whole blood samples were stained with antibodies for cell surface markers: CD45, CD3, CD4, CD8, CD19, CD56 and CD16 as well as for two activation markers: HLA-DR and CD27. Samples were lysed and fixed with 1x FACSLysing solution (BD Biosciences, USA). Upon centrifugation, leukocyte pellets were resuspended in 1% paraformaldehyde/PBS. Acquisition of samples was performed using the Navios flow cytometer (Beckman Coulter, USA). Data was analyzed using FlowLogic™ software package (Inivai Technologies, Australia).

**Results:** Peripheral blood from more than 70 patients aging from 2-18 years was analysed in the same manner. Dual-panel platform that involved two six color antibody combinations and extensive analysis of FCS data files resulted with the finding of several interesting immunophenotypes: CD19mono–CD27mono– B cells, CD3+CD8+CD16hi/hi–T cells and CD3+CD4+–lymphocytes. In addition, three subpopulations of monocytes were resolved in each patient: CD14+hi/hi, CD16+14hi/hi, CD16+14dim and CD16+14hi.

**Conclusions:** Polychromatic flow cytometry using expanded immune monitoring panel in routine immunophenotyping enabled the detection of mononuclear subsets that could not be resolved in procedures where typical four-color immunophenotyping assays are used. In studies of immune disorders, such as primary immunodeficiencies, a polychromatic approach using even only six colors may allow deeper insight and understanding of the complex interplay among immune cells and improve diagnostic procedures.
284/B153

**Methodology Effect on Flow Cytometry-Based MHC-Multimer Assay**

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Immudex provides the MHC-multimer Proficiency Panel, for any laboratories with a wish to compare its performance to other laboratories, detecting antigen specific CD8 T-cells. This poster will assess results from the 2013 MHC-Multimer Proficiency Panel, evaluating the effect of new methodologies, accessory reagents, and protocols, used by the 51 different participating labs. The frequency of CD8 positive antigen specific T-cells, in 4 controlled donor sample were analyzed, and will be evaluated according to the differences in protocols used.

In particular, the use of gating reagents, such as anti-CD3, anti-CD4, live/dead staining, and the effect of inclusion or exclusion gating strategy will be assessed. Furthermore, an evaluation of the effects of used staining protocols, such as incubation time, temperatures, and number of washes, will be included.

A preliminary look on the results indicates that the MHC-Multimer assay is very robust. Interestingly, the different protocols applied seems not to have a major influence on the outcome of the multimer assay, which is surprising, and may be extrapolated to other flow cytometry assays.

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**IMMUNOLOGY (B154 – B171)**

**285/B154**

**Increased Expression of NKG2C on T Cells and Stable Plasma Perforin Levels in Rhesus Macaques Immunized with a Live-Attenuated Retroviral Vaccine Is Associated with Protection after Challenge with Pathogenic SIV**

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**Introduction:** Vaccines based on live-attenuated retroviruses confer the most effective protection in the SIV/rhesus macaque animal model, and offer the possibility of studying correlates of protection against infection with virulent virus.

**Methods:** We utilized a tether system for studying the acute phase of infection in naive macaques and in animals vaccinated with a live-attenuated SIV vaccine. The use of this tether system allowed for the frequent sampling of small blood volume in the absence of sedation or restraining of the animals, thus reducing the confounding effect of the stress associated with these sampling methods. We analyzed in blood the phenotype, proliferating and activating markers of lymphocytes by 5-color flow cytometry, and plasma cytokines using 32-plex Luminex assays

**Results:** We hypothesized that, in comparison to naive macaques, vaccinated animals would show a transient peak of inflammation followed by control of challenge virus. We previously reported that control of infection in vaccinated animals was associated with a small expansion of SIV-specific CTL responses. Here we analyzed the phenotype of immune cells and the systemic cytokine profile of the challenged animals. Surprisingly, vaccinated animals resisted infection and showed absence of the NK and CD8 T cell activation and proliferation, loss of memory CD4 T cells, and elevation of proinflammatory cytokines and perforin in plasma observed in naive animals. The only cellular changes observed in vaccinated animals after challenge were increases in the activating NKG2C receptor on the surface CD8 and CD4 T cells.

**Conclusions:** These studies indicate that cellular components of both the innate and adaptive immune systems of animals inoculated with a live-attenuated SIV vaccine respond to and control infection with virulent virus; additionally, our report underpins the utility of the tether system for the intensive study of acute immune responses to viral infections.

**286/B155**

**Regulatory T Cells Inhibit Efficient Antitumor Immune Responses by Setting Up a Trap for CTLs in Tumor Draining Lymph Node**

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Regulatory T cell (Treg cell) is one of the major mechanisms that are responding for tumor cells to escape from immune surveillance, whereas the precise mechanism underlying this is still poorly understood. Our previous study found that Treg cells could alleviate mouse allergic asthma symptoms by setting up a trap to capture effector T cells in lung draining lymph node. So, we wonder if Treg cells exhibit their suppressive effects to resist antitumor immune responses in the same way. We first found that CD4+ CD25+ FOXP3+ Treg cells in tumor draining lymph node (TDLN), but not that in non-tumor draining lymph node (NT-DL), are correlated with the process of tumor progression. And
position-specific deletion of Treg cells by injecting anti-CD25 (PC61.5) directly into TDNL could inhibit tumor growth in a mouse fibrosarcoma model. To further confirm the role of Treg cells in trapping CDB+ cytotoxic T cells (CTCs) in TDNL, we are now using confocal microscopy and two-photon microscopy to observe the behavior of CTCs after deletion of Treg cells and tumor cells in vivo. Once this mechanism that Treg cells occupying TDNL as a fortress to defend the tumor cells is confirmed, it will help us to better understand the pivotal role that Treg cell plays in anti-tumor responses.

287/B156
Determination of Alterations in the Cytoplasmic Concentration of Calcium Ions in Immune Cells upon Activation with Thymic Peptides
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Background: Alterations in cytoplasmic calcium concentrations ([Ca2+]i) play a key-role in signalling pathways following cell activation. Herein, we assessed to detect such changes, implicated in the intracellular signalling pathways downstream TLR-4 in immune cells, following their activation with the immunoreactive peptides prothymosin alpha (proTα) and its carboxy-terminal decapetide proTα(100-109). Both peptides signal through TLR-4, as previously shown by our research group.

Methods: Total human peripheral blood mononuclear cells (PBMCs), isolated monocytes and monocytes differentiated in vitro to immature dendritic cells (iDCs) and macrophages were activated with LPS (model ligand of TLR-4), proTα and proTα(100-109), directly or after 24 h incubation with the same molecules. Isolated neutrophils were challenged directly due to their short lifespan. The ionophore A23187 was used as positive control. Following loading of the cells with Fluo-4, cytoplasmic calcium alterations were recorded by flow cytometry over 120-180 sec.

Results: Direct challenge with LPS induced a measurable increase in [Ca2+]i, only in macrophages (MFI 30913 vs 18145 of the background). Moreover, macrophages incubated for 24 h with LPS and subsequently challenged with LPS, displayed acceleration of the appearance of the signal (at 30 sec vs 120 sec). Total PBMCs incubated for 24 h with LPS or proTα displayed a fast increase in cytoplasmic calcium levels after LPS challenge, accompanied by cell activation. Isolated monocytes (CD14+ cells) showed an immediate (starting at 30 sec) and significant (ca 2-fold) increase in [Ca2+]i, (max MFI 86291, 61896 and 58774, vs background 43731, 33897 and 33249, respectively). In contrast, samples containing the CD14- cells and iDCs exhibited no alterations in any incubation with LPS or proTα. Moreover, macrophages incubated for 24 h with LPS and subsequently challenged with LPS, displayed accelerated calcium ion influx. A23187 was used as positive control. Following loading of the cells with Fluo-4, cytoplasmic calcium alterations were recorded by flow cytometry over 120-180 sec.

Conclusion: We established a new flow cytometry method for monitoring calcium ion influx alterations implicated in the endocytic pathway of TLR-4. We showed that incubation of PBMCs for 24 h with LPS or proTα resulted in sufficient calcium ion influx alterations thereof. Moreover, incubation of monocytes and macrophages with proTα or proTα(100-109) generated an activating background, similar to that induced by LPS, which enables us to record [Ca2+]i alterations upon their subsequent stimulation with LPS.

288/B157
A Novel Approach to Measuring Cell Mediated Lympholysis Using Quantitative Flow and Imaging Cytometry
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In this study, we established a novel cell-mediated lympholysis (CML) using multiparameter flow and imaging cytometry. CML is an established in vitro assay to detect the presence of cytotoxic effector T-lymphocytes (CTLp) precursors. Current methods employed in the identification of CTLp are based upon the quantification of chromium (116Cr) released from target cells. In order to adapt the assay to flow cytometry, primary porcine lymphocyte targets were labeled with eFluo670 and incubated with fully major histocompatibility complex (MHC) mismatched effectors. With this method, we were able to detect target-specific lysis that was comparable to that observed with the 116Cr-based assay. In addition, the use of quantitative cell imaging demonstrated the presence of accessory cells involved in the cytotoxic pathway. This innovative technique improves upon the standard 116Cr release assay by eliminating the need for radioisotopes and provides enhanced characterization of the interactions between effector and target cells. This technique has wide applicability to numerous experimental and clinical models involved with effector-cell interactions.

289/B158
Assessing Autophagic Flux by Measuring LC3, p62 and LAMP1 Co-localization Using Imaging Flow Cytometry
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Autophagy is a catabolic pathway in which normal or dysfunctional cellular components that accumulate during growth and differentiation are degraded via the lysosome; for instance, during starvation it is a survival mechanism that reallocates nutrients from unnecessary processes to more vital processes in the cell. In autophagy, cytoplasmic LC3 protein is processed and recruited to the autophagosomal membranes. The autophagosome then fuses with the lysosome to cause the breakdown of the autophagosomal vesicle and its contents. The ubiquitin-associated protein p62 which binds to LC3 is also used to monitor autophagic flux. Immunofluorescence microscopy has been used to visually identify LC3 puncta, p62 and/or lysosomes on a per-cell basis; however, an objective and statistically rigorous assessment can be difficult to obtain. To overcome these problems, the ImageStreamX Mark II imaging cytometry platform was used to collect large numbers of cell images. Using a new analytical feature (tri-colocalization) the localization of 3 autophagy markers; LC3, p62 and lysosomal LAMP1 could be assessed in an objective, quantitative, and statistically robust manner. The ability to co-localize 3 markers of autophagy in a single assay will lead to novel insights into the induction and regulation of autophagy.
290/B159
Rapid Recovery from Severe H7N9 Disease Requires a Diversity of Response Mechanisms Driven by CD8+ T Cells

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The avian origin A/H7N9 influenza virus causes high hospitalisation rates (>99%) and mortality (>30%), with ultimately favorable outcomes ranging from rapid recovery to prolonged hospitalisation. Using a novel multi-colour assay for monitoring adaptive and innate immunity, we dissected the kinetic emergence of different effector mechanisms across the spectrum of H7N9 disease and recovery, to find that a diversity of response mechanisms contribute to resolution and survival. Immune effector cells were quantified in 160 H7N9 patients and the patients were divided into three recovery groups, R1(d14-d18), R2(d21-d27), R3(d31-d35) and RD(fatal outcome). Immune analysis of PBMCs used a 13-colour IFN-γ-based flow cytometric assay that quantified H7N9-specific CD8+ CTLs and CD4+ TH cells, NKT cells, γδ T cells and MAIT cells following incubation with the H7N9 virus and subsequent 18-hour cell culture. Patients discharged with 2-3 weeks had an early peak in H7N9-specific CD8+ T-cell responses, while individuals with prolonged hospital stays had late recruitments of CD8+CD4+ cells and antibodies simultaneously/recover by week 4), augmented even later by prominent NK cell responses(recovery>30 days). In contrast, those who succumbed had minimal influenza-specific immunity and little evidence of T cell activation. In general, our data suggest that in the absence of substantial pre-existing, H7N9-specific CD8+ T-cell memory(R1 group), the recruitment of naive (or low frequency memory) CTLs, together with TH cells, Nabs and NK effectors, provides a protective mechanism that becomes more obvious with the persistence of severe disease in those who will eventually recover. Our study illustrate the importance of robust CD8+ T-cell memory for protection against severe influenza disease caused by newly-emerging influenza A viruses.

291/B160
Development of a Polychromatic Flow Cytometry Panel for Analysis of Antigen Presenting Cell Populations in Human Blood and Lymph Nodes

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Background: Recent data have begun to classify the distinct subsets of dendritic cells (DCs) and other professional antigen presenting cells (APCs) in human tissues and polychromatic flow cytometry has played a major role in making this possible. The DC compartment, whether in blood or tissue, is significantly heterogeneous and is reported to require a minimum of 8 fluorochromes to distinguish these different cell types. As well as identifying these populations with the minimal characterising antigens, it is also of great interest to be able to further decipher the hierarchy, especially in tissue, as well as track these populations following short in vitro challenges. We have developed a 15 colour panel to identify and track activation of the major APC populations (eg monocytes, dendritic cells, macrophages) applicable to both human blood and tissue. Simultaneous detection of up to 15 colors is achieved by careful selection and testing of cell-subset-defining antibodies in various fluorochrome combinations to first establish a base panel containing 11 colors. This base panel can then be used to identify and compare the APC populations in human blood with those found in tissues such as lymph node and liver. APC populations in blood are largely well characterized, however APCs found in tissue are likely more heterogeneous and therefore require further markers for their characterization. In addition, this panel can be used to test the targeting of FITC-labelled candidate vaccine constructs to APC populations both in the blood and tissue. This 15 color panel allows for an 11 color base panel of key antigens, a FITC-labelled construct, plus 3 activation markers or alternatively, ‘drop-in’ positions for other antigens of interest.

Methods: A number of antibodies were first tested to develop an optimized base panel of 11 antigens. The antigens required to detect all populations were CD14, CD16, BDCA2 (CD203), CLEC9a, HLADR, lineage cocktail (CD3/CD19/CD20/CD56), CD11c, CD11c, CD34 and CD45. DAPI was used as a viability marker. CD80, CD86 and CD83 were also included in the panel optimization to assess activation/maturity and various macrophage markers were also assessed for tissue APCs. As CD11c is a key DC marker, 3 different antibody clones (3.9, Bly6, Bu15) were tested. Panel optimization was performed on a 4 laser BD SORP FACS Aria II (5 blue, 3 red, 2 UV, 7 violet) using optimized voltages and application settings for day-to-day consistency.

Results: Using commercially available antibodies, we have optimized a panel for the identification and characterization of the major APC populations in human blood and tissue. We did this by first training a number of antibodies, assessing the stain index achieved and identifying any problematic fluorophores and staining combinations. An 11 color base panel was first established to allow flexibility in testing earlier antigens. We also revealed that the choice of antibody clone for CD11c is important for maximum staining index.

Conclusions: We have optimized an 11 color base panel to identify and characterize the major APC populations in human blood and lymph nodes. This panel allows for up to 15 colors to be detected simultaneously with the addition of macrophage markers for deeper characterization in tissue or activation/maturity markers, depending on the application required.

292/B161
Modulation of Regulatory T Cell by Differential Ratios of Fish Oil and Corn Oil in Experimental Colon Carcinoma

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Introduction: Regulatory T cells have been reported to play an important role in cancer by suppression of anti-tumor immune response. The involvement of these cells in colon cancer can help in understanding chemoprevention of cancer by natural dietary polyunsaturated fatty acids (PUFA).

Objectives: This study was designed to understand the effect of different ratios of fish oil (FO) and corn oil (CO) on induced T regulatory cell populations in colon carcinoma.

Methodology: Male Wistar rats were divided into six groups: Ethylenediamine-tetra acetic acid (EDTA) treated group, dimethylhydrazine-dihydriodichloride (DMH) treated (DMH/week for four weeks), FO:CO(1:1)+EDTA, FO:CO(1:1)+DMH, FO:CO(2:5)+EDTA, FO:CO(2:5)+DMH. The animals were studied for initiation (4 weeks) and post initiation (16 weeks) phases. nTregs(CD4+CD25+Fox3) and intracellular cytokines in CD4+CD25+ T cells were analyzed in intraepithelial lymphocytes (IELs).

Results: Treatment with DMH resulted in nTregs reduction in both the phases in comparison to the EDTA treated group. Group receiving FO:CO(1:1)+DMH treatment showed considerable increase in nTregs in post initiation phase only but FO:CO(2:5)+DMH treatment exhibited substantial elevation of nTregs population in both the phases. Intracellular cytokines (IL-4, IL-6) in CD4+CD25+ T cells increased considerably on DMH treatment in both the phases, however their expression reduced in post initiation phase in FO:CO(2:5)+DMH treated groups.
Conclusion: Fish oil affects the number of regulatory T cells and cytokine expression in isolated IELs in rat colon cancer model in dose and time dependent manner.

293/B162
PKCθ Is Involved in Macrophage Polarization Both Directly and by Affecting T Cell/Macrophage Cross-Talk
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Background: PKCθ is a kinase involved in T cell activation, proliferation and survival, thus identified as a potential pharmacological target to treat immune disorders, such as muscular dystrophy. Besides T cells, essential for orchestrating systemic immune response, it is increasingly becoming evident that macrophages, CD25+ T cells and T cells cooperate in determining local inflammatory milieu. Macrophage can polarize in a wide and continuum spectrum of phenotypes ranging from the pro-inflammatory (M1) to the anti-inflammatory (M2) phenotype, different in terms of function and cytokines expressed. Moreover, T cell/macrophage cross talk can affect each other phenotype, thus the inflammatory environment and the onset/progression of the immune response. Although PKCθ role in T cells has been widely described, there is a lack of information on its involvement in the above mentioned mechanisms. Our aim was to verify PKCθ involvement in macrophage polarization by assessing its role both in macrophages directly and in T cell/macrophage cross talk.

Methods: F4/80+/CD11b+ macrophages have been sorted by flow cytometry from hindlimb muscles derived from mdx and mdx-mdx™ mice (the mouse model of Duchenne muscular dystrophy, expressing or not expressing PKCθ). Raw 264.7 macrophage cell line has been cultured alone or in co-culture with splenic murine T cells derived from C57BL6/PKCθ-/- or C57BL6/PKCθ+/- mice. M1/M2 polarization stimuli (LPS and IL4 respectively) have been administrated to Raw 264.7, pre-treated or not with PKCθ inhibitor (C20), and to T cell/Raw co-culture. T cell phenotype has been characterized by flow cytometry with CD25 (activation marker), CD4 (T helper), CD8 (CTL) and Foxp3 (T regulatory) antibodies, while muscle-derived macrophages and Raw 264.7 have been analyzed for M1/M2 markers expression by RT-qPCR (iNOS and IL6 for M1; Arg1 and CD206 for M2).

Results: Lack of PKCθ in mdx resulted in higher iNOS and IL6 expression in muscle-derived macrophages. Raw 264.7 cells C20-mediated PKCθ inhibition resulted in higher LPS-induced iNOS expression and IL4-induced CD206 expression, thus resulting in a higher macrophage responsiveness. Analysis of co-culture-derived T cells showed that Raw presence did not affect CD25+ T cells percent and greatly increased CD25 expression in muscle-derived macrophages and Raw 264.7 have been analyzed for M1/M2 markers expression by RT-qPCR (iNOS and IL6 for M1; Arg1 and CD206 for M2).

Conclusion: Our work demonstrated that PKCθ in T cell is involved in T cell/macrophage cross-talk mechanism and, noteworthy, that it is directly involved in macrophage responsiveness to polarization stimuli.

294/B163
CD163 mRNA Detection Using PrimeFlow™ Flow Cytometry Detection Assay; Analysis for Monocyte Pathobiology in Systemic Juvenile Idiopathic Arthritis
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Systemic Juvenile Idiopathic Arthritis, SJIA, is a chronic inflammatory disease of childhood with prominent extra-articular features, including prolonged spiking fevers, rash, lymphadenopathy and serositis. Macrophage activation syndrome (MAS) occurs in approximately 10% of SJIA patients, and is a potentially fatal episode of overwhelming inflammation characterized by fever, cytokines, coagulopathy, liver and central nervous system dysfunction, and emergence of hemophagocytic macrophages. SJIA is an autoinflammatory disease, marked by activation of innate immune effector cells; however, the precise phenotype of monocytes and macrophages in this disease has not been fully characterized. Classical activated M1 macrophages produce inflammatory cytokines and exhibit increased microbicidal activity. In contrast, several forms of alternatively activated macrophages, known collectively as M2, are involved in immune modulation, tissue repair and scavenger functions. SJIA monocytes have features reflective of M1 activation, including the presence of proinflammatory cytokines; however, increasing evidence suggests that markers associated with alternative activation are also present in these cells, including CD206, MS4A4A and CD163. CD163 expression is strongly associated with both overt and subclinical MAS and levels remain elevated in SJIA even in the absence of MAS clinical features. We hypothesize that monocytes in SJIA manifest a novel phenotype with features of classical and alternative activation. To test this hypothesis we are developing in vitro assays to assess the regulation of specific cell markers in response to macrophage differentiation. We are utilizing THP-1 cells to develop a flow cytometry panel for markers of macrophage differentiation that would then be utilized on human peripheral blood mononuclear cells. Our study addresses the use of PrimeFlow technology to detect CD163 mRNA since protein levels, assessed by CD163 PE antibody binding, showed a minimal shift in cell surface expression. THP-1 cells were stimulated with IL-10 (50ng/ml) for 24 hours. CD163 mRNA+ THP-1 cells were increased by approximately 3-fold after IL-10 stimulation compared with unstimulated cells, while CD163 protein levels were minimally detected in the same cells. This correlates with real time PCR analysis of CD163 mRNA indicating an approximate 3-fold increase in CD163 mRNA in these cells. This assay will allow for analysis of CD163 mRNA levels which, when increased, are indicative of hemophagocytic macrophage phenotypes and will allow us to explore macrophages cultured under various polarizing conditions. This assay can then be translated to analysis of peripheral blood monocytes in SJIA and MAS, to obtain a more thorough understanding of the pathobiology of these diseases.
examined the intracellular expression of indoleamine 2,3-dioxygenase (IDO) applying flow cytometry and plasma levels of tryptophan (TRP), kynurenine (KYNA) and kynurenic acid (KYNA) using high-performance liquid chromatography.

A significant increase in the prevalence of CD28+ T cells was observed in HP compared to NP women. At the same time a decrease was shown in the expression of CTLA-4 on these cells. The frequency of CD80+ monocytes was lower in HP women. The prevalence of IDO-expressing T cells and monocytes was higher in HP compared to NP women. Plasma KYNA, KYNA and TRP levels were lower, while at the same time, the KYN/TRP ratio was higher in HP than in NP women.

Costimulation via CD28 may not contribute to the immunosuppressive environment, at least in the third trimester of pregnancy. The development of the pregnancy-specific specific immune tolerance in the mechanism of B7 costimulation may be more related to the alteration of B7 proteins on APCs rather than that of their receptors on T cells. The elevated intracellular IDO expression in monocytes and T cells, as well as higher plasma enzymatic IDO activity are likely to contribute to the systemic immunosuppressive environment in the third trimester characteristic for healthy gestation.

296/B165
Assessment of Mast Cell/Basophil Degranulation by Combined Cell Surface Protein Analysis and Intracellular Histamine Measurement
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Mast cells and basophils are considered as primary effector cells in allergies. Clinically relevant mast cell/basophil degranulation can be assessed by the measurement of both secreted mediators and translocation of secretory granule proteins to the plasma membrane. Histamine is preformed in mast cell/basophil granules and immediately released from cells e.g. upon activation through cell surface antigen-specific IgE bound to high affinity FcεRI or G-protein-coupled receptors. Here we assess mast cell/basophil functionality on single cell level by a combination of multiparametric cell surface protein analysis with intracellular histamine measurement. Murine peritoneal mast cells (PMCs) were enriched and degranulated by specific stimuli. Activation dependent cell surface expression of granule membrane proteins (CD107a, CD63) was combined with measurement of histamine-specific mesoionic acid fluoride - Histamine Blue (HB) – fluorescence by flow cytometry. In activated PMCs a decrease of HB fluorescence correlated with CD107a and CD63 upregulation and β-hexosaminidase release. PMC degranulation leads to changes in cell size and granularity. Subcellular live-cell analysis of PMCs indicated granular pattern of HB fluorescence. Highly purified human basophils were activated and subsequently, HB-fluorescence was measured. In human basophils HB staining indicate heterogeneity in histamine content; basophil activation also induced reduction of HB-fluorescence. Thus, simultaneous analysis of receptor expression/translocation and measurement of intracellular histamine content by flow cytometry will complete degranulation-related mast cell/basophil signature, provide additional information about stimuli-specific degranulation requirements and dissect new functional populations of mast cells and basophils.
CD8+ T cell subset (CTL). More interestingly, CD3+CD4+CD8+ (double positive, DP) effector/memory T cells were involved. Depending on the intensity of tumor regression graded by the physical appearance of individual pigs, DP T-cells represented gradually growing and then slowly disappearing T cell subpopulation in circulation. Intensive phase of melanoma regression and depigmentation was characterized by the presence of vast prevalence of DP T-cells over their CD4+ single positive (SP) counterparts in peripheral blood. Interestingly, mouse isotype-specific indirect CD4/CD8 immunofluorescence is more sensitive for detection of melanoma/melanocyte-specific DP T-cells from other DP circulating CD4+ lymphocytes based on their relatively higher CD8 and lower CD4 expression. This conclusion was verified by cost-effective bulk sorting and TCR expression analysis that revealed superimposed oligoclonal patterns of expanded TCRβ clones. More detailed flow cytometric characterization has confirmed uniform CD3+CD4+CD5+CD8+CD25-CD45RC- phenotype of the DP T-cells with no MHC class II (SLA-DQ) expression. Finally, screening of a panel of anti-pig monoclonal antibodies has revealed an anti-CD45R monoclonal antibody that may be used for unambiguous identification of tumor-specific lymphocytes within CD3+CD8+ cells. In conclusion, our results support the hypothesis that DP T-cells in pigs play an important role in the anti-viral and anti-tumor immunity. According to our results we believe that DP T-cells are critically involved in melanoma regression in MeLiM.

This work was supported by grant DZRO-ZHN and project EXAM.

299/B168
A High-Throughput Direct Cell Counting Imaging Cytotoxicity Method for Natural Killer Cell-Mediated Cytotoxicity Detection
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Cytotoxicity assays play a central role in studying the function of immune effector cells such as cytolytic T lymphocytes (CTL) and natural killer (NK) cells. Traditionally, cytotoxicity assays have been performed using 51Cr and Calcein release assays. The assays involve labeling tumor cells (target) with radioisotope or fluorescent dyes, when the target cells are subjected to cytolysis by CTLs or NK cells (effector), they release entrapped labels into the media upon lysis. The amount of labels in the media is measured to determine the level of cytotoxicity the effectors have induced. These traditional methods may generate inconsistent results due to low sensitivity caused by poor loading efficiency and high spontaneous release of the reagents. In this work, we demonstrate a novel cytotoxicity assay using the Celigo imaging cytometry method. Utilizing imaging cytometry, direct cell counting of live fluorescent target cells can be performed, which is a direct method for assessment of cytotoxicity. Human NK cells from one healthy donor were used as effectors, and K562 (suspension) and IMR32 (adherent) were used as the target cells. Both target cells were first stained with Calcein AM, and seeded at 10,000 cells/well in a standard 96-well microplate. The donor NK cells were then added to each well at Effector-to-Target (E:T) ratios 10:1, 5:1, 2.5:1, 1.25:1, 0.625:1, and 0.3125:1. The 96 well plate was then scanned and analyzed using Celigo imaging cytometer at t = 1, 2, 3, and 4 h to measure the % lysis of target cells. The results showed increasing % lysis as incubation time and E:T ratio increased. The propose Celigo imaging cytometry is an accurate and simple method for direct quantification of cytotoxicity, which can be an attractive method for both academic and clinical research.

300/B169
Multi-color Flow Cytometry to Analyze Co-Inhibitory Molecule Co-expression Levels on Antigen-Specific T Cells Using Brilliant VioletTetramer Technology
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Allogeneic stem cell transplantation (alloSCT) can be a curative treatment for patients with a hematological malignancy due to alloreactive T cell responses recognizing minor histocompatibility antigens (MiHA) expressed by the patient’s malignant cells. Despite the long-term presence of memory T cells, many patients eventually relapse. The failure to launch productive tumor-reactive T cell responses results from immunosuppressive mechanisms in the tumor micro-environment, such as altered expression of co-inhibitory and co-stimulatory molecules on tumor cells, as well as tumor-reactive T cells. The aim of this study was to set-up a 13-color flow cytometry panel to analyze co-expression levels of multiple co-inhibitory and co-stimulatory molecules on antigen-specific T cells using dual color tetramer technology.

Tetramers were produced by overnight incubation of biotinylated peptide/HLA-monomers at a ratio of 1:6 with streptavidin-Brilliant Violet (BV) 605 and BV711 conjugates at 4°C. Thereafter, tetramers were stored at 4°C and analyzed in time for their binding efficacy using antigen-specific T cell cultures. In addition, all antibodies for co-inhibitory and co-stimulatory molecules were validated and titrated on healthy donor peripheral blood mononuclear cells (PBMCs), monocyte-derived dendritic cells, cell lines and/or activated T cell blasts. Subsequently, we set-up a 13-color flow cytometry panel to analyze co-expression levels of various co-inhibitory and co-stimulatory markers in FITC, PE, PE-Cy7, BV421, APC, on antigen-specific CD3+CD8+ T cells (BV510, PerCP) using dual color tetramer staining (BV605 and BV711). Viable cells were gated using a UV live/dead stain. Currently, we are examining healthy donor and patient peripheral blood mononuclear cells in our well optimized flow cytometry protocol. Data will be analyzed using Kaluza, BD Diva, and SPICE software.

The results of this study will provide insight in interesting target molecules for blocking therapy, in order to restore tumor-reactive T cell functionality and prevent tumor relapse in alloSCT patients.

301/B170
Cytotoxic Involvement of NKT Cells in the Immunopathogenesis of Human Cutaneous Leishmaniasis
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Introduction: Cutaneous leishmaniasis (CL) is a parasitic infection affecting about 12 million people worldwide. Treatment options are limited and no effective vaccines exist. The clinical course of CL depends on the interaction between the parasite and the
immune response of patient. Natural killer T (NKT) cells are a conserved innate-like lymphocyte population with immunomodulating effects in various settings, but their role in immune processes of infectious diseases is not well understood. Some studies have addressed the role of NKT cells in the immune response to *Leishmania infection*, but the results are variable and do not provide a coherent understanding.

Methods: In our study we set out to assess the frequency and the cytotoxic activity of NKT cells and their subsets through immunophenotyping and evaluation of CD107a expression, in peripheral blood samples from patients: with active CL (ACL); during antimicrobial treatment (DT); and recently healed (RH); as well as from healthy individuals (HI). The markers used were: CD3-Pacific Blue, CD4-APC Alexa Fluor 750, CD8-APC, CD56-PE (all from Beckman Coulter) and CD107a-FITC (BD Biosciences).

Results: The frequency of total circulating NKT cells varied between individuals (4.3% ± 0.67) and no statistical difference was detected. A lower CD107a expression was observed in RH. Although NKT cells are a small proportion of circulating lymphocytes, expression of CD107a by NKT cells represent around 10% among cytotoxic cells (T CD8+ and T CD4+ lymphocytes and NKT cells). Based on the expressions of CD4 and CD8, NKT cells are subdivided into: CD8+, CD4+, CD8/CD4+ (double negative - DN) and CD8+/CD4+ (double positive - DP). In all subjects CD8+ NKT cells represent about half of total circulating NKT; CD4+ and DN NKT subsets had similar intermediate proportions; and DP NKT cells were detected in small amounts. Although the DP NKT cells always show a lesser extent, there were fluctuations in their percentage among the groups. DP NKT cells showed lower percentage in ACL compared to HI; but these levels tend to increase in DT and they restore to normal levels in RH. We also observed fluctuations in DN NKT cells percentage, which were higher in RH. The CD107a expression by NKT cell subsets varied widely, but had highlighted the low amount of CD107a+ DP NKT cells in ACL.

Conclusion: These results point to the cytotoxic involvement of NKT cells in CL and the importance of evaluating NKT subsets. Patients with ACL had impaired the number and cytotoxic activity of DP NKT cells, which may be related to a specific weakening of such subset by the infection. NKT cells can influence the course of CL and therapeutic modulation of their functions could be beneficial both to new treatments as well as potentially develop vaccines.

302/B171 Regulatory Macrophages Derived from Peritoneal Dialysate Reduce Renal Injury in Adriamycin Nephropathy

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Background: Patients undergoing peritoneal dialysis (PD) discard huge numbers of peritoneal macrophages in dialysate daily. Macrophages have shown great promise as a therapeutic strategy for different types of experimental kidney disease and human kidney transplantation. This study aimed to examine the potential of using peritoneal macrophages (PMs) from peritoneal dialysate to treat kidney disease.

Methods: Peritoneal macrophages derived from PD patients were examined. Peritoneal dialysate was collected also from mice undergoing PD daily for 7 days via a silicon catheter in the peritoneal cavity. Murine Adriamycin nephropathy (AN) was induced by 10 mg/kg Adriamycin in BALB/c mice. Adoptive transfer of modulated macrophages derived from PD mice into mice with AN was used to assess their in vivo functions.

Results: PMs from PD patients and mice accounted for about 40% of total peritoneal leukocytes. PMs from PD patients and mice expressed lower levels of CCR2, and higher levels of CD86 and MHC-II compared to that in blood monocytes. 90% of PMs from PD patients maintained their viability after storage in liquid nitrogen for at least one year. PMs from PD patients and mice displayed normal macrophage function and could be modulated into an M1 (effect) or M2 (protective) phenotype. In vivo, adoptive transfer of M2 PMs from PD mice protected against kidney injury in mice with AN.

Conclusions: M2 macrophages derived from PD mice are able to reduce renal injury in AN, suggesting that peritoneal macrophages from PD patients may have the potential for clinical therapeutic application.

Infectious Diseases (B172 – B176)

303/B172 High Throughput Multiplexed Assays to Facilitate Detection of Shiga Toxin Producing *E. coli* in the Beef Chain

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Introduction: Shiga toxin-producing *Escherichia coli* (STEC) have been identified by the USDA as a serious threat to the nation’s health stemming from contaminations in the food supply, specifically, the beef chain. We have developed an assay that is able to identify STEC in a quick and multiplexed format. Multiplex oligonucleotide ligation-PCR (MOL-PCR) is a nucleic acid based assay patented at Los Alamos National Laboratory (LANL) that uses flow cytometry and microarray bead arrays for detection of nucleic acid based signatures. By using MOL-PCR for detecting unique STEC DNA signatures in the beef supply chain we will be able to offer a multiple and high throughput alternative to the multiplex PCR currently in use. This research will be focused on DNA detection of 8 STEC serotypes (STEC-8): O26, O45, O103, O104, O111, O121, O145, and O157:H7. The goal is to produce a multiplex panel of MOL-PCR probes for identifying DNA signatures corresponding to each of the STEC-8 serotypes and ultimately strain specific identification.

Methods: MOL-PCR oligonucleotide pairs, or MOLigo pairs were designed starting with published unique sequences specific for STEC serogroups as well as toxin genes utilizing unique software for designing MOLigo pairs: MOLigoDesigner. The first round of design was centered on a multiple hit matrix identifying each of the eight STEC serotypes and also testing for signatures for toxin genes (stx1, stx2, eae). which allowed for distinction of STEC from non-STEC samples. MOLigo pairs designed in this way are evaluated using the Luminex system for highly multiplexed analysis. Initial assay panels are tested for specificity by running against isolated genomic DNA from commercial sources as well as reference strains from collaborators and compared against their known profile of toxin genes (stx1, stx2, eae), which allowed for distinction of STEC from non-STEC samples. MOLigo pairs designed in this way are evaluated using the Luminex system for highly multiplexed analysis. Initial assay panels are tested for specificity by running against isolated genomic DNA from commercial sources as well as reference strains from collaborators and compared against their known profile of toxin genes (stx1, stx2, eae). Samples are run in triplicate with data analysis scheme done with Wilcoxon rank sum test to determine reactive serogroups. Work has also been started on an assay that is capable of distinguishing STEC and non-STEC serogroups based on single MOLigo pairs using unique single nucleotide polymorphisms.
Conclusion: The designed 11-plex MOLigo pair assay is able to identify the presence of each of the STEC-8 serotypes of interest as well as identify the correct toxin gene markers based off of the strains known expression profile. The assay correctly identifies STEC-8 samples from both commercial sources as well as purified samples from collaborators to the serotype level with correct toxin profiles. The MOL-PCR assay and algorithm has been evaluated against a set of blinded samples with very positive results. Limits of detection are being evaluated currently. The assay is also being tested against samples that contain bovine fecal background.

304/B173
A New Phagocytosis Assay in Whole Blood by Flow Imaging Cytometry with GFP-Expressing Bacteria
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Introduction: Phagocytosis is essential for the clearance of pathogens, apoptotic bodies and necrotic debris. Defects in the phagocytic process are diagnostic and prognostic biomarkers in deficiencies of the innate immune system, including disorders such as chronic granulomatous disease. Also, novel cell-based assays may complement current approaches for in vitro immunotoxicology. We have developed a flow cytometric method based on the use of green fluorescent protein (GFP) labeled Escherichia coli (GFP-E. coli) and dihydroethidium (DHE) to assess phagocytosis and the subsequent oxidative burst in human whole blood samples and the human monocytic cell line U937.

Materials and Methods: For the whole blood study, GFP-E. coli was added at different bacterial:leukocyte ratios (1.05 and 0.25) to 50 mL of heparinized whole blood incubated with 2.5 mL of CD45-APC antibody. After incubation at 37° C for 30 min, 1 mL of BD FACS lysing solution was added. For the U937 study, GFP-E. coli was added to U937 cells at bacterium:leukocyte ratio of 30:1. After incubation at 37°C for 90 min, 400 mL of RPMI was added. For oxidative burst quantitation in both assays, 2 mL DHE at 1 mg/mL was added prior incubation at 37°C. Positive controls were samples stimulated with PMA. Negative controls included cytochalasin A and 0°C incubation. Samples were analyzed in three different cytometers: FACSVerses, Accuri C6 and Aminis ImageStream.

Results: A bacterium:leukocyte ratio as low as 0.25 is enough to trigger phagocytosis and oxidative burst by neutrophils in whole blood samples. The capability of GFP-E. coli to elicit radical oxygen species is nearly that induced by PMA. Contrarily, cytochalasin A decreased markedly the phagocytic potential of blood and U937 cells. Analysis by imaging flow cytometry of U937 cell line and whole blood samples allowed us to discriminate between surface-adherent and internalized bacteria.

Conclusions: This assay has been applied successfully to estimate the phagocytic potential of leukocytes in whole blood samples of normal subjects and it may provide a fast, simple and accurate protocol for diagnosing phagocytic disorders and toxic effects on phagocytic cells.

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305/B174
13-Color Immunophenotypic and Functional Analysis of Whole Blood and Spleen from Human T-Lymphotropic Virus Type-1 (HTLV-1) Infected Humanized BLT Mice
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Adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) are caused by infection with HTLV-1. Because of the inability of HTLV-1 to infect animals other than humans and non-human primates, development of animal models to study the pathogenesis of these diseases has been difficult. In order to study HTLV-1 infection in vivo, nonobese diabetic (NOD) severe combined immunodeficiency (SCID) common gamma chain knockout (NSG) mice were humanized. NOD/SCID mice were surgically implanted with human fetal bone marrow/liver/thymus tissue (BLT) and injected with donor matched CD4+ cells. To determine if these mice were susceptible to HTLV-1 infection, BLT mice were injected with lethally-irradiated CD4+ HTLV-1 producing cells, and then whole blood and tissue samples were collected, frozen and shipped overnight. The samples were thawed, a portion saved for DNA extraction for viral load and the remainder stained with a viability dye and a panel of anti-human antibodies against CD45, CD3, CD4, CD8, CD19, CD14, CD16, CD45RA, CD45RO, CD25, CD3, CD27, CD2 and CD25 were chosen as markers because of their association with HTLV-1 infection. We found that BLT mice can be infected by HTLV-1 and observed an increase in CD4+CD25+CD70 T-cells as seen in the human infection. To determine the functional consequences of the HTLV-1 associated changes in the phenotypic profile we designed a T-regulatory cell assay that uses sorted splenic cells and measures their effect on T-cell proliferation in response to viral antigens.

306/B175
iNKT Cells and Their Subpopulations Are Not Restored in HIV+ Patients with Low CD4/CD8 ratio after Prolonged Effective Therapy, and Display a Pronounced Th-1 and Th-17 Pro-inflammatory Profile
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Introduction: In HIV+ patients treated with combined antiretroviral therapy (cART), the ability to normalize the CD4/CD8 ratio is assuming a growing importance and clinical relevance. Recently it has been shown that in patients who suppress viral production after cART low CD4/CD8 ratio are associated with increased risk of serious events and deaths, and that this ratio could be used to identify patients at risk of non-AIDS-related event (Mussini C. et al., Lancet HIV, March 2015). CD4 molecule can be expressed by a population of lymphocytes defined ‘invariant natural killer T cells’ (iNKT), that express an invariant T cell receptor (TCR, Vα24Jβ18) and produce proinflammatory cytokines upon stimulation. We have thus
analyzed their phenotype and polyfunctional in HIV+ patients with low (<0.5) and high/normal (>1.0) CD4/CD8 ratio after successful cART.

**Methods:** We studied a total of 20 HIV+ adults who started cART with ratio <0.5 and had undetectable plasma viral load for 2 years. Among them, 8 maintained a CD4/CD8 ratio <0.5 while 12 reached a ratio >1; 27 age- and sex-matched subjects were used as healthy controls (CTR). PBMC were stained with mAbs recognizing Vα24jul18 TCR, CDS, CD4, CD8, CD14, CD19 or CD161. Due to lack of cells, iNKT cells ability to simultaneously produce up to 4 cytokines was measured in 19 HIV+ patients and in 21 CTR. PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with Live-dead and with mAbs recognizing Vα24jul18 TCR, CD3, CD4, CD8, IL-17A, TNF-α, IFN-γ and IL-4. Up to 20 million cells per sample were acquired on two acoustic focusing flow cytometers (Attune and Attune NKT, Thermo Fisher).

**Results:** HIV patients with CD4/CD8 ratio >1 were able to restore the percentage of total iNKT cells. The percentage of iNKT cells expressing CD8 was higher in those with low CD4/CD8 ratio, while that of CD4+ iNKT cells was lower. Double negative (CD4-CD8-) iNKT cells were lower in both groups of HIV patients. We analyzed cytokine production by different iNKT cell subsets. CD4+ iNKT cells from HIV patients with high CD4/CD8 ratio produced less IFN-γ, and they had also less CD4+ iNKT cells able to produce simultaneously IFN-γ, TNF-α and IL-17. Patients with low CD4/CD8 ratio had more CD4+ iNKT cells able to produce simultaneously IFN-γ and IL-17, IL-4 production was nearly undetectable in all iNKT cell subsets in all patients.

**Conclusions:** Total iNKT cells, which have an effector-memory phenotype, were restored in patients who were able to increase CD4/CD8 ratio after cART. The presence of a high percentage of CD4+ iNKT cells in patients with low CD4/CD8 ratio likely indicates a persistent level of activation among such cells, and could reflect what happens in the T cell compartment. Finally, the pronounced Th-1 and Th-17 pro-inflammatory profile of iNKT cells from patients with low CD4/CD8 ratio could contribute to the inappropriate and persistent immune system activation.

**307/B176 Exploring the Potential for Immune Correlate Discovery in Human Survivors of Filovirus Infection**

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In the last four years, Africa has recorded five confirmed filovirus outbreaks. The 2014 Zaïre ebolavirus outbreak in West Africa highlights the unpredictable nature of this infection as well as the challenges of managing and limiting the outbreak. With the high lethality rates seen in all of these outbreaks (generally ~40-90%), and the potentially dramatic disease sequelae, filovirus outbreaks can devastate local communities and present great health risks for affected regions. These issues render obvious the need for effective vaccines and therapeutics to limit and manage future outbreaks. Though vaccine development has been underway for many years, establishing immune memory parameters based on human immune responses could uncover correlates of immunity that can be applied to the development process. Furthermore, advancing our understanding of immune correlates against filoviruses will better enable us to protect the warfighter in deployment situations as well as in biological threat scenarios. A Sudan ebolavirus outbreak in 2000-2001 in Uganda involved over 400 patients with a fatality rate of 53%. Our group previously determined numerous survivors maintained strong neutralizing antibody titers and could elicit antigen-specific cytokine responses greater than 10 years after the outbreak. In 2012, three new outbreaks occurred in Uganda: two Sudan virus and one Marburg virus. We collected PBMC and serum samples from the majority of survivors from each outbreak to evaluate the presence of immune memory and its composition. Serum antibodies displayed specificity to the infecting agent as measured in relation to Marburg virus and Sudan virus glycoprotein in addition to other proteins. The large majority of serum samples displayed neutralizing activity 6 months to 2 years after infection. PBMCs stimulated with irradiated virus and recombinant antigen were evaluated by flow cytometry for functional parameters as well as level of antigen-responsive T cells. Additionally, cytokines in supernatants of these cultures were determined by multiplex ELISA. Cytokine responses were Th1-biased and produced predominantly by CD4 T cells. These studies are a valuable resource for filovirus vaccine development and provide immune correlate information of direct relevance to human immunology.

**Live Cell Imaging/Tracking (B177 – B179)**

**308/B177 Unexpected Homodimerization of the c-Fos Transcription Factor in Live Cells Revealed by FRET, SPIM-FCCS and MD-Modeling**

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The transcription factors c-Fos and c-Jun, members of the activator protein 1 (AP-1) family, play a key role in the regulation of proliferation, differentiation, apoptosis and oncogenesis. They belong to the basic-region leucine-zipper proteins binding as heterodimers to palindromic AP-1 sites in the promoter or enhancer regions of mammalian genes. Whereas purified Jun can also form homodimers (less stable than the heterodimer), earlier in vitro studies showed that Fos was mainly monomeric. The instability of Fos homodimers was attributed to the electrostatic and hydrophilic properties of the leucin-zipper.

We used Förster resonance energy transfer (FRET) and fluorescence correlation spectroscopy (FCS) applied to HeLa cells transfected with fluorescent protein-tagged c-Fos and c-Jun to investigate their homo- and heterodimerization. Both FRET results and molecular brightness analysis by FCS indicated that c-Fos formed homodimers. We worked out a method to determine the absolute concentrations of transfected and endogenous c-Fos and c-Jun by combining FCS and immunofluorescence, which allowed us to analyze protein association quantitatively. From FRET data of cells expressing various concentrations of donor- and acceptor-tagged proteins, we determined the dissociation constant of c-Fos homodimers and c-Fos-c-Jun heterodimers in live cells. Single plane illumination microscopic (SPIM) FCCS studies confirmed that c-Fos homodimers were stably associated and could bind to the chromatin. Molecular modeling simulation also supported that stable homodimers could form. Our results introduce c-Fos homodimers as a new form of the AP-1 complex, which might be an autonomous transcription factor in c-Fos overexpressing tissues, and as an oncogene it could contribute to tumor development.

**309/B178 Combining Kinetic Ligand Binding and 3D Tumor Invasion Technologies to Assess Drug Residence Time and Anti-metastatic Effects of CXCR4 Inhibitors**

Dominic Herring, Brad Larson, Leonie Rieger, Nicolas Pierre, Hilary Sherman

1Invasion Technologies to Assess Drug Residence Time and Anti-metastatic Effects of CXCR4 Inhibitors
identify subpopulations of cells, such as cancer stem cells. In fluorescence microscopy, subpopulations of cells can be labeled and tracked, but with the risk for phototoxic effects on the cells. When combining HM and fluorescence, it is possible to both label subgroups of cells and to follow them over time without undue phototoxicity. Fluorescence images are captured with long intervals, while holographic images are captured every couple of minutes. Morphology and movement information can be attributed to subgroups of cells and compared to the rest of the cells.

Methods: JIMT-1 human breast cancer cells and MCF10A human normal like breast cells were studied with both fluorescence microscopy and HM. The cells were fluorescently labeled for CD24 and ESA, which are cancer stem cells markers.

We have built a combined holographic and fluorescent cell analysis instrument. We have used this instrument to follow the JIMT-1 cells and the MCF10A cells. Photos were taken every four minutes during twelve hours, for each experiment and repeated twice for each cell line.

Results: JIMT-1 cells positive for CD24, CD24+ESA or ESA or negative for both CD24 and ESA display no morphological differences. In morphology investigations of MCF10A, no specific subpopulations could be found. Although no morphological differences were found, we know that there are significant differences between how those cell subpopulations act in the body or when treated with a drug. We will continue to investigate the differences during the spring to determine whether the subgroup of cancer stem cells show altered motility compared to other cells in the cell culture.

Conclusions: The combined fluorescence microscope and HM instrument will allow for long term studies of living individual cells and cell populations as well as cell subpopulations. The results will aid in the understanding of live cell behavior.

**Microbiology and Aquatic Sciences (B180 – B184)**

**311/B180**

The Use of RiboTagger FISH Probes and Cell Sorting to Isolate and Characterise Novel Microbial Populations in Complex Microbial Communities

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Today metagenomics is frequently being used as a tool in the study of the ecology of complex microbial communities. Metagenomics uses shotgun sequencing which generates short reads of small DNA sequences of approximately 100 base pair length. Previously, extracting taxonomically-valuable information from these short reads was difficult and time consuming. To overcome these limitations we used a computer program called RiboTagger, developed by SCELSE, to scan the shotgun data sets for taxonomically-valuable information located in the V6 variable region of the 16S rRNA gene (called RiboTags). Fluorescence in situ hybridization (FISH) probes specific for these RiboTags can then be designed and used to identify different taxa populations. Using these probes we can identify novel microbial groups of interest in any ecosystem analysed.

The concept of RiboTagger was applied to a complex microbial community in activated sludge from a municipal wastewater treatment plant in Singapore with the aim of understanding the diversity of the bacteria present in the sludge. Illumina Hi Seq shotgun sequencing of the DNA extracted revealed several uncharacterized microbial groups which had no taxonomic classification in the 16S rRNA Silva database. To visualise these groups a FISH probe was designed to target the most abundant

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Metastasis, the spread of cancer cells from the original tumor to secondary locations within the body, is linked to approximately 90% of cancer deaths (Saxe, 2013). The expression of chemokine receptors, such as CXCR4 and CCR7, is tightly correlated with the metastatic properties of breast cancer cells. In vivo, neutralizing the interaction of CXCR4 and its ligand, SDF-1α (CXCL12), significantly impaired the metastasis of breast cancer cells and cell migration (Muller et al., 2001). Traditionally, the discovery of novel agents has been guided by the affinity of the ligand for the receptor under equilibrium conditions, largely ignoring the kinetic aspects of the ligand-receptor interaction. However, awareness of the importance of binding kinetics has started to increase due to accumulating evidence (Swiny, 2004; Copeland et al., 2006; Tummino and Copeland, 2008; Zhang and Monsma, 2009) suggesting that the in vivo effectiveness of ligands may be attributed to the time a particular ligand resides at its receptor (Drug-Target Residence Time).

Similarly, appropriate in vitro cell models have also been lacking to accurately assess the ability of novel therapies to inhibit tumor invasion. Tumors in vivo exist as a three-dimensional (3D) mass of multiple cell types, including cancer and stromal cells (Mao et al., 2013). Therefore, incorporating a 3D spheroid-type cellular structure that includes co-cultured cell types forming a tumoroid, provides a more predictive model than the use of individual cancer cells cultured on the bottom of a well in traditional two-dimensional (2D) format.

Here we examine the drug-target residence time of various CXCR4 inhibitors using a direct, homogeneous ligand binding assay and CXCR4 expressing cell line in a kinetic format. The inhibitor panel was further tested in a 3D tumor invasion assay to determine the eventual phenotypic effect of each molecule. MDA-MB-231 breast adenocarcinoma cells, known to be invasive, and metastasize to lung from primary mammary fat pad tumors (Kamath et al., 2001), were included, in addition to primary human dermal fibroblasts. The cells were aggregated into 3D structures using spheroid microplates containing an ultra low attachment surface. A novel cell imaging multi-mode reader was incorporated to provide PMT-based assessment of drug-target residence time, as well as automated image-based monitoring of tumor invasion through a basement membrane matrix. Cellular analysis algorithms provided accurate quantification of changes to the original tumoroid structure, as well as invadopodia development. The combination presents an accurate, yet easy-to-use method to assess target-based and phenotypic effects of new, potential anti-metastatic drugs.

**310/B179**

Holographic Image Cytometry and Tracking of Fluorescently Labeled Cell Subpopulations

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**Background:** In traditional cell analysis methods, cells are often disturbed or even killed in the analysis processes. Digital holographic microscopy (HM) retrieves cytometric values as well as 3D images of live cells without disturbing labels or stains. Live cells can be analyzed for days without phototoxicity due to the low energy of the light.

HM uses a laser beam split in two, one that travels undisturbed (the reference beam) and one that passes through the sample. The beams will then merge, and an interference pattern called a hologram is created. A computer is used to extract data about cell morphology as well as a holographic 3D image of the cells from the hologram. It is possible to extract values for e.g. cell area, volume, thickness, roughness and irregularity. As HM is non-invasive and non-disruptive it is very suitable for time-lapse studies. Cells can be followed over time for studies of cell migration and motility.

HM provides the researchers with an abundance of data on both individual cells and cell populations, but it lacks the ability to

**Results:** We have built a combined holographic and fluorescent cell analysis instrument. We have used this instrument to follow the JIMT-1 cells and the MCF10A cells. Photos were taken every four minutes during twelve hours, for each experiment and repeated twice for each cell line.

**Conclusions:** The combined fluorescence microscope and HM instrument will allow for long term studies of living individual cells and cell populations as well as cell subpopulations. The results will aid in the understanding of live cell behavior.
uncharacterized microorganism (0.77%) in our metagenomics dataset. A second FISH probe was designed 3 base pairs downstream of the target site of the first RiboTag FISH probe, but labelled with a different fluorophore. By using confocal microscopy, it was possible to visualise this unknown microorganism, which appeared to have a filamentous morphology, by the overlap of the 2 FISH probes.

Next we wanted to obtain the partial genome of this group of unknown filamentous bacteria and to screen it for functional genes. To sequence and purify this unknown microorganism from the mixed microbial community we used FISH-Fluorescence Activated Cell Sorting (FACS). To increase the specificity of the sorting two FISH probes were used; the specific RiboTag FISH probe and a general bacterial probe EUB-338, labelled with Cy5 and Cy3 respectively. A total of 3 sorting gates: side scatter versus forward scatter to pick up suspected cell population from debris, high Cy3 fluorescence of the EUB-338 probe and high Cy5 fluorescence of the specific RiboTag FISH probe was used. One of the difficulties encountered was the high auto-fluorescence of sludge samples. Therefore, it was important to include a no probe control to estimate the level of auto-fluorescence for subsequent gating. Also to boost the signal fluorescence intensity of the FISH probes over auto-fluorescence, dual-labelled probes were used in hybridization experiments. The isolation of the unknown bacteria was successful, and the partial 16S RNA sequence suggests the placement of new species in a new genus. We believe that this technique could be applicable to the discovery and study of other novel microbiological species in environmental samples.

312/B181
Advanced Volumetric Cytometric Analysis of Marine Phytoplankton Bacteria, and Viral Concentrations Utilizing the Acea NovoCyte

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Recent advances in cytometer design and functionality have modernized certain marine biology applications creating a more accurate, data-rich, and timely assessment of microscopic marine organisms. Analyses of the phytoplankton, bacterial, and viral fractions of seawater were determined using the NovoCyte, a relatively low cost, three-laser (405, 488, and 640 nm), syringe driven flow cytometer from Acca Biosciences. Samples were collected during a time series investigation at the Scripps Pier (La Jolla, CA) and during transits across the NW Weddell Sea continental shelf. Photysynthetically plankton groups (eukaryotic and prokaryotic) were assessed using natural pigment fluorescence and scatter. Bacterial and viral populations were distinguished using the nucleic acid stain SYBR Green I. Abundance estimates were compared with epifluorescence microscopy direct counts. Accurate characterization and quantification of these populations in near shore and oligotrophic environments demonstrates the utility of the NovoCyte in oceanographic studies. These refined methodologies demonstrate a more accurate and efficient means in assessing phytoplankton, bacterial, and viral quantities in marine ecosystems.

313/B182
Isolation of Ant-cancer Compounds Found within Marine Tunicates

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The marine biosphere is an enormous source of structurally diverse compounds contained within organisms with a long evolutionary history. Tunicates have a documented history of 520 million years. Ocean bio-prospecting is in its infancy. Therefore, we have embarked upon developing a system suitable for location and identification of potential life forms associated with anti-tumor compounds. Our targets are the protochordate tunicates that possess the human traits of a notochord during larval stage and a vertebrate type immune system throughout its life cycle. They have survived predation by evolving unique, cell-killing compounds making them deadly for consumption. These complex compounds are in the form of secondary metabolites generated by actinobacteria within specific species biomes. We describe a marine survey apparatus consisting of high resolution CMOS, GPS and GPS/photo integration software used to construct image-time stamps and geo-spatial maps. A diver armed with a SeaLife MicroHD camera scanned 3 habitats within the Apo Reef system of the Philippine Islands. The resulting gallery of tunicate images revealed animals belonging to the class Ascidiae which contains 3000 species including a few within the Didemniidae family. Generally, members of this family of tunicates are known to produce alkaloids and peptides with anti-tumor activity. Specifically, Trabectedin (commercial name Yondelis®) a marine derived extract of Ecteinascidia turbinata is now being used for the treatment of soft-tissue sarcoma. Tunicates may also be a useful model organism for the study of cancer because neoplastic disease has not been documented in these animals, yet oncogenes such as ras and src are conserved within tunicates and humans. Bacterial extracts from tunicates inhabiting waters of Maryland, South Carolina and Florida, USA were used to establish a model for bacterial flow cytometry and cell sorting. A modular flow cytometer at the Medical University of South Carolina and a Vantage cell sorter at the University of Maryland were altered to enhance signal to noise ratio and resolve differences among small particles. Resulting signal patterns originating from hyphae-endowed actinobacteria were established within this model mixture of submicron particles. We continue our efforts to improve resolution and enrichment of tunicate-derived actinobacteria by applying cell sorting, microscopy and multispectral cytometry.

314/B183
Flow Cytometry as a Platform for Probiotic Enumeration in Encapsulation and Matrix Technologies

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Encapsulation and matrix technologies offer great advantages for cell protection against processing and gastrointestinal transit in addition to shelf-life and storage. Concomitantly, encapsulation adds an additional layer of complexity to flow cytometry (FACS) as adapted techniques require resuspension / recovery of cells prior to cytometric enumeration. The ability to distinguish different physiological states is especially important for assessing cell survival, growth in oligotrophic environments and the effects of toxic substances on microbial activity. Hence, the development of a high throughput cytometric screening methodology for accelerated enumeration of encapsulated bacteria represents a potential time- and cost-cutting process for academia and industry, respectively. This is particularly true for bacteria incorporated into food matrices/agricultural applications whereby cells are exposed to rapid and dynamic environmental conditions and processes. Accurate determination of survival/resistance is important since viability at point of consumption/application is an essential consideration for their efficacy. Our approach involves the encapsulation of single bacterial cells (10^3 CFU/ml) in independent matrices and enables the digital detection, differentiation and selection of live, injured and dead populations during the process and storage of encapsulation systems. Flow Cytometry was employed for rapid viability assessment of various strains of bacteria stabilised or encapsulated in complex food-grade matrices. Survival of the bacteria was examined using FACS compared with traditional plate count techniques and specific gating and compensation approaches were applied using fluorescent probe combinations inclusive of SYTO9, PI and TO. Cell extraction and digestive pre-treatments were used to release cells and reduce protein background. Hence, the use of this method represents the reduction of traditional laboratory plate counting, which would reduce the environmental impact of industrial bioprocess management and exponentially increase the efficiency of laboratory work in the area of cell stabilisation and encapsulation screening.
315/B184
Flow Cytometric Analysis of Protozoan Grazing on Marine Cyanobacteria: Bulk and Single Cell Sorting Tools to Determine the Molecular Diversity of Grazers

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Synechococcus is a genus of marine cyanobacteria that is ubiquitous across all marine waters. It is a primary producer that accounts for nearly 40% of primary productivity in the oceans and is a critical player in the global carbon cycle. Using flow cytometry, Synechococcus has been detected as part of a long-term time-series in Booth Bay, Maine. For over 14 years (2001-2014), Synechococcus, has bloomed annually in late summer (August-September), with rapid onset of the bloom over 1-2 weeks whereas, the decline typically lasts for many months. It is hypothesized that the bloom decline is associated with grazing by protists, however, little is known about these grazers and the role they play in the bloom termination and the ecology of Synechococcus.

Using both natural field samples and field samples spiked with cultured Synechococcus, putative mixotrophic and heterotrophic protist grazers were identified via flow cytometry by targeting the autofluorescence of naturally occurring photosynthetic pigments. Samples were analyzed for the presence of grazers over a 24-48 hour period using a Becton Dickinson FACScan and Influx Mariner as part of the J.F. MacIsaac Facility for Aquatic Cytometry at Bigelow Laboratory. Over the course of these experiments, the abundance of Synechococcus and putative grazers was monitored and members of these populations were sorted. Sorted cells were identified from both ‘bulk-sorted’ and ‘single-cell’ isolations using a variety of molecular techniques. Within the spiked field experiments, the concentration of Synechococcus decreased after 48 hours, while putative grazers populations increased in abundance within specific regions on cytometric dot plots. In the ‘Synechococcus-spiked’ experimental controls (no grazers present), Synechococcus abundance increased steadily. Using bulk cell sorting, putative grazer populations were identified and sorted, followed by whole genome amplification (WGA) and 18S rRNA gene sequence analysis. Ciliates, heliozoans, cryptophytes, stramenopiles and cercozoans were identified as the potential protist grazers of Synechococcus in Booth Bay, ME. In the late fall and summer of 2012 during a natural Synechococcus bloom, putative grazer regions were sorted for both bulk and single-cell analysis. Single cells were isolated at the Single Cell Genomics Facility at Bigelow Laboratory, DNA was extracted, amplified (WGA), and sequenced for both 18S rRNA and 16S rRNA genes. Single cell sorts that contained sequences for both the 18S and 16S rRNA Synechococcus genes were identified as Synechococcus grazers. Using this combined approach of bulk and single-cell grazer analysis, the diversity of specific grazers in natural phytoplankton communities can be revealed to uncover trophic linkages among microbial predators and their prey.

316/B185
Observing Surface Glycosylation Changes on Apoptotic B-Cells Using the Lab in a Trench Platform

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Lab in a Trench (LiaT) is a microfluidic system that allows repeated probing of cells captured in a shear-free environment. We have demonstrated a novel method of sequentially glycoprofiling live B-cells using LiaT and an epifluorescent microscope.

Lectins are carbohydrate binding proteins that can identify specific glycans on a cell surface. Cells were captured with LiaT and incubated in appropriate buffer. Commercially available plant lectins were labelled with compatible fluorophores, introduced into the system and allowed time to bind to the cells. These lectins can then be eluted using the appropriate free sugar and further lectins were used to probe the cell.

Lab in a Trench analyses can be carried out in parallel, with the analysis of up to four trenches of cells at a time. Each trench captures between 5 and 30 cells. The compact platform is compatible with standard laboratory reagents and microscopes.

Measurements of the cell fluorescence were taken at various timepoints after addition of the lectin probe and its elution by sugar. Images were analysed using ImageJ. Sequential images were overlaid to demonstrate localisation of the lectins and the glycans to which they bind. Image analysis allows semi-quantitative comparison of glycan density on cells.

By inducing apoptosis while cells are in the trenches, the lectin binding profile of the cells can be monitored in real time. It has been reported (Meesmann, 2010) that de-sialylation of cell surface glycans exposes terminal N-acetylglucosamine. Here, we can probe this exposure of terminal GlcNAc as we observe the cells in the trench. Confirmation of this lectin specificity can be controlled by eluting the lectins with free GlcNAc.

Reference:

Multi-dimensional Flow Cytometry (B186 – B196)

317/B186
Use of Mass Cytometry to Identify Circulating T Cell Populations Involved in Pathogenic Responses to Human Rhinovirus

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Background: Infection with rhinovirus (RV) exacerbates allergic asthma through immune mechanisms that are not well understood. This is due in part to practical and technical limitations in human studies. It has been proposed that virus-induced asthma arises from deficient anti-viral Th1 responses. The objective of the present study was to apply mass cytometry to an experimental model of rhinovirus infection in humans, in order to identify the T cell populations that contribute to anti-viral responses in allergic asthmatics.

Methods: Allergic asthmatic subjects were inoculated intranasally with RV-16. Peripheral blood mononuclear cells (PBMCs) were collected immediately before inoculation, and then at days 4 and 21 post-inoculation. A 35-parameter mass cytometry phenotyping panel was developed to monitor CD4+ T cell, CD8+ T cell, and B cell populations in a longitudinal fashion. Data was analyzed using viSNE.
Results: During the acute phase of infection, loss of cells expressing the Th1 transcription factor, T-bet, was observed, suggesting their egress from the periphery. These cells included memory CD4+ T cells with lung-homing potential (CCR5+), and comprised a mixture of discrete lineage-specific phenotypes, including novel Th2-like subtypes that expressed CCR4. While Tc2-like cells were observed prior to infection, CD8+ T cell populations expressed lower CCR5 as compared to CD4+ T cells, and were only modestly reduced in numbers during the acute phase. Both Th2- and Tc2-like cells increased in the blood 3 weeks after infection, consistent with their sustained expansion.

Conclusions: Longitudinal monitoring using mass cytometry identifies previously unrecognized T cell subtypes that contribute to pathogenic responses to rhinovirus. These subtypes resemble conventional and novel T cell types with lung-homing signatures. Taken together, our findings point to a dominant pathogenic role for complex Th2-like populations in rhinovirus-infected allergic asthmatics.

318/B187
Big Data in Single Cell Biology: Analytic Approaches and Pipelines to Handle the Growing Multi-parameter Cytometry Space
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A major challenge in biomarker discovery leveraging single-cell cytometry has been due to an increasing amounts of high-dimensional and high-throughput data. In cases of discovering rare cell types, pouring through 2D biaxial plots has proved to be extremely difficult or nearly impossible. To discover cell subsets that are rare and hard to find, as well as automatically discover and profile new cell subsets of interest, I will present an overview of analytic methods and steps which are critical to support this new inundation of data. I will also give examples of how automated biomarker discovery can find small cellular subsets with unique markers (e.g. CD34), ranging from 0.25-3% of the total initial sample size. These examples leverage dimensionality reduction using tSNE as well as clustering via SPADE to identify cell subsets that may not have been easily identifiable by hand. We utilize methods for identifying these cell subsets both manually and visually as a comparator and support for automated cell discovery. In addition, we extend these cell subsets to automated statistical analysis in R in order to illustrate an end-to-end automated workflow for automatic cell subset discovery.

319/B188
Standardizing and Packaging a High-Content Discovery Analysis Workflow with Plug-and-Play Application Architecture
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The data stream from high-content flow and mass cytometers represents an opportunity to derive actionable insights about cellular communication, development, and disease. This challenge has led to the development of several open-source (e.g. FlowMeans, SPADE, ACCENSE, Vortex) and proprietary algorithms which facilitate data discovery and visualization. However, the required dependencies for each application, nuances of clustering and input, various programming languages used to create them (e.g. R, C++, Java), lack of a standard data exchange format, and interpretation of data transformation puts these beyond the reach of the bench scientist by requiring computer programming skills to tie into a standardized analysis pipeline. Herein, we sought to remove the barriers to entry in data discovery and to integrate these applications seamlessly into a user-friendly platform with common hooks and well-documented application program interface (API). In this manner, the various discovery algorithms could be leveraged by bench scientists and workflows shared in a large user base; an approach employed by similar bioinformatics platforms (e.g. the Galaxy genomics platform). Using Flowjo version 10 as a platform and a diverse discovery analysis suite including SPADE, ACCENSE, FlowMeans, and Vortex, we built a reproducible, shareable analysis workflow allowing a deep dive into phenotypic results from large data sets. Herein, we generated a discovery protocol for >25 parameter mass cytometry data leveraging FlowJo templates with plug-and-play clustering algorithms, mapping human-named to algorithm-identified clusters, and tested the pipeline’s reproducibility with varying experimental conditions. Our work brings ease and reproducibility to single cell phenomics and democratizes discovery tools for the bench scientist.

320/B189
Real-Time Cytometry Reveals Biochemical Interactions between Fluorescent Probes in the Analysis of Oxidative Stress
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Background: Analysis of reactive oxygen species (ROS) is a relevant application of flow cytometry. ROS analysis is complicated because of their very short-half life and the extensive interplays among them. Moreover, the specificity of fluorogenic substrates for ROS analysis, and their possible interferences with ROS metabolism are still unresolved issues. We were interested in developing multicolor real-time flow cytometry (RT-FCM) assays for ROS by combining spectrally-compatible fluorogenic substrates for ROS. While setting up such assays we found discrepancies between the responses of fluorogenic substrates alone and in combination, suggestive of biochemical interferences of the probes with ROS metabolism.

Methods: Suspensions of N13 rat hepatoma cells were incubated with several fluorogenic substrates commonly used in oxidative stress studies, namely: Mitosox Red Dye, dihydrodihydroamine 123, dihydroethidium and dihydro-dichlorofluorescein. Cells were incubated with the individual probes or with spectrally-compatible pairs of them. For setting up RT-FCM experiments, appropriately stained cells were run on an Accuri C6 cytometer and live cells gated based on their FSC vs SSC features. After defining baseline fluorescence for about 30 sec, ROS generation was triggered by cumene hydroperoxide, and fluorescence emission was continuously measured for 10 minutes. Listmode files were replayed and fluorescence compensation matrices were defined at different run times. Such matrices were found to be similar, independent of the sampling time and were implemented in the RT-FCM running protocols. Under these conditions, similar RT-FCM experiments were performed with hydrogen peroxide, NOR-1, propanolol and epinephrine.

Results: Our results showed that kinetics of individual probes responded as expected to the addition of prooxidant compounds. However, the same probes, when paired to other spectrally-compatible ROS probes, showed important increases or reductions in the rate of fluorescence generation, induced by the same pro-oxidant. Variations were complex, and dependent on fluorochrome pairs and prooxidants, but not on fluorescence compensation, as they were similar in compensated and uncorrected runs.

Conclusions: These results suggest biochemical interactions among ROS and fluorogenic substrates commonly used in oxidative stress studies and recommend caution when several probes are to be combined in multiparametric analysis of ROS.

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Mass cytometry is the meeting point of conventional flow cytometry and time-of-flight (TOF) mass spectrometry. While the two parent technologies have been embraced fervently in different fields for more than thirty years, the newborn hybrid has yet to find its place in the toolbox of scientists and clinicians. Using lanthanide isotopes to label antibodies, mass cytometry aims to overcome the limitations of conventional flow by increasing the resolution to more than 40 metal probes with minimal signal overlap, hence enabling comprehensive profiling of cellular phenotypes, signaling states, cytokine/chemokine expression, and viability. Despite these advances, there is still considerable hesitation in adopting this new technology, which can be due to a number of reasons such as: 1) limited numbers of commercially-labeled antibodies, 2) high initial costs linked to large antibody panels and specific reagents, 3) lacking experience in design and validation of CyTOF antibody panels, 4) low density of CyTOF based research to date, the need to 5) adapt and optimize different staining protocols, and 6) standardize experimental guidelines, especially when automating sample introduction.

To address some of these challenges, at the Ragon Institute of MGH, MIT and Harvard Mass Cytometry Core, we have determined guidelines to standardize and streamline mass cytometry experimental design. We show that applying these guidelines minimize cell loss and optimizes the detection of rare cell populations, especially while automating sample introduction into the CyTOF2 mass cytometer during long runs using the autosampler. We have also developed an organizational framework aiming to optimize pooled resources in an attempt to decrease initial costs of adopting the technology. These protocols and guidelines can be acquired by any shared core facility as well as new users to overcome some of the barriers of implementing the technology as part of routine analysis and diagnostic tools used in lab and clinic.

Cell surface protein signatures have been successful to discriminate hematopoietic progenitor populations allowing major advances in understanding blood cell production, to define pathways in hematologic malignancies and to foster new therapeutic approaches (1). Limited knowledge on the phenotype of cells that participate in heart formation impairs our understanding of progenitors of the cardiac cell lineages and their eventual persistence in the adult heart. As a consequence, therapies to restore heart function after injury have been unsuccessful. A number of membrane proteins have been identified on cardiomyocytes (2, 3, 4); on cardiac fibroblasts (5, 6); and on endothelial cells, however a multi-parametric analysis of the phenotype of the different cardiac cell compartments along development is still missing.

We combined multi-parametric flow cytometry with transcriptional characterization, based on well-known gene expression patterns, to describe major cardiac cell-subsets.

The expression of CD24, CD54, Sca-1 and CD90 allowed defining cardiac populations in the non-hematopoietic and non-endothelial cell fraction by conventional flow cytometry. Transcriptional profiling of the sorted populations enabled the identification of cardiomyocytes, in the CD24+ population, while differential expression of CD54, Sca-1 and CD90 defined four cardiac stromal compartments. The identified subsets exhibited specific distributions in three analyzed regions (atria, auriculo-ventricular junction and ventricles). Nonetheless, analysis at the single cell level indicated heterogeneity in the populations. We hypothesized that by increasing the number of parameters analyzed unique signatures could be resolved.

To this end, spectral cytometry (Sony Analyzer) was used for the analysis of the previous subsets empowering us to further discriminate subsets of cells in our previous populations by adding three additional markers (CD166, CD140a and CD146). These new subsets are presently undergoing transcriptional analysis.

Moreover, we find the same subsets as in conventional cytometry with similar frequencies while accounting for the autofluorescence in the analysis permitted a better definition of the subsets.

We have thus identified a panel of surface markers, some of which novel in the cardiac context, that allowed assigning surface signatures to different cellular fractions by their unique transcriptional profiles. This work is the foundation for comprehensive studies on the role of different cell fractions of the heart in physio-pathological conditions.


321/B190

The Hitchhiker's Guide to CyTOF: Standards, Guidelines and Everything

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322/B191

Multi-dimensional Flow Cytometry Unravels Novel Populations in the Developing Mouse Heart

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New Fluorescent Dyes for Sensitive Detection of Regulatory T Cells by Flow Cytometry Analysis

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Regulatory T cells (Tregs) play a crucial role in the suppression of unspecific or excessive immune responses. Therefore, the detailed characterization of these cells and their function gains an increasing interest in basic and translational research. Tregs generally express the surface markers CD4 and CD25, which are mainly used for initial identification purposes. Additional cell markers, like CD45RA, CD127, FoxP3 and/or Helios, are usually used to distinguish respective subpopulations in order to analyze the diverse functional potential of the cells. Due to the varying expression levels of these surface markers, the identification of cell subsets can be very challenging. Multicolor flow cytometry is a well-suited tool for the profiling of Tregs but the limited brightness of most commercial available fluorochromes can delimitate the accurate analysis of this cell type. In order to overcome these limitations novel fluorochromes are introduced that furnish very high fluorescence intensities with minimal spillover values in adjacent detection channels, and are of similar utility to the phycoerythrin proteins phycoerythrin (PE) and allophycocyanin (APC). Using these new fluorochromes, a multicolor panel and a gating strategy is presented that facilitates the profound discrimination and characterization of Tregs in peripheral blood. These findings provide a nice example how researchers can benefit from superior fluorochromes in multiparameter flow cytometry analysis towards regulatory T cell profiling.
324/B193
A Biomarker Discovery Approach for Urinary Cells in Lupus Nephritis
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Background: Systemic lupus erythematosus (SLE) is a multisystem autoimmune inflammatory disease. 50-80% of SLE patients develop end-stage renal failure (lupus nephritis LN) that is one of the major causes of morbidity and mortality. Currently, histopathological results from renal biopsy are the basis for categorizing patients according to the ISN/RPS classification scheme into LN classes I to VI and for defining therapeutic plans. Renal biopsy is an invasive procedure not free from risk and it is not applicable to monitor LN activity over time. The goal is to detect and define cell-based urinary abnormalities and signatures circumventing a critical intervention. Recently, conventional flow cytometry was used to detect immune cells in urine of LN patients. We apply mass cytometry technology to urine samples of LN patients to accomplish a more comprehensive immunophenotyping.

Methods: Urine and heparin anti-coagulated blood samples were simultaneously collected from 4 LN patients (one was followed-up) and prepared for mass cytometry analysis. All patients had clinically active SLE and had already started an immunosuppressive therapy. Urine samples were adjusted with 30% v/v PBS/BSA immediately after collection to preserve cell viability. Peripheral blood cells were obtained after erythrocyte lysis. A 29 surface marker staining protocol including live/dead discrimination (Ph/Ir intercalators) was performed and fixed cells were analyzed on CyTOF1 instrument. Data were further analyzed by manual gating using FlowJo 10.0.7 software.

Results: In order to preserve as many cells as possible for mass cytometric analysis, the viability of cells in urine was significantly improved by simply adding 30% v/v PBS/BSA to the fresh collected samples. Mass cytometry allows a comprehensive immunophenotyping of immune cells washed out in urine circumventing problems regarding sample size, autofluorescence-induced artefacts and spectral overlap inherent in conventional flow cytometry. In urine of LN patients monocytes or granulocytes were identified as the dominating leucocyte subsets. Monocytes were characterized by a coexpression of CD14 and CD16 – a phenotype not found in peripheral blood. Although T cells were below 3% of CD45+ cells, we were able to observe a decreased CD4/CD8 ratio as already described in the literature for LN blood. Additionally, a T cell subpopulation expressing the activation marker CD69 was identified in urine but not in blood. Almost no B cells, NK cells or dendritic cells have been detected in urine samples.

Conclusion: First patient data shown indicate that leucocytes detectable in urine of LN patients are promising biosensors reflecting chronic inflammation in the kidneys. Obviously, the LN histopathologic heterogeneity seems to be better reflected by urinary cell populations than by peripheral blood. Therefore, larger patient cohorts and additional epithelial markers will be analyzed to identify robust biosignatures that can be used diagnostically for a classification of lupus nephritis.

Background: Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease which preferentially affects the axial skeleton and is strongly associated with the pro-inflammatory cytokine tumor necrosis factor alpha (TNF). Therapeutic targeting of TNF is approved to be highly effective in patients who fail to respond to conventional anti-inflammatory drugs. However, only around two-thirds of anti-TNFa treated AS patients show an adequate response according to the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) independently from the biological used. Therefore, there is an urgent need for biomarkers which would aid in treatment choice and treatment outcome separating responders and non-responders to such expensive therapies and to avoid side effects induced by inefficient drugs. Thus, the aim of this study was to identify cell-based biosensors in peripheral blood by multiparametric flow cytometry that can be used for an early treatment stratification of AS patients.

Methods: A multiparametric flow cytometric approach, including 50 monoclonal antibodies combined to 10 staining cocktails, was applied to identify useful baseline predictors in 38 AS patients with active disease before treatment with the TNF blocker Adalimumab, Etanercept, Golimumab or Infliximab. BASDAI response criteria were used to determine therapeutic success after 1 to 6 month. Automated clustering of flow data, correlation analysis and receiver operator characteristics were accomplished to appoint auspicious candidate phenotypes.

Results: Out of multiple potentially significant parameters, which are involved both in acquired and adaptive immunity the NK cell compartment revealed most promising subsets that are predictive for a successful therapy response to Etanercept in AS. Correlation analyses showed an errorfree classification of responders and non-responders for Etanercept but not for Adalimumab-treated patients.

Conclusions: This is the first study demonstrating that the composition of the NK cell compartment has predictive power with respect to classify AS patients whether they will respond or fail to the treatment by Etanercept. These results also shed some new light on the mode of action comparing TNF-alpha neutralizing antibodies and soluble TNF alpha-receptors. In conclusion, these data make it reasonable to assume that monitoring of particular NK cell phenotypes can be used in terms of a companion diagnostic to realize the concept of personalized medicine in the field of rheumatology. But we are aware that the results presented have to be validated by independent and larger patient cohorts.

326/B195
Classification of Flow Cytometry Samples with a Dimension Reduction and Binning Approach
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Motivation: The automated analysis of multiple follow cytometric dataset with respect to a covariate has been at the forefront of attention in the past 5 years. It is not a straightforward problem, as the data format does not fit into any priorty available statistical framework. Efforts have focused on clustering of the samples - either each individual sample or pooled. However, a clustering-based approach has the drawback of cluster validation.

Results: We propose a scheme of inter-sample comparison that is based on binning dimension-reduced data using a uniform grid. For a given covariate, we choose the best dimensionality and grid size by training Support Vector Machines with half the data (randomly selected), validating with the test set and replicating this procedure 100 times for each parameter combination. We demonstrate the approach on a dataset consisting of tonsil, bone marrow, spleen and blood samples (53 in total), and the selected model has a median classification rate of 85%. Additionally, this approach allows for the embedding of the samples in a vector space, highlighting the relationship between the samples and bins. Finally, this approach can be extended to regression in the case of a continuous covariate.

325/B194
NK Cells as Biosensors for Responsiveness to Etanercept in Ankylosing Spondylitis (Morus Bechterew)
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327/B196
The Immunophenome of the Mouse: An Integrative Approach to Identify New Key Genes of the Immune System
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One of the main challenges in biology is to characterize the function of each of the 20,000 genes composing our genome and determine their implication in human biology. The possibility to edit ‘at will’ the mouse genome via targeted mutagenesis is currently one of the most reliable and fast way for functional genomics in higher eukaryote organism. Within the last 5 years, the IMPC (International Knock-out Mouse Consortium) performed serial inactivation of all genes of the mouse genome. The second phase of this approach consists in subjecting resulting mouse mutant lines to arrays of standardized tests in order to establish the first truly comprehensive, functional catalogue of a mammalian genome. The IMPC (International Mouse Phenotyping Consortium) is planning to release the clinical status of 4000 mutant lines by the end of 2016.

The Centre of Immunophenométrie (CIPHE) and other institutes of the research infrastructure PhenomIn (ICS, TAAM) takes part to this global effort to identify the function of genes. By using standardized experimental procedures, the immunophenotyping module of CIPHE specifically analyzes the functional status of the immune system of these mice at steady state and under inflammatory conditions. Using Cytoprepate and Batlab, integrative data analysis are performed on multiple high-content flow and mass cytometry panel in order to link cell population dynamics to several organs, multiple immune assays and functional phenotyping. The resulting immune-phenome signatures for each mutant line are then compared to control samples to maximize the chances to find a phenotype and get an insight on the function of a gene. Across practical examples, the strength of this screening methodology will be presented.

MULTI-DIMENSIONAL IMAGE CYTOMETRY (B197 – B202)

328/B197
Quantification and Characterization of Viral Infection Cycle Using Imaging Flow Cytometry
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The recently discovered Acanthamoeba polyphaga Mimivirus (APMV) is a giant virus that infects Acanthamoeba species. It belongs to the Nucleo-Cytoplasmic Large DNA Viruses (NCLDVs) clade, large dsDNA viruses sharing the unique feature of replicating (entirely or partially) at their host’s cytoplasm. Cytoplasmic replication is associated with the formation of a viral factory (VF) at the host cytosol. The VF is a large and highly elaborate compartment within which central infection events occur, including DNA replication, transcription, DNA packaging and capsid assembly. Using the unique imaging and analysis abilities of the ImageStream, a high-throughput imaging flow cytometer, we present for the first time a method to track and characterize the progression of the Mimivirus infection cycle in a quantitative manner. We designed a series of features describing the morphological changes of the infected cell and the formation and maturation of the VF throughout the infection cycle. This allows us to monitor changes in the infected amoeba under different experimental conditions and to unravel the involvement of key elements in the infection cycle. The features used to track the progression of the infection cycle include the identification of the early formation of the VF, its localization within the infected cell, VF development and progression, as well as quantifying changes in the cells’ morphology and granularity. In addition, we developed a method for directly counting viral particles. The characterization and quantification methods presented here for the first time may serve as a valuable and general tool in studies of viral infections.

329/B198
Intestinal Epithelial Cells Fragment upon Isolation, and Their Debris Is Taken Up by Intestinal Epithelial Lymphocytes or Other Epithelial Cells
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Gastrointestinal tissue has a complex milieu of cells, with a relatively high proportion of “unconventional” immune cells. Intraepithelial mononuclear phagocytes and intraepithelial lymphocytes (IELs) normally maintain tolerance to food and commensal bacteria-derived antigens, while protecting against pathogens. However, in response to inflammatory signals, IELs can also induce epithelial damage directly, and by recruiting and stimulating inflammatory leukocytes.

In standard tissue preparations, a significant proportion of IELs are positive for EpCAM+ by FACS. Peyer’s patch leukocytes, however, are only rarely EpCAM+. By imaging cytometry, EpCAM on T cells has a punctate appearance at the cell membrane, often colocalizing with a bleb in bright field or scatter images. These EpCAM+ blebs are not themselves positive for Annexin V or for cell-impermeable live/dead stains, but are often found on cells positive for Annexin V or a live/dead stain. We suspect that this EpCAM is in fact derived from epithelial cell-derived blebs, not from the IELs themselves.

When we examined the cytotoxicity of various IEL subsets, in some experiments we unexpectedly found high frequencies of dead cells in the absence of stimulation. By imaging cytometry, over 80% of the cells positive for live/dead staining in some samples were in fact conjugates of a live target cell with a dead bleb. Doublet discrimination in conventional flow cytometry, using time-based parameters fails to distinguish these conjugates from actual dead cells. However, imaging cytometry is able to unambiguously resolve dead cells from conjugates of live cells and dead cells. These results indicate that flow cytometric assays of cytotoxicity should be interpreted with caution, especially when several different populations are being compared simultaneously, and underline the importance of imaging cytometry for these assays.

330/B199
Phenotypic and Epigenetic Mechanism of Action Determinations of Histone Methylation and Demethylase Inhibitors Using Digital Widefield Microscopy
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Histone lysine methylation is a reversible process, dynamically regulated by both lysine methyltransferases and demethylases. In general, methylation of histone H3 lysine 4 (H3K4me), H3K36, or H3K79 is associated with active transcription, whereas...
methyltransferase that selectively induces apoptosis in cancer cell lines. MCF-7 breast cancer cells stably expressing GFP, were incorporated to create a more 3D cell model. Induction of apoptosis was monitored using fluorescent probes, while the photoproducts allowed differentiation of the final cytotoxic effect on the two cell types in the co-culture. Mechanism of action studies of the inhibitors were then performed using antibodies to the specific histone H3 lysine residues and their methylated state. All assessments were made via digital microscopy using a novel cell imaging multi-mode reader.

331/B200
A Rapid 3D Tumor Spheroid Imaging Cytometric Analysis Method for Drug Discovery
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The current 2D methods for cancer drug discovery have had some difficulty in identifying potential drug candidates that can be used for clinical testing. To overcome this challenge, there has been an increase in research of 3D tissue culture that facilitated the development of new in-vitro tumor model assays. Traditional 2D and 3D analysis method relied heavily on visual observation using microscopy. However, the method is time-consuming and has high variations. Automated plate-based imaging cytometer can be employed to rapidly analyze and characterize 3D tumor spheroids, which can be used to generate both quantitative and qualitative results. In this work, we demonstrate a novel 3D tumor spheroid analysis method using the Celigo imaging cytometer for spheroid counting, size analysis, tumor migration and invasion, tumor viability, and dose response of drug induced/inhibited tumor growth. The plate-based imaging cytometer utilizes bright-field and three fluorescence channels (Blue, Green, and Red) for multi-channel analysis. By utilizing the F theta lens technology, uniform bright-field image is captured for more accurate cell counting and analysis of the entire well. In addition, Celigo analysis software is used to report numerous parameters allowing detailed spheroid characterization. In addition to direct spheroid counting in the

well, the use of specific fluorescent dyes and probes allow the researcher to define viable and hypoxic areas within spheroids and monitor migration and invasion on or into supporting cells and/or tumor tissues. The results showed that Celigo imaging cytometer can accurately count and measure spheroid sizes in response to drug induction. Furthermore, tumor migration and invasion were clearly observed and quantified in the captured images. By utilizing the 3D spheroid imaging cytometry method, researchers can quickly characterize and quantify tumor spheroids, which can improve the efficiency of identification of potential cancer drug candidates.

332/B201
Multiplex Human Whole Blood Phenotyping with Cell Signaling Assessment Using Imaging Flow Cytometry
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The studies of signal transduction pathways that regulate immune cell activation have become very important in the understanding of human health and disease. In particular, the activation of cell signaling molecules and their translocations to the nucleus have become a focal point for studying cellular functions, signal transduction pathways, disease mechanisms and drug discovery efforts. Current methods to study nuclear translocations of signaling molecules rely on manual microscopy or Western blots, which are cumbersome and do not permit statistically robust analysis of translocation events on a per cell basis in the heterogeneous samples. In this study, we present results on the phenotyping of human whole blood in combination with antibodies against cell signaling molecules such as nuclear factor-kB (NFκB), mitogen-activated protein kinases (MAPK) and nuclear factor of activated T-cells (NFAT1) utilizing the Amnis imaging flow cytometry and assess nuclear translocation of these molecules in cell sub-sets. This method combines the quantitative power of flow cytometry with the spatial information provide by microscopy in one system allowing the rapid quantitative assessment of nuclear translocation in white blood cell sub-populations. The ability to study NFκB, MAPK or NFAT1 translocation in specific immune cell subtypes in the whole blood can be a powerful tool to evaluate the effectiveness of host immune responses and in understanding disease mechanisms.

333/B202
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The main objective of this study was to develop a method for assessing sepsis-related senescence and fragility of human red blood cells (RBC). The presence of senescent RBC is linked with the development of microvascular dysfunction observed during sepsis. It is hypothesized that accelerated senescence and excessive fragility of RBC might be manifested by the higher number of RBC-derived microparticles, increased exposure of phosphatidylserine on the surface of RBC, and general changes in their shape and size. If this hypothesis is correct, measurements of those parameters could be used to predict clinical outcomes of patients with sepsis. In order to develop an assay that measures RBC senescence markers simultaneously we decided to take advantage of the capabilities offered by ImageStream (Amnis/Millipore) imaging flow cytometer. Using this platform in conjunction with IDEAS analysis software allows gaining information regarding cells’ size and shape, as well as multispectral fluorescent signals in large numbers of events. In a single sample we were able to perform Annexin-V binding for detection of phosphatidylserine, use RBC specific antibody to

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enumerate microparticles, and also identify RBC with abnormal shape (echinocytes). The preliminary tests performed on samples from 12 sepsis patients and 6 healthy controls revealed higher frequencies of BCR-derived microparticles and Annexin-V binding cells in the sepsis patients samples. We also observed significant spread of measured parameters, especially for sepsis samples, therefore further testing on a larger number of samples will be required to fully validate this assay. The sample preparation, staining protocol and analysis strategy using IDEAS analysis software is presented.

**New Probes and Assays (B203 – B208)**

**334/B203**
Spatial Information on Receptor Clustering in Flow Cytometry via FRET

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Flow cytometry is commonly used to detect the overall fluorescent signal of a cell and therefore to analyze whether a specific target molecule is present. However, no information on the distribution or clustering of the molecules of interest can be gained from these signals. Yet, the clustering of receptors or the interaction of proteins plays a key role in almost all biological functions.

Foerster resonance energy transfer (FRET) is a well-suited method to identify protein-protein interactions in cells. Here, we established an automatic FRET-based program, which allows for the identification of protein interactions and receptor clusters on the cell by flow cytometric analysis.

Using this automatic FRET-based method, we could measure the clustering of the T cell marker CD3 on the cell surface after activation by a strong superantigen. The CD3 receptor clustering on the T cell surface is essential for the downstream signaling of activation signals inside the cell. As a proof of principle, we could successfully demonstrate that the FRET efficiency increased along with the formation of CD3 clusters. Therefore, using this technique it is possible to detect the T cell activation on the flow cytometer.

This novel FRET-based method is a powerful tool which allows to acquire spatial information of protein clusters. It can be used on large cell numbers in a minimum of time and is easy to use even in high throughput screenings.

**335/B204**
DARPins as Analytical Reagents for Mass Cytometry – Increased Performance at a Reduced Price

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Currently, available flow cytometry technologies based on fluorescence detection are limited to around 20 simultaneous parameters measured due to the limited availability of different fluorochromes and spectral overlap between them [1]. Vastly improving this limitation, mass cytometry is a novel technological platform primarily aimed at the multiparametric analysis of protein expression profiles of living cells. Instead of using fluorochromes for labeling of binding agents (e.g. antibodies), they are labeled with a polymer carrying metal chelates and the metals are detected by mass as a unique identifier [2].

Traditionally, monoclonal antibodies are used for labeling, but their performance has been unsatisfactory, due to their limited stability during labeling, interference of the coupled polymer with binding, or lack of performance of the antibody in the first place. Despite poor performance [3], many antibodies are marketed very expensively. We therefore sought to create more robust specific binding molecules that can be labeled with the polymer of metal chelates.

Designed ankyrin repeat proteins (DARPins) have been developed as powerful alternatives to conventional antibodies [4]. Combinatorial libraries and ribosome-display selection of DARPin give rise to target-specific binders of very high stability, affinity and specificity. DARPins can for recombinantly produced in E. coli at low cost with high reproducibility. Most importantly, DARPin can be engineered to contain unique lysine residues for metal-tag labeling without affecting their structural integrity, and can be designed in many different multivalent formats and with multiple sites for attachment of detection molecules, resulting in increased sensitivity of detection.

We have completed proof-of-principle experiments with several DARPin, examining their performance as analytical reagents under various conditions in CyTOF assays, thereby proving the ability of DARPin to be used to detect cellular surface and intracellular antigens. In these experiments we showed the excellent functionality of DARPin binding human Her2, MAP Kinase 8, EGFR and CD4. Importantly, their production and labeling has turned out to be much cheaper, faster and more reproducible than that of antibodies. Thus, we believe that we can develop much more robust and sensitive probes for mass cytometry on a DARPin basis.


**336/B205**
Measurement of Monocyte-Platelet Aggregates by Imaging Flow Cytometry

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Platelets are subcellular blood elements with a well-established role in haemostasis. Upon activation platelets express P-Selectin (CD62P) on the cell membrane and bind to P-Selectin glycoprotein ligand 1 expressing monocytes, influencing them toward a pro-adhesive and inflammatory phenotype. It is well established that elevated circulating monocyte-platelet aggregates (MPAs) are linked to atherothrombosis in high risk patients. However, whole blood flow cytometry (FCM) has recently shown that circulating MPAs may also occur in the absence of platelet activation, particularly in healthy children. A potential limitation of conventional FCM is the potential for coincident events to resemble monocyte platelet aggregates. Here we report a novel imaging cytometry approach to further characterize monocyte-platelet aggregate formation by P-Selectin dependent and P-Selectin independent mechanisms and distinguish circulating MPAs from coincidental events. Monocytes were identified by expression of the lipopolysaccharide receptor (CD14 BV421), while platelets were identified by expression of the glycoprotein
lb (CD42b APC). Differentiation of P-Selectin dependent and P-Selectin independent binding was achieved with AF488 labeled CD62P. Overall analysis of circulating and in vitro generated MPAs by conventional and imaging cytometry methods showed very strong correlation (r(2) = 0.99, P < 0.01). The Bland-Altman bias of -1.72 was not significantly different to zero. However, when measuring only P-Selectin negative MPAs, a lack of correlation (r(2) = 0.27, P = n.s.) likely reflects better discrimination of coincidence events using imaging cytometry. Our data demonstrate that IFC is more accurate in enumerating MPAs than conventional FCM, which over-estimates the number of MPAs due to the presence of coincident events.

337/B206
New High-Sensitivity Fluorescent Dyes for Immunofluorescence Applications

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The versatility of flow cytometry as means to analyze complex biological systems is still limited by the variety of bright fluorochromes currently available. The sensitivity of fluorescent probes in cell staining is usually limited by several factors including, the ability of the dye for absorbing energy (extinction coefficient) and the conversion of the absorbed light to fluorescence (quantum yield), the degree to which extend the intrinsic autofluorescence of the system interferes with the dye emission, and for certain applications the resistance of the dye to fixation agents and photolytic degradation.

Herein, we present a new set of fluorescent probes that address the FITC and APC detection channels of common flow cytometers. We demonstrate that these fluorescent reporters are superior to commercial available alternatives, e.g., Viobright™ FITC, BD Horizon Brilliant™ Blue 515 or APC, and can even exceed the fluorescence signal of PE-conjugated antibodies. While offering phycobiliprotein-like brightness they provide higher stability towards photolytic degradation and fixation conditions than phycobiliproteins. The intrinsic low unspecific binding allows for low background staining even in the absence of buffer additives unlike conducting organic polymer-based fluorescent molecules. They are thus ideally suited for cytometric assays requiring high sensitivity for the detection of especially low expressed cell markers. Their extraordinarily bright signal, desirable spectral properties, compatibility with existing measurement technology, low unspecific binding, and reasonable resistance to fixation agents as well as photolytic degradation make them suitable for a variety of immunofluorescence applications. This is an exciting and powerful addition to the comprehensive arsenal of fluorescent molecules that should pave the way for the design and implementation of new multicolor panels towards high-sensitivity cell profiling approaches.

338/B207
Flow Karyotyping and Sorting Chromosomes from Drosophila melanogaster

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Chromosome sorting is a very useful tool for directed analysis of individual chromosomes, either singly or in bulk. While the general technique has been in use for multiple decades, it has not been successfully applied to some organisms, including the ubiquitous model system Drosophila melanogaster. The small genome of D. mel. makes whole genome analysis much more feasible than for many other organisms. However, there are still some techniques that would be improved or made possible by sorting the chromosomes prior to downstream assays (e.g., single-chromosome analysis, chromosome-specific genomic libraries, and identification of genomic aberrations). Here we present a method for the fluorescence-activated sorting of D. mel. chromosomes from an S2 cell line.

After arresting cultured S2 cells in metaphase, the chromosomes are collected using a polyamine buffer protocol and then stained with Hoechst 33342 and chromomycin A3 to allow for bivariate analysis. Each population from the resulting flow karyotype is sorted at low numbers and libraries are prepared using the PicoPLEX™ DNA-seq kit from Rubicon Genomics. Next-generation sequencing is used to assign a chromosome identity to each karyotype population.

With this method, a distinctive bivariate flow karyotype pattern has been reproducibly achieved, and multiple populations are identified by next-generation sequencing. Thus, the described method can be used to sort enriched D. mel. S2 chromosomes for a variety of downstream applications. Unexpectedly, the flow karyotype revealed more chromosome populations than naturally occur in D. mel., demonstrating the high rate of chromosomal aberrations that occur in cultured S2 cells. Therefore, flow karyotyping of primary and immortalized Drosophila cell lines may prove to be a useful tool in the study of genomic instability, an important hallmark of cancer.

339/B208
Anti-glycophorin A Labeling of Human Red Blood Cells to Screen P. vivax Malaria Vaccine Candidates

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The difficulty culturing the malaria parasite Plasmodium vivax has hampered any progress towards an effective vaccine. While there can be some parasite invasion in vitro the small number of invasion events and high percentage of parasite death makes evaluation of invasion inhibition very difficult. We have developed a novel flow technique to label target human red blood cells with Glycophorin A (FITC), to allow for the enumeration of new P. vivax invasions. P. vivax arrives frozen from the ATCC in the blood of Aotus monkeys which are Glycophorin A negative. Therefore, the observation of a parasitized Glycophorin A red blood cell can only be the result of a new invasion event. Coupling this event identification with high throughput rare event analysis allows for analysis of invasion mechanisms to be studied for the first time. Reticulocyte enriched human blood samples were exposed to different strains of P. vivax which have several key genetic variations in the ligand the parasites use to bind to red blood cells called the Duffy Binding Protein (DBP). New invasions were measured on a BD LSRII using Hoechst 33342 to identify DNA positive red blood cells when in culture with and without anti-DBP blocking antibodies. The results showed that the ability of the anti-DBP antibodies to prevent invasion of human red blood cells was highly dependent upon the parasite strains used which has been theorized but never definitively shown until now. Anti-DBP antibodies directed against the DBP variant found in the Thailand strain showed a 97.0% inhibition of invasion. Conversely, these same anti-DBP antibodies only showed 1.4% inhibition of the P. vivax Nicaragua strain. These results demonstrate that polymorphisms in the parasite’s DBP may play a larger role in invasion success than previously believed.

NEW SOFTWARE DEVELOPMENT (B209 – B212)

340/B209
Sneak Peek at New Features in Flow Repository

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Data associated with publications should be available and easily accessible. In order to address this need in flow cytometry, we developed FlowRepository for authors to deposit and share their annotated datasets. FlowRepository was launched in 2012 with the support of ISAC and the Wallace H. Coulter Foundation, and
it quickly became one of the main sources of public flow cytometry data. Currently, there are 212 public datasets; 94 of these are related to Cytometry A papers. FlowRepository downloads (16,000 datasets in 3 years) illustrate the community’s need for this resource. FlowRepository facilitates the integrated review of datasets referenced in published literature and supports storage, MFlowCyt compliant annotation, online data analysis and sharing. Over the last year, we have developed several new features that will be released in the upcoming months, including FlowRepository’s integration with R, support for development of high content panels, and integration with the International Mouse Phenotyping Consortium (IMPC) data.

Earlier this year, we added authentication to FlowRepository’s Application Programming Interface (API) and extended it with new searching capabilities and programmatic access to both, public and private datasets. We then leveraged this API to develop FlowRepositoryR - an R library that allows for a convenient access to FlowRepository from within R. FlowRepositoryR is easy to use even for researchers with little programming experience. Basic operations, such as locating a dataset, reviewing metadata and downloading files can be done with as little as two simple lines of code. After that, data can be analyzed either directly in R, or other third-party software. FlowRepositoryR is publicly available on GitHub and will be submitted to BioConductor. The use of the new API is open to third-party software developers.

A new ‘Phenoverse’ (the universe of phenotypes) subsection of FlowRepository has been developed and is currently being tested in collaboration with Drs. Chattopadhyay and Roederer. It has been designed as a resource for the development of high content flow cytometry panels for immunophenotypic analysis using 27+ color fluorescence flow cytometry. Phenoverse allows registered users to design complex panels and discuss those within FlowRepository with other experts in the field.

IMPC centers are generating mutant mouse targeted knockouts for all known and predicted mouse genes, and collecting phenotypic data for each mutation. This effort is expected to generate over 77,000 FCS files in the next 5 years. We have partnered with IMPC to extend FlowRepository to accommodate primary IMPC data and metadata, to integrate FlowRepository in IMPC’s automated data acquisition and analysis workflows, and to provide additional capabilities facilitating mining of such a large and complex dataset.

342/B211 rFRET: A User-Friendly Matlab Application for the Analysis of Intensity-Based Microscopic FRET Experiments

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Background: Förster (or fluorescence) resonance energy transfer (FRET) is a widely used technique for the investigation of protein clustering. An intensity-based measurement in which the donor-acceptor double-labeled sample is measured in three fluorescence channels (donor, FRET and acceptor) is probably the easiest way to detect FRET. Since careful determination of many compensation factors is required for the quantitative evaluation of such a FRET experiment, most analysis tools designed for ordinary users are capable of performing only a semi-quantitative evaluation.

Results: We have developed a GUI (graphical user interface)-based application, rFRET, for the rigorous quantitative analysis of intensity-based microscopic FRET experiments in Matlab. The program performs the analysis in four steps: (i) conversion of spectral overspill factors for the donor and acceptor dyes; (ii) determination of a, a parameter describing the sensitivity of the detection system for excited acceptors compared to excited donors; and (iv) calculation of the FRET efficiency. Images stored in formats Matlab can handle can be read and analyzed by the program. The application can perform analysis of overspill factors and the FRET efficiency on a pixel-by-pixel basis, using summed intensities, regression analysis and maximum-likelihood estimation. In each section of the program a mask image can be used to restrict analysis to pixels of interest. Pixels can be gated on one-dimensional and two-dimensional histograms (dot plots) to eliminate outlier pixels.

Conclusion: rFRET combines rigorous analysis with an easy-to-use graphical user interface to make quantitative analysis of intensity-based microscopic FRET experiments available to ordinary cell biologists.
Acute Lymphoblastic Leukemia (ALL) is the most frequent leukemia entity in children and adolescents. Despite continued progress and refinement of therapeutic approaches, disease relapse due to insufficient extinction of leukemic blasts still remains the number one cause of treatment failure. About 15-20% of pediatric patients with the disease still suffer from relapse. Flow cytometry (FCM) is one of the methodologies most useful in this respect, because it is widely available and applicable to most patients. While FCM acquisition can be harmonized straightforwardly, data analysis and interpretation rely largely on operator skills and experience. Hence, these requirements represent the current bottleneck of safely applying the FCM-Minimal Residual Disease (MRD) methodology in a growing community of diagnostic laboratories. Thus, Machine Learning (ML) methods represent an objective methodology for automated analysis of FCM data to cluster/classify cell populations and determine MRD.

Although automatic clustering methods based on mixture models are suitable for general FCM data there are currently no comprehensive evaluations, particularly for ALL data. Therefore, we concentrate on the evaluation of different strategies for automatic FCM analysis on a newly collected database: the annotated data set derives from samples of ALL patients that have been treated uniformly according the AIEOP-BFM 2009 treatment protocol. In detail, the data set will initially hold about 200 leukemia cases for B cell precursor ALL and will be expanded to T-lineage ALL. Representatives of all known sample appearances including phenotypic shifts, subclone formation, and parallel occurrence of normal regenerating precursor cells are present in the dataset. Many samples of the 200 cases hold low MRD-rates <1%, since reaching state-of-the-art detection accuracy below this proportion is crucial. Each leukemia case is measured at 3 time points during leukemia treatment (d15, d33 and d78), with 2 tubes per time point, and each containing 300,000 cells with currently 9 FCM-parameters. All samples have been annotated using a standard gating strategy to define the operator based ground truth of blast cells.

The data set is used for an evaluation of different learning approaches (Mean-Shift clustering with a Bayes decision, Bayes decision based on Kernel Density Estimation (KDE), and a Bayes Classifier based on mixture models).

The usage of a Gaussian Mixture Model allows a compact representation of the high dimensional FCS data so we propose a model based on GMM, learned on a training set of 150 patients. Blasts (leukemic) cells are modeled separately from the non-blasts in order to prevent that the first group is missed in the modeling phase. The model is then represented using a set of basis functions in order to reduce the computational complexity in the testing phase.

The dataset will be extended by ALL data of the same leukemia types from different medical centers and different FCM devices, to develop a device independent methodology. Thus, the data will be normalized to be independent of different channels and setup variations.

A summary of the evaluation on our ALL data set is presented and future perspectives are discussed.
Among hundreds of proteins involved in apoptosis, Bcl-2 family members are of great significance in controlling cell's fate. Antiapoptotic proteins such as Bcl-2 and Bcl-XL inhibit apoptosis in response to a wide variety of stimuli. Conversely, proapoptotic proteins, such as Bad, Bax, and Bcl-XS, can accelerate this process. Over expression of antiapoptotic proteins, or inadequate expression of proapoptotic proteins, can result in the lack of cell death. Taking advantage of the superior sensitivity of HSFCM, we have developed a quantitative approach to measure the copy number of Bcl-2 and Bax on individual mitochondria via immuno-fluorescence staining. SYTO 62 was used to label mitochondrial DNA to distinguish the intact mitochondria from mitochondrial fragments and contaminates of other organelle debris. A series of fluorescent nanospheres with known molecules of equivalent soluble fluorochrome (MESF) were used to construct a calibration curve that can convert the fluorescence intensity of single mitochondria to the copy number of the specific protein. The average Bcl-2 copy number measured for individual mitochondria is ~1250 with a large distribution ranging from 100 to 10,000. This approach allows us to monitor the change of Bcl-2 and Bax abundance on individual mitochondria induced by apoptotic stimulation. It turned out that the content of Bax increased about 4 fold while Bcl-2 remained the same in response to 9 h of staurosporine treatment. Enzyme linked immunosorbent assay (ELISA) and Western blot analysis were carried out in parallel to verify the reliability of our HSFCM assay. Besides Bcl-2 family proteins, the as-developed approach is applicable to the quantitative measurement of other mitochondrial proteins at the single-organelle level. It is believed that HSFCM will provide an effective tool for the study of mitochondrial proteins and their roles in mitochondria related process.

### 346/B215
Developing a High Throughput Estrogen Receptor Screening Assay Based on FRET between Fluorescent Estradiol and Enhanced Green Fluorescent Protein

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**Background:** Förster resonance energy transfer (FRET) is a fluorescence-dependent phenomenon that is regularly implemented in biological assays. FRET measurements help to determine intracellular protein-protein interactions, ligand-receptor binding, or protein folding. In this project, we evaluate whether or not FRET is occurring between the fluorophores, E2HPY (triazaborolopyridinium) and enhanced green fluorescent protein (EGFP). E2HPY is a fluorescent estradiol compound; it passes through the membrane of viable cells, binds to, and activates estrogen receptor alpha (ER-α), and fluoroceses at approximately 420-nm. In order to screen at a high throughput the activation of ER-α by E2HPY, we have designed an EGF-ER-α fusion protein. Our hypothesis is that treatment of E2HPY in cells expressing ER-α-EGFP fusion protein will result in FRET between the EGFP and E2HPY. We test this at different time points and concentrations to optimize E2HPY as it enters the cell, binds to ER-α, and potentially engages in energy transfer with the EGFP. The implication of this work is the development of a rapid, intracellular, and viable screening assay that measures competitive binding of other compounds to ER-α.

**Method:** To accomplish this we culture Chinese hamster ovarian (CHO-K1) cells, transiently express ER-α fused to EGFP, and incubate for 24 hours. Then E2HPY is dissolved in absolute ethanol and added to the cells for 30 minutes. After washing the cells with PBS, fluorescence intensity of each fluorophore is measured using fluorescence microscopy and flow cytometry to search for fluorescent quenching of the donor fluorophore (EGFP). We also look for FRET occurrence using time-resolved flow cytometry whereby we measure fluorescence lifetime changes of EGFP. Additional control experiments are accomplished such as taking non-fluorescent estradiol (17β-estradiol, E2), dissolving in ethanol and treating cells that express ER-α-EGFP fusion. Control cells with and without E2HPY are also measured by microscopy and flow cytometry.

**Results:** Our microscopy results indicate that CHO-K1 cells uptake E2HPY within 30 minutes. Also, microscopy images show the expression of EGFP fused to ER-α in CHO-K1. Our results from cytometry show that the fluorescence intensity of EGFP fused to ER-α decreases in the presence of E2HPY. Also, E2HPY fluorescence intensity increases in the presence of EGFP fused to ER-α. Preliminary lifetime results suggest there is no change in fluorescence lifetime of EGFP when E2HPY is added and future work is planned to verify this, including measurement of FRET efficiency.

**Conclusion:** The results from this project suggest that both fluorophores can be successfully introduced to CHO-K1 cells. When both fluorophores are present, the fluorescence intensity of EGFP-ER-α fusion protein decreases when the appropriate emission channel is observed, and likewise the fluorescence intensity of E2HPY increases. The fluorescence changes suggest possible FRET. After further work is accomplished to verify and design an ideal FRET pair, we anticipate a reliable, viable, screening assay for a variety of drug targets to estrogen receptor alpha.

### 347/B216
Characterization of Cell Alterations Caused by FACS: A Functional Verification of Cell Sorting Approaches

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**Introduction:** The biological status of cells, their viability, vitality and functionality after a sort, is crucial for the reliability and outcome of subsequent experiments. So far no attempts have been made to systematically evaluate sorting parameters and their influences on the quality of sorted cells.

**Methodology:** Preliminary data indicate that sorting parameters such as temperature, pressure, charging and shearing effects during the sort, as well as antibody staining and buffer composition, can severely affect cell functionality. We established a sorter setup, in which each sorting parameter can be changed and its influence can be analyzed like in an equation, with just one variable. In this approach we used cell subpopulations of highest purity, by using a new method, which minimizes cell alterations by antibody staining.

**Results:** FACS is a technique that influences cells dependent on their origin. At the beginning, temperature influences were believed to be the parameter with the biggest importance, jet primary cells showed to be surprisingly stable concerning this parameter. Since MAP kinase activation is induced in stress related cell signaling, we chose p38 phosphorylation as a read out. A phosphorylation of p38 was observed in all cell types, due to cell sorting. However, buffer composition, antibody staining, pressurizing of the sample, temperature or droplet charging, showed no effects on p38 activation so far.

**Conclusion:** We were able to observe sorting induced cell alterations in different cell types. Though no changes could be observed, which led to dramatic cell physiological changes in proliferation or apoptosis.
Material and Methods: The comparison of different analyzers and different cell sorters was performed by analyzing fluorescence beads as well as stained cell material. Since sorted cells are often part of further experiments, other questions arise concerning purity but also viability. Hence, we did a bench to bench comparison of three different commercially available cell sorters, and analyzed the purity, viability and induction of apoptosis of different cell populations.

Results: Important criteria of the performance of a flow cytometer are sensitivity and resolution. All analyzers showed similar performances for standardized fluorescence beads and cell material. On the other hand, for comparing three different cell sorters, the instruments were all operated in one room at the same time with the same operator and with an identical sample. All three tested instruments showed overall a similar high purity and viability of the sorted cell populations.

Discussion and Conclusion: Although the tested instruments all showed only small variations in their performance, individual experimental outcomes should be considered by the instrument choice.

349/B218
Using Multispectral Imaging Flow Cytometry to Assess Ricin Trafficking in Human Lung Epithelial-Like A549 Cells
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Ricin is a toxin that is expressed in the seeds of the castor oil plant *Ricinus communis*. It is a heterodimeric protein composed of a cell binding B chain (RTB) and a catalytic cytotoxic ribosome-inactivating A chain (RTA). The endocytosis and trafficking of ricin in mammalian cells is an important area of research for those producing ricin anti-toxins and other ricin therapeutics. Previous ricin trafficking research utilised confocal microscopy techniques. This gives good resolution and leads to a detailed understanding of the internal movement of ricin within cells. However, microscopy techniques often lack large scale quantification and the ability to look at ricin trafficking in large populations of cells. In these studies we have directly labelled ricin and assessed if trafficking can be seen and quantified using Multispectral Imaging Flow Cytometry (MiFC). The advantage of using MiFC is the ability to make image based morphological and spatial calculations from large cell populations. Ricin was directly labelled with Alexi Flour 647 and cells exposed to 0.2µg (1µg/ml) over a time course of 5 mins to 6 hours. Treated cells were harvested and nuclear counter stained using 4',6-diamidino-2-phenylindole (DAPI) before data capture. Alternatively treated cells were harvested and their Golgi apparatus stained using a mouse anti-human golgin-97 antibody followed by a goat anti-mouse Alexi Flour 405 secondary, before being nuclear counter stained with SYTOX®B-green. Data was captured using MiFC via an ImageStream X MkII. A minimum of 10,000 in focus, single cell events were captured at x60 magnification, using 405 nm (2 mW and 120 mW dependent on sample), 488 nm (100 mW) and 642 nm (150 mw) excitation lasers and powers. Using the IDEAS data analysis software the specific fluorescence location of the ricin within the cells was analysed. Then using cytoplasmic masking techniques to quantify the number of cells with endocytosed cytoplasmic ricin the kinetics of ricin endocytosis was determined. We have shown that ricin gains access to the cytoplasm within 5 minutes of exposure in approximately 20% of cells and after 60 minutes over 90% of cells have cytoplasmic ricin. Following counter staining of the Golgi apparatus three different populations of cells were identified, those with no Golgi associated ricin, those with Golgi associated ricin and cytoplasmic ricin, and cells where all the ricin is located in the Golgi apparatus. In these studies we have demonstrated that it is possible to visualise AF647 labelled ricin using MiFC and identify the intracellular location of the ricin using co-localisation studies.

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**OTHER CLINICAL APPLICATIONS (B219 – B225)**

350/B219
Leishmanial Sphingolipid Induces Apoptosis in Melanoma Cells via Mitochondrial Signaling Pathways through Regulation of Tumour Growth by Involving Angiogenesis
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Cancer appears to be a major cause of death worldwide and the situation may take a turn for the worse due to the development of resistance in therapeutic approaches. Now a days it needs to be designed several strategies for targeting cancer cell growth. Moreover microbes, their cellular components, or metabolites mainly azurin, myriocin, 6-MFA from various microbial origin which afford protection against many human deadly diseases including several carcinoma. Other way apoptosis induction is the major target to develop anti cancer agents to restrict tumour growth.

Lipids from attenuated *Leishmania donovani* in rheumatoid arthritis and also in sepsis associated inflammatory injury is well documented in our previous finding. As we know sphingolipids, may play pivotal role in cellular functions including proliferation, differentiation, growth arrest and apoptosis in system. So, in this study, we explored the apoptotic effect of LSPL-1 (Leishmanial sphingolipid-1) in panel of cancer cell line but highest level of cytotoxicity found in the murine and human melanoma cells with also restrict the tumour growth in murine system.

*In vitro*, we demonstrate that upon LSPL-1 treatment in B16F10 cells manifested nuclear shrinkage, condensation, and fragmentation based on DAPI and AO/EtBr staining. Also it may process phosphatase exposure with cell cycle arrest in G0/G1 phase with its modulatory protein like cyclin D1, E, cyclin-dependent kinase 2,4 in selective time and dose. Additionally, exposure of B16F10 cells with LSPL-1 resulted in activation of the caspase-3, -8, -9 and cleavage PARP and the release of cytochrome c. In the upstream, LSPL-1 increased the expression of Bax, BID, Smac/Diablo, Apaf-1, decreased the expression of Bid and Bcl-2, Bcl-xl and augmented the Bax/Bcl-2 ratio alteration of mitochondrial membrane potential subsequently generation of reactive oxygen species (ROS). It may lead to activation of p53 at serum anchor region via up-regulation of p21 subunit along with PUMA and NOXA signalling cascade which may be assayed flow cytometrically, western blot and ELISA based techniques. *In vivo*, it may improve the survival rate of B16F10...
cells induce tumour bearing mice alters the associated pathological changes including tumour volume, microvascular-density. LSPL-1 may also suppress the tumour associated inflammatory responses. It may regulate several angiogenic factors including VEGF, Ang-2, CD34 and HIF-1α in angiogenic events generated in cell induced tumour microenvironment with the involvement of apoptosis, evidence from histopathology, immunohistochemical and flowcytometry based assay.

These studies underline the significance of anti-neoplastic potential of LSPL-1 through apoptosis induction and abrogation of angiogenic and inflammatory responses in B16F10 cell associated tumour microenvironment.

351/B220
Age-Dependent Activation of Leukocytes in Childhood during and after CPB Surgeries of Congenital Heart Disease
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Background: Surgical trauma induces systemic inflammation both in children and adolescents affecting the postoperative course and response to therapy. This response may be age dependent. To this end we analyzed the innate cellular immune response of neutrophils, monocytes, and natural killer (NK) cells by 4-color flow cytometry in children with congenital heart disease who underwent corrective cardiac surgery with cardiopulmonary bypass (CPB).

Methods: Retrospectively, data of 72 children who underwent surgery between 1995-2002 were analyzed. These pediatric patients were grouped by age: newborns (N) with up to 28d (n=7), infants (I) between 29d up to 1y (n=16), toddlers (T) with 1-5y (n=15), schoolchildren (S) with 6-10y (n=26) and juveniles (J) with 11-16y (n=8). EDTA blood samples were collected at 11 time points: (1) 24 h preoperatively; (2) after anesthesia; (3) thoracotomy; (4) CPB contact; (5) CPB end; (6) immediately and (7) 4h, (8) 24h, (9) 48h after surgery; (10) at discharge from hospital; and (11) after 2-6m at outpatient follow-up. Leukocyte cell numbers were determined, and the expression of surface markers, e.g. mean fluorescent intensity of CD11a, CD11b, CD18, CD45 on neutrophils; CD11a, CD11b, CD18, CD45, CD14, HLA-DR on monocytes; and CD16, CD56 on NK cells was characterized and compared between the age groups statistically.

Results: Neutrophil counts increased 2x (I), 3x (T) and up to 3.5-4x (S, J) during and after surgery compared to baseline (time points 1 and 11), but remained stable for N. Monocyte counts increased after surgery dramatically for T, S and J but only moderately in N and I. NK counts increased 2-3x for I, T, S and J during surgery too, dropped to 0.2x (I) or 0.5x (N, T, S, J) 4-48h post-operative and reached the baseline at time point 11. In contrast, the expression of all activation markers decreased during the CPB surgery for all age groups (p<0.05). The increased superoxide levels and membrane depolarization of the mitochondria in lupus patients showed mitochondria dysfunction in SLE. It might be influencing the mitochondria to produce higher levels of intracellular Cytochrome c, then activating the caspase pathway. This study suggests mitochondrial dysfunction in SLE patients as well as leading way to apoptosis.

353/B222
Altered Early T Cell Activation in Pediatric Crohn’s Disease
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Introduction: Crohn’s disease is a transmural inflammation of the intestinal system, which is developed by many factors. Immunologically it is characterised by the disbalance of the pro- and anti-inflammatory cell populations, which leads to the progression of the autoimmune process. Data on the functional alterations of the involved lymphocyte subsets are rare. Based on earlier studies in other autoimmune diseases, we hypothesised that modulation of the calcium influx during the early phase of T cell activation by potassium channel inhibition may serve as a novel therapeutic approach. We aimed to investigate the activation kinetics of the peripheral Th1, Th2 and Tc

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352/B221
Study of Mitochondria Dysfunction in Systemic Lupus Erythematous
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Introduction: Systemic Lupus Erythematous (SLE), an autoimmune disease is characterized by the production of autoantibodies. The pathogenesis of lupus multifactorial including genetic factors, environmental factors etc. Mitochondria dysfunction is found to be associated with various diseases. The study has been designed to study the association of mitochondria in SLE.

Methodology: SLE patients (n=15) diagnosed by a single physician along with age and sex matched healthy controls (n=15) were collected from outpatient department of PGIMER, Chandigarh, India after informed consent. Ethical clearance was also taken from institutional ethics committee. PBMCs were isolated from whole blood using Ficoll histopaque. Mitochondrial reactive oxygen species was detected by using 1µM of DCFH-DA in flow cytometry. The polarized state of mitochondria i.e Mitochondria membrane potential (MMP) was also assessed in PBMCs using JC1 dye. Intracellular production of Cytochrome c levels and the levels of activated Caspase 3 were also studied using specific antibody by flow cytometer. Statistical analysis was done using Graph Pad prism software. P value less than 0.05 was considered as significant. Values was expressed in Mean±Standard deviation.

Results: Mitochondrial ROS production (1.5 fold increase) and mitochondria depolarization was found to be increased in cells from patients as compared to controls significantly (p<0.05). Cytochrome c production (1.4 fold) as well as caspase 3 productions (1.7 fold) were also found to be higher in lupus patients significantly (p<0.05).

Conclusion: The increased superoxide levels and membrane depolarization of the mitochondria in lupus patients showed mitochondria dysfunction in SLE. It might be influencing the mitochondria to produce higher levels of intracellular Cytochrome c, then activating the caspase pathway. This study suggests mitochondrial dysfunction in SLE patients as well as leading way to apoptosis.
Results: We observed Th2 cell activation predominance in the peripheral blood samples of children with Crohn’s disease. This elevated cytokastic calcium concentration was decreased to the levels seen in healthy children by both potassium channel inhibitors. The calcium influx modulatory effect of the inhibitors proved to be selective to Th2 cells in our study, as values of Th1 and Tc cells were not affected. Interestingly, infliximab treatment also decrease the elevated levels of intracellular calcium levels. This effect of the chimeric anti-TNF-α antibody cannot be further enhanced by potassium channel inhibitors. Calcium influx parameters and the PCDAI score (marker of clinical severity), as well as other clinical parameters correlate well.

Conclusions: Kv1.3 and IKCa1 potassium channels are potential novel therapeutic targets for reducing the effects of abnormal T cell activation in pediatric Crohn’s disease.

354/B223
Development of a Cell-Based Flow Cytometric Assay to Detect the Presence of AAV5 Neutralising Antibodies in Human Matrix
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Introduction: Gene therapy, the use of nucleic acid polymers as a drug to treat disease by genetic transfer to patient cells, has shown massive promise in the treatment of a variety of genetic disorders in recent years. The adeno-associated virus (AAV) family has emerged as a promising vehicle in the development of gene therapies as they can be engineered to contain any nucleic acid cargo, targeted to specific genes and can infect both quiescent and dividing cells without inducing disease in humans. Several considerations are needed in preclinical development of such gene therapies. Firstly, the emergence of immunogenic response and development of neutralising antibodies towards the vector is a concern. Secondly, natural AAV infections, leading to naturally occurring seropositive AAV antibodies can limit the type of vector used as well as the treatment efficiency of the gene therapy.

Aims: The purpose of this study was to develop and validate a cell-based assay to detect the presence of neutralising antibodies against AAV serotype 5 (AAV5) vector in human matrix.

Methods: HEK-293 cells were used as the model cell line and were transduced with AAV5-eGFP to optimise multiplicity of infection (MOI). Matrix effects from blood collection tubes on eGFP transduction in HEK-293 cells were measured in the presence/absence of Lithium Heparin, Sodium Heparin, K2 EDTA, K3 EDTA and Sodium citrate. For developing the neutralising antibodies assay, plasma was collected from Rhesus Macaques before and after infusion with an AAV5 vector to generate negative and positive plasma (containing AAVS neutralising antibodies) respectively. Negative/positive plasma and eGFP vector were incubated at a 1:1 volume ratio for 1 hour prior to addition to serial dilution and addition to HEK-293 cells for 1 hour. Cells were trypsinised, fixed and analysed by flow cytometry.

Results and Discussion: Initially cell culture and AAV transduction conditions were optimised. The optimal MOI, generating a transduction efficiency of 15 ± 10% in HEK-293 cells was assessed as 1 x 10^12 genome counts/cell. Since high concentrations of matrix in plasma following blood collection is known to inhibit transduction, the transduction efficiency of eGFP was determined in the presence/absence of Lithium Heparin, Sodium Heparin, K2 EDTA, K3 EDTA and Sodium citrate at a dilution of 1:50, 1:500 and 1:5000 respectively. At the lowest dilution (1:50) there was no inhibitory effect of matrix on transduction. For the AAV5/eGFP neutralisation assays, we found that negative plasma from Macaques (not infused with AAV5 vector) had no inhibitory effect on eGFP vector transduction in HEK-293 cells as determined by flow cytometry, however positive plasma, containing AAV5 neutralising antibodies, inhibited eGFP transduction in a concentration-dependent manner, with maximal inhibition observed at 1:50 dilution. These data indicate that AAV5 neutralising antibodies can be successfully detected in plasma.

Conclusions: We have optimised a cell based assay and AAV transduction conditions and developed a reliable flow cytometry assay for assessing the presence of neutralising antibodies in matrix.

355/B224
Optimising Human and Computer Performance in Reproducible Analyses of High Content Cytometric Data
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Clinical trials and high-throughput flow cytometry produce large amounts of data, leading to an analysis bottleneck unaddressed by available analysis software. Herein, we directly compare manual analysis to both command-line and server-based analysis using a subset of data from the KIRALA study (R01AI080129 PI Barbour), which examined an 11 color functional intracellular calcium staining panel in 60 acquisition runs of 10-20 patients per run, evaluated under 10 stimulation conditions for a total of 9943 individual samples. The NKFCF sub-study of the KIRALA study examined the phenotype (KIR3DL1/S1 frequency) and function (CD107alpha and IFNGamma production) of NK cells in early untreated HIV-1 infection. The volume of raw data (91GB) produced in this study prompted us to search for effective methods to reproduce analyses effectively and to generate statistical reports. Utilizing FlowJo v10 software as a platform, we created modular templates to apply compensation, to generate gating nodes and to generate statistical reports. Four reports were generated: two CSV tables evaluating sample quality and reporting sample statistics, and two layouts reviewing sample gates and illustrating the functional responses of NK cells under different stimulation conditions. We analyzed the ability of off-the-shelf FlowJo v10 software to reduce the time necessary to analyze this data set by running via command line FlowJo and FlowJo Server. Command line FlowJo is the way to use FlowJo without a user interface (‘headless’) or human intervention; FlowJo Server has high-performance calculation engines leveraging multiple cores on dedicated hardware. Importantly, users can utilize command line or FlowJo Server to execute FlowJo templates on data sets and produce output files such as batched CSV and PDF reports. First, data files were processed manually through on a 2013 Macbook Pro with 2.7GHz Intel Core i7 processor and 16GB of RAM. Second, command line interface was used to process data through the identical templates on the same Macbook Pro. Finally, analysis was performed using FlowJo Server running on a MacMini with 2.6GHz Intel Core i7 and 16 GB RAM. We found that manual processing of data, which requires opening the FlowJo client interface, manually loading data and initiating batching of reports one by one, was nearly 2-fold slower than processing templates through command line interface, primarily due to the extra time spent manually executing batching tasks. Processing templates on a dedicated FlowJo Server further improved processing time to reports generation - leading to results greater than 5-fold faster than...
command line, resulting in a more efficient and time effective workflow. Overall, we observed that hands-on time spent repetitively analyzing large high content data sets can be reduced by processing FlowJo templates through command line FlowJo, and processing speed can be further optimized by utilizing a dedicated FlowJo Server.

**356/B225**

Assessment of Bacterial Membrane Fluidity by Flow Cytometry

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Owing to its location as the barrier between the external environment and the intracellular medium, the cytoplasmic membrane is particularly vulnerable to damage caused by changes in external conditions during the culture and harvesting processes of industrial microorganisms. Cellular functions that occur at the membrane level require the maintenance of a proper membrane fluidity and liquid crystalline lamellar phase. For example, in response to stress such as temperature, pH or medium composition, bacteria adapt their membrane fatty acid composition by several processes, including alterations in saturation degree, carbon chain length, branching position, cis/trans isomerisation and cyclopropanation in order to preserve proper physical membrane properties.

As such, it can be interesting to evaluate the membrane fluidity of lactic acid bacteria to understand the impact of stresses during fermentation and harvesting processes and to predict the behavior of the bacterial starter cells at the time of use after storage.

With membrane probe 1, 6-diphenyl-1,3,5-hexatriene (DPH), changes in fluorescence anisotropy represent changes in membrane fluidity. The higher the anisotropy, the more rigid the membrane. To date, only spectrofluorimetry has been used to measure anisotropy of bacterial membrane. In the 1990s, several authors used flow cytometry to assess membrane fluidity on eukaryotes such as lymphocytes and vegetal cells. The associated cell by cell analysis delivers significant benefits as performed by flow cytometry compared to spectrofluorimetry.

In this work we develop a method for measuring membrane fluidity by CFM on bacteria.

First we adapt the flow cytometer to measure fluorescence anisotropy: Our cytometer (CyFlow® Space Sysmex Partec) was initially equipped with a solid blue laser emitting at 488 nm, and four band-pass filters: FSC, SSC and two fluorescence signals FL1 and FL2.

To measure fluorescence anisotropy, Sysmex Partec added a UV laser that is polarised vertically emitting at 375nm, vertical and horizontal polarizers, a half-wave retarder plate placed in a mount and two photomultipliers for IH (FL3) and IV (FL4).

After balancing, the means of the horizontal (FL3) and vertical (FL4) fluorescence histograms were used to calculate anisotropy from \( r = \frac{IV - IH}{IV + 2IH} \).

Second we measured DPH staining and anisotropy: the results showed that the anisotropy measurements on bacteria can be performed by flow cytometry as well as by spectrofluorimetry (\( R2 = 0.99 \)). Young bacterial cells were more fluid than old cells, and membrane fluidity depends on culture medium composition. Absolute value depended on the strain.<br data-list=>

Thirdly, we developed multi-staining membrane fluidity/viability/mortality: the co-staining viability/mortality/membrane fluidity let us separately measure the anisotropy of viable cells and dead cells by gating the population according to viability staining. Viable cells have more fluid membranes than dead cells.

In conclusion, we have managed to adapt the Sysmex Partec CyFlow® Space cytometer to measure bacterial membrane fluidity. This measure is interesting for taking into account the changes of the cellular state of bacteria in fermentation processes.

**OTHER TECHNOLOGY ADVANCES (B226 – B230)**

357/B226

Longitudinal Intravital Multiphoton Endomicroscopy in the Murine Bone Marrow (LIMB)

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Key Words: longitudinal intravital microscopy, GRIN, bone marrow, internal fixator

The immune system and its interaction with other bone cells show high plasticity both in normal tissue and during bone regeneration after injury. In vivo multiphoton fluorescence microscopy is the method of choice to gain insight into the key mechanisms of dynamic interactions between cells of the immune and skeletal system, such as cell migration or the activation of the hematopoietic system.

To date, intravital microscopy in the bone marrow has been limited to the time frame of a few hours and an imaging depth of 100 - 150 µm. Since immune reactions and bone healing processes may last for several days or weeks and need to be observed with high spatial and temporal resolution, the dynamic analysis of the functional aspects of the skeletal and immune system has proven to be a major technical challenge. In order to improve these methods, we have currently developed a reproducible longitudinal microendoscopy technique based on gradient index (GRIN) rod lenses.

An internal fixation system used for osteotomy on the murine femur (Matthys & Perren 2009) was further modified in order to allow the precise guidance of GRIN endoscopes (350 µm in diameter), which have been successfully employed in many other microendoscopic systems (Jung & Schnitzer 2003; Messerschmidt & Matz 2014).

In a lateral approach, the right femur is exposed and pilot holes are drilled for the introduction of a tunneling assembly for the rod lens endoscope, as well as for bicortical bone screws that fix the implant. The guiding tube (650 µm outer diameter) reaches through the skin and ends up in the marrow cavity where it is sealed by a 160 µm thin sapphire glass. This window separates the two compartments of the marrow cavity and the environment, thus preventing any invasion of external contaminants. Thereby we are able to repeatedly access the marrow cavity with a GRIN rod lens (~4 mm in length) from the exterior without the need for further surgical procedures.

We have proven by ex vivo µCT that the precise positioning of this chronic window at the center of the marrow cavity is ensured in living mice over a time period of 21 days.

The lateral approach for the exposure of the femoral shaft and the fixation of the implant were evaluated with regard to iatrogenic impact by immunofluorescence-based histology and histochemical methods. The wound healing was completed after about one week.

Furthermore, we demonstrated that the optical performance of the imaging system allows for longitudinal intravital multiphoton microendoscopy in the murine bone marrow (LIMB). As a first approach, we repeatedly imaged B-cells in CD19:RFP reporter mice for several weeks following the implantation procedure, in order to monitor the initial immune reaction and to check for eventual rejection reactions. Vascularization in the field of view
was displayed by i.v. injection of FITC-dextran prior to each imaging session.

Thus, the newly developed imaging technique, LIMB, for the first time allows longitudinal, dynamic, in vivo studies of the osteo-immune system in the murine bone marrow.

358/B227
Light Scattering Simulation for Depolarization Measurement of Birefringent Crystals via Flow Cytometry
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Polarized light scatter is a very interesting parameter in flow cytometry (FC). When linearly polarized laser beam is scattered by isotropic particles at 90°direction (‘side scattering’), the polarization of the incident beam is mostly conserved in its scattered light. However, the polarization of the incident beam is lost (thus depolarized) when scattered by anisotropic particles such as cells with complex internal structures. Since the depolarization by particles reflects their optical anisotropy, the depolarized ratio of side scattering has been measured in FC applications such as discrimination of eosinophils and neutrophils. In addition, urine crystals are representative anisotropic particles of interest. Crystals have birefringence, which exhibits different refractive indices for different polarizations of incident light, and it leads to depolarization. Compared to cells, the light scattering of crystals has not been well investigated despite it being routinely measured by FC-based urine particle analyzers in clinical laboratories. Crystals can be observed in human urine sediments and often lead to incorrect counts of red blood cells (RBCs) that are clinically important. Therefore, we have theoretically and experimentally investigated the light scattering of crystals in FC by using computational simulation to explore the potential of depolarization measurement for specific counts of crystals.

Discrete Dipole Approximation was used to simulate the light scattering of particles with arbitrary shape and refractive index. Four simulation models were developed: bead, RBC (isotropic), calcium oxalate and uric acid (anisotropic). The intensities of side scattering (SS) were calculated by integrating the angle-resolved light scattering within the detection area. The depolarized side scattering (DSS) were also calculated by multiplying the transmission and absorption rate of polarizing filter to vectors of electric field at each point of observation. The models were rotated by 45°steps along two orthogonal axes respectively to observe the orientation dependence. The simulated result was experimentally validated by the FC measurement of human RBCs and calcium oxalate crystals suspended in PBS.

The simulated result showed that the degree of depolarization (DOD, which is the intensity ratio of DSS/SS) relies both on optical anisotropy and on orientation. The DODs of isotropic particles were low and uniform (Mean: 10-15%, SD: 3%) while those of crystals were relatively high and spread (Mean: 19-28%, SD: 5-13%). The experimental data agreed with the simulated result in terms of the average DODs.

The obtained results suggest that the DODs of birefringent crystals are 10-20% higher than those of isotropic particles on average, while they are highly spread (CV <60%) owing to the strong dependence on shape and orientation. In conclusion, the depolarization measurement in FC is expected to be able to separate >60% of crystals from other isotropic particles.

359/B228
Incorporating Spectral Information of Multiple Autofluorescent Signals Supports the Improved Resolution of Cell Populations in Inflamed Tissue
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Flow cytometry is one of the most fundamental tools available to immunologists for querying immune cell population dynamics in naive and injured tissues, in both pre-clinical and clinical samples. The capacity to identify infiltrating and differentiating immune cell populations through labeling with fluorescent antibodies is complicated by the autofluorescent properties of the cells and tissue under investigation. One such example is the bladder, which is known to possess a strong autofluorescent signature due to vacuoles present in the luminal urothelial cell layer. We recently completed a survey of the resident immune cell population of this tissue in mice, finding that autofluorescent signals from nonimmune cells overlapped with immune cell specific markers, masking ‘true positive’ populations and complicating our approach and analysis. This was particularly important with respect to phenotyping macrophage populations in the bladder.

In this study, we assessed the ability to overcome the technical limitations of conventional flow cytometry, employing the Sony Spectral Analyzer to measure immune cell infiltration. Specifically, we evaluated whether this new spectral unmixing-based analytical approach could enable the use of autofluorescence in the bladder as a distinguishing characteristic of cells. We stained resting and inflamed bladder with a panel of 13 antibodies; additionally identifying 5 different autofluorescent signals (AF1-AF5) defined by their unique spectral shapes and intensities in bladders 24 hours after injury. Using each of these signals alone or in combination, we generated 32 different spectral unmixing matrices and applied them to our data to determine the optimal method to resolve conflicting autofluorescent signals. Finally, we evaluated whether any of the AF signals could be assigned to a specific cell type.

Preliminary results indicate that the use of multiple autofluorescent signals in the unmixing of spectral data resulted in unmixing conflicts potentially stemming from the similarity in spectral shapes of the autofluorescent signals. We could assign the spectral patterns of AF2 and AF3 to bladder resident macrophages. Of note, not all macrophages exhibited the same autofluorescence intensity, suggesting that these cells make up a heterogeneous population. AF3 could be attributed to non-immune cells, and likely emerges from the urothelial cells that line the lumen of the bladder. From these data, we conclude that the multiple staining panel of inflamed bladders including spectral information of autofluorescent signals in both immune cells and non-immune cells revealed that the proper assignment of autofluorescent information may have the potential to achieve more precise analysis of complex or inflamed tissue.

360/B229
CyTOF Tuning: How Low Can You Go
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Introduction: The CyTOF is a mass spec cytometer used to measure single cells labeled with metal conjugated probes. Ion clouds of each cell are generated allowing time of flight measurements for each of the metal tags based on ion mass. This allows for quantitative measurements for over 100 different parameters. Tuning solution provided by DVS/Fluidigm is used to run daily calibration of the CyTOF. One of the many measurements taken to test performance is dual count of Tb. We have seen these counts vary depending on alignment and cleanliness of the glassware and cones. Tb dual counts when above 450,000 are considered passing, but often counts can be as high 1,500,000. These counts represent variations in instrument performance that can have an impact when comparing data...
throughout the day or long term. These counts drop through out the day and over time, mainly due to cellular debris on glassware and cones, and changes in ion efficiency. To adjust for these variations a single lot of 4 metal polystyrene beads are added to the samples. Using software provided by Fluidigm data is normalized for variation in instrument performance, insuring a drop in signal throughout a run will not negatively affect the data. Because there can be a large loss of signal over time, weaker labeled epitopes still may not be resolve able even after normalization. Here we evaluate how data is affected by continuous drop of signal through out the day or days, even after normalization, and at what level is a minimal detectable threshold reached in a run.

Methods: Whole Blood was stained with multiple metal conjugated markers, both weakly and strongly expressed epitopes. Some of the same epitopes were labeled with different masses to see the effect over the mass range collected by the CytoF. Cells were then resuspended in DI water with DVS/Fluidigm Eu 4 elemental calibration beads, added to the sample. Aliquots of the same sample were run on the CytoF throughout the day while monitoring instrument performance using tuning solution and monitoring Tb dual counts. Raw data and normalized data was analyzed and correlated. A lower level threshold was established base off biological data and compared to the intensity of the Eu beads in the sample.

Conclusion: The lower passable limit during tuning for Tb dual counts 450,000 may be much lower then what is actually acceptable to resolve weaker antibody markers. There is a tolerable drop in range during a run that bead normalization is effective. This lower threshold was determined by using low expressing markers across the mass range correlated with a loss in Eu bead intensity. Monitoring the intensity of Eu beads after each sample is collected allows the operator to determine when to rerun tuning solution through out a run or clean the instrument to reestablish an acceptable sensitivity.

361/B230
Immunoprofiling of Rare Pathogen-Specific Responses Using a Streamlined Analysis Approach in an Effort to Identify Correlates of Protection
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The lack of known immune correlates of protection has been a major hurdle for the development of effective vaccines against various disease models. What is urgently needed are novel readout methods and advanced technologies for an in-depth immunoprofiling of responses induced by protective vaccines. The work presented here applies a two-step approach to the characterization of vaccine-induced responses against two major infectious diseases, namely, ebola and malaria and provides a first glimpse of the landscape of antigen-specificity and type of vaccine-induced immune responses. The first step is to establish a broad cytokine profile of antigen-specific responses in ex vivo stimulated PBMC using a multiplex testing platform (Mesoscale). These results are the basis for the design of the subsequent flow cytometric analysis whereby the antigen-specific cytokine-producing cells are characterized in detail. The targets of the flow-based analyses are rare subpopulations of cells, which have slowed progress in this area of research. To overcome this obstacle, we are enriching activated, antigen-specific cells using an automated system for magnetic bead separation and then analyzing these cells based on their lineage, activation and differentiation state. By combining these state-of-the-art technologies, we expect to generate unprecedented insights into vaccine-induced protective immune mechanisms.

PLANT SCIENCES (B231 – B232)

362/B231
Rapid Purification of Microfossils from Lacustrine Sediments by Flow Cytometry
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Sedimentary deposits are an important palaeoenvironmental archive but producing robust chronological frameworks for reconstructing past atmospheric, climatic and environmental conditions can be a challenge, particularly during periods of abrupt and extreme climate change. The application of stable isotope geochemistry to the four main elements (Oxygen, Carbon, Nitrogen and Silicon) can help identify changes in past environmental conditions. Terrestrial pollen grains and aquatic diatoms are common in sedimentary deposits. In addition, pollen grains are suitable for 14C radiocarbon dating as well as being naturally auto-fluorescent. Diatoms, equally, are an important achieve of past environmental change. The purification of microfossils from sediment is a laborious procedure. Flow cytometry has been shown previously to sort extant pollen and a variety of living phytoplankton from aquatic environments. Using flow cytometry we rapidly purified preserved pollen grains and fossil diatoms from sedimentary deposits. The purified microfossils were suitable for 14C radiocarbon dating and stable isotope geochemistry allowing a more accurate understanding of palaeoenvironmental change.

363/B232
Analysis of Progressively Partial Endoreplication in Orchids
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Background: In many plant species, cell differentiation is frequently accompanied by endoreduplication, a process in which cell nuclei undergo one or more rounds of DNA synthesis without intervening mitosis, resulting in nuclei with multiples of 2C DNA amounts (4C, 8C, 16C, etc.). However, in orchids, a disproportional increase in nuclear DNA content has been observed, where successive endoreplication cycles result in nuclear DNA contents 2C+P, 2C+3P, 2C+7P, etc., where P is the DNA content of the replicated part of 2C nuclear DNA, and is always smaller as compared to regular endoreduplication. This phenomenon was termed "progressively partial endoreplication" (PPE). At present, nothing is known about the processes involved in PPE, and sequences excluded from replication and/or eliminated after replication have not been identified.

Methods and Results: We chose Ludisia discolor, a species belonging to the family Orchidaceae, as a model to investigate the nature and mechanism of PPE. We first carried out cell cycle analysis using flow cytometry on nuclei pulse-labelled with EdU (5-ethyl-2'-deoxyuridine, a nucleoside analog of thymidine). In contrast to cell cycle analysis in other plant species, we did not observe the typical 'horseshoe' pattern of S-phase nuclei ranging from 2C to 4C DNA contents, but rather two cell cycles, one ending above the population of PPE nuclei with 2C+P, and the other with a regular pattern as observed in cycling diploid cells. We then flow-sorted both 2C and hyporeplicated (2C+P) nuclei and paired-end sequenced their DNA on Illumina MiSeq. Sequence reads were analyzed to identify possible differences in copy number (presence/absence) of repetitive DNA sequences between the 2C and 2C+P nuclei. The most abundant DNA repeats were also assembled and characterized. The comparative analysis did not confirm a lack of specific types of repetitive DNA in the 2C+P nuclei. The sequence data were also assembled and searched for homology to genomic sequences in the whole genome.

364/B233
Rapid Purification of Pollen Grains from Flowering Plants by Flow Cytometry
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Dolomites (Northern Italy) represents a unique high altitude palaeoenvironmental archive but producing robust chronological frameworks for reconstructing past atmospheric, climatic and environmental conditions can be a challenge, particularly during periods of abrupt and extreme climate change. The application of stable isotope geochemistry to the four main elements (Oxygen, Carbon, Nitrogen and Silicon) can help identify changes in past environmental conditions. Terrestrial pollen grains and aquatic diatoms are common in sedimentary deposits. In addition, pollen grains are suitable for 14C radiocarbon dating as well as being naturally auto-fluorescent. Diatoms, equally, are an important achieve of past environmental change. The purification of microfossils from sediment is a laborious procedure. Flow cytometry has been shown previously to sort extant pollen and a variety of living phytoplankton from aquatic environments. Using flow cytometry we rapidly purified preserved pollen grains and fossil diatoms from sedimentary deposits. The purified microfossils were suitable for 14C radiocarbon dating and stable isotope geochemistry allowing a more accurate understanding of palaeoenvironmental change.
assembly of orchid Phalaenopsis with the aim to identify possible differences in the gene space.

**Conclusions:** Our observations provide the first insights into the nature and mechanisms of the unique phenomenon of progressively partial reduplication which has been observed only in some species of the family Orchidaceae. Cell cycle analysis indicates that the process leading to nuclei with lower DNA amounts does not involve elimination of specific sequences after a complete round of DNA synthesis, but rather incomplete replication of the whole genome. The preliminary data obtained after DNA sequencing suggest that there are no specific DNA sequences which are under-replicated. Rather, it seems that all types of repetitive DNA sequences are affected.

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**RARE EVENT DETECTION (B233 – B236)**

**364/B233**

Does CyTOF® Need Flow? Approaching Mass Cytometry from the Perspective of a New Flow Cytometrist

**Kristen Leone**

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I am approaching Mass Cytometry from the perspective of someone new to Flow Cytometry (1.5 years) and ultimately exploring how applicable methods I have learned in flow cytometry are of use in mass cytometry. To accomplish this I will plan and execute my own mass cytometry experiments from experimental design, cell labeling, instrument tuning, acquisition and data analysis. My introduction and experiences with mass cytometry will, in addition to being an example for others to learn from, explore how certain elements of mass and flow cytometry can be integrated. One of my objectives of this presentation is to address the issues I encountered while venturing into the world of mass cytometry and to share these experiences with others in the field of flow cytometry who are interested in utilizing new assays and technologies. One area of concern for mass cytometry is its limitations in analyzing rare event samples. I suggest that fluorescent activated cell sorting (FACS) prior to CyTOF® analysis can improve certain mass cytometry experiments by first sorting out specific subpopulations based on well-established fluorescent CD markers. This will enrich for rarer events and allow for additional markers to be used in the mass cytometry panel. The effects of electronic sorting on cell integrity will also be discussed.

Human peripheral blood cells positive for CD45/CD3/CD4 or CD45/CD3/CD8 will be sorted and then labeled for CyTOF® analysis in parallel with unsorted cells for additional CD markers. Enrichment of these markers in sorted samples will be compared to the unsorted controls. Cells will be sorted at 3 different levels of precision to determine which precision is the best for CyTOF® staining. To determine the ideal preparation of human peripheral blood samples I will compare two isolation methods: Ficoll and whole blood red cell lysis. I will ultimately run a comprehensive T cell, B cell and NK cell panel on a sorted sample to compare to an unsorted sample to show any changes in the expression and frequency distribution of surface antigen makers to determine if sorting negatively effects the cells. If sorting does have a negative effect on sample quality, the use of other methods will be explored. CyTOF® cell labeling is a long process that involves many washes, it is recommended to aspirate the supernatant after centrifugation rather than pouring it off to avoid cell loss. To determine if cells are being lost during the wash steps the supernatant of each wash will be recorded along with the original sample on a BD FACS Aria Ilu for comparison.

As someone who only first learned to stain cells for flow cytometry a year and a half ago, I can attest to how quickly a seemingly complex process can become second nature. The staining process is long and like staining for flow cytometry it gets easier each time. Sharing my experiences can help others to smoothly transition into using the CyTOF® and mass cytometry technology from a flow cytometry background. If sorting prior to CyTOF® analysis can improve the resolution of small populations and allow additional markers to be used on a given panel then it will be beneficial to others in the flow cytometry community. I feel that my project is very accessible to those with any level of Flow Cytometry experience and will encourage other new comers to the field to explore what they find interesting and execute their own experiments.

**365/B234**

Single Cell Copy Number Variation Analysis of Flow Sorted Primary Tissue and Cell Line

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**Introduction:** Single cell technologies have exploded over the last decade making the impossible experiments now possible. Flow Cytometry is a powerful tool that has enabled this. Index sorting of especially rare population’s i.e. stem cells has allowed us to match phenotype with genotype to try to gain a better understanding of heterogeneous populations.

**Aim:** To collaborate with our genomics facility within our institute and to provide a novel, reliable, affordable single cell service. Single cell copy number variation analysis was chosen, as this was not currently being performed within our flow cytometry/ genomic facilities.

**Flow Cytometry:** Next generation sequencing (NGS) from bulk populations has been performed very successfully but it is far more challenging from just a single cell. There are five factors that will affect the quantity and quality of the DNA that will be extracted, amplified and sequenced. Flow cytometry can overcome these factors making sure that the cells sorted are: intact/live cells by use of live/dead discrimination dye, have the same amount of DNA (G1) cells by use of live cell cycle dye, free from contaminating DNA from serum and sorted at +4°C within a cabinet. The gating strategy insured that the cells were singlet’s, live, population of interest and within the G1 phase of the cell cycle.

**Samples:**
1. Two different MCF7 cell lines from within the Institute were stained with CD24, PI and Hoechst 33342 and then flow sorted into the appropriate wells.
2. A healthy human breast tissue sample was dissociated and stained with a Lineage cocktail (CD45 and CD31) and sorted for a luminal population (CD49+ and EpCAM+) and then sorted into the appropriate wells.
3. A 10 cell control well was used for each of the populations.
4. A no cell NTC and a commercial MCF7 gDNA at 16pg were used as extra controls.

**Method:** The BD Influx was used to sort the samples; the Influx is housed in a Class II Containment hood, as one of the samples was primary tissue. The Amis ImageStream 100 was used to collect images of the MCF7 cells after the sort to investigate the morphology of the cells. The Rubicon PicoPLEX DNA Seq Kit was used to extract and amplify the DNA from the single cells. The Illumina Hi Seq 2500 was used to sequence 50bp single end.

**Results:** In total the whole project took a week to complete, this included a day of staining, sorting and extraction, a second day of library preparation and three days of sequencing. The raw data was then passed over to our bio-informatics core to analyse. The analysis of the raw data was the most difficult and time consumed...
part of this collaboration. The cell populations were successfully sorted and DNA libraries were generated from all samples except two wells containing singular MC7’s. Almost all samples generated between 2 and 3 million reads of data. CNV analysis can successfully be performed with a single cell. The total cost of the project was under £3,000 for a 48 well plate including controls.

**Conclusions:** This was a proof of principle experiment to see if this service could be provided within our Institute by the collaboration of two core facilities and this was proved to be a success.

**366/B235**

**Water-in-Oil Emulsion Sorting for Digital PCR and Single Cell Analysis Using Air Pressure Pulse Flow in Disposable Microfluidic Chip**

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Droplet digital polymerase chain reaction (ddPCR) using droplets of water-in-oil (W/O) emulsion is gaining interest for detection of nucleic acids of specific cells, or free DNA or RNA of low concentration suspended in sample containing high concentration of non-target particles. This method, which involves running PCR for amplification of nucleic acids and expression of fluorescent signals while each individual target is encapsulated in W/O droplets, allows for detection of, for instance, extremely rare cells blended in 1 million other cells. However, the genetic sequencing of the targets that exhibit positive results is still considered inevitable due to the appearance of false positives or false negatives as outcome of point mutations. Therefore, a sorting system for post-PCR emulsion droplets according to the fluorescence intensity is required. Collection of emulsions in oil using dielectrophoresis has been demonstrated, but an instrument that integrated such technology is not yet commercialized.

In 2012, we developed a flow cytometric cell sorter ‘On-chip Sort’ that sort cells using gentle pulse flow generated by air-over-liquid pressurization within disposable microfluidic chip. Its unique sorting method allows for the use of various liquids as sheath liquid including culture media, sea water and oil. In this work, sorting of W/O emulsion droplets of mineral oil and fluorocarbon oil was investigated using On-chip Sort and a disposable microfluidic chip with the channel width of 80 μm. The emulsion droplets used in this investigation were generated using ‘On-Chip Droplet Generator’, an emulsion droplet generating system recently developed with droplet size adjustment function. With this, we have achieved sorting of emulsions flowing at a rate of 50 droplets per second with high sorting efficiency and purity. These droplets can be used to trap single cells in the scale of 10 μm, and are useful tools for single cell analysis.

**367/B236**

**Label-Free Detection of Tumor Cells by Auto-fluorescence Intensity and Lifetime Measurements Using Flow Cytometry**

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**Introduction:** The accumulation of reduced nicotinamide adenine dinucleotide (NADH) correlates with increased metabolic rates in cells. NADH is a coenzyme that is abundant in cells and mainly involved in redox reactions during metabolism. On interesting feature of NADH is that it is highly fluorescent, compared to its oxidized counterpart (i.e. NAD+).

NADH has been found to be a useful marker in cancer diagnostics because transformed have increased metabolism. This correlates to elevated NADH levels, and thus higher autofluorescent signatures. Cellular autofluorescence intensity measurements due to NADH accumulation can be used to differentiate tissue sections and margins where there is a clear delineation between normal and cancerous cells. In addition to autofluorescence intensity measurements, the fluorescence lifetime of NADH shifts when this cofactor is protein-bound. Therefore the fluorescence lifetime, or average time the fluorophore spends in the excited state, can be used as a quantitative ‘marker’ of the presence of cancer. Many literature reports have shown that the average autofluorescence decay time associated with NADH measured from cancer cells markedly decreases compared to non-cancerous cells. Previous studies using fluorescence lifetime imaging microscopy (FLIM) have demonstrated this by measuring fluorescence lifetime changes across a cell. In this work we replicate the fluorescence lifetime measurements of protein-bound NADH in cancer cells and evolve these measurements using time-resolved flow cytometry.

**Methods:** We have constructed a time-resolved flow cytometer capable of measuring the fluorescence lifetime of single cells. In a variety of experiments we show how this system can screen for autofluorescence lifetimes of cancer cells using 405-nm excitation and >410-nm emission. Average fluorescence lifetime and mean fluorescence intensity are measured for a variety of cell types (breast cancer, normal breast, lung cancer, normal lung) before and after metabolic changes upon treatment with potassium cyanide (KCN) or by serum starvation. Finally we measure the MFI and fluorescence lifetime of these samples. Lastly, we mix non-altered cells with altered cells as well as different permutations and ratios of cancer, normal, as well as peripheral blood mononuclear cells.

**Results and Conclusions:** We measured distinct MFI and fluorescence lifetime changes of metabolically altered cells as well as normal cancer and non-cancerous cells. These values range and were cataloged by us after many repeats. The autofluorescence lifetimes match those expected (between 1 and 2 nanoseconds). Specifically we measured an increase in the autofluorescence intensity and a decrease in the fluorescence lifetime of treated cells when compared to untreated control cells. These results allowed us to discriminate between mixtures of cells using the autofluorescence lifetimes but not generally by the autofluorescence intensity. Overall we seek to demonstrate that this is an ideal way to detect and sort rare tumor cells that might be circulating among a heterogeneous blood cell population. Our approach does not require tumor cell enrichment, exogenous labeling, and it not tumor cell-type specific. We hope this work has future implications for cell sorting to separate a circulating tumor cell for future studies. Additionally this technique might be useful for cancer diagnostics in a minimally invasive manner.

**Regenerative Medicine (B237 – B242)**

**368/B237**

**IIB or Not IIB: Development of a Screening Method by Flow Cytometry to Characterize Diverse Sources of Human Mesenchymal Stem Cells and to Evaluate Their Potential for Cartilage Reconstruction**

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**Background:** Articular cartilage is not vascularized and presents poor intrinsic healing potential. Consequently, traumatic and
degenerative lesions of articular cartilage eventually progress to osteoarthritis, a worldwide leading source of disability. In this context, interest in cell therapy and tissue engineering for cartilage repair is increasing and currently, autologous chondrocytes represent the most common source of cells that are transplanted although they imply tissue morbidity at the donor site. The use of mesenchymal stem cells (MSCs) represents a promising alternative cellular model but satisfactory protocols allowing proper chondrogenic conversion together with sufficient cartilage matrix production are still missing. Moreover, it is difficult to rank the MSCs in regard of their potential of proliferation and differentiation from the studies published in the literature since the cell culture conditions used by the research groups are often very different. A main project of our laboratory is to identify the best source of MSCs for cartilage repair. In this line, and as cells must be capable of synthesizing a proper cartilage-specific matrix, we recently developed and characterized the first antibody capable of detecting the IIB isoform of human type II procollagen, the only chondrocyte-specific isoform of this type of collagen.

Methods: In this study, we compared MSCs isolated from Whartons' jelly, adipose tissue, dental pulp, and bone marrow. MSCs were cultured in entirely xeno-free conditions, allowing more easily their use in clinical application. At the end of the isolation phase, cells were further processed for flow cytometry characterization or differentiation studies. A polychromatic (8 colors/6 tubes) panel of 30 surface markers was developed from the literature that includes conventional positive or negative selection markers of MSCs and a set of additional markers of interest regarding chondrogenic potential.

We further developed a xeno-free medium containing BMP-2 and TGF-β3 for chondrogenic differentiation of MSCs in a 3-dimensional model. The assessment of chondrogenesis was achieved by fluorescently labeling the anti-procollagen IIB, and measuring its internal expression by chondrogenically-induced MSCs with flow cytometry. The cells were also stained for mesenchymal surface markers in addition to internal Col-IIB during chondrogenesis to monitor their differentiation.

Results: We demonstrate here that MSCs from various adult or perinatal tissues, even though cultured in the same xeno-free conditions and perfectly complying with ISCT guidelines in terms of immunophenotype, osteogenic and adipogenic potential, show significant differences in the expression of specific markers of interest and in their chondrogenic potential. We also show for the first time that the anti-collagen-IB antibody can be used in flow cytometry to reveal intracellular IIB procollagen expression in MSCs and therefore be applied as a quality control tool to quantitatively assess the degree of MSCs' chondrogenic conversion.

Conclusion: The results of our study will help to select the most appropriate source of MSCs capable of producing high quality cartilage matrix in a clinically-translatable protocol.

369/B238
mRNA Detection Using SmartFlare Gold Nanoparticles in Normal Human Keratinocytes In Vitro
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Background: Gene expression analysis is a time- and sample-consuming procedure, generating not always efficient results. As an alternative, a direct detection of mRNA sequences in the living cell can be performed using some of the available kits. We have tried SmartFlare Merck-Milipore detection kit, which comprises usage of gold nanoparticles coated with anti-mRNA recognition sequences.

Materials and Methods: Keratinocytes were isolated from skin biopsy via enzymatic digestion with dispase, following epidermis dissociation using trypsin. The cells were seeded and a primary culture was started. When the culture reached its desired confluence (around 60-80%), adherent cells were stained with SmartFlare detection probes complementary to TRP and MYC mRNA sequences. Additionally unspecific controls were performed. Nanoparticles are coated with oligonucleotides containing recognition elements to a specific RNA transcript and are hybridized with short cyanine (Cy3 or Cy5) dyes which act as ‘flares’ when displaced by a longer target (mRNA in the cell). The samples were analyzed with flow cytometry using FACS Aria I (BD) or FACS Canto (BD). To assess how the gold nanoparticles (SmartFlare particles) are incorporated into the cells and whether it affects the condition of the cells, we performed electron microscopy. Specimens were coated with gold in a sputter-evaporator (Balzers), and stored under vacuum until they were examined and imaged in a Philips XL 30 scanning electron microscope operated at an accelerating voltage of 10 kV.

Results: Using nanoparticles conjugated with anti-sequences, we managed to stain the cultured adherent cells and analyze them with the cytometer. Cells efficiently incorporated nanoparticles (40-60%) in the medium supplemented with 10% serum. The imaging with electron microscopy revealed no major changes to the morphology of the cells nor to the cellular metabolism.

Conclusions: The mRNA detection with gold nanoparticles probes can be a great alternative to gene expression analysis. Yet, the uptake of the gold nanoparticles is dependent on the cell type. Therefore, an optimisation of the procedure must be conducted, in relation to culture conditions, especially the concentration of serum.

370/B239
Alkaline Phosphatase Live Stain for Stem Cell Research
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Background: Alkaline phosphatase (AP) is a phenotypic marker of pluripotent stem cells (iPSCs), including undifferentiated embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and EGCs. While AP is expressed in most cell types, its expression is highly elevated in PSCs. Therefore, an optimisation of the procedure must be conducted, in relation to culture conditions, especially the concentration of serum.

Materials and Methods: In this study we have used human brain LN405 astrocytoma cells, which contain low numbers of cancer stem cells. Data were collected using the Attune® Acoustic Focusing Cytometer (Life Technologies) equipped with two lasers operating at 405 and 488 nm. Filter combination for APLS analysis was: 555 DLP, BP 530/30 (green). DCV signal was measured using linear scale at 50 mW. APLS was measured using logarithmic scale at 20 mW.

Results: APLS is not effluxed by multidrug resistance pumps, making feasible this stain for the identification of differentially expression within stem-like Side Population (SP) cells. In addition, alkaline phosphatase is expressed differentially under normoxic and hypoxic conditions, and overexpressed when SP cells were incubated at low O2 concentrations.

Conclusions: Current available alkaline phosphatase substrates are toxic to the cells, which prevent them from propagating once stained. However, APLS can be used to easily distinguish primitive stem cells, since the stain maintains stem cell viability.
371/B240
Side Population Stem Cell Identification by Vybrant® DyeCycle™ Violet Stain
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Background: The side population (SP) has become an important hallmark for the definition of the stem-cell compartment, especially for the detection of stem cells and for their physical isolation by fluorescence-activated cell sorting (FACS). More recently, a new assay based on the efflux of Vybrant® DyeCycle™ Violet stain (DCV) has been documented to discriminate SP cells.

Material and Methods: In this study we have used different human brain astrocytoma cell lines (MOG- G-CCM, GOS 3, LN4105, SW1783 and U87-MG). Data were collected using the Attune Acoustic Focusing Cytometer (Life TechnologiesTM) equipped with two lasers operating at 405 and 488 nm. Filter combination for DCV analysis was: 500 DLP, BP 450/40 (blue), and BP 603/48 (red). DCV signal was measured using linear scale at 50 mW.

Results and Discussion: DCV has been validated to study and visualize DNA in living cells as well as to allow side population analysis on flow cytometers with violet lasers. Using DCV and violet excitation, the SP displays almost identical distributions as for the Ho342 SP assay.

372/B241
In Search of Hematopoietic Stem Cells: Functional Heterogeneity of CD34+ Cells by Co-staining with Vybrant® DyeCycle™ Violet
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Background: We previously developed a new method for counting CD34+ cells in whole blood samples, based on nucleic acid viable staining to discriminate erythrocytes and debris. We have adopted this protocol to study the numbers of circulating CD34+ cells in rat peripheral blood. Rat erythrocytes have increased resistance to osmotic lysis, making our method ideal for such research studies.

Material and Methods: In this study we have used rat peripheral blood cells. Rat blood cells were obtained from samples taken for hypoxic training studies. DyeCycle Violet stain was used to discriminate nucleated cells from non-nucleated cells and debris. PE-CD34 and FITC-CD45 monoclonal antibodies were used in this study for simultaneous staining with DCV. Data were collected using the Attune® Acoustic Focusing Cytometer (Life TechnologiesTM) equipped with two lasers operating at 405 and 488 nm.

Results and Discussion: DCV allows the identification of relevant and reproducible subsets of rat hematopoietic progenitors. Panels of immunomarkers provide greater information in combination with functional analysis of unlysed whole blood specimens. The distribution of DCV-CD34+ cells identifies two main cluster groups, with significantly different fluorescent intensities.

373/B242
Negative Cell Sorting for Removal of Undifferentiated Cells for Regenerative Medicine
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In the field of regenerative medicine involving embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, differentiated cells are often cultured to tissues and implanted to a patient. However, there is a serious problem that some remaining undifferentiated cells in the implanted tissue may cause tumors, and thus undifferentiated cells must be completely removed prior to implantation.

As a method of undifferentiated cell removal, we have developed a novel method called ‘negative cell sorting’ using a disposable microfluidic chip-based cell sorter (On-chip Sort). On-chip Sort can be installed with up to 3 lasers and 6 color detectors, and has a sorting function using gentle liquid pulse flow in a microfluidic channel. The flow speed is about 1 mm/s at 2 kPa (0.29 psig) of air pressure over sample. The advantages of On-chip Sort include damage-free sorting, sterile sorting environment and use of culture media as sample buffer and sheath liquid: crucial factors for regenerative medicine.

For this application, the sample is loaded to sample reservoir of the chip, and undifferentiated cells that flow past the sorting chamber are removed by the formation of gentle pulse flows to waste. Differentiated cells and some remaining undifferentiated cells flow through the sorting area into the reservoir located downstream. These cells are then recovered and reloaded to the sample reservoir for further clean-up. We repeat this process of negative sorting until all the undifferentiated cells are removed from the sample. The maximum negative sorting speed is 300 cells/sec. Therefore, if the population of undifferentiated cells is 1%, the maximum flow rate of differentiated cells is about 30,000 cells/sec. In this work, we report the efficiency of undifferentiated cell removal and the purity of the collected neuron cells differentiated from iPS cells.

**SIGNAL TRANSDUCTION (B243)**

374/B243
Aberrent TGFβ Superfamily Signalling Maintains Mesenchymal Phenotype of Breast Cancer Cells
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Plasticity of cancer cells, characterised by dynamic and reversible transition between different phenotypes of cancer cells, is a very common cause of malignant cancer progression. EMT (epithelial-to-mesenchymal transition) and MET (mesenchymal-to-epithelial transition) are two of the most occurring phenotype changes in adenocarcinomas in vivo. These events together with clonal selection are driven by various factors, e.g. immune system and microenvironment, and are responsible for metastasis formation. Unfolding the mechanisms of cancer cell plasticity might contribute to future therapeutic approaches. Breast cancer stem cells (BCSC) are suggested to be drivers of metastasis, resistance to chemotherapy and radiation. BCSC were previously shown to co-exist in diverse mesenchymal and epithelial states in different tumor sites. To understand the plasticity in BCSC we used HER2/neu-overexpressing primary mouse mammary carcinoma cell line and its relapsed HER2/neu antigen-negative variants.
SMALL MOLECULE DISCOVERY (B244)

375/B244 Phenotypic Chemotaxis by the Use of Flow Cytometry

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Introduction: Chemotaxis assays have been traditionally set up to analyse gross migration of cells across a membrane in response to a chemotactrant either by manual counting or by the use of a cellular dye measuring gross cellular movement. For simple assay systems, where a recombinant cell line is being used for basic efficiency measurement this system works well, however chemotaxis assays can also be used to monitor the migration of specific cell types from within a mixed population of cells. At Huntingdon Life Sciences we have worked with AstraZeneca on the development of a phenotyping chemotaxis assay by the use of flow cytometry to monitor the migration of the specific cell types within human Peripheral Blood Mononuclear Cells (PBMCs) in response to MDC.

Method: The assay uses the HTS Transwell-96 System to allow the chemotaxis of isolated PBMCs in response to MDC, and subsequent inhibition with CCR4 small molecule antagonists. The resultant cell suspension was then stained with markers for CD3, CD4, CD8, CLA and CD195 (aka CCR4) and the number of CD3+CD4+CLA+CD195+ lymphocytes in a set volume of supernatant were measured using a BD FACSCanto II flow cytometer.

Results: The results showed a clear dose response in the CLA+CD195+ T lymphocytes in response to MDC, which showed a bell-shaped profile. The addition of a CCR4 antagonist caused a dose-dependent reduction in the levels of chemotaxis observed.

Conclusion: A method was developed at HLS for the phenotypic analysis of the migrated cells, using flow cytometry and the Transwell system. This has enabled the chemotaxis assay not only to be used as a primary efficacy assay, but to be also used as an assay system with the potential to investigate disease mechanism. The nature of this novel assay would allow the use of disease state PBMC samples or conditioned media from separate primary assay systems in mechanism of action (MOA) studies with compounds, or allow the biological investigation of disease state samples and further investigation of the specific cell types involved.

SOLID TUMORS (B245 – B247)

376/B245 Near-Haploidiisation in Thyroid Cancer Significantly Associates with the Oncocytic Phenotype but Not with Mitochondrial DNA Mutations

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Background: Oncocytic mitochondria-rich tumours are most frequently found in the thyroid. The accumulation of mitochondria is thought to be caused by disruptive and/or damaging mutations in the mitochondrial genome, which initiates uncoupling and subsequent mitochondrial proliferation. We demonstrated recently that oncocytic thyroid tumours frequently show near-haploidiisation and endoreduplication which manifests as a near-homozygous genome (NHG). Now we extended our observations to a large series of thyroid tumours and included mitochondrial DNA (mtDNA) mutation analysis and also studied nuclear genes directly or indirectly associated with cellular metabolism.

Experimental Design: In total 88 thyroid tumours of varying histology were studied. Genome-wide high-density SNP array and flow cytometry: DNA content analysis were combined to determine the chromosomal dosage (allelic state). The entire mitochondrial genome of 45 tumours (oncocytic and non-oncocytic) was studied for mutations. These tumours were also studied for IDH1/2 mutations. All tumours were also characterized for frequently observed somatic mutations in EGFR, BRAF (V600E), RAS, PIK3CA.

Results: An NHG was identified in 16/88 thyroid tumours. An oncocytic metaplasia was found in 33/88 thyroid tumours. Strikingly, all 16 tumours with a NHG were oncocytic. This correlation showed to be highly significant (P = 0.0001, two-tailed Fisher’s exact). One or more damaging or disrupting mtDNA mutations were found in 62.2% (28/45) of the tumour samples. No correlation was found between these mtDNA mutations and the oncocytic phenotype or a NHG (P = 0.3414 and P = 0.1401, respectively). The BRAF V600E mutation was found in 22 cases (20 PTC, 2 ATC). The NRAS Q61R was found in 7 cases (2 PTC, 5 ATC). The IDH1, PIK3CA and HRAS mutations were found sporadically and EGFR mutations were not found. None of the mutations in the nuclear encoded genes correlated with the oncocytic phenotype or a NHG.

Conclusions: A NHG correlates significantly with oncocytic thyroid tumours. In contrast, nuclear encoded genes associated with cellular metabolism do not correlate with the oncocytic phenotype or with a NHG. This also accounted for damaging and/or disrupting mtDNA mutations which were frequently found in oncocytic and non-oncocytic thyroid tumours. A combination of unknown factors likely underlies a NHG in a subset of oncocytic thyroid tumours.

377/B246 Tritterpenoids Alter Mitochondria Function and Cause Cell Death in RKO Human Colon Cancer Cells

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(AV). FACS-based screen using 252 validated antibodies against selected surface antigens identified distinct interplay between cell phenotype and expression of molecules responsible for interaction with extracellular matrix. Precise analysis of TGFβ superfamily pathway revealed stabilised expression of Inhibitor of differentiation 1 (ID1) as a consequence of aberrant signalling in mesenchymal cancer cells, but not in their epithelial counterparts. We also observed different expression of surface stem cell markers, transcription factors in general responsible for stemness, EMT regulators and mediators of metastasis amongst cell lines. In summary we showed that regulation of stemness and cancer-associated plasticity is tightly related and under context-dependent control of TGFβ superfamily signalling pathway.

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Background: The anticancer triterpenoid 2-cyano-3, 12-dioxoolean-1,9(11)-dien-28-oic acid (CDDO), and its C-28 methyl ester derivative (CDDO-Me) inhibit mitochondrial Lon protease (Lon), which is able to modulate several mitochondrial functions. Using RKO human colon carcinoma cell line, we investigated the effects of these drugs on mitochondria activity and functionality, by analyzing intramitochondrial reactive oxygen species (iROS), mitochondrial membrane potential (MMP) and mitochondrial mass. Finally, since Lon is a stress protein inductible by multiple stressors, we asked whether Lon overexpression could protect RKO cells from CDDO-induced cell death.

Methods: The apoptosis of mitochondria, mitochondrial hydrogen peroxide and mitochondrial superoxide production was performed by staining cells with Annexin V Alexa Fluor Pacific Blue conjugate, TO-PRO-3 iodide, mitochondria peroxyl yellow 1 (MitoPyro) and MitoSOX Red Superoxide Indicator. MMP was studied by using JC-1 (that was excited by two lasers, i.e., 488 nm for monomers and 561 nm for aggregates), mitochondrial mass by MitoTracker Green. All samples were analysed by using an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher). Mitochondria morphology was studied by confocal microscopy. To investigate the importance of Lon hyperexpression, we generated a retroviral vector harbouring the cDNA encoding for Lon protease (pMSCV-Lon), and we used this to stably transduce RKO cells.

Results: CDDO and CDDO-Me decreased proliferation, induced apoptosis in RKO cells, and increased mitochondrial hydrogen peroxide rather than mitochondrial superoxide anion. CDDO and CDDO-Me caused MMP depolarization and altered mitochondrial morphology, without influencing mitochondrial mass. In all cases, CDDO-Me was much more potent than CDDO. In pMSCV-Lon cells, a 3-fold increase and a 2-fold increase was observed for Lon mRNA and Lon protein, respectively. When treated with CDDO and CDDO-Me, cells overexpressing Lon were less prone to undergo apoptosis than control cells. Interestingly, also in this case CDDO-Me was more effective than CDDO in causing cell death.

Conclusion: In RKO cancer cells, CDDO and CDDO-Me exert antiproliferative and anticancer activities and inhibit cell growth in a dose-dependent manner by triggering apoptosis and inducing MMP depolarization. The overexpression of Lon reduced apoptotic cell death induced by CDDO and CDDO-Me, indicating that Lon is a key nononcogenic protein that is required for the maintenance of mitochondrial functions in cancer cells. Further studies are in course to better understand the mechanisms linking Lon protease inhibition and mitochondrial alterations induced by both CDDO and CDDO-Me.

378/B247

Polychromatic Flow Cytometry in Cancer Stem Cells Characterization from Lung Cancer Samples

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Background: Non-squamous lung cancer (NSCLC) is almost 80% of lung cancer and the overall survival does not exceed 15% at five years. Resistance to treatment may depend on Cancer Stem Cells (CSCs), a rare cell population with ability to self-renewal and differentiation. To date, specific markers for lung-CSCs have not been identified. The aim of our study was to characterize the expression of surface markers related to stem cell phenotype in NSCLC cancer, and the distribution of cell subpopulations generated by the combination of different markers.

Methods: We performed flow cytometry immunophenotyping of NSCLC cell lines and tumors from patients to analyze the distribution of different subpopulations, based on the expression of EpCAM, CD90, CD166, CD44, CD133 and E-cadherin. Moreover, assays to evaluate the ability to grow in spheres in vitro and in vivo tumor formation were performed.

Results: Flow cytometry analysis showed high heterogeneity in the expression of the stem-related surface markers in NSCLC cell lines and tumor from patients. Two main subpopulations were characterized in cell lines (EpCAM+ and CD90+). Only EpCAM+CD90− cells were able to generate spheres in vitro. The potential of tumor spheres as tumor initiating cells (TIC) was explored by injecting different dilutions and subpopulation of cells in NOD/SCID mice. The EpCAM+CD166− spheres showed the highest tumor induction frequency.

Conclusions: Our data suggest that EpCAM could be related with a high ability to form spheres in vitro and to generate tumors in immunosuppressed mice in vivo. Blocking CD126 signalling pathway appear to be rationale therapeutic strategy for eliminating NSCL stem cells.

STANDARDS AND CALIBRATION (B248 – B254)

379/B248

Quantitative Light Scattering of Extracellular Vesicles for Flow Cytometry Standardization

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Introduction: Although flow cytometry is the most widely used method to study single vesicles, comparison of flow cytometry results between laboratories remains challenging. At present, most laboratories select vesicles by setting an inclusion gate based on the scatter signal from two polystyrene bead sizes, resulting in a coefficient of variation (CV) of the vesicle concentration of 91%. This moderate reproducibility is attributed to the variety of optical configurations in flow cytometers and the refractive index difference between vesicles and polystyrene. Due to this refractive index difference, polystyrene beads scatter light 10-fold more efficiently than vesicles.

Methods: A well-defined polystyrene beads mixture (metves.eu) and two vesicle standards are measured on 45 flow cytometers in 31 laboratories worldwide. The relation between particle size, refractive index, and scatter is obtained from the variety of optical configurations in flow cytometers and the refractive index difference between vesicles and polystyrene. Using this relation, the relative concentration of vesicles can be determined. This unique approach provides a profound understanding of vesicle detection by flow cytometry, which is essential to inter-laboratory data comparison.
Background: Systemic autoimmune diseases (SADs) affect 1% of the population. However, due to the heterogeneity of molecular mechanisms resulting in the same disease class or due to misclassification to a different disease class despite similar etiological mechanisms, patients are being denied access to potentially beneficial novel and approved agents. The ‘Molecular Reclassification to Find Clinically Useful Biomarkers for SADs’ (PRECISE SADS) IMI project will study 2.500 individuals affected by SADs into clusters of molecular, instead of clinical entities. All these patients will get flow cytometry analyses performed in 11 different centers.

Methods: These centers are provided with distinct flow cytometers (Canto II, Aria III, Fortessa, Verse, Navios and Gallios). The prerequisite of the integration of their data in bioinformatical and biostatistical analyses is a fine inter-cytometer calibration before starting the inclusions. VersaComp AB capture beads and Rainbow 8-peak beads were acquired to determine identical sensitivity and subsequent individual QC control, respectively. Duraclane tubes (Beckman Coulter) have been designed for the daily flow cytometry analyses of the blood samples.

Results: One Navios was used as internal reference for the whole project. Acquisition of VersaComp AB capture beads by this Navios was used to fix the MFIs of 8 different fluorochrome-conjugated Abs (FITC, PE, PC5.5, PC7, APC, APC-AF750, PB and KR0). The 10 other centers have then fixed the PMT values of their flow cytometers to reach the same MFIs of the 8 fluorochrome-conjugated Abs. Using their fixed PMT values, each center have then acquired Rainbow 8-peak beads to determine their own internal QC control. One control blood sample has been dispatched in all centers to be concomitantly stained with their own internal QC control. One control blood sample has been dispatched in all centers to be concomitantly stained with their own internal QC control, respectively. Duraclane tubes (Beckman Coulter) have been designed for the daily flow cytometry analyses of the blood samples.

Conclusions: Versa Comp Antibody Capture Beads allowed to reach very close sensitivity for the 11 flow cytometers. The Rainbow 8-peak beads must now be acquired as internal QC control before any inclusion of patient in each center to validate the stability of each flow cytometer. The very first comparisons of the expression of cell surface markers on cells from the same blood control stained with the Duraclane tubes are very promising. The inter-calibration of the 11 flow cytometers is now achieved. The inclusion of patients in the different centers can start, knowing that the comparison of the flow cytometry data will be possible. Identification of specific molecular signatures in patients with SADs will enable clinicians to tailor therapies accordingly to the specific pathways to be targeted in individual cases. In short, to implement precision medicine strategies. (This work has received support from EU/EFPIA Innovative Medicines Initiative Joint Undertaking PRECISESADS grant n°115565).

Accurate cell counting - including the correct identification of cell subpopulations and enumeration - is essential for a wide range of clinical applications. There is a demand for comparability and reliability of measurement in different (clinical) laboratories, which can be assured by traceability of the measurement results to the international system of units (SI).

To enable manufacturers and end-users to easily assess the accuracy of their routine results, a secondary reference method based on relative concentration measurements with respect to traceable calibrators is being investigated. The calibrator, e.g. beads of known concentration, used in secondary reference measurements, is calibrated by applying the primary reference procedure. The quantification of counting losses due to random coincidences in relative counting is particularly important when using the secondary reference procedures. These individual counting losses are investigated during blood cell counting and bead counting, mimicking blood samples with respect to relative concentrations and signal amplitudes of erythrocytes and immune-labelled platelets. All methods are evaluated using CD4 positive lymphocytes as a model system.

Microscopic cell counting offers an alternative to flow cytometry with the advantage of more accurate identification of cells by immunological and morphological parameters. However, the sample volume that can be analyzed is usually much lower even when using counting chambers and slide scanning. This may lead to high contributions to uncertainty from counting statistics, in particular, when measuring samples with low particle concentration. Results for microscopic and flow cytometric measurements on cerebrospinal fluid will be presented.

The research receives partly funding from the EMRP project SIB-54 Bio-SITrace. The EMRP is jointly funded by the EMRP participating countries within EURAMET and the European Union.
We have developed a new class of stable, synthetic cell mimics for flow cytometry and cell sorter calibration using a novel hydrogel particle technology. These mimics can be tailored based on polymer and monomer composition to more precisely match the differential optical and fluorescent properties of cell subpopulations such as monocytes, lymphocytes, granulocytes, and neutrophils. The optical properties of the new mimics include the generally measured parameters of forward (FSC) and side (SSC) scattered light. FSC and SSC are not well matched by beads which suffer from two primary limitations: (1) polystyrene beads have completely different optical and (2) hydrodynamic properties when compared to cellular material. These drawbacks create dramatic differences when looking at the behavior of polystyrene particles in a flow cytometer in comparison with that of cells and force researchers to further calibrate instruments with cell lines or biological controls to achieve correct interpretation of immunophenotyping data. We will be able to show how our new cell mimics create assay specific reference templates for normal controls with consistent assay patterns including scattered light (e.g., FSC and SSC gating for lymphs, monos, neuts and granis). This new standard promises to increase measurement accuracy across labs, instruments and time, decrease setup costs and the use of precious sample material while improving detection limit controls for low biomarker expression. In other words, these new particles represent the first flow cytometry standards that will simultaneously verify instrument performance with assay-specific parameter pass/fail criteria.

Recent applications of nanoparticles (NPs) in consumer products have induced concerns to their adverse effects on environment and human health, due to the possibility of their exposure as well as potential hazards [1]. With these concerns, understanding the interaction between NPs and biological system became one of the most important scientific issues to be addressed. Currently, although various analytical techniques have been used for the measurement of cellular association of NPs, conventional electron microscopic instruments (e.g., TEM, SEM) have limitations in quantification of cellular NPs, while direct measurement techniques (e.g., ICP-MS) are destructive and labor-intensive, since they require extensive sample preparation procedures.

In this study, we have proposed a simple and easy prediction model for the estimation of cellular NPs based on side scattering intensities (SSC) of flow cytometry (FCM). In FCM, the SSC are known to depend on cellular granularity and forward scattering intensities (FSC) are related to cell size.[2] Therefore, the SSC has been often used to estimate the amount of cell-associated NPs. Recently, we have reported similar, but semi-quantitative approach for SiO2 NPs using FCM and x-ray fluorescence technique [3]. Building upon this previous work, we have further investigated the effects of various parameters, such as SSC’s normalized with three different methods, mass- and number-based dosimetry, volume- and area-based dosimetry, core and hydrodynamic diameters, surface charges, agglomeration and sedimentation and developed a 3D regression model for the estimation of cellular Au NPs.

References:

Biosafety of Cell Sorting: A New Rapid Standardized Procedure to Evaluate Biocontainment Quality
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Due to the ever-growing use of high-speed cell sorters for sorting of potentially infectious samples, operator protection has to be assured. Hence, aerosol formation during sorting in normal or accidental situations may present a risk of infection to the operator. Providers offer level 2 biosafety cabinets for the containment of cell sorters, but no rapid standardized procedure has been proposed to confirm containment integrity. Here, we describe a simple and original procedure to verify the efficacy of containment, and we demonstrate its importance. We used the...
Coriolis® μ AirSampler system (Bertin Technologies) and we assessed the containment level of InFlux (BD Biosciences, International NSF-49 standard) and FACS ARIA (BD Biosciences, European EN12469 standard) cell sorters equipped with a Baker Hood system (Baker) and a HBOX117 (Norkit) level 2 biosafety cabinet, respectively. The tests were performed using 2 micron diameter fluorescent beads in normal and accidental situations. The Coriolis μ AirSampler system captures aerosolized particles in a liquid by aspiration of a pre-determined and calibrated volume of air. This liquid can subsequently be easily and quantitatively analysed for the content of fluorescent beads using flow cytometry. We demonstrate that FACS LSR II and MACSQuant analysers provide a quantitative measure of the aerosol dispersion rate into the environment. Our measurements lead us to conclude that cell sorting associated with biological risk, such as infected cells, has to be systematically conducted under level 2 biosafety cabinets. This method holds promise for the evaluation of the effectiveness of containment in both normal and accidental conditions on cells sorters. In conclusion, we propose to consider standardizing this procedure for use as a common international basis for the development of norms and rules in containment control of cell sorters.

**STEM CELLS (B255 – B263)**

386/B255

**Exploiting High Dimensional Flow Cytometry for the Identification and Isolation of Hematopoietic Stem and Progenitor Cell Populations in Human Bone Marrow**

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Over the last decades, studies of the hematopoietic system have been greatly facilitated through technical advances in flow cytometry powered by an increased availability of monoclonal antibodies. Thereof, surface phenotypes of major human hematopoietic stem and progenitor cell (HSPC) populations have become increasingly refined. Conversely, the application of high-dimensional flow cytometry for the prospective isolation of these rare cell subsets lags far behind. One of the major obstacles is the lack of consensus in the choice of markers for isolation using cell sorting. While major subsets of human HSPC are currently classically isolated based on combinations of markers including lineage markers, CD34, CD38, CD90, CD45RA and CD123, the identified populations undoubtedly remain far from phenotypically and functionally homogeneous.

To allow further refinement in the isolation strategy of human bone marrow derived HSPC and hematopoietic stem cells (HSC) in particular, we first identified key markers reported to identify those subsets and systematically evaluated their concomitant use to achieve higher-resolution identification of HSC. Phenotypically defined lineage-CD34+CD38–CD90+CD45RA–HSC appeared to be heterogeneous with regards to expression of alpha-6-integrin, CD114, and efflux of Rhodamine 123, a substrate for the ABC-transporter ABCB1/MDR1. Further sub-fractionating of HSC based on expression of CD114 or alpha-6-integrin showed no strong segregation. Indeed, CD114+ cells contained higher frequency of alpha-6-integrin+ cells, but not the inverse. Interestingly, apart from negative markers, no overlap in expression was observed when comparing CD34+ HSC and their more primitive CD34-counterparts, supporting their distinct entities. Our exploration of markers on HSCC enabled the devise of a 12 color panel allowing the identification and concomitant isolation of both the recently described surface CD34+CD93+HSC as well as the canonically-defined CD34+HSC together with their myeloid descendants including common myeloid progenitor (CMP), megakaryocyte-erythrocyte progenitor (MEP) and granulocyte-monocyte progenitor (GMP) cells. By allowing a more refined and simultaneous isolation of human HSPC subsets, our strategy can contribute to achieving a more precise molecular and functional characterization of stem and progenitor thereby helping to advance current understanding of the developmental relationships within the hematopoietic system both in health and disease.

387/B256

**Unraveling the Complexities of Human Skin: Optimization of a 16 Colour Panel to Enable Characterization of Mesenchymal and Vascular Cell Populations by Multicolour Flow Cytometry**

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**Background:** The skin is a large and complex organ composed of many cell types. Identifying and characterizing key cells with regenerative potential is important in understanding tissue regeneration and abnormal wound healing, such as keloid scarring. We recently reported the most comprehensive characterization of human skin (dermal) cell populations using an 11 color flow cytometry panel and immunohistochemical techniques. This panel enabled the detection and isolation of dermal mesenchymal progenitor cells (dMPCs) which were shown to have regenerative capacity. However, due the vast complexity and detection of numerous cell types, we sought to re-design this 11 color base panel to include 14 colors plus leave 2 further ‘drop in’ channels empty for future-proofing the panel.

**Methods:** Due to a recent increase in commercially available antibodies specific for mesenchymal markers, the 11-color panel was completely redesigned and extended up to 16 colors. This enabled enhanced cell characterization of both the mesenchymal and vascular populations to help improve our understanding of the composition of the human skin. Designing a 16 color panel is technically challenging particularly when many antigens are not mutually exclusive and the dissociated tissue preparation contains many cell types. The quality of the data is also highly dependent on machine setup, therefore voltages of PMTs were optimized to maximize the stain index of each fluorophore and application settings were utilized to ensure day-to-day consistency.

**Results:** A BD SORP FACS Aria II was optimized to perform analyses of a 16 color panel on human dermis. We describe the process of redesigning and subsequently testing a number of panel iterations, where possible, taking into consideration antigen expression levels, fluorophore brightness, reagent availability, and spectral overlap. Many of the key mesenchymal antigens that are co-expressed at high density on dMPCs in the dermis (CD34, CD90, CD73) were also expressed on sub-populations of non-mesenchymal cells, which meant assigning high-density antigens to dimmer fluorophores was not a foolproof strategy. Instead a number of antibodies were chosen based on reagent availability and various combinations tried to determine the optimal combination to enhance both mesenchymal and vascular cell characterization.

**Conclusions:** The development of a new, optimized panel resulted in better resolution of some markers and more extensive characterization of the populations present. This enabled better classification of the vascular populations and highlighted further heterogeneity within the mesenchymal populations. This panel can now be applied to keloid scar samples to dissect which populations may be involved in keloid scarring.

388/B257

**Loss of Trop-2 Expression Is Associated with Mesenchymal Phenotype in Prostate Cancer Cells**

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**Background:** The Trop-2 transmembrane protein is a member of the integral membrane glycoprotein family, which is highly expressed on normal and neoplastic cells of keratinizing epithelia of skin, and in breast cancer epithelial and stromal cells. It has been shown to be overexpressed in many carcinomas of skin, breast, colon, lung and prostate. Recent studies indicate that Trop-2 expression is associated with tumor progression and metastasis. The presence of Trop-2 is also linked with poor clinical outcome and shorter overall survival in patients with prostate cancer. Thus, it has been proposed as a potential therapeutic target.

**Results:** We have determined the expression of Trop-2 in a panel of prostate cancer cell lines using immunohistochemistry, flow cytometry, Western blotting and immunoassays. In situ hybridization (ISH) studies were performed to verify expression of Trop-2 mRNA. We also performed a genome-wide analysis of microRNA expression in these cell lines.

**Conclusions:** Our results suggest that Trop-2 expression is associated with mesenchymal phenotype in prostate cancer cells. This is supported by the finding that Trop-2 expression is inversely correlated with the expression of mesenchymal markers, such as SMA and αSMA. Furthermore, our microRNA expression analysis reveals that Trop-2 expression is associated with a mesenchymal gene expression signature. These findings provide new insights into the role of Trop-2 in prostate cancer and suggest potential therapeutic targets for the treatment of this disease.
Trop-2 (trophoblast antigen 2) protein is a transmembrane glycoprotein that is involved in calcium signalling and is overexpressed by most human cancers. Role of Trop-2 has been described during development and tumorigenesis, including self-renewal (McDougall et al., 2014). It has been demonstrated that Trop-2 is a marker of stem/progenitor cells and functionally regulates adult tissue self-renewal and prostate regeneration (Stoyanova, et al., 2012). The cancer stem cells-like cells (CSC-like cells) are believed to be a source of heterogeneity within the tumours and represent an attractive target for anticancer therapy. Prostate CSC-like cells can be characterised by expression of several tissue specific surface markers, such as CD44, Trop-2, CD49f (Garraway et al., 2010). However, the exact role of Trop-2 in prostate cancer remains to be revealed.

In our study, we analysed expression of Trop-2 in panel of prostate cancer cell lines and discovered subpopulation of the Trop-2 positive cells in metastatic prostate cancer cell line DU-145. Therefore, we examined the role of Trop-2 expression on cytokinetics and cellular plasticity in DU-145 cell line. Cell cycle analysis of DU-145 showed that Trop-2- cells are predominantly in G2 phase compared to Trop-2- cells. We further analysed clonogenic capacity of sorted single cells and observed significant higher clonogenic capacity of Trop-2- cells. Moreover, there was a characteristic difference in colony morphology - Trop-2- typically formed meroclones (loose colonies), whereas Trop-2- formed predominantly holoclonies (compact colonies). Next, we established stable Trop-2- and Trop-2+ cell lines from sorted single cells and analysed EMT status of these cell lines using western blot and qRT-PCR E-cadherin, N-cadherin, Slug, Snail. Based on these analyses the Trop-2 cell line was found to present more mesenchymal phenotype in comparison to Trop-2+ cell line.

In conclusion, we found that Trop-2- is a marker that determines cells with different EMT status and that cells defined by expression of Trop-2 differ in cell cycle progression and clonogenic capacity.


389/B258 Tissue-Associated M2 Macrophages Localize to CD90+ Cancer Stem Cells in Barrett’s Esophagus but Not in Normal Esophagus and Invasive Adenocarcinoma
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The epithelial-mesenchymal transition (EMT), a process important in embryonic development and wound healing, also plays a role in the transition from preinvasive to invasive cancer. In breast cancer tumor associated macrophages (TAM) promote EMT via juxtapacrine signaling with CSCs. It is unknown whether TAM-tumor interactions precede transition to invasive cancer and are already present in pre-invasive stages. Frank esophageal malignancy often includes areas of preinvasive Barrett’s esophagus, providing an opportunity to answer this question. Here, we combine the use of immuno-histology and multidimensional flow cytometry to examine TAM-tumor interactions in invasive adenocarcinoma of the esophagus and its precursor Barrett’s esophagus. In normal esophagus, very rare CD90+CD44+-vimentin+-cytokeratin dim cells are localized to the basal layer or to the papillae which are finger-like invaginations of the mucosal epithelium. Ki67+ proliferating cells also concentrate in papillae of the basal epithelium and are likely to give rise to these proliferating and differentiating cells. CD90+, CD44+ and Ki67+ cells are absent in the mature luminal epithelium, suggesting that only differentiated cells comprise the luminal mucosa. Immune cells, including lymphoid nodules and scattered CD68+ tissue macrophages cells are limited to the submucosa. Macrophages in the lymphoid follicles are both CD68+HLADR+CD163- (M1) and CD68+HLADR-lowCD163+ (M2). CD90+vimentin+cytokeratin dim cells are present in Barrett’s but not in invasive carcinoma. In both Barrett’s and invasive cancer, elements of the epithelial and mucosal layers are admixed, providing the opportunity for direct interaction between tumor cells and TAMs, which are present at significantly higher density than in normal submucosa. TAM in Barrett’s are exclusively CD163+HLADRlow or neg and therefore M2. Across the spectrum from normal esophagus to invasive adenocarcinoma, the prevalence of M2 macrophages and CD90+ cells are highly correlated (p=0.000000). Flow cytometric analysis of disaggregated single cells isolated from primary tumors and metastatic pleural effusions (MPE) confirmed the presence of EpCAM+CD90+ tumor cells coexpressing mesenchymal markers CD44, CD73 and CD105. Such mesenchymal tumor cells are rare in primary tumors and prevalent in MPE. In Barrett’s esophagus and MPE, CD90+ stem-like cells have the greatest opportunity to interact with TAMs to amplify the EMT program. This is not as apparent in primary invasive adenocarcinoma. This interpretation from static observations in freshly isolated tissues requires confirmation by mechanistic TAM-tumor co-culture experiments, using sort-purified TAM and tumor subpopulations from freshly isolated esophageal tissues.

390/B259 Validation and Monitoring of 2nd Reprogrammed iPSC Colonies Using a Rapid Plate-Based Imaging Cytometry Method
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Flow cytometry (FC) and fluorescent microscopy (FM) have been commonly used for the detection of induced pluripotent Stem Cell (iPSC) reprogramming, which often have limitations, where flow cytometry requires disruption of adherent iPSCs by trypsinization, and fluorescent microscopy requires manual quantitative analysis that is low throughput. In the recent years, image-based cytometry systems have been utilized to perform direct whole well based assays in microplates without trypsinization and with comparable sensitivity as current fluorescence detection methods. In this work, we developed an automated method for detection of iPSC colonies utilizing the combination of the Celigo S Imaging Cytometer and secondary iPS reprogramming. This approach is based on the fluorescent identification of iPSC colonies that express the four reprogramming factors, Oct4, Sox2, Klf4 and e-Myc expressing mOrange following ires. The reprogramming progress is also monitored using fluorescent detection of the pluripotency reporter Nanog-GFP+ cells within these colonies. Results demonstrated the capability of the imaging cytometer showing the increase in Nanog-GFP+/mOrange+ iPSC colonies in respect to time and also increase in iPSC colonies when treated with shRNA X. The Celigo Imaging Cytometer allowed high throughput whole well fluorescence imaging and analysis of the iPSC colonies, which provided accurate direct measurement of colony numbers as well
as improve research efficiency through automation. This method can be used to not only follow the reprogramming kinetics, but could also be used to examine the effect of extrinsic factors, thus, providing a strong tool to investigate molecular mechanisms of reprogramming.

**391/B260**

The Low-Affinity Neurotrophin Receptor p75 Identifies Cell Line Subpopulations Displaying Stem-Like Properties In Vitro and Increased Tumorigenicity In Vivo

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**Background:** The low-affinity neurotrophin receptor (p75-LNGFR) is a putative marker of neuroectodermal stem generator cells. We investigated whether p75 could identify a cancer stem cell subpopulation in a panel of Melanoma, Neuroblastoma and Glioblastoma cell lines.

**Methods:** All experiments were performed on SNB-75 (Glioma), SK-MEL-28 (Melanoma) and LAN-5 (Neuroblastoma) cell lines. For the immunophenotype, approximately 2x10⁸ cells, resuspended in PBS with 1% FBS to minimize unspecific binding of antibodies, were stained 30 minutes on ice, in the dark, with conjugated primary antibodies, and analyzed by flow cytometry. To detect intracellular markers, cells were previously fixed in a formaldehyde solution, permeabilized in saponin and then stained 20 minutes at room temperature, in the dark, with antibodies against intracytoplasmic markers. Fluorescence-activated cell sorting was performed on cells stained with monoclonal antibody against human CD271 (p75); pulse width versus forward scatter height profiles were used to eliminate cell doublets; dead cells were eliminated by excluding DAPI positive population. Evaluation of apoptotic activity was performed by staining approximately 2x10⁶ cells with Annexin V FITC-conjugated for 20 minutes, at room temperature, in the dark; Annexin V was used in conjunction with a vital dye such DAPI, to distinguish viable cells to early apoptotic, late apoptotic and dead populations. The DNA content and cell cycle were assessed by incubating cells 3 hours at 4°C in a solution containing 50mg/ml propidium iodide and 100mg/ml RNase A. 1x10⁹ cells were injected into the adrenal gland of NOD/SCID mice to perform orthotopic transplantation.

**Results:** We found that p75 is abundantly expressed in subpopulations of cell lines from tumors of neuroectodermal origin. In the case of glioblastoma and neuroblastoma, p75 expression was found to colocalize with stem markers and was increased in neurospheres formed by cell lines kept in culture conditions and selected for stem cells. In sorted subpopulations, unlike p75⁺, p75⁻ cells could form neuro- or melanomas, and, in standard culture conditions, reconstituted a mixed population including both p75⁺ and p75⁻ cells. Moreover, the p75⁺ subpopulation was slower cycling and more resistant to apoptosis than the p75⁻ subpopulation. After orthotopic transplantation (adrenal gland) in immunocompromised mice, the p75⁺ subpopulation sorted from neuroblastoma LAN-5 displayed significantly enhanced tumorigenic ability as compared with the p75⁻ subpopulation, resulting in reduced mouse survival, increased tumor volume and metastatic dissemination.

**Conclusions:** These data indicate that, in cell lines of neuroectodermal origin, the p75⁺ subpopulation holds stem-like properties in vitro and increased tumorigenicity in vivo

**392/B261**

Identification and Characterization of a Label-Retaining Stem-Like Subpopulation in Primary Glioblastoma-Initiating Cell

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Gliomas are the most frequent type of brain tumor in adults. Among gliomas, glioblastoma multiforme (GBM) is the much aggressive type and the majority of patients relapse or become refractory to conventional therapies. Therapy inefficacy is due, in part, to the high degree of tumor heterogeneity, contributing to both tumor progression and therapeutic resistance. Accordingly to the cancer stem cell paradigm, a small subpopulation of cancer cells with stem cell-like characteristics has been revealed in several central nervous system (CNS) tumors, including GBM. We aimed to identify and characterize a subpopulation of stem-like cells within primary glioblastoma-initiating cells (GICs), undifferentiated tumor cells considered to be responsible for tumor initiation, relapse and therapeutic resistance.

To this aim, we explored a recently described label retention approach based on the use of the small fluorescent lipophilic molecules Vybrant® Dil, which irreversibly binds the lipid cell bilayer and get proportionally distributed among daughter cells. This method allowed us to discriminate between fast (Dil low) and a small subpopulation (< 7%) of slow (Dil bright) growing cells in primary GICs, followed by fluorescence-activated cell sorting and evaluation of their phenotype and function.

Expression of stem cell markers, including CD24, CD133, nestin and SOX2 was evaluated by flow cytometry and the neurosphere and multipotency assays were performed using fluorescence microscopy and image analysis software.

Our preliminary data show that: i) the Dil bright subpopulation exhibited only a partial overlap with nestin and SOX2 expression compared to the Dil low subpopulation; ii) the frequency and average size of the neurospheres generated by the Dil bright subpopulation is lower than that of the neurospheres generated by the Dil low subpopulation; iii) under differentiation conditions the Dil bright subpopulation showed an elongated fibroblast-like cell shape and was negative for neuronal differentiation markers.

Our study provides additional data supporting the use of approaches based on label retention to identify, isolate and characterize subpopulations of GICs.

**Keywords:** GBM, CNS, GICs, cancer stem cells, Vybrant® Dil.

**393/B262**

Isolation, Selection and Culture Methods to Enhance Clonogenicity of Murine Bone Marrow-Derived Mesenchymal Stromal Cells

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**Background:** Mesenchymal stromal cells, or MSCs, are multipotent stromal cells that can differentiate into a variety of cell types including osteoblasts, chondrocytes and adipocytes. Conventional methods for MSC isolation from murine bone marrow (mBM-MSC) involve cell culture selection based on adherence to plastic tissue culture dishes, resulting in a heterogeneous population of cells. Prospective isolation has been reported but is rarely undertaken.

**Aim:** The aim of this study is to optimise the isolation, culture conditions and selection of mBM-MSCs.
Methods: Two methods were used to isolate bone marrow derived cell suspensions from the femurs and tibias of 6-8 week old C57Bl/6 mice. Method 1: cells were flushed from the bone marrow using 18 gauge needles - 'flushed mBM' fraction; method 2: the bones were crushed and enzymatically digested - trabecular bone or 'compact mBM' fraction. Cell suspensions were incubated under normoxic (21% O₂) and hypoxic conditions (5% or 2% O₂). Multi-colour flow cytometry and fluorescently activated cell sorting (FACS) was used to identify and to isolate specific MSC populations directly from mBM, using a combination of antibodies to CD45, Ter119, Sca-1, PDGFRα and CD90 surface antigens. Clonogenic potential was assessed via CFU-F formation and qRT-PCR.

Results: The CFU-F frequencies of CD45/Ter119/Sca-1- populations of cells isolated from flushed mBM and compact mBM was significantly higher in the compact mBM fraction. Incubation under hypoxic conditions improved the CFU-F frequency in the CD45/Ter119/Sca-1- population. qRT-PCR analysis indicated 100 fold increase in the expression of transcripts characteristic for stem cells (NANOG & TERT), osteocytes (BMP2) and myogenic/adipocytes (Myf5) in the CD45/Ter119/Sca-1- versus the CD45/Ter119/Sca-1+ population isolated from compact mBM. The inclusion of two additional selection markers, PDGFRα and CD90, resulted in the isolation of CFU-F with a frequency of 1/4 from compact mBM.

Conclusion: Using a combination of antibodies to CD45, Ter119, Sca-1, PDGFRα and CD90 and hypoxic culture conditions, this study has successfully developed a method to prospectively isolate MSCs with enhanced clonogenicity from murine bone marrow.

394/B263 Novel Fluorescent Reporters CDy1 and CDr3 Enrich for Neural Stem Cells Derived from the Murine Brain

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Neurogenesis occurs continuously in two brain regions of adult mammals, the subventricular zone (SVZ) in the wall of lateral ventricles and the subgranular zone (SGZ) in the hippocampus. Optimal brain function is dependent on adequate numbers of Neural Stem Cells (NSC’s), as reduction in neurogenesis during aging or in neurodegenerative diseases can result in sensory and/or cognitive impairments. As such, in order to advance our understanding of NSC’s function and to develop therapeutic and diagnostic approaches, it is important to accurately identify and enrich for NSC’s.

To date there are no definitive markers for the identification of NSC’s in the mouse brain. While there are reports of very high enrichment for SVZ NSC’s by combining expression of GFAP, with Prominin 1 (CD133) to yield an enrichment of 1 NSC in 4 cells, this method is limited in its application to the transgenic mouse line used to select for the intracellular proteins of interest (e.g. GFAP). Two intracellular methods that enrich for NSC’s have also shown utility, AldeFluor and the so-called Side Population (SP). Given that the literature shows variability in practical utility, additional probes for defining the NSC population in primary tissue are critically important.

In a recent study, Kang and colleagues 1) identified a fluorescent molecule, designated CDy1, which upon incubation with tissue culture cell lines of either mouse or human embryonic stem cells (ESC), as well as human induced pluripotent stem cells (iPS) cells, specifically selected for pluripotent cells (1). We have recently shown 2) that CDy1 labels a population of primary brain cells and enriches for neurosphere-forming cells from primary mouse SVZ tissue. We then examined the effectiveness of CDy1 to select for NSC’s derived from the SVZ of aged animals, where the total pool of NSC’s present is significantly lower than in young animals and showed that CDy1 positive cells numbers reflect the expected trend of higher numbers in young animals and decreased numbers in aged animals.

We then sought to build on this work utilizing the recently commercially available NeuroFluor™ CDr3 (Stemcell technologies). NeuroFluor™ CDr3 binds specifically to mouse, rat and human fatty acid binding protein 7 (FABP7) and selectively labels live primary and pluripotent stem cell-derived neural progenitor cells generated using an embryoid body protocol. Here we present our results showing the effectiveness of CDr3 in the enrichment of NSC’s from primary murine SVZ tissue. Further we demonstrate, using a combined antibody labelling and CDr3 staining protocol, that selective removal of sub populations of cells enriches for NSC’s.


THERAPEUTICS (B264)

395/B264 Autotransplantation of Bone Marrow-Derived Mesenchymal Stem Cells in R6/2 Mouse Model of Huntington's Disease

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A major goal of research at the Field Neurosciences Institute (FNI) Laboratory at Central Michigan University is developing optimal methods for the use of stem cell transplantation in alleviating functional deficits in rodent models of Huntington’s Disease (HD). The main focus of this research study is on characterizing bone-marrow derived mesenchymal stem cells (MSCs) from R6/2 wild-type (HD-), and R6/2 transgenic (HD+) mouse models and studying the effects of syngeneic vs. autologous MSC transplantsations into the striatal region of the mouse brain. The working hypothesis is that such implanted cells will provide trophic support that promote regenerative effects via the inclusion of a specific population of MSCs, the multipotent adult progenitor cells (MAPCs), which have the potential to be engrafted, and subsequently, survive, proliferate, and differentiate into neuronal phenotypes. Screening for a more robust cell population is needed in order to distinguish the most pertinent cell markers for optimal transplantation. Identification of these optimal cell markers may, putatively, distinguish a potentially more efficacious subset of MSCs that are more prone to differentiate into neurons than the general population of MSCs.

TISSUE CYTOMETRY/MORPHOMETRY (B265)

396/B265 Image Cytometry Analyses of Hepatocyte Nuclei Size in the Liver Histological Sections of Patients with Different Outcome of Alpha-1-Antitrypsin Deficiency

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Clinical experience indicate derangement of the hepatocyte proliferation in response to alpha-1-antitrypsin deficiency (α1ATD) that associates with increased risk chronic liver disease and hepatocellular carcinoma. Although it is known that the AT mutant Z protein triggers the accumulation of liver damage, however, neither laboratory tests nor histopathological data carried out in the early stages of the disease does not allow to presage further course of the disease. Therefore the aim of the presented analyses was to determine whether the variability in hepatocytes nuclei size can be a differentiating feature of patients with good and bad prognosis.

Liver tissue samples divided into groups: I - α1ATD pts with good prognosis; II - α1ATD pts with unfavourable prognosis, were investigated. Paraffin 4 μm thick sections were labelled with multipurpose sets of antibodies and next with DAPI (1:1000, 5 minutes). Image cytometry method Scan-W, Olympus was used to quantify hepatocytes nuclei size.

Results of investigations indicated the presence of three populations of hepatocytes with small (500-2500); medium (2500-4000); large (4000-6500) nuclei. Simultaneously showed that more hepatocytes with nuclei of medium size and with large nuclei were in group II compared to the group I. Significant, that only single cells from large nuclei populations was found in group I.

In conclusion this could suggest that occurrence of hepatocytes with increased area section of nuclei at early α1ATD phase could be an important marker of liver further unfavorable remodeling. Moreover, image cytometry analyses could be a useful tool for this research.

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Tools: Chemical Probes and Fluorescent Proteins (B266 – B269)

397/B266
Methyl Green: Old Dye with New Applications
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Cell cycle analysis as a flow cytometry application is based on the ability to stain the cellular DNA in a stoichiometric manner. Although PI is the gold standard method it has methodological and biosafety disadvantages. PI stains also RNA and for multicolor analysis the spectral overlap with others fluorophores cannot always be compensated. Methyl green (MG) stain is a simple, inexpensive, safe, method for DNA labeling. MG binds to DNA, but not RNA, in a non-intercalating manner, interacts with the major groove and with preferential binding for AT-rich regions. Also it is suitable for multicolor analysis, for example, when R-PE antibodies labeled are used.

The aim of this work was to validate the use of MG as a DNA binding dye to perform cell cycle analysis.

NMU (ATCC - CRL-1743), CHO-GM-CSF and CHO-GM-CSF-YFP recombinant cells were used which express cytoplasmic yellow fluorescent protein (YFP). For fixation and permeabilization different protocols were used: 70% ethanol at 4°C for 1 h and 0.5% formaldehyde solution in PBS at RT for 1 h and then permeabilized with 70% ethanol or 0.1% Triton X100 in a 2% PBS solution. PI, 7-AAD, TO-PRO-3 and MG were incubated overnight at 22°C in the dark. Cells were treated with RNase I as needed. A CyAn ADP flow cytometer with 488 nm and 633 nm laser was used. YFP, PI, 7-AAD, emissions were collected through a 530/40 BP, 613/20 and a 680/30 BP filter, respectively. MG, and TO-PRO-3 emissions were collected through a 665/20 BP filter. The fluorescence derived from DNA stain was acquired using linear amplification. To exclude cell doublers pulse area versus pulse height were set up and a flow rate of 100 events per second was used. 10,000 counts gated on an FSC versus SSC dot plot, excluding doublers were recorded. Summit v4.3 software was used for data acquisition and FlowJo vX.0.7 for analysis.

MG ability to penetrate ethanol fixed cells and bind the DNA was checked. The lowest assayed concentration produced the best DNA histogram quality. The coefficient of variation (CV) for MG was similar to that obtained for PI and below 5%. MG was assayed in cells expressing YFP and the best condition was 0.5% formaldehde and then 70% ethanol, which yielded DNA histograms of adequate resolution. It was possible to analyze cell cycle distribution in CHO-GM-CSF-YFP cell line with the MG staining, evaluating both markers at the same time, and were comparable to that obtained using the gold standard method.

Studies are on course in order to increase the use of MG in different cells, as well as in more complex. We believe that the MG method for cell cycle analysis can be an economical alternative and suitable for use in those laboratories equipped with standard cytometers and when the color mix is not compatible with the use of PI.

398/B267
Automated High-Throughput Method for Assessing Pathogenic Infectious Dose (TCID50) Using Celigo Imaging Cytometer
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Qualitative assessment of pathogenic infections, as well as the protective effects of candidate therapeutics, often relies on the observation of specific pathologcal changes within the host cell. These phenotypic changes, such as cell rounding, swelling/shrinking, granularity, etc., are known as a Cytotoxic Effect (CPE) and can be visualized via light microscopy. As the magnitude and localization of the CPE may vary considerably, careful examination of replicate samples at various titers is required for reliable, qualitative results. This subjective approach which is specific to the infectious agent as well as the host cell is tedious, time consuming and low throughput, requiring manual well-by-well examination by highly skilled personnel. In this study, the Celigo imaging cytometer has been used to rapidly assess viral infectivity in a range of plate formats. The infectivity level was assessed by characterizing the cell count and cell morphology when induced with different MOI levels. In addition, the viral infectivity at 0, 6, 12.5, 25, and 50 ng of Lentivirus concentration was assessed by using a GFP fluorescence reporter to measure % GFP expression in the cell population. The total number of cells were counted using the Hoechst stain, and GFP of each was assessed to determine the % GFP in the population. The Celigo imaging cytometer uses the f-theta optics, which can generate uniform whole well bright-field and fluorescent images. Image-based analysis can rapidly and objectively quantify output of CPE based on characteristic changes to the host cell monolayer. By directly counting cells in whole well on microplates, the viral infectivity can be quickly quantified for both suspension and adherent cells without trypsinization, which can reduce assay time and improve research efficiency.

399/B268
Single Channel Pharmacokinetic - Pharmacodynamic profiling of Bax Oligomerisation in B-Cell Lymphoma
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**Introduction:** BAM7 has been identified as an apoptosis activator molecule that directly and selectively activates BAX (Gavathiotis et al. 2012 Nature Chemical Biology, 8, 639-645). This previous study has shown that the interaction between BAM7 and BAX induces structural changes that yield functional BAX oligomerization. In order to elaborate on the capacity of this agent to act as a reporter for mitochondrial pore formation in single cells we have developed and implemented an integrated flow cytometry approach. The first aim therefore was to determine the fluorescent spectral characteristics of this molecule; and second to decipher the linked PK-PD response of the B-cell lymphoma cell lines DOHH2 and SU-DHL-4.

**Methods:** Absorption spectra for 0.2 ml samples in 1-cm path length micro quartz silica cuvettes were determined using a Beckman Coulter DU 800 UV/Visible spectrophotometer. Wavelength scans were collected from 200 to 800 nm. Emission spectra for 0.5 ml solution of BAM7 in 1-cm path length semi-micro quartz silica cuvette were determined using a Perkin-Elmer LS50B spectrofluorometer, with a spectral bandwidth of 5 nm. We used one key readout to quantify the cell death response, membrane permeability of DRAQ7. DRAQ7 (also a far-red probe) was detected across the first decade, a mono-PD readout. DRAQ7 (cell death) was detected third decade. We undertook an assay development phase to co-detected in the same channel [logFL3-H; >670L] a BAM7/DRAQ7 signal detection to effectively provide a coPK-PD readout. DRAQ7 (cell death) was detected third decade of the log scale, double peak to indicate DNA content; while BAM7 was detected across the first-second decade, a mono distribution. We were able to optimize the single channel readout to derive the multiple dose-dependent patterns across the whole detection range. We measured these readouts for both cell lines. Using wide-field microscopy BAM7 was located in a highly localized perinuclear region in the cell similar to the typical mitochondrial pattern of these cells. Further co-localisation studies will provide validation of this.

**Results:** We show for the first time that BAM7 is a far-red fluorescent agent, with an emission spectra between 620-700 nm; this provided the capacity to detect the drug in single cells. The fluorescent read out per cell increased in a dose dependent way between 2-50 nM. Treatment of DoHH2 cells with BAM7 led to an early detectable increase in cell death (3% above background) after 4 hrs at 50 nM, maximal levels (50%) were obtained after 48 hours at the same dose. SU-DHL-4 cells showed only a residual cell death response to BAM7. We undertook an assay development phase to co-detected in the same channel [logFL3-H; >670L] a BAM7/DRAQ7 signal detection to effectively provide a coPK-PD readout. DRAQ7 (cell death) was detected third decade of the log scale, double peak to indicate DNA content; while BAM7 was detected across the first-second decade, a mono distribution. We were able to optimize the single channel readout to derive the multiple dose-dependent patterns across the whole detection range. We measured these readouts for both cell lines. Using wide-field microscopy BAM7 was located in a highly localized perinuclear region in the cell similar to the typical mitochondrial pattern of these cells. Further co-localisation studies will provide validation of this.

**Conclusions:** We have demonstrated a unique approach to quantify simultaneously the drug uptake and linked apoptotic studies will provide validation of this.

**Results:** We found that the higher the F/P ratio, the lower the affinity of the antibody to its antigen was, since the F/P of the unbound fluorescent antibody was higher than that of the pre-labeling solution. The magnitude of this effect was even more significant in the case of higher F/P ratios. Increasing the F/P ratio does not lead to a proportional increase in the fluorescence emission of antibodies, therefore if the number of fluorescent dyes on an antibody is high, their quantum efficiency is decreased. In addition, systematic changes were observed in the emission spectra of AlexaFluro647 as a function of the degree of labeling, fluorescence emission spectra were also recorded in order to determine the relative brightness of the antibodies with different degrees of labeling.

**ToxicoLOGY (B270 - B271)**

**401/B270 Flow Cytometric Study of Melphalan-Induced Cytotoxicity in the Bone Marrow of Rats**

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**Background:** Bone marrow (BM) that contains hematopoietic cells of various lineages is a sensitive target for a number of cytotoxic agents including chemotherapy drugs. Therefore, in the present work, this object was chosen to be examined for cytotoxicity of an alkylating chemotherapy drug such as melphalan.

**Methods:** Both intravenous (i.v.) and intraperitoneal (i.p.) routes of melphalan administration were used in the experiment. While one group of adult female rats received melphalan i.v. with a single dose of 3 mg/kg followed by BM examination on the 3rd and 7th day after drug administration, another group of animals received this drug i.p. every other day with single doses of 3 mg/kg followed by BM examination on the next day after 3rd and 5th injection of the drug. BM cells were isolated from femurs, stained with acridine orange and analyzed by flow cytometry. Cytotoxicity was assessed by determining percentages of total nucleated cells (TNC<sub>BM</sub>) of whole BM cell population and percentages of polychromatic erythrocytes (PCE<sub>BM</sub>) of all nucleated erythrocytes.

**Results:** Regardless of the administration dose and regimen, either i.v. or i.p. administered melphalan caused a significant reduction of TNC<sub>BM</sub>. On the average i.p. administered melphalan resulted in about 2.0-fold decrease of TNC<sub>BM</sub> while i.v. administered drug resulted in about 1.3-fold decrease of TNC<sub>BM</sub>. As for erythrocytes, i.p. administered melphalan resulted in about 1.4-fold decrease of PCE<sub>BM</sub> whereas i.v. administered drug did not cause any significant changes in PCE<sub>BM</sub>. Nevertheless, on the 7th day after i.v. drug delivery there was some trend in BM recovery, particularly at the expense of erythropoiesis.

**Conclusions:** Under the present experimental conditions i.p. administered melphalan is considerably more cytotoxic than i.v. administered drug, and this effect is preferentially manifested due to impaired erythropoiesis. However, the i.v. delivery of melphalan of this particular dose (3mg/kg) is likely to be more appropriate in terms of monitoring recovery trends and selecting anti-myelosuppressive strategies.
402/B271
Giving Flow Cytometry and Cell Based Assay Validations Power

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Flow cytometry validations are still in their infancy with regard to having structured analytical method validation guidelines similar to a regulated PK validation. There has been a high level of industry discussion about best practice and the focus of this discussion has been very much aligned to replicate numbers and assessing variability and stability, so mirroring where possible the FDA and EMA guidelines for method validation. Huntington Life Sciences has recently been building upon these discussions and using statistics to power Flow Cytometry and cell based assay methods to the appropriate level to take into account the ‘live’ biological nature of these types of assay. Each assay is broken down into the key parameters and, once identified; statistical analysis is used to ensure the correct number of variables for each parameter has been included.

Method: Statistical power is the ability of a study to detect the effect of interest, if that effect exists. The closer the statistical power to 100% the more powerful the study is. For example, if the study has a power of 80% then a scientist will be able to detect the effect of interest 8 out of 10 times.

A nested design power analysis was used to check whether 3 donors with 3 replicates per donor provide enough data for the detection of a difference of at least 100% deviation from the control mean for the parameters of interest. Proc MIXED (SAS Institute Inc., 2002) was used to obtain variance components for Donor and Replicate nested within the Donor for each pair of Analyst/Analyser combinations.

This analysis was carried out on a Flow Cytometry validation in whole blood from non-human primates. The validation parameters assessed were: Intra and Inter-assay precision; Inter-operator; Inter-animal; and Stability (Fresh & Processed).

Results: A design with three individuals and three replicates per individual appeared to have 100% power to detect 100% deviation from the mean for the counts and proportions of B cells, monocytes, NKT cells, T cells, CD8 T cells, proportions of CD4 T cells and LSUM and TSUM parameters. The design has approximately 85% power to detect 100% deviation from the mean for the counts of NK cells and is slightly underpowered (65% power) for counts of CD4 T cells and proportions of NKT cells.

Conclusion: Percentages of improvement in power from a design with one replicate show the benefit of having three replicates per individual. The design enables a deviation from the mean of less than 100% to be detected and guards against studies which may have increased variability compared to this validation study. This approach can be used to design Flow Cytometry and Cell based assay validations to give them the appropriate level of statistical significance to ensure assay quality.

VACCINES (B272 – B274)

403/B272
Boosted T Cell Immunity to Conserved Epitopes by Sequential Priming and Boosting with Heterologous HIV Immunogens in Rhesus Monkeys

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Background: Genetic diversity among different HIV isolates is one of the major challenges to develop an effective HIV vaccine. It has been considering that boosting the immune response to the conserved epitope might be a promising solution. We have previously shown in mouse that sequential priming and boosting with heterologous HIV immunogens could predominately elicit T cell immune response against the conserved epitopes. Here we tested the concept in nonhuman primate model.

Methods: The humanized fusion genes of gag-env, pol and TRIVN (Fusion gene of Tat, Rev, Integrate-C half, Vif, Nef) derived from Thai B (B’), CB’ recombinant and A/E recombinant were synthesized and inserted into DNA and recombinant Pichia vaccinia vectors as described before. 14 rhesus monkeys were randomly divided into three groups. All monkeys received three inoculations. Monkeys in group 1 were primed with two inoculations of DNA vaccines which expressing immunogens derived from clade CB’ and A/E recombinant respectively with an interval of 4 weeks, and then boosted by rTTV vaccine expressing immunogens derived from clade B’. Monkeys in group 2 were immunized with vaccines derived from A/E recombinant. Group 3 was used as control and received sham vaccines. Peptides of A/E recombinant, consensus B (Con B) and consensus C (Con C) which span the entire immunogens were used as stimul, and Elispot assay and ICS were used to assess the T cell immunity.

Results: Sequential priming and boosting could predominantly stimulate T cell immunity to the epitopes which are conserved not only between clade B, CB’ and AE, but also between them and Con B and Con C. Whereas a single vaccine derived from one clade primarily raised T cells against less conservative or non-conservative epitopes. The conservation was reflected in the consistency of amino acid sequence of the epitope, and the similarity of key processes during antigen presentation, such as similar cleavage site of the proteasome and TAP binding site predicted online.

Conclusion: Sequential priming and boosting with heterologous immunogens was a promising practical strategy to raise immune responses against conserved epitopes. This strategy has important implications for vaccine development against HIV and other pathogens that have high genetic diversity, such as influenza.

404/B273
Development and Optimization of an 18-Color Immunophenotyping Panel and Intracellular Cytokine Staining Assay for the Measurement of T Cell Responses from Rhesus Macaques in Pre-clinical Vaccine Studies

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Background: Experimental vaccines are often tested in nonhuman primates (NHP) for immunogenicity and protection in pre-clinical trials before proceeding to trials in humans. Historically, vaccine-induced T cell responses have been measured by IFN-g ELISpot or intracellular cytokine staining (ICS) for IFN-g, IL-2, and TNF. However, antibody rather than T cell responses correlate with protection for many vaccines. As such, measuring only Th1 responses may not be adequate. Therefore, we designed an 18-color assay to measure Th1, Th2, Th17, and Th17 cells to cover a fuller spectrum of T cell responses. This assay was designed in conjunction with a similar panel for humans to allow for comparison of responses across NHP and humans.

Methods: NHP cross-reactive clones were chosen for all antibodies. Antibodies for subsetting T cells by memory (CD45RA and CCR7) and follicular helper T cell markers (CXCR3, CXC5, PD-1, and ICOS) were selected. For functional responses, antibodies to these cytokines were used: IL-2 and IFNg for Th1 responses; IL-4, IL-5, and IL-13 for Th2 responses; IL-17 for Th17 responses; and IL-21 for Th17 responses. Since expression of Th2 and Th17 cytokines is dim, these antibodies were conjugated to the recently developed bright brilliant blue and brilliant violet dyes. Three conjugates are not commercially available and were produced as custom conjugates. Optimal
stimulation conditions were tested for Th2 and Th1 cells as these cytokines are more difficult to detect than Th1 cytokines.

**Results:** The following stimulation conditions were tested: absence or addition of anti-CD28 and CD49d co-stimulatory antibodies; 6 or 12 hour stimulation; and brefeldin A versus monensin as Golgi apparatus inhibitors. We determined that use of co-stimulation, 6 hour stimulation, and monensin were optimal for the detection of Th2 and Th1 cytokines. While these conditions were less ideal for Th1 cytokines, both the frequency and MFI of these measurements is generally greater and easier to detect, so we chose to optimize the conditions for detection of Th2 and Th1 cytokines.

**Conclusions:** The development and optimization of a preclinical assay to measure cytokines from four T cell subsets at once is unprecedented. The limited number of cells available from NHP vaccine studies makes it necessary to have assays that can measure multiple functions simultaneously. This assay will provide better immune correlate information and thus help inform vaccine design.

**405/B274**

**Induction of Interferon-gamma and Interleukin-17 in T Cells by a Novel Vaccine for Tuberculosis**

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In 2013, nine million people fell ill with tuberculosis (TB) and 1.5 million died from it. Much remains to be done to improve our ability to induce protection from Tuberculosis infection via vaccination. The current standard in TB vaccination, the BCG vaccine, can be effective by preventing at least the most severe cases in children up to 15 years of age; however, after that protection fades and cannot be boosted by revaccination. A new approach to vaccination development that we are pioneering is the production of polyhydroxybutyrate polyester beads (biobeads) in mycobacteria. These mycobacterial biobeads (MBB) can be produced expressing known tuberculosis antigens on their surface but will also display many yet undiscovered antigens which have the potential to induce protective immunity. In this preliminary study, MBB were produced in M. smegmatis. Using flow cytometric analysis of intracellular cytokines interferon gamma (IFN-γ) and interleukin 17 (IL-17) we measured the ex vivo recall response of splenic T cell subsets after MBB vaccination of mice. We also measured activation (CD25) and homing (CD194) receptors on the same cells. We found a strong induction of IFN-γ and IL-17 production from non-classical CD3 T cells (CD4 and CD8 negative) in cells from mice receiving MBB vaccination. This contrasts with the response from the same cell subset from mice that received BCG vaccination, where the cytokine response was low. In the same experiment, parallel groups of mice were challenged with M. bovis. Some protection from infection was observed in the MBB vaccinated group, although not as protective as that observed in the MBB vaccinated group, where the cytokine response was significantly decreasing with increasing age of SHRs. To validate a relationship between the level of CD8+ and CD8+ naïve T cells and ability to respond to foreign antigen, we immunized SHR rats with active vaccine directed against tub protein. Statistical analysis revealed a positive correlation (p = 0.009; r = 0.5577) between the level of CD8+ and CD8+ naïve T cells and level of humoral immune response in the periphery.

In conclusion, we demonstrated that determination of the level of CD8+/CD28+ naïve T cells in the periphery of healthy subjects could be used as a potential biomarker of immunosenescence. Moreover, our results indicate that individuals with physiologically higher levels of CD8+/CD28+ naïve T cells seem to be a better responders to active vaccine in further immunotherapy than the others.

**407/B276**

**Effect of Eosinophils on Wnt-5A and TGF-β, Expression by Airway Smooth Muscle Cells in Asthma**

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**Background:** Airway eosinophilic inflammation and remodeling are main pathological features in asthma. Process of airway remodeling is associated with transforming growth factor β (TGF-β) induced extracellular matrix production by airway smooth muscle (ASM) cells and commonly related with higher Wingless/integrate 1 (Wnt) 5A ligand gene expression (Kumawat et al., 2013). At present, the impact of eosinophils on ASM cells and role of Wnt signaling in asthma remains elusive.

**Aim:** To investigate the effect of eosinophils on Wnt-5A and TGF-β ligands genes expression in ASM cells in asthma patients.

**Methods:** Human airway smooth muscle cell lines immortalized by human telomerase reverse transcriptase (hTERT) were used for the experiments. Eosinophils using magnetic separation were isolated from peripheral blood of eight stable, steroid-free asthmatic patients and seven healthy subjects. For each study subject an individual co-culture was prepared. The expression of Wnt-5A and TGF-β ligands in ASM cells and eosinophils after 24 h co-culture was measured using quantitative real-time PCR.

**Results:** Eosinophils isolated from asthmatic patients significantly increased Wnt-5A ligand gene expression in ASM cells by 11.63 folds (range 0.23 - 603.11, p<0.05) comparing with control (non
co-cultured cells). However, no effect on Wnt-5A expression in ASM cells was detected after co-culture eosinophils isolated from healthy subjects with ASM cells (0.83 folds, range 0.06 - 3.49, p<0.05). Meanwhile, eosinophils of asthmatic patients and healthy subjects upregulated TGF-β, gene expression in ASM cells by 16.70 folds (range 2.16 - 5.31, p<0.05) and 8.64 folds (range 0.98 - 5.63, p<0.05), respectively, comparing with control.

**Conclusion:** Eosinophils and their binding to ASM cells may contribute to airway remodeling by augmented Wnt-5A and TGF-β, genes expression in asthma.

408/B277  
**Purification of Replicating Pancreatic β-Cells for Gene Expression Studies**  
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**Background:** β-cell proliferation is a rare event in adult pancreatic islets, less than 10 β-cells per islet replicate in 24h. To study the replication-related β-cell biology we sought to design a simple and reliable replicating beta cell sorting system with good quality RNA recovery for gene expression experiments.

**Methods:** Replicating cells were identified by EdU incorporation and Newport green in cultured rat islets. β-cell viability and replication of islets exposed to EdU were determined by TUNEL and Newport green in cultured rat islets. Sorting of dispersed islet cells resulted in insulin staining of the resulting sorted populations. Total RNA was isolated from purified cell-sorted populations for gene expression analysis.

**Results:** β-cell viability and replication were not affected by islet exposure to EdU in culture. Sorting of dispersed islet cells resulted in 96.2% purity in the collected β-cell fraction and 100% efficiency of the EdU-based cell separation. RNA yield and integrity as well as gene expression was analyzed to compare fresh and fixed cells after sorting.

**Conclusions:** The method presented herein is efficient, reliable and may allow expression studies of replicating beta cells.

409/B278  
**Correlation of UV-Excited, Blue NADH Autofluorescence with Probes of Plasma Membrane Alterations for Measurement of Apoptosis**  
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**Background:** Excitation of cells with ultraviolet (UV) light and recording their blue autofluorescence has been used to assess the intracellular levels of reduced nicotinamide adenine dinucleotide (NADH). Previous studies have also investigated the relationship between the amount of NADH and mitochondrial function in apoptosis. We sought to apply multi-parameter flow cytometry to the investigation of NADH levels in correlation with apoptosis measurements using probes that detect changes in plasma membrane permeability or asymmetry.

**Methods:** CCRF-CEM and Jurkat cells were cultured under suboptimal conditions. For different experiments, samples were stained with Hoechst 33342 and 7-amino-actinomycin D (7-AAD), with Annexin V and propidium iodide (PI), and with the ratiometric probe F2N12S and PI. Flow cytometric analysis was performed on a BD LSRII analyzer equipped with a UV laser. Blue NADH-associated autofluorescence was collected after a 450/50 nm bandpass filter. For morphologic examination, cells isolated by cell sorting were collected on slides, stained by the Jenner-Giemsa method and observed under a light microscope.

**Results:** Aged cultures of CCRF-CEM and Jurkat cells both showed a distinct subpopulation of cells with low blue autofluorescence with reduced forward and increased side scatter profile indicative of dying or dead cells. Furthermore, the frequencies of cells with intact membranes that excluded PI and had decreased NADH-associated levels of fluorescence were comparable with the number of early apoptotic, 7-AAD+ cells characterized by increased take-up of Hoechst 33342. Similarly, PI− cells with low blue autofluorescence overlapped with Annexin V+ PI− cells and with a PI− cell population with a decreased ratio of orange/green fluorescence exhibited after staining with F2N12S. Detailed multiparametric analysis revealed that on average >90% of Annexin V+ PI− were contained in the PI− cell population with low blue autofluorescence; in contrast, variable, but considerable numbers of cells with low NADH levels showed the Annexin V− PI− phenotype. This pattern was also observed with the ratiometric probe which considered cells ‘live’, when they were classified as early apoptotic by blue autofluorescence. When CCRF-CEM cells that excluded PI were sorted either based on low blue autofluorescence or Annexin V positivity and examined by microscopy, Annexin+ cells displayed more of the advanced morphological features associated with apoptosis compared to the cells with decreased NADH levels, supporting the findings by flow cytometry.

**Conclusions:** Data indicate that under our experimental conditions, measurement of low blue autofluorescence is a useful method of assessing the frequency of cells undergoing apoptosis. Moreover, results show that decreased NADH levels appear to precede phosphatidylserine exposure and alterations in plasma membrane asymmetry, making this method more sensitive for the detection of early apoptotic changes.

410/B279  
**New Application of CyTOF Technology: Quantitative Analysis of Specific Cell Proliferation by Click-it EdU**  
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The CyTOF (Cyttometry Time of Flight) Mass Spectrometry simultaneously resolves multiple parameters and phenotypic complexity of biological system at the single cell level. CyTOF uses element isotope tagged antibodies for protein detection and allows to measure up to 40 parameters per cell overcoming the limitations of emission spectral overlap associated with fluorochromes utilized in conventional flow cytometry. In this study CyTOF technology was applied to study the T cell antigen-specific proliferation based on the ‘Click-it EdU’ assay. In conventional flow cytometry the 5-ethyl-2′-deoxyuridine (EdU) incorporated into DNA during active DNA synthesis is detected by ‘click reaction’ which is a reaction between an alkyne (EdU) and an azide (Alexa-488-azide). In order to reveal Edu-Azide Alexa 488 fluorescent complex by CyTOF, we have tagged an anti-Alexa-488 with an element isotope (156 Gd).

Different experimental conditions were tested for setting up Click-it EdU assay for CyTOF. To investigate antigen-specific T cell response upon immunization Click-it EdU assay was done on murine splenocytes and CyTOF analysis performed. The results showed an increase of the percentage of active DNA synthesis in immunized mice in comparison with the mice that received the adjuvant alone. Mass cytometry was directly compared with fluorescence cytometry and yielded equivalent results confirming our hypothesis that we can measure a T cell specific response using the Click-it EdU assay by CyTOF. These results suggested that CyTOF technology could be used as an alternative approach
to measure the specific antigen T cell proliferation and simultaneously the expression of different markers.

411/B280
Microfluidic Separation of Blood Components Using Novel t-PFF-v Device
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Due to the rising demand of medical diagnostic and therapeutic application study, the biological fluids, including blood components, are needed to be separated each other prior to analysis. Blood is one of the most important biological indicators because of its specific roles such as oxygen carrier, defending and curing systems. However, blood is a complex mixture containing cellular components of erythrocytes, leukocytes and platelets as well as a lot of plasma proteins. The efficient separation of these components may be considered key factor to enhance the precision of analysis in medical research area.

Microfluidics may be alternative solutions for biological mixture separation. The mixture sample can be manipulated at the micro-scale by miniaturized microfluidic device. Pinched flow fractionation (PFF) is well-known size-based separating system proposed by Yamada et al. [1]. Different sized particles are separated according to their sizes by fluid mechanism and steric exclusion within PFF device consisted of two inlets, pinched segment and broadened segment [2]. PFF is appropriate to biological samples in terms of passive system without external forces such as electric field which may induce cellular damage. In conventional PFF, the separating trajectory of non-spherical erythrocytes is affected by their minor axis of 2–3 μm. It is similar to diameter of platelets so that these two blood cells are hardly sorted.

In this study, we demonstrated novel type PFF with tilted sidewall and vertical focusing channels (t-PFF-v) to improve the separation efficiency of blood cells. Due to the tilted sidewall, the difference of effluent positions between erythrocytes and platelets at pinched segment is increased although their minor axis and diameter are similar to each other. Moreover vertical focusing channels can prevent broad dispersion of components induced by tilted sidewall [3]. Therefore the separating resolution of t-PFF-v is enhanced 2.5 times compared with conventional PFF device in spite of same width of pinched segments.

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412/B281
Diurnal Variations in Blood Extracellular Vesicles
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The identification of extracellular vesicles (EVs) as intercellular conveyors of biological information has recently emerged as a novel paradigm in paracrine signaling. However, whether there are diurnal variations in the size, number, and tissue of origin of blood EVs is currently not known, and could have significant implications when using EVs as biomarkers for disease progression. Traditional flow cytometers are capable of measuring particles down to 500 nm, which is significantly larger than the average and median sizes of plasma EVs. Utilizing a Beckman Coulter MoFlo XDP cell sorter with NanoView module, we examined the relative number and scatter distribution of plasma EVs at three different time points during the day in 6 healthy adults. Flow cytometric analysis of liposomes and plasma EVs as small as 100 nm was achieved with this instrument configuration. With this high resolution FSC configuration, we observed significant diurnal variations in the relative size (FSC/SSC scatter distributions) and concentration (proportions) of EVs in healthy adult plasma.

Our results suggest that diurnal variations of biological systems impact the number and size distribution of circulating EV populations. Whether diurnal variations impact protein and nucleic acid composition, and the consequent biological activity, of these particles warrants further investigation. The use of high resolution flow cytometers provides a valuable tool for the study of EVs in both health and disease. Additional refinement of nanoscale flow cytometric methods is needed for use of the instruments for quantitative particle counting and sizing.

413/B282
Comparison of a Techniques and Instrumentation for Flow Cytometric Methods for Microparticle Quantification and Gating
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Introduction: Reliable enumeration of microparticles/microparticles (MVs/MPs) remains a critical methodological challenge in flow cytometry, which is largely viewed as the best suited method for studying MPs. Presently, cytometers must often on MP gating strategies utilizing either or beads fluorescence thresholding with enumeration being accomplished by additional bead-based MP counting methods. Here, we propose a novel strategy using a fixed biological membrane bilayer for both gating and enumeration and compare its accuracy to established gating strategies and enumeration methodologies.

Methods: Samples were analyzed on a BD Aria III flow cytometer with small particle detection module allowing MP enumeration via several strategies. Gating was established via either 1) fluorescently positive MP events, 2) light scatter-based strategies using either polystyrene beads (300-1000 nm) or 3) by excluding debris/instrument noise on the low end and purified fixed bilayer membranes on the upper end. MPs sampled from bronchoalveolar lavage fluid, plasma or cell culture were enumerated within these defined gates based on known concentrations of: i) fluorescent beads, ii) fixed bilayer membranes or iii) actual volume consumed during acquisition (MP/μL).

Results: In comparison to MP/μL assayed (volume determined using the sample weight pre and post analysis and the specific gravity of the analyte) the technique with the greatest agreement was the novel fixed bilayer membrane based strategy, while bead based enumeration frequently under- or overcounted. A comparison of laser power, cytometer type and annexin V + staining was also done to identify which strategies and instrumentation provided the best MP enumeration.

Conclusion: Fixed bilayer membranes as an MP enumeration strategy was effective in all samples tested, often more accurate than alternative strategies and provided both gating and enumeration support. This further allows for a larger effective MP gate which makes fluorescence labels such as CFSE or annexin V
To interpret the SPADE tree, manual gating on the SPADE tree is needed to annotate which parts of the tree correspond to what cell phenotypes. This is much less labor-intensive compared to manual gating on 2D plots, but can still benefit from automated algorithms. We developed a tree partitioning algorithm, which is able to automatically suggest the best partitioning based on variance reduction of marker expressions. We have implemented the algorithm into software with graphical user interface. With this software, users can conveniently verify interpretations of SPADE tree in an interactive and semi-automated fashion.

In conclusion, we have made several improvements on SPADE in terms of its robustness, reproducibility and automated annotation. Together, these improvements will greatly enhance the capability of SPADE for analyzing high-dimensional single-cell data.

416/B285
New Distinct Compartments in the G2 Phase of the Cell Cycle Defined by the Levels of γH2AX

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Induction of DNA double strand breaks leads to phosphorylation and focus-formation of H2AX. However, foci of phosphorylated H2AX (γH2AX) can appear during DNA replication also in the absence of exogenously induced DNA damage. We measured the levels of γH2AX in different phases of the cell cycle by flow cytometry and compared with microscopy analysis for the number of foci of γH2AX in flow cytometry sorted G1, S, G2 and M phase cells in 4 malignant B-lymphocyte cell lines. There were no detectable γH2AX and no γH2AX-foci in G1 cells in exponentially growing cells or after 24 h continuous PARP inhibitor-treatment (PARPi, Olaparib). Olaparib is known to trap PARP1 onto nicked DNA, causing replicative stress, as well as delaying PARP1-associated DNA repair. The amount of γH2AX increased immediately upon S phase entry, and about 10 and 30 γH2AX foci per cell were found in mid S phase control and PARPi-treated cells, respectively. The γH2AX-labeled damage caused by DNA replication was not repaired before entry into G2. Intriguingly, for G2 cells there was a continuous distribution of γH2AX levels, from G2 cells with a high content of γH2AX and the same number of foci as S phase cells (termed 'G2/H' compartment), to cells that were almost negative and had about 2 foci (termed 'G2/L' compartment). Labeling of S phase cells with EdU revealed that G2/H was directly populated from S phase cells, while G2/L was populated from G1/H and (only in control cells) some directly from S phase. The length of G2/H in particular increased after PARPi treatment, compatible with longer time needed for repair. Our results show that cells repair replication-induced damage in G2/H, and enter mitosis after a 2 h delay in G2/L.
commercially available, but many contain practical limitations such as costs of service and are not always specific or customizable to the needs of a fast paced flow cytometry core laboratory.

Methods: With the help of an in house software developer we have built an evolving web-based software tailored to our needs. The user interface serves for instrument scheduling, communication of specific sort-set ups (nozzle or configuration change), sort documentation (yield, purity, dot plots), daily instrument quality control, along with a message board for announcing relevant upcoming seminars and conferences. The Primary Investigator (PI)/administration interface provides tracking of all instrument usage with billing information for lab managers and PIs, allocation of grant activities to users and labs, etc. From a core perspective, the software provides a framework and tools to conveniently archive and access for example daily/monthly maintenance documentation, quality controls, trouble shooting Q&As, instrument and service contract information. Changes in instrumentation such as filter configuration or laser upgrade can be conveniently updated. The software is accessible from any electronic instrument with internet capacity.

Results and Conclusions: Over the past 3 years we have been able to streamline and optimize our daily operations within the core, using software tools to address challenges of more and more complex and growing user and administrative needs without additional resources to the core. Our interface has evolved over the years and continues to do so primarily due to the flexibility and availability of our in-house software developer. Finally, this approach allowed us to implement tools to improve our quality performance and engage users and staff alike in advancing flow cytometry at our research institution.

418/B287
Violet SSC: An Alternative to FSC PMT of Fluorescence in the Detection of Extracellular Vesicles

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Over the past decade, there has been a rapid growth in studies of secreted membrane vesicles, collectively called extracellular vesicles (EVs). (1) The release of EVs has been reported in the pathologies of cancer (2-5), neurological, hematological (6), cardiovascular (7), autoimmune and rheumatological diseases (8), and viral infections such as malaria (9). The study of EVs is gaining increasing interest within both the medical and scientific communities due to the diagnostic and therapeutic possibilities. However, the identification and classification of EVs has been problematic. Although advances in various fields, including microscopy, have addressed some of the preliminary hindrances, flow cytometry remains the dominant approach for the characterization of submicron cell-derived particles. The primary hurdle in analyzing particles at the submicron level has been to accurately represent their size distribution and light scatter profiles. Instrumentation thresholds were originally designed using whole blood as the standard, thereby excluding cellular profiles. Instrumentation thresholds were originally designed to distinguish populations spanning the <400nm to 1um range.

In this independent study, several of those technologies are evaluated and compared. As most of the hardware adjustments are accomplished by enhancements to the FSC parameter, the study will also evaluate the use of Violet SSC on Beckman Coulter’s Cytoclix as a novel approach to small particle detection. It is theorized that Violet SSC will give comparable results, as the lower wavelength will allow for detection of smaller particles.

The interest in the identification and detection of submicron particles has increased in recent years. The ability to study them has been hindered by available techniques to measure particles at sizes below 1um. Flow Cytometry has become an important tool in EV research with instrumentation being developed to identify particles at the submicron level. Instrumentation such as cytometers optimized to improve light scattering collection (10, 11) and image cytometers (12). However, most equipment designed for the detection of EVs is expensive and complex. Hardware enhancements have focused around the development of the FSC PMT. While the FSC PMT enhancements have proven to enable the flow cytometer to detect particles <200nm in size, the instrumentation is not practical for all lab settings. In this comparison study, it has been shown that the Cytoflex VSSC is compatible to 488nm SSC and to the results obtained from the AstrostarQ and NanoView enhanced FSC. The ability to resolve and distinguish the populations as effectively as its counterparts, has proven the Cytoflex Violet SSC to be a viable alternative to the FSC PMT to detect EVs.

419/B288
A System Overview of Process Workflow and Analysis for 13-Color on the CytoFLEX with Plate Loader Research Flow Cytometer

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Background: The CytoFLEX Flow Cytometer with Plate Loader option is the newest cytometer offered by Beckman Coulter Life Sciences that provides up to 3-laser, 13-colors for detection in a compact system. This instrument incorporates the latest fiber array photodiode (APD) technology to provide high resolution, dynamic range, and sensitivity in signal detection. We present here a 14-marker, 13-color study to demonstrate the CytoFLEX with Plate Loader option functionality and workflow as well as its multi-color detection capability.

Methods: A workflow was designed for a typical multi-color study (13-color) including Daily Startup, QC, Instrument Settings and Single Color Compensation, Multi-Color Samples and Daily Clean. The CytExpert software offers flexible sample management to accommodate all processes noted above, thereby maximizing plate usage. To identify the TBNK populations, samples were prepared from normal whole blood stained with single color reagents and lysed with Versalyse Lysing Solution. Data were collected on the CytoFLEX with Plate Loader option and analyzed using CytExpert software.

Results: The results are presented here with special emphasis on optimizing on plate loader workflow. We show that all TBNK populations are well resolved, notably the CD25+ subset in CDA- T Cells. The CytoFLEX with Plate Loader option delivers sound detection and resolution for even the most dim versus negative population while providing users the flexibility to design a streamlined workflow.

420/B289
Sorting and Maintaining Haploid Stem Cells Using a Beckman Coulter MoFlo Legacy and a Bio-Rad S3 by Light Scatter

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Maintenance of viable mammalian haploid stem cell lines constitute a difficult challenge. Sorts using traditional DNA dyes to identify the haploid population result in heavy losses in post-sort cell cultures. It was reported that attrition could be as much as 70%. This is possibly due to the cytotoxic effects of standard supra-vital DNA dyes, in particular the vital stain Hoechst 33342 and others (1,2). The time taken in preparing the cells for sorting and the sorting itself may also induce cell damage leading to poor
viability. Typically sorting live cells with Hoechst 33342 required incubation times between 20-90 minutes (3,4). Also the near UV laser illumination of the cells raises concerns about of viability after sorting. We assumed that if we can minimize the time the cells are out of culture and their exposure to potential mutagenic conditions, we could increase their survival post sorting. Haploid cells can be acquired by carefully adjusting the Forward Scatter (FSC) and Side Scatter (SSC) collection optics on a Beckman Coulter MoFlo and with precise gating, an enriched population for G0/G1 cells can be found without staining (5,6). We applied this technique to the haploid sort that significantly reduced the total processing time -incubator to incubator cycle. There was a rise in the survival rates of the cells post sort using this method to near 100% when compared to plating without sorting.

The Bio-Rad S3 sorter in the flow cytometry facility has two collinear lasers, a 488nm and a 561nm. Initial tests on the system to confirm the FSC-SSC haploid sort as performed on the MoFlo were successful. The basic elements of the assay were very simple to port.

It has been shown that scattering intensity at angle is dependent on wavelength (7). The co-linearity of the S3’s laser configuration means that there is only one laser intersection point on the jet. This configuration is ideal in constructing a second side scatter but from the yellow laser. By implementing this, one of the fluorescence channels must be converted to this function in this case, FL4. The extra SSC detector improved the resolution of the Haploid sort over the MoFlo at no cost to the cells survival post sort, and was very easy to implement.

5) The European Cytometry Network: http://www.eurocyt.net/page-sorting-out-g0g1-cells-without

421/B290 Using a Spectra Physics Tsunami Infrared Pulsed Laser and Second Harmonic Generation on a Beckman Coulter MoFlo Legacy Sorter: A Tunable Light Source

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For maximal excitation of fluorochromes used in flow cytometry, it would be advantageous if we could tune the laser to the absorption maxima of the fluorochrome. This would improve the probability of an absorption/emission event and therefore improve overall resolution of the assay as more signal would be generated.

Standard diode or gas lasers found on most flow cytometers have discreet lasing transitions and do not provide a continuum of excitation wavelengths. They are typically set up near to the excitation maxima of the fluorochromes they excite. However, most fluorochrome absorption maxima is not at the wavelengths emitted by these lasers (1). Gas lasers such as the argon ion laser have limited tuning, but less than a dozen of the discrete laser lines are useful in flow cytometry (2). The diode laser systems, for example, the 323nm DPSS system uses high-powered diodes to pump a lasing material such as Neodymium doped Yttrium Aluminum Garnet (Nd:YAG) to produce laser light at 1064nm. The laser light is then frequency doubled in a nonlinear crystal such as a KTP crystal. The high energy from the diodes induces Second Harmonic Generation (SHG) in the KTP crystal that results in green 532nm laser light (3). Other wavelengths are possible, but the laser lines are discrete and cannot be tuned. There have been reports of tunable low power super continuum white light lasers used on cuvette based flow cytometer systems (4,5,6). But generally they are not useful on jet-in-air sorters because particle excitation time of flight is lower -about 1-2usec-7, and the light collection optics are not as efficient as the cuvette based flow cytometers.

Here I demonstrate an alternative method to produce a tunable laser system that can be used in jet-in-air flow cytometer sorters. A Spectra Physics Tsunami laser was configured in femto second mode and tuned to twice the desired wavelength. It was then used to pump a Beta Barium Borate (BBO) crystal for SHG. The SHG light was collimated and directed into a Beckman Coulter MoFlo sorter to be used as a light source. The wavelength produced by SHG in this system spans 350nm to 540nm. The broadband optics of the Tsunami limit the power output of the SHG above 530nm to about 100mW or so in theory. However, using long wavelength mirrors on the Tsunami should improve the SHG power above 530nm. Because the power output of the SHG system is so much greater than the supercontinuum white light lasers, it is therefore possible to use it in jet-in-air flow cytometry sorters experiments.

1) http://www.spectra.arizona.edu/
2) https://www.coherent.com/Products/355/Innova-9
3) http://www.repairfaq.org/sam/lasercds.htm

422/B291 Simple Method for Characterizing Cytometer Noise

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One of the most important parameters of how well a cytometer is functioning is its noise level. Cytometer noise has both an electronic and optical contribution, which together can decrease a cytometer’s low-end resolution capabilities.

This method uses commonly available blank beads and existing cytometer hardware to characterize and assist in trouble-shooting problem areas. Using the four steps of checking electronic, PMT, ambient, and blank bead noise, we qualify noise contribution in a cumulative way.

We will present case studies of cytometers with noise problems, how they were characterized, and how this characterization aided in resolving the cause of the high noise. This method can be easily implemented as part of a laboratory’s routine quality assurance efforts.

423/B292 Reducing Background in MHC Tetramer Assays: Characterization of a New Commercially Available MHC Tetramer for the Violet Laser - Brilliant!

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Results: Using SPADE we reproducibly identified canonical cell subsets, as well as identified potential visual outliers to exclude from further analysis. In addition, we generated heatmap visualizations of these cell subsets to visually evaluate this consistency and identify any unusual samples. Secondary identification of these populations using viSNE allowed for consistent identification of larger cell subsets which could then be gated and statistically compared across samples. We observe that although the cell counts vary for each subset, the ratio of cell events in each subset to total lymphocytes are consistent with frequencies in human peripheral blood, and few outliers exist across cell subset event counts.

Conclusion: We show that automated methods can be leveraged to reliably identify canonical cell subsets. These methods can be used both qualitatively, as in the visualization of SPADE and viSNE plots for patient-specific anomalies, or quantitatively, as is the generation of statistical differences between samples.

425/B294
Effect of IL10, IFNy, and IL4 Receptor Knockouts on Macrophage Response to Stimulation In Vivo and to Peripheral Nerve Injury In Vivo
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Emerging evidence suggests that driving macrophages toward an alternatively activated phenotype improves axon regeneration following nerve injury. We hypothesized that mice with cytokine receptor deletions would have alterations in macrophage responses to stimulation in vitro and in vivo that would make them useful for studying the effect of macrophage phenotype on nerve regeneration.

Bone marrow derived macrophages were generated from juvenile IL10-R−/−, IFNγ-R−/−, IL4-R−/−, and wild-type mice (n=5 each). Cells were stimulated with IFNy and LPS to generate classically activated, or IL4 to generate alternatively activated macrophages. Cells were labeled with CD11b-Pacific Blue and CD16/32-Pacific Blue for macrophages, and Nos2 and Arg1 as markers of phenotype. Adult mice of the same strains (n=3 each) underwent sciatic nerve transection and repair with a 3mm gap. The regenerative bridge was harvested and labeled as above 10 days after injury.

IFNy-R−/− reduced and IL10-R−/− marginally increased Nos2 response to classical stimulation compared to wild type (30%, 82%, 77% respectively, P<0.02). IL4-R−/− blunted the Arg1 response to alternative stimulation compared to wild-type (10% vs. 25%, P=0.008). No significant differences were detected in Arg1 or Nos2 expression between strains after nerve repair.

The in vitro data suggests these strains could be useful for future investigation of the effect of macrophage phenotype on peripheral nerve repair. The lack of effect in vivo could be due to small numbers in this pilot project or because mice have short duration of classical activation, which could have been missed at the 10 day post-repair time point.

426/B295
Live/Dead Bacteria Discrimination by Styrly Fluorescence Staining and Spectral Intensity Ratio Analysis
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The present work provides a method for quantitatively validating the efficiency of different disinfection procedures that are abundant in the food and water industries such as pasteurizing, chlorinating, UV, Ozone, etc. by utilizing spectral intensity ratio
analysis. This was performed by fluorescence spectroscopy, FACS and microscopic analysis. The method described here based upon staining the bacteria with membrane dyes such as the Styryl dye (FM 1-43 / FM 4-64), where the outcome fluorescence of live bacteria vs inactivated bacteria is weaker and blue shifted. Such phenomena might be the results of the interaction of the dyes in the lipophilic membrane environment in the live cells, versus the inactivated cells where the dyes are inserted to the more hydrophilic environment of the cytoplasm. As these dyes contain major hydrophobic region in their chemical structure, it is reasonable to assume the dependency of their spectral emission on the hydrophobicity / hydrophilic characterization of the surrounding. As demonstrated in this work, the spectral difference between the viable to the inactivated bacteria is evident in both in the maximum emission peak and emission intensity, where live bacteria exhibit "blue" shifted spectra and lower intensity, compared to inactivated / dead bacteria. The new discovered phenomena has been observed by fluorescence spectroscopy, flow-cytometry and fluorescence microscopy. As quantitative parameter the ratio of two distinct wavelength in the fluorescence spectrum intensity ratio was measured to evaluate the Spectral Intensity Ratio – SIR. The ratio between SIR of a bacterial sample to a known viable bacteria population raise the Non Viability Parameter – NVP. This phenomena had been demonstrated and quantitated for both Gram negative (E.coli, Ps. aeruginosa, E.colaceae and more) and Gram positive (E.faecalis, E.faecium….) bacteria in several inactivation methods as pasteurization, chlorine, UV-medium pressure and alcohol treatments.

We believe this could be key component in the development of new fast tool to detect viable bacteria in samples by microscopic or other fluorescent tools.

Reference:
Zahavy, E. Spectral Intensity Ratio (SIR) Analysis For Rapid Live Microbial Enumeration. Provisional patent application #62102506, Jan 2015

427/B296 Optimisation of Polychromatic Flow Cytometry Panels for T Cell Analysis in a Clinical Research Project
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Polychromatic immunophenotyping using more than 10 colours has become standard practice in many research laboratories. The optimisation of these panels requires careful planning and testing to maximise the quality of the data obtained. This is especially important when using precious clinical samples. The research focus of our group is to investigate any potential relationship between T cell subsets and clinical outcome in rectal cancer patients.

The aim of this project was to optimise two polychromatic flow cytometry panels (12 and 13 colours), to analyse T cell subsets including CD4+ helper (T_h), CD8+ cytotoxic (T_c) regulatory T cells (T_r) and T helper 17 cells (T_h17) in the peripheral blood of rectal cancer patients over multiple time points during treatment. Both panels include both extracellular and intracellular antigens, and care was taken in the panel design to consider antigen density, co-expression and spectral overlap. Cells were stained sequentially with a fixable viability dye, followed by extracellular antibodies, permeabilisation and intracellular antibodies. Optimisation experiments were carried out on cryopreserved human PBMCs isolated using a Ficoll–Paque gradient and analysed with a BD LSR Fortessa (SRP).

Instrument settings for the BD LSR Fortessa were optimised to maximise signal to noise ratios and limit spectral overlap. All antibodies included in the panels were titrated and in some cases additional optimisation steps were required to improve staining. Minor adjustments to the fluorphores were required to improve panel functionality and resolution of dim populations. Finally, the complete panels were tested for reproducibility both intra- and inter-experiment, and relevant controls were selected. Both flow cytometry panels and instrument settings are now optimised for analysis of clinical research samples and are now being used to analyse populations of T_h, T_c, T_r and T_h17 subsets and to assess their activation status.

428/B297 Demonstration of a 7-Color TBNK Application with Viability Dyes Using Beckman Coulter CytoFLEX Flow Cytometer
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Identification of T cells, B cells, and Natural Killer (NK) cells is a common immunophenotyping method in flow cytometry analysis. Necrotic, apoptotic, and/or damaged cells can often interfere with data interpretation. These cells can be identified and excluded from viable cells through the use of viability dyes. Damaged and permeable cellular membranes permit the intercalation of impermanent viability dyes into nuclear DNA. Beckman Coulter currently offers three impermanent viability dyes which are excited by three different lasers: 7-AAD (excited by the blue laser), DRAQ7® (excited by the red laser), and DAPI (excited by the violet laser).

To demonstrate the utility of these viability dyes for flow analysis, we designed an 8-marker 7-color panel leaving channels available for the drop-in of each viability dye. The panel contained the following antibodies: HLA-DR FITC, CD16 PE, CD56 PE, CD19 ECD, CD4 PC7, CD8 APC, CD3 APC-Alexa Fluor® 750, and CD45 Krome Orange®. Whole blood specimens from 3 normal donors were stained with the 7-color combination 24-hours post-venipuncture and then lysed using Versalysis®. Compensation was performed by single color staining. The viability dye was added following lysis. The flow analysis was performed on a CytoFLEX instrument, an ultra-flexible, high performance flow cytometer that offers detection of up to 3 lasers (488nm, 638nm, and 405nm) and 13-colors with high sensitivity and resolution. CytExpert software was used to analyze the collected data.

All three viability dyes identified non-viable cells and allowed live/dead discrimination. The results showed that a similar percentage of non-viable cells were detected using any of the viability dyes, and some of the non-viable cells showed non-specific binding to antibody conjugates. Data was also analyzed to assess percent positive and Mean Fluorescent Intensity (MFI) of specific lymphocytes populations (CD3+ T-cells, CD8+ T-cells, CD4+ T-cells, NK cells, and B-cells). All parameters recovery and MFI were comparable after excluding non-viable cells, regardless of the viability dye used. There was no impact on MFI of gated populations by the use of a viability dye. In conclusion, 7-AAD, DRAQ7® or DAPI viability dyes can be used interchangeably to identify and exclude non-viable cells on the CytoFLEX instrument yielding an accurate analysis of viable leukocyte populations.
This presentation will discuss the commercial development of metal-tagged label oligonucleotide probes by Fluidigm Corp. for multiplex, quantitative detection of RNA and proteins in single-cells combining Mass Cytometry and the RNAseqce® in situ hybridization technology pioneered by Advanced Cell Diagnostics. This novel assay consists of the sequential hybridization of RNA-specific target probes, Target Probe-specific PreAmplifiers, PreAmplifier-specific Amplifiers, and Amplifier-specific Label Probes, which are tagged with isotopically pure metal atoms for mass cytometry detection. The proprietary probe design strategy of RNAseqce® allows detection of single RNA molecules by significantly amplifying target-specific signals while suppressing background within individual cells. Antibody staining for the protein targets in the same sample can be performed before and after RNA staining.

The work of Van Hoof et al. described RNA and protein analysis based on flow cytometry. We have demonstrated that Mass Cytometry can also be utilized for both RNA and protein analysis. As part of this process a number of factors were optimized and issues solved. Basic protocols were formalized for the production of the metal-labeled detection oligos. The metal-oligo in situ hybridization (MISH) protocol and the simultaneous staining of biomarkers with Maxpar® Antibodies were optimized for ease of use. Applications will be discussed that enable researchers to profile a targeted number of genes and proteins (up to 40) across millions of single cells to monitor gene and protein expression profiles and their regulations. Co-detection of RNA/protein in individual cells within distinct populations of PBMc is a significant enablement compared to alternative methods in the market today.


430/B299
Flow Cytometry and Single Cell Genomics
Bootcamp: A Case Study at UC Davis
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Background: Fluorescence activated cell sorting is the gold-standard method used to isolate and identify single target cells for subsequent functional or genetic analysis. Recent advances in microscale nucleic acid amplification chemistries now make it possible to amplify DNA and RNA from single cells with excellent reproducibility and fidelity. These new reagents allow researchers to investigate the genomic and transcriptional heterogeneity within a population of cells to determine, for instance, the specific characteristics of stem cells, to evaluate genomic changes in tumor cells in response to treatment in mouse PDX models, and to characterize tumor-specific immune cells.

Method: To meet these evolving research needs, the flow cytometry core at UC Davis developed methods to facilitate the successful execution of single cell studies. The isolation and preparation of high-quality cDNA is a necessary but technically challenging step for many single cell studies. A new instrument, the Fluidigm C1 system, is designed to capture and lyse up to 96 individual cells for cDNA amplification using microscale fluidic channels and chambers within a chip. The C1 system simplifies single-cell cDNA preparation, however protocols to perform total genomic or transcriptional studies using cDNA isolated from single cells that derived from liquid or solid tissues were developed and tested to ensure success.

Result: To this end, the UC Davis FC Core planned and executed a ‘boot camp’ designed to lead select investigators through the entire process of cell sorting, collection, loading the C1 chip, harvesting cDNA which was then transferred to specialists for library preparation and sequencing, all at a highly subsidized, affordable cost.

Conclusions: The boot camp provided a unique opportunity for investigators to gain technical expertise in single cell genomics and high content data analysis. Important lessons learned including methods to improve C1 chip loading efficiency and alternative approaches such as index sorting followed by manual cDNA scale up are discussed along with future plans for additional workshops.

431/B300
Purification of Macrophages and Schwann Cells from Injured Murine Peripheral Nerve for Gene Expression Analysis
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Current techniques to treat peripheral nerve injury produce moderate results that seldom reach full functional recovery. Driving macrophages toward an alternatively activated phenotype improves axon regeneration, although little is known about how this effect is mediated. We used flow cytometry to isolate macrophages and Schwann cells (SC) from repaired peripheral nerves to elucidate the mechanism by which macrophage phenotype affects early events after nerve injury. Adult C57BL/6 mice underwent sciatic nerve transection and repair with a 5mm silicone conduit to create a 3mm gap. The regenerative bridge within the conduit was harvested at days 3, 5, 9, 12, 22, and 26 after surgery. Cells were labeled with anti-mouse antibodies CD11b-Pacific Blue and CD16/32-BV605 for macrophages, p75NTR-FITC for non-myelinating SC (NMSC) and GFAP-PE for myelinating SC (MSC). Macrophages (CD11b^+CD16/32^+ and SC (CD11b^+CD16/32^+ and p75NTR^+ and/or GFAP^+ were sorted on a BD FACS Aria. Sort purity was evaluated using RT-PCR.

Macrophages and SC were successfully sorted from the regenerative bridge over the early phase of nerve repair. The ratio of p75NTR^+GFAP^- SC decreased over time (P<0.003). This temporal relationship is consistent with de-differentiation of MSC to NMSC during early Wallerian degeneration, and then the later return of a MSC phenotype. Total RNA isolated from sorted cells had adequate quality for Nanostring mRNA analysis of a panel of >90 genes relevant to the interaction between macrophages, SC, and regenerating axons. This approach will be used to investigate the mechanism by which altering macrophage phenotype affects peripheral nerve regeneration.

432/B301
Analysis of Intracellular Signaling in Different T-Cell Subpopulations Using CytoFLEX and Perfix EXPOSE
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Introduction: Intracellular signaling cascades comprise complex series of alterations in protein interactions, activation levels, modifications, multimerizations, folding, expression levels and subcellular localizations. Combining the analysis of intracellular signaling with complex cellular systems (e.g., whole blood) presents additional complexity, with the necessity of first parsing out the populations of interest prior to allowing further signaling analyses. Minimizing this complexity is aided largely by the performance of both reagents and the instrument used for data analysis.
acquisition. In this set of experiments, we have used the new 13-color CytoFLEX flow cytometer to enable robust and high quality detection of TCR signaling in different T-cell subsets, together with PerFix EXPOSE, a fixation/permeabilization kit that enables the efficient staining of even the most difficult-to-detect intracellular signaling molecules.

Methods: Normal whole-blood samples were collected on site daily. The samples were stimulated with CD3 and/or CD28 antibodies combined with a crosslinking antibody. The stimulation was stopped by the addition of formaldehyde, and the samples were further processed using PerFix EXPOSE (Beckman Coulter). The antibodies used for subset immunophenotyping included: CD3, CD4, CD8, CD56, y6 TCR, and a variety of activation and homing markers (e.g., CD25, FoxP3, CCR4, etc.); while the antibodies used for signaling responses included: pERK T202/Y204, pCREB S133, pAKT S473, pRelA S536, IκBα, pSTAT3 Y705 and/or pSTAT5 Y694. All samples were acquired using a CytoFLEX flow cytometer (Beckman Coulter), and the data were analyzed using CytExpert or Kaluza software (Beckman Coulter).

Results/Conclusions: The CytoFLEX flow cytometer was found to perform well for the analysis of multicolor panels, while PerFix EXPOSE was found to enable the efficient staining and analyses of all of the markers used in this study, including pSTAT3 and pSTAT5. As a result, multiple T-cell subset populations were found to have different optimal stimulation concentrations, as well as different signaling responses to such stimulation. Notably, even general CD4 and CD8 cells had dramatically different responses to CD3 and/or CD28 stimulation. These results suggest that consideration should be directed toward optimization of stimulation parameters for each specific T-cell subpopulation when analyzing and comparing signaling responses between the different subsets.

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433/B302
Genomic Alterations in Invasive Melanoma Cell Lines
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Malignant melanoma is one of the most aggressive human cancers with a highly metastatic potential and resistance to current therapeutic treatments. Invasion of melanoma cells is the first step in metastasis, resulting in increased cell migration through tissue compartments.

In this study, we aimed to evaluate genomic alterations specifically associated with the invasive characteristics of melanoma cells. Array comparative genomic hybridisation (aCGH) analyses were performed on 19 human melanoma cell lines originated from primary and metastatic melanomas. Matrigel invasion chambers were used to define the invasive properties of cells. Cerebral recurrent components (CNAs) were found that affected cancer-related genes, such as CNTNAP2, IGF1, LRRC28, SYN1, MAP2K3 and FAM27L. Invasive cell lines displayed high frequencies of CNAs, including losses of a high number of genes (PTPN12, ADAM22, FZD1, TFF2, GNG11, COL1A2, SMURF1, VGF and RELN). Gain of the GLP1R gene occurred significantly more frequently in invasive cell lines than in non-invasive ones, confirming the key role of the gene during melanoma progression. Deletion of the RBFOX1 gene was found in both invasive and metastatic cell lines, suggesting a functional role of this gene in melanomagenesis. DNA samples of paired cell lines (invasive and the original cell line) were hybridised to Affymetrix CytoScan 750K array (high-resolution coverage of cancer and constitutional genes of interest) in order to define the molecular alterations related to metastasis and to determine similarities as well as differences of the corresponding tumour pairs. Significantly altered regions were observed on 3q13.2, 8p23, 10q11, 15q26, 17p11.2, similarly to the results of the current study using CytoChip ISCA array. Furthermore, loss of RBFOX1 gene was detected in 3/4 of metastatic cell lines. Furthermore, in invasive cell lines carrying the BRAF V600E mutation, gains of the NEDD9 and KREB1 genes were frequently observed. The present study describes remarkable genomic differences between invasive and non-invasive melanoma cell lines that may contribute to the aggressive phenotype of human melanoma cells. Our genomic analysis of human melanoma cell lines and the classification of CN alterations associated with melanoma invasiveness thus provide novel candidate genes for further functional studies.

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Optimisation of Cell Counting Using the MACS Quant Flow Cytometer
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Introduction: The development of immune-mediated therapies for cancer requires investigation of the changes in the immune response in order to examine the mechanism of action of the candidate drug, develop predictive hypotheses to help understand which patient population to treat and develop rationales for combination therapies. This has led to the use of new flow cytometric staining panels on several tissues from tumour-bearing mice after treatment, including blood, spleen, tumour-draining lymph node and tumour. Optimised flow cytometric staining requires the use of a fixed number of cells per stain, so the cell count of each sample must be determined. This is a rate-determining step in the experiment since often up to 200 samples must be counted. In order to rapidly process all of these fresh samples we sought to optimise and validate an automated cell counting strategy using the MACS Quant Analyser 10 flow cytometer, which is equipped with a high precision syringe and a 96 well plate reader.

Methods: We compared the live cell counts obtained using the MACS Quant flow cytometer to those obtained with the previous method of haemocytometer enumeration. We also assessed the reproducibility of the counts using triplicate repeats, the range that can accurately be counted using several dilutions of the splenocytes and the similarity to haemocytometer counts of the MACS Quant using 3 different flow rates, 3 different sample uptake volumes and 3 different washing modes to determine the fastest method that still gave acceptable accuracy of counting. We have also used the accurate counting capabilities of this machine to investigate the quality of several types of cell counting beads for absolute cell number determination on the BD Fortessa flow cytometer.

Results: We determined that the cell counts at a low or medium flow rate were very similar to haemocytometer counts for splenocytes and even using only a 5μl sample uptake volume were very reproducible – with a mean coefficient of variation under 10% compared to 14% with the haemocytometer. The data also shows low carryover in the fast wash mode and the surprising accuracy of the 5μl samples allowed us to optimise the cell counting protocol suggested by Miltenyi Biotech (25μl sample uptake volume on low flow rate with a standard wash) to a 5μl sample uptake volume on the low flow rate with a fast wash cycle – reducing acquisition times from 1 minute to 40 seconds to merely 42 seconds per sample as well as reducing cell loss during the counting before flow cytometric staining and analysis.
Conclusions: We have optimised a cell counting method using the MACS Quant flow cytometer that yields rapid and reproducible cell counts while reducing the sample volume required and the need for counting beads. This has greatly simplified our endpoint sample processing and eliminated the need for labor-intensive manual counting, thus increasing the numbers of samples that can be processed whilst requiring fewer scientists.
The number following each name is the abstract Program Number.

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## CYTO 2015 PRELIMINARY PROGRAM AT A GLANCE - As of May 19, 2015

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<th>Saturday, June 27</th>
<th>Sunday, June 28</th>
<th>Monday, June 29</th>
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<tr>
<td><strong>Exhibitor Move-in/Setup</strong></td>
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<td>Robert Hooke Lecture</td>
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<td>2014 Nobel Prize Winner</td>
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*Ticket required for admittance.
SAVE THE DATE!

31st Congress of the International Society for Advancement of Cytometry

June 11 – 15, 2016
Washington State Convention Center
Seattle, Washington, USA