Current Trends in Flow Cytometry

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Department of Anatomy and Department of Medicine

University of Hong Kong
• The power of flow cytometry:
  – To measure independent properties on individual cell.
  – To use quantitative measurements to classify and isolate cells of interest.
Flow Cytometry

• Early 1970s – To perform analysis and sorting of viable cells.

• 1980s - Identification of leukocyte cell subsets based on unique cell surface markers by staining with fluorochrome-conjugated antibodies. –Immunophenotyping and cell cycle analysis.

• Nowadays – Multiparameter flow cytometry allows detection of intracellular functional markers (e.g. cytokines) and cell-signaling molecules and more...
Applications in Flow Cytometry

• Multicolor Flow Cytometry; up to 18 independent color parameters
  – Many New Dyes/Fluorochromes
• Multiplex bead-based assays
• Stem cells and Side Population Cells
• Cell Tracking/Labeling/Sorting
• Cell Signaling Analysis
• Imaging flow cytometry and more...
Multicolor Flow Cytometry; up to 18 independent color parameters

**BD Aria SORP cell sorter is equipped with:**
- Blue laser (488nm)
- Yellow-green laser (561nm)
- Red laser (633nm)
- Violet laser (405nm)
- UV laser (355nm)

**BD FACSAria I Cell Sorter**
1. BD FACSAria I cell sorter:
   - 488nm blue laser:
     i. FITC (530/30nm)
     ii. PerCP-Cy5.5/P1 (695/40nm)
   - 640nm red laser:
     i. APC (660/20nm)
     ii. APC-Cy7 (780/60nm)
   - 561nm yellow-green laser:
     i. PE (575/28nm)
     ii. PE-Texas Red/mCherry (610/20nm)
     iii. PE-Cy5 (660/20nm)
     iv. PE-Cy7 (780/60nm).
   - 405nm violet laser:
     i. Pacific Blue/Horizon V450 (450/50nm)
     ii. Pacific Orange/Horizon V500 (510/50nm)
   - 355 nm UV laser:
     i. Hoechst blue (450/20nm)
     ii. Hoechst red (670LP)
Faculty Core LB6-11

Email: corefac@hku.hk

Charges

For Cell Sorter (minimum booking: 0.5 hr):
Office hours (per hour of usage including set-up and clean-up without technical support)
Aria I: $100
Aria SORP: $130

Non-office hours (per hour of usage including set-up and clean-up without technical support)
Aria I: $80
Aria SORP: $100

For Analyzer:
$40 per hour

* Same charges will applied for over-run usage

| Technical Support | $100/hr (minimum half hour; pro-rata charge will apply after the 1st half hour) |
Multicolor Flow Cytometry - Fluorochromes/Dyes

BD Horizon V450 (Em- Max448 nm -Violet)
Pacific Blue (Em- Max452 nm -Violet)

BD Horizon V500 (Em-Max 500 nm –Violet)
Pacific Orange (Em- Max452 nm -Violet)
Alexa Fluor 488 (Em- Max 519 nm –Blue)
FITC (Em- Max520 nm -Blue)
PE (Em- Max578 nm –Blue/Yellow-Green)
PE-Texas Red (Em- Max 615 nm –Blue/Yellow-Green)

Alexa Fluor Dyes – highly photostable
FITC – sensitive to pH and photobleaching
PE-Cy5 – sensitive to photobleaching
APC-Cy7 – sensitive to formaldehyde
PE-Cy7 – extremely sensitive to light

Quantum dots (UV/violet) –
By Invitrogen/Molecular Probe
Flexible; Stable; Reproducible; Minimal compensation
Violet laser Reagents – cell cycle; apoptosis

Multicolor fluorescent protein – tracking cells

APC (Em- Max 660 nm –Red) Alexa Fluor 647 (Em- Max 668 nm –Red)
PE-Cy5 (Em- Max 667 nm –Blue/Yellow-Green)

APC Cy7 (Em- Max 785 nm –Red)
APC H7(Em- Max 785 nm –Red)

PE- Cy7 (Em- Max 785 nm –Blue/Yellow-Green)

www.tsienlab.ucsd.edu
**BD Biosciences Fluorochrome Reference Chart**

Visit [bdbiosciences.com/colors](http://bdbiosciences.com/colors) for detailed information about our newest fluorochromes and instrumentation. To select your optimal combination of fluorochromes, visit [bdbiosciences.com/spectra](http://bdbiosciences.com/spectra) to use an interactive fluorescence spectrum tool.

### BD FACSAria™ bioanalyzer
- **Green Diode**: 532 nm
- **Red Diode**: 635 nm

### BD FACSCalibur™ flow cytometry system
- **Argon**: 488 nm
- **Red Diode**: 635 nm

### BD FACSCanto™ flow cytometry system
- **Solid State**: 488 nm
- **HeNe**: 633 nm

### BD FACSCanto™ II flow cytometry system
- **Solid State**: 488 nm
- **HeNe**: 633 nm

### Preconfigured BD™ LSR II (typical setup)™
- **Solid State**: 488 nm

### Special Order BD™ LSR II Special Order BD LSRFortessa™ (typical setup)™
- **Solid State**: 532 or 640 nm

### BD FACSAria™ cell sorter family (typical setup)™
- **Solid State**: 488 nm

#### Fluorochromes provided by BD Biosciences
- **FITC**: Alexa Fluor® 488, PerCP-Cy7™, Pacific Blue™
- **PE**: PE-Cy7™, PE-Cy5.5™, APC-Cy7™
- **APC**: BD APC-H7™, BD Horizon™ V450™
- **PerCP**: PerCP-Cy5.5™, BD Horizon™ V500™
- **Texas Red®**: PE-Texas Red®
- **Cy™5**: APC-Cy7™
- **Cy™7**: Alexa Fluor® 647
- **PE-Cy™5.5**: PE-Cy5.5™
- **PE-Cy™7**: PE-Cy7™
- **PerCP-Cy™5.5**: PerCP-Cy5.5™
- **PerCP-Cy™7**: PerCP-Cy7™
- **Pacific Blue™**: Pacific Blue™
- **Alexa Fluor® 647**: Alexa Fluor® 647
- **Alexa Fluor® 700**: Alexa Fluor® 700
- **AmCyan**: AmCyan
- **PerCP-Cy™5.5**: PerCP-Cy5.5™
- **BD Horizon™ V500™**: BD Horizon™ V500™
- **BD APC-H7™**: BD APC-H7™

#### Stain index of various fluorochrome conjugates on a BD™ LSR II

<table>
<thead>
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<th>Reagent</th>
<th>Clone</th>
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<td>PE</td>
<td>RP-A4</td>
<td>575/26</td>
<td>305</td>
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<td>APC</td>
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<td>660/20</td>
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<td>PE-Cy™7</td>
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<td>780/60</td>
<td>122</td>
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<td>BD APC-H7™</td>
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</table>

*Fluorochromes listed with the same superscript number are read in the same detector, and thus would not normally be used in combination.*
## Fluorochromes for Flow Cytometric Analysis

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Excitation Max (nm)</th>
<th>Excitation Laser Lines (nm)</th>
<th>Emission Max (nm)</th>
<th>Instrument(s)</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Cascade Blue®</td>
<td>405</td>
<td>360, 405</td>
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<td>FITC (fluorescein)</td>
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<td>Viability Probe</td>
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<tr>
<td>7-AAD (7-aminoactinomycin D)</td>
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<td>488</td>
<td>647</td>
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<td>PI (Propidium Iodide)</td>
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- inFlux™ is a trademark of Cytopeia, Inc.
- CyAn™ ADP and MoFlo™ are trademarks of Dako Denmark A/S
- Cascade Blue®, Alexa Fluor®, Texas Red® are registered trademarks of Molecular Probes, Inc.
- Pacific Blue™ is a trademark of Molecular Probes, Inc.

* These fluorochromes can be used as single color configurations or multi-colored configurations depending on instruments, available lasers and filters, and appropriate color compensation settings.

** These instruments are provided as general information. User must check instrument capability and choose appropriate setting.
Flow Cytometry

Immunophenotyping

Intracellular Cytokines Staining

Cell Proliferation/In Vivo Tracking

CFSE – dilution of dye
Multicolor Flow Cytometry

- Multifunctional Cells
- Heterogeneous Populations
- Clinical Samples: Cells from biopsies or pediatric samples

**Single-Cell Functional Characterization:** Importance of Multifunctional T Cells

_Darrah et al., Nat Med 2007, 13:843_
Advancement in laser and dye technology: Side Population Cells

- Stem cells and early progenitors in hematopoietic tissues.
- Efflux of DNA dye Hoechst33342 under UV excitation; stem cells preferentially exclude this dye

http://science.cancerresearchuk.org/sci/facs/facs_major_apps/stem_cells/SPSChtml/?version=1
Measurement of a variety of soluble and intracellular proteins, including cytokines, chemokines, growth factors, and phosphorylated cell signaling proteins.

Luminex - xMAP Technology built on flow cytometry and microsphers
  - Cancer markers, Cadiac markers; cellular signaling, etc.
BD Biosciences – Cytometric Bead Array
  - cytokines, cell signaling, etc.
CBA Kit
- preconfigured panels for ultimate ease of use

CBA FlexSet
- Multiplexed for up to 30 analytes in a single assay
- For human, mouse, rat signalling proteins (phospho proteins) & soluble proteins (cytokines, chemokines)
Multiplex Microbead Assay

• Advantages
  – Analyze multiple analytes simultaneously
  – Reduced sample volume requirements
  – Reduced hands-on time by parallel analysis of samples
  – Wide dynamic range of fluorescence detection (requires fewer sample dilutions)

• Limitations
  – Not as sensitive as ELISA
  – Requires a flow cytometer
Multiflex microbeads Flow Cytometry

Soluble Proteins

- ELISA
- ELISPOT
- In vivo Capture
- BD CBA

Intracellular Proteins

- FCM (ICC)
- WB
- IHC
- BD CBA
BD CBA Assay Overview

Bead Specificity

Soluble Cytokines

- IL-2
- IL-4
- IL-5
- IFNγ
- TNF

Capture Beads

Bead + Cytokine

PE Detection Reagent

Wash & Analyze
Protein kinases mediate most of the signal transduction in eukaryotic cells.

Traditional methods – Western blot, immunoprecipitation, and immunofluorescence microscopy.

Limitations – cannot distinguish subpopulations of cells.

Flow cytometry-based analysis – measure phosphorylation status of both homogenous and heterogeneous cell populations.

BD Phosflow
Signaling Pathways in Single Cells

• Single-cell resolution
  – Collect single-cell activation state information from heterogeneous samples
  – Detect and identify phospho-protein activities in small cell subpopulations

• Multi-parameter analysis
  – Correlate multiple cell surface or intracellular markers and phosphorylation events simultaneously

• Rapid, sensitive, and scalable
  - Uncover signaling events in rare cell populations
  - Increase sample throughput with 96-well plate analysis

BD Phosflow
Signaling Pathways in Subpopulations of Cells


BD Phosflow
Signaling Pathways in Single Cells

Cell Signaling Technology

Phospho-Histone H3 during mitosis
Increase in mitotic cells
http://www.cellsignal.com/products/9716.html
Acute Myeloid Leukemia
- Changes in signaling pathways:
  - PI3 kinase, ERK MAP kinase, and Jak/STAT pathways
- Monitor:
  - PI3 kinase, ERK & STAT5

Beckman Coulter
Pharmacodynamic Monitoring of Molecular-Targeted Agents in the Peripheral Blood of Leukemia Patients Using Flow Cytometry
– Hedley et al., 2008 36:133-139

Use phosphospecific Ab to measure activation states of intracellular signaling elements after drug application.

Beckman Coulter
LPS activation of PI3 kinase in PBMC:
PBMC - stained by PE-Cy7 anti-CD14 Ab (monocytes; 3-5%)
Gate on CD14 then P-ERK

Shankey et al. Flow Cytometry-Based Biomarkers for Molecular Therapeutics
Drugs Discovery & Development 2010
<table>
<thead>
<tr>
<th></th>
<th>BD Phosflow</th>
<th>Western blot</th>
<th>BD CBA</th>
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<td>Multiplexing – multiple phospho-proteins in one sample</td>
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<td>–</td>
<td>++</td>
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<td>Multiplexing – combination with surface markers</td>
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<tr>
<td>Requires skilled operator</td>
<td>+/-</td>
<td>–</td>
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</table>
Signaling Pathways in Subpopulations of Cells

BD Phosflow

Multiple phosphoproteins in different cell types studied by BD Phosflow

Simultaneous analysis of multiple phosphoproteins in complex cell mixtures. In this experiment, BD Phosflow was used to analyze the effect of four different stimuli on cell signaling pathways in mouse splenic cell subsets (B cells, T cells, and CD11b<sup>hi</sup> cells). Differences in signaling pathway responses were uncovered between mouse splenocyte cultures stimulated in vitro and splenic cells stimulated in vivo.

http://wwwbdbiosciences.com/research/phosflow/features/rapidanalysis.jsp
Phosphoprotein Analysis in a Variety of Sample Types

- Whole Blood
- PBMCs
- BD IMag
- Ficoll Gradient
- T-/B-Cells

**Preparation Steps:**
1. **Lyse/Fix/Perm**
2. **Lyse**
3. **Fix/Perm**

**Examples:**
- A. Untreated
- B. Treated with IL-4 and IL-6

**Imaging and Analysis:**
- Alexa Fluor® 488-STAT 6 (pY641)
- Alexa Fluor® 467-STAT 3 (pY705)
- PE-CD3
- PerCP-Cy5.5 – CD20
• It combines the statistical power & fluorescence sensitivity of standard flow cytometry with the spatial resolution and quantitative morphology of digital microscopy.
• Quantifying cellular structure in health & disease
• Identification of tumor cells
• Localization of targets inside individual cells
• In situ hybridization

Basiji et al. Cellular Image Analysis and Imaging by Flow Cytometry
Clin Lab Med. 2007. 27:653
Cell Proliferation

1. Changes in Cell Cycle
2. Follow # of cell divisions over a period of time
Advancement in violet reagents
Apoptosis Assays

- Apoptotic Plasma Membrane Assays
- Mitochondrial Assays
- Caspase Assays
- Nuclear Apoptosis Assays
- Multitparametric Apoptosis Assays
Useful Websites

BD Spectrum Viewer
http://wwwbdbiosciencescomspectra

Invitrogen Spectrum Viewer
http://probesinvitrogencomresourcesspectraviewer

Purdue Cytometry Lab
wwwcytopurdueedu

Mario Roederer’s Home Page
wwwdrmrcom