Cytometry Basics and New Developments

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http://www.desatoya.com
Why Single Cell Analysis

Intensity Histogram for Single Particles

Intensity per Sample

Cell by cell intensity analysis detects population heterogeneity.
Single Cell Cytometry vs. Bulk Analysis
Benefits of Subset Analysis

Subpopulation analysis detects changes better, especially for rare subpopulations.
From Early to Present Microscopy and Flow Cytometry

Source: BD Biosciences
Flow and Imaging Cytometry Features

Single cell analysis with
- High sensitivity (single molecule sensitivity) I,F
- Wide dynamic range ($10^0$ to $7$ mL$^{-1}$) F
- High analysis rates to $\sim 10^5$ sec$^{-1}$ F
- Light scatter F
- Direct size and 3D information I
- Multi-parameter analysis I,F
- Direct kinetic measurements I
- Live/dead discrimination I,F
- Viable cells can be re-covered F,(I)
- Measurement of adherent cells I
- Good ease-of-use F,(I)
Physical parameters

- Light scatter
- Fluorescence
- Phosphorescence
- Raman
- Element mass
- Electrical properties e.g. impedance
- ...

http://www.dvssciences.com/technical.html
Flow Cytometer Fluidics

Hydrodynamic or acoustic focusing

Cells after analysis, available for culture

V. Kachel, H. Fellner-Feldegg & E. Menke - MLM Chapt. 3

Fluorescence signals

Focused laser beam
Flow Cytometer Optical Systems

BD FACSCalibur™ Optics

- detectors
- side scatter
- yellow
- dark red
- red
- filters
- beam shaper and combiner
- blue laser and combiner
- red laser
- focusing lens
- waste
- forward scatter
Filter Arrangement and Spectral Overlap

Cytometry 77A, 410ff

Nature Reviews Immunology 4, 648ff
Basic Data Processing

Flow Cytometry

Numbers in Memory

- FSC
- SSC
- FITC
- APC

Laser delay

Digital microscopy

Intensity 0.255

Cell | P1 | P2 | P3 | P4 | P5 | Pop# |
--- | --- | --- | --- | --- | --- | --- |
1   | 242| 135| 704| 175| 612| 1    |
2   | 146| 132| 690| 178| 566| 1    |
3   | 269| 147| 89 | 206| 580| 3    |
4   | 442| 143| 399| 250| 255| 4    |
5   | 212| 167| 155| 926| 526| 2    |
6   | 269| 2  | 659| 207| 575| 1    |
7   | 204| 232| 112| 171| 679| 3    |
8   | 152| 74 | 160| 828| 532| 2    |
... | ...| ...| ...| ...| ...| ... |
9997| 215| 119| 138| 936| 662| 2    |
9998| 244| 50 | 72 | 261| 543| 3    |
9999| 214| 137| 174| 1014|597 | 2    |
10000|312|87 |110 |904 |560 |2    |
“Droplet-based” Sorting

Source: BD Biosciences
Sorting for gene expression analysis in single cells

Figure 9: C, heat map from 96 PCR assays, are shown on a 96.96 chip. Each point represents gene expression within a single cell. Some genes are more highly expressed in SP High than SP Low cell population.
Sorting for Cell Surface Proteomics

Cell surface proteome by FACS sorting, followed by LC MS

(in collaboration with Thermo Finnigan, San Jose, CA)

The dot plots show the sorting strategy used for stained peripheral blood cells and population purity after sorting for CD4- and CD8-positive cells. CD4 cells were gated on scatter and FITC fluorescence, CD8 bright cells were gated on scatter and RPE fluorescence. Sorted populations showed >95% purity.

Peptide masses were separated by reverse phase HPLC (A) as described in Methods. Eluted peptides were subjected to electrospray injection into the mass spectrometer and analyzed for their mass/charge ratio (m/z value) (B). Selected ions were collected in the ion trap. These parent ions were cracked by collision ion dissociation to produce a range of fragment sizes (D) that were compared to predicted peptide sequences in the human database using TurboSequest (G).
Subset Analysis Example

profiling a lymphoma patient

Slide provided by Nikesh Kotecha, CytoBank Inc, Mountain View CA

Data: J. Irish, Stanford
BeadArray Assays

Step 1: Antigen Capture

Capture Antibody

Antigen Capture Bead

Step 2: Detector Antibody

15 Minutes @ RT / Dark

Detector Antibody (biotinylated)

Step 3: Reporter Molecule

15 Minutes @ RT / Dark

Streptavidin-Dye

Dr. Rudi Varro, BD Biosciences
Relative B (Br) is a measure of true optical background in the fluorescence detector.
Instrument Evaluation Qr

Source: Joe Trotter, BD Biosciences

$$Q_r = \frac{\# \text{ photoelectrons}}{\# \text{ fluorescence molecules}}$$
Optimizing cytometry measurements (I)

- **Background light**
  - The total measurement SD is the sum of the error contributions from all sources:
    \[ SD = \sqrt{SD_{\text{total}}^2 + SD_{\text{noise}}^2 + SD_{\text{dim}}^2 + SD_{\text{bias}}^2} \]
  - When two dyes (PE and FITC) are measured by a single detector the SD is additive:
    \[ SD_{\text{total}} = \sqrt{SD_{\text{PE}}^2 + SD_{\text{FITC}}^2 + SD_{\text{background}}^2} \]

- **Dye properties (brightness and spectral overlap)**

Next slides:
- Multi-color dye overlap
- Gain (PMT, CMOS, CCD) settings
- Data Display
- Controls

Reagent performance

\[ \text{Stain index} = \frac{\text{Medium}_{\text{pos}} - \text{Medium}_{\text{neg}}}{2 \times SD_{\text{neg}}} \]

Source: Joe Trotter, BD Biosciences
Optimizing cytometry measurements (II)

- **Gain** (PMT, CMOS, CCD) settings

- **Data Display**

- **Controls**

Source: Joe Trotter, BD Biosciences and others
Marker Brightness

Brighter markers resolve rare populations better
### Rare Subpopulation Analysis

Counting Statistics

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
</tr>
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<tbody>
<tr>
<td>6</td>
<td>2</td>
<td>6</td>
<td>8</td>
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<table>
<thead>
<tr>
<th></th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
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<tbody>
<tr>
<td>Mean</td>
<td>2.4</td>
<td>4</td>
<td>4.6</td>
<td>5.2</td>
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<tr>
<td>St.Dev</td>
<td>2.2</td>
<td>1.9</td>
<td>2.1</td>
<td>2.2</td>
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<table>
<thead>
<tr>
<th>Overall</th>
<th>Mean</th>
<th>St.Dev</th>
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</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4.1</td>
<td>2.2</td>
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</table>

Ignoring Counting Statistics Can Lead to Erroneous Conclusions
Eliminating system noise from fluidics perturbations and other sources improves the limit of detection for rare cell analysis.

Gross HJ et al, Cytometry 1993
Particle Carry-Over

**Carry-over specification examples**  
(from manufacturer’s info)

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Specification</th>
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<tr>
<td>Beckman Coulter FC500</td>
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<tr>
<td>Beckman Coulter</td>
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<td>BD FACSCanto II</td>
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<td>BD FACSVerse</td>
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<td>Guava EasyCyte</td>
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<tr>
<td>Life Technologies Attune</td>
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<tr>
<td>Miltenyi MACSQuant</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td></td>
<td>loader &lt;0.5%</td>
</tr>
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</table>

Eliminating fluidic system particle carry-over is vital for reliable rare cell analysis.
Multi-parameter Fluorescence Cytometry Points To Consider

Summary

- Know your instrument e.g. Qr & Br for different channels, carryover, …
- Use high enough gain settings to maximize sensitivity
- An antibody/dye combination that marginally allows discrimination of positives/negatives in a single color assay is unlikely to contribute anything helpful in a multicolor experiment.
- Avoid spillover from bright cell populations into channels requiring high sensitivity
- Beware of tandem dye degradation
- Internal controls are essential
- Take advantage of bright markers for “dim markers”
New Developments for Multi-parameter Cytometry


- **SERS-Label Flow Cytometry** (uses spectral fine-structure to distinguish labels, Cytometry, 2008, 73A(2), pp 119-128)

- **Sequential Stain Destain Cytometry** (Cytometry, 2009, 75A(4), pp 362-370)

- **SONY spectral analysis**

John Nolan, La Jolla Bioengineering Institute

Christian Hennig, ChipCytometry
Hannover Medical School
New Developments for Sample Preparation

Removing erythrocytes and/or free reagent without centrifugation

Silicon filter for leukocyte/ erythrocyte separation

Acoustic particle focusing

Leukocyte recovery

<table>
<thead>
<tr>
<th>Flow rate</th>
<th>0.18ml/min</th>
<th>0.33ml/min</th>
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<tbody>
<tr>
<td>Cell load</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10ul</td>
<td>0.98±0.04</td>
<td>0.92±0.07</td>
</tr>
<tr>
<td>(51.1±7.5)×1000 cells</td>
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<tr>
<td>50ul</td>
<td>0.75±0.18</td>
<td>0.35±0.15</td>
</tr>
<tr>
<td>(550±14.1)×1000 cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2011 Liping Yu et al, BD Biosciences and Aviva Inc.

Lyse no wash  Chip wash  Centr. wash

2010, Laurell group, Lund University & Brian Warner, BD Biosciences
Intra-vital Cytometry
Single cell analysis in living animals

Flow cytometry in blood vessels

2010, Zharov VP and coworkers

2010, Chang Y-C et al

Signals from
• 2-photon fluorescence
• bioluminescence
• photo-acoustic effect
• …

Microscopy

2011, Runnels JM et al; homing of multiple myeloma cells in bone marrow

Review paper:
Niesner RA, Cytometry 79A (2011)
Conclusions

New technologies from many fields are rapidly generating new opportunities for cytometry.
Acknowledgements

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- Collette Rudd, Thermo

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