Outline

Influx overview:
1. Principle of flow cytometry
2. BD Influx 6-way sorter

Sort theory and application:
1. Principle of sorting
2. Accurdrop technology: Decide drop delay
3. Sort Mode: Purity, Recovery and Speed
4. Sorting Strategy and tip
5. Application
What is Flow Cytometry?

- Flow = Fluid
- Cyto = Cell
- Metry = Measurement

- A variety of measurements are made on cells, cell organelles, and other objects suspended in a liquid and flowing at rates of several thousands per second through a flow chamber.

System Overview

Fluidics
Optics
Electronics
Software

BD Biosciences
Influx System Overview

Subsystems

Fluidics
To introduce and focus the cells for interrogation and create a stable breakoff for sorting

Optics
To generate and collect the light signals

Electronics
To convert the optical signals to proportional digital signals, process the signals, and communicate with the computer
Influx Fluidics

Hydrodynamic Focus

Sample

Sheath fluid

Sample needle

Hydrodynamic focusing zone

Laser beam

-Fluorescent light

-Scatter light
Subsystems

Fluidics
To introduce and focus the cells for interrogation and create a stable breakoff for sorting

Optics
To generate and collect the light signals

Electronics
To convert the optical signals to proportional digital signals, process the signals, and communicate with the computer
Scatter Light

SSC
Internal Complexity
Granularity

FSC
Size
Shape
Surface
Refractive index

Scatter Light indicating physical properties of cell

Cell Line

Side Scatter
Cell debris

Major Cell population

Forward Scatter
Scatter Light indicating physical properties of cell

Peripheral blood

Threshold

Debrise

8 to 10 µm Lymphocyte

10 to 14 µm Neutrophil

12 to 14 µm Monocyte

Fluorescent Light

Fluorescence
Fluorescent Light

Fluorescent Dye-Excitation and Emission

Excitation: Which laser can generate signals

Emission: Which detector to collect signals
Stream-in-Air Excitation

Collection Optics

- nozzle & stream
- objective
- collimating lens
- pinhole = spatial filter
- chromatic filter
- detector
**Detector Block**

- 488-nm, 4-color mirror block (example)

**Optical Filters**

- **Shortpass**
  - 460, 500, 540

- **Longpass**
  - 460, 500, 540

- **Bandpass**
  - 460, 500, 540
  - \( BP500/50 = 500 \pm 25 = 475-525 \text{nm} \)
# Influx Optics Configuration

3 lasers-14 color system (5B-6V-3R)

<table>
<thead>
<tr>
<th>Excitation laser</th>
<th>Detection Range</th>
<th>Example Fluorochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>488 Blue laser (200mW)</td>
<td>530/40</td>
<td>FITC</td>
</tr>
<tr>
<td></td>
<td>580/30</td>
<td>PE</td>
</tr>
<tr>
<td></td>
<td>610/70</td>
<td>PE-TR</td>
</tr>
<tr>
<td></td>
<td>682/40</td>
<td>PerCP-Cy5.5</td>
</tr>
<tr>
<td></td>
<td>750LP</td>
<td>PE-Cy7</td>
</tr>
<tr>
<td>405 Violet laser (100mW)</td>
<td>425/26</td>
<td>BV421</td>
</tr>
<tr>
<td></td>
<td>520/35</td>
<td>BV500</td>
</tr>
<tr>
<td></td>
<td>610/20</td>
<td>BV605</td>
</tr>
<tr>
<td></td>
<td>660/20</td>
<td>BV650</td>
</tr>
<tr>
<td></td>
<td>710/50</td>
<td>BV711</td>
</tr>
<tr>
<td></td>
<td>780/60</td>
<td>BV786</td>
</tr>
<tr>
<td>640 Red Laser (120mW)</td>
<td>670/30</td>
<td>APC</td>
</tr>
<tr>
<td></td>
<td>720/40</td>
<td>APC-Alexa700</td>
</tr>
<tr>
<td></td>
<td>750LP</td>
<td>APC-Cy7</td>
</tr>
</tbody>
</table>

**Subsystems**

**Fluidics**
To introduce and focus the cells for interrogation and create a stable breakoff for sorting

**Optics**
To generate and collect the light signals

**Electronics**
To convert the optical signals to proportional digital signals, process the signals, and communicate with the computer
Quantification of a Voltage Pulse

![Diagram showing pulse height, pulse width, and pulse area (integration)]

Linear vs Log Amplification

![Graphs showing linear and log amplification comparison]

[BD Biosciences logo]
**Sortware Software display data**

**BD Influx 6-way sorter**

Purify target cell or particle population into various device
With different choice of nozzle size 70µm, 86µm, 100µm, 140µm, 200µm (optional)

Collection device:
5ml tube, 15ml tube, 50ml tube
96 well, 384 well, slide, customized device

Sort Mode:
2 way, 4 way, 6 way, single cell, index sorting

Optional:
BSC, AMO, temperature control
### Sorting Overview

1. Pass cells through the laser one at a time
2. Collect and analyze signals from each cell to determine which cells to sort
3. Charge the stream as the droplet containing a target cell breaks off
4. Deflect the charged droplet into the appropriate collection tube or device
5. Allow uncharged droplets to pass to waste

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### Drop Formation

- Oscillating Voltage
- Piezo Disc
- Acoustic Wave

**Nozzle Size:**
- 70µm, 86µm, 100µm, 140µm, 200µm
**Pressure/Frequency**

Optimal pressure frequency

Recommended pressure/frequency combinations

**BD Influx**

**Nozzle/Pressure/Frequency setting**

<table>
<thead>
<tr>
<th>Nozzle size</th>
<th>Pressure (psi)</th>
<th>Frequency (kHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>35</td>
<td>~75-76</td>
</tr>
<tr>
<td>70</td>
<td>40</td>
<td>~80</td>
</tr>
<tr>
<td>70</td>
<td>52</td>
<td>~86.8</td>
</tr>
<tr>
<td>70</td>
<td>60</td>
<td>~99.2</td>
</tr>
<tr>
<td>70</td>
<td>65</td>
<td>~98.2</td>
</tr>
<tr>
<td>86</td>
<td>25</td>
<td>~50</td>
</tr>
<tr>
<td>86</td>
<td>30</td>
<td>~48</td>
</tr>
<tr>
<td>86</td>
<td>33</td>
<td>~58</td>
</tr>
<tr>
<td>86</td>
<td>40</td>
<td>~65</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>~36</td>
</tr>
<tr>
<td>100</td>
<td>16</td>
<td>~29.2</td>
</tr>
<tr>
<td>100</td>
<td>17-18</td>
<td>~27</td>
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<tr>
<td>100</td>
<td>20</td>
<td>~37</td>
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<tr>
<td>100</td>
<td>20</td>
<td>~39</td>
</tr>
<tr>
<td>100</td>
<td>24</td>
<td>~39.00</td>
</tr>
<tr>
<td>100</td>
<td>27</td>
<td>39.2</td>
</tr>
<tr>
<td>100</td>
<td>27</td>
<td>45.6</td>
</tr>
<tr>
<td>100</td>
<td>32</td>
<td>~48.6</td>
</tr>
<tr>
<td>140</td>
<td>5</td>
<td>~13.9</td>
</tr>
<tr>
<td>200</td>
<td>2.8</td>
<td>~6.2</td>
</tr>
</tbody>
</table>
**Drop Charging**

BD FACS™ technology

- Accudrop beads
- Diode laser
- Camera
- Optical filter

**Drop Delay**

BD FACS™

Accudrop technology

- Accudrop beads
- Diode laser
- Camera
- Optical filter
Drop Delay using BD FACS Accudrop

- When drop delay is set correctly, Accudrop beads will be depleted from center stream.
- Use the Accudrop filter so that only the beads show in the stream camera.

Accudrop

Correct Drop Delay
Accudrop Optical Filter on

Not Correct Drop Delay
Accudrop beads still in the center stream

Correct Drop Delay
Accudrop beads all in deflected side stream

Sort Mode
<table>
<thead>
<tr>
<th>Setting</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drops</td>
<td>Sort an additional drop if the cell is on edge of drop</td>
</tr>
<tr>
<td>Objective</td>
<td>Enrich (no coincidence)</td>
</tr>
<tr>
<td></td>
<td>Purify (coincidence with override)</td>
</tr>
<tr>
<td></td>
<td>Single (coincidence no override)</td>
</tr>
<tr>
<td>Extra Coincidence</td>
<td>Adjust how much phase into adjacent drops to abort due to non-target cells</td>
</tr>
<tr>
<td>Phase Mask Current Drop</td>
<td>Will only sort if cell is in designated portion of droplet</td>
</tr>
</tbody>
</table>

**Number of Drops**

1.0-drop setting
- Sort 1 drop, regardless of the cell’s position within the drop.
- There’s a chance that cell is actually in other drops.
**Number of Drops**

1.5-drop setting (50%×1 drop+50%×2 drop=1.5 drop)

- If the cell is in the center of the drop, sort 1 drop.
- If the cell is at the edge of the drop, sort 2 drops.
- You could get more accurate cell count compared to 1.0 drop.

1.5 drop is equivalent to Yield mask of 16 on the Aria.

**Objective**

The Objective setting influences sort **purity** and sort **efficiency**.

- **Enrich.** Disables all coincidence. Sort as much as you can, for rare cell population.

- **Purify.** Enables coincidence and coincidence override (2 target cells in same drop or within the extra coincidence zone).

- **Single.** Enables coincidence and disables coincidence override to ensure that only one target event can be sorted. (Single cell sorting for plate)
## Enrich Setting

**Enrich On**
- Get 1 target cell
- Also 1 non-target cell

**Enrich Off (Ex: Purify)**
- No cell was sorted

### Standard Sort Modes

| Sort Mode  | Settings                                      | When to use                                                                 |
|------------|-----------------------------------------------|                                                                            |
| 1.0 Drop Yield | 1-drop sort  
  1.0 Drop Coincidence  
  No phase gate | If you need to get the highest combination of purity and yield               |
| 1.5 Drop Pure | 1.5-drop sort  
  1.5/2.5 Drop Coincidence  
  No phase gate | If you need an exact count and high purity                                   |
| 2.0 Drop Enrich | 2-drop sort  
  No Coincidence  
  No phase gate | If the recovery of target cells is more important than purity                |
| 1.0 Drop Single | 1-drop sort  
  1.5 Drop Coincidence  
  10/16 Drop Phase Mask | If you need to sort large or sticky cells and need an exact count  
  If you need to sort single cells into plate wells                          |
Sorting Tips

Sort Performance

• Depends on what you want:
  – Purity
  – Recovery
  – Yield
  – Viability

Speed !!
Sort Performance

- Speed vs. Yield/Recovery

The Importance of Frequency

- More empty drops
- Greater chance of coincidence

Same event rate, higher frequency will have higher efficiency and recovery.
The Importance of Event Rate

- High event rate
- Low event rate

Example: 39.0KHz/s

Same Frequency, lower events rate will give you higher recovery but will consume more time.

Sample Preparation Considerations

- Enrich rare cell population if possible
- Avoid cell clumps
  - Always filter your cells before sort!
  - Use Accutase instead of Trypsin
  - Treat cells with DNAse
- Use appropriate sample buffer
  - PBS, HBSS or phenol-red free culture media w/ 25mM HEPES, 5mM EDTA and 1~2% FBS or 0.1~0.2% BSA to maintain cell viability
- Use viability dye to confirm cell viability before sort
Summary: Sorting Considerations

- Collection tube:
  Pre-coated with 1% BSA or 10% FBS overnight and filled
  with appropriate collection buffer
  - 5ml Falcon tube: 2ml
  - 15ml centrifuge tube: 7ml
- Change collection tubes periodically
- Temperature control
- Event (Threshold) rate:
  1/10~1/4 drop drive frequency for better yield

Compensation Theory
Compensation Examples

Incorrect Compensation

Correct Compensation

Undercompensation

Overcompensation

Application

- Sort different target cell
- Life or death
- Morphology
- Surface antigens
- Gene expression
- Cell functions
- DNA content
Sorting Cells By Surface Markers

- **Sorting NK Cells**
  - CD3 FITC to exclude T cells
  - CD56+CD16 PE to include all NK Cells.

### Sorting NK Cells for Cytotoxicity Studies

![Graph showing cytotoxicity results](image)
Regulatory T Cell Sorting

Figure 1: Schematic representation of the role of regulatory T cells in immune function.

CD4/CD25 Treg sorting

Sort on R1 and R2  sort on R1 and R3

BD Biosciences
CD4/CD25 High/CD127 Lo

A. Pre Sort

CD25-FITC vs CD127-PE

Post Sort

CD25high/CD127lo

Percentage

Sorting by Gene Expression

Add viability check.

BD Biosciences
Figure 12. Organelles targeted by BD Living Colors™ Subcellular Localization Vectors.
Rare Cell Sorting tip

- Rare cell sorting usually requires pre-enrichment steps:
  - Bring the starting purity to > 5 %
  - Ficoll
  - Immune Panning
  - Magnetic Beads (Positive/Negative)
Stem cell sorting

Schematic adapted from http://stemcells.nih.gov/index.asp

Hematopoietic Stem Cells

Sca-1, c-kit and CD34 expression in mouse bone marrow
Four color analysis

BD Biosciences
Bone Marrow Stem Cell

Before Sorting

C-Kit

Sca-1

LSK 0.7%

After Sorting

C-Kit

Sca-1

LSK 97%

Thank You