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
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

- 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

10 to 14 µm Neutrophil

12 to 14 µm Monocyte

8 to 10 µm Lymphocyte

Fluorescent Light

Side scatter

Forward scatter

Incident light source

Fluorescence
Fluorescent Light

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-c1 m1 1 ck (example)

Optical Filters

- **Shortpass**
  - SP 500
  - 460 500 540

- **Longpass**
  - LP 500
  - 460 500 540

- **Bandpass**
  - BP500/50
  - 460 500 540
  - BP500/50 = 500 ± 25 = 475-525 nm
Influx Optics Configuration

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

<table>
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<th>tlectionRang1</th>
<th>Examp1 Fluxo1ohm1</th>
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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
Electronics System Overview

BD Influx Instrument Electronics  Instrument Interface Server  BD Software Client Computer

Electronics

Detector (PMT)  Amplifier (Linear or Log)  Processing

Photon in  Signal out  Voltage in

PMT power supply

Peak hold level

PMT Signal Curve

BD Biosciences
Quantification of a Voltage Pulse

Volts

Pulse Height

Pulse Width

Time (µ Seconds)

Pulse Area (Integration)

Linear vs Log Amplification

Cytometer

Cytometer
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 assay one at a time.
2. Collect and analyze signals from each cell to determine which cells to sort.
3. Change the stream as the plate contains a target cell each.
4. Defect the charged droplets into the plate containing target cell devices.
5. Allow uncharged droplets to pass through waste.

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**Drop Formation**

- Oscillating Voltage
- Piezo Disc
- Acoustic Wave

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

- Wave Becomes Drops
BD Influx Nozzle/Pressure/Frequency setting

<table>
<thead>
<tr>
<th>Nozz1</th>
<th>size1</th>
<th>P1</th>
<th>u1 (p1)1</th>
<th>F1 freqcy (kHz)1</th>
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Drop Charging

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
Sort Mode

Accudrop beads still in the center stream

Accudrop beads all in deflected side stream

Not Correct Drop Delay
Correct Drop Delay
<table>
<thead>
<tr>
<th>Drops</th>
<th>Sort an additional drop if the cell is on edge of drop</th>
</tr>
</thead>
<tbody>
<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>

**Numb1 of 1 op1**

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% x 1 drop + 50% x 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.

Sort 1 drop
Sort 2 drops
Sort 2 drops

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

sort

Enrich Off (Ex: Purify)
No cell was sorted

Standard Sort Modes

<table>
<thead>
<tr>
<th>Mode</th>
<th>Conditions</th>
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<tbody>
<tr>
<td>0.0 D1 p YeH1</td>
<td>-d1 p s1 t1 .0 D1 p C1 nC1ence1 N1 phase gatel</td>
</tr>
<tr>
<td></td>
<td>If you need to get the highest 1 cell and the 1 cell in phase gate 1</td>
</tr>
<tr>
<td>0.5 D1 p PuH1</td>
<td>.5-d1 p s1 t1 .5/2.5 D1 p C1 nC1ence1 N1 phase gatel</td>
</tr>
<tr>
<td></td>
<td>If you need an exact cell and the 1 cell in phase gate 1</td>
</tr>
<tr>
<td>2.0 D1 p En1cl1</td>
<td>2-d1 p s1 t1 N1 C1 nC1ence1 N1 phase gatel</td>
</tr>
<tr>
<td></td>
<td>If the very first target cell is 1 in phase gate 1</td>
</tr>
<tr>
<td>0.0 D1 p ShgH1</td>
<td>-d1 p s1 t1 .5 D1 p C1 nC1ence1 0/16 D1 p Phase Mask1</td>
</tr>
<tr>
<td></td>
<td>If you need to get a high percent target cell and an exact cell</td>
</tr>
<tr>
<td></td>
<td>If you need to select cells in phase gate 1 in plate well 1</td>
</tr>
</tbody>
</table>
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 late, high frequency w1 have 1
h ghe1eff c ency and 1e1ve1y1
The Importance of Event Rate

Example: 39.0 KHz/s

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/ 25 mM HEPES, 5 mM 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
Emission Spectra: Spectral Overlap

Normalized Intensity

Wavelength (nm)

Spillover

Relative Intensity

Wavelength (nm)
FITC Compensation

![Graph showing FITC Compensation](image)

Comp1n1ation Mat1ix1

![补偿矩阵](image)
Compensation Examples

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 t1 exclude T cells
  - CD56+CD16 PE t1 include a1 NK Cells.

Sorting NK Cells for Cytotoxicity Studies

![Image of sorting NK cells](image-url)
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
CD4/CD25 High/CD127 Lo

A. Pre Sort

B. Post Sort

CD25^hi/CD127^lo

Add viability check.

Sorting by Gene Expression

R2
Figure 12. Organelles targeted by BD Living Colors™ Subcellular Localization Vectors.
Rare Cell Sorting tip

- Rare cells typically undergo enrichment steps:
  - Binding the starting purity to >5%1
  - Fc1
  - Immune Panning1
  - Magnet c Beads (P1s t ve/Negat ve)1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD123+ DCs</th>
<th>CD11c+ DCs</th>
<th>Basophile</th>
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<tbody>
<tr>
<td>Lin 1</td>
<td>–</td>
<td>+/+</td>
<td>–</td>
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<tr>
<td>Anti-HLA-DR</td>
<td>++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>CD11c</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
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Immunophenotype of CD11c, CD123 DCs and baso (fluorescence intensities)

- 4-Color Staining
  - Lineage Cocktail FITC
    - CD3, 14, 16, 19, 20, 56
  - CD123 (IL-3Re) PE
  - HLA-DR PerCP
  - CD11c APC
  - Controls
  - FACLyzing Solution
Stem cell sorting

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

After Sorting

Thank You