Sort

Best practices in panel design to optimize the isolation of cells of interest
Cell sorting

**Cell sorting:** isolation of a population of interest for downstream analysis
- Cell enrichment
- Cell transplantation
- Downstream functional and genomic analysis

**Goal:** to obtain a pure population with maximum yield
Increasing resolution for cell sorting

• Clear resolution of a population of interest is critical for an optimal sort.

• How to increase resolution in a sorting setting?
  – Eliminate the impact of unwanted cells
  – Increase the ability to visualize from population of interests.
Increasing resolution for cell sorting

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Considerations for cell sorting

Cell analysis
• Fluorochromes
• Biology
• Instrument setup

Cell sorting
• Fluorochromes
• Biology
• Instrument setup
• Sample preparation
• Gating strategy
Considerations for cell sorting: overview

- Experiment setup
  - Sample preparation
  - Instrument settings

- Gating strategy
  - Histogram vs plots
  - Biexponential scale
  - Doublet discrimination
  - Data display

- Panel design
  - Dead cell exclusion
  - Lineage exclusion/depletion
  - Antibody titration
  - Fluorochrome choice
Considerations for cell sorting
Sample preparation, instrument setup
Sample preparation

- Cell dissociation/detachment
- Cell resuspension buffer
- Staining volume and antibody concentration
- Cell sorting buffer and cell density
- Optional use of DNAsé
- Temperature, pH

Sample collection:
- Cell collection buffer (cell culture, transplantation, genomic analysis)
- Temperature, pH
Instrument settings

• Nozzle size/sheath pressure
  – 70 µm/70 psi for lymphocytes
  – 100 µm/20 psi for larger and/or fragile cells

• Event rate (number of events/second)
  – Low speed for higher sorting efficiency

• Sort setup
  – Bulk sorting
  – Purity vs yield
  – Single cells

• Laser alignment

• Drop delay
Instrument settings: speed vs yield vs purity

- Influenced by:
  - Drops per second, events per second, sort “mask” and target population frequency

- Good rule of thumb:
  - Maximum recommended event rate = drops per second / 5
Considerations for cell sorting

Gating strategy

Histogram vs plots, biexponential scale, doublet discrimination, data display
Different options for data display

- **Histogram**
- **Dot plot**
- **Contour plot**

Count

CD-X

CD-Y
Histograms vs plots: How many populations do you see?
Dot plots vs contour plots: How many population do you see?
Histograms vs bivariate plots: where to draw the gate?

- Transfected cells express different levels of GFP.
- Bivariate plots better reveal the separation from negative/dim to positive cells.
- What if GFP is expressed at low levels?
Gating low GFP expressing cells: what is real and what is not?

- High background in the GFP channel is usually due to autofluorescence.
- Negative controls are instrumental for proper gating.
Gating low GFP expressing cells: what is real and what is not?

• High background in the GFP channel is usually due to autofluorescence.
• Negative controls are instrumental for proper gating.
Gating low GFP expressing cells: leveraging autofluorescence

- Autofluorescence is detected in multiple channels.
- Plot GFP against another channel with autofluorescence.
- Autofluorescent cells will be “double positive” (diagonal), revealing true GFP single positives.
The biexponential scale: the best way to look at compensated data

Visualization of compensated data is greatly improved using the biexponential scale.

This example showing different displays of the same data shows the value of the biexponential scale, a mostly logarithmic scale on the upper end, linear at the low end and symmetrical about the negatives.

- Compensated single positives are continuous.
- All populations are visible.
Gating strategy: biexponential scale
Doublet discrimination

Sort check
Electronic pulse

- Height
- Area
- Width
Doublet discrimination

Signal vs. Time for 1x Width and 2x Width events.
Doublet discrimination

- **Cells**
  - **Gate on cells**
  - **Gate on doublets**
  - **Gate on singlets**

- **Gate on doublets**
  - 2n
  - 4n
  - Doublets >4n

- **Gate on singlets**
  - Singlets

- **BD Horizon™ Global Tour**

- **DAPI**

- **FSC-H**
  - **Gate on doublets**
  - **Singlets**
Doublet discrimination

Bulk sort

Single-cell sort

Total cells

Singlets

Doublets
Gating strategy:
low antigen density populations
Gating strategy:
low antigen density populations

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Gating strategy: low antigen density populations

Unstained

Pre-sort

Post-sort
Gating strategy: summary

• Use bivariate plots rather than histograms.
• Use contour plots for a clearer identification of populations of interest.
• Manually adjust the biexponential scale to gate all the cells of interest.
• Use proper controls to identify and eliminate background (autofluorescence).
• Use a doublet discrimination strategy for proper isolation of a single-cell suspension.
Considerations for cell sorting

Panel design
Fluorochrome choice, dead cell exclusion, lineage exclusion/depletion
Why is it relevant to design an optimized panel for cell sorting?

- Best practices to build an optimized panel for analysis apply to the cell sort as well.
- Additional considerations may be taken in account when designing a panel for cell sorting to obtain:
  - Highest purity and yield
  - Clear resolution from unwanted cell populations
How to build a panel for cell sorting?

• A good panel for sorting relies on the use of negative as well as positive markers.
• Properly choose fluorochromes.
  – Antigen density
  – Spillover
  – Co-expression
• Know the biology.
  – Minimize spillover into the most critical markers to maximize the resolution of your population of interest.
• Exclude unwanted cells to increase the resolution of the target cells.
  – Dead cells
  – Lineage
Choosing fluorochromes for a cell sorting panel: exclude unwanted cells

- Dead cells
- Lineage cells
- Cells of interest
Dead-cell exclusion

The presence of dead cells impacts cell sorting.
- Inaccurate quantification of the population of interest
- Reduced purity

- Dead cells can be excluded using:
  - Light scatter properties
  - Viability dyes
Dead-cell exclusion by light scatter

- Scatter alone can be used to identify heat-killed HeLa cells.
- A viability dye is required to detect and gate out dead cells.
Dead-cell exclusion using viability dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Unfixed cells</th>
<th>Fixed cells</th>
<th>Detector</th>
<th>Laser</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>✓</td>
<td>X</td>
<td>BV421</td>
<td>UV/Violet</td>
</tr>
<tr>
<td>Via-Probe Green</td>
<td>✓</td>
<td>X</td>
<td>FITC</td>
<td>Blue</td>
</tr>
<tr>
<td>PI</td>
<td>✓</td>
<td>X</td>
<td>PE</td>
<td>Blue/YG</td>
</tr>
<tr>
<td>7-AAD</td>
<td>✓</td>
<td>X</td>
<td>PerCP-Cy™5.5</td>
<td>Blue/YG</td>
</tr>
<tr>
<td>DRAQ7™</td>
<td>✓</td>
<td>X</td>
<td>APC</td>
<td>Red</td>
</tr>
<tr>
<td>Via-Probe Red</td>
<td>✓</td>
<td>X</td>
<td>APC</td>
<td>Red</td>
</tr>
<tr>
<td>FVS450</td>
<td>✓</td>
<td>✓</td>
<td>BV421</td>
<td>Violet</td>
</tr>
<tr>
<td>FVS510</td>
<td>✓</td>
<td>✓</td>
<td>BV510</td>
<td>Violet</td>
</tr>
<tr>
<td>FVS575V</td>
<td>✓</td>
<td>✓</td>
<td>BV605</td>
<td>Violet</td>
</tr>
<tr>
<td>FVS520</td>
<td>✓</td>
<td>✓</td>
<td>FITC</td>
<td>Blue</td>
</tr>
<tr>
<td>FVS570</td>
<td>✓</td>
<td>✓</td>
<td>PE</td>
<td>Blue/YG</td>
</tr>
<tr>
<td>FVS620</td>
<td>✓</td>
<td>✓</td>
<td>PE-CF594</td>
<td>Blue/YG</td>
</tr>
<tr>
<td>FVS660</td>
<td>✓</td>
<td>✓</td>
<td>APC</td>
<td>Red</td>
</tr>
<tr>
<td>FVS700</td>
<td>✓</td>
<td>✓</td>
<td>AF700</td>
<td>Red</td>
</tr>
<tr>
<td>FVS780</td>
<td>✓</td>
<td>✓</td>
<td>APC-H7</td>
<td>Red</td>
</tr>
</tbody>
</table>

- Nucleic acid dyes bind nucleic acids non-covalently
- No-wash stain procedure
- Recommended for sort of unfixed samples
- Fixable Viability Stains bind amine moieties covalently
- Wash is required after stain
- Recommended for sort of fixed samples
Choosing fluorochromes for a cell sorting panel: exclude unwanted cells

- Choices for dead-cell exclusion
- Choices for lineage exclusion

- Dead cells
- Lineage cells
- Cells of interest
Lineage-cell exclusion

The presence of lineage cells impacts cell sorting.
• Inaccurate quantification of the population of interest
• Reduced purity
• Increased time necessary to sort a rare population

• Lineage cells can be excluded using:
  – Light scatter properties
  – Lineage cocktails
Lineage exclusion by light scatter

- In peripheral blood, different cell lineages can be easily discriminated based on light scatter.
Lineage exclusion by light scatter is not sufficient for rare population detection

- In samples such as mouse bone marrow, detection of rare stem cells is confounded by the overwhelming presence of lineage cells.
- The use of lineage markers is needed to clearly detect rare populations of interest.
### Examples of lineage marker cocktails

<table>
<thead>
<tr>
<th>Lineage cocktail</th>
<th>Marker</th>
<th>Fluorochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>CD3</td>
<td>FITC</td>
</tr>
<tr>
<td>B cells</td>
<td>CD19</td>
<td>APC</td>
</tr>
<tr>
<td>NK cells</td>
<td>CD56</td>
<td>PerCP-Cy5.5</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
<td>CD14</td>
<td>BV421</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>CD235a</td>
<td>BV786</td>
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- Take into consideration instrument configuration and available detectors.
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- Take into consideration instrument configuration and available detectors.
- Combine all lineage markers in the same format to overcome configuration limitations and to increase panel design flexibility.
Choosing a fluorochrome for a lineage cocktail

- Match the fluorochrome for the lineage cocktail with a viability dye detected in the same channel.
- In a single channel (dump channel), lineage and dead cells can now be excluded.
- Choose moderate dyes with high spillover into other detectors for the dump channel.
- Reserve dyes with bright signal and low spillover for the population of interest.

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Viability dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC</td>
<td>BD Via-Probe Green, FVS520</td>
</tr>
<tr>
<td>PerCP-Cy5.5</td>
<td>7-AAD, FVS620</td>
</tr>
<tr>
<td>APC</td>
<td>BD Via-Probe Red, FVS660</td>
</tr>
<tr>
<td>Alexa Fluor® 700</td>
<td>DRAQ7, FVS700</td>
</tr>
<tr>
<td>BV421</td>
<td>DAPI, FVS450</td>
</tr>
</tbody>
</table>
Building a lineage dump channel

- Lineage 1: PerCP-Cy5.5
- Lineage 2: PerCP-Cy5.5
- Lineage 3: PerCP-Cy5.5
- All lineages: PerCP-Cy5.5 + 7-AAD
- No lineage exclusion
- Lineage exclusion

- 2.4%
- 15.4%
Advantages of depleting lineage cells prior to cell sort

• Lineage cells can be removed from the sample prior to sort using multiple rounds of magnetic selection or cell sorting
  – Cell enrichment
  – Cell depletion

• Lineage depletion can improve cell sorts of rare population of cells
  – Increased sort efficiency
  – Increased purity
  – Reduced sort time
Magnetic depletion of lineage cells reduces sorting time...

No lineage depletion

- Theoretical time to sort $10^5$ pDCs
  - 6.5 hours

1st round of lineage depletion

- Theoretical time to sort $10^5$ pDCs
  - 2.5 minutes
...and increases purity

No lineage depletion

<table>
<thead>
<tr>
<th>Population</th>
<th>#Events</th>
<th>%Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Events</td>
<td>293</td>
<td>100.0</td>
</tr>
<tr>
<td>Region cells</td>
<td>249</td>
<td>85.0</td>
</tr>
<tr>
<td>pDC</td>
<td>218</td>
<td>74.4</td>
</tr>
</tbody>
</table>

1st round magnetic lineage depletion

<table>
<thead>
<tr>
<th>Population</th>
<th>#Events</th>
<th>%Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Events</td>
<td>1,908</td>
<td>100.0</td>
</tr>
<tr>
<td>Region cells</td>
<td>1,856</td>
<td>97.3</td>
</tr>
<tr>
<td>pDC</td>
<td>1,818</td>
<td>95.3</td>
</tr>
</tbody>
</table>
Choosing fluorochromes for a cell sorting panel: resolve the population of interest

- **Dead cells**
- **Lineage cells**
- **Cells of interest**
Detection of murine hematopoietic stem and progenitor cells

- Hematopoietic stem cells (HSCs) and common lymphoid progenitors (CLPs) are rare cell populations (<1%) in mouse bone marrow.

- HSCs: bright expression of c-kit and Sca-1

- CLPs: dim expression of c-kit and Sca-1

- Clear resolution of dim and bright c-kit and Sca-1 populations is critical for the isolation of HSCs and CLPs.

Impact of fluorochrome choice on HSC and CLP resolution

<table>
<thead>
<tr>
<th>Antigen Specificity</th>
<th>Assignment</th>
</tr>
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<tbody>
<tr>
<td>Lineage/7-AAD</td>
<td>Fluorochrome</td>
</tr>
<tr>
<td>Sca-1</td>
<td>BV421</td>
</tr>
<tr>
<td>c-kit</td>
<td>BV650</td>
</tr>
<tr>
<td>CD127</td>
<td>PE</td>
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<tr>
<td></td>
<td>PE-CF594</td>
</tr>
<tr>
<td></td>
<td>BB515</td>
</tr>
<tr>
<td></td>
<td>APC/Alexa Fluor® 647</td>
</tr>
<tr>
<td></td>
<td>BV605</td>
</tr>
<tr>
<td></td>
<td>BV786</td>
</tr>
<tr>
<td></td>
<td>BV510</td>
</tr>
<tr>
<td></td>
<td>FITC/Alexa Fluor® 488</td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5</td>
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<tr>
<td></td>
<td>V450</td>
</tr>
<tr>
<td></td>
<td>V500</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor® 700</td>
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<tr>
<td></td>
<td>APC-H7</td>
</tr>
</tbody>
</table>
Fluorochrome choice: panel 1

Antigen density: APC-H7 is not bright enough to clearly separate dim CLPs. Adjacent spillover: Co-expression of c-kit and CD127 was not taken into consideration.
## Impact of fluorochrome choice on HSC and CLP resolution

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<td></td>
<td>APC-H7</td>
</tr>
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</table>
Fluorochrome choice: panel 2

**Antigen density:** BV421 clearly separates dim and bright c-kit positive cells.

**Adjacent spillover:** Fluorochromes were spread across different lasers, for minimal spectral overlap, maximum resolution.
Fluorochrome choice: panel comparison
Recipe for best panel for sorting

• Use a dump channel to exclude dead and lineage cells.
• Magnetic depletion of lineage cells further improves the cell sort of rare populations.
• Take into consideration co-expression and spillover.
• Match the brightest fluorochromes with the antigens with lower antigen density.
Conclusion

Choices for dead-cell exclusion

Choices for lineage exclusion

Choices for populations of interest

Dead cells
Lineage cells
Cells of interest