Multicolor Flow Cytometry

Principles of Panel Design

Presented by
Mark Edinger, Scientist, BD Biosciences
Key Concepts for the Design of Multicolor Flow Cytometry Panels

- Multicolor flow cytometry is a powerful tool enabled by development of advanced flow cytometers.
- Robust assays can detect and monitor multiple analytes to produce a tremendous amount of data while conserving sample.
- Availability of different conjugates and putting the colors in the appropriate channels makes developing multicolor (>6) panels challenging.
- Data quality (results) depends greatly on proper panel design and optimization of instrument setup.
Principles of Panel Design

1. Match fluorochromes by brightness (values from the stain index) to the lowest density and smeared antigens (published values or TDSs).

2. Minimize spillover.

3. Use tandem dyes with consideration of their technical limitations.

4. Know your instrument and use the BD™ CS&T system.

5. Use appropriate controls.
1 Match Fluorochromes by Brightness

- Know the stain index of the instrument for which you are designing a panel.
  - Specific to a set of lasers and filters
- Refer to the ranking of fluorochromes by stain index for a platform.
Normalized Signal to Background: Stain Index

Stain Index (SI) = \frac{D}{W}

Goal: Normalize the signal to the spread of background where background may be autofluorescence, unstained cells, or compensated cells from another dye dimension.

\[ \text{SI} = \frac{1.645 \times (\text{positive} - \text{background})}{\text{background}_{95\%} - \text{background}_{5\%}} = \frac{\text{positive} - \text{background}}{2 \times \text{SD}_{\text{background}}} \]

Stain Index: metric used by David Parks, Stanford, presented at ISAC 2004
# CD4 Conjugates – Average Stain Index on the BD™ LSR II and BD FACSCanto™ II for CD4 Antibody

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Stain Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE-Cy™5</td>
<td>353</td>
</tr>
<tr>
<td>PE</td>
<td>302</td>
</tr>
<tr>
<td>APC</td>
<td>278</td>
</tr>
<tr>
<td>Alexa Fluor® 647</td>
<td>214</td>
</tr>
<tr>
<td>PE-Cy™7</td>
<td>139</td>
</tr>
<tr>
<td>PerCP-Cy™5.5</td>
<td>107</td>
</tr>
<tr>
<td><strong>BD Horizon™ V450</strong></td>
<td>85</td>
</tr>
<tr>
<td>Pacific Blue™</td>
<td>80</td>
</tr>
<tr>
<td>Alexa Fluor® 488</td>
<td>73</td>
</tr>
<tr>
<td>Alexa Fluor® 700</td>
<td>61</td>
</tr>
<tr>
<td>FITC</td>
<td>56</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>37</td>
</tr>
<tr>
<td>PerCP</td>
<td>37</td>
</tr>
<tr>
<td>AmCyan</td>
<td>25</td>
</tr>
<tr>
<td>APC-H7</td>
<td>24</td>
</tr>
</tbody>
</table>
Antigen Density

- Level of antigen expression on a cell:
  - Antigen expression can vary due to cell activation level and functional differences
  - Antigen density can be a range (ie, smeared population)

<table>
<thead>
<tr>
<th>Cell</th>
<th>Antigen</th>
<th>Molecules per Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell</td>
<td>TCR</td>
<td>100,000</td>
</tr>
<tr>
<td>CD2</td>
<td>55,000</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>124,000</td>
<td></td>
</tr>
<tr>
<td>CD5</td>
<td>90,000</td>
<td></td>
</tr>
<tr>
<td>CD7</td>
<td>20,000</td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td>&gt;200,000</td>
<td></td>
</tr>
<tr>
<td>CD4+ T cell</td>
<td>CD4</td>
<td>100,000</td>
</tr>
<tr>
<td></td>
<td>CD28</td>
<td>20,000</td>
</tr>
<tr>
<td></td>
<td>CCR5</td>
<td>4,000-24,000</td>
</tr>
<tr>
<td>CD8+ T cell</td>
<td>CD8</td>
<td>90,000</td>
</tr>
<tr>
<td></td>
<td>CD28</td>
<td>15,000</td>
</tr>
<tr>
<td>B cell</td>
<td>CD19</td>
<td>18,000</td>
</tr>
<tr>
<td></td>
<td>CD20</td>
<td>109,000</td>
</tr>
<tr>
<td></td>
<td>CD21</td>
<td>210,000</td>
</tr>
<tr>
<td></td>
<td>CD22</td>
<td>14,000</td>
</tr>
<tr>
<td></td>
<td>HLA-DR</td>
<td>85,000</td>
</tr>
<tr>
<td></td>
<td>CD11a</td>
<td>10,000</td>
</tr>
<tr>
<td></td>
<td>CD40</td>
<td>2,000</td>
</tr>
<tr>
<td></td>
<td>CD86</td>
<td>16,000</td>
</tr>
<tr>
<td></td>
<td>CD80</td>
<td>2,000</td>
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<tr>
<td>Dendritic cell</td>
<td>CD11a</td>
<td>27,000</td>
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<tr>
<td></td>
<td>CD40</td>
<td>17,000</td>
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<td>CD80</td>
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<tr>
<td></td>
<td>CD86</td>
<td>208,000</td>
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<tr>
<td>Monocyte</td>
<td>CD14</td>
<td>110,000</td>
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<tr>
<td></td>
<td>CD32</td>
<td>21,000</td>
</tr>
<tr>
<td></td>
<td>CD64</td>
<td>13,000</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>CD14</td>
<td>3,500</td>
</tr>
<tr>
<td></td>
<td>CD16</td>
<td>225,000</td>
</tr>
<tr>
<td>NK cell</td>
<td>CD56</td>
<td>10,000</td>
</tr>
<tr>
<td>Red Blood Cell</td>
<td>Glycophorin A</td>
<td>340,000</td>
</tr>
<tr>
<td>Basophil</td>
<td>CD23</td>
<td>15,000</td>
</tr>
</tbody>
</table>
Determine Antigen/Fluorochrome Combos

- Match brightest fluorochromes with dimmest antigens.
  - Antigen density
  - Intracellular antigens are usually dimmer and/or less discrete populations than surface antigens.
- Review antibody/fluorochrome combinations in TDSs.
  - Visually compare all antigens conjugated to same fluorochromes.

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Example

Bright antibodies go on dim fluorochromes

Example:
CD8 bright → Pacific Blue™ (SI = 80)
CD7 less bright → PE (SI = 302)

CD8 = 90,000 molecules per cell
CD7 = 20,000 molecules per cell
Principles of Panel Design

1. Match fluorochromes by brightness (values from stain index) to the lowest density and smeared antigens (published values or TDSs).

2. Minimize spillover.

3. Use tandem dyes with consideration of their technical limitations.

4. Know your instrument and use the BD™ CS&T system.

5. Use appropriate controls.
2 Minimize Spillover

- Minimize the potential for spectral overlap
  - Spillover estimates available in the BD fluorescence spectrum viewer

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Data Spread Due to Spillover

\[ \uparrow \text{spillover} = \downarrow \text{resolution} \]

Population Lost Due to Spillover

CD45 FITC spills over into the PE detector. CD4 PE dim cells cannot be separated.

CD45 PerCP DOES NOT spill over into the PE detector. Dim CD4 cells to be separated from background.
Dual Excitation Reduces Resolution

Fluorochromes that are excited by more than one laser cause high spillover.

- AmCyan excited by the violet and blue lasers spills into the FITC detector.
- PE-Cy5 excited by the blue and red lasers spills into APC detector.

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Spread Antigens Across Lasers

If multiple antigens are present on a cell, spread them across as many lasers as possible to minimize spillover.

Example:
CD3 bright        APC-Cy7 (SI = 42.2)
CD7 less bright   PE (SI = 356.3)

Both antigens expressed on same cell, low spillover of CD3 into CD7 and vice versa.

CD3 = 124,000 molecules per cell
CD7 = 20,000 molecules per cell
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Use Tandem Dyes with Consideration of Their Technical Limitations

- Compensation requirements for tandem dye conjugates can vary, even between two experiments with the same antibody.
  - Require compensation that is: lot specific, experiment specific, and label specific.

- Certain tandem dye conjugates (APC-Cy7, PE-Cy7) can degrade with exposure to light, elevated temperature, and fixation.
  - Minimize exposure to these conditions.
  - Use BD™ Stabilizing Fixative for final fixation.
Using Tandems with Intracellular Staining

- The fixation/permeabilization protocol of intracellular staining makes spillover of all Cy5 and Cy7 tandems unpredictable unless you:
  - Treat compensation controls the same as sample cells.
    - In this case you would not use BD™ CompBeads for compensation as you compensate with fixed/permed cells.
  - Stain with tandems post-fixation and permeabilization.
    - BD CompBeads can be used for compensation.
False Positives Due to Tandem Degradation

A. With CD8 APC-Cy7 and CD4 PE-Cy7

CD8 APC-Cy7+ cells  CD4 PE-Cy7+ cells

B. Without CD8 APC-Cy7

False positives in APC channel reduced in absence of APC-Cy7
False positives in PE channel remain
False Positives Due to Tandem Degradation

C. Without CD4 PE-Cy7

- False positives in APC channel remain
- False positives in PE channel reduced in absence of PE-Cy7
New Tandems Are More Stable

APC-H7 to replace APC-Cy7:

Comparison of Sample Stability
(in BD Stabilizing Fixative at RT)
Principles of Panel Design

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2. Minimize spillover.

3. Use tandem dyes with consideration of their technical limitations.

4. Know your instrument and use BD™ CS&T Setup system.

5. Use appropriate controls.
BD FACSDiva™ 6.1 Software with BD CS&T System: Automatic Instrumentation Optimization and Setup

- A revolutionary new tool that automates cytometer setup and optimizes all the key parameters for superior multicolor performance.
- The BD Cytometer Setup and Tracking (CS&T) system results in consistent instrument setups day-to-day and decreased data variability due to instrument setup.
- Application settings within BD CS&T allow you to adjust the instrument PMT voltage and laser power settings optimally for a particular assay.
  - Reproducible instrument setup and assay settings will result in superior data quality (reproducibility), especially in higher order multicolor assays.
Know Your Instrument

BD CS&T Values:

- Linearity
- Qr
- Br
- Electronic Noise (SD_{EN})
Performance Parameter: Linearity

- Defined as the proportionality of output to input (Signal:number of photons)
- Important for fluorescence compensation
- Important for quantitative measurements
  - DNA measurements
  - Antigen to antibody binding
Linearity: Effect on Compensation

- Compensation of data in the last decade involves subtraction of large numbers.
- Errors (non-linearity) in one or both large numbers can cause a large absolute error in the result.

### Detector Median Fluorescence Intensity (MFI)

<table>
<thead>
<tr>
<th>Detector</th>
<th>FITC</th>
<th>PE</th>
<th>FITC</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>68</td>
<td>80</td>
<td>1796</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>5921</td>
<td>79</td>
<td>5921</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>73,000</td>
<td>365</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BD CompBeads stained with varying levels of FITC-Ab. Compensation was set using samples A and C. This cytometer had a 2% deviation from linearity above 50,000 units.
Keeping Signals within Linear Range

- Run the brightest staining samples for each channel.
  - If the brightest populations are off scale, lower the PMT voltage to put the population on scale within the linear range.
- Save application settings.
What is Q?

Q is the efficiency of the system to detect fluorescence.

\[ Q = \frac{\text{No. of photoelectrons}}{\text{No. of fluorescence molecules}} \]

\[ Q = \frac{2 \text{ photoelectrons}}{8 \text{ fluorescence molecules}} = 0.25 \]

\[ Q = \frac{1 \text{ photoelectron}}{8 \text{ fluorescence molecules}} = 0.125 \]
Why is Qr Important?

Qr is the relative efficiency of each channel to detect fluorescence.

A system with a higher Qr has a better resolution than a system with a lower Qr.

Low Qr value = high CV = lower resolution

High Qr value = low CV = higher resolution
What is Relative Background (Br)?

Relative B (Br) is a measure of the true optical background in the fluorescence detector.

- Unbound antibody or fluorochrome
- Spectral overlap on a cell
- Scatter from the flow cell and ambient light
- Raman scatter
- Cell autofluorescence
What Factors Affect Br?

- Dirty flow cell
- Damaged optical component

<table>
<thead>
<tr>
<th>Laser</th>
<th>Detector</th>
<th>Parameter</th>
<th>Br</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>FSC</td>
<td>FSC</td>
<td>N/A</td>
</tr>
<tr>
<td>Blue</td>
<td>F</td>
<td>SSC</td>
<td>N/A</td>
</tr>
<tr>
<td>Blue</td>
<td>E</td>
<td>FITC</td>
<td>135.09</td>
</tr>
<tr>
<td>Blue</td>
<td>D</td>
<td>PE</td>
<td>320.66</td>
</tr>
<tr>
<td>Blue</td>
<td>B</td>
<td>PE-Cy5</td>
<td>3.94</td>
</tr>
<tr>
<td>Blue</td>
<td>A</td>
<td>PE-Cy7</td>
<td>29.88</td>
</tr>
<tr>
<td>Red</td>
<td>C</td>
<td>APC</td>
<td>6.24</td>
</tr>
<tr>
<td>Red</td>
<td>A</td>
<td>APC-Cy7</td>
<td>0.00</td>
</tr>
<tr>
<td>Violet</td>
<td>B</td>
<td>Pacific Blue</td>
<td>1,137.38</td>
</tr>
<tr>
<td>Violet</td>
<td>A</td>
<td>AmCyan</td>
<td>1,212.19</td>
</tr>
</tbody>
</table>

High Br due to Raman light scatter
What is the Standard Deviation of Electronic Noise (SD\textsubscript{EN})?

Electronic noise (SD\textsubscript{EN}) is the constant low level background noise contributed by the electronics system.

<table>
<thead>
<tr>
<th>Laser</th>
<th>Detector</th>
<th>Parameter</th>
<th>Electronic Noise Robust SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>FSC</td>
<td>FSC</td>
<td>N/A</td>
</tr>
<tr>
<td>Blue</td>
<td>F</td>
<td>SSC</td>
<td>N/A</td>
</tr>
<tr>
<td>Blue</td>
<td>E</td>
<td>FITC</td>
<td>23.19</td>
</tr>
<tr>
<td>Blue</td>
<td>D</td>
<td>PE</td>
<td>23.24</td>
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<tr>
<td>Blue</td>
<td>B</td>
<td>PE-Cy5</td>
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<tr>
<td>Blue</td>
<td>A</td>
<td>PE-Cy7</td>
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<tr>
<td>Red</td>
<td>C</td>
<td>APC</td>
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<td>Red</td>
<td>A</td>
<td>APC-Cy7</td>
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<tr>
<td>Violet</td>
<td>B</td>
<td>Pacific Blue</td>
<td>22.90</td>
</tr>
<tr>
<td>Violet</td>
<td>A</td>
<td>AmCyan</td>
<td>28.99</td>
</tr>
</tbody>
</table>
Decreasing Window Extension Lowers Electronic Noise
Sensitivity: Resolution vs. Background

1. Negative population has low background. Populations well resolved.

2. Negative population has high background. Populations not resolved.

3. Negative population has low background and high CV. Populations not resolved.

The ability to resolve populations is a function of both background and spread of the negative population.
4 Cytometer Setup – Application Settings

A pre-defined application settings worksheet provides a guide to setting PMT voltages based on a baseline definition.

- **Crosshair** indicates the target value for the negative population based upon $10 \times SD_{EN}$.

- Adjust the negative population to fit:
  - Within the gray box
  - Center at the crosshair
Why is PMT Optimization Important?

Finding PMT settings that maximize resolution sensitivity for each experiment:

- Move fluorescent populations out of electronic noise

<table>
<thead>
<tr>
<th>Voltage</th>
<th>CD4 dim monocytes</th>
<th>CD4 negative</th>
<th>CD4+ lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>550 volts</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>650 volts</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>750 volts</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>
4. **BD CS&T Workflow with Application Settings**

1. **Run** the BD CS&T Baseline Definition and Performance Check procedures to set the voltages.

2. **Run** fully stained cells using the Application Settings Worksheet.

3. **Run** single-stained BD CompBeads to see if each bead is brightest in its primary detector. Make a spill index calculation if necessary.
   - If not, increase the voltage in the primary detector.

4. **Record** single-stained BD CompBeads and calculate compensation.

5. **Run** samples.
Principles of Panel Design

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2. Minimize spillover.

3. Use tandem dyes with consideration of their technical limitations.

4. Know your instrument and use BD™ CS&T system.

5. Use appropriate controls.
Use FMO Controls for Accurate Data Analysis

- Fluorescence Minus One (FMO) controls contain all the lineage markers except the one of interest.
- For low density or smeared populations (e.g., activation markers), FMOs allow accurate delineation of positively vs negatively stained cells.
  - Regulatory T cells (Tregs) are a good example.
Blue = Tregs
How to Titrate Antibodies?

Oversaturated

Titrate down

Negative  Positive
How to Titrate Antibodies?

Saturated

Titrate up

Negative  Positive
When Not to Titrate?

- Do **not** titrate antibodies when the cell concentration expressing the marker is unknown.
  - Leukemias and Lymphomas
  - Many activated cells
One Last Caveat: Quantum Yield

- All fluorochromes have a potential quantum yield.
- This yield can vary with conjugation.
- This means tube combinations must be validated.
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BD Horizon™ V450 Dye
A New Violet-Excited Fluorochrome

- Several violet-excited dyes have been developed over the last few years and are currently in limited use.
- As a part of a new dye development program, BD has synthesized several new violet fluorochromes.
- One of these, BD Horizon™ V450, proved to be as bright or brighter than any of the currently existing violet-excited organic fluorochromes.
- The BD Horizon V450 dye matched 405-nm excitation better than any other current violet-excited fluorochrome.
Excitation and Emission

BD Horizon V450 better fits the laser excitation line and emission filter sets on current flow cytometers.

Figure 1. Spectral overlay comparison of BD Horizon V450 and Pacific Blue™
Brighter Than Pacific Blue™ by 15%

- Eighteen matched pairs of BD Horizon V450 vs Pacific Blue™ conjugates.
- Peripheral blood prepared by lyse/wash method with BD FACS™ Lysing solution.
- On average, V450 reagents are 15% brighter than corresponding Pacific Blue™ conjugates.
Stable pH-Independent Fluorescence

Brightness is pH independent over the biological range, 6–10.
BD Horizon V450 vs Pacific Blue™
Conjugates of CD3 (SK7) vs CD4 (SK3)

Brightness is clone specific.

V450 vs. Pacific Blue: CD3 (SK7)

V450 vs. Pacific Blue: CD4 (SK3)

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BD Horizon V450: Photostability Data

V450 is as photostable as Pacific Blue™ and much better than other commonly used fluorochromes.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Pacific Blue</th>
<th>V450</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.4 μM</td>
<td>96.3 ± 1.02</td>
<td>98.4 ± 0.35</td>
</tr>
<tr>
<td>4.7 μM</td>
<td>97.6 ± 0.32</td>
<td>98.0 ± 0.36</td>
</tr>
</tbody>
</table>

Comparative evaluation of photostability for BD Horizon V450 vs Pacific Blue™ after 12 hours of illumination (ambient light) in PBS, pH 7.4 (average of 3 percentage values ± SD)
Minimal Lot-to-Lot Variation

Results suggest that there will be minimal lot-to-lot variation.

Labeling Efficiency of 3 different lots of V450 with CD45; antibody at 2 mg/ml

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Sample Data: BD Horizon V450 hFoxP3 (259D) vs Competitor’s hFoxP3 (259D) Pacific Blue™ Both Gated on CD4⁺
If you have further questions:

Contact your US Reagent Sales Rep
or e-mail: ResearchApplications@bd.com

Please visit our BD Colors page at:
www.bdbiosciences.com/colors

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Cy™ is a trademark of Amersham Biosciences Corp.
Class I (1) laser product.
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