Incorporating New, Bright Fluorochromes into Multicolor Panel Design

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BD Biosciences
Overview

Multicolor flow: successful application prerequisites for immunophenotyping:

A. Careful reagent selection and sample preparation
   • New fluorochromes: more colors, more choices that improve population discrimination in multicolor flow cytometry

B. Proper cytometer performance, setup, and data collection

C. Proper data analysis
Immunophenotyping by Flow Cytometry (1)

- **Ultimate Goal**
  Resolve distinct subsets of leucocytes expressing one or more unique key markers

- **Method**
  The proper classification of many of the large number of distinct leucocyte subsets (phenotypic and functional) often requires simultaneous labeling with several markers: a multicolor panel.
Typical Problems and Challenges

- Some markers are highly expressed, others are expressed at low levels.
- Some dyes are much brighter than others.
- Significant emission spillover from non-primary fluorescent reagents contributes to optical background, which can often diminish the resolution of dim markers (due to spread after compensation).
- Some markers may be available only in certain colors.
- New fluorochromes are not as bright or stable as the original ones.
- Instrument setup considerations.
- Additional complexity increases error possibilities.
Multicolor flow assays and reagent panel combinations need to be very carefully assembled to obtain reliable and interpretable data.

What factors affect reagent performance on a cytometer?

What is the best approach to assemble a multicolor panel?
Multiparametric Flow Cytometry Assays

Example: TNK panel
Several markers used to define T- and NK-cell subsets:

- T-cell markers: CD3, CD8, and CD7
- NK-cell markers: CD16, CD56, and CD57
- CD8 and CD7 are also expressed by some subsets of NK cells
Principles of Panel Design: Reagent Selection

2. Match fluorochromes by brightness (values from stain index) according to antigen density and distribution (published values or TDS).
3. Minimize spectral overlap.
4. Use tandem dyes with consideration of their technical limitations.
5. Run appropriate controls.
## Instrument Configuration for 6, 8, 10, and More Colors

<table>
<thead>
<tr>
<th>6-color</th>
<th>8-color</th>
<th>10-color</th>
<th>Additional</th>
</tr>
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<td>Brilliant Violet™ 421, BD Horizon™ V450</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Q dots</td>
</tr>
</tbody>
</table>
Checking Reagent Availability

- New online tool available at bdbiosciences.com/paneldesigner

- Clone selection. Consider:
  - Cell type (example: CD16 staining for NK vs granulocytes)
  - Sample preparation (LW vs LNW)
  - TDS and literature
Principles of Panel Design: Reagent Selection

2. Match fluorochromes by brightness (values from stain index) according to antigen density and distribution (published values or TDS).
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2. **Determine Antigen/Fluorochrome Combos (1)**

- Use the brighter fluorochromes for dimly expressed markers.

- Use the dimmer fluorochromes for more highly expressed markers.
Determine Antigen/Fluorochrome Combos (2)

Classify the antigens you would like to measure.1

**Primary:** Well characterized, easily classified positive or negative (CD3, CD4, CD8, etc). Get them in as many colors as possible if you need to do testing.

**Secondary:** Well characterized, also expressed at a higher density, often over a continuum (CD27, CD28, CD45RA/RO, IFN-γ). Get some of them.

**Tertiary:** Expressed at low levels only (CD25), also uncharacterized antigens. Often available in only one or two colors.

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

<table>
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<th>Cell</th>
<th>Antigen</th>
<th>Molecules per Cell</th>
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<tbody>
<tr>
<td>T cell</td>
<td>TCR</td>
<td>100,000</td>
</tr>
<tr>
<td></td>
<td>CD2</td>
<td>55,000</td>
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<tr>
<td></td>
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<td></td>
<td>CD5</td>
<td>90,000</td>
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<tr>
<td></td>
<td>CD7</td>
<td>20,000</td>
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<tr>
<td></td>
<td>CD45</td>
<td>&gt;200,000</td>
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<tr>
<td>CD4+ T cell</td>
<td>CD4</td>
<td>100,000</td>
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<tr>
<td></td>
<td>CD28</td>
<td>20,000</td>
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<td></td>
<td>CCR5</td>
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<tr>
<td>CD8+ T cell</td>
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<td>B cell</td>
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<td>CD20</td>
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<td></td>
<td>CD21</td>
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<tr>
<td></td>
<td>CD22</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>CD11a</td>
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<tr>
<td></td>
<td>CD40</td>
<td>2,000</td>
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<td></td>
<td>CD86</td>
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<td></td>
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<td>CD11a</td>
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<td></td>
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</tr>
<tr>
<td>Basophil</td>
<td>CD23</td>
<td>15,000</td>
</tr>
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</table>
**Glossary**

**Stain Index**: 

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

- **D**: difference between positive and negative peak medians
- **W**: the spread of the background peak (\(= 2 \times rSD_{\text{negative}}\))

**Resolution sensitivity**: the ability to resolve a dim positive signal from background. “Bright” = Good Resolution Sensitivity.
Estimated Stain Index Ranking
Available from TDS

- Review antibody/fluorochrome combinations in the TDS.
- Visually compare all antigens conjugated to same fluorochromes.
Example

“Bright” antibodies go on “dim” fluorochromes

Example:
CD8 “bright” → V450 (SI = 80)
CD7 “less bright” → PE (SI = 302)
CD8 = 90,000 molecules per cell
CD7 = 20,000 molecules per cell
New Fluors are Brighter: Brilliant Violet 421

- Polymer-based dye developed by Sirigen
- Ex Max: 407 nm
- Em Max: 421 nm and 448 nm

Compatible with the standard BD Horizon V450 filter: 450/50 nm
Brilliant Violet 421: the Brightest Signal Available

PE has been the brightest fluor available...until now.

Brilliant Violet 421 is 3 to 4 X brighter than PE on equivalent clones.
New Fluors are Brighter: BD Horizon™ PE-CF594

- Tandem dye combining PE and CF594
- Ex Max: 496 nm and 564 nm
- Em Max: 612 nm
- Excited by blue (488-nm), green (532-nm) and yellow-green (561-nm) lasers
- Compatible with the standard PE-Texas Red® filter: 610/20 nm
BD Horizon PE-CF594: Improved Brightness

Improved brightness and spectral characteristics over other dyes in this detector
4. BD Horizon PE-CF594: Brightness Comparable to PE
Brighter Fluors Facilitate Discrimination of Dim Populations

Brighter = Better Resolution Sensitivity
2. PE-CF594 and Brilliant Violet 421

- Brighter than other dyes available
- Are fixable dyes
- Compatible with all BD buffers
- Useful for intracellular staining
- Support 10-color experiments
Examples: Intracellular Staining

Intracellular Cytokine Staining

- CD8 Brilliant Violet 421
- IFN-γ-PE
- CD8 Brilliant Violet 421
- IL-2 APC

Regulatory T cells: FOXP3 Staining

- CD127 A647
- CD25 Brilliant Violet 421
- FOXP3 PE-CF594
## Stain Index Comparison of Bright Fluorochromes

<table>
<thead>
<tr>
<th>Relative Brightness</th>
<th>Reagent</th>
<th>Filter</th>
<th>Stain Index</th>
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<tbody>
<tr>
<td><strong>BRIGHTEST</strong></td>
<td>Brilliant Violet™ 421</td>
<td>450/50</td>
<td>800</td>
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<tr>
<td></td>
<td>PE</td>
<td>575/26</td>
<td>232</td>
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<tr>
<td></td>
<td>BD Horizon PE-CF594</td>
<td>610/20</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>PE-Cy5</td>
<td>670/14</td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>APC</td>
<td>660/20</td>
<td>193</td>
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<tr>
<td><strong>BRIGHT</strong></td>
<td>PE-Cy7</td>
<td>780/60</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor® 647</td>
<td>660/20</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5</td>
<td>695/40</td>
<td>82</td>
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</tbody>
</table>

- Brilliant Violet 421 is the **brightest fluor** currently available.
- PE-CF594 provides a brighter alternative to ECD and PE-Texas Red®.
### Stain Index Comparison

<table>
<thead>
<tr>
<th>Relative Brightness</th>
<th>Reagent</th>
<th>Filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightest</td>
<td>Brilliant Violet™ 421</td>
<td>450/50</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>575/26</td>
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<tr>
<td></td>
<td>BD Horizon PE-CF594</td>
<td>610/20</td>
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<td></td>
<td>PE-Cy5</td>
<td>670/14</td>
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<tr>
<td></td>
<td>APC</td>
<td>660/20</td>
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<tr>
<td>Bright</td>
<td>PE-Cy7</td>
<td>780/60</td>
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<tr>
<td></td>
<td>Alexa Fluor® 647</td>
<td>660/20</td>
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<tr>
<td></td>
<td>PerCP-Cy5.5</td>
<td>695/40</td>
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<tr>
<td>Moderate</td>
<td>Alexa Fluor® 488</td>
<td>530/30</td>
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<td></td>
<td>Pacific Blue™</td>
<td>450/50</td>
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<tr>
<td>Dim</td>
<td>Alexa Fluor® 700</td>
<td>730/45</td>
</tr>
<tr>
<td></td>
<td>PerCP</td>
<td>695/40</td>
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<tr>
<td></td>
<td>APC-Cy7</td>
<td>780/60</td>
</tr>
<tr>
<td></td>
<td>AmCyan</td>
<td>525/20</td>
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<tr>
<td></td>
<td>BD Horizon V500</td>
<td>525/20</td>
</tr>
<tr>
<td></td>
<td>BD APC-H7</td>
<td>780/60</td>
</tr>
</tbody>
</table>
Principles of Panel Design: Reagent Selection

2. Match fluorochromes by brightness (values from stain index) according to antigen density and distribution (published values or TDS).
3. Minimize spectral overlap.
4. Use tandem dyes with consideration of their technical limitations.
5. Run appropriate controls.
3 Fluorescence Spillover

- Is the single most important factor affecting resolution sensitivity (SI) in multicolor flow cytometry experiments.

- Fluorescence spillover from other channels:
  - Directly and irreversibly reduces the resolution sensitivity of that channel.
  - Contributes to background.

- This “background” is subtracted in the process called compensation.
Population resolution for a given fluorescence parameter is decreased by increased spread due to spillover from other fluorochromes.
Spillover Decreases Resolution Sensitivity (2)

- Spread is **NOT** eliminated by compensation.

- More colors = more spillover = higher background.

- To improve resolution (**sensitivity**) of subpopulations, including dim subpopulations, you want to minimize the amount of spillover from other fluorochromes.
### Percent Spillover of Fluorochromes

Spillover Column into Row (calculated using BD FACS™ 7-color setup beads and BD FACSCanto™ clinical software)

<table>
<thead>
<tr>
<th>Detector</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP</th>
<th>PE-Cy7</th>
<th>APC</th>
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</thead>
<tbody>
<tr>
<td>FITC</td>
<td></td>
<td>1.57%</td>
<td>0%</td>
<td>0.22%</td>
<td>0.01%</td>
</tr>
<tr>
<td>PE</td>
<td>18%</td>
<td></td>
<td>0.32%</td>
<td>2.06%</td>
<td>0.01%</td>
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<tr>
<td>PerCP</td>
<td>2.67%</td>
<td>16.16%</td>
<td></td>
<td>4.00%</td>
<td>1.05%</td>
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<tr>
<td>PE-Cy7</td>
<td>0.32%</td>
<td>1.44%</td>
<td>10.40%</td>
<td></td>
<td>0.19%</td>
</tr>
<tr>
<td>APC</td>
<td>0%</td>
<td>0.12%</td>
<td>5.04%</td>
<td>0.08%</td>
<td></td>
</tr>
</tbody>
</table>

Before designing a multicolor experiment, **know all** the % spillovers for the fluorescence parameters to be used in your experiment.
Spillover: Brilliant Violet 421

Minimum spillover into V500/AmCyan
Dual Excitation Reduces Resolution

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

- AmCyan excited by violet and blue (FITC detector)
- PE-Cy5 spills into the APC detector

Only an issue when the two markers (CD45 and CD19) are co-expressed on the same cell population
3 Strategies to Minimize Spillover Issues

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

BD Fluorescence Spectrum Viewer
A Multicolor Tool
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
Principles of Panel Design: Reagent Selection

2. Match fluorochromes by brightness (values from stain index) according to antigen density and distribution (published values or TDS).
3. Minimize spectral overlap.
4. Use tandem dyes with consideration of their technical limitations.
5. Run appropriate controls.
4. 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.
  - Treat compensation controls the same as sample cells.

- 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.
3 Spillover and PE-CF594 Reagents

PE-CF594 reagents have consistent spillover values between lots and specificities, minimizing the need for lot specific compensation controls.

<table>
<thead>
<tr>
<th>BD Horizon PE-CF594 Reagents</th>
<th>FITC (530/30 nm)</th>
<th>PE (572/26 nm)</th>
<th>PerCp (695/40 nm)</th>
<th>PE-Cy7 (780/60 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>0.06</td>
<td>5.47</td>
<td>13.99</td>
<td>11.43</td>
</tr>
<tr>
<td>CD4</td>
<td>0.06</td>
<td>5.90</td>
<td>14.10</td>
<td>11.42</td>
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<td>CD8</td>
<td>0.07</td>
<td>5.72</td>
<td>14.26</td>
<td>11.20</td>
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<td>CD14</td>
<td>0.12</td>
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<td>CD16</td>
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<td>Average</td>
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<tr>
<td>Standard Deviation</td>
<td>0.04</td>
<td>0.24</td>
<td>0.28</td>
<td>0.42</td>
</tr>
</tbody>
</table>
Effect of Light on Tandem Degradation

CD8 PE-Cy7
CD3 PE-Cy5

0 hours
2 hours
22.5 hours
4 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: Reagent Selection

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5. Run appropriate controls.
What Controls Do You Need and Why?

Controls:

- Instrument setup controls (eg, BD™ CompBead particles)
- Gating controls (eg, FMO)
- Biological controls (eg, unstimulated samples, healthy donors)

This will allow you to:

- Obtain consistent setup and compensation
- Gate problem markers reproducibly
- Make appropriate biological comparisons and conclusions
5 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.
FMO Example

Gated on lymphs, CD3⁺ CD4⁻

Gated on lymphs, CD3⁺ CD4⁺

Full 9-color cocktail

FMO AmCyan
Coming Back to TNK Panel

Antigen Density:
High → Low

Fluorochrome brightness
Low → High

CD45 V500

CD8 V450

CD3 APC H7

CD16 PerCP-Cy5.5

CD56 APC

CD57 FITC

CD7 PE

Fluorochrome brightness changes from Low to High for each antigen.
TNK Panel (1)
Using New Fluors for TNK Panel

Antigen Density: High → Low

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Fluorochrome brightness</th>
<th>Fluorochrome</th>
<th>Corresponding Marker</th>
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<tr>
<td>CD45</td>
<td>V500</td>
<td>V500</td>
<td>CD45 V500</td>
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<td>V450</td>
<td>V450</td>
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<td>CD8</td>
<td>APC-H7</td>
<td>PerCP-Cy5.5</td>
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<td>PE-CF 594</td>
<td>PE-CF 594</td>
<td>CD16 PE-CF 594</td>
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<td>PE</td>
<td>PE</td>
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<tr>
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<td>Brilliant Violet 421</td>
<td>Brilliant Violet 421</td>
<td>CD56 BV-421</td>
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TNK Panel (2)
Acknowledgments

- Alan Stall
- Homero Sepulveda
- Kimberly Duffy
- Cynthia Lane
- Christopher Boyce
- Margaret Inokuma
- Oliver Crespo
- Patricia Collins
- Ming Yan

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