Multicolor Flow Cytometry

Principles and Examples of Panel Design

Presented by
Christopher Alfonso, PhD, Scientist, BD Biosciences
Basic Principles

- Reagent selection starts with your instrument configuration.
- Laser and optical filter choices determine dye selection.
- Optical design impacts signal detection and selection.
- Instrument setup and QC influence performance.
- Proper fluorochrome choices lead to success.
## Color Choices for 6, 8, 10, and More Colors

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<tr>
<th>6-color</th>
<th>8-color</th>
<th>10-color</th>
<th>Additional</th>
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<td>FITC or Alexa Fluor® 488</td>
<td>FITC or Alexa Fluor® 488</td>
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<td>PE</td>
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<td>PerCP-Cy™ 5.5</td>
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<td>BD Horizon™ V500 or AmCyan</td>
<td>BD Horizon V500 or AmCyan</td>
<td>BD Horizon V500 or AmCyan</td>
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<td>BD Horizon™ V450 or Pacific Blue™</td>
<td>BD Horizon V450 or Pacific Blue™</td>
<td>BD Horizon V450 or Pacific Blue™</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Q-dot 655, 705…</td>
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</tbody>
</table>

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Instrument Setup

- Determine PMT settings that maximize resolution sensitivity for each experiment.

- Track instrument performance to ensure consistency over time.
Why is PMT Optimization Important?

Finding PMT settings that maximize resolution sensitivity for each experiment: move fluorescent populations out of electronic noise.

550 volts  
CD4 dim monocytes  
CD4 negative  
CD4+ lymphocytes

650 volts  
CD4 dim monocytes  
CD4 negative  
CD4+ lymphocytes

750 volts  
CD4 dim monocytes  
CD4 negative  
CD4+ lymphocytes
BD™ Cytometer Setup & Tracking (CS&T) Beads

- Fully automated software and reagent system for BD digital flow cytometers
  - Defines and characterizes baseline performance
  - Optimizes and standardizes cytometer setup
  - Tracks cytometer performance

- Benefits:
  - Consistent, reproducible data
  - Simplifies multicolor experiment design
  - Higher quality multicolor experiment data
  - Flags degrading cytometer performance
Important QC Parameters

CS&T beads and software automatically derive and store baseline and application-specific settings.

CS&T values:

- Linearity
- Qr
- Br
- Electronic Noise ($SD_{EN}$)
QC Parameters Defined

- **Linearity**: Defined as proportionality of input to output (signal to # of photons)
  - Important for fluorescence compensation and quantitative measurements (e.g., DNA analysis, antigen/antibody binding)

- **Qr**: Relative efficiency of the system to detect fluorescence
  - Low Qr value = high CV = lower resolution
  - High Qr value = low CV = higher resolution

- **Br**: A measure of true optical background in the fluorescence detector
  - Unbound antibody, scatter from the flow cell and ambient light, Raman scattering, spectral overlap, and cell autofluorescence

- **SD_{EN}**: The constant level background noise contributed by the electronics system
A pre-defined application settings worksheet provides guides to setting PMT voltages based on 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
Rules for Proper Fluorochrome Choice

• Rule 1: Choose the brightest set of fluorochromes for your particular instrument configuration.

• Rule 2: Choose fluorochromes to minimize the potential for spectral overlap.

• Rule 3: Reserve the brightest fluorochromes for dim antibodies and vice versa.

• Rule 4: Avoid spillover from bright cell populations into detectors requiring high sensitivity for those populations.

• Rule 5: Take steps to avoid tandem dye degradation, and consider its impact upon results.
Relative Brightness

Stain Index\(^1\) (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.

Where

\[ D = \text{Difference between positive and negative peak medians} \]

\[ W = 2 \times rSD \text{ (robust standard deviation)} \]

\(^1\)Stain Index: Metric used by Dave Parks, Stanford – Presented at ISAC 2004
CD4 Conjugates: Average Stain Index on BD™ LSR II and BD FACSCanto™ II for CD4 Antibody

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Stain Index</th>
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<tr>
<td>PE-Cy™5</td>
<td>353</td>
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<tr>
<td>PE</td>
<td>302</td>
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<tr>
<td>APC</td>
<td>278</td>
</tr>
<tr>
<td>Alexa Fluor® 647</td>
<td>214</td>
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<tr>
<td>PE-Cy7</td>
<td>139</td>
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<td>PerCP-Cy5.5</td>
<td>107</td>
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<tr>
<td>BD Horizon V450</td>
<td>85</td>
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<tr>
<td>Pacific Blue™</td>
<td>80</td>
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<tr>
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<tr>
<td>AmCyan</td>
<td>25</td>
</tr>
<tr>
<td>APC-H7</td>
<td>24</td>
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</table>
Spectra Predict Spillover

- Minimize the potential for spectral overlap
  - Spillover estimates available with the BD Fluorescence Spectrum Viewer
Determine Antigen/Fluorochrome Combos

• Match brightest fluors with dimmest antigens
  – Antigen density
  – Intracellular antigens are usually dimmer and/or less discrete populations than surface antigens

• Review antibody/fluorochrome combinations in Technical Data Sheet (TDS)
  – Visually compare all antigens conjugated to the 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 = 90K molecules/cell
CD7 = 20K molecules/cell
Tandem Dyes

CD8
PE-Cy7

CD3
PE-Cy5

Time Sample
Left in Light

0 hours

2 hours

22.5 hours

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False Positives Due to Tandem Degradation

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

- False positives in APC channel reduced in absence of APC-Cy7
- False positives in PE channel remain

B. Without CD8 APC-Cy7

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

APC-H7 to replace APC-Cy7:

Comparison of Sample Stability
(in BD™ stabilizing fixative at RT)

% Spillover

CD4 APC-H7
CD8 APC-H7
CD4 APC-Cy7
CD8 APC-Cy7

Hours of light exposure

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Introducing BD Horizon V500

- Novel organic dye for the violet laser
  - Reduced spillover into the FITC channel when compared to AmCyan
  - Brighter than Pacific Orange™
  - Maximizes choice and flexibility in multicolor panel design
- Excitation max of 415 nm, emission max of 500 nm
Horizon V500 Features

Improved Brightness over Pacific Orange™

Reduced Spillover into the FITC Channel

<table>
<thead>
<tr>
<th></th>
<th>% Spillover into FITC</th>
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<tbody>
<tr>
<td>V500</td>
<td>AmCyan</td>
</tr>
<tr>
<td>Hu CD4</td>
<td>3</td>
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<tr>
<td>Hu CD19</td>
<td>5</td>
</tr>
<tr>
<td>Hu CD45</td>
<td>3</td>
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</table>

Freshly isolated lymphocytes stained with CD4 (RPA-T4) conjugated to V500 or AmCyan, CD19 (SJ25C1) V500 or AmCyan, or CD45 (HI30) V500 or AmCyan. Run on a BD LSR II, spillover detected in the FITC channel.

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# Available Products

## Ordering Information
**BD Horizon™ V500 RUO Reagents**

<table>
<thead>
<tr>
<th>Description</th>
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<td>SP34-2</td>
<td>Ms IgG₁, κ</td>
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Visit [bdbiosciences.com/colors](http://bdbiosciences.com/colors) for more information.
Panel - Naïve and Memory Human T Cells
(Cell Surface Staining)

Blue (488 nm)
• CD57 FITC
• CD11a PE
• CD28 PE-Cy5
• CD27 PE-Cy7
• CD8 PerCP-Cy5.5

Red (633 nm)
• CCR7 A647
• CD45RA APC-H7

Violet (405 nm)
• CD3 V450
• CD4 V500
CD4⁺ T-Cell Subsets
CD8$^+$ T-Cell Subsets

A Naive cells  B Experienced-1  C Experienced-2  D Experienced-3  E Experienced-4
Panel - Naïve and Memory Human T cells (Cell Surface and Intracellular Staining)

- IFN-γ FITC
- CD11a PE
- CD28 PE-Cy5
- CD27 PE-Cy7
- CD8 PerCP-Cy5.5

Blue (488 nm)

- CCR7 A647
- CD45RA APC-H7

Red (633 nm)

- CD3 V450
- CD4 V500

Violet (405 nm)
Functional Correlation with Cell Surface Markers in CD4 and CD8 Subsets after Stimulation

2h stim: CD4 cells

5h stim: CD4 cells

5h stim: CD8 cells

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Considerations with Cell Stimulation In Vitro

Down-regulation of CD4: Stimulation followed by fixation down-modulates CD3 & CD4

Partial solution to down-regulation of CD4: Stain before stimulation
Tools and Resources Available at bdbiosciences.com/colors

Fluorescence Spectrum Viewer

Absorption and Emission Spectra

- **BD Horizon™ V450 (448 nm)** is a new violet-laser excitable coumarin dye that exhibits spectral properties similar to Pacific Blue™. The BD Horizon V450 dye has an excellent quantum yield of nearly 1 (0.99), and conjugates are typically as bright or brighter than comparable reagents conjugated to Pacific Blue™.

- **Pacific Blue™ (457 nm)**, a violet-laser excitable dye, is based on the 6,8-difluoro-7-hydroxycoumarin fluorophore, and is strongly fluorescent, even at neutral pH.

- **AmCyan (493 nm)** is a 100-kDa protein derived from Anemonia majano. With an excitation peak of 493 nm and an emission peak of 493 nm, it can be used in combination with BD Horizon V450 dye or Pacific Blue™ on violet laser-equipped flow cytometers.

- **Alexa Fluor 488 (495 nm)** conjugates are highly photostable and remain fluorescent over a broad pH range. Due to its extraordinary photostability, this fluorochrome is also highly suitable for fluorescence microscopy.

- **FITC (515 nm)**, fluorescein isothiocyanate, is a fluorochrome with a molecular weight of 391 kDa. The isothiocyanate derivative (FITC) is the most widely used form for conjugation to antibodies and proteins, but other derivatives are available. FITC has a high quantum yield (efficiency of energy transfer from absorption to emission fluorescence), and approximately half the absorbed photons are emitted as fluorescent light. The number of FITC molecules per conjugate partner (antibody, avidin, streptavidin, etc.) usually in the range of three to five molecules. FITC is sensitive to pH changes and photobleaching.

- **Posters**
  - Fluorochrome Specifications Chart and the newly updated Fluorochrome Reference Guide

- **Application Note**
  - Selecting Reagents for Multicolor Flow Cytometry

- **eLearning Courses**
  - Introduction to Flow course available online

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• Bob Balderas
• Immunocytometry Research group at BD Pharmingen
• BD Pharmingen Flow-Core Facility
If you have further questions, ask us!

We are fully committed to helping you achieve the most from multicolor flow cytometry.

Contact your US Reagent Sales Rep, e-mail ResearchApplications@bd.com, or call 877.232.8995.

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

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Class I (1) laser product.

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