Multicolor Flow Cytometry: Setup and Optimization on the BD Accuri™ C6 Flow Cytometer

Presented by Clare Rogers, MS
Senior Marketing Applications Specialist
BD Biosciences
Webinar Overview

• Multicolor flow: successful application prerequisites
  o Clear definition of your experimental goals
  o Careful reagent selection and sample preparation
  o Proper cytometer performance, setup, and data collection
  o Data analysis: Proper classification of positive and negative populations
Example: HPB 4-Color T-Cell Immunophenotyping

• Goals
  o Identify **lymphs**, **monos**, and **grans** by **CD45** (common leukocyte antigen) and **SSC** properties
  o Within the **lymphocyte** population, determine the percentage of helper (**CD3**<sup>+</sup>**CD4**<sup>+</sup>) and cytotoxic (**CD3**<sup>+</sup>**CD8**<sup>+</sup>) T cells
  o Within the **monocyte** population, determine the percentage of **CD4**<sup>+</sup> cells
Principles of Panel Design: Reagent Selection

1. Identify required markers and reagents (CD45, 3, 4, 8).
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. Check reagent availability.
6. Lay out experimental plan with appropriate controls included.
Understand Relative Expression Level for Markers of Interest

- 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 (i.e., smeared population).
  - Check the literature.

Pick Possible Fluorochromes Based on Your Instrument Configuration

<table>
<thead>
<tr>
<th>Detector Position</th>
<th>Filter Options</th>
<th>Fluorophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL1</td>
<td>533/30</td>
<td>FITC, GFP, YFP, CFSE, Alexa Fluor® 488</td>
</tr>
<tr>
<td></td>
<td>510/15</td>
<td>GFP</td>
</tr>
<tr>
<td>FL2</td>
<td>585/40</td>
<td>PE, PI, PE-CF594</td>
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<tr>
<td></td>
<td>540/20</td>
<td>YFP</td>
</tr>
<tr>
<td>FL3</td>
<td>670 LP</td>
<td>PerCP, PE-Cy™5, PerCP-Cy™5.5, PE-Cy™7, PI, 7-AAD</td>
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<tr>
<td></td>
<td>610/20</td>
<td>RFP, PI</td>
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<tr>
<td></td>
<td></td>
<td>PE-CF594</td>
</tr>
<tr>
<td>FL4</td>
<td>675/25</td>
<td>APC, Alexa Fluor® 647</td>
</tr>
<tr>
<td></td>
<td>780/60</td>
<td>APC-H7, APC-Cy7</td>
</tr>
</tbody>
</table>
Choose Antigen-fluor Pairing Based on Relative Intensities

Antigen density

- CD45_{lym}
- CD3
- CD8
- CD4_{lym}
- CD45_{mon}
- CD4_{mon}
- CD45_{gran}

C6 fluor intensity

- high
  - PE
  - APC
  - PerCP-Cy5.5
- low
  - PE-Cy7
  - FITC
  - PerCP
Choose Antigen-fluor Pairing Based on Relative Intensities

<table>
<thead>
<tr>
<th>Antigen density</th>
<th>Fluor intensity</th>
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<tr>
<td>CD45\textsubscript{lym}</td>
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<tr>
<td>CD8</td>
<td>PerCP-Cy5.5</td>
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<tr>
<td>CD4\textsubscript{lym}</td>
<td>PE-Cy7</td>
</tr>
<tr>
<td>CD45\textsubscript{mon}</td>
<td>FITC</td>
</tr>
<tr>
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<td>PerCP</td>
</tr>
<tr>
<td>CD45\textsubscript{gran}</td>
<td></td>
</tr>
</tbody>
</table>

- PE → CD4 PE
- APC → CD3 APC
- PerCP-Cy5.5 → CD45 PerCP-Cy5.5
- PE-Cy7 → CD8 FITC
- FITC → CD8 FITC
Principles of Panel Design: Reagent Selection

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Fluorescence Spillover

• The single most important factor affecting resolution sensitivity (SI) in multicolor flow cytometry experiments.

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

• This “background” is mathematically accounted for in the process called compensation.
Spillover Irreversibly Decreases Resolution Sensitivity

Population resolution for a given fluorescence parameter is decreased by increased spread due to spillover from other fluorochromes.

- The dim CD4+ cells, when negative for CD8 (orange ball), are easily resolved from the double negative.
- The same dim CD4+ cells, when positive for CD8, cannot be resolved from CD4-, CD8+ cells.
- To improve resolution (sensitivity) of subpopulations, including dim subpopulations, one must minimize the amount of spillover from other fluorochromes.
Spillover Irreversibly Decreases Resolution Sensitivity

This spread is NOT eliminated by compensation.

More colors = more spillover = higher background
Average Compensation Values for Various Fluor Combos:
BD Accuri C6

FITC, PE, PerCP-Cy5.5, APC, Compensation dialog layout:

<table>
<thead>
<tr>
<th>Correct FL1 by:</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.20</td>
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<td>0.00</td>
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<tr>
<td>Correct FL2 by:</td>
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<td>PerCP-Cy5.5</td>
<td>APC</td>
</tr>
<tr>
<td></td>
<td>7.50</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>Correct FL3 by:</td>
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<td>PE</td>
<td>APC</td>
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<tr>
<td></td>
<td>0.5</td>
<td>19.50</td>
<td>0.80</td>
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<tr>
<td>Correct FL4 by:</td>
<td>FITC</td>
<td>PE</td>
<td>PerCP-Cy5.5</td>
</tr>
<tr>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td>12.00</td>
</tr>
</tbody>
</table>
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
Principles of Panel Design: Reagent Selection

1. Identify required markers and reagents (CD45, 3, 4, 8).
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3. Minimize spectral overlap.
4. Use tandem dyes with consideration of their technical limitations.
5. Check reagent availability.
6. Lay out experimental plan with appropriate controls included.
Use Tandem Dyes with Consideration of their Technical Limitations

- Compensation requirements for tandem dye conjugates can vary.

- 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.
  - Use APC-H7 when possible.

- PE-CF594 (PE-Texas Red® replacement) very stable
  - FL3 of the C6 (670 LP)
Principles of Panel Design: Reagent Selection

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Availability: Use BD FACSelect™ Multicolor Panel Designer

Antigen density

| CD45_{lym} | CD3 | CD8 | CD4_{lym} | CD45_{mon} | CD4_{mon} | CD45_{gran} |

Fluor intensity

| PE | APC | PerCP-Cy5.5 | PE-Cy7 | FITC | PerCP |

→ CD4 PE
→ CD3 APC
→ CD45 PerCP-Cy5.5
→ CD8 FITC
Tools: BD FACSelect Multicolor Panel Designer

bdbiosciences.com/research/multicolor
Step 1: Select target species
Step 2: Select specificities
Step 3: Select lasers available or fluors desired
Step 4: Search

BD FACSelect Multicolor Panel Designer

Welcome to the public beta version of the BD FACSelect™ Multicolor Panel Designer. This tool is designed to help you find antibodies for your multicolor assays. If you experience any issues, or have suggestions, please send us your feedback.

1. Search for antibodies
2. Specificities or Clones
3. Search by...
   - Lasers
   - Fluorochromes
   - Violet 405nm
   - Blue 493nm
   - Green 532nm
   - Yellow - Green 561nm
   - Red 633/640nm

4. (Buttons: Search, Clear, Save my search)
### Build your panel

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>CD45</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
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<tbody>
<tr>
<td><strong>488nm Blue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>519 FITC</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>510 Alexa Fluor® 488</td>
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<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>578 PE</td>
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<td>6</td>
<td>4</td>
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<tr>
<td>667 PE-Cy™5</td>
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<td>4</td>
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<tr>
<td>678 PerCP</td>
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<tr>
<td>695 PerCP-Cy™5,5</td>
<td>1</td>
<td>2</td>
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</tr>
<tr>
<td>785 PE-Cy™7</td>
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<td>3</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td><strong>633nm Red</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>660 APC</td>
<td>3</td>
<td>5</td>
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<td>4</td>
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<tr>
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<tr>
<td>785 APC-H7</td>
<td>3</td>
<td>3</td>
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</table>

*Note: Laser and fluorochrome compatibility vary based on the configuration of your flow cytometer. To help ensure you select products that are compatible with your setup, consult our Fluorochrome Specifications Chart, the Instrument and Fluorochrome Guide, and the Fluorochromes/Laser Reference Chart, and ask your flow lab manager about your instrument configuration.*

**Tools:** BD FACSelect Multicolor Panel Designer

**View available conjugates**
Tools: BD FACSelect Multicolor Panel Designer

Select desired clone

Close window
Tools: BD FACSelect Multicolor Panel Designer

Review the selected panel

<table>
<thead>
<tr>
<th>Build your panel*</th>
</tr>
</thead>
<tbody>
<tr>
<td>em/Max Fluorochrome</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td><strong>488nm Blue</strong></td>
</tr>
<tr>
<td>1 519 FITC</td>
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<tr>
<td>519 Alexa Fluor® 488</td>
</tr>
<tr>
<td>2 578 PE</td>
</tr>
<tr>
<td>4 667 PE-Cy™5</td>
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<tr>
<td>678 PerCP</td>
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<tr>
<td>695 PerCP-Cy™5,5</td>
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<tr>
<td>5 785 PE-Cy™7</td>
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<td><strong>633nm Red</strong></td>
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<td>1 660 APC</td>
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<tr>
<td>668 Alexa Fluor® 647</td>
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<tr>
<td>2 719 Alexa Fluor® 700</td>
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<tr>
<td>3 785 APC-H7</td>
</tr>
<tr>
<td>785 APC-Cy™7</td>
</tr>
</tbody>
</table>

**Note:** Laser and fluorochrome compatibility vary based on the configuration of your flow cytometer. To help ensure you select products that are compatible with your set-up, consult our Fluorochrome Specifications Chart, the Instrument and Fluorochrome Guide, and the Fluorochromel Laser Reference Chart, and ask your BD representative for advice.

Human CD8 FITC
RPA-T8 25 tests
Cat. No. 561947

Human CD4 PE
RPA-T4 25 tests
Cat. No. 561843

Human CD45 PerCP-Cy™5,5
2D1 50 tests
Cat. No. 340953

Human CD3 APC
SK7 100 tests
Cat. No. 340440

Add to Cart
Bookmark
Print
Before you perform your first experiment:
Optimize your new antibodies!

Optimization data: CD4$^+$ to CD4$^-$

5 µL Ab/50 µL whole blood (1/10)

1 µL Ab/50 µL whole blood (1/50)
Principles of Panel Design: Reagent Selection

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3. Minimize spectral overlap.
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6. Lay out experimental plan with appropriate controls included.
What Controls Do You Need and Why?

- Instrument setup controls (eg, BD™ CompBead particles)
- Gating controls (eg, “fluorescence minus one”:FMO)
- Biological controls (eg, unstimulated samples)

This will allow you to:

- Obtain consistent setup and compensation
- Gate populations reproducibly
- Make appropriate biological comparisons and conclusions
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 (eg, activation markers), FMOs allow accurate delineation of positively vs negatively stained cells.
Example: 4-color T-cell Immunophenotyping

• Goals
  
  o Identify lymphs, monos, and grans by CD45 and SSC properties
  
  o Within the lymphocyte population, determine the percentage of helper (CD3⁺CD4⁺) and cytotoxic (CD3⁺CD8⁺) T cells
  
  o Within the monocyte population, determine the percentage of CD4⁺ cells
## Lay Out Your Experimental Plan

<table>
<thead>
<tr>
<th>Filter</th>
<th>533/30</th>
<th>585/40</th>
<th>670 LP</th>
<th>675/25</th>
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<tbody>
<tr>
<td></td>
<td>FL1</td>
<td>FL2</td>
<td>FL3</td>
<td>FL4</td>
</tr>
<tr>
<td>Tube</td>
<td>FITC</td>
<td>PE</td>
<td>PerCP-Cy5.5</td>
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<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>CD3</td>
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</tbody>
</table>

6 - - - CD45 CD3 Gating control: FMO (Fluorescence Minus One)  
7 CD8 - CD45 CD3 FMO  
8 - CD4 CD45 CD3 FMO  
9 CD8 CD4 CD45 CD3 Test sample

Population Classifier
Webinar Overview

• Multicolor flow: successful application prerequisites
  o Clear definition of your experimental goals
  o Careful reagent selection and sample preparation
  o Proper cytometer performance, setup, and data collection
  o Data analysis: Proper classification of positive and negative populations
The BD Accuri C6 Flow Cytometer: Unique System Attributes

An affordable, full-featured, easy-to-use flow cytometer
Two lasers and six detectors
BD Accuri C6 Features that Simplify Multicolor Analysis

- Four fluorescence detectors
- Totally digital system
- Easy setup for data collection
  - Locked down optical alignment
  - No laser delay to set
  - Validate system with beads and collect data
- Predictable fluorescence spillover values
- Flexibility in fluorochrome choice using:
  - Optional optical filters
  - Selectable Lasers option
System Innovation: Alignment and Signal Detection are Optimized and Locked Down at Manufacture

- 488-nm solid state laser
- 640-nm diode laser
- PMTs for fluorescence detection
- Diodes for scatter detection
Fluorescence Detection Sensitivity is Pre-optimized, Obviating the Need for Voltage Control

SPHERO™ Rainbow Calibration Particles (Cat. No. 653144)

Allophycocyanin (APC) Calibration Particles (Cat. No. 653145)
Validate System Performance Before each Experiment, Set Appropriate Threshold, Collect Data

Keep daily bead runs all together in one BD Accuri C6 software file. View data in the Statistics Tab.
Pre-optimizing Voltage and Gain Settings does not Reduce the Fluorescence Detection Range

Dynamic Range Comparison of 3 Cytometers with Different Signal Processing (DSP) Resolution

**4-log**
(Adjustable Voltage)

**5.2-log**
(Adjustable Voltage)

**6.2-log**
(Fixed Voltage)

**Sample**: Stained CHO cells plus non-fluorescent 1.0 micron beads
Fixing Optical Alignment and Pre-optimizing Voltage and Gain Settings Results in Predictable Spillover

Suggested Compensation Values for the BD Accuri C6

<table>
<thead>
<tr>
<th></th>
<th>FITC</th>
<th>PE</th>
<th>PerCP</th>
<th>PerCP-Cy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL1 (533 BP)</td>
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<tr>
<td>FL2 (585 BP)</td>
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<td>1.50</td>
<td>0.0</td>
</tr>
<tr>
<td>FL3 (670 BP)</td>
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<td>19.5</td>
<td></td>
<td></td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>FL4 (675 BP)</td>
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<td>3.00</td>
<td>12.00</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>
Flexible Fluorochrome Choice: Optional Filters

User changeable optical filters

- 510/15
- 540/20
- 565/20
- 610/20
- 780/60

Separation of GFP and YFP signals

Standard Filters

FL1: 533/30
FL2: 585/40

GFP/YFP Filters

FL1: 510/15
FL2: 540/20

GFP
YFP
Flexible Fluorochrome Choice: Selectable Lasers Option

Selectable Lasers Module:
Reassign laser and detector associations

Standard: 488 FL1,2,3 640 FL4
2-blue 2-red: 488 FL1,2 640 FL3,4
4-blue: 488 FL1,2,3,4

<table>
<thead>
<tr>
<th></th>
<th>FL1</th>
<th>FL2</th>
<th>FL3</th>
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<td>PE</td>
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<td>PE-Cy7</td>
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</table>
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  o Data Analysis: Proper classification of positive and negative populations
Using the Controls to Analyze the Data

(1) Apply compensation using single-color controls
Using the Controls to Analyze the Data

(2) Set the gate on the desired population using a primary classifier:

In this case it is the lymphocytes, defined as $\text{CD45}_{\text{bright}} \text{SSC}_{\text{low}}$

Goal 1: Identify lymphocytes, monocytes, and granulocytes
(3) Apply the primary classifier gate to the appropriate FMO plots to determine gate placement.
Perform Calculations

(4) Subtract the background from the FMO plots to obtain percent positives for each population.

Goal 2: Of the lymphocyte population, what percentage are:

CD3⁺CD4⁺: 60.1 - 0.2 = 59.9%  
CD3⁺CD8⁺: 12.7 - 0.1 = 12.6%
Goal 3: Of the monocyte population, what percentage are CD4+?

96.13\% - 0.3\% = 95.83\%
Webinar Summary

- Multicolor flow: successful application prerequisites
  - Clear definition of experimental goals
  - Proper cytometer performance, setup, and data collection
    - The BD Accuri C6 is well suited for these applications.
    - Fluorescence detection and the optical bench are optimized at manufacture.
    - Easy to use: validate and collect samples
  - Careful reagent selection and sample preparation
    - BD FACSelect multicolor panel designer, along with antigen density and stain index charts, are BD tools that simplify this step.
  - Proper classification (analysis) of multiple combinations of positive and negative populations
Acknowledgments

- Maria Dinkelmann
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- Bob Hoffman
- Pat Collins
- Joerg Hildmann
- Holden Maecker
- Mirion Schultz
- Barny Abrams
- Laurel Nomura
- Dennis Sasaki

Thank you!

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