Panel Design: “Multi-Sizing” Your Multi-Color

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Beckman Coulter, Miami
“Multi-Sizing” into Your Multi-Color

• Fluor selection
• Tandem dyes
• Considerations for cocktail design
• Krome Orange™ - A new violet fluor
How Many Colors Needed?

TetraCHROME™ CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 with normal blood

CD45-FITC  CD4-PE  CD3-PC5
How Many Colors Needed?

T-reg cells:  
\[ \text{CD4}^+ \]
\[ \text{CD25}^+ \]
\[ \text{FoxP3}^+ \]
\[ \text{CD127}^{lo/-} \]

Up to 6-8 colors with addition of CD45 and markers for other leukocyte subpopulations
High Complexity Multi-Color Flow

Benefits
- Correlated Data
  - Single cell interrogation with multiple markers
  - Better population definition
- Labor Efficiency
  - Higher throughput with fewer tubes
  - Minimize sample volume used

Challenges
- More Colors = Greater Complexity
- Complex antibody and fluor combinations
- Requires optimization
- Requires greater expertise
Prerequisites and Pitfalls for 8+ Colors

- Clone selection: specific & avid antibodies
- Bright fluorochromes with range of Stoke’s shifts
- “Well-behaved” conjugates (stable binding; low spectral overlap; low background)
- Higher-plex flow cytometers with efficient light paths
  - BD Canto™ cytometer (8-colors)
  - BC Cyan™ cytometer (9-colors)
  - BC GALLIOS™ cytometer (10-colors)
Fluorochrome Landscape

Intrinsic Characteristics
• Extinction Coefficient
• Quantum Yield
• Emission Spectral Overlap

Instrument Optics
• Filter Selection
• PMT Sensitivity
• Laser Wavelength & Power

Comparative Intensities of CD8 Conjugates

Excitation

Blue
FITC
PE
ECD
PC5
PECy5.5
PC7

Red
APC
Alexa 700
APCAlexa 700
APC-H7
APCAlexa 750

Violet
Pacific Blue
Pacific Orange

+ Nanocrystals

Brighter Fluors

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Dye Options: 10-Colors

- 405nm: Pacific Blue
- 488nm: FITC, PE, ECD, PE-Cy5.5, PE-Cy7
- 635nm: APC, APC-AF700, APC-Cy7, APC-AF750

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Dye Options: Conventional Fluors

- 405nm
- 488nm
- 635nm

Dye Options: Conventional Fluors

- Pacific Blue
- Krome Orange
- FITC
- PE
- APC or AF647
Spectra of Common Fluorochromes

• Critical Fluorescence Properties
  – Extinction Coefficient
  – Quantum Efficiency
  – Stoke’s Shift
  – Excitation wavelength

• Consider
  – Available excitations
  – Emission filters

R-PE (565/576)
\[ \varepsilon = 2,000 \, \text{K} \]

Fluorescein (495/518)
\[ \varepsilon = 78 \, \text{K} \]

APC: (650/662)
\[ \varepsilon = 700 \, \text{K} \]
Conjugation Chemistry

Brightness: Optimization of F/P molar ratio
- Minimize impact on antibody binding affinity
- Maximize fluorescence at saturation dosing

Performance: Influenced by multiple factors
- Site of covalent linkage to the antibody
  - Fc – minimal impact on binding affinity
  - F(ab) Region – competition with antigen binding
- Molecular weight (size) of dye molecule
- Hyperconjugation
  - Fluorescence quenching due to close coupling proximity
  - Non-specific binding
  - Dye/Cell aggregation
Performance Impact: Organic Dye Ratio

CD4-Alexa Fluor 488

CD3-Alexa Fluor 488

Fluorimeter
Flow Cytometry

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Dye Options: Tandem Dyes

- 635nm
- 488nm
- 405nm

Dye Options: Tandem Dyes
Fluorescence Resonance Energy Transfer

• Definition
  – Excitation is transferred from donor to acceptor \textit{without emission of a photon}.
  – Donor / acceptor molecules in close proximity (10–100 Å)
  – Acceptor absorption spectrum must overlap donor emission spectrum
  – Donor and acceptor transition dipole orientations must be approximately parallel.

• Advantages
  – Expands fluorochrome choices from single laser source
  – Enhanced fluorescence intensity versus organic dyes
Fluorescence Resonance Energy Transfer

- **Definition**
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- **Advantages**
  - Expands fluorochrome choices from single laser source
  - Enhanced fluorescence intensity versus organic dyes

- **Limitations**
  - Lot-to-lot variation
    - Fluorescence sensitivity
    - Energy transfer efficiency
    - Non-specific binding to myeloid populations
  - Photo- and chemical instability
Patented Tandem Dye Process

Native State Phycobiliprotein → Unfold Protein → Couple Acceptor Dye → Refold to Native State

Conjugation process delivers optimum fluorescence intensity
Patented Tandem Dye Process

Three Lot Comparison of PC5
Process controls variability

Dye Coupling Step

HIC Purification Step

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**Impact on Compensation**

*must* treat different vendor tandems as different fluorochromes for compensation set-up!
Tandem Dye Selection: Dual Laser

Minimized Spectral Overlap = Better Resolution
Enhanced on photo-stability of APC-Alexa Fluor 750 conjugate regardless of paraforaldehyde
Performance Impact: Antibody & Dye

Non-Specific Binding

Binding due to Fc receptor
CD14-PECy5

Binding due to Cy dye binding
CD3-PECy5

Conjugate/Dye Aggregation

CD15-FITC (IgM)

0.25 µg

0.125 µg

0.063 µg

0.031 µg
Advancing the Science of Cytometry

Proprietary Chemistry / Enhanced Specificity

- Low background fluorescence on negative populations
- Optimal signal to noise
- Low affinity binding of cyanine dyes to monocyte populations eliminated
Fluor Choice

- **Surface Antigens**
  - Large selection of conjugates
  - Limited by detection sensitivity and proximity of co-expressed antigens
  - Tandem dyes provide ↑ sensitivity over organic dyes
  - Moderately bright organic fluors (FITC, Alexa Fluor dyes, violet-excited dyes) good for subset gating

- **Intracellular Antigens**
  - Cytoplasmic antigens
    - Phycobiliproteins and organic dyes may be better
    - Alexa Fluor 488 better than FITC – lower background
  - Nuclear antigens
    - Phycobiliproteins or tandem dyes hindered due to conjugate size?
    - Close proximity can lead to FRET between dyes
Instrument Contributions

- Channels available
- Sensitivity in channels

GALLIOS™ cytometer
- 3 lasers
- 10 colors

Comparative Fluorochrome Sensitivity between Platforms

- FITC
- PE
- ECD
- PECY5
- PECY5.5
- PECY7
- APC
- Alexa Fluor 700
- APCA700
- APCA750
- Pacific Blue
- Pacific Orange
Optimizing the Combination

• Determine fluorochrome/conjugate strategy
  – Organic dyes to maximize spectral separation for gating reagents
  – PE & APC used for antigens with continuum of expression
  – Tandems dyes for mid-density to bright antigens
• Perform titration curves for each conjugate
  – Determine Signal/Noise Ratio
  – Choose optimal dose: Saturation, Highest S/N
• Prepare combination, verify performance
  – Always use controls – approach can vary
    • Negative Control: negative population, FMO, isotype controls
    • Positive controls:
      – Each antibody as single color
      – Known positive control material
  – Evaluate performance for major interactions
What Can Go Wrong?

• Potential conjugate interactions
  – Non-specific binding
    • Aggregate formation between conjugates
    • Cyanine & Alexa Fluor dye binding to myeloid populations
  – Steric Hindrance
    • Ligand – receptor binding blocked due to physical interference
  – Fluorescence Quenching
    • Over conjugation of antibody
    • Concentration and proximity on the cell
  – Unwanted FRET
Dose Optimization: Multi-Step Process

Single color titrations:
- Optimal S/N
- Saturation binding when possible

Combination Matrix to finalize dosing
- Target optimal S/N dose for each component
- Evaluate for potential interactions
- Evaluate multiple doses:
  Simple matrix or DOE

<table>
<thead>
<tr>
<th>CDxx</th>
<th>2X</th>
<th>1x</th>
<th>½ x</th>
</tr>
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<tbody>
<tr>
<td>2X</td>
<td>2,2</td>
<td>2,1</td>
<td>2, ½</td>
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<tr>
<td>1x</td>
<td>1,2</td>
<td>1,1</td>
<td>1,½</td>
</tr>
<tr>
<td>½ x</td>
<td>½, 2</td>
<td>½, 1</td>
<td>½, ½</td>
</tr>
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</table>
Spectral Overlap

Impact on co-expressed antigens

- Spectral Overlap/Compensation
  - Loss of low end resolution
  - Display artifacts

- If bright signal overlaps into PMT containing dimmer signal
  - Increased “noise”
  - Spread of the negative population
  - Difficulty in accurate determination of low level positivity

ECD shows higher overlap into PE than PC7

![Graphs showing spectral overlap and compensation](image-url)
Effect of Antigen Proximity

- APC conjugates of CD3, CD8, and CD45 versus PE-labeled tetramer
- FRET from PE to APC results in FL3 signal (PerCP channel)
- CD3 & CD8 close to TCR; CD45 antigen spatially separated from TCR
Assay Interferants: Washing

Kappa/Lambda Resolution

- Requires high sensitivity
- Dependant on sample preparation methodology
- Pre-wash required to remove plasma immunoglobulins
### Beckman Coulter Solastra™ Panels

<table>
<thead>
<tr>
<th>B-cell Kit</th>
<th>FITC</th>
<th>PE</th>
<th>ECD</th>
<th>PC5.5</th>
<th>PC7</th>
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<td>Lambda</td>
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<td>CD10</td>
<td>CD19</td>
<td>CD38</td>
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<td>CD2</td>
<td>CD56</td>
<td>CD7</td>
<td>CD5</td>
<td>CD45</td>
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<tr>
<td>CD8</td>
<td>CD4</td>
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<td>CD3</td>
<td>CD45</td>
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<table>
<thead>
<tr>
<th>T-cell Kit</th>
<th>CD15</th>
<th>CD11b</th>
<th>CD16</th>
<th>CD14</th>
<th>CD45</th>
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<tr>
<td>CD7</td>
<td>CD13</td>
<td>CD34</td>
<td>CD13</td>
<td>CD33</td>
<td>CD45</td>
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<table>
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<tr>
<th>Myeloid Kit</th>
<th>CD15</th>
<th>CD11b</th>
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<th>CD14</th>
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<td>HLADR</td>
<td>CD56</td>
<td>CD34</td>
<td>CD117</td>
<td>CD45</td>
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</tr>
</tbody>
</table>

Aligned with Bethesda Recommendations

* Not available for sale in US
Peripheral Blood: Solastra B-cell Kits

**B-CLL #1:**
CD45++/CD19+/CD5+/−/CD20++/Kappa<sub>bright</sub>+
New Violet-Excitable Dye

- **GALLIOS™ Configuration:**
  - 3 laser 10 color instrument
    - 405nm laser – 2 colors
    - 488nm laser – 5 colors
    - 635nm laser – 3 colors

- **Krome Orange™ dye**
  Second violet-excitable fluor to pair with Pacific Blue™ dye
Krome Orange Spectrum

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Molar Extinction Coefficient (M⁻¹·cm⁻¹)</th>
<th>Absorbance Maximum (nm)</th>
<th>Emission Maximum (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krome Orange Dye</td>
<td>17,685</td>
<td>398</td>
<td>528</td>
</tr>
<tr>
<td>Pacific Blue Dye</td>
<td>46,000</td>
<td>410</td>
<td>455</td>
</tr>
<tr>
<td>Pacific Orange Dye</td>
<td>24,500</td>
<td>400</td>
<td>551</td>
</tr>
<tr>
<td>AmCyan</td>
<td>NA</td>
<td>458</td>
<td>489</td>
</tr>
<tr>
<td>V500 Oye</td>
<td>NA</td>
<td>415</td>
<td>500</td>
</tr>
</tbody>
</table>

- KO
- V500
- Pac Or
- AmCyan
Krome Orange Conjugation

CD45-Krome Orange Titration Curve

- CD45-Krome Orange Titration Curve
  - Median Fluorescence
  - µg Conjugate per Test
  - F:P 17.3
  - F:P 15.9
  - F:P 14.0
  - F:P 10.8
  - F:P 6.1

CD14 (RMO52)-Krome Orange
- SI = 101.3
- Lymphocytes
- Monocytes

CD16 (3G8)-Krome Orange
- SI = 18.6
- Lymphocytes

CD19 (J4.119)-Krome Orange
- SI = 9.2
- Lymphocytes

Krome Orange Conjugation
Krome Orange vs Other Violet Fluors

**Krome Orange Dye** (550/40)

- CD3: UCHT1 SI = 33.2
- CD4: 13B8.2 SI = 36.6
- CD8: B9.11 SI = 81.7
- CD45: J.331 SI = 29.65

**Pacific Orange Dye** (575/26)

- CD3: UCHT1 SI = 26.1
- CD4: S3.5 SI = 13.5
- CD8: 3B5 SI = 34.7
- CD45: HI30 SI = 16.03

**AmCyan** (525/50)

- CD3: SK71 SI = 43.5
- CD4: SK3 SI = 20.6
- CD8: SK1 SI = 48.5
- CD45: 2D1 SI = 21.20

**V500 Dye** (525/50)

- CD3: SK71 SI = 8.67
- CD4: SK3 SI = 4.19
- CD8: SK1 SI = 13.32
- CD45: RPA-T8 SI = 3.54

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# Krome Orange vs Other Violet Fluors

Relative compensation values

<table>
<thead>
<tr>
<th>Violet Dye</th>
<th>Target</th>
<th>Pacific Blue - % Violet Dye (FL9 - %FL10)</th>
<th>Fluorescein - % Violet Dye (FL1 - %FL10)</th>
<th>PE - % Violet Dye (FL2 - %FL10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Krome Orange dye</strong></td>
<td>CD3</td>
<td>1.7</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>1.9</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>3.3</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Pacific Orange dye</strong></td>
<td>CD3</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<td></td>
<td>CD8</td>
<td>1.8</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>AmCyan</strong></td>
<td>CD3</td>
<td>22.6</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>21.7</td>
<td>&gt;100</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>29.5</td>
<td>&gt;100</td>
<td>19.3</td>
</tr>
<tr>
<td><strong>V500</strong></td>
<td>CD8</td>
<td>15.1</td>
<td>8.5</td>
<td>2.8</td>
</tr>
</tbody>
</table>
Krome Orange: 4-Color Stain

FL9-%FL10 = 0.5%
FL10-%FL9 = 5.8%

FL9-%FL10 = 0.0%
FL10-%FL9 = 5.8%

CD4-Pacific Orange
CD8-Fluorescein
CD19-PE
CD4-Krome Orange

CD4-Pacific Orange
CD8-Fluorescein
CD19-PE
CD4-Krome Orange

CD4-Pacific Orange
CD8-Fluorescein
CD19-PE
CD4-Krome Orange

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Krome Orange: 10-Color Stains

- CD3+ gated
- Side Scatter
- CD8-Pacific Blue
- CD4-Pacific Orange
- CD3-APC
- CD45-Krome Orange
- CD14-PC5
- FL9 - %FL10: Krome Orange™ 1.5, Pacific Orange™ 0.0
- FL10 - %FL9: 9.1, 9.3
Krome Orange: CD45/Side Scatter

CD45-ECD

CD45-Pacific Orange

CD45-V500

CD45-Krome Orange
Summary

- Multi-parametric flow analysis provides a powerful tool
  - Dissection of complex cell populations
  - Identification of underlying mechanisms of disease states
  - Increased efficiency in laboratory testing

- Optimal design is critical for scientifically valid results
  - More colors = Greater complexity
  - Match fluorochrome choices to the platform capability
  - Pair dye intensity with antigen density
  - Violet-excited fluors can easily add 2 parameters

- Validate your applications prior to initiating studies
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  Mark Sandison
  Rhonda Federspiel

Collaboration
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Questions and Answers?

Upcoming Webinars

2 Sep 2010
_T. Vincent Shankey_, Ph.D, Beckman Coulter
Cytometry of Cell Signaling: Monitoring Signal Transduction
Monitoring Signal Transduction Pathways in Human Disease

9 Sep 2010
_Robert Zigon_, Beckman Coulter
Data “Flow” – Rethinking Data Analysis for Flow Cytometry

16 Sep 2010
_John Norman_, Beckman Coulter
The Perfect 10: A Technology Overview of Beckman Coulter’s New Multi-color Solution for Flow Cytometry

23 Sep 2010
_Laura Nieto Gligoroski_, Ph.D., Beckman Coulter

30 Sep 2010
_Bill Kirouac_, Beckman Coulter
Light Forward Scatter Innovation in the Gallios Flow Cytometer

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