Spectral Flow Cytometry

Conventional flow cytometry uses mirrors and filters to select specific wavelength ranges for detection of signal from different fluorophores on individual PMTs. Spectral flow cytometry uses dispersive optics, such as prisms or gratings, to disperse the collected light across a detector array, allowing the full spectra from each particle to be measured.


Spectra of dyes excited by blue (488 nm) laser
Cytek Aurora’s Optical Design

Unique Optical Design
• High Sensitivity Collection Optics
• Lasers are spatially separated. Each excitation laser has an associated solid state multi channel semiconductor array detector module

Full Spectrum Analysis
• Entire emission spectrum is captured across the different modules and then stitched together to create a spectral signature that combines emission information from all three excitation wavelengths

Spectral Unmixing
• Spectral unmixing algorithms calculate the contribution of each known fluorophore’s spectra to the total collected emission signal
5 Laser Aurora: Optical Design
The entire emission spectra of fluorescent dyes excited by the onboard lasers is measured.

Emission spectra excited by the UV, Violet, Blue, Yellow-Green and Red lasers are measured from the laser line to the infrared region.

Full spectrum capture enables the use of novel unmixing algorithm for data analysis.
Markers that are co-expressed can effectively be used in combination.

Plot gated on singlet lymphocytes.

Full Spectrum Enables Use of Highly Overlapping Dyes.
# 5 Laser Aurora: Detector Arrays

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Ultraviolet Laser Unique Signatures

- BUV395
- Live/Dead Blue
- BUV496
- BUV563
- BUV661
- BUV737
- BUV805
Violet Laser Unique Signatures

- BV421: Alexa Fluor 405, Super Bright 436, Zombie Violet
- BV570: Pacific Orange, Live/Dead Yellow
- Qdot655: Super Bright 702, BV711
- BV480: eFluor 450, VioBlue, Pacific Blue, Live/Dead Violet
- Super Bright 600, BV605
- Qdot705
- BV480: Zombie Yellow
- BV750
- eFluor 506
- Qdot605
- BV510, VioGreen, Zombie Aqua, Live/Dead Aqua
- BV650, Super Bright 645
- Qdot800
Blue Laser Unique Signatures

BB515, Vio 515, sVio 515

Alexa Fluor 488, FITC, VioBright FITC, Zombie Green, Live/Dead Green

Alexa Fluor 532

PerCP-Cy5.5

PerCP-Vio700, PerCP-eFluor710

PerCP
Yellow Green Unique Signatures

- PE
- PE-eFluor 610, PE/Dazzle 594, PE-TxRed, PE-CF594, Live/Dead Red
- PE-Alexa Fluor 610
- Zombie Red

- PE-Cy5
- PE-Cy5.5
- PE-Alexa Fluor 700
- PE-Cy7, PE-Vio 770
Red Laser Unique Signatures

- **APC**
- **Alexa Fluor 647, Vio 667, sVio 667, eFluor 660, Live/Dead Far Red**
- **APC-Cy5.5**
- **Alexa Fluor 700, APC-R700**
- **Zombie NIR**
- **APC-Alexa 750, APC/Fire 750, APC-Cy7, APC-Vio 770, APC-eFluor 780, APC-H7**
- **Live/Dead NIR**
Compensation vs. Spectral Unmixing

Conventional Cytometer - Compensation

- Each fluorochrome is associated with a primary detector. For an n color assay, n detectors are needed.
- Using single stained controls, spillover is mathematically removed by subtracting out the % photons of light contribution from the non-primary color into the primary detector, a mathematical process called compensation.
- A compensation matrix is calculated: it is a square matrix, nxn.

Spectral Analyzer - Unmixing

- Each fluorochrome is detected in multiple channels. In the 5 laser Aurora analyzer, there are 64 fluorescent channels.
- The number of detectors has to be higher than the number of fluorochromes.
- Single stained controls are used to establish the signatures of each fluorochromes.
- Unmixing is used to determine which combination of reference controls best fits the multicolor spectral signature of a multicolor sample.
- An unmixing matrix is calculated: it is an nx64 rectangular matrix.
1. Run **UNSTAINED control**
2. Run **individual dye spectra controls (Reference Controls)**
3. Unmix (equivalent to Compensation step in conventional cytometer)

**Reference Spectra from Single Stain Controls**

**Raw Worksheet** -> **Unmixing Algorithm** -> **Unmixed Worksheet**
**Raw vs. Unmixed Data**

**RAW DATA**
- Parameters are the instrument channels (V1, V2, etc)
- Visualized in raw worksheet
- Large fcs file size: up to 64 parameters + FSC and SSC
- Can be unmixed as many times as desired

**UNMIXED DATA**
- Parameters are the fluorochromes included in the assay
- Visualized in unmixed worksheet
- Smaller fcs file size: number of fluors + FSC and SSC
- Can not be used to unmix
Need to calculate spillover (slope) between fluorochromes

**How to get an accurate calculation?**

- The more separate the two data points are, the better the calculation
  - Bright particles are necessary for this
- Both particles need to have IDENTICAL autofluorescence characteristics
  - If negative particles are beads, then the positive particle need to be the exact same beads (same lot)
- There is need to have enough events for both data points
  - Stopping rules need to be adjusted according to the sample type and marker used
- The fluorescence spectrum of the positive data points needs to be IDENTICAL to the one in the multicolor sample
  - Special considerations when using tandem dyes
  - The spectrum of the reagent binding to beads may be different to the spectrum of the same reagent when bound to cells!
Unstained Control vs Negative Population in Reference Control

- In addition to the Reference Controls, an **Unstained Control is mandatory for Spectral Unmixing**
  - This control is NOT needed for spillover calculation
  - This control is used for measurement of autofluorescence
    - ALWAYS needed for unmixing even without autofluorescence extraction
  - This control needs to exactly match the particle type and sample prep procedure used in the multicolor samples

- **If Reference Controls do not have a negative population:**
  - New software 2.1 allows for additional unstained controls
    - Negative cells MUST match the cells used as reference controls for spillover calculation
    - Negative beads MUST match beads used as reference controls for spillover calculation
Unstained control troubleshooting, human PBMCs

Expected

Provided by User
Reference Controls QC Examples (2)

Qdot 605 control troubleshooting

![Expected](image1.png)

![Provided by User](image2.png)
**Should I use beads or cells as controls?**

- Beads are easy to use and it is very likely that they will have a bright positive signal. It’s also easy to collect enough events.
- HOWEVER, users need to assess whether the signature of the reagents used to stain the beads matches the one when stained on cells
  - If possible, compare unmixing results using beads vs cells as reference controls
  - Users also need to assess how forgiving a specific assay is if there are errors in the calculations

**I want to use cells, but my marker is rare or very dim. What can I do?**

- If a fluorochrome is NOT a tandem, replace with a marker highly expressed in a distinct population (CD3, CD4, CD8, B220 etc). Example: instead of using CD25 PE, use CD4 PE.
- If fluorochrome is a tandem, only option is to use beads stained with exactly same reagent (same lot)
Rules for Using Beads as Controls

• Fluorochrome spectrum signature needs to be IDENTICAL to be one when antibody is bound to cells
  • Beads should be treated as the cells in order to ensure fluorochromes have been in the same “environment” (exposure to same buffers, for same amount of time, etc)

• Intensity does matter: beads need to be equally bright or brighter than cells to be an adequate control for a given fluor

• Each of these requirements are equally important
Panel Design: Gathering Information

1. STARTING POINT: BIOLOGY!!!
   a) Antigen Classification: primary, secondary and tertiary
   b) Antigen co-expression

2. What fluorochromes should I use for my assay?
   a) How many antigens I want to detect?
   b) What are the best X number of fluors that I can use?

3. What antibodies are commercially available?
   Make a table, antigens vs. fluor
Antigen Classification

- **Primary**: high density, on and off expression
- **Secondary**: relatively high density, continuous expression
- **Tertiary**: Uncharacterized or expressed at low levels.

Antigen Classification

**Level of Antigen Expression**

**PRIMARY**
- CD3
- CD19
- CD56
- CD14
- CD16
- CD11b
- CD4
- CD8

**SECONDARY**
- CD45RA
- IgD
- CD27
- CD28
- CD127
- HLA-DR
- CD27
- CD38
- CD1c
- CD95

**TERTIARY**
- CD1c
- CD123
- CD127
- PD-1
- CD25
- TCR γ/δ
- CD11c
Assessing Antigen Resolution

**Main Contributors for Resolution Reduction**
- Instrument Performance
- Instrument Setup
- Fluorochrome Brightness

**Main Contributors for Resolution Reduction**
- SPREAD!!!
- Antibody titer
Quantification of Impact of Spread in Resolution

**SINGLE COLOR SCENARIO**

- **Marker A Fluor X**
  - Stain Index Marker A Fluor X
  - MFI Fluor X

- **Marker A Fluor Y**
  - Stain Index Marker A Fluor Y
  - MFI Fluor X

**MULTICOLOR SCENARIO**

- **Fluor X**
  - Does not spread into **Fluor Y**
  - MFI Fluor Y

- **Fluor Y**
  - Does not spread into **Fluor X**
  - MFI Fluor X

**Considerations**

- **CO EXPRESSION**
- **ANTIGEN LEVEL OF EXPRESSION**
- Data used for calculations has to be unmixed using a certain combination of fluorochromes
Co-expression and antigen classification are needed for correct fluorochrome choice.

FMO

antigen and overlapping fluor co-expressed
Panel Design: Fluor Assignment

Same rules apply as conventional cytometry!

1. Fluorochrome assignment for tertiary antigens
   a) Assess reagent availability (often not too many options available)
   b) Assign brightest fluor available (use fluor brightness ranking)

2. Fluorochrome assignment for secondary antigens
   a) Based on CO-EXPRESSON of antigens expressed at intermediate levels
   b) If no co-expression, use any bright dye still available
   c) If co-expression:
      • if available, use a bright dye that does not spread into selected fluor for tertiary antigens
      • If only available dyes have spread, use a dim dye to minimize spread impact

3. Fluorochrome assignment for primary antigens
   a) Often available in many colors
   b) Try to assign to dyes that are dim and that have minimal spread in other dyes (examples: FITC, Pacific Blue, BV510, Alexa 532, APC H7)
Cross Stain Index Matrix for 30 Fluorochromes

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How to read this table: the fluor in the row impacts the one in the column. Red means the fluor in that row has significant spread into the dye in the column (for example PE into BV570). Areas in bright pink and red is where more attention to panel design is needed.
1. **Strongly suggested settings to use as a starting point for any application**

2. What are Cytek assay settings?
   a) Settings established using biological samples
   b) Ensure optimal resolution for each detector
   c) Leave enough room to accommodate bright markers
   d) Ensure unique spectrum with accurate emission peak for all currently tested dyes
   e) Spread minimized as much as possible (remember.. there will always be spread!)

3. When to modify these settings?
   a) ONLY if signals are off scale
   b) Increasing the gains will not result in more resolution and in contrast can result in increased spread!
**Issue:** BV421 signal in V1-V3 is off scale

1. Decrease V1 (primary channel of BV421) gain until V1 is on scale
2. Change V2 and V3 gains proportionally to maintain the minor differences in the spectrum of BV421, Super Bright 436, eFluor 450

*Only three channels gain needs to be changed so that we don’t sacrifice other dyes resolution while keeping reasonable spectrums for all dyes.*