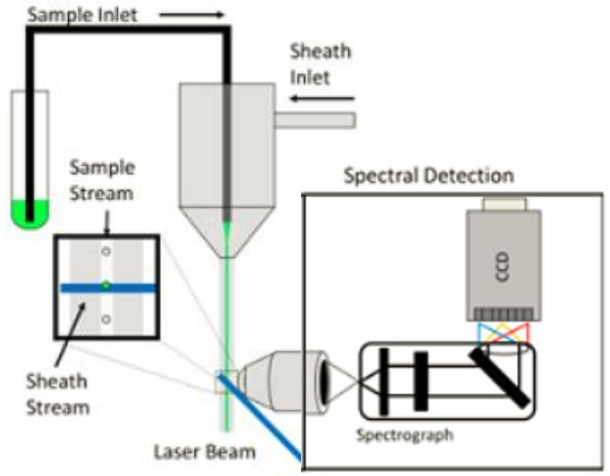
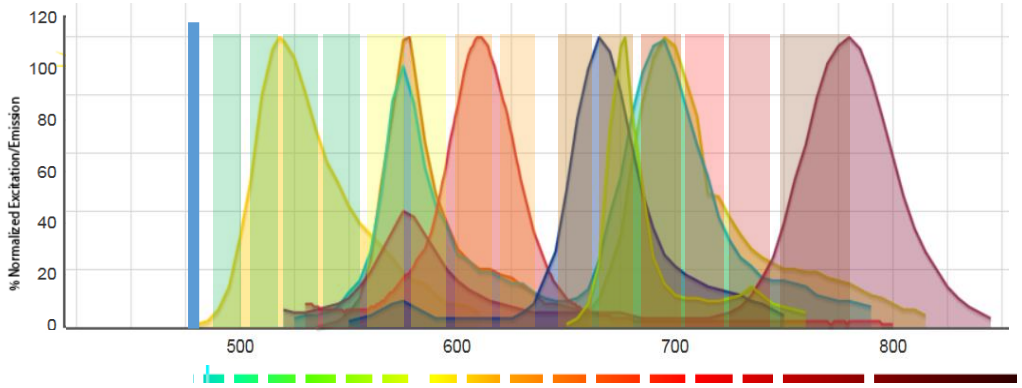
Spectral Flow Cytometry Definition

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.

Nolan and Condello (2013) Current Protocols in Cytometry





Spectra of dyes excited by blue (488 nm) laser

Cytek Aurora's Optical Design

Unique Optical Design

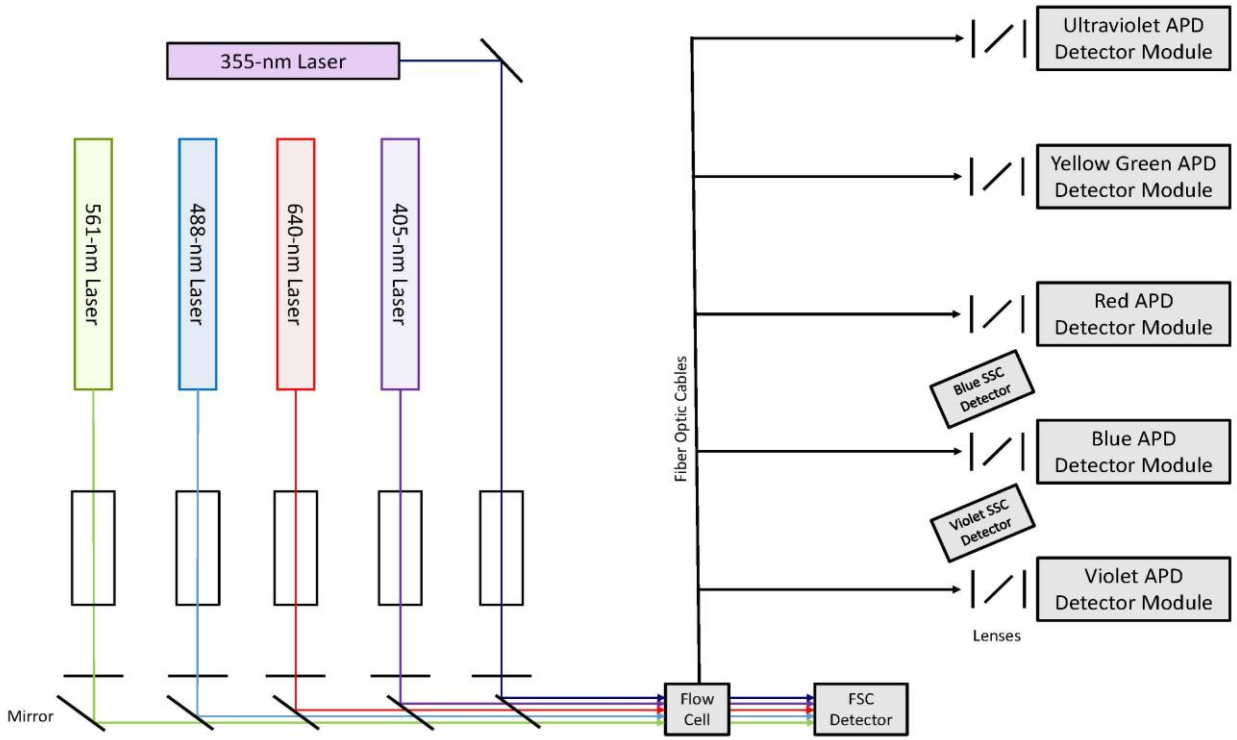
- High Sensitivity Collection Optics
- Lasers are spatially separated. Each excitation laser has an associated solid state multi channel semiconductor array detector module

Full Spectrum Analysis

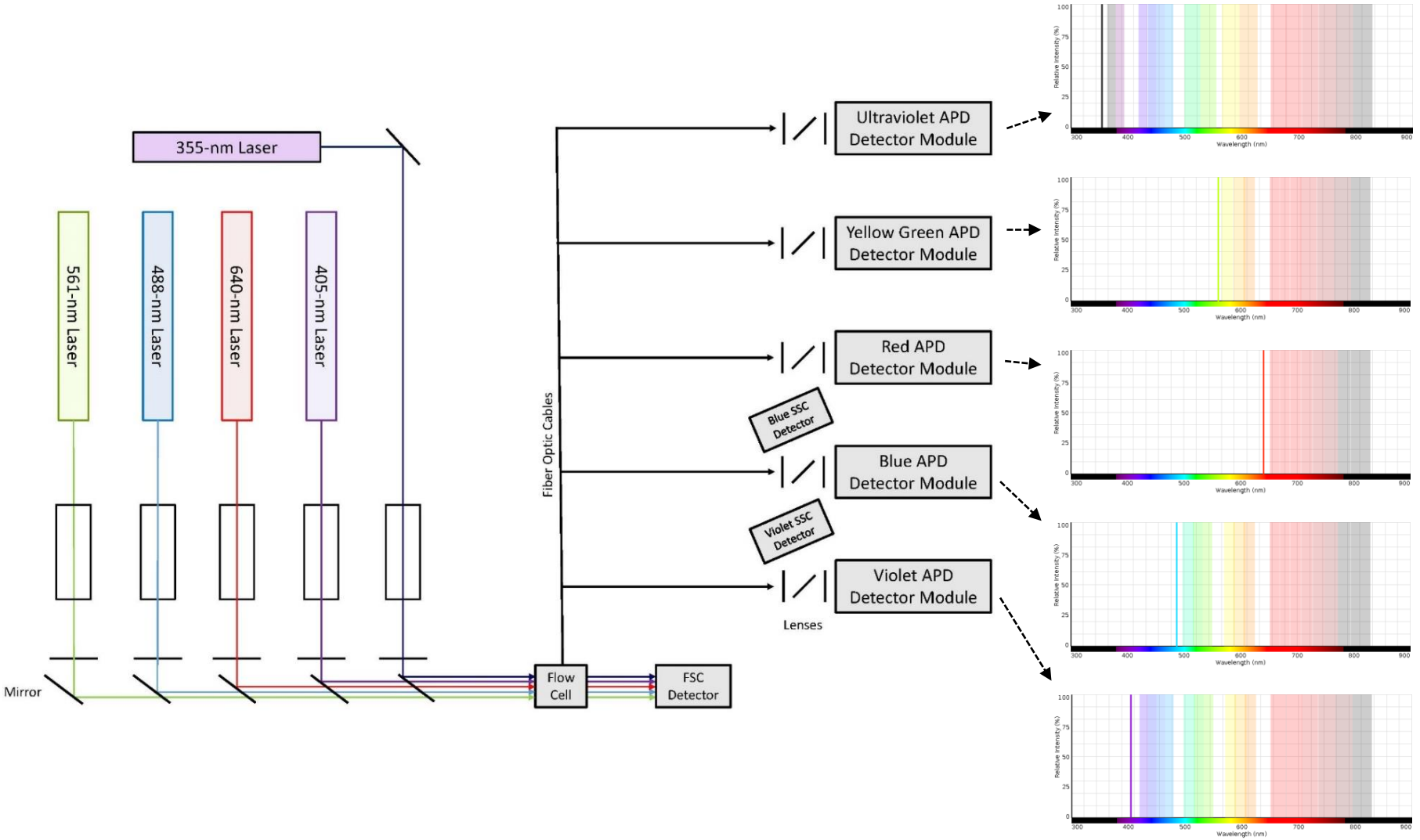
- Entire emission spectrum is captured across the different modules and then stitched together to create a spectral signature that combines emission information from all three excitation wavelengths

Spectral Unmixing

- Spectral unmixing algorithms calculate the contribution of each known fluorophore's spectra to the total collected emission signal

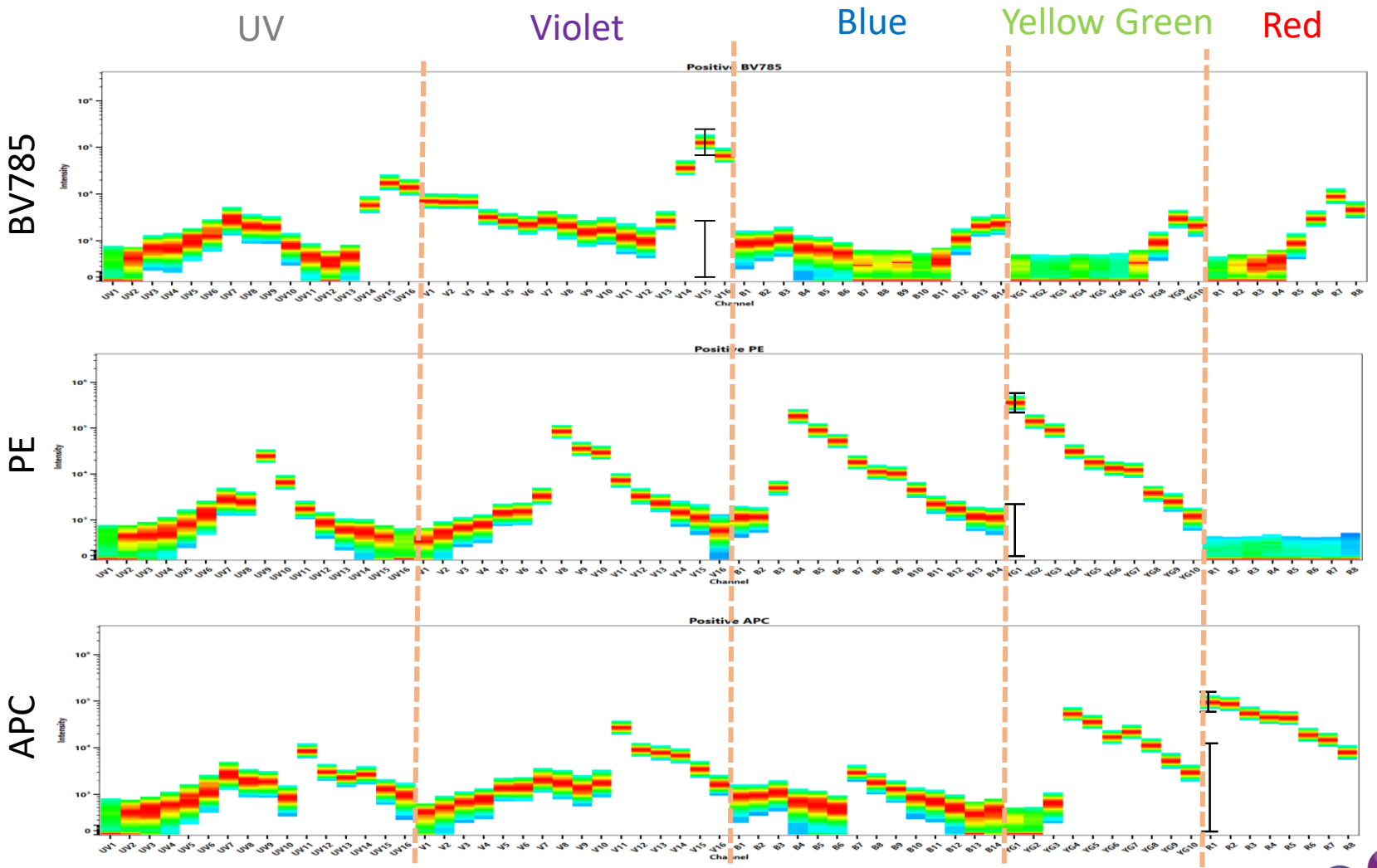


5 Laser Aurora: Optical Design

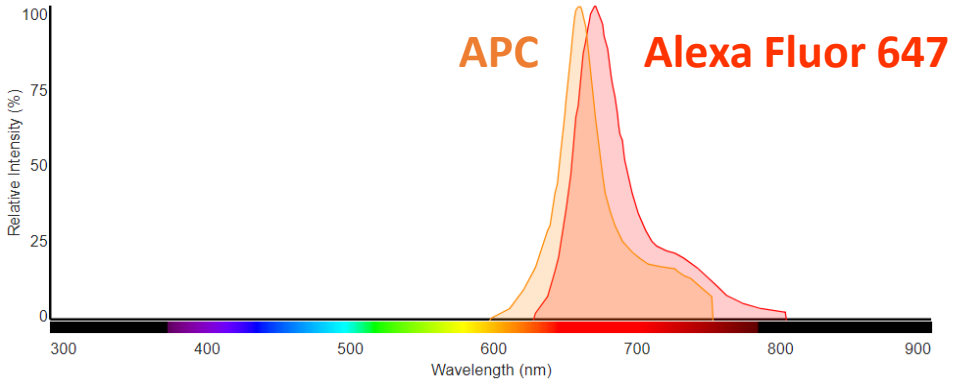


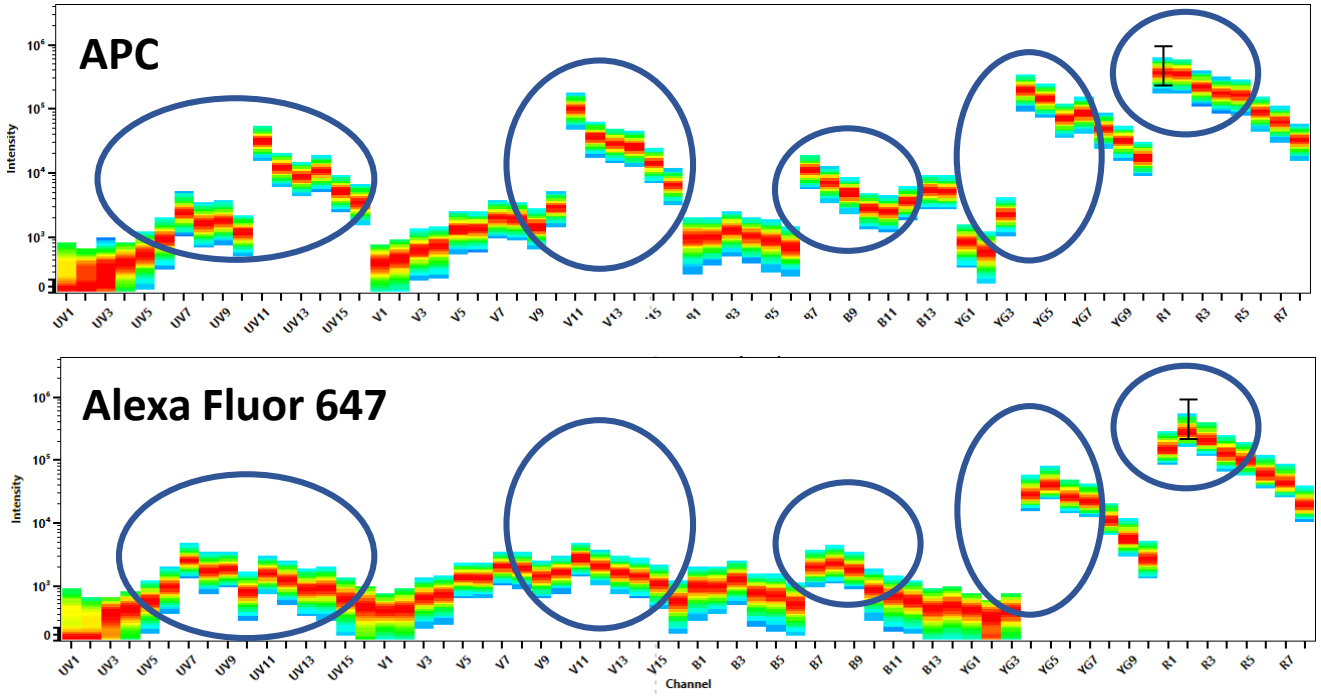
Full Spectrum Signatures

- 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.

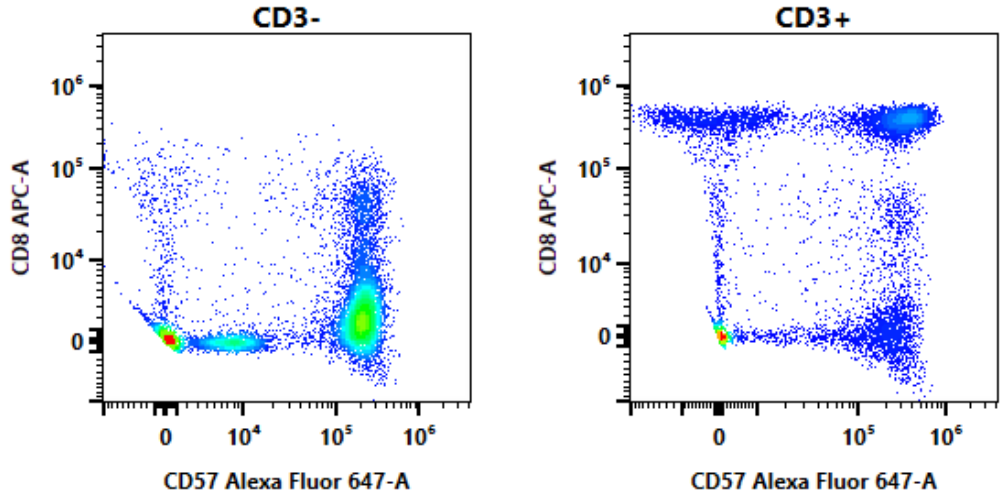


Full Spectrum Enables Use of Highly Overlapping Dyes





Plot gated on singlet lymphocytes



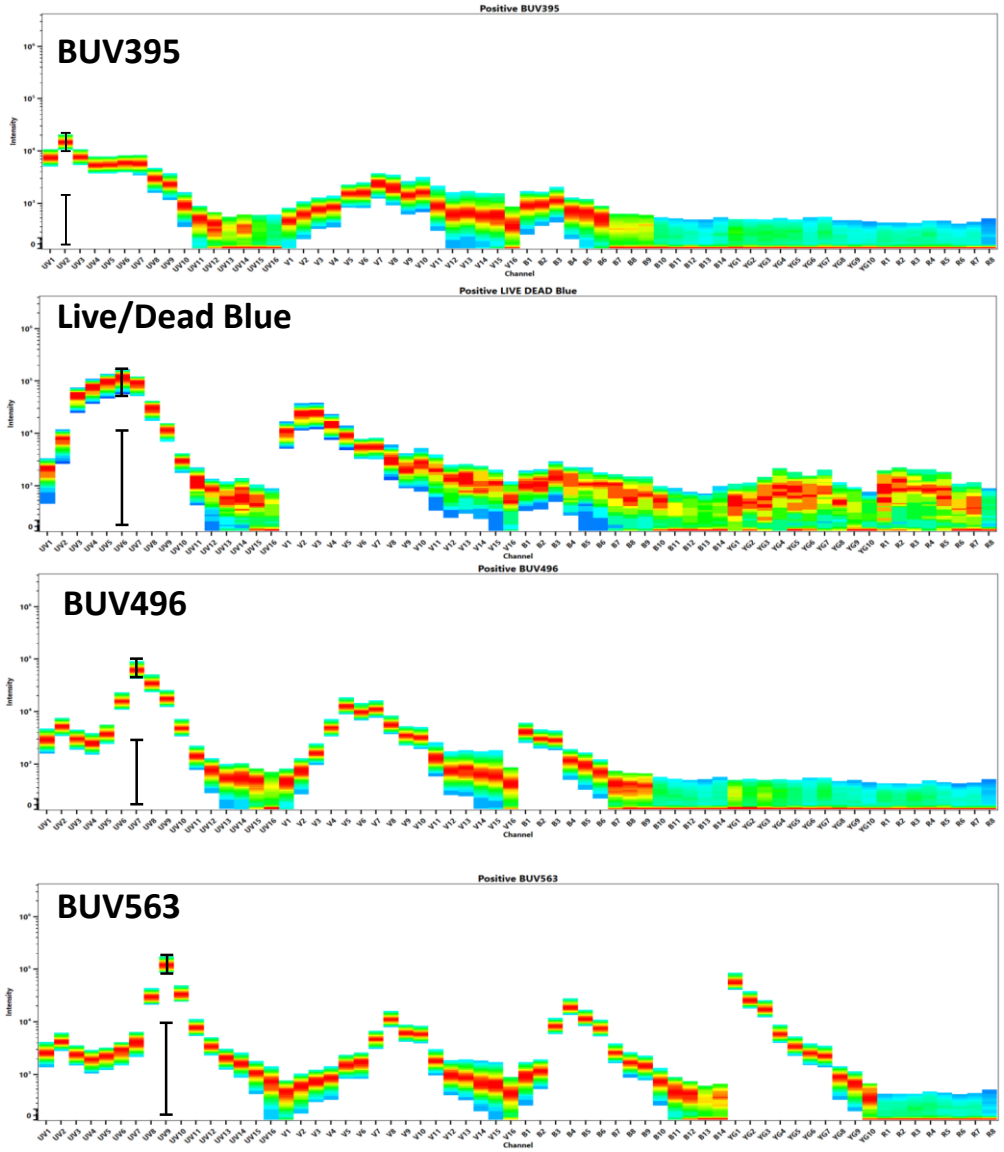
Markers that are co expressed CAN effectively be used in combination

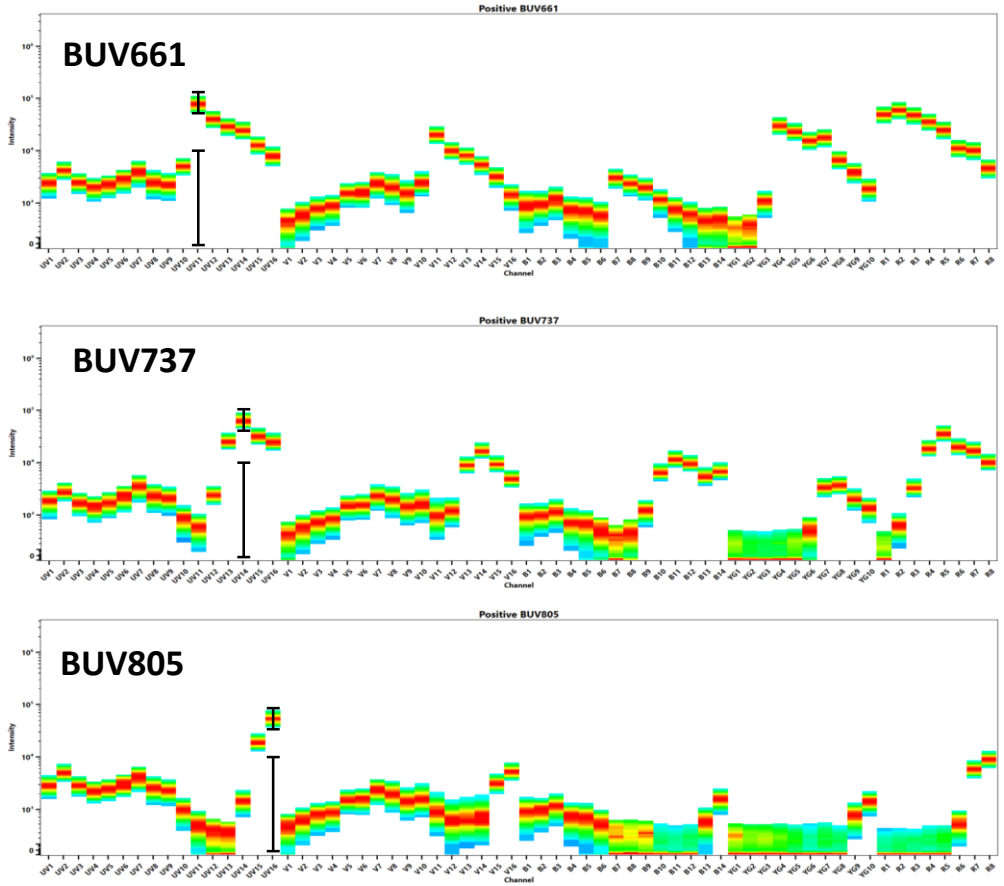
5 Laser Aurora: Detector Arrays

Laser	Channel	Center Wavelength (nm)	Bandwidth (nm)	Wavelength Start (nm)	Wavelength End (nm)
Ultraviolet	UV1	372	15	365	380
	UV2	387	15	380	395
	UV3	427	15	420	435
	UV4	443	15	435	450
	UV5	458	15	450	465
	UV6	473	15	465	480
	UV7	514	28	500	528
	UV8	542	28	528	556
	UV9	581	31	566	597
	UV10	612	31	597	628
	UV11	664	27	650	677
	UV12	691	28	677	705
	UV13	720	29	705	734
	UV14	750	30	735	765
	UV15	780	30	765	795
	UV16	812	34	795	829
Violet	V1	428	15	420	435
	V2	443	15	436	451
	V3	458	15	451	466
	V4	473	15	466	481
	V5	508	20	498	518
	V6	525	17	516	533
	V7	542	17	533	550
	V8	581	19	571	590
	V9	598	20	588	608
	V10	615	20	605	625
	V11	664	27	651	678
	V12	692	28	678	706
	V13	720	29	706	735
	V14	750	30	735	765
	V15	780	30	765	795
	V16	812	34	795	829

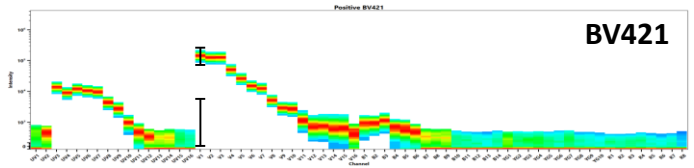
Laser	Channel	Center Wavelength (nm)	Bandwidth (nm)	Wavelength Start (nm)	Wavelength End (nm)
Blue	B1	508	20	498	518
	B2	525	17	516	533
	B3	542	17	533	550
	B4	581	19	571	590
	B5	598	20	588	608
	B6	615	20	605	625
	B7	660	17	652	669
	B8	678	18	669	687
	B9	697	19	688	707
	B10	717	20	707	727
	B11	738	21	728	749
	B12	760	23	749	772
	B13	783	23	772	795
	B14	812	34	795	829
Yellow Green	YG1	577	20	567	587
	YG2	598	20	588	608
	YG3	615	20	605	625
	YG4	660	17	652	669
	YG5	678	18	669	687
	YG6	697	19	688	707
	YG7	720	29	706	735
	YG8	750	30	735	765
	YG9	780	30	765	795
	YG10	812	34	795	829
Red	R1	660	17	652	669
	R2	678	18	669	687
	R3	697	19	688	707
	R4	717	20	707	727
	R5	738	21	728	749
	R6	760	23	749	772
	R7	783	23	772	795
	R8	812	34	795	829

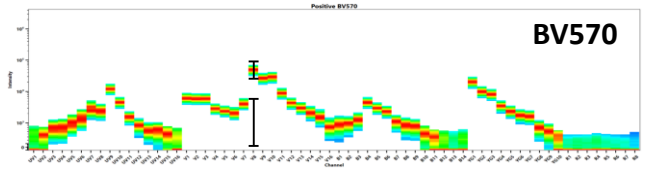
Ultraviolet Laser Unique Signatures

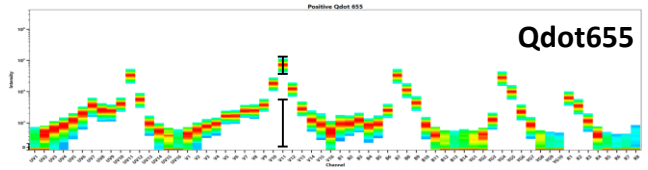


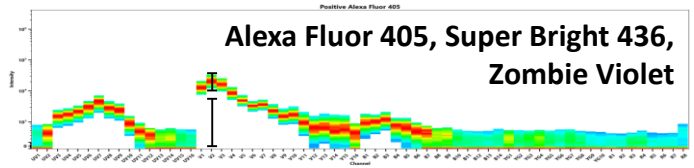


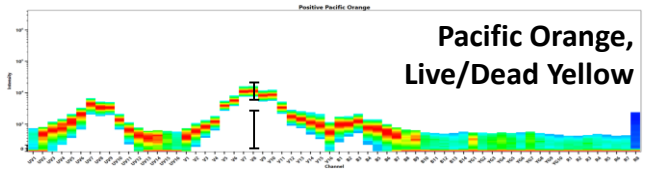
Violet Laser Unique Signatures

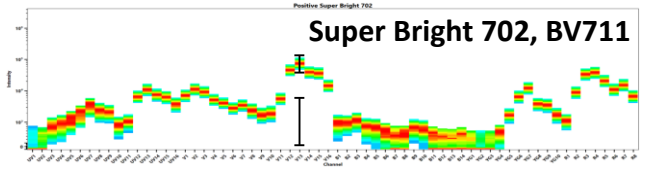


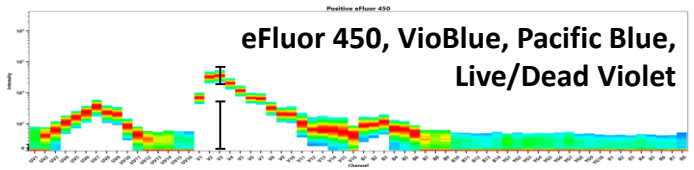


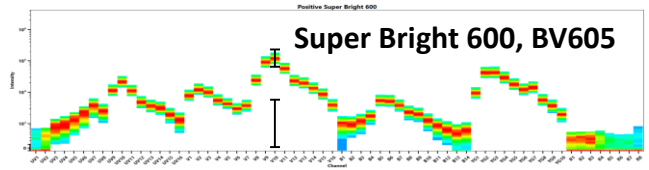


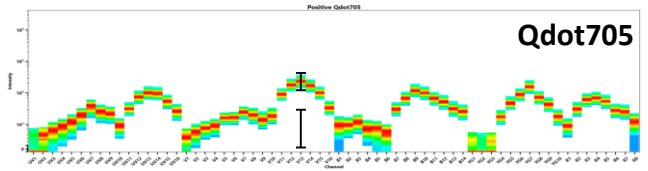


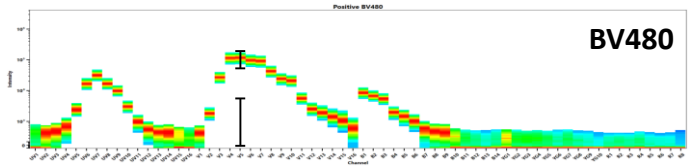


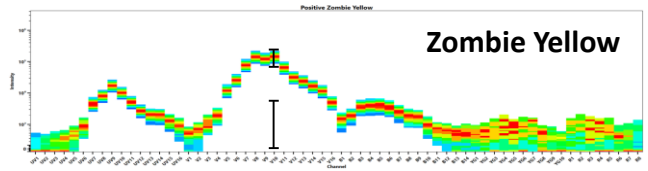


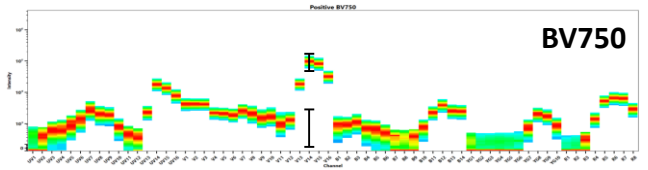


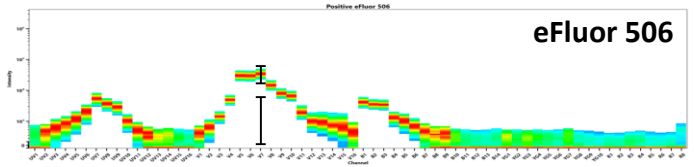


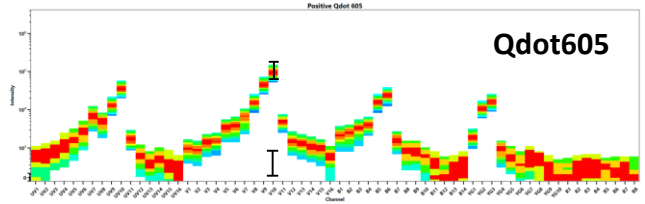


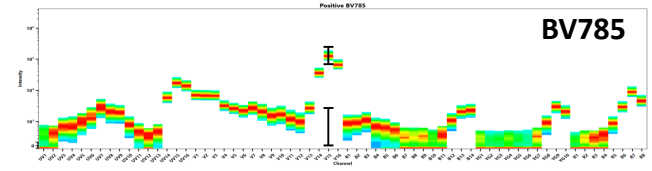


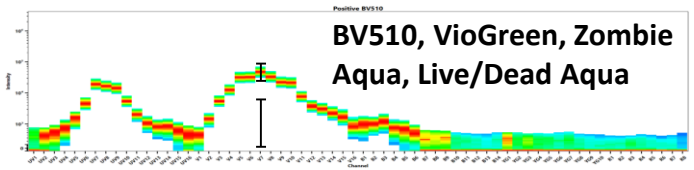


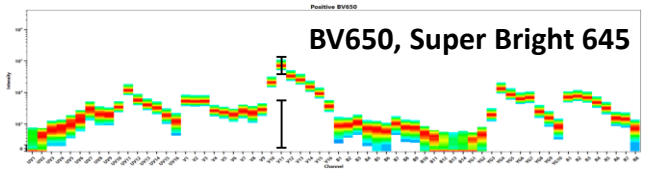


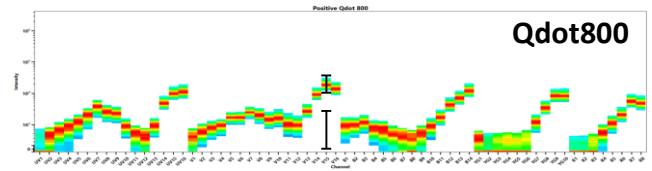




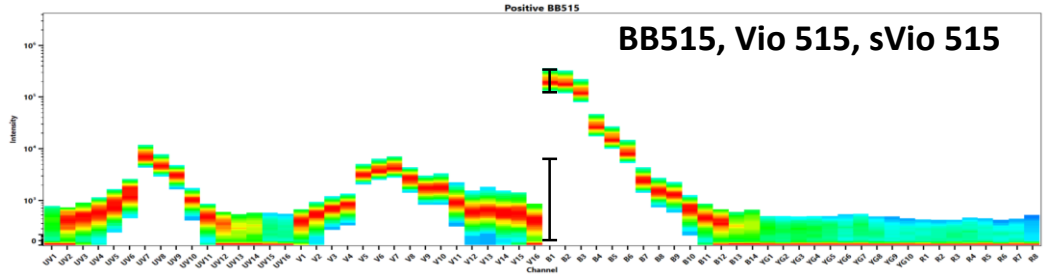


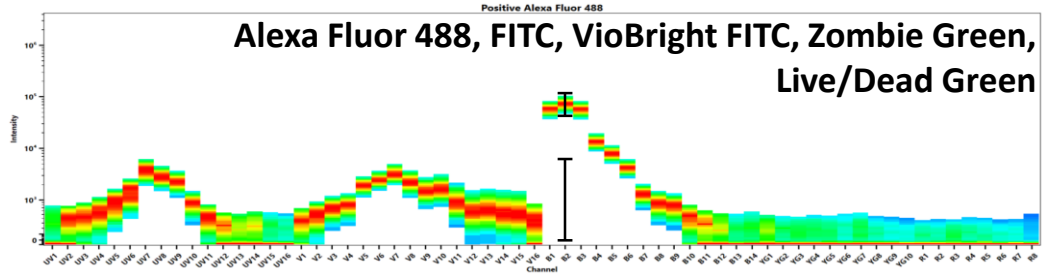


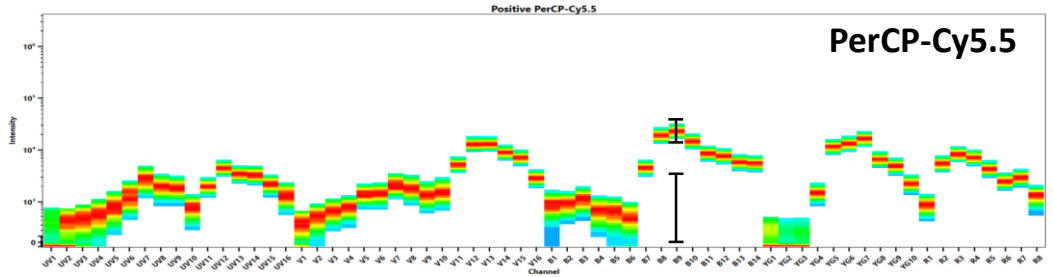


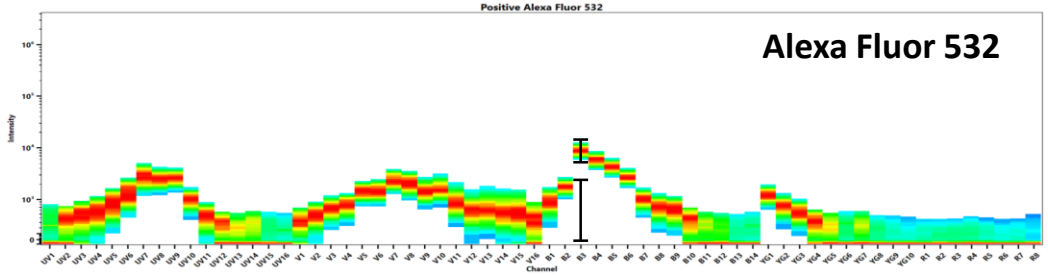


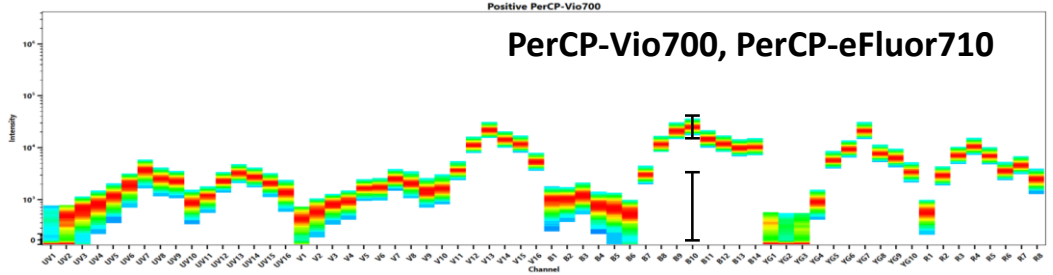
Blue Laser Unique Signatures

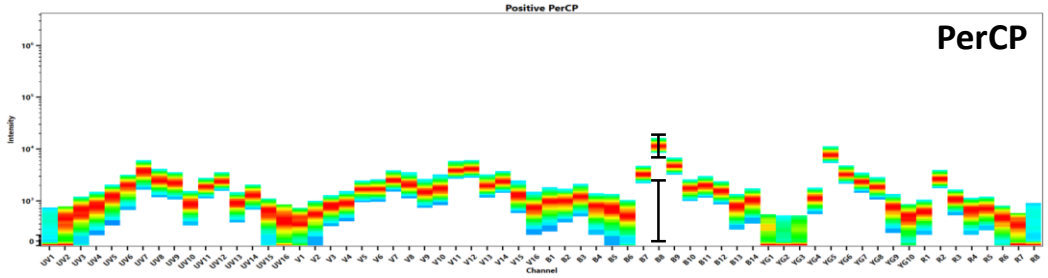




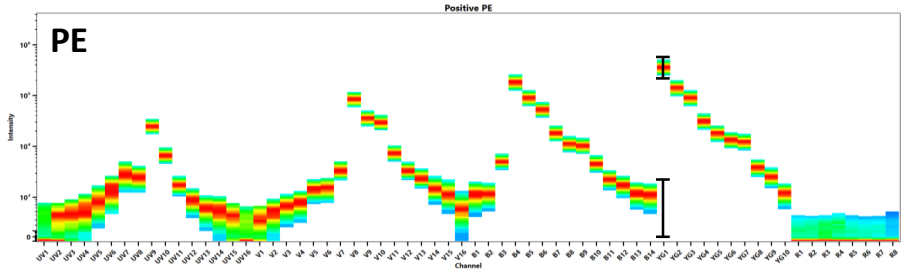


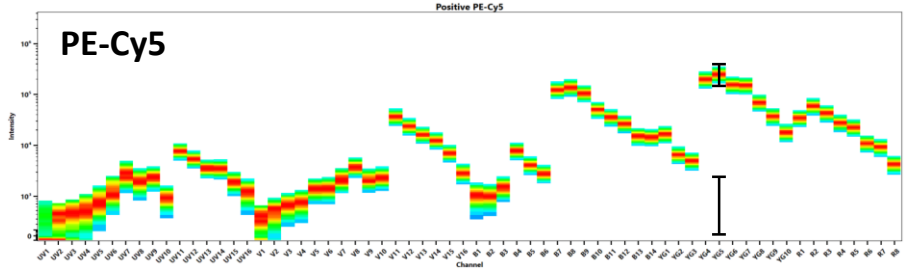


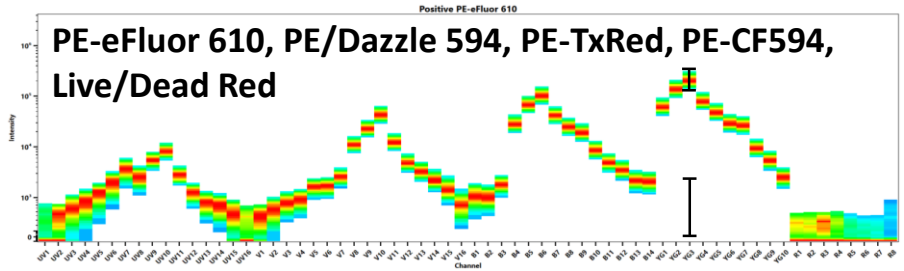


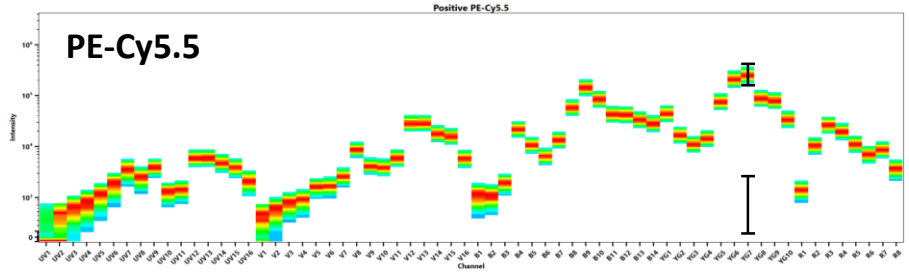


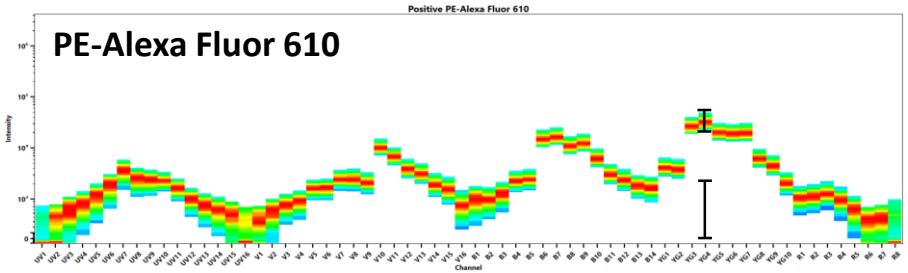
Yellow Green Unique Signatures

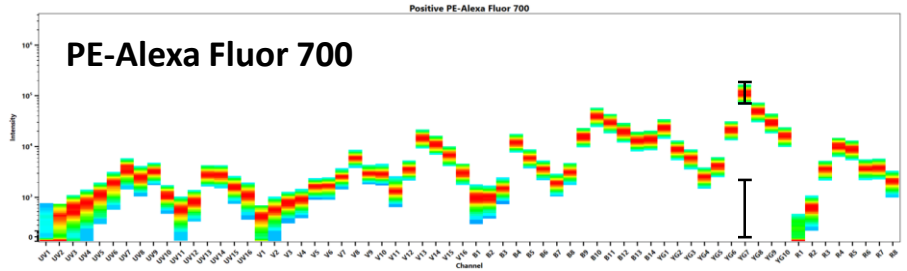


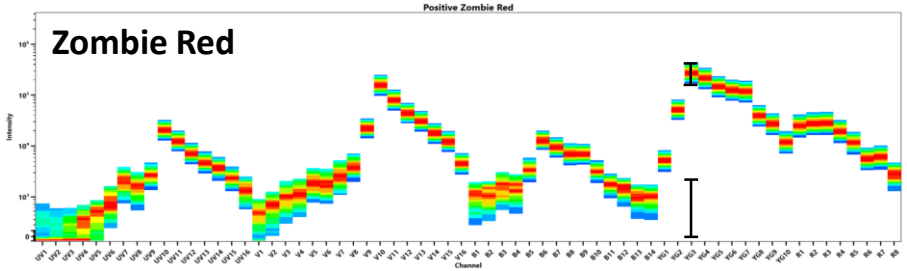


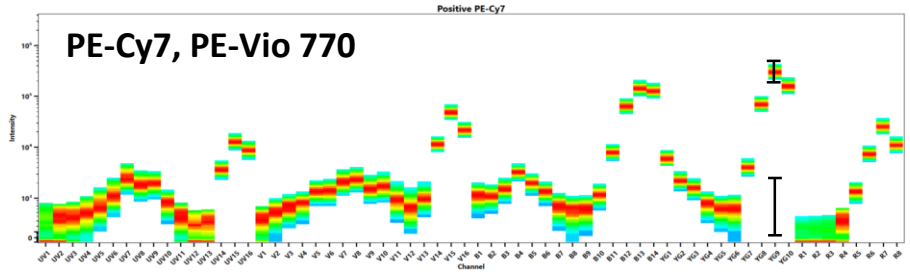




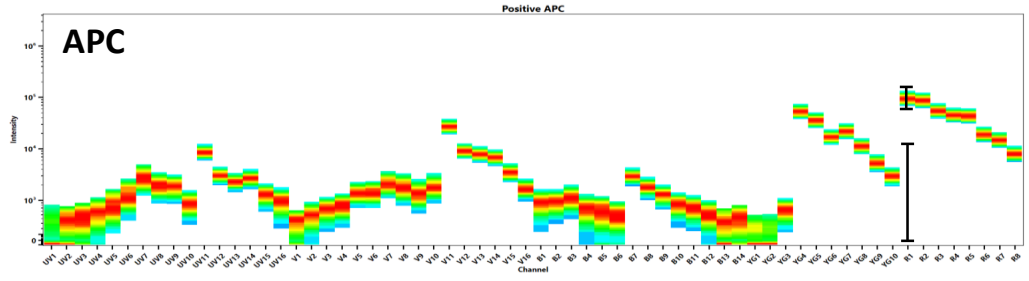


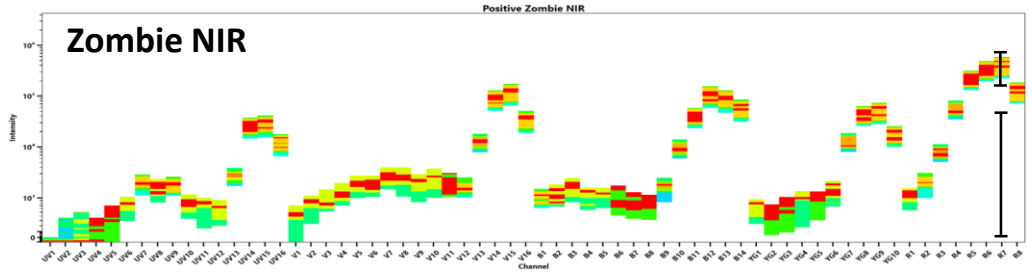


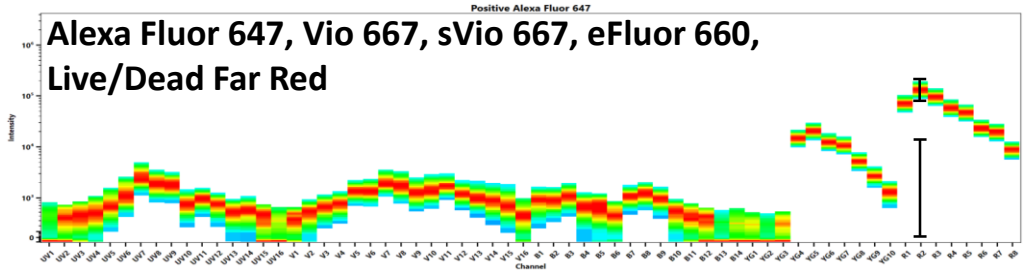


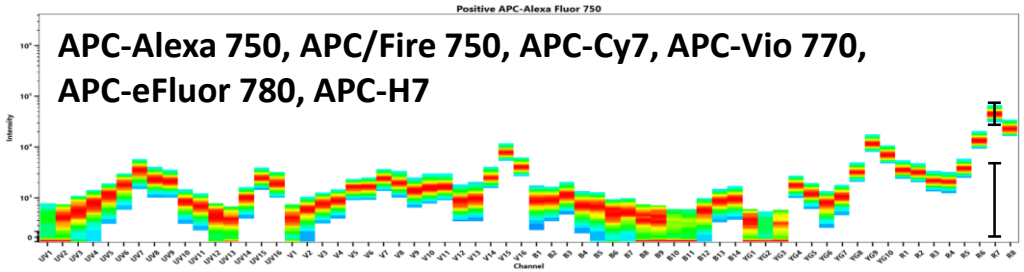


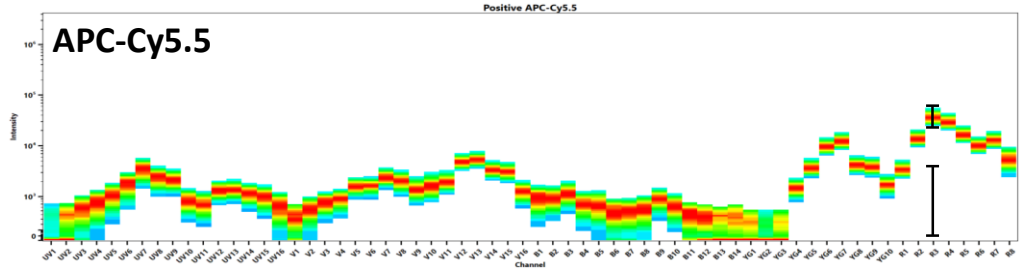
Red Laser Unique Signatures

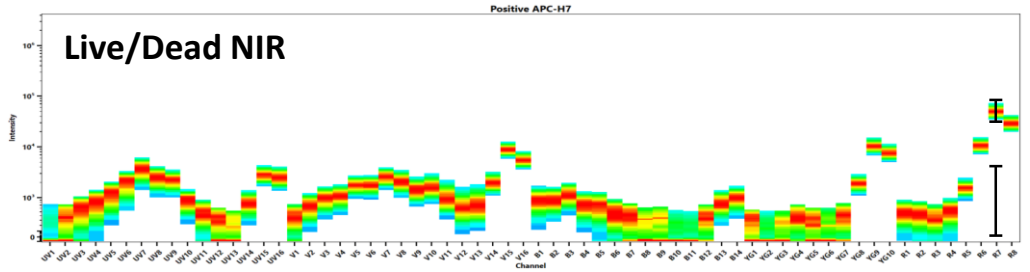


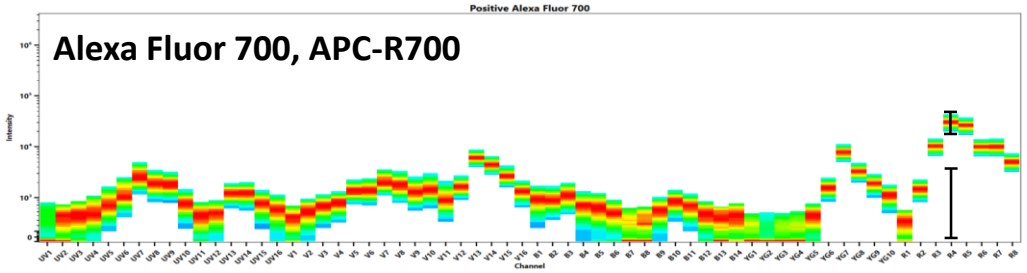






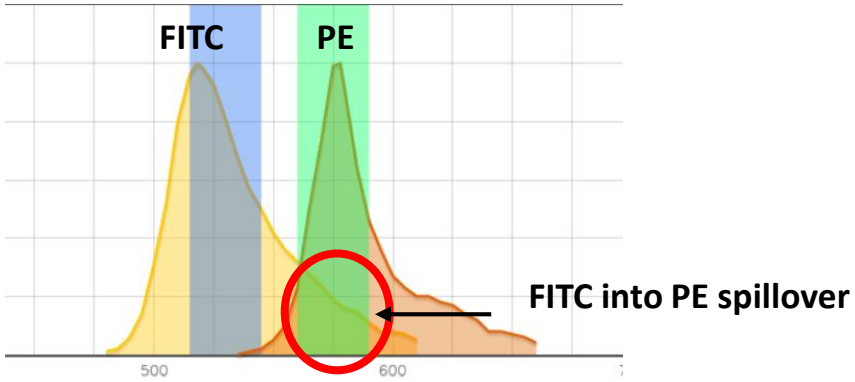






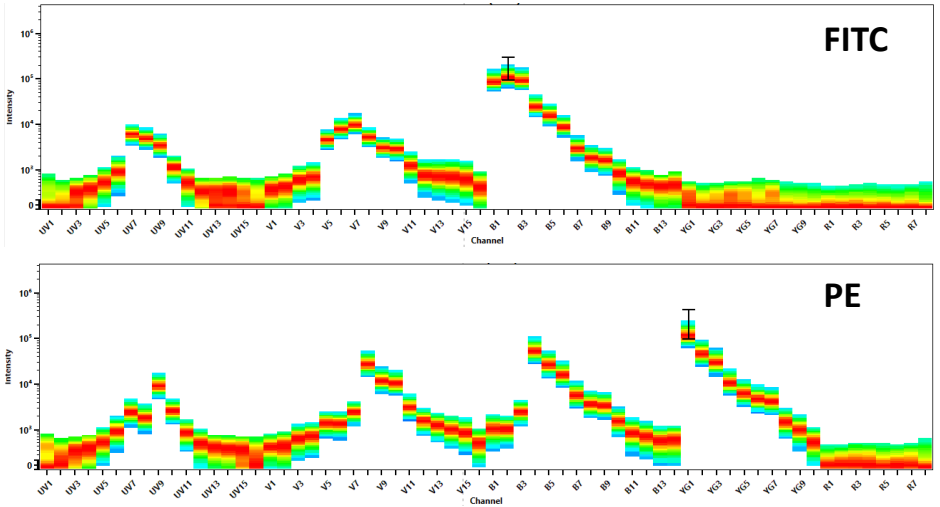
Compensation vs. Spectral Unmixing

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

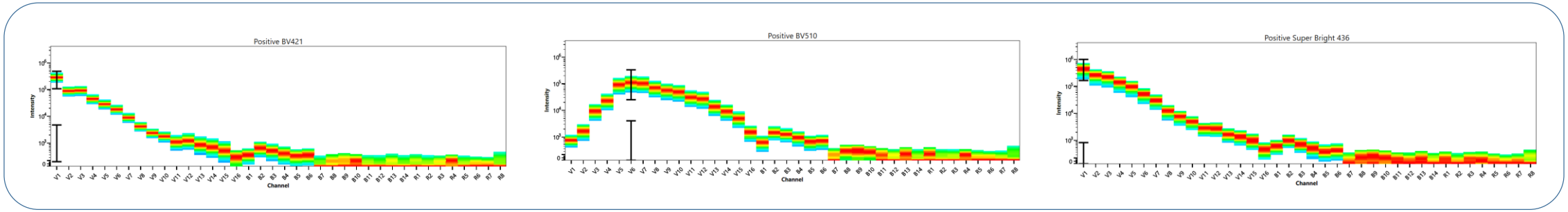


- Each fluorochrome is detected in multiple channels. In the 5 laser Aurora analyzer, there are 64 fluorescent channels.
- The number of detectors has to be higher than the number of fluorochromes.
- Single stained controls are used to establish the signatures of each fluorochromes
- Unmixing is used 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

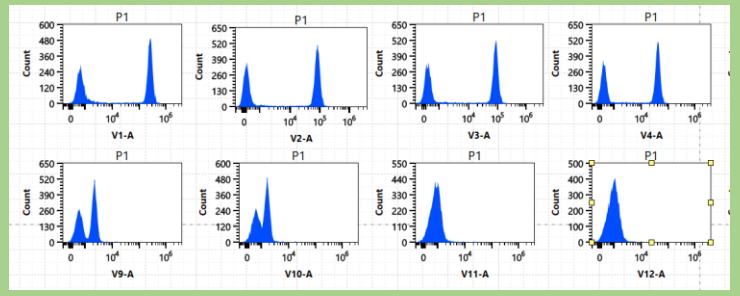
Unmixing Workflow in Aurora

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

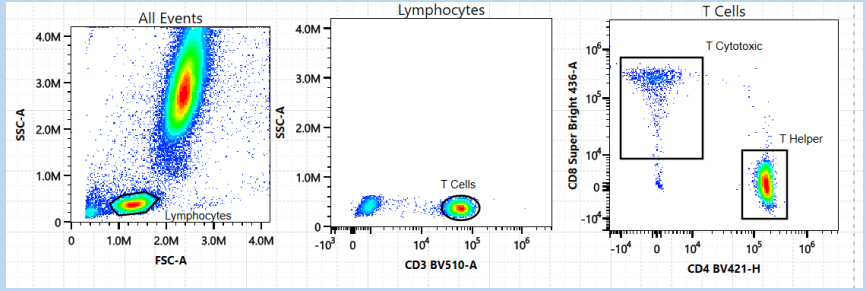


Raw Worksheet



Unmixing Algorithm

Unmixed Worksheet



Raw vs. Unmixed Data

RAW DATA

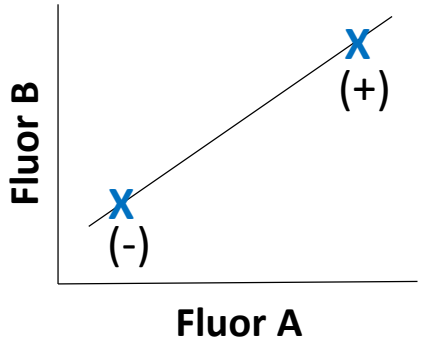
- Parameters are the instrument channels (V1, V2, etc)
- Visualized in raw worksheet
- Large fcs file size: up to 64 parameters + FSC and SSC
- Can be unmixed as many times as desired

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

Requirements for Optimal Reference Controls

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!

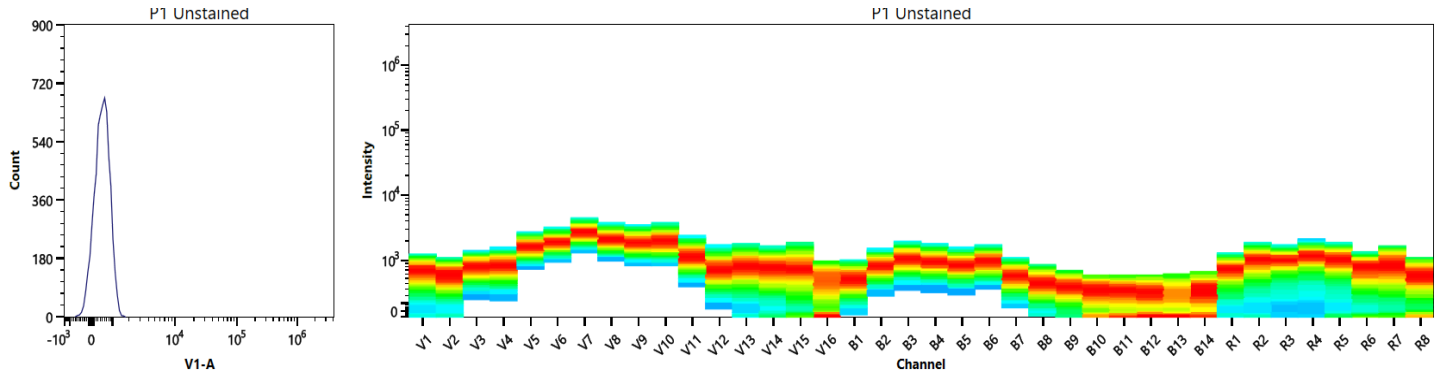
Unstained Control vs Negative Population in Reference Control

- **In addition to the Reference Controls, an <u>Unstained Control is mandatory for Spectral Unmixing</u>**
  - 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

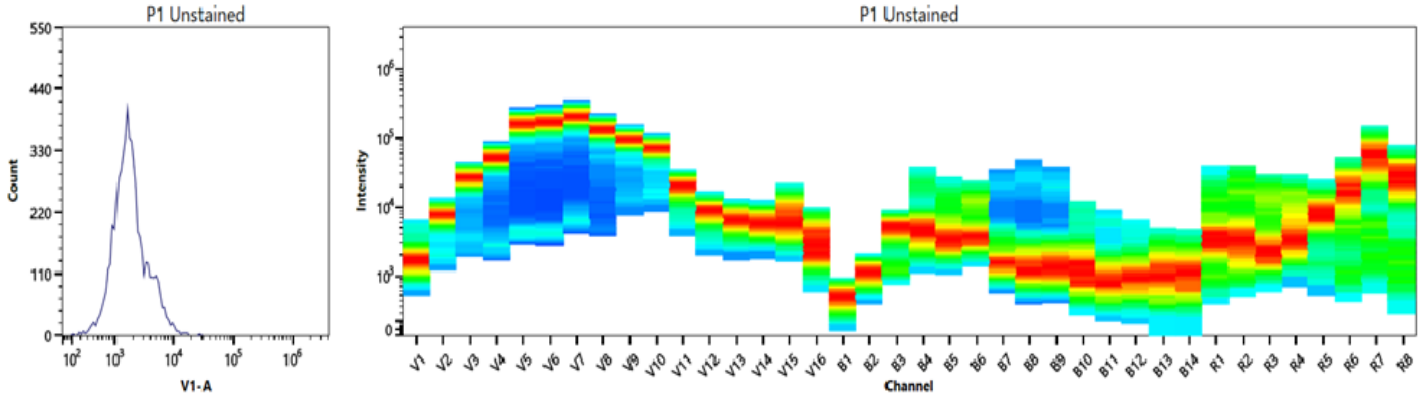
- **If Reference Controls do not have a negative population:**
  - New software 2.1 allows for additional unstained controls
    - Negative cells MUST match the cells used as reference controls for spillover calculation
    - Negative beads MUST match beads used as reference controls for spillover calculation

Reference Controls QC Examples (1)

Unstained control troubleshooting, human PBMCs



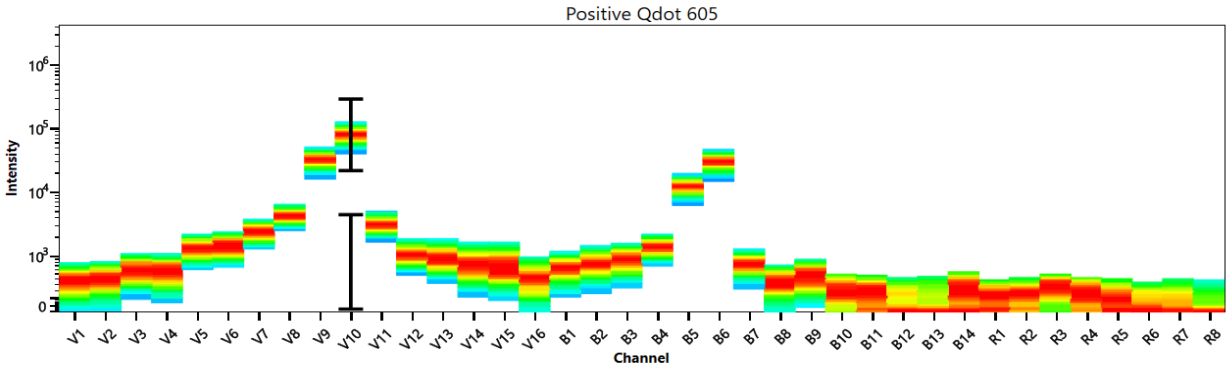
√ **Expected**



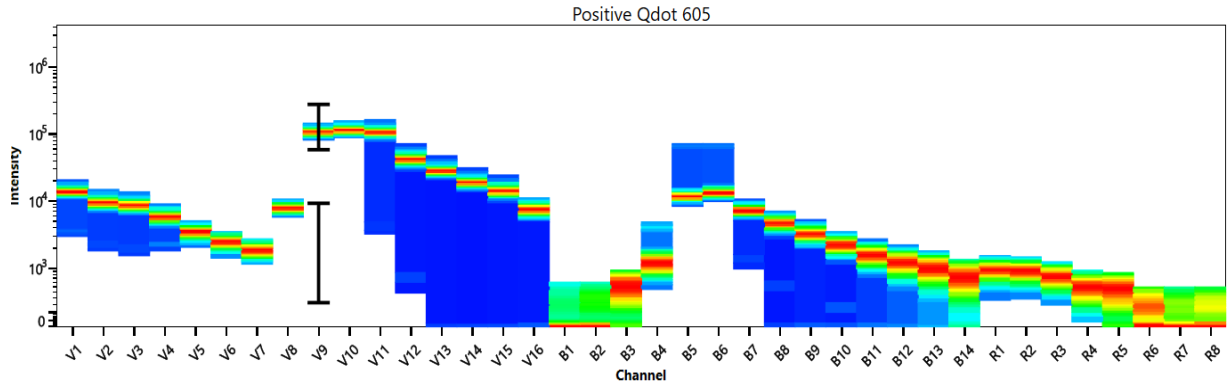
X **Provided by User**

Reference Controls QC Examples (2)

Qdot 605 control troubleshooting



√ **Expected**



X **Provided by User**

Reference Controls: Making Good Choices

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?

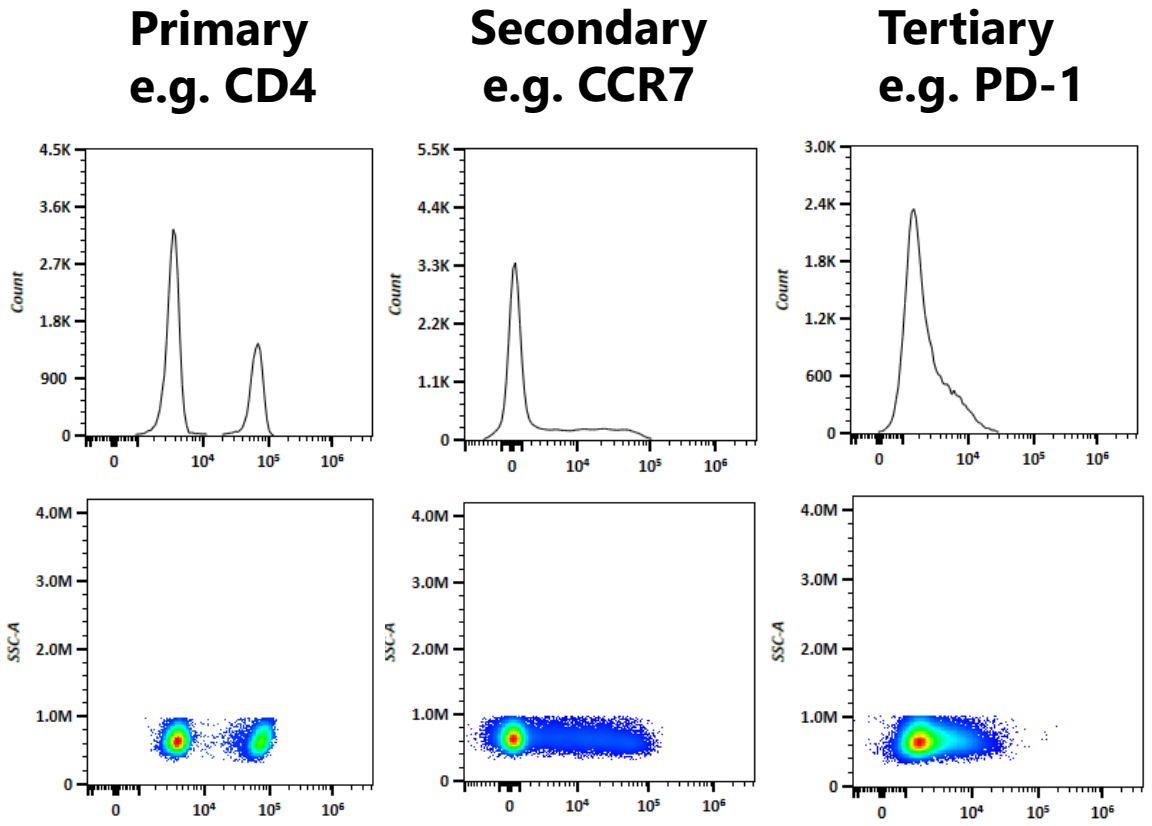
3. What antibodies are commercially available?

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.

Y. Mahnke and M. Roederer. Clin Lab Med:2007. 27:469



Antigen Classification

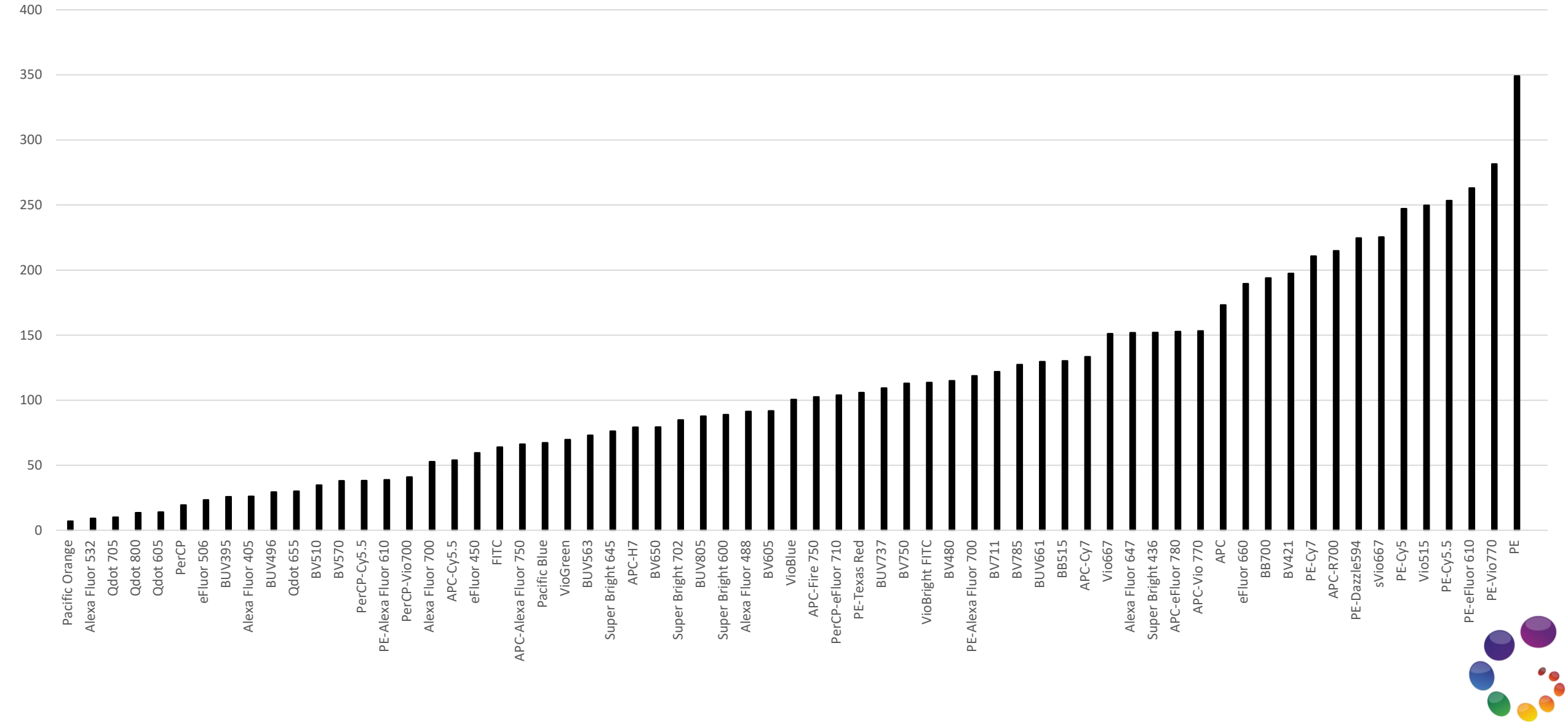
PRIMARY			
CD3	CD19	CD56	CD14
CD4		CD16	CD16
CD8			CD11b

SECONDARY		
CD45RA	IgD	CD8
CCR7	CD27	CD57
CD27	CD38	
CD28	CD1c	
CD127	CD95	
HLA-DR		

TERTIARY		
PD-1	CD1c	CD123
CD25		CD11c
TCR γ/δ		

Level of Antigen Expression

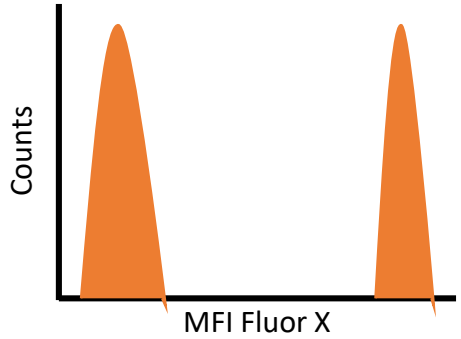
Fluorochrome Brightness Ranking



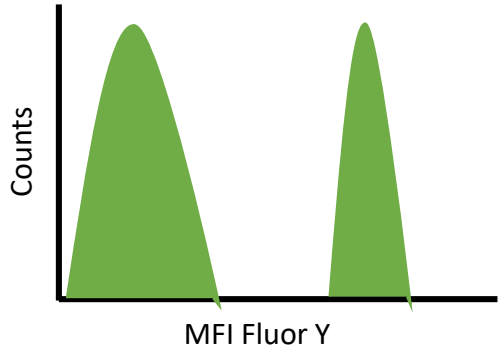
Assessing Antigen Resolution

SINGLE COLOR SCENARIO

Marker A Fluor X



Marker A Fluor Y

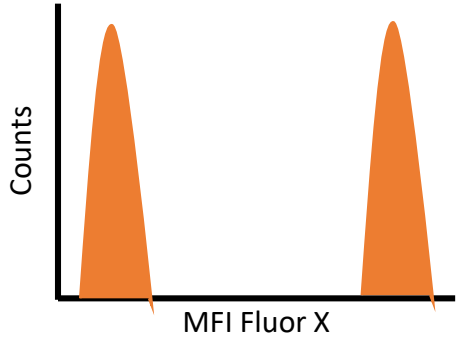


Main Contributors for Resolution Reduction

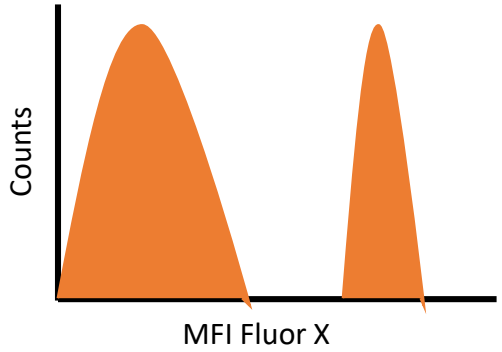
- Instrument Performance
- Instrument Setup
- Fluorochrome Brightness

MULTICOLOR SCENARIO

Marker A Fluor X SINGLE STAINED



Marker A Fluor X MULTICOLOR TUBE



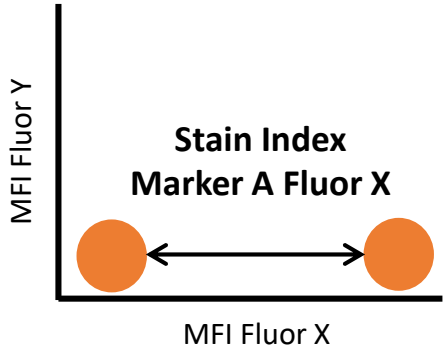
Main Contributors for Resolution Reduction

- **SPREAD!!!**
- Antibody titer

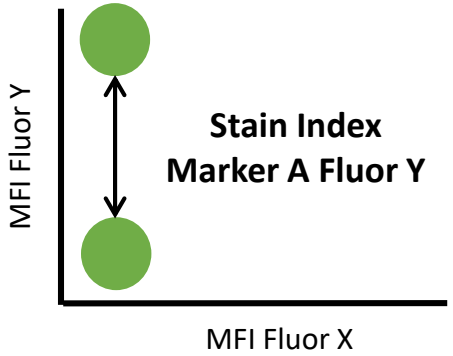
Quantification of Impact of Spread in Resolution

SINGLE COLOR SCENARIO

Marker A Fluor X



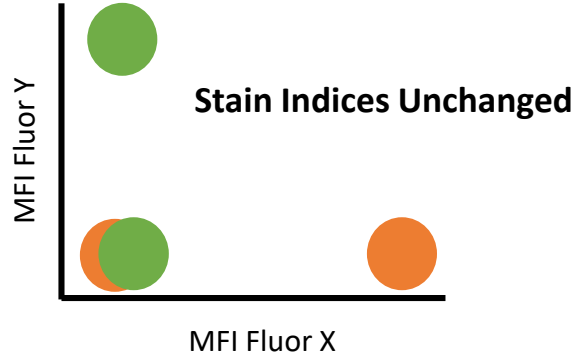
Marker A Fluor Y



MULTICOLOR SCENARIO

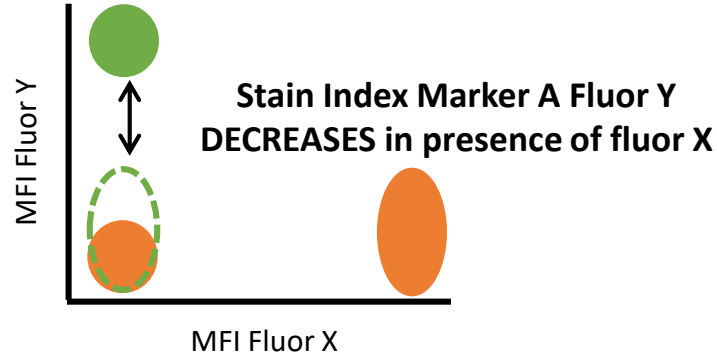
Fluor X DOES NOT spread into Fluor Y

Fluor Y DOES NOT spread into Fluor X



Fluor X DOES SPREAD into Fluor Y

Fluor Y DOES NOT spread into Fluor X

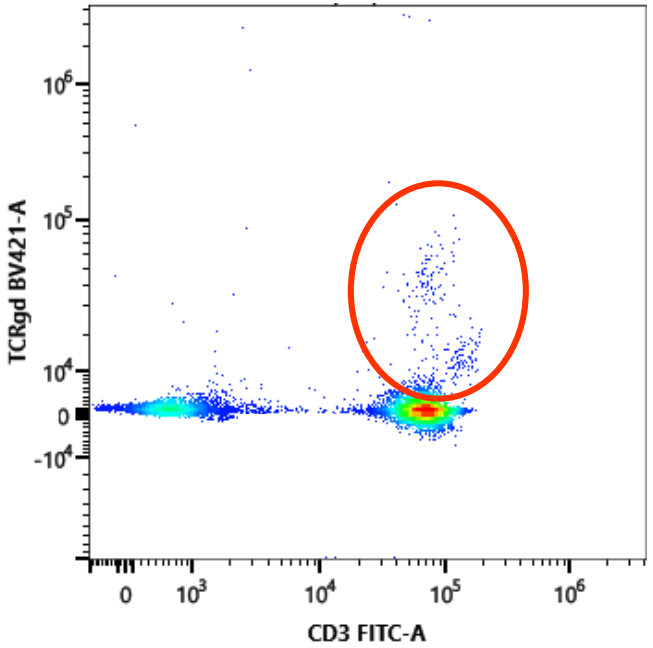


Considerations

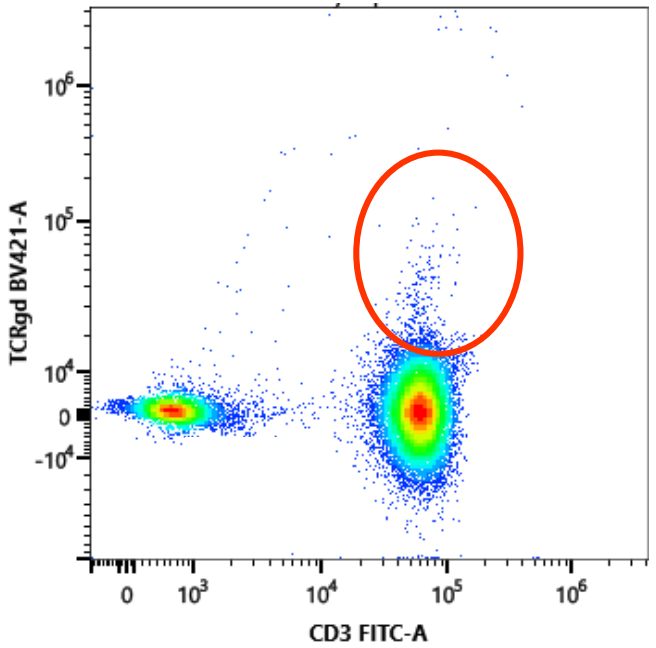
- CO EXPRESSION
- ANTIGEN LEVEL OF EXPRESSION
- Data used for calculations has to be unmixed using a certain combination of fluorochromes

Panel Design and Highly Overlapping Dyes (1)

Co-expression and antigen classification are needed for correct fluorochrome choice.



FMO



antigen and overlapping fluor co-expressed

Panel Design: Fluor Assignment

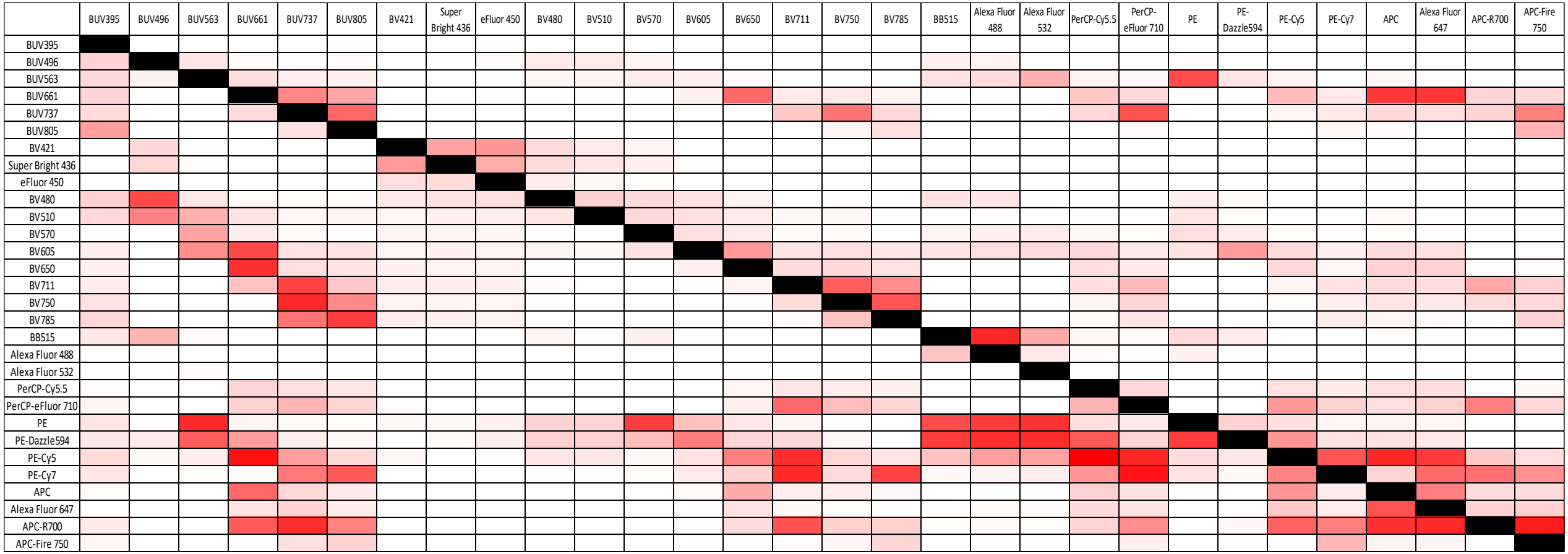
Same rules apply as conventional cytometry!

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-EXPRESSION 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



	BUV395	BUV496	BUV563	BUV661	BUV737	BUV805	BV421	Super Bright 436	eFluor 450	BV480	BV510	BV570	BV605	BV650	BV711	BV750	BV785	BB515	Alexa Fluor 488	Alexa Fluor 532	PerCP-Cy5.5	PerCP-eFluor 710	PE	PE-Dazzle594	PE-Cy5	PE-Cy7	APC	Alexa Fluor 647	APC-R700	APC-Fire 750
BUV395																														
BUV496																														
BUV563																														
BUV661																														
BUV737																														
BUV805																														
BV421																														
Super Bright 436																														
eFluor 450																														
BV480																														
BV510																														
BV570																														
BV605																														
BV650																														
BV711																														
BV750																														
BV785																														
BB515																														
Alexa Fluor 488																														
Alexa Fluor 532																														
PerCP-Cy5.5																														
PerCP-eFluor 710																														
PE																														
PE-Dazzle594																														
PE-Cy5																														
PE-Cy7																														
APC																														
Alexa Fluor 647																														
APC-R700																														
APC-Fire 750																														

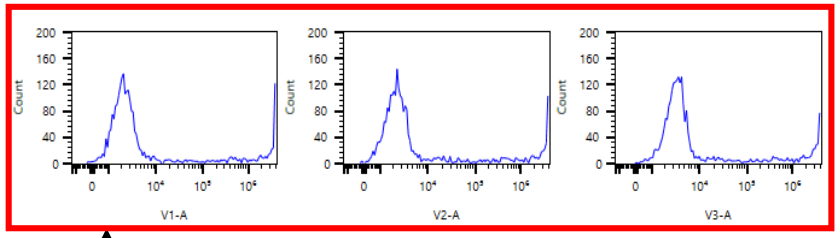
How to read this table: the fluor in the row impacts the one in the column. Red means the fluor in that row has significant spread into the dye in the column (for example PE into BV570). Areas in bright pink and red is where more attention to panel design is needed.

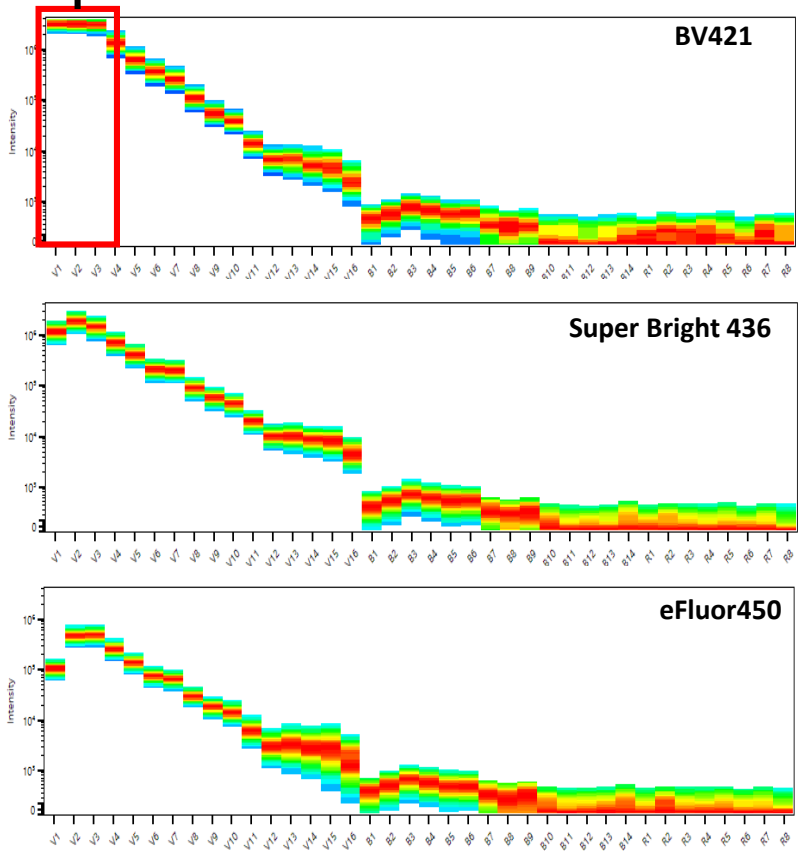
Cytek Assay Settings

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!

Instrument Setting Adjustment: Example 1

Off scale





Fluorochrome
CD8 BV421
CD4 Super Bright 436
CD4 eFluor450

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.

In scale