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INTRODUCTION TO SPECTRAL CYTOMETRY

USING CYTEK® AURORA, AURORA CS, and NORTHERN LIGHTS™ SYSTEMS

Cytek® Biosciences, Inc.
47215 Lakeview Blvd
Fremont, CA 94538

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You can answer polls

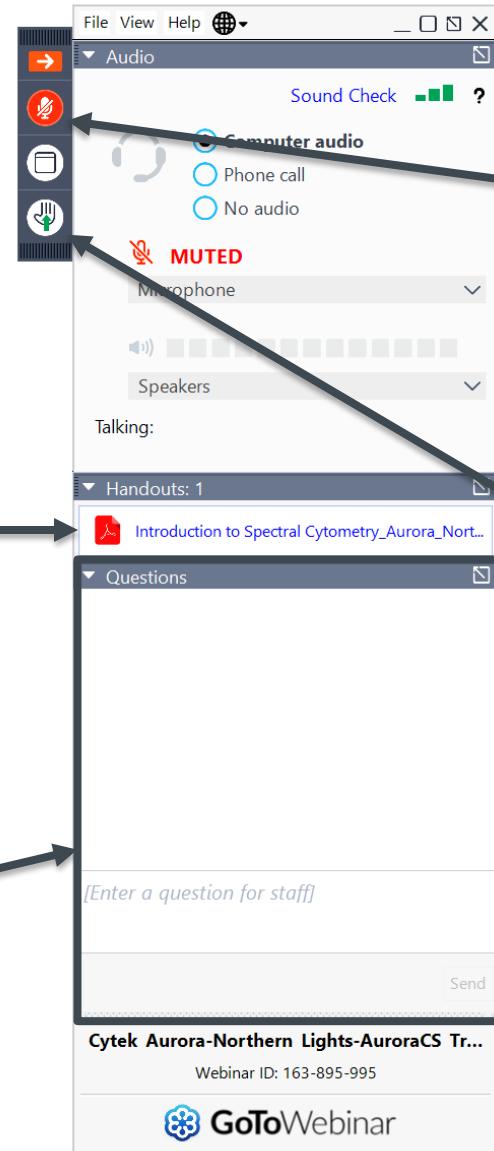
What is your favorite color?

- Red
- Blue
- Green

Download the PDF



You can enter questions
for us, and we will answer
them during the class



You may be invited to
use your microphone at
the end of this training

Raise your hand
if experiencing
any issues with
GoToWebinar





Course Overview

- 1 Full Spectrum Cytometry Basics
- 2 Full Spectrum Experiment Workflow
- 3 Tips for Planning, Running, and Evaluating Assays
- 4 Working with Optimized Assays



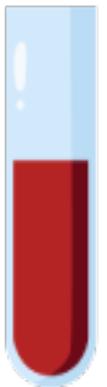
Full Spectrum Cytometry Basics

- Flow Cytometry Fundamentals
- Generating Full Spectrum Signatures
- Benefits of Cytek® Full Spectrum Profiling™ Technology (FSP™)

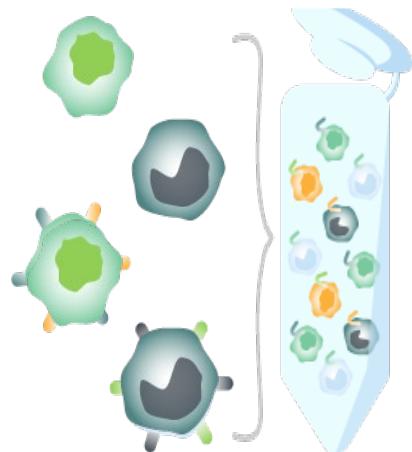


Sample Preparation: Basics

Collect and prepare samples



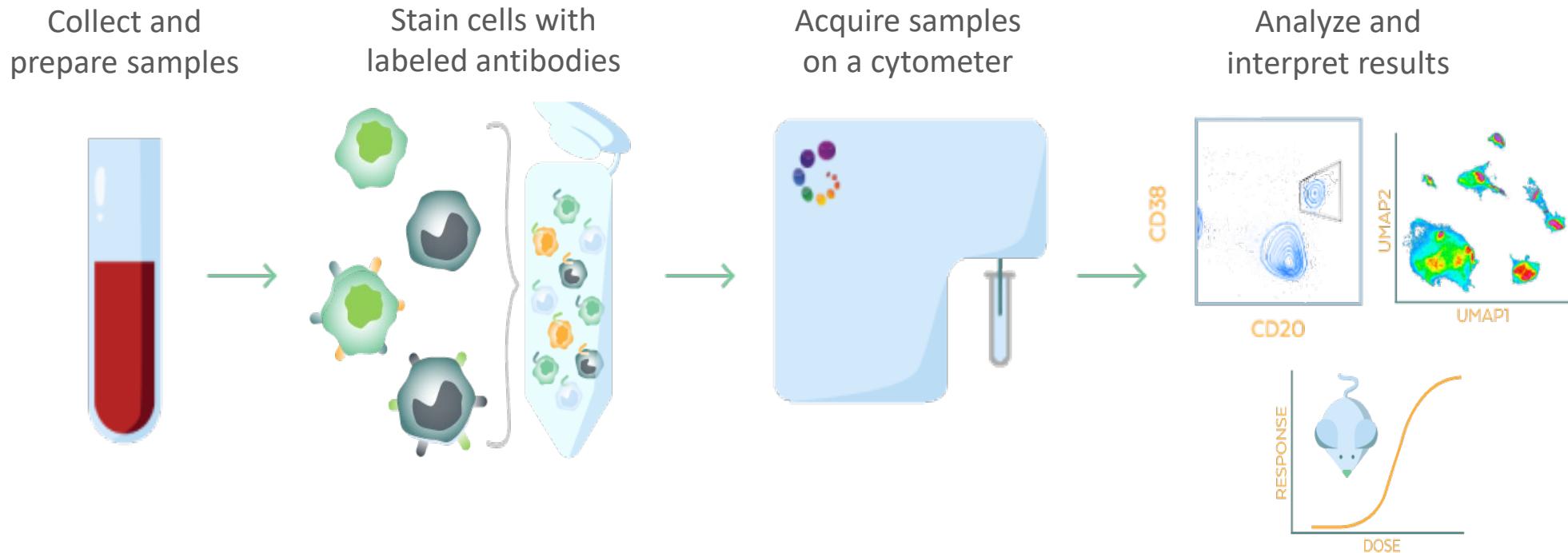
Stain cells with labeled antibodies



- 1 Collect and prepare single cells in suspension
- 2 Add fluorescent-tagged antibodies, fluorescent dye(s), and/or utilize a fluorescent protein
- 3 Wash and resuspend cells in buffer



Flow Cytometry: Basics



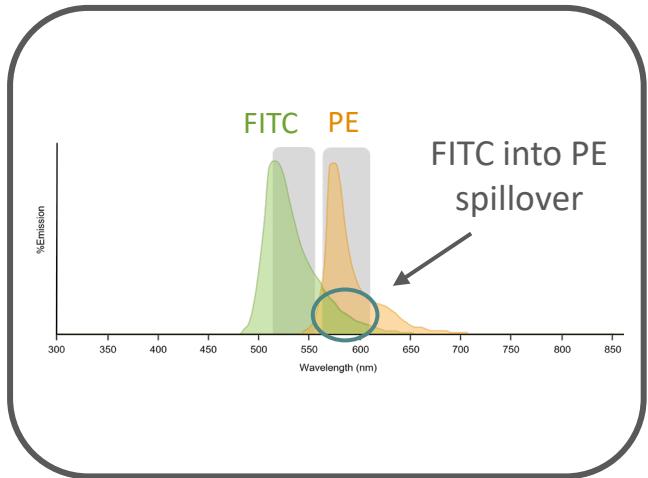


Conventional vs. Full Spectrum: Similarities

Run controls to define fluorescent signal on the cytometer



Account for spectral overlap with a mathematical calculation



Run multicolor samples with calculation applied



Conventional Flow Cytometry Terminology

Compensation Controls

Input

Compensation

Output

Compensated data

Full Spectrum Flow Cytometry Terminology

Reference Controls

Input

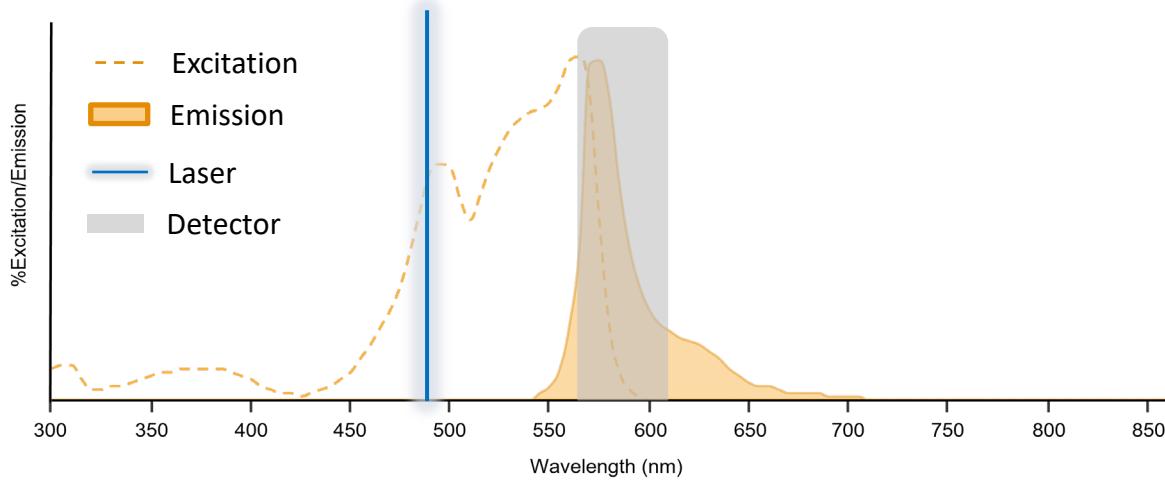
Spectral Unmixing

Output

Unmixed data



What Are We Capturing?

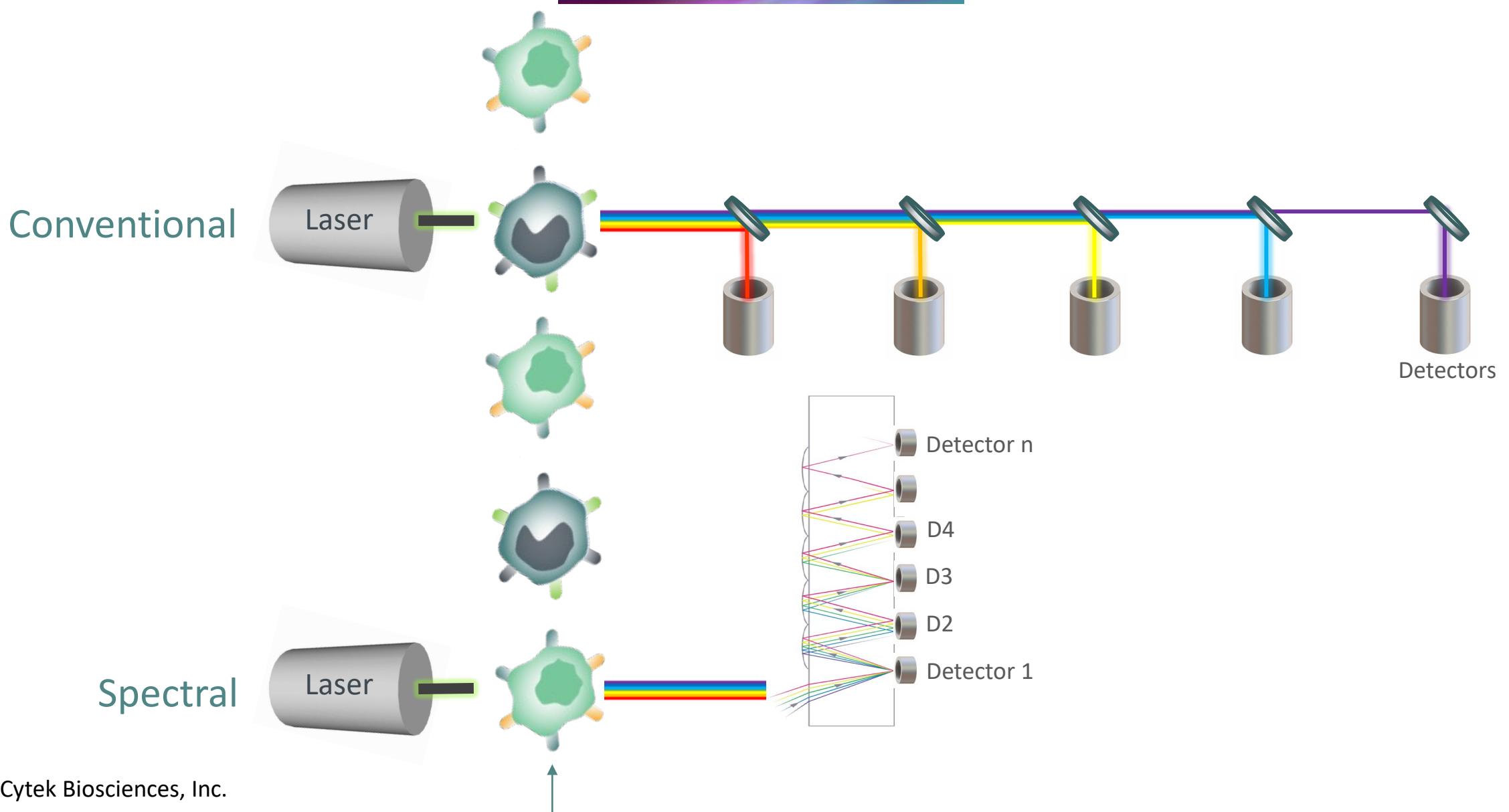


Is this the whole picture?





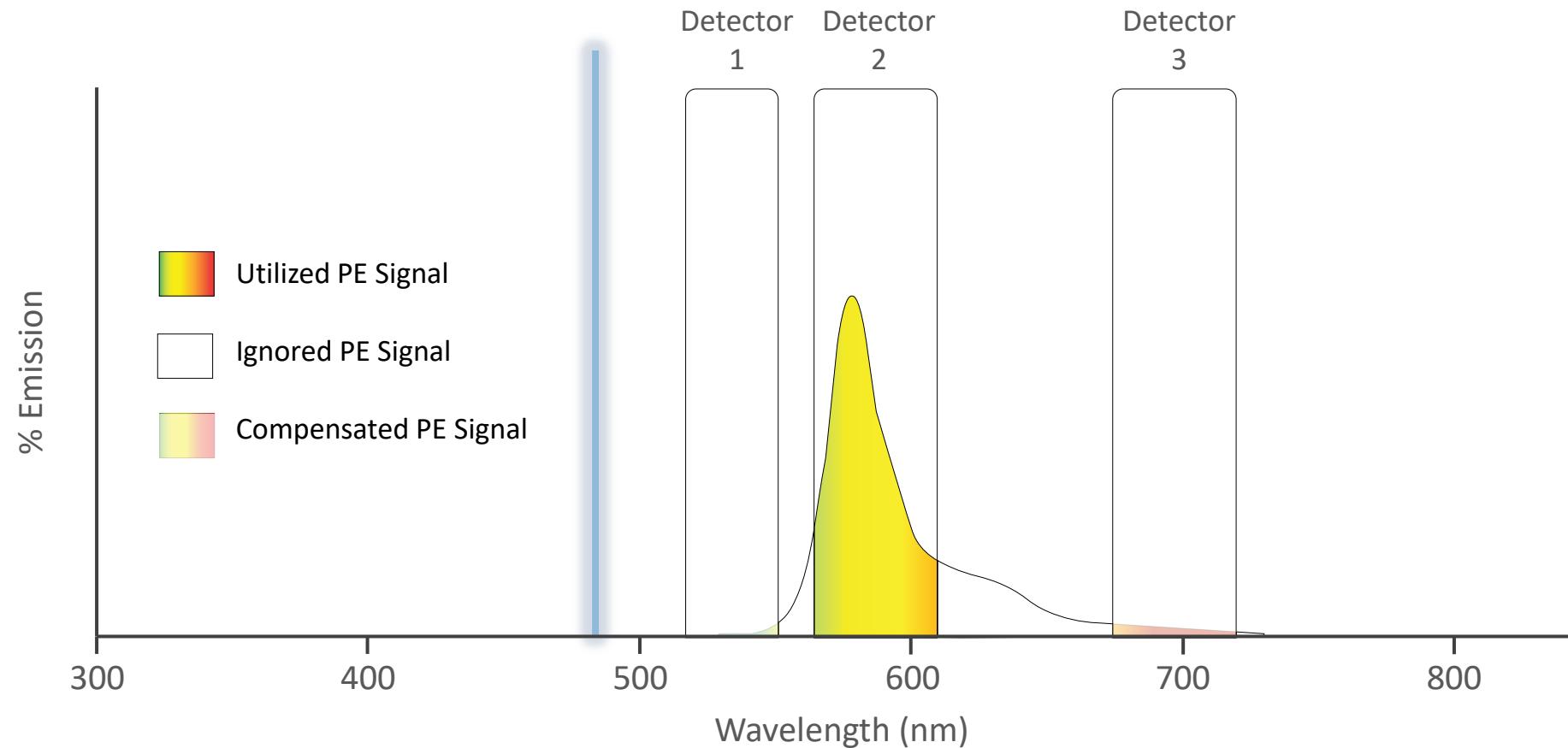
Flow Cytometry Optics





Conventional vs. Full Spectrum: Differences

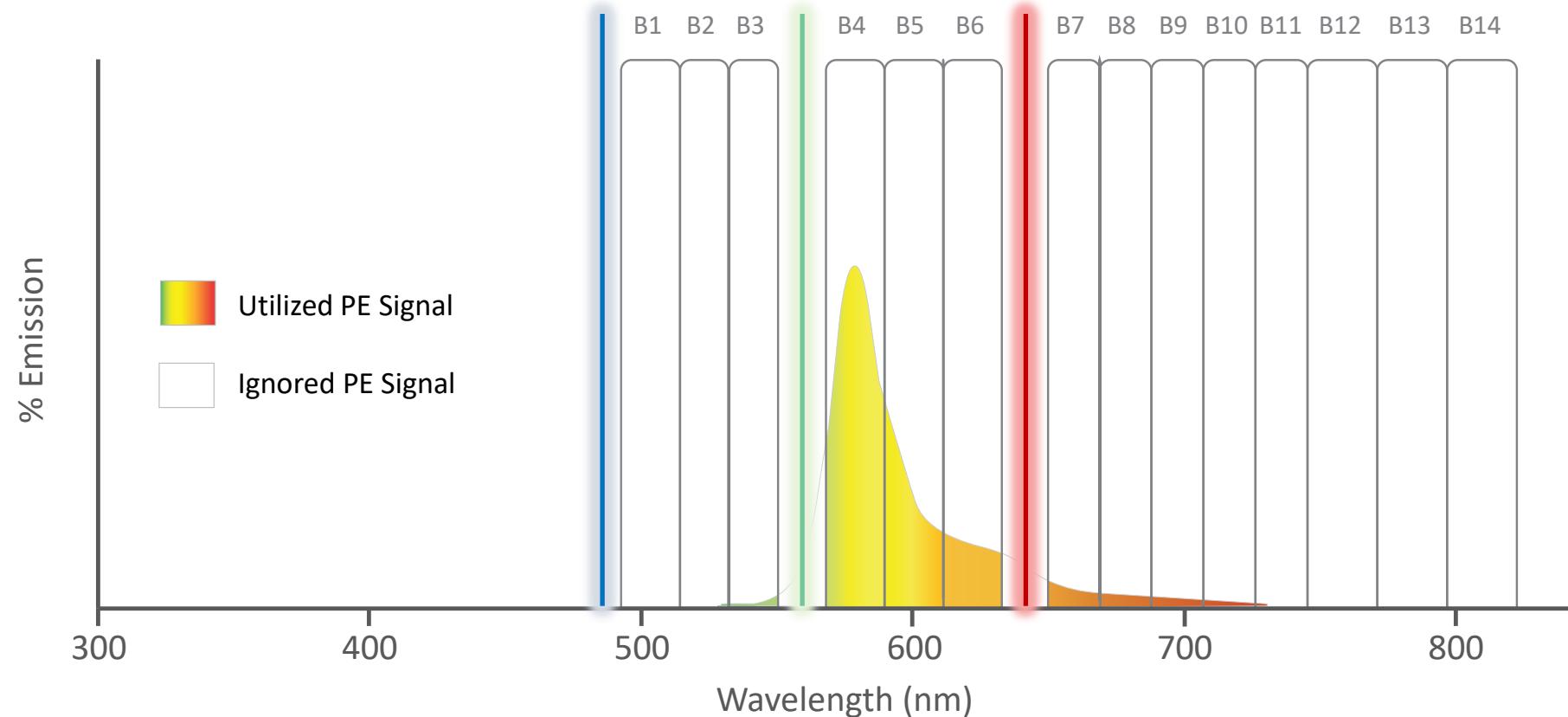
Detecting PE on a conventional cytometer





Conventional vs. Full Spectrum: Differences

Detecting PE on a Cytek® System





Interactive Poll #1

What is different about full spectrum cytometry?



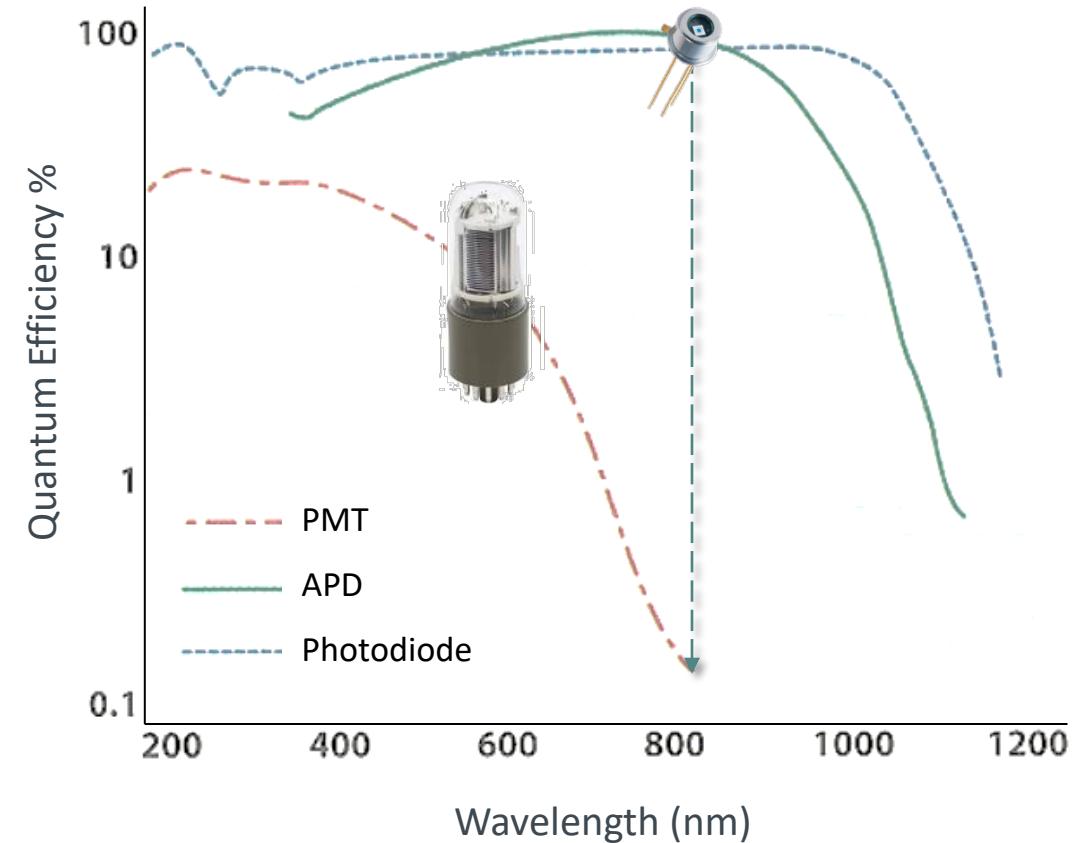
Unique Capabilities of Cytek® Systems

- Achieve better resolution with higher sensitivity detectors
- Define fluorochromes by their full spectrum signatures
- Easier fluorochrome selection
- Extract autofluorescence



Achieve Better Resolution With Higher Sensitivity Detectors

- Cytek® Systems use detectors called Avalanche Photodiodes (APDs), whereas many other cytometers use Photomultiplier Tubes (PMTs)
- Quantum efficiency (QE) is the ability to convert photons to electrons
- APDs have higher QE which translates to better resolution, especially with fluorophores that emit at longer wavelengths



Data from Hamamatsu Photonics



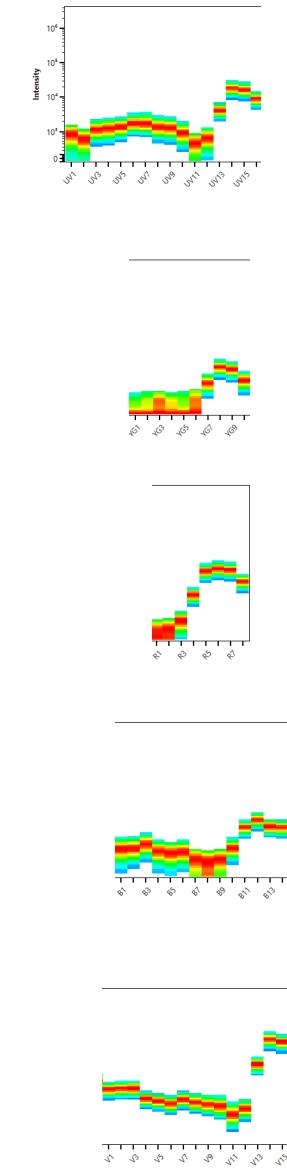
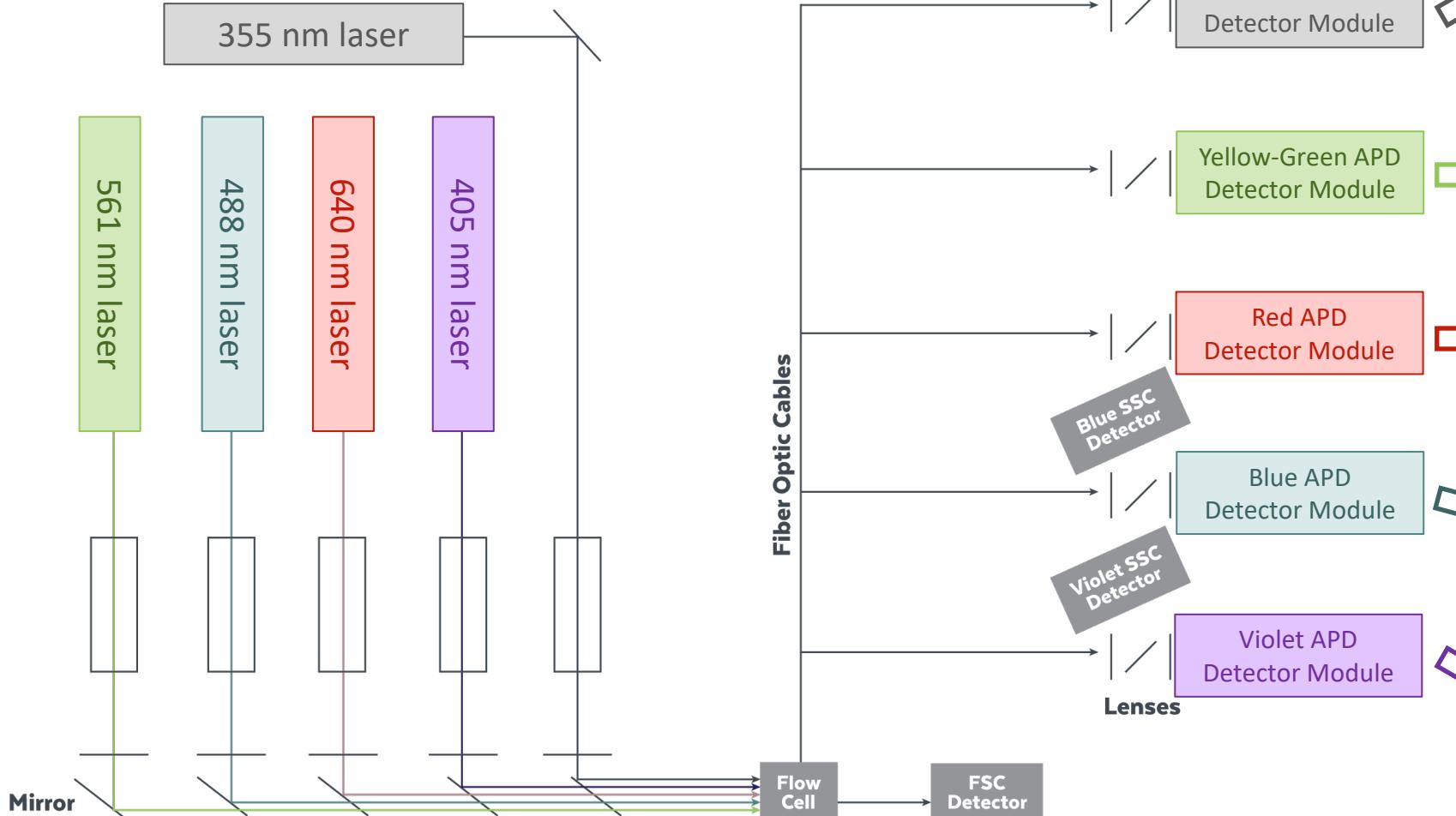
Generating Full Spectrum Signatures

Components of a full spectrum signature

Options for viewing signatures

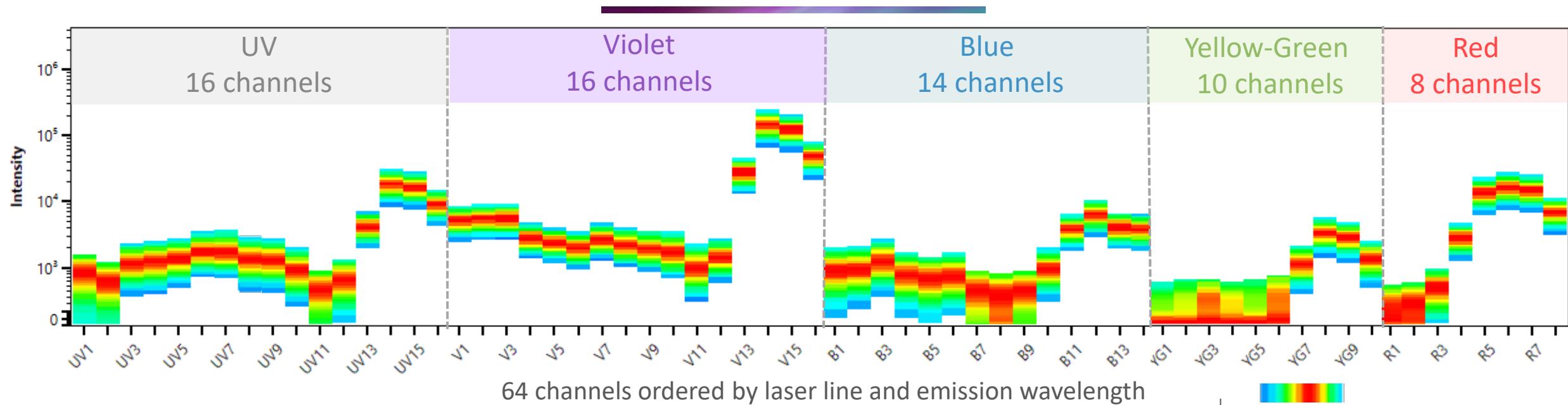


Generating a Full Spectrum Signature

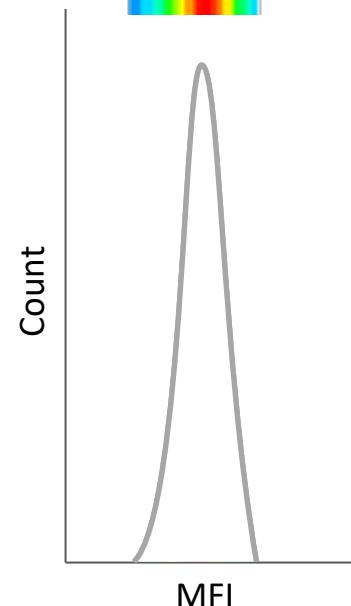




Building a Full Spectrum Signature

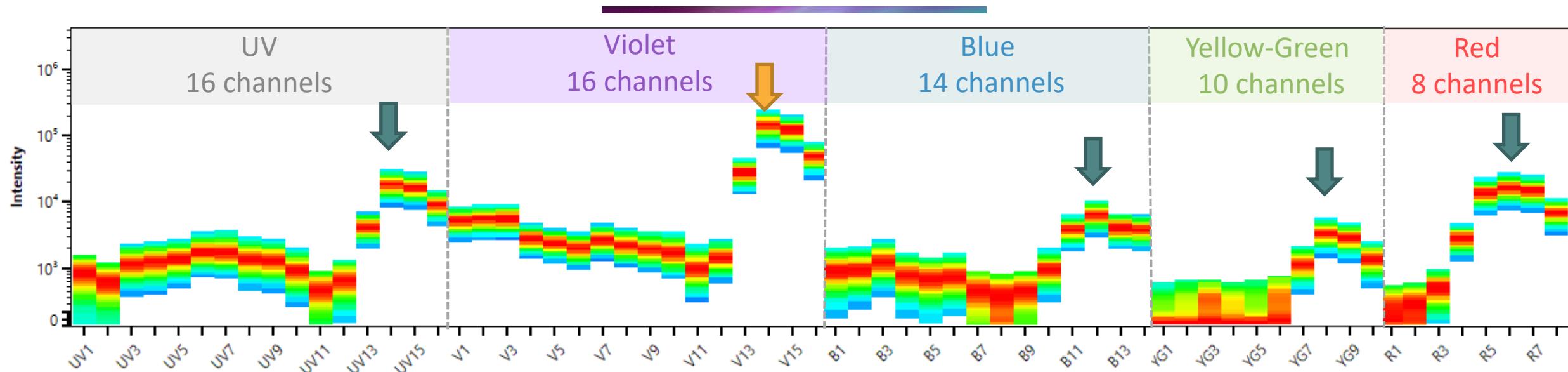


The signals are captured from each of the different modules and stitched together to create a single spectral signature





Building a Full Spectrum Signature

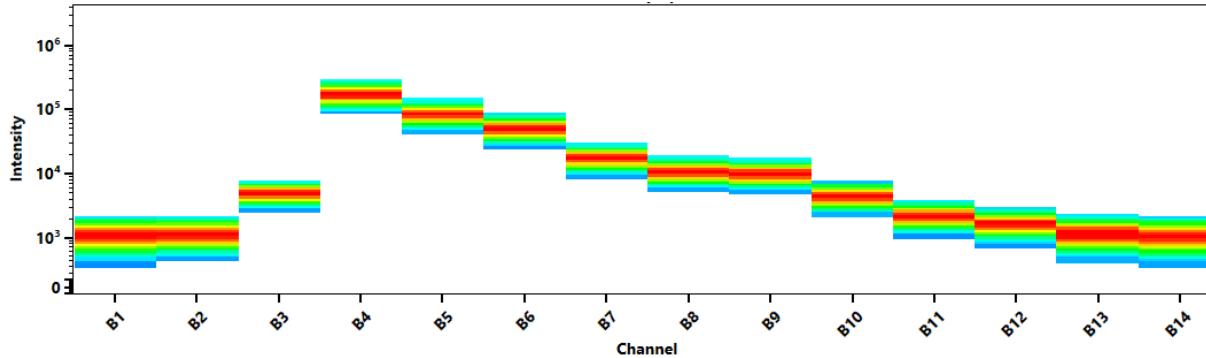


Primary Emission Channel: Captures the overall emission maxima. Occurs in detector array matching the primary excitation laser.

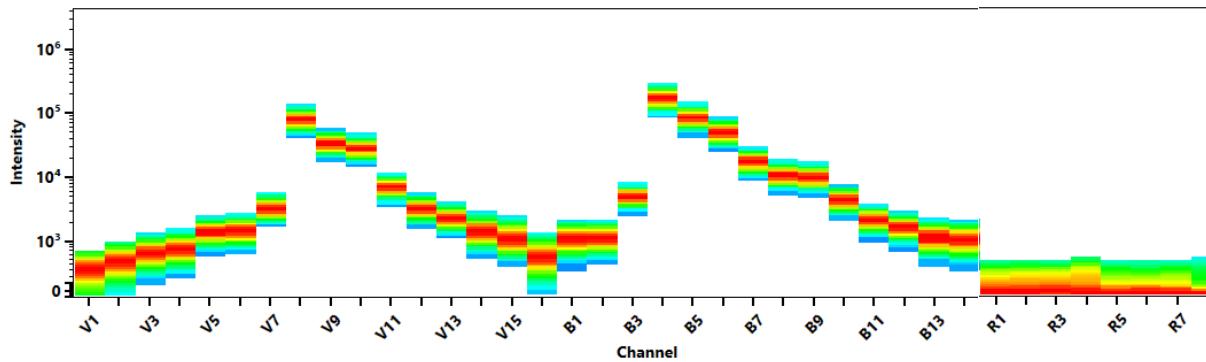
Secondary Emission Channel: Captures any secondary emission maxima. Occurs in detector array(s) matching any secondary excitation laser(s).



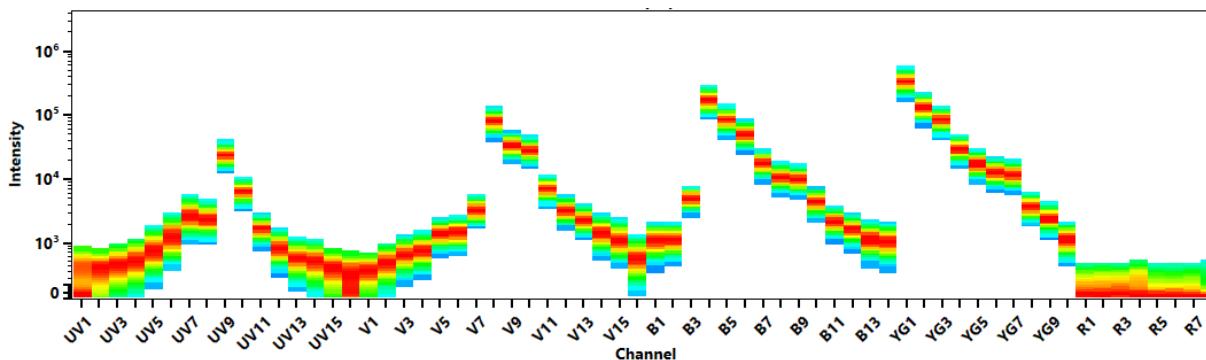
Signatures From Different Configurations



cFluor® BYG575 (PE) – 1 Laser (B) system



cFluor® BYG575 (PE) – 3 Laser (VBR) system



cFluor® BYG575 (PE) – 5 Laser system



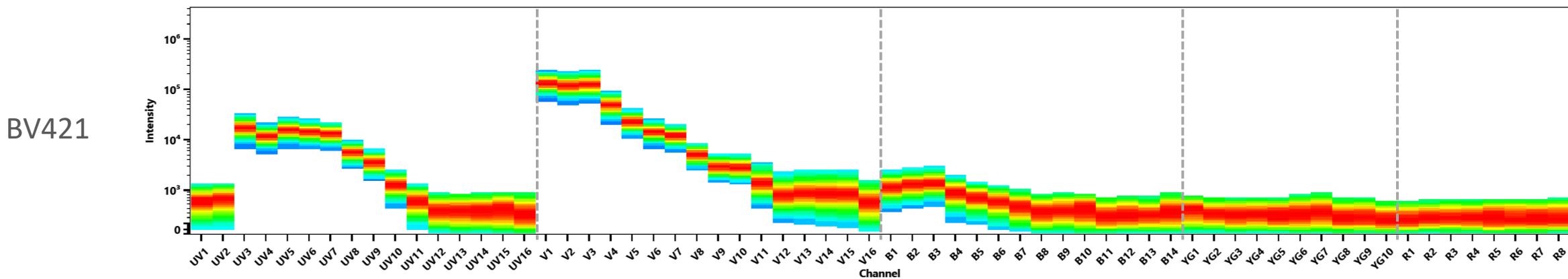
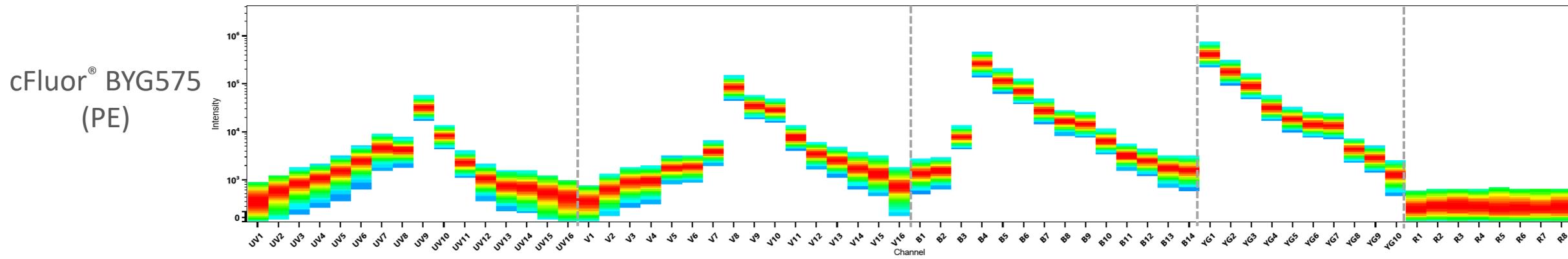
Exercise 1: Reading a Full Spectrum Signature

Goals

- Identify peak emission channel
- Identify secondary peak emission channels

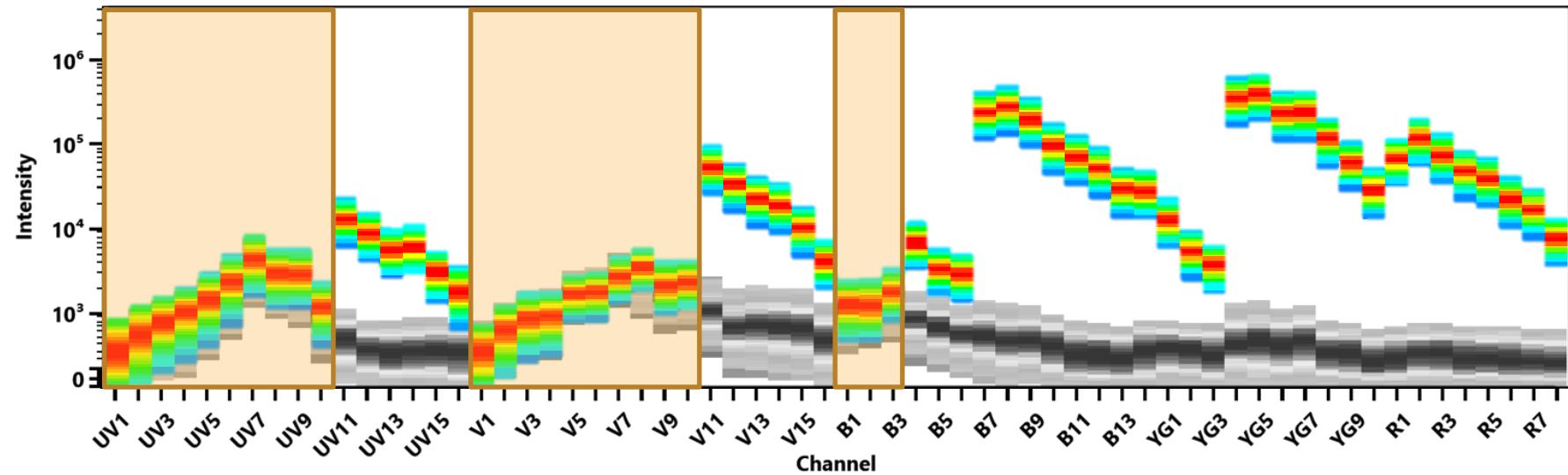
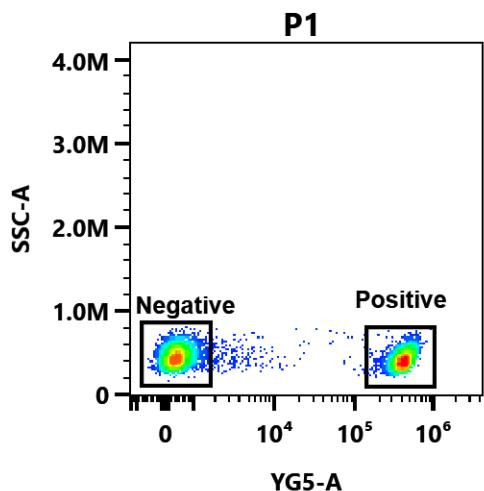


Exercise 1: Full Spectrum Signatures





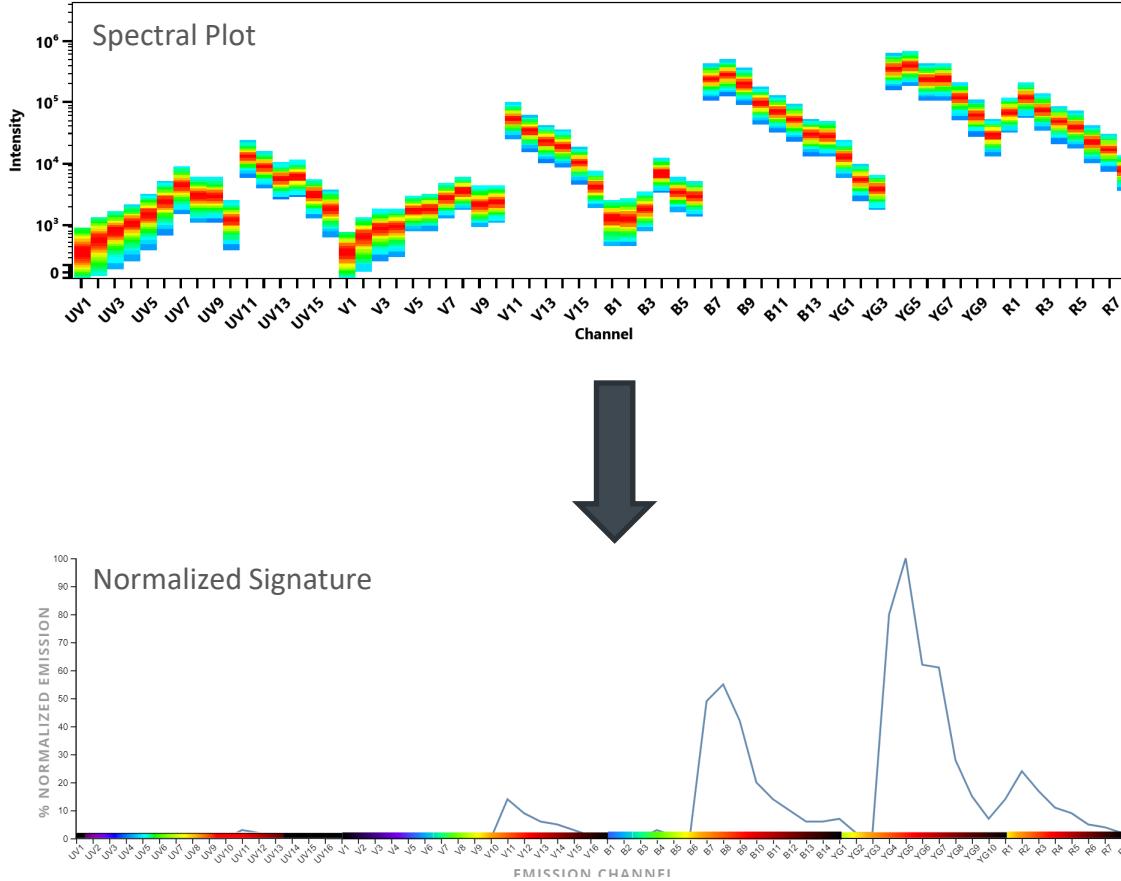
Spectral Plots



Spectral plots capture both the fluorophore signature
and the autofluorescence signature



Normalized Signatures



Normalized signatures capture the fluorophore signature *without* the autofluorescence signature and can be helpful for comparison

Normalized signatures are created by:

- calculating the median
- subtracting out autofluorescence
- setting the peak emission channel to 100%



Access All Tools in Cytek® Cloud



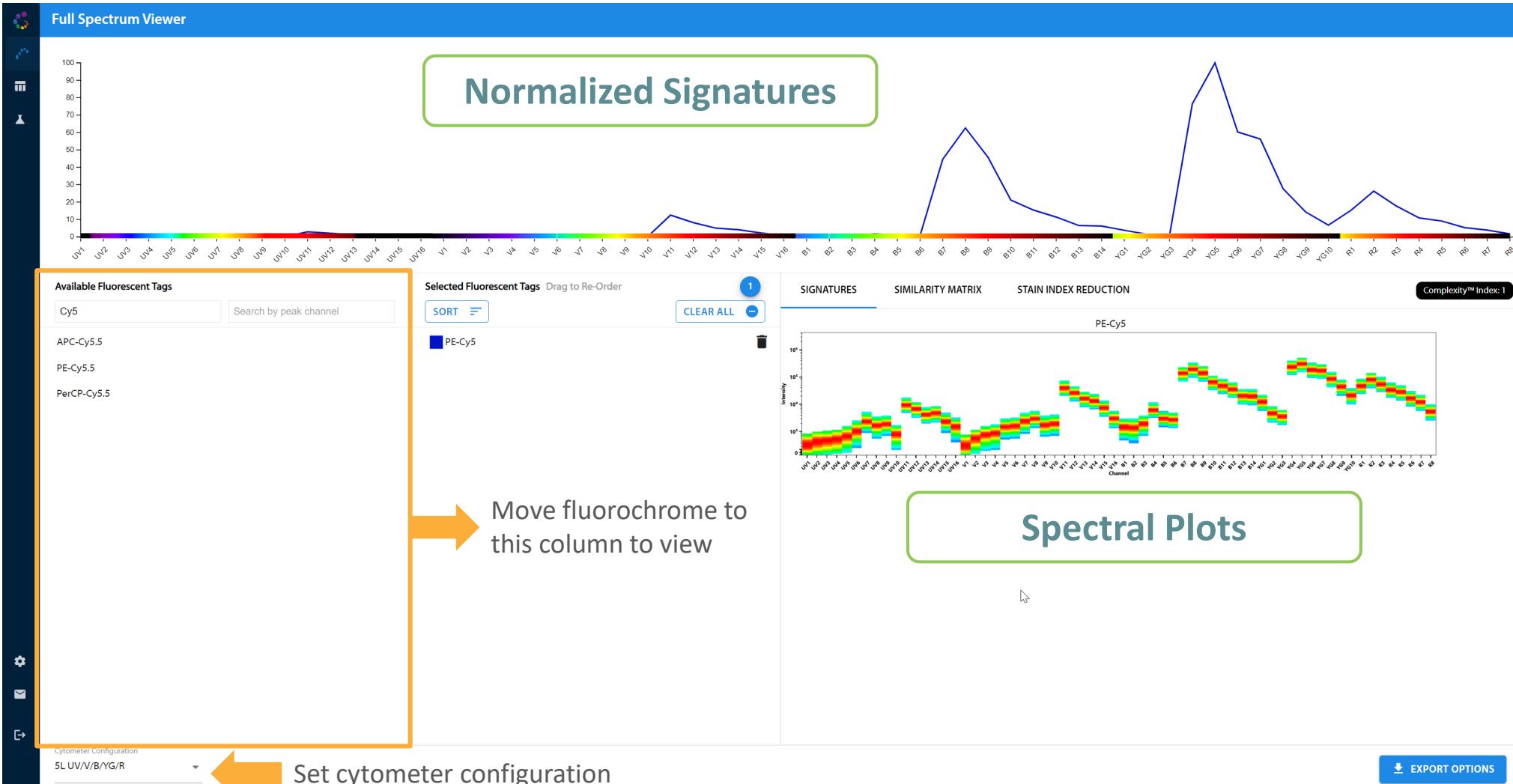
- Three integrated online tools:
 - Full Spectrum Viewer
 - Panel Builder
 - Experiment Builder
- Tailored for each Cytek® instrument configuration (1 to 5 laser)

Sign up for a free account at
<https://cloud.cytekbio.com/>



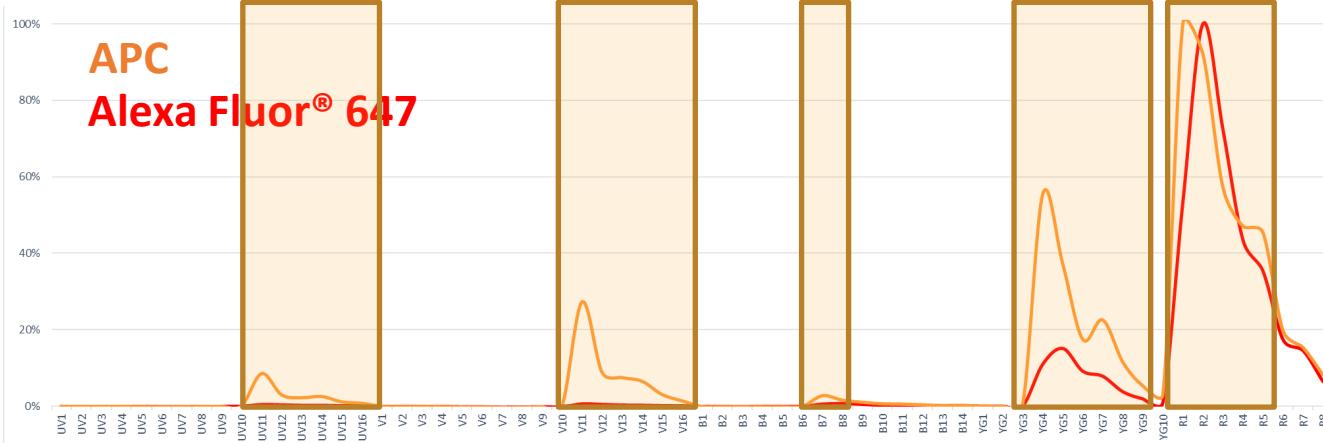
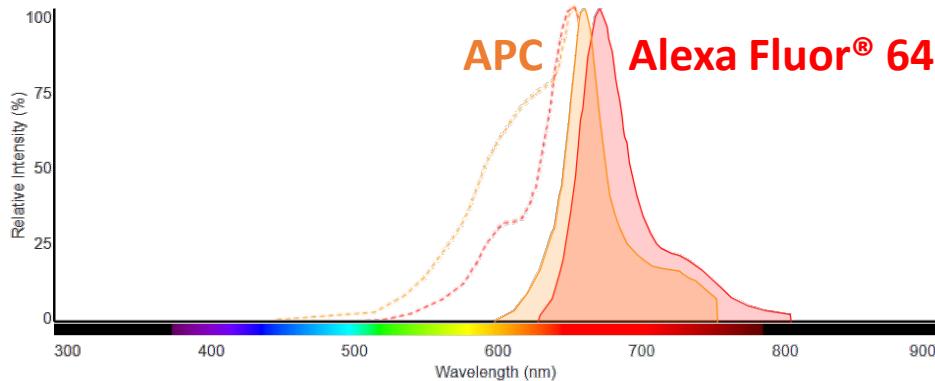
Cytek® Cloud – Full Spectrum Viewer

Signatures can be found in the Full Spectrum Viewer

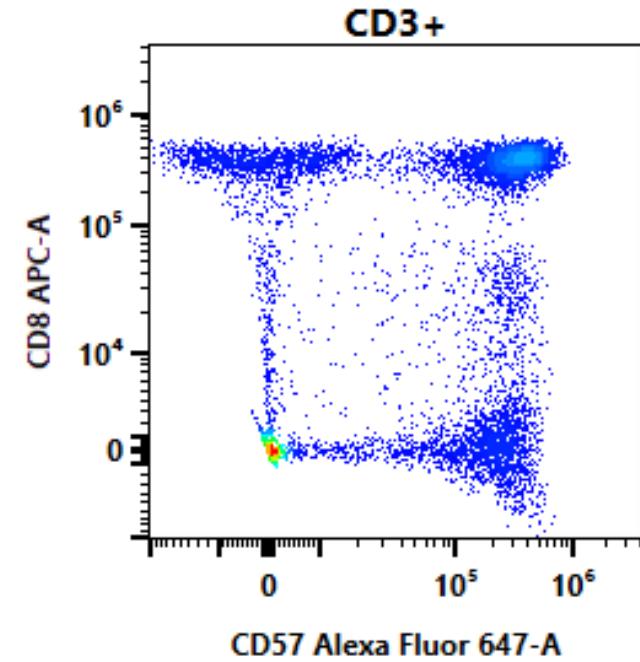




Use of Highly Overlapping Dyes in Panels



Plot gated on singlet lymphocytes

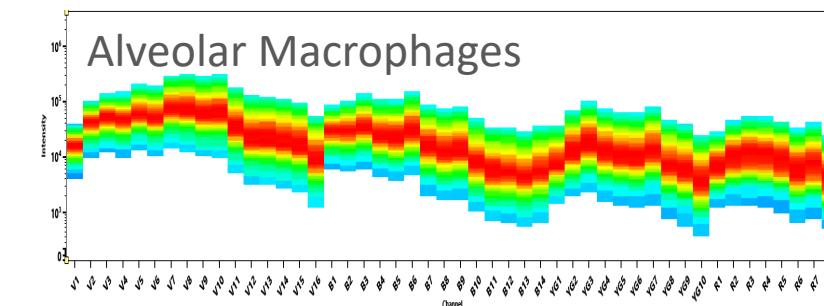
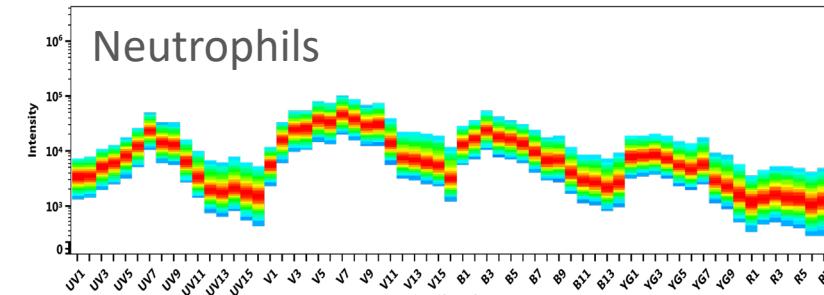
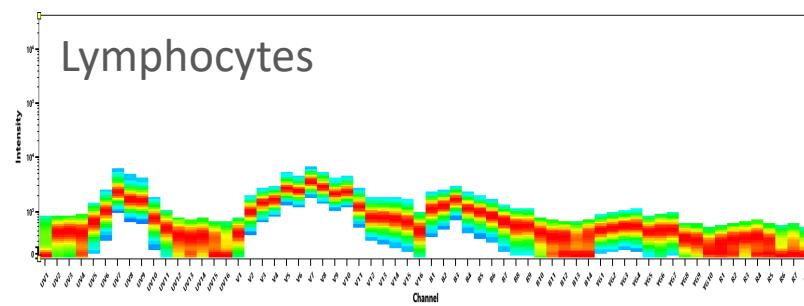
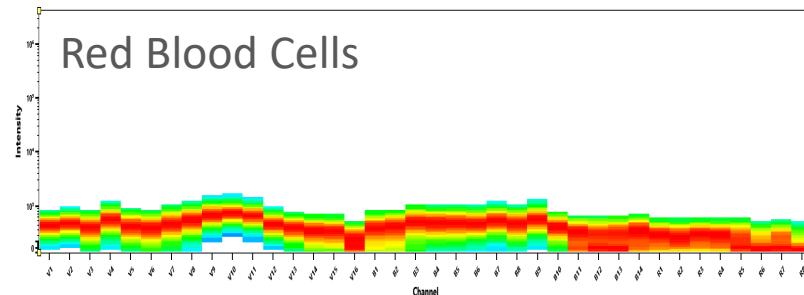


Fluorochromes with highly overlapping emission spectra can be used effectively on co-expressed markers



FSP™ Technology Easily Defines Autofluorescence

Autofluorescence is the native emission of light that comes from cellular components observed in unstained cells

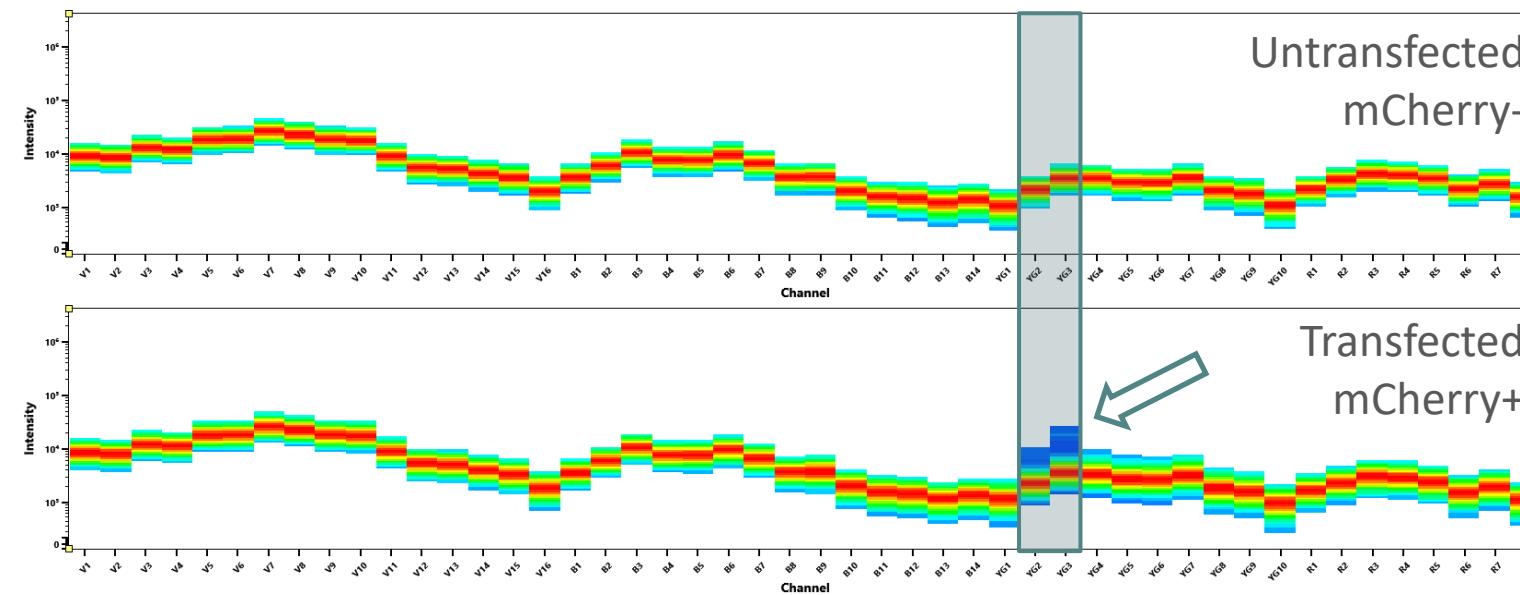
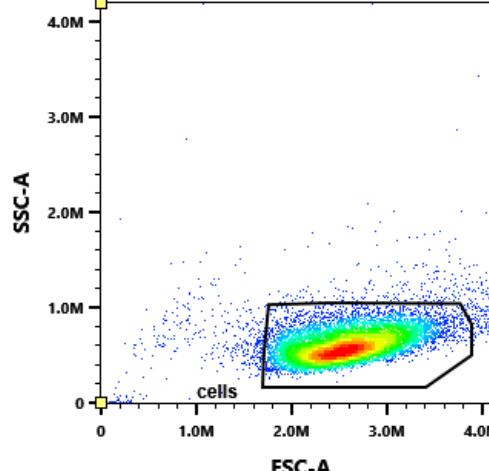


Cytek® Full Spectrum Profiling™ Technology can extract
autofluorescence and potentially improve marker resolution

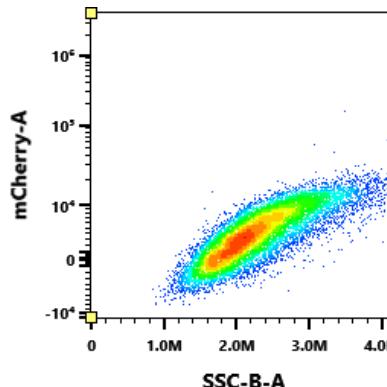


Benefits of Autofluorescence Extraction

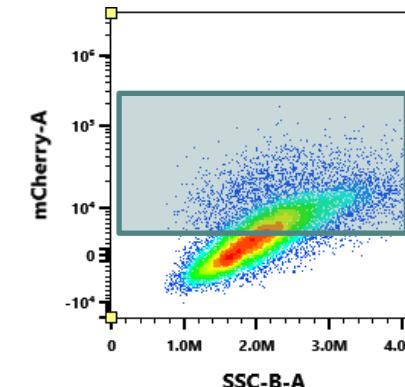
HeLa human cells were transfected with a vector carrying an mCherry reporter



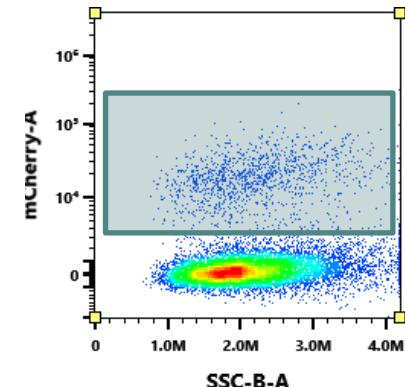
Untransfected
Without
Autofluorescence
Extraction



Transfected
Without
Autofluorescence
Extraction



Transfected
With
Autofluorescence
Extraction

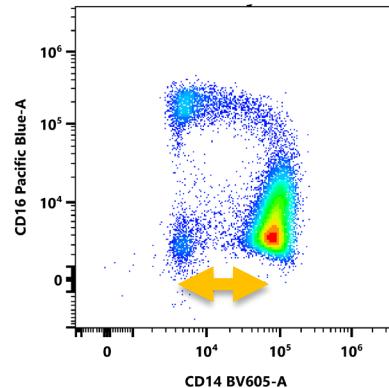




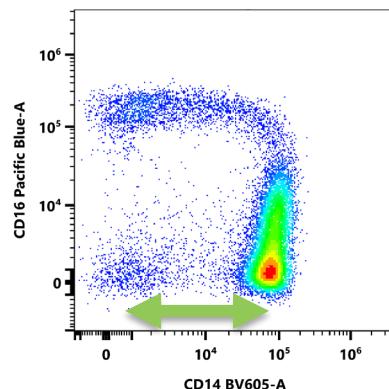
Autofluorescence Extraction Benefits Are Assay-Dependent

PBMCs stained with a 16-color assay

Without
Autofluorescence
Extraction

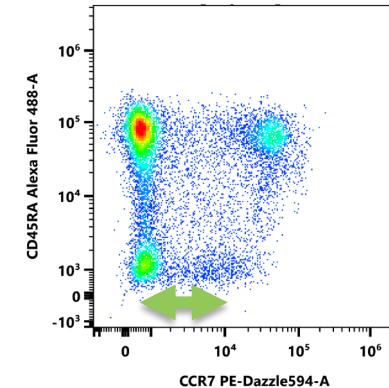


With
Autofluorescence
Extraction

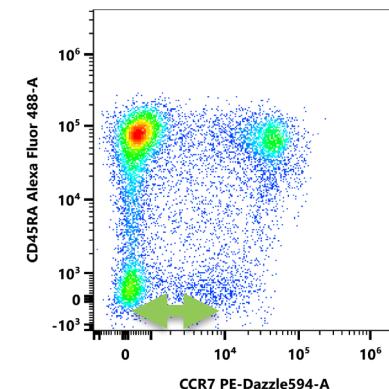


Software setup:

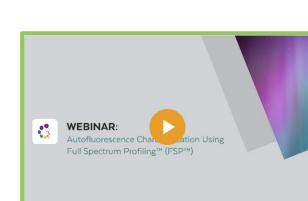
Unmixing Model: Spectral Unmixing



Unmixing Model: Spectral Unmixing With AF Extraction



Dyes emitting in high AF regions have improved resolution
Dyes emitting in low AF regions are unimpacted





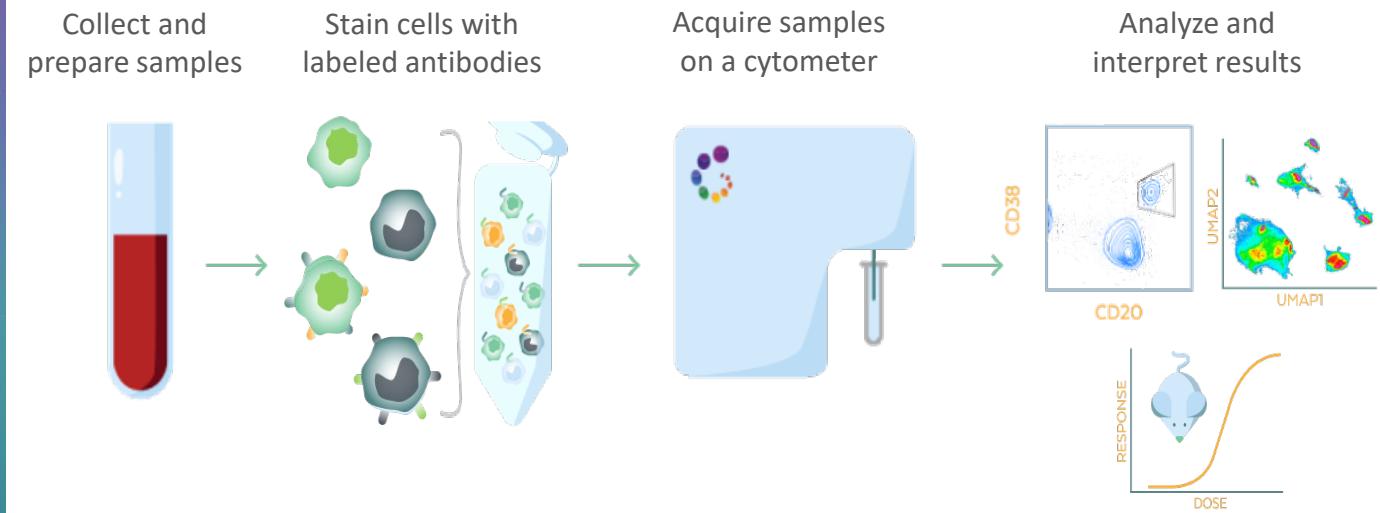
Interactive Poll #2

Benefits of Full Spectrum Profiling™ Technology (FSP™) include:



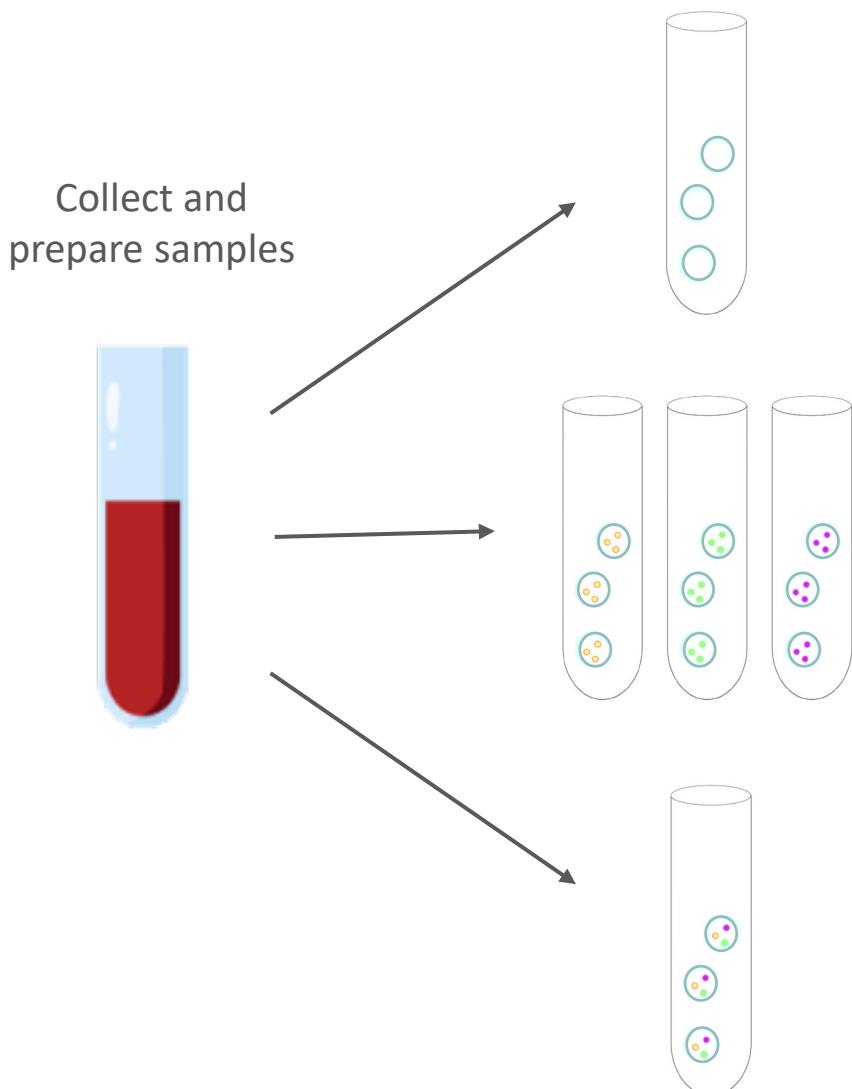
Full Spectrum Experiment Workflow

Let's review...





What Samples Need to Be Prepared?



Unstained Control

- No added fluorophores (fluorescent proteins, etc.)
- Must match cell type with multicolor assay (may need multiple!)

Reference Control

- Need one control for each fluorochrome present in the multicolor assay
- Defines a *single* fluorophore
- Not required to match cell type with multicolor

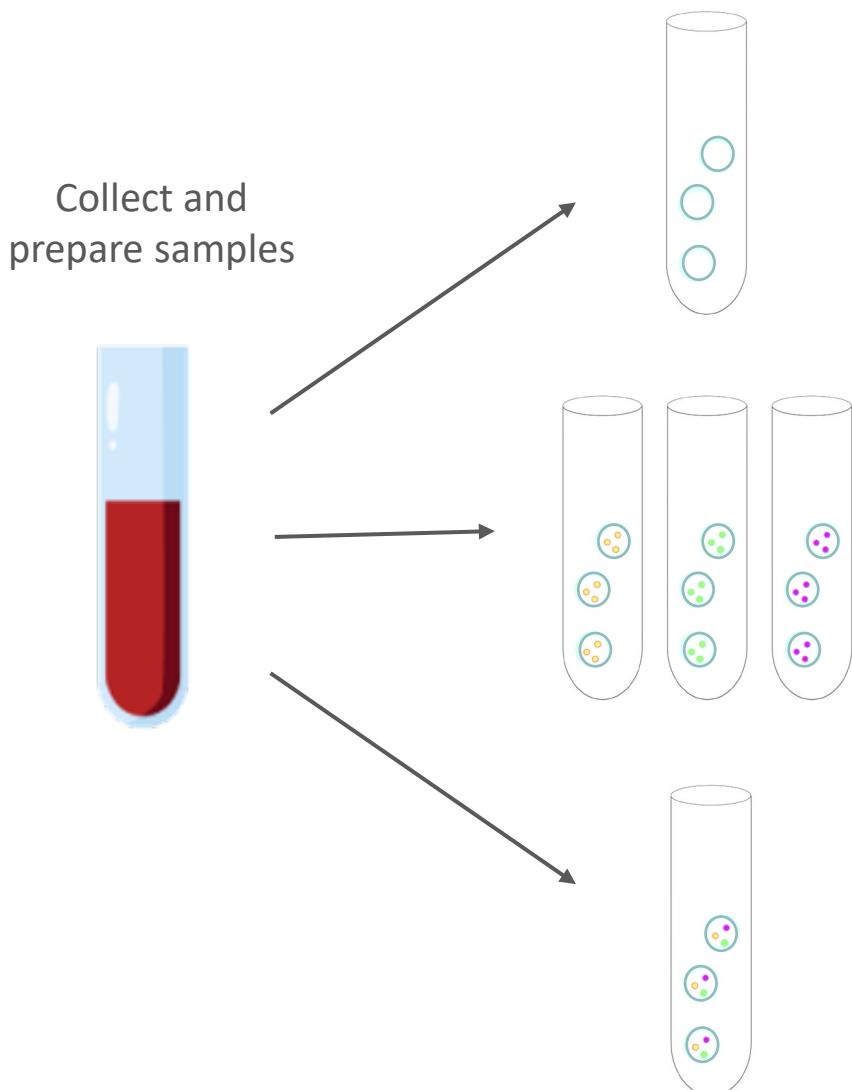
Multicolor Assay

- Contains all fluorophores in one tube/well

Additional experimental controls may be prepared depending on the assay



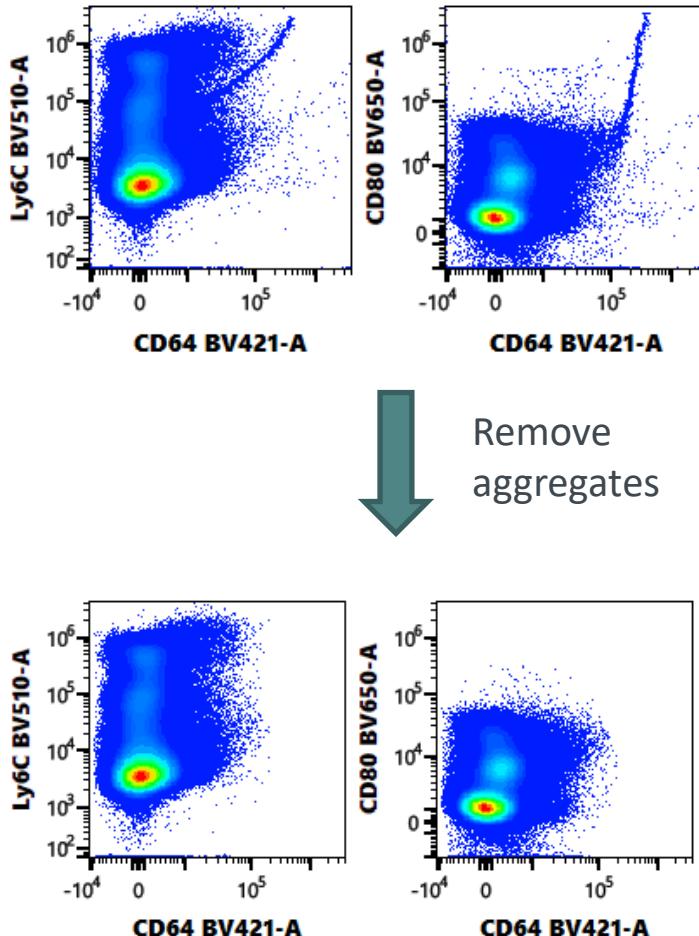
What Samples Need to Be Prepared?



- Prepare single cells in suspension
- All unstained and reference controls should be prepared using the same buffers and staining protocol as the multicolor



Tips for Staining Samples



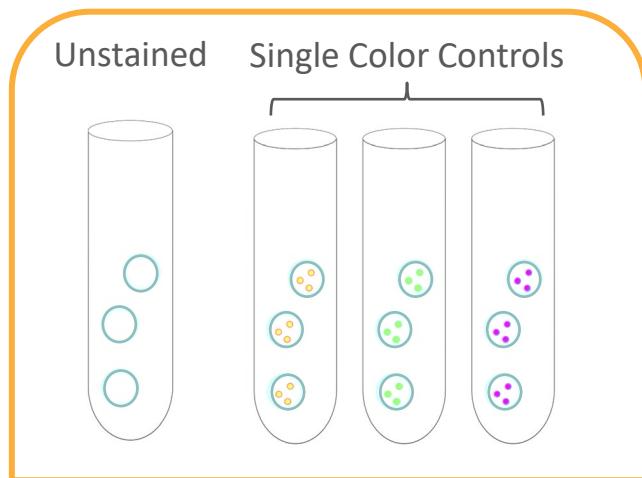
- 1 Resuspend single cells in blocking agents
 - FACS buffer with BSA or FBS, Fc Block, monocyte blocker, etc.
- 2 Prepare antibody cocktail
 - Centrifuge antibodies at maximum speed (10,000-14,000xg for 5 minutes at 4°C) to remove aggregates
 - Include Brilliant Stain Buffer/Super Bright Stain Buffer if the panel contains more than one polymer dye (BV, BUV, BB etc.)*
- 3 Protect fluorophores from light exposure

Optimize protocol for your own assay



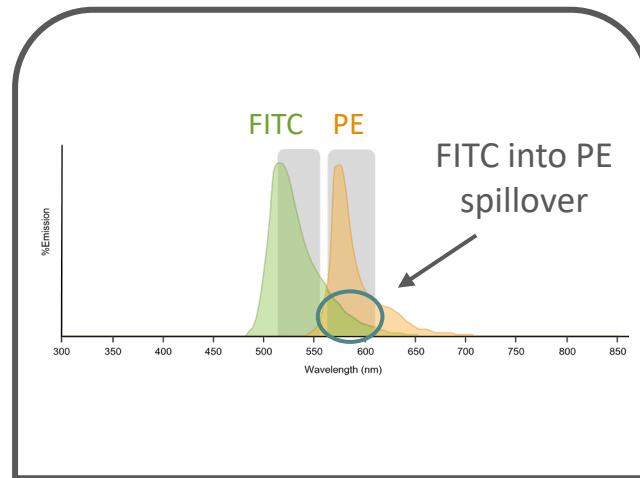
Acquisition Overview

Run compensation/reference controls



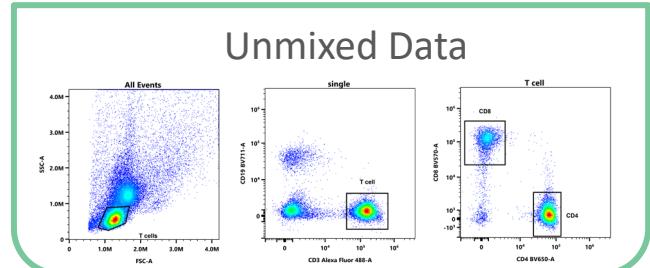
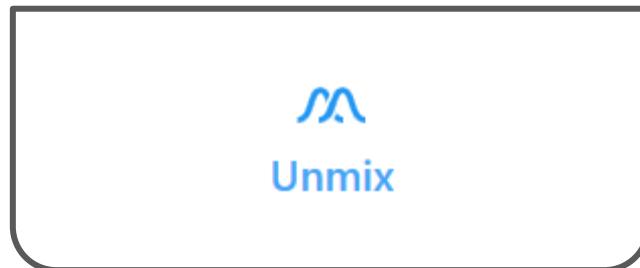
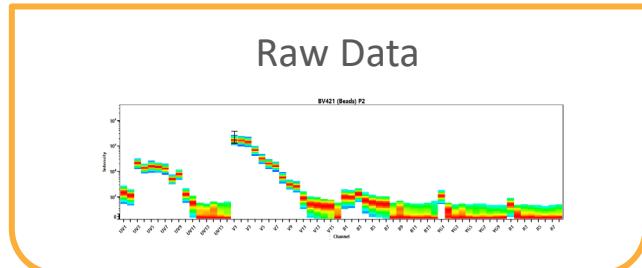
Input

Calculate compensation/unmixing



Output

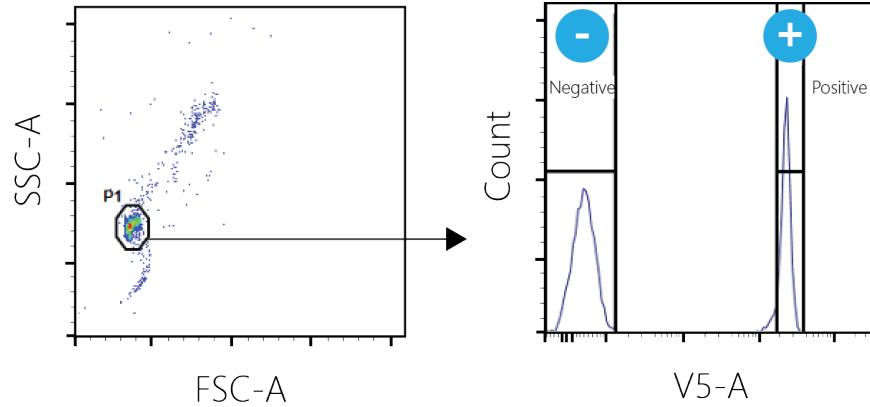
Run multicolor samples with compensation/unmixing applied



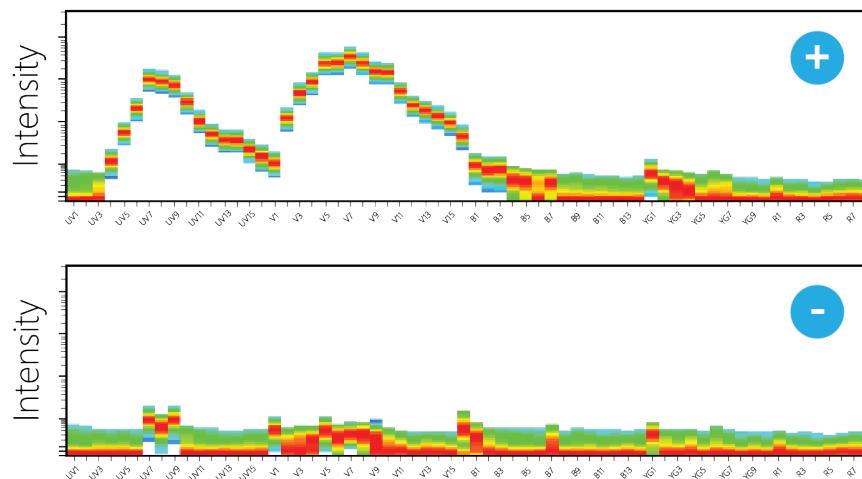
Unmixing converts raw data to unmixed data



Setting Up Spectral Unmixing in SpectroFlo® Software

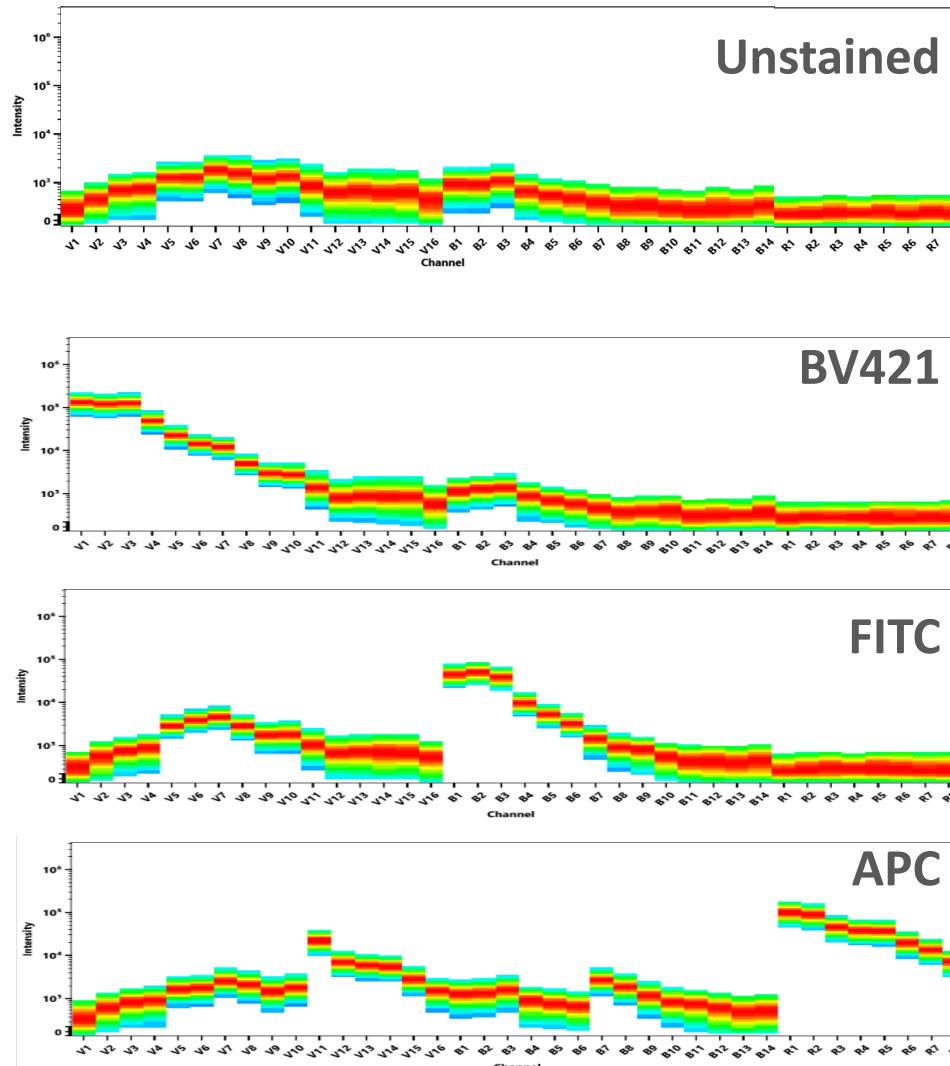


- 1 Set P1 gate on population expressing marker
- 2 Set positive and negative gates





Setting Up Spectral Unmixing in SpectroFlo® Software

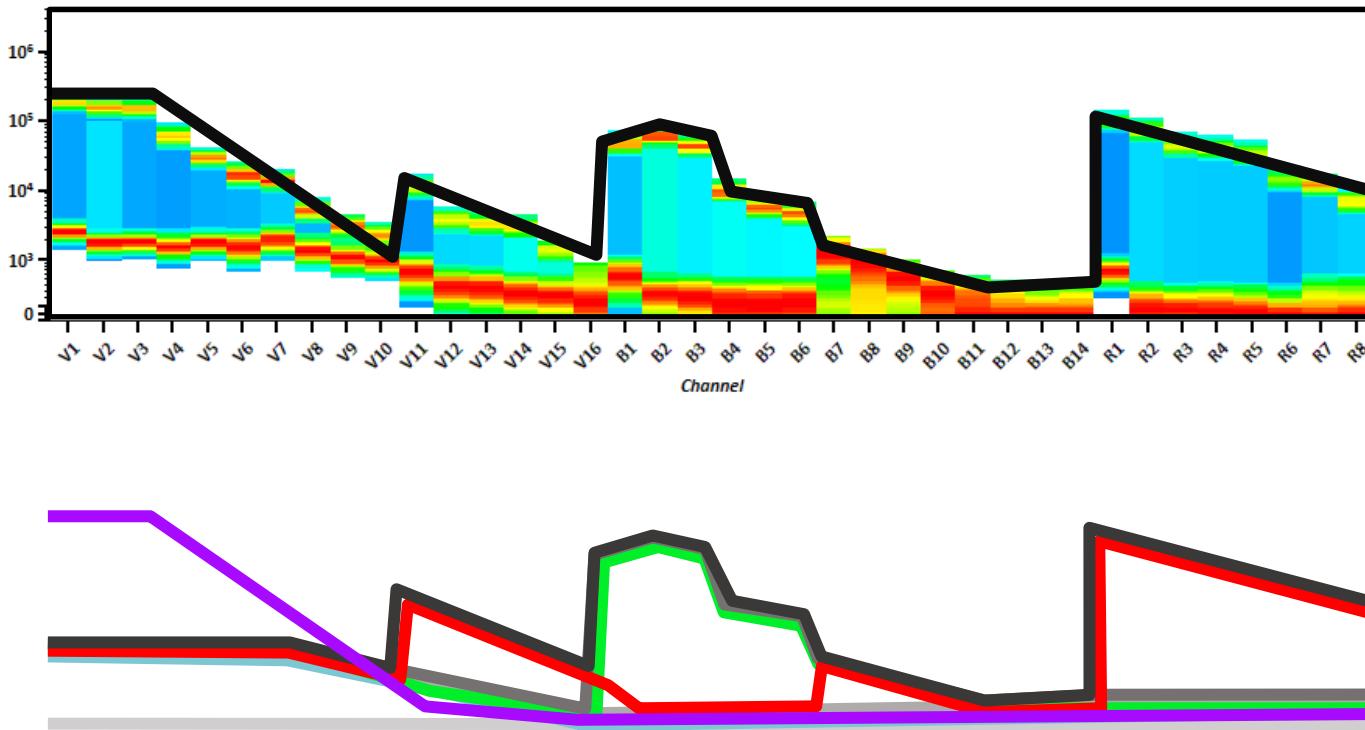


- 1 Set P1 gate on population expressing marker
- 2 Set positive and negative gates
- 3 Confirm signatures meet expectations
- 4 Click unmix



Spectral Unmixing - Ordinary Least Squares (OLS)

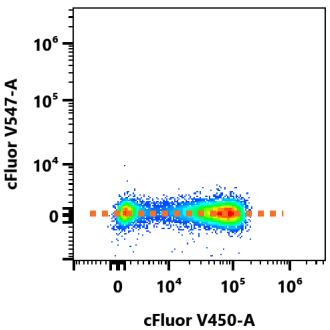
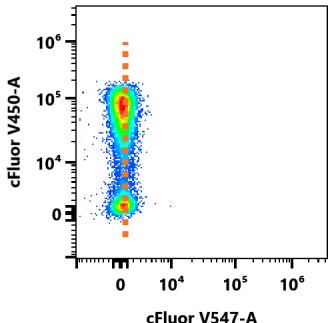
The spectral unmixing algorithm uses the provided controls to calculate the contribution of each fluorophore in the multicolor assay.



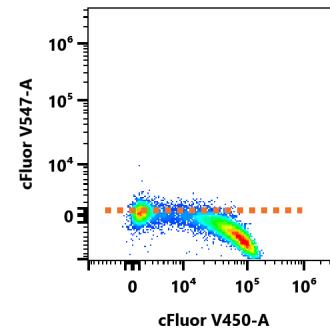
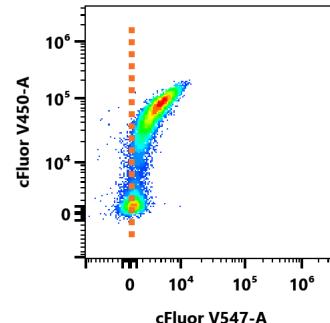
We can think of this as extracting or deconvoluting each component until we have nothing left.



Spectral Unmixing Applied to Data



MFI of the positive matches the MFI of the negative



MFI of the positive DOES NOT match the MFI of the negative

Unmixing/compensation errors can be either above or below the negative MFI

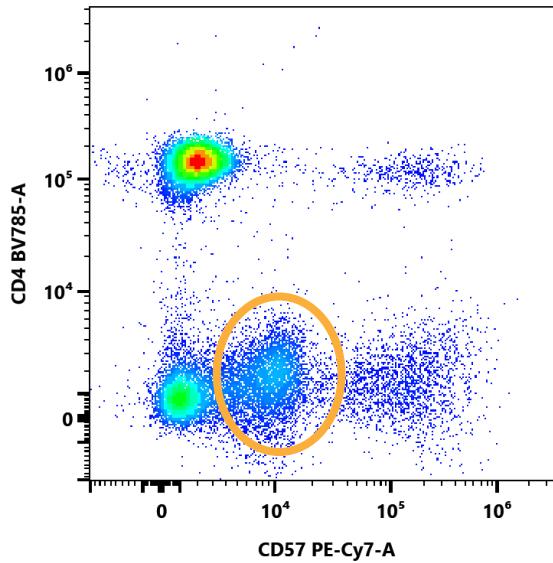
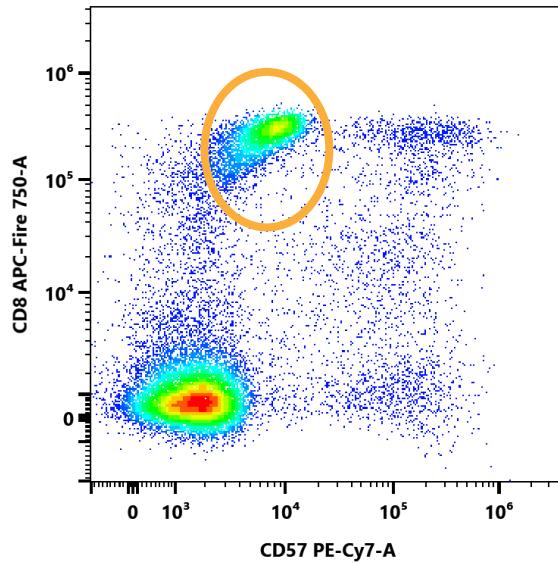


Unmixing/Compensation Errors Lead to Wrong Conclusions

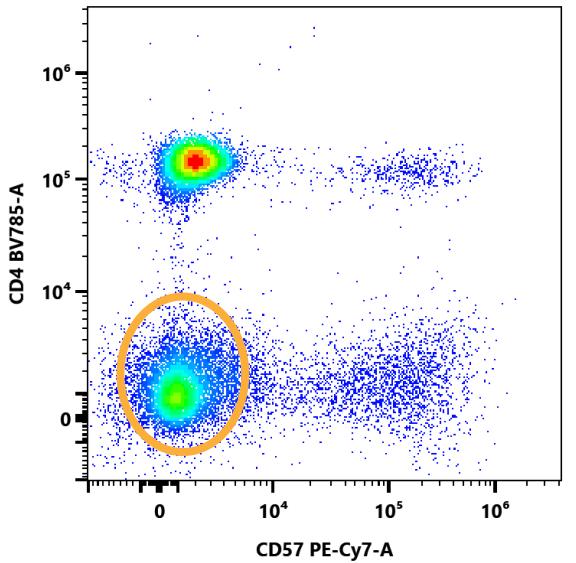
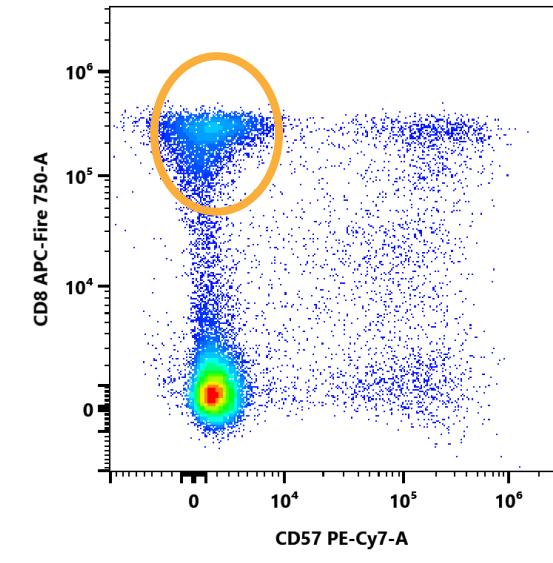
Impacts data accuracy and result interpretation: false populations!



Data WITH Unmixing/Compensation Error



Correct Unmixing/Compensation

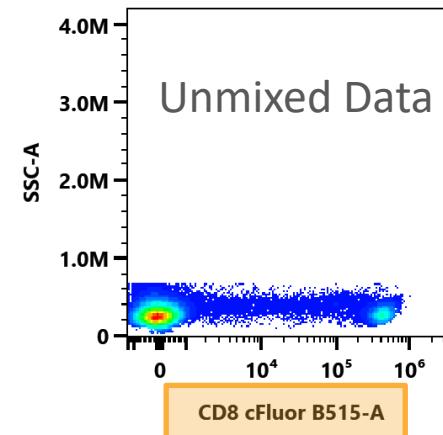
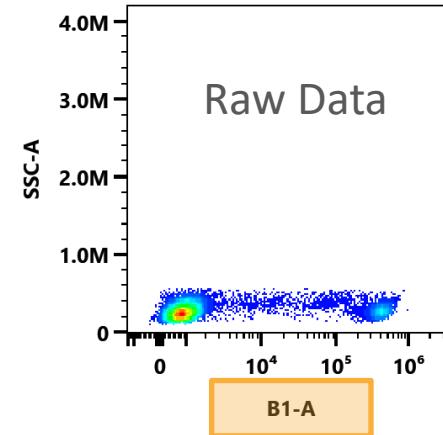




Options for Analyzing Data

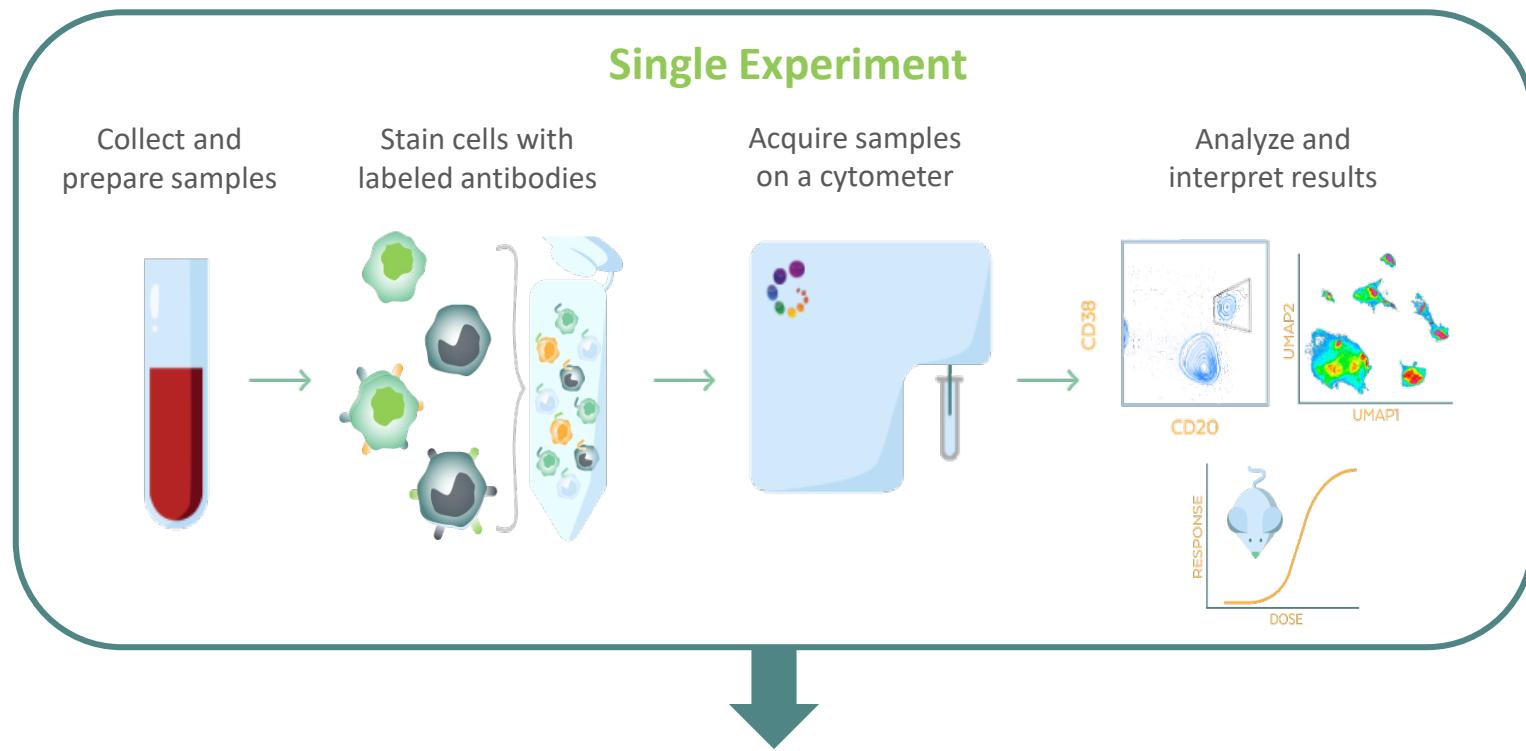
- Can analyze **raw data** when:
 - Only one fluorophore analyzed
 - Fluorophores in panel have no spectral overlap
- Analyze **unmixed data** when:
 - Multiple fluorophores in panel with spectral overlap

Any third party applications that accept FCS files can be used for analyzing data from Cytek® Systems





Developing an Assay to Answer a Scientific Question



Is my assay ready to answer my scientific question?

Three components to assay development:

Plan Your Assay

Run Your Assay

Evaluate Your Assay



Plan Your Assay

Build Your Panel

- Cytek® Tools for panel design

Select Appropriate Reference Controls

- How controls impact unmixing quality



Cytek® Panel Design Videos

SpectroLearn

<https://cytekbio.com/blogs/spectrolearn>

Educational Portal

SpectroLearn™

Cytek® SpectroLearn™ is our new educational portal where you can find content that will cover all things spectral cytometry.

FSP™ Panel Design Series

The Cytek® FSP™ Panel Design Series covers a range of topics that will help you learn fundamentals of flow cytometry. This series will dive deep into Full Spectrum Profiling™ Technology (FSP™) so that you can get the most out of your Cytek® Aurora, Northern Lights™ and Aurora CS systems.

Videos

Flow Cytometry Panel Design Best Practices Step 1: Marker Expression and Co-Expression

Flow Cytometry Panel Design Best Practices Step 2: Fluorochrome Selection

Flow Cytometry Panel Design Best Practices Step 3: Spillover-Spreading Error

Flow Cytometry Panel Design Best Practices Step 4: Designing a Panel

Flow Cytometry Panel Design Best Practices Step 5: Evaluate Panel Performance

Webinars

<https://cytekbio.com/blogs/videos>

WEBINAR: Building Panels for Flow Cytometry: Key Steps for Success

Building Panels for Flow Cytometry: Key Steps for Success

Click here to view this webinar led by Laura Johnston, where she covers the fundamentals of panel design, using examples in each step to demonstrate how to apply best practices of panel design to generate a...

WEBINAR: Debunking Antibody Titration Myths

Webinar: Debunking Antibody Titration Myths

Click here to watch this webinar, led by Diana Bonilla Escobar, PhD as she covers the fundamentals of antibody titrations, a critical step in the development and optimization of multicolor flow cytometry assays.

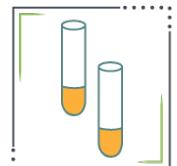


Panel Design Methods Apply to All Cytometers

Panel Design Should be An Informed Process



The
Biology



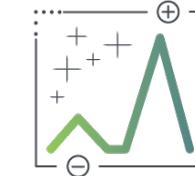
The
Fluorochromes



The
Design



The
Optimization





Tools for Panel Design



The
Fluorochromes



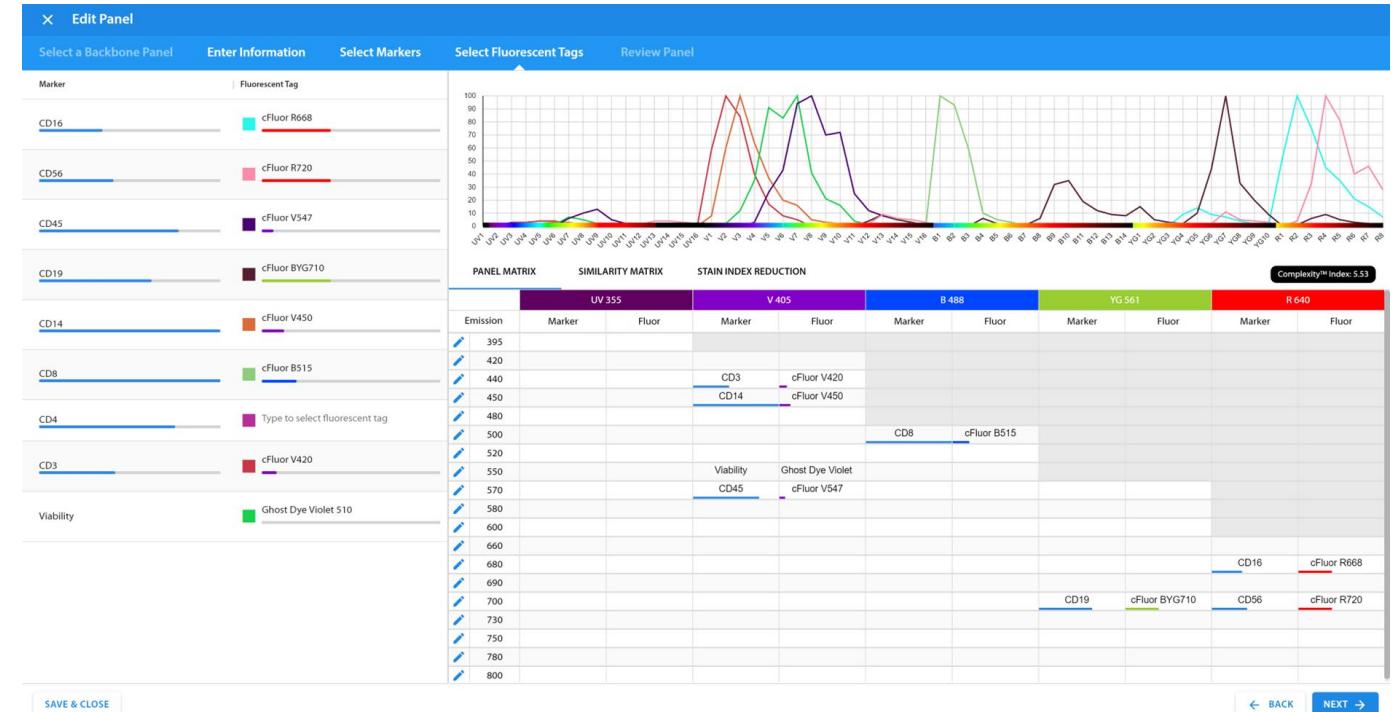
The
Design





Cytek® Cloud – Panel Builder

- Combines all full spectrum design tools in one place
- Options to:
 - Build custom panels from scratch
 - Modify pre-designed panels
 - Enter a previous panel from another cytometer





Cytek® Cloud – Panel Builder

X Edit Panel

Select a Backbone Panel Enter Info

Marker	Fluorescent Tag
CD16	BUV395
CD56	BUV496
CD45	BV421
CD19	Super Bright 436
CD14	cFluor V420
CD8	cFluor V450
CD4	violetFluor 450
CD3	cFluor V505
Viability	cFluor V500
	cFluor V547
	Super Bright 600

Type to select fluorescent tag

SAVE & CLOSE

BACK

NEXT →

Select Fluorescent Tags Review Panel

PANEL MATRIX SIMILARITY MATRIX STAIN INDEX REDUCTION Complexity™ Index: 5.53

UV 355 V 405 B 488 YG 561 R 640

Emission	Marker	Fluor	Marker	Fluor	Marker	Fluor	Marker	Fluor	Marker	Fluor
395			CD3	cFluor V420			CD8	cFluor B515		
420			CD14	cFluor V450						
440										
450										
480										
500										
520										
550										
570										
580										
600										
620										
640										
660										
680										
700										
730										
750										
780										
800										



Cytek® Cloud – Panel Builder

X Edit Panel

Select a Backbone Panel Enter Information Select Markers Select Fluorescent Tags Review Panel

Marker | Fluorescent Tag

CD16 cFluor R668

CD56 cFluor R720

CD45 cFluor V547

CD19 cFluor BYG710

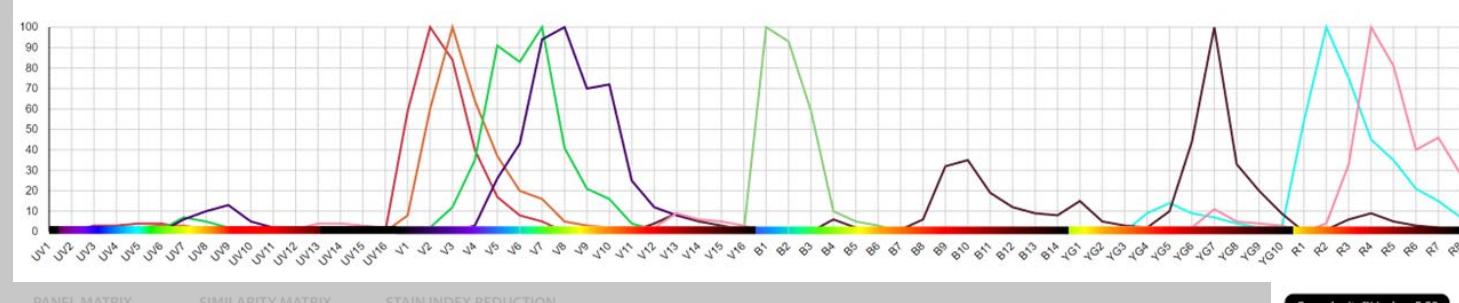
CD14 cFluor V450

CD8 cFluor B515

CD4 Type to select fluorescent tag

CD3 cFluor V420

Viability Ghost Dye Violet 510

 Complexity™ Index: 5.53

Spectrum Viewer

UV 355 Emission Marker Fluor

395 420 440 450 480 500 520 550 570 580 600 660 680 690 700 730 750 780 800

CD3 cFluor V420
CD14 cFluor V450
CD8 cFluor B515
Viability Ghost Dye Violet
CD45 cFluor V547
CD16 cFluor R668
CD19 cFluor BYG710
CD56 cFluor R720

SAVE & CLOSE BACK NEXT →



Cytek® Cloud – Panel Builder

X Edit Panel

Select a Backbone Panel Enter Information Select Markers Select Fluorescent Tags Review Panel

Marker | Fluorescent Tag

CD16 cFluor R668

CD56 cFluor R720

CD45 cFluor V547

CD19 cFluor BYG710

CD14 cFluor V450

CD8 cFluor B515

CD4 Type to select fluorescent tag

CD3 cFluor V420

Viability Ghost Dye Violet 510

UV1 UV2 UV3 UV4 UV5 UV6 UV7 UV8 UV9 UV10 UV11 UV12 UV13 UV14 UV15 UV16 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 YG1 YG2 YG3 YG4 YG5 YG6 YG7 YG8 YG9 YG10 R1 R2 R3 R4 R5 R6 R7 R8

Panel Matrix

PANEL MATRIX SIMILARITY MATRIX STAIN INDEX REDUCTION Complexity™ Index: 5.53

	UV 355		V 405		B 488		Y 561		R 640	
Emission	Marker	Fluor	Marker	Fluor	Marker	Fluor	Marker	Fluor	Marker	Fluor
395										
420										
440			CD3	cFluor V420						
450	CD14	cFluor V450								
480										
500										
520										
550			CD45	cFluor V547						
570			CD45	cFluor V547						
580										
600										
660										
680										
690										
700										
730										
750										
780										
800										

The colored bar below the fluor indicates the exciting laser and fluor brightness

CD8 cFluor B515

CD16 cFluor R668

CD56 cFluor R720

CD19 cFluor BYG710

CD56 cFluor BYG710

CD16 cFluor R668

CD56 cFluor R720

SAVE & CLOSE BACK NEXT →



Cytek® Cloud – Panel Builder

X Edit Panel

Select a Backbone Panel Enter Information Select Markers Select Fluorescent Tags Review Panel

Marker | Fluorescent Tag

CD16

CD56

CD45

CD19

cFluor BYG710

CD14

cFluor V450

CD8

cFluor B515

CD4

Type to select fluorescent tag

CD3

cFluor V420

Viability

Ghost Dye Violet 510

UV1 UV2 UV3 UV4 UV5 UV6 UV7 UV8 UV9 UV10 UV11 UV12 UV13 UV14 UV15 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 YG1 YG2 YG3 YG4 YG5 YG6 YG7 YG8 YG9 YG10 R1 R2 R3 R4 R5 R6 R7 R8

Use **Similarity™ Index** to measure degree of uniqueness between two dyes

Use **Complexity™ Index** to assess all dyes used in the panel

PANEL MATRIX SIMILARITY MATRIX STAIN INDEX REDUCTION Complexity™ Index: 5.53

Double click the similarity panel to expand.

FULL MATRIX

Complexity Index

	cFluor V420	cFluor V450	Ghost Dye Violet 510	cFluor V547	cFluor B515	cFluor BYG710	cFluor R668	cFluor R720	cFluor V420	cFluor V450	Ghost Dye Violet 510	cFluor V547	cFluor B515	cFluor BYG710	cFluor R668	cFluor R720						
cFluor V420	1	0.87	0.22	0.06	0	0	0	0	1	1	0.02	0.01	0.01	1	0.15	0.18	0.53	1				
cFluor V450	0.87	1	0.44	0.17	0.73	0.01	0.02	0.01	0.01	0.01	1	0.01	0.01	0.01	1	0.15	0.18	0.53	1			
Ghost Dye Violet 510	0.22	0.44	1	0.73	0.02	0.01	0.01	0.01	0.01	0.01	1	0.01	0.01	0.01	0.01	1	0.15	0.18	0.53	1		
cFluor V547	0.06	0.17	0.73	1	0.02	0.01	0.01	0.01	0.01	0.01	1	0.01	0.01	0.01	0.01	1	0.15	0.18	0.53	1		
cFluor B515	0	0	0.02	0.01	0.01	1	0.01	0	0	0	0	0.01	0	0	0	0	1	0.15	0.18	0.53	1	
cFluor BYG710	0	0	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	1	0.15	0.18	0.53	1
cFluor R668	0	0	0	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0.15	0.18	0.53	1	
cFluor R720	0	0	0	0	0	0.01	0.01	0.01	0.01	0	0	0	0	0	0	0	0	0.18	0.53	1		

Complexity™ Index: 5.53

SAVE & CLOSE BACK NEXT →



Cytek® Cloud – Panel Builder

X Edit Panel

Select a Backbone Panel Enter Information Select Markers Select Fluorescent Tags Review Panel

Marker | Fluorescent Tag

CD16 cFluor R668

CD56 cFluor R720

CD45 cFluor V547

CD19 cFluor BYG710

CD14 cFluor V450

CD8 cFluor B515

CD4

CD3

Viability

SpectroLearn | Cytek Biosciences

PANEL DESIGN E PRACTICES: Step 2: Fluorochrome Selection

Flow Cytometry Panel Design Best Practices Step 2: Fluorochrome Selection

Watch

SAVE & CLOSE

Use Stain Index Reduction (SIR) to assess potential areas of spread

PANEL MATRIX SIMILARITY MATRIX STAIN INDEX REDUCTION Complexity™ Index: 5.53 SHOW #

Double click the stain index reduction matrix to expand.

	cFluor V420	cFluor V450	Ghost Dye Violet 510	cFluor V547	cFluor B515	cFluor BYG710	cFluor R668	cFluor R720
cFluor V420								
cFluor V450								
Ghost Dye Violet 510								
cFluor V547								
cFluor B515								
cFluor BYG710								
cFluor R668								
cFluor R720								

BACK NEXT →



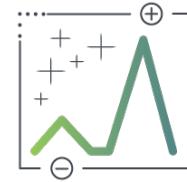
Interactive Poll #3

Where can I find more information from Cytek® on panel design?



How to Determine if a Panel Is Successful

A panel is successful if we can resolve all populations and markers of interest



Steps for Assay Optimization

- 1 Optimize individual reagents
 - Select best clone, fluorophore, and antibody concentration (titer) for optimal single color resolution
- 2 Optimize reference controls
 - Achieve accurate unmixing in multicolor sample
- 3 Optimize multicolor staining
 - Confirm all populations and markers are resolved in multicolor sample

The Optimization

4

See additional resources on antibody titration and assay optimization on cytekbio.com



Select Appropriate Reference controls

How controls impact unmixing quality



Factors to Consider When Selecting Controls

Compensation and Reference Controls should account for:

- Fluorescence intensity
- Accuracy of fluorophore signature – must match multicolor
- Collecting enough events to appropriately define the fluorophore

Two approaches for selecting controls:

- 1 Use a control that matches the multicolor (cell type, reagent, etc.)
- 2 Use a different sample type or reagent while still considering selection factors



Selecting the Best Controls for Successful Unmixing

Compensation and Reference Controls should account for:

- Fluorescence intensity
- Accuracy of fluorophore signature – must match multicolor
- Collecting enough events to appropriately define the fluorophore



1

Guidelines for Best Controls

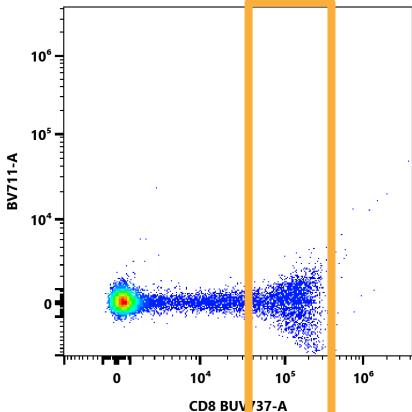
- Must be as-bright or brighter than the multicolor sample with positive and negative particles clearly separated



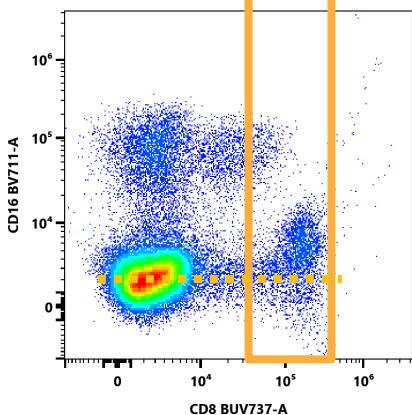
1

Fluorescence Intensity Affects Unmixing

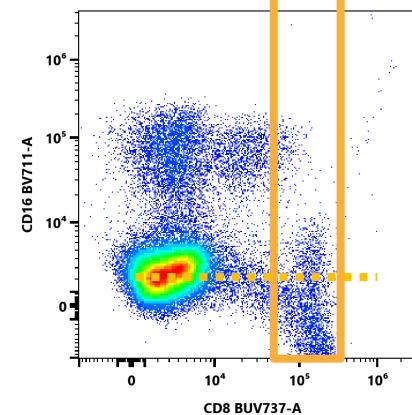
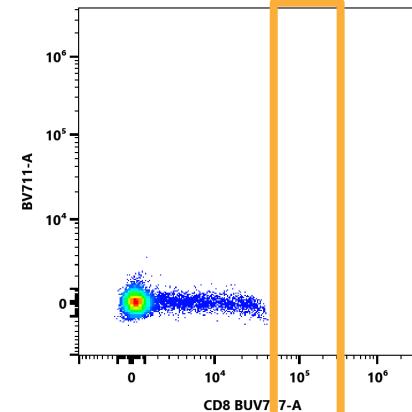
Reference
Control



Multicolor
Sample



Reference has **same** fluorescence
intensity as multicolor



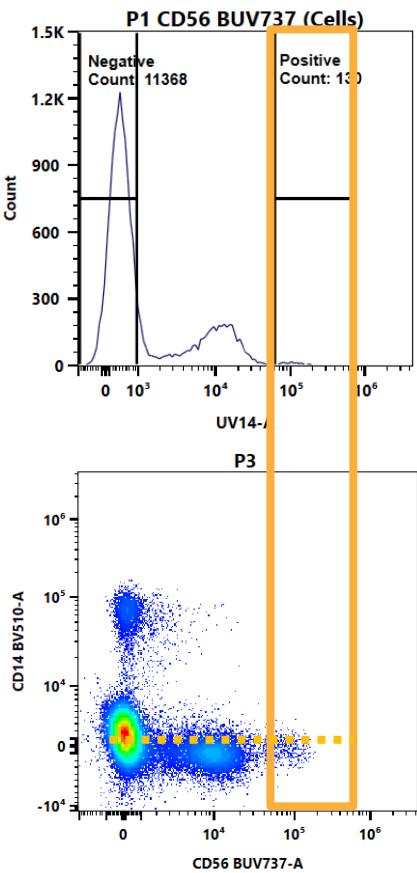
Reference has **lower** fluorescence
intensity than multicolor



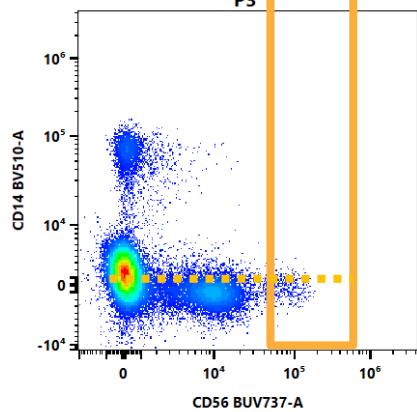
1

Reference Control Gating Affects Unmixing

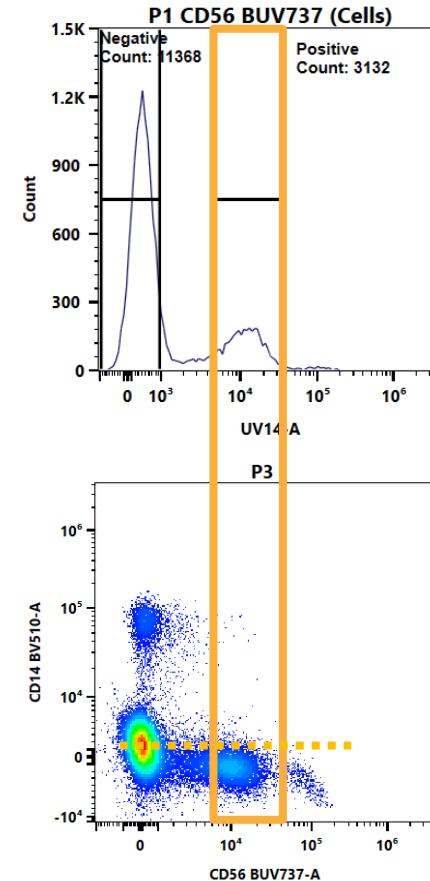
Reference
Control



Multicolor
Sample



Gating captures population with
same brightness as multicolor



Gating captures population
dimmer than multicolor



Selecting the Best Controls for Successful Unmixing

Compensation and Reference Controls should account for:

- Fluorescence intensity
- Accuracy of fluorophore signature – must match multicolor
- Collecting enough events to appropriately define the fluorophore

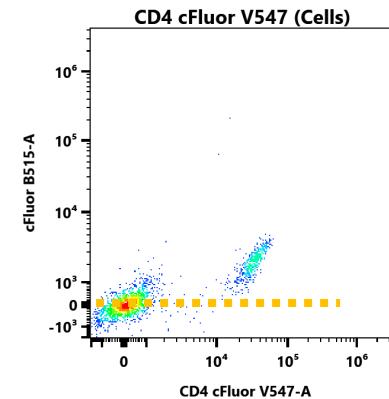
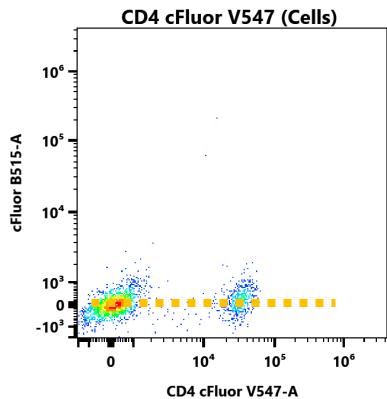
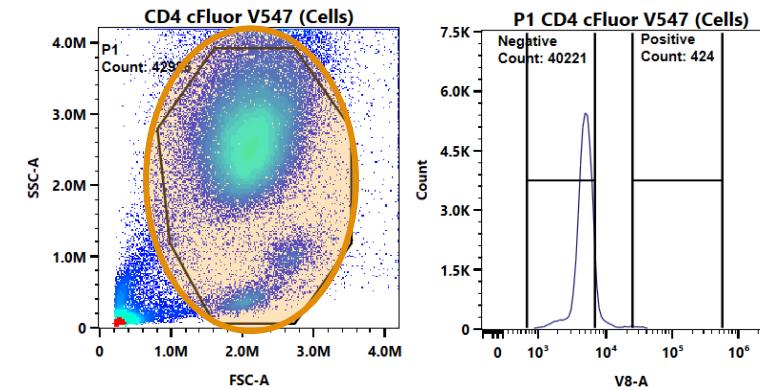
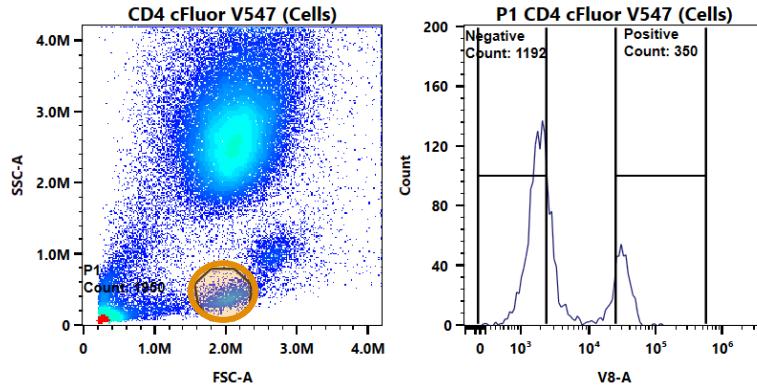
Guidelines for Best Controls

- 1 Must be as-bright or brighter than the multicolor sample with positive and negative particles clearly separated
- 2 Negative and positive particles must have IDENTICAL autofluorescence characteristics
- 3 Fluorescence spectrum of reference control needs to be accurate and IDENTICAL to the one in the multicolor samples



2

Negative Signatures Affect Unmixing



Positive and negative
autofluorescence match

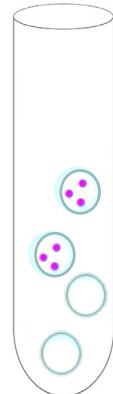
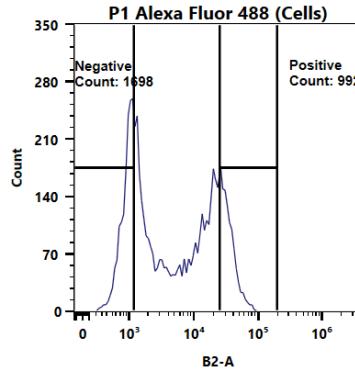


Positive and negative
autofluorescence do not match



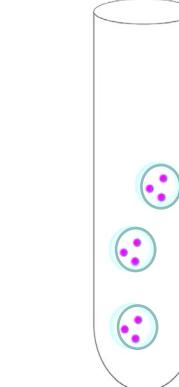
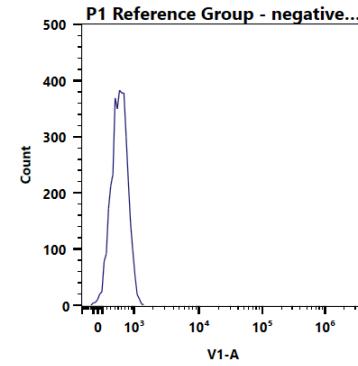
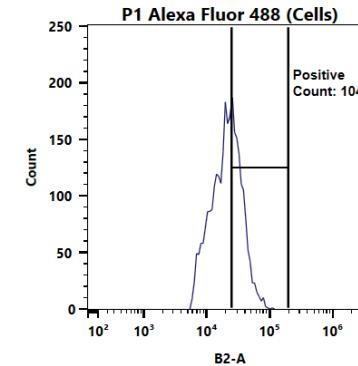
What Are Internal vs. Universal Negative Controls?

Internal negative is found in the same tube as the positive population

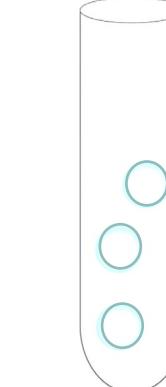


Internal negative in reference control

Universal negative is needed when reference control does not contain a negative



Reference control



Universal negative



Exercise 2: Assigning Negative Controls

Goals

- Identify appropriate negative control
- Identify appropriate unstained control



Exercise 2: Assigning Negative Controls

Bone marrow cells stained with:

- CD4 BV421
- CD45 FITC
- CD19 PE-Cy7

I do not have enough bone marrow sample for controls, I'll use PBMCs instead

Define Fluorescence Signature		
Reference Control	Particle type	Universal or Internal Negative?
CD4 BV421	PBMCs	
CD45 FITC (all cells +)	PBMCs	
CD19 PE-Cy7	PBMCs	

Define Autofluorescence of Multicolor	
Unstained control	

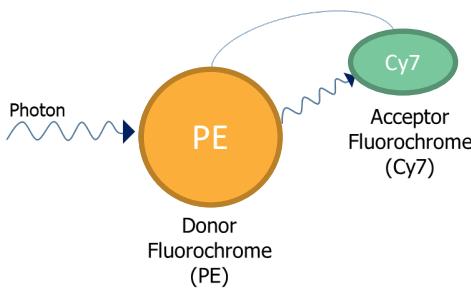


3

Fluorescence Spectrum Affects Unmixing

Factors That Can Alter Fluorescence Spectrum

Lot Variation in Tandem Dyes



Guideline

Stain controls and samples with the same reagent lot

Using Compensation Beads



Guideline

Experimentally determine if spectrum from beads is accurate for unmixing

Staining/Fixation Conditions

Staining Considerations

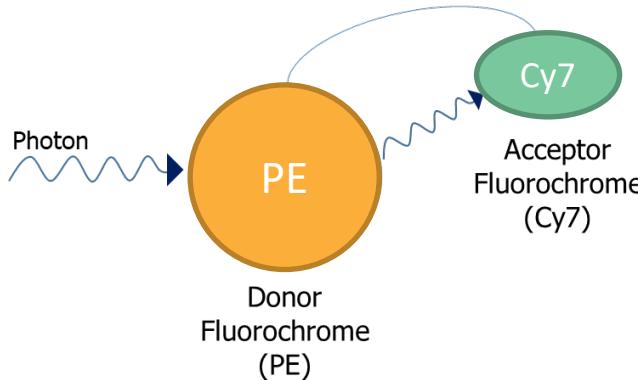
- Stain Buffers
- Fixatives
- Temperature
- Time

Guideline

Prepare all controls and samples using the same protocol



What Is a Tandem Dye?



Tandem Dyes

- Two covalently attached fluorescent molecules
- The donor-acceptor pair behaves like a fluorophore with the excitation properties of the donor and the emission properties of the acceptor

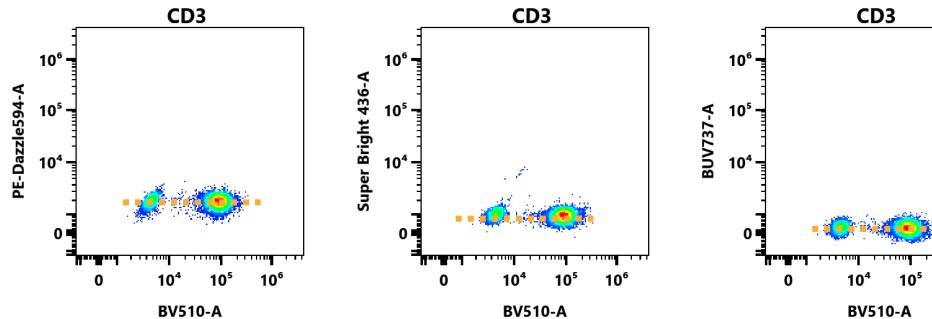
Base dye	Tandem from base dye
cFluor BYG575 (PE)	PE-Cy5.5 cFluor BYG610, BYG667 (PE-Cy5), BYG710, BYG750, BYG781 (PE-Cy7)
cFluor R659 (APC)	APC-R700, APC-Cy7, APC-H7, cFluor R780 (APC-Fire 750), cFluor R840
BV421	BV570, BV605, BV650, BV711, BV750, BV785
BV480/BV510	



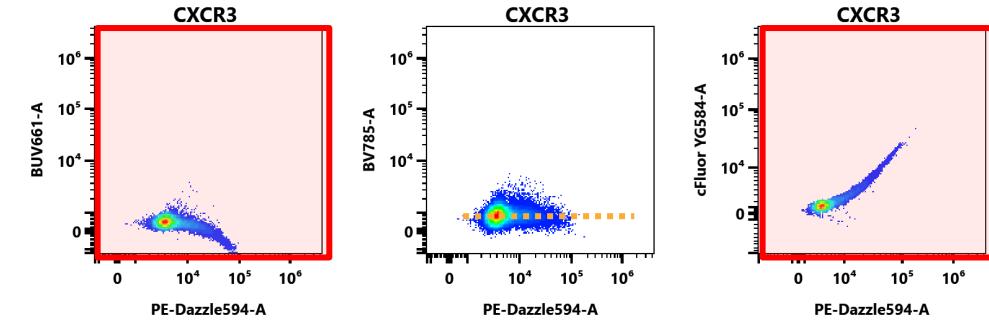
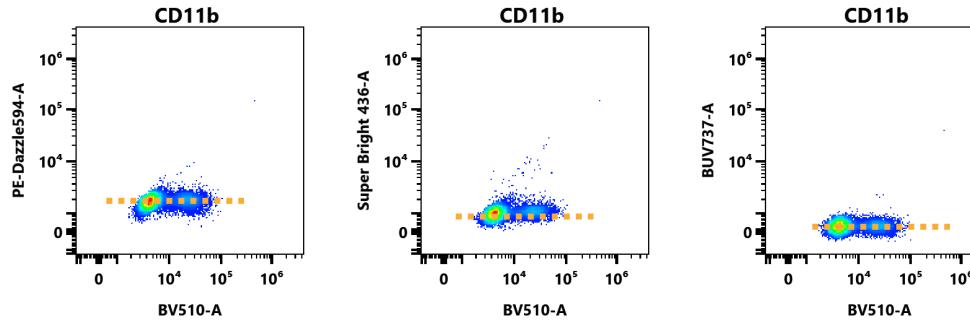
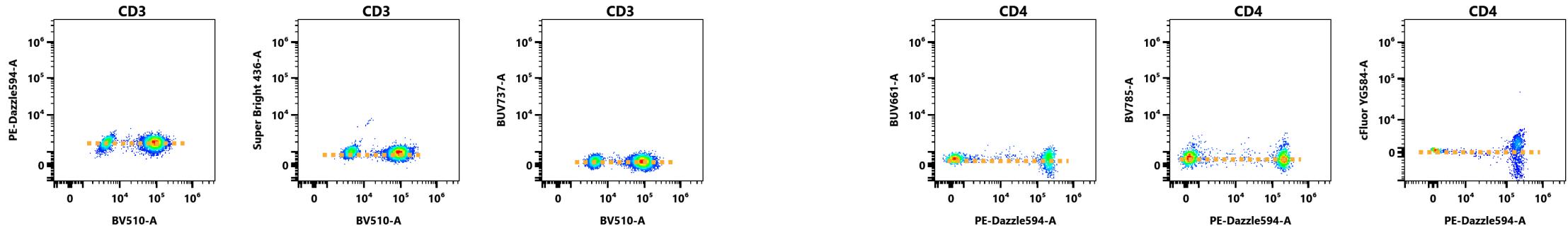
Reference Control Signatures and Tandem Dye Variation

Inaccurate Unmixing Results When Signatures Do Not Match

CD3 BV510 used to unmix CD11b BV510 (base dye)



CD4 PE-Dazzle594 used to unmix CXCR3 PE-Dazzle594 (tandem dye)



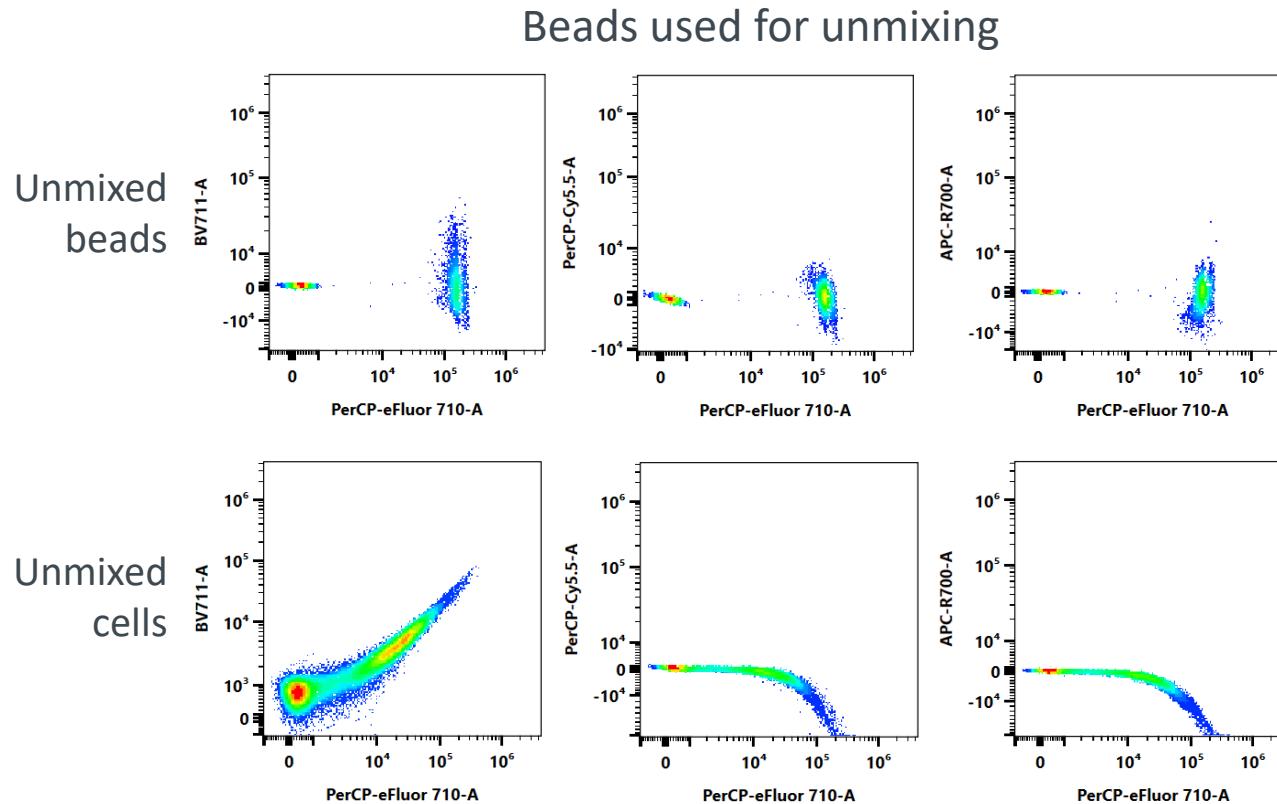
✓ Correct unmixing for both conjugates

✗ CD4 PE-Dazzle594 cannot unmix CXCR3 PE-Dazzle594 because of spectral mismatch



Comparison of Beads vs. Cells As Reference Controls

Inaccurate Unmixing Results When Signatures Do Not Match



Best Practice: Optimal controls should be experimentally determined for each assay

In Cytek's 40-color panel OMIP-069, 29 out of 40 reference controls were made using beads



Selecting the Best Controls for Successful Unmixing

Compensation and Reference Controls should account for:

- Fluorescence intensity
- Accuracy of fluorophore signature – must match multicolor
- Collecting enough events to appropriately define the fluorophore

Guidelines for Best Controls

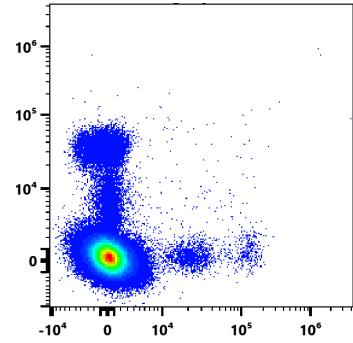
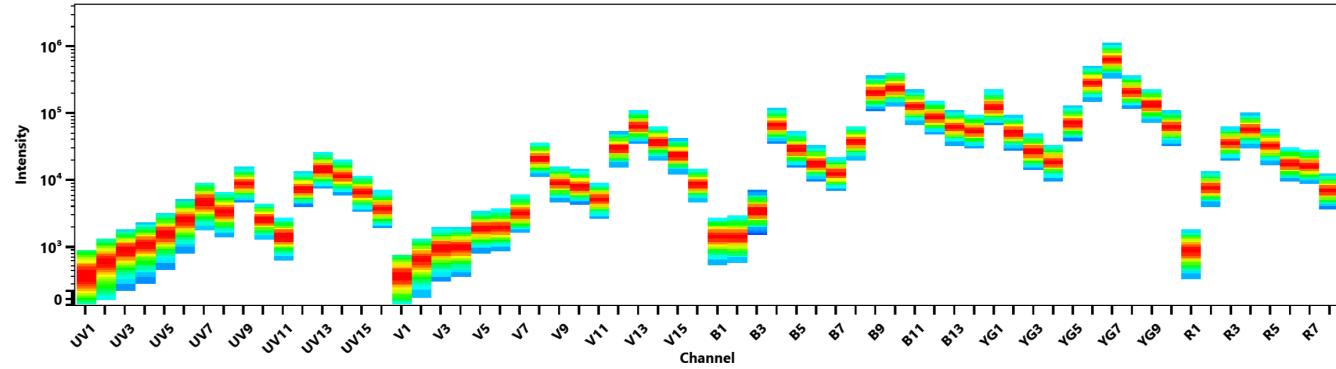
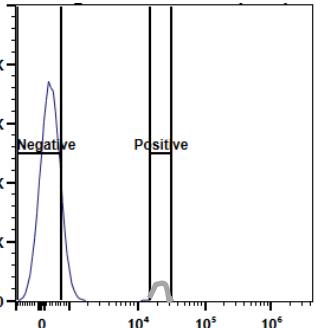
- 1 Must be as-bright or brighter than the multicolor sample with positive and negative particles clearly separated
- 2 Negative and positive particles must have IDENTICAL autofluorescence characteristics
- 3 Fluorescence spectrum of reference control needs to be accurate and IDENTICAL to the one in the multicolor samples
- 4 Sufficient events in both positive and negative populations



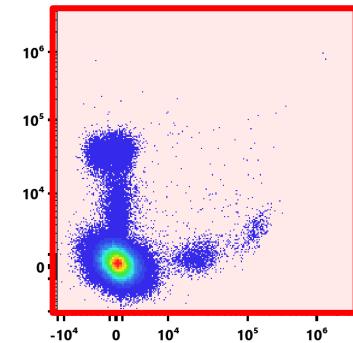
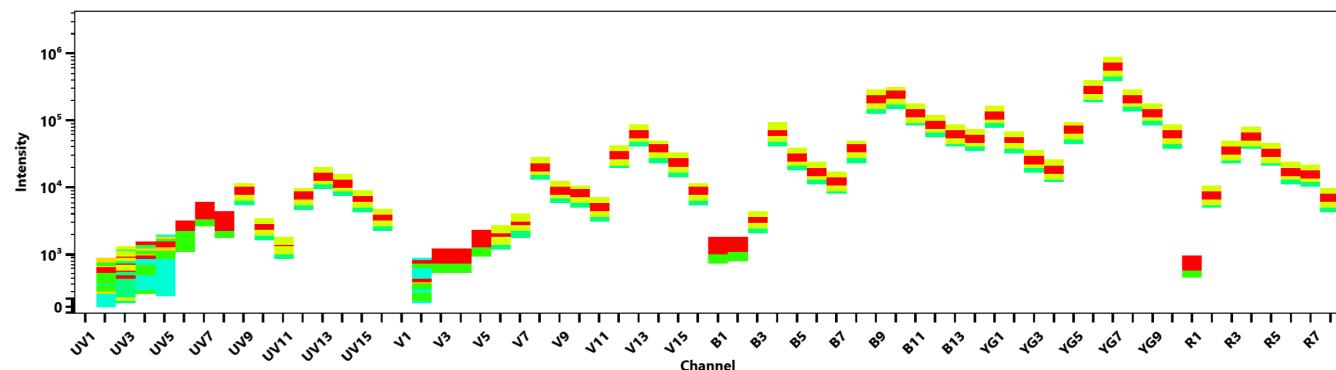
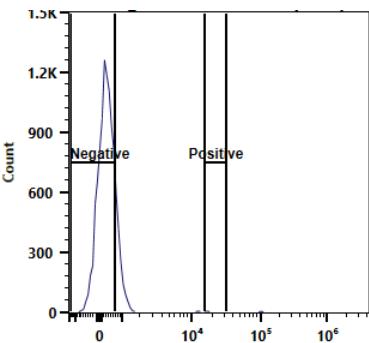
4

Event Count Affects Unmixing

Sufficient Events



Insufficient Events





Reference Controls: Making Good Choices

Any alternative cells or beads can be used to calculate unmixing as long as **ALL** guidelines are followed.

I don't have enough cells to stain controls...What can I do?

Alternative Cells

Select another tissue that expresses the marker

Beads

Stain with same reagent (same lot). Beware of possible signature mismatch

The marker is dim...What can I do?

Alternative Cells

Select another tissue that expresses the marker

Beads

Stain with same reagent (same lot). Beware of possible signature mismatch

Alternative Reagents

Only for non-tandem dyes: Use a highly expressed marker in a distinct population (CD3, B220, etc.)
Alternative reagents are not recommended for tandem dyes.

[SpectroLearn™](#) | Cytek Biosciences



Best Practice: Optimal controls should be experimentally determined for each assay



Interactive Poll #4

True/False: The guidelines for compensation and reference controls are the same



Exercise 3: Reference Control Selection

Goals

- Identify most appropriate reagent for controls
- Understand considerations for experimental condition



Exercise 3: Reference Control Selection

Tissue: Isolated T Cells

Which reagents need to stay lot-matched, and which can be swapped for any marker?

Marker	Fluor	Control Stained With	Level of Expression	Experimental Condition?
CD3	APC			
CD4	PE-Cy7			
CD45	cFluor® BYG610			
IFN γ	BV480			
Viability	LIVE DEAD Blue			

*if changing reagent for any bright stain think about level of expression & cell type



Exercise 4: Staining Protocol

Goals

- Apply staining protocol to controls



Exercise 4: FoxP3 Reference Control Staining Protocol

Protocol	Multicolor Cells	Beads for Surface Antibodies	Beads for FoxP3 IC Antibody
1. Stain Buffer Wash	✓		
2. Add Surface Abs and Incubate	✓		
3. Stain Buffer Wash	✓		
4. Fix and Permeabilize	✓		
5. Perm Wash	✓		
6. Add IC Ab and Incubate	✓		
7. Perm Wash/resuspend in final volume	✓		



Run Your Assay

Overview of Experiment Workflow

- Use Cytek® Cloud and SpectroFlo® Software to create experiments



Acquisition Workflow – The Big Picture

Workflow for New Experiment

- 1 Start up cytometer
- 2 Set up experiment on cytometer
- 3 Record reference controls
- 4 Unmix
- 5 Record samples



[Video Tutorials on YouTube](#)



Interactive Poll #5

At what point in the workflow can unmixing be calculated?



Cytek® Cloud – Experiment Builder

Option 1: Select “Create Experiment” in the **Panel builder** to transfer to the Experiment Builder

Edit Panel

Select a Backbone Panel Enter Information Select Markers Select Fluorescent Tags Review Panel

Review Panel Information

Name: Cytek_cFluor_8CTBMNK
Cytometer Configuration: SL UV/V/B/YG/R
Staining Region: Intracellular (Cytosolic), Surface
Sample Preparation Method: Blood, Gut
Panel Description: S1AUG
Notes: --

Marker **Clone Name** **Fluorophore Tag** **Target Species** **Dilution** **Supplier** **Size** **Catalog #**

CD16	3G8	cFluor R668	Human	—	Cytek Biosciences	100 Tests	R7-20009
CD56	S.1H11	cFluor R720	Human	—	Cytek Biosciences	100 Tests	R7-20089
CD45	HIB30	cFluor V547	Human	—	Cytek Biosciences	100 Tests	R7-20011
CD19	HIB19	cFluor BYG710	Human	—	Cytek Biosciences	100 Tests	R7-20009
CD14	M8E2	cFluor V450	Human	—	Cytek Biosciences	100 Tests	R7-20003
CD8	SK1	cFluor 8S15	Human	—	Cytek Biosciences	100 Tests	R7-20035
CD4	SK3	cFluor V420	Human	Cytek Biosciences	100 Tests	R7-20035	

SAVE & CLOSE **BACK** **COMPLETE**

Fluorophores and markers will be transferred to Experiment Builder

Option 2: Select “Send to Experiment Builder” under Export Options in the **Full Spectrum Viewer**

Full Spectrum Viewer

Experiment Builder

Available Fluorescent Tags **Selected Fluorescent Tags** **Signatures** **Similarity Matrix** **STAIN INDEX REDUCTION**

Export **Send to Experiment Builder** **EXPORT OPTIONS**

Fluorophores will be transferred to Experiment Builder

Option 3: Select “Create Experiment” in the **Experiment Builder** to start from scratch

My Experiments

CREATE EXPERIMENT **IMPORT**

Experiment **Description** **Configuration** **Date Created** **Date Modified**

Cytek_cFluor_8CTBMNK

Experiment Builder

Account Settings **Contact Support** **Log Out**



Cytek® Cloud – Experiment Builder

1

Fluorescent Tags

Add fluorophores to the experiment

2

Groups

Add and organize tubes/plate(s)

3

Markers

Add labels to fluorophores

4

Acquisition

Add stopping parameters for recording files

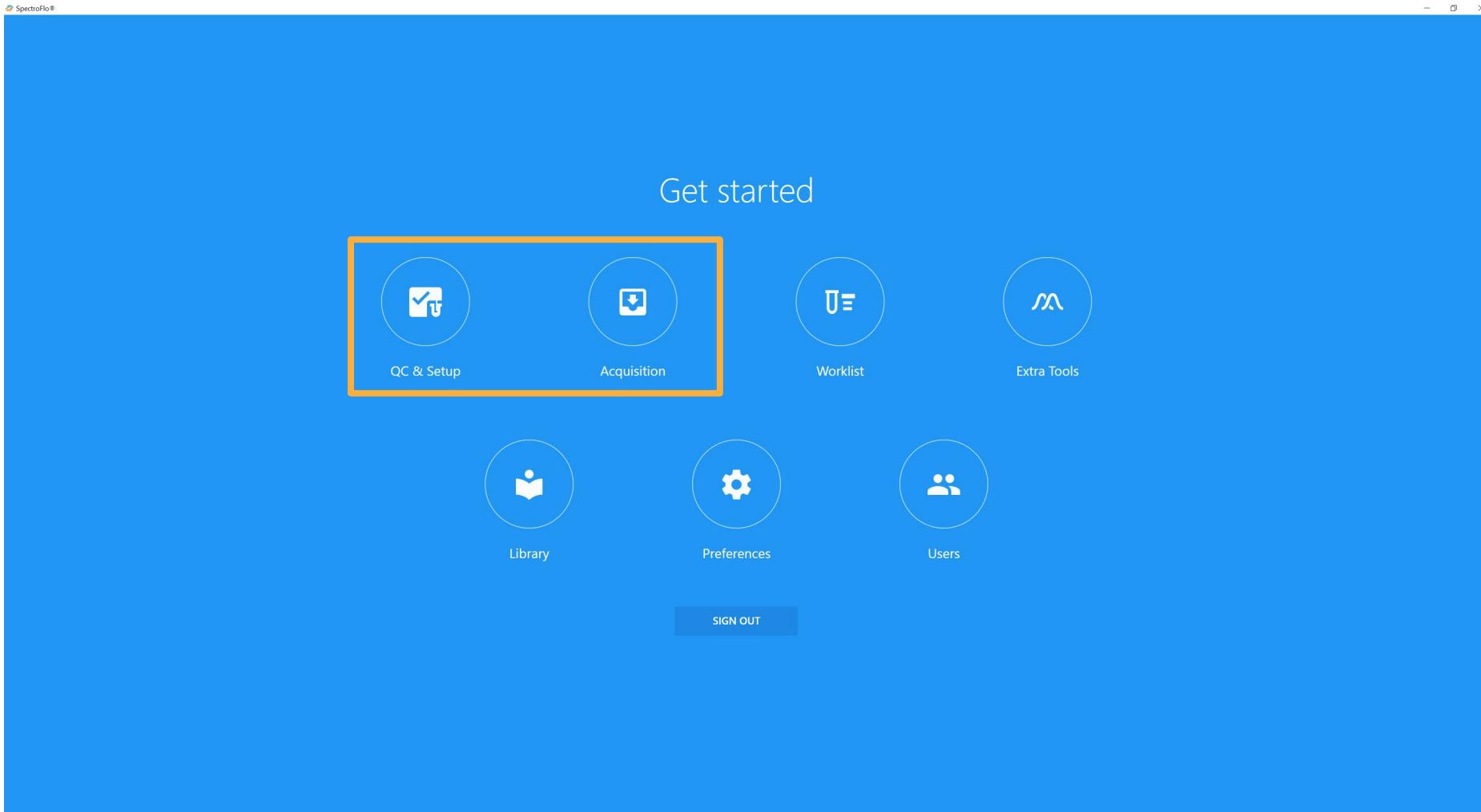
Edit Experiment							
Fluorescent Tags	Groups	Markers	Acquisition				
Import Worksheet							
Name	Worksheet		Stopping Gate	Events to Record	Storage Gate	Stopping Time (sec)	Stopping Volume (µl)
▼ Cytek_cFluor_BCTBMNK	Default Raw Worksheet (Raw)		P1	1-10,000,000	All Events	10000	150
▼ Reference Group	Default Raw Worksheet (Raw)		P1	1-10,000,000	All Events	10000	150
Unstained (Cells)	Default Raw Worksheet (Raw)		P1	500000	All Events	10000	150
CD3 cFluor V420 (Cells)	Default Raw Worksheet (Raw)		P1	10000	All Events	10000	150
CD14 cFluor V450 (Cells)	Default Raw Worksheet (Raw)		P1	10000	All Events	10000	150
CD45 cFluor V547 (Cells)	Default Raw Worksheet (Raw)		P1	20000	All Events	10000	150
CD8 cFluor B515 (Cells)	Default Raw Worksheet (Raw)		P1	20000	All Events	10000	150
CD19 cFluor B7110 (Cells)	Default Raw Worksheet (Raw)		P1	20000	All Events	10000	150
CD16 cFluor R668 (Cells)	Default Raw Worksheet (Raw)		P1	20000	All Events	10000	150
CD56 cFluor R720 (Cells)	Default Raw Worksheet (Raw)		P1	30000	All Events	10000	150
CD4 cFluor R780 (Cells)	Default Raw Worksheet (Raw)		P1	20000	All Events	10000	150
▼ Samples	Default Raw Worksheet (Raw)		P1	500000	All Events	10000	150
Donor A	Default Raw Worksheet (Raw)		P1	500000	All Events	10000	150
Donor B	Default Raw Worksheet (Raw)		P1	500000	All Events	10000	150
Donor C	Default Raw Worksheet (Raw)		P1	500000	All Events	10000	150
Donor D	Default Raw Worksheet (Raw)		P1	500000	All Events	10000	150
Donor E	Default Raw Worksheet (Raw)		P1	500000	All Events	10000	150

Any information entered in the Experiment Builder can be changed anytime, even after importing to the cytometer workstation



SpectroFlo® Software

SpectroFlo® Software was designed to be intuitive and user-friendly





SpectroFlo® Software – Run Daily QC

QC & Setup Cytometer QC

Cytometer QC

Daily QC: **PASSED**

Performed on March 01, 2023 - 10:32 AM

Bead Lot: 2004 Carrier Type: Manual Tube

Running

Start Abort



Acquisition Experiment

Instrument Control

User Settings: CytekAssaySetting (Cytek)

GAIN THRESHOLD SIGNAL LASERS

FSC	SSC	SSC-B
479	182	190

UV	Violet	Blue	YellowGreen	Red
292	207	363	451	409
317	425	328	453	295
415	332	532	911	742
871				

All Channels %: 0

- Run Daily QC to track cytometer performance
- Gains in CytekAssaySetting (CAS) will be automatically updated
- Complete every day the instrument is used

Daily QC promotes consistent assay performance day-to-day

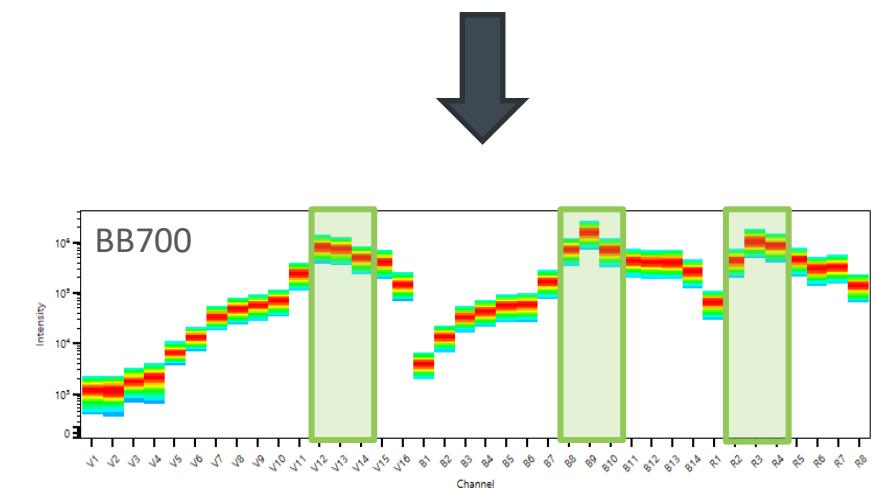
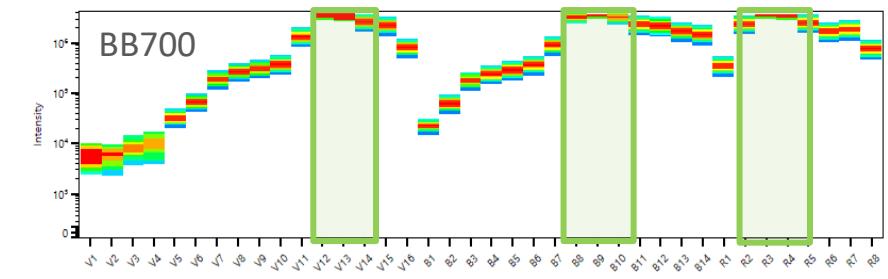


What Is CAS?

- Settings established on biological sample performance to:
 - Preserve spectral characteristics of each dye
 - Provide optimal resolution of each fluorochrome

CAS is useful for most applications

- When would CAS not be used?
 - If signals are off scale using CAS - lower all gains proportionally*



*For future experiments, consider adjusting antibody concentration or panel design



Interactive Poll #6

CAS provide an optimal set of detector gains to use with most flow cytometry applications



SpectroFlo® Software – Set Up Experiment

The screenshot shows the SpectroFlo Software interface. The top navigation bar includes tabs for 'QC & Setup', 'Acquisition', 'Extra Tools', 'Library', 'Preferences', 'Users', 'Help', and 'Sign Out'. The main menu bar has 'Acquisition' and 'Experiment' selected. On the left, a sidebar titled 'Select an experiment' offers options: 'Default' (selected), 'New', 'Template', 'Import' (highlighted with an orange box), and 'My Experiments'. A central message reads 'Import the experiment file from Cytek® Cloud'. At the bottom, status indicators show 'Sheath', 'Waste', 'Cytometer', and 'Loader' are active.

Import the experiment file from Cytek® Cloud

QC & Setup Acquisition Extra Tools Library Preferences Users Help Sign Out

Acquisition Experiment

Select an experiment

- Default
- New
- Template
- Import**
- My Experiments

Sheath Waste Cytometer Loader



SpectroFlo® Software – Record Reference Controls

SpectroFlo® (Admin)

Acquisition Experiment

QC & Setup Acquisition Extra Tools Library Preferences Users Help Sign Out

*3 Color Dry Kit

Save Save As Edit Unmix Manual Tube

Tube Group Collapse All

Reference Group

Unstained (Cells)

CD3 FITC (Cells)

CD56 APC (Cells)

CD8 Alexa Fluor 647 (Cells)

Samples

3c Multicolor

All Events

FSC-A

SSC-A

P1

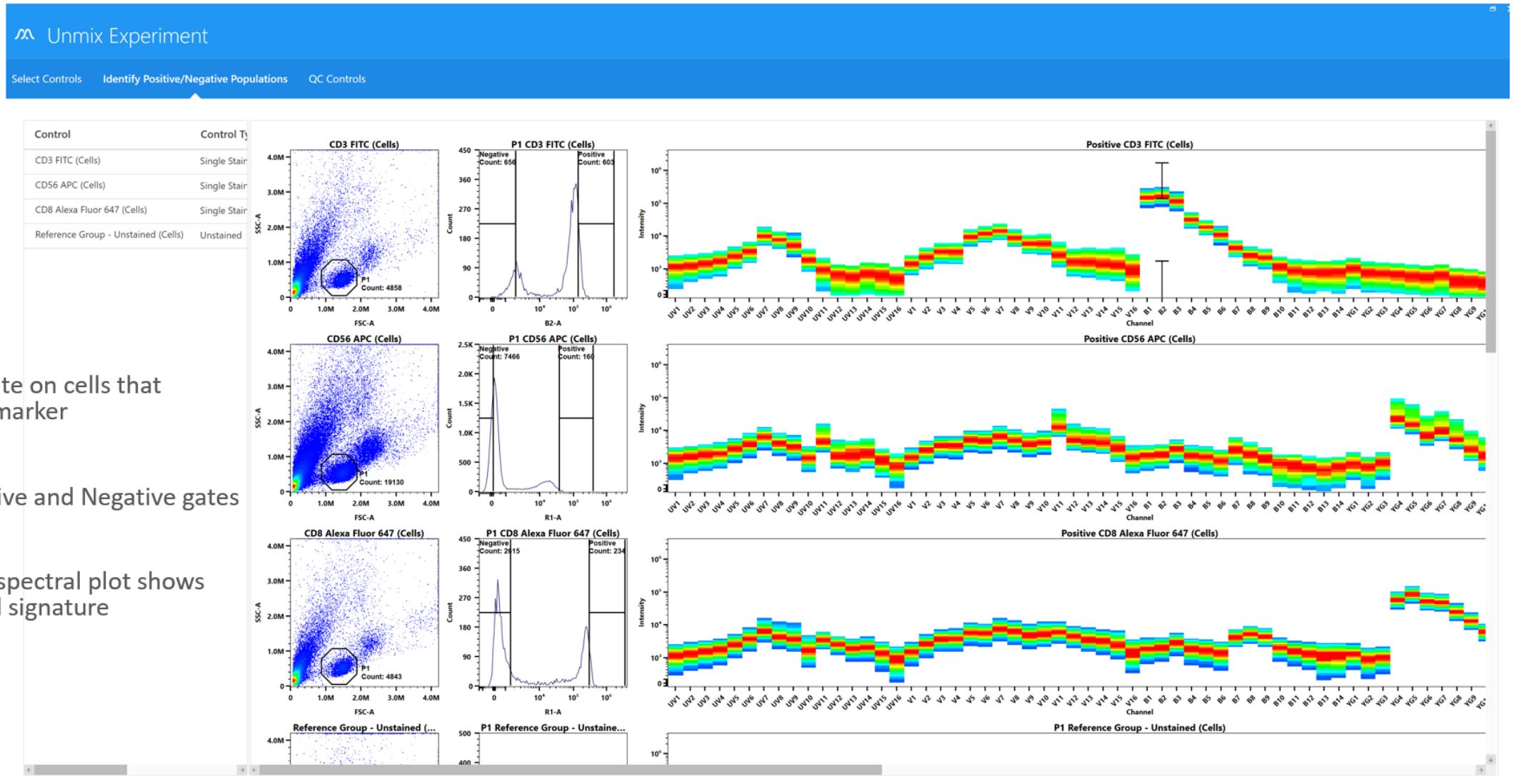
Intensity

Channel

UV1 UV3 UV7 UV9 UV11 UV13 UV15 V1 V3 V5 V7 V9 V11 V13 V15 V17 V19 V21 V23 V25 V27 V29 V31 V33 V35 V37 V39 V41 V43 V45 V47 V49 V51 V53 V55 V57 V59 V61 V63 V65 V67 V69 V71 V73 V75 V77 V79 V81 V83 V85 V87 V89 V91 V93 V95 V97 V99 V101 V103 V105 V107 V109 V111 V113 V115 V117 V119 V121 V123 V125 V127 V129 V131 V133 V135 V137 V139 V141 V143 V145 V147 V149 V151 V153 V155 V157 V159 V161 V163 V165 V167 V169 V171 V173 V175 V177 V179 V181 V183 V185 V187 V189 V191 V193 V195 V197 V199 V201 V203 V205 V207 V209 V211 V213 V215 V217 V219 V221 V223 V225 V227 V229 V231 V233 V235 V237 V239 V241 V243 V245 V247 V249 V251 V253 V255 V257 V259 V261 V263 V265 V267 V269 V271 V273 V275 V277 V279 V281 V283 V285 V287 V289 V291 V293 V295 V297 V299 V301 V303 V305 V307 V309 V311 V313 V315 V317 V319 V321 V323 V325 V327 V329 V331 V333 V335 V337 V339 V341 V343 V345 V347 V349 V351 V353 V355 V357 V359 V361 V363 V365 V367 V369 V371 V373 V375 V377 V379 V381 V383 V385 V387 V389 V391 V393 V395 V397 V399 V401 V403 V405 V407 V409 V411 V413 V415 V417 V419 V421 V423 V425 V427 V429 V431 V433 V435 V437 V439 V441 V443 V445 V447 V449 V451 V453 V455 V457 V459 V461 V463 V465 V467 V469 V471 V473 V475 V477 V479 V481 V483 V485 V487 V489 V491 V493 V495 V497 V499 V501 V503 V505 V507 V509 V511 V513 V515 V517 V519 V521 V523 V525 V527 V529 V531 V533 V535 V537 V539 V541 V543 V545 V547 V549 V551 V553 V555 V557 V559 V561 V563 V565 V567 V569 V571 V573 V575 V577 V579 V581 V583 V585 V587 V589 V591 V593 V595 V597 V599 V601 V603 V605 V607 V609 V611 V613 V615 V617 V619 V621 V623 V625 V627 V629 V631 V633 V635 V637 V639 V641 V643 V645 V647 V649 V651 V653 V655 V657 V659 V661 V663 V665 V667 V669 V671 V673 V675 V677 V679 V681 V683 V685 V687 V689 V691 V693 V695 V697 V699 V701 V703 V705 V707 V709 V711 V713 V715 V717 V719 V721 V723 V725 V727 V729 V731 V733 V735 V737 V739 V741 V743 V745 V747 V749 V751 V753 V755 V757 V759 V761 V763 V765 V767 V769 V771 V773 V775 V777 V779 V781 V783 V785 V787 V789 V791 V793 V795 V797 V799 V801 V803 V805 V807 V809 V811 V813 V815 V817 V819 V821 V823 V825 V827 V829 V831 V833 V835 V837 V839 V841 V843 V845 V847 V849 V851 V853 V855 V857 V859 V861 V863 V865 V867 V869 V871 V873 V875 V877 V879 V881 V883 V885 V887 V889 V891 V893 V895 V897 V899 V901 V903 V905 V907 V909 V911 V913 V915 V917 V919 V921 V923 V925 V927 V929 V931 V933 V935 V937 V939 V941 V943 V945 V947 V949 V951 V953 V955 V957 V959 V961 V963 V965 V967 V969 V971 V973 V975 V977 V979 V981 V983 V985 V987 V989 V991 V993 V995 V997 V999 V1001 V1003 V1005 V1007 V1009 V1011 V1013 V1015 V1017 V1019 V1021 V1023 V1025 V1027 V1029 V1031 V1033 V1035 V1037 V1039 V1041 V1043 V1045 V1047 V1049 V1051 V1053 V1055 V1057 V1059 V1061 V1063 V1065 V1067 V1069 V1071 V1073 V1075 V1077 V1079 V1081 V1083 V1085 V1087 V1089 V1091 V1093 V1095 V1097 V1099 V1101 V1103 V1105 V1107 V1109 V1111 V1113 V1115 V1117 V1119 V1121 V1123 V1125 V1127 V1129 V1131 V1133 V1135 V1137 V1139 V1141 V1143 V1145 V1147 V1149 V1151 V1153 V1155 V1157 V1159 V1161 V1163 V1165 V1167 V1169 V1171 V1173 V1175 V1177 V1179 V1181 V1183 V1185 V1187 V1189 V1191 V1193 V1195 V1197 V1199 V1201 V1203 V1205 V1207 V1209 V1211 V1213 V1215 V1217 V1219 V1221 V1223 V1225 V1227 V1229 V1231 V1233 V1235 V1237 V1239 V1241 V1243 V1245 V1247 V1249 V1251 V1253 V1255 V1257 V1259 V1261 V1263 V1265 V1267 V1269 V1271 V1273 V1275 V1277 V1279 V1281 V1283 V1285 V1287 V1289 V1291 V1293 V1295 V1297 V1299 V1301 V1303 V1305 V1307 V1309 V1311 V1313 V1315 V1317 V1319 V1321 V1323 V1325 V1327 V1329 V1331 V1333 V1335 V1337 V1339 V1341 V1343 V1345 V1347 V1349 V1351 V1353 V1355 V1357 V1359 V1361 V1363 V1365 V1367 V1369 V1371 V1373 V1375 V1377 V1379 V1381 V1383 V1385 V1387 V1389 V1391 V1393 V1395 V1397 V1399 V1401 V1403 V1405 V1407 V1409 V1411 V1413 V1415 V1417 V1419 V1421 V1423 V1425 V1427 V1429 V1431 V1433 V1435 V1437 V1439 V1441 V1443 V1445 V1447 V1449 V1451 V1453 V1455 V1457 V1459 V1461 V1463 V1465 V1467 V1469 V1471 V1473 V1475 V1477 V1479 V1481 V1483 V1485 V1487 V1489 V1491 V1493 V1495 V1497 V1499 V1501 V1503 V1505 V1507 V1509 V1511 V1513 V1515 V1517 V1519 V1521 V1523 V1525 V1527 V1529 V1531 V1533 V1535 V1537 V1539 V1541 V1543 V1545 V1547 V1549 V1551 V1553 V1555 V1557 V1559 V1561 V1563 V1565 V1567 V1569 V1571 V1573 V1575 V1577 V1579 V1581 V1583 V1585 V1587 V1589 V1591 V1593 V1595 V1597 V1599 V1601 V1603 V1605 V1607 V1609 V1611 V1613 V1615 V1617 V1619 V1621 V1623 V1625 V1627 V1629 V1631 V1633 V1635 V1637 V1639 V1641 V1643 V1645 V1647 V1649 V1651 V1653 V1655 V1657 V1659 V1661 V1663 V1665 V1667 V1669 V1671 V1673 V1675 V1677 V1679 V1681 V1683 V1685 V1687 V1689 V1691 V1693 V1695 V1697 V1699 V1701 V1703 V1705 V1707 V1709 V1711 V1713 V1715 V1717 V1719 V1721 V1723 V1725 V1727 V1729 V1731 V1733 V1735 V1737 V1739 V1741 V1743 V1745 V1747 V1749 V1751 V1753 V1755 V1757 V1759 V1761 V1763 V1765 V1767 V1769 V1771 V1773 V1775 V1777 V1779 V1781 V1783 V1785 V1787 V1789 V1791 V1793 V1795 V1797 V1799 V1801 V1803 V1805 V1807 V1809 V1811 V1813 V1815 V1817 V1819 V1821 V1823 V1825 V1827 V1829 V1831 V1833 V1835 V1837 V1839 V1841 V1843 V1845 V1847 V1849 V1851 V1853 V1855 V1857 V1859 V1861 V1863 V1865 V1867 V1869 V1871 V1873 V1875 V1877 V1879 V1881 V1883 V1885 V1887 V1889 V1891 V1893 V1895 V1897 V1899 V1901 V1903 V1905 V1907 V1909 V1911 V1913 V1915 V1917 V1919 V1921 V1923 V1925 V1927 V1929 V1931 V1933 V1935 V1937 V1939 V1941 V1943 V1945 V1947 V1949 V1951 V1953 V1955 V1957 V1959 V1961 V1963 V1965 V1967 V1969 V1971 V1973 V1975 V1977 V1979 V1981 V1983 V1985 V1987 V1989 V1991 V1993 V1995 V1997 V1999 V2001 V2003 V2005 V2007 V2009 V2011 V2013 V2015 V2017 V2019 V2021 V2023 V2025 V2027 V2029 V2031 V2033 V2035 V2037 V2039 V2041 V2043 V2045 V2047 V2049 V2051 V2053 V2055 V2057 V2059 V2061 V2063 V2065 V2067 V2069 V2071 V2073 V2075 V2077 V2079 V2081 V2083 V2085 V2087 V2089 V2091 V2093 V2095 V2097 V2099 V2101 V2103 V2105 V2107 V2109 V2111 V2113 V2115 V2117 V2119 V2121 V2123 V2125 V2127 V2129 V2131 V2133 V2135 V2137 V2139 V2141 V2143 V2145 V2147 V2149 V2151 V2153 V2155 V2157 V2159 V2161 V2163 V2165 V2167 V2169 V2171 V2173 V2175 V2177 V2179 V2181 V2183 V2185 V2187 V2189 V2191 V2193 V2195 V2197 V2199 V2201 V2203 V2205 V2207 V2209 V2211 V2213 V2215 V2217 V2219 V2221 V2223 V2225 V2227 V2229 V2231 V2233 V2235 V2237 V2239 V2241 V2243 V2245 V2247 V2249 V2251 V2253 V2255 V2257 V2259 V2261 V2263 V2265 V2267 V2269 V2271 V2273 V2275 V2277 V2279 V2281 V2283 V2285 V2287 V2289 V2291 V2293 V2295 V2297 V2299 V2301 V2303 V2305 V2307 V2309 V2311 V2313 V2315 V2317 V2319 V2321 V2323 V2325 V2327 V2329 V2331 V2333 V2335 V2337 V2339 V2341 V2343 V2345 V2347 V2349 V2351 V2353 V2355 V2357 V2359 V2361 V2363 V2365 V2367 V2369 V2371 V2373 V2375 V2377 V2379 V2381 V2383 V2385 V2387 V2389 V2391 V2393 V2395 V2397 V2399 V2401 V2403 V2405 V2407 V2409 V2411 V2413 V2415 V2417 V2419 V2421 V2423 V2425 V2427 V2429 V2431 V2433 V2435 V2437 V2439 V2441 V2443 V2445 V2447 V2449 V2451 V2453 V2455 V2457 V2459 V2461 V2463 V2465 V2467 V2469 V2471 V2473 V2475 V2477 V2479 V2481 V2483 V2485 V2487 V2489 V2491 V2493 V2495 V2497 V2499 V2501 V2503 V2505 V2507 V2509 V2511 V2513 V2515 V2517 V2519 V2521 V2523 V2525 V2527 V2529 V2531 V2533 V2535 V2537 V2539 V2541 V2543 V2545 V2547 V2549 V2551 V2553 V2555 V2557 V2559 V2561 V2563 V2565 V2567 V2569 V2571 V2573 V2575 V2577 V2579 V2581 V2583 V2585 V2587 V2589 V2591 V2593 V2595 V2597 V2599 V2601 V2603 V2605 V2607 V2609 V2611 V2613 V2615 V2617 V2619 V2621 V2623 V2625 V2627 V2629 V2631 V2633 V2635 V2637 V2639 V2641 V2643 V2645 V2647 V2649 V2651 V2653 V2655 V2657 V2659 V2661 V2663 V2665 V2667 V2669 V2671 V2673 V2675 V2677 V2679 V2681 V2683 V2685 V2687 V2689 V2691 V2693 V2695 V2697 V2699 V2701 V2703 V2705 V2707 V2709 V2711 V2713 V2715 V2717 V2719 V2721 V2723 V2725 V2727 V2729 V2731 V2733 V2735 V2737 V2739 V2741 V2743 V2745 V2747 V2749 V2751 V2753 V2755 V2757 V2759 V2761 V2763 V2765 V2767 V2769 V2771 V2773 V2775 V2777 V2779 V2781 V2783 V2785 V2787 V2789 V2791 V2793 V2795 V2797 V2799 V2801 V2803 V2805 V2807 V2809 V2811 V2813 V2815 V2817 V2819 V2821 V2823 V2825 V2827 V2829 V2831 V2833 V2835 V2837 V2839 V2841 V2843 V2845 V2847 V2849 V2851 V2853 V2855 V2857 V2859 V2861 V2863 V2865 V2867 V2869 V2871 V2873 V2875 V2877 V2879 V2881 V2883 V2885 V2887 V2889 V2891 V2893 V2895 V2897 V2899 V2901 V2903 V2905 V2907 V2909 V2911 V2913 V2915 V2917 V2919 V2921 V2923 V2925 V2927 V2929 V2931 V2933 V2935 V2937 V2939 V2941 V2943 V2945 V2947 V2949 V2951 V2953 V2955 V2957 V2959 V2961 V2963 V2965 V2967 V2969 V2971 V2973 V2975 V2977 V2979 V2981 V2983 V2985 V2987 V2989 V2991 V2993 V2995 V2997 V2999 V3001 V3003 V3005 V3007 V3009 V3011 V3013 V3015 V3017 V3019 V3021 V3023 V3025 V3027 V3029 V3031 V3033 V3035 V3037 V3039 V3041 V3043 V3045 V3047 V3049 V3051 V3053 V3055 V3057 V3059 V3061 V3063 V3065 V3067 V3069 V3071 V3073 V3075 V3077 V3079 V3081 V3083 V3085 V3087 V3089 V3091 V3093 V3095 V3097 V3099 V3101 V3103 V3105 V3107 V3109 V3111 V3113 V3115 V3117 V3119 V3121 V3123 V3125 V3127 V3129 V3131 V3133 V3135 V3137 V3139 V3141 V3143 V3145 V3147 V3149 V3151 V3153 V3155 V3157 V3159 V3161 V3163 V3165 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V3499 V3501 V3503 V3505 V3507 V3509 V3511 V3513 V3515 V3517 V3519 V3521 V3523 V3525 V3527 V3529 V3531 V3533 V3535 V3537 V3539 V3541 V3543 V3545 V3547 V3549 V3551 V3553 V3555 V3557 V3559 V3561 V3563 V3565 V3567 V3569 V3571 V3573 V3575 V3577 V3579 V3581 V3583 V3585 V3587 V3589 V3591 V3593 V3595 V3597 V3599 V3601 V3603 V3605 V3607 V3609 V3611 V3613 V3615 V3617 V3619 V3621 V3623 V3625 V3627 V3629 V3631 V3633 V3635 V3637 V3639 V3641 V3643 V3645 V3647 V3649 V3651 V3653 V3655 V3657 V3659 V3661 V3663 V3665 V3667 V3669 V3671 V3673 V3675 V3677 V3679 V3681 V3683 V3685 V3687 V3689 V3691 V3693 V3695 V3697 V3699 V3701 V3703 V3705 V3707 V3709 V3711 V3713 V3715 V3717 V3719 V3721 V3723 V3725 V3727 V3729 V3731 V3733 V3735 V3737 V3739 V3741 V3743 V3745 V3747 V3749 V3751 V3753 V3755 V3757 V3759 V3761 V3763 V3765 V3767 V3769 V3771 V3773 V3775 V3777 V3779 V3781 V3783 V3785 V3787 V3789 V3791 V3793 V3795 V3797 V3799 V3801 V3803 V3805 V3807 V3809 V3811 V3813 V3815 V3817 V3819 V3821 V3823 V3825 V3827 V3829 V3831 V3833 V3835 V3837 V3839 V3841 V3843 V3845 V3847 V3849 V3851 V3853 V3855 V3857 V3859 V3861 V3863 V3865 V3867 V3869 V3871 V3873 V3875 V3877 V3879 V3881 V3883 V3885 V3887 V3889 V3891 V3893 V3895 V3897 V3899 V3901 V3903 V3905 V3907 V3909 V3911 V3913 V3915 V3917 V3919 V3921 V3923 V3925 V3927 V3929 V3931 V3933 V3935 V3937 V3939 V3941 V3943 V3945 V3947 V3949 V3951 V3953 V3955 V3957 V3959 V3961 V3963 V3965 V3967 V3969 V3971 V3973 V3975 V3977 V3979 V3981 V3983 V3985 V3987 V3989 V3991 V3993 V3995 V3997 V3999 V3001 V3003 V3005 V3007 V3009 V3011 V3013 V3015 V3017 V3019 V3021 V3023 V3025 V3027 V3029 V3031 V3033 V3035 V3037 V3039 V3041 V3043 V3045 V3047 V3049 V3051 V3053 V3055 V3057 V3059 V3061 V3063 V3065 V3067 V3069 V3071 V3073 V3075 V3077 V3079 V3081 V3083 V3085 V3087 V3089 V3091 V3093 V3095 V3097 V3099 V3001 V3003 V3005 V3007 V3009 V3011 V3013 V3015 V3017 V3019 V3021 V3023 V3025 V3027 V3029 V3031 V3033 V3035 V3037 V3039 V3041 V3043 V3045 V3047 V3049 V3051 V3053 V3055 V3057 V3059 V3061 V3063 V3065 V3067 V3069 V3071 V3073 V3075 V3077 V3079 V3081 V3083 V3085 V3087 V3089 V3091 V3093 V3095 V3097 V3099 V3001 V3003 V3005 V3007 V3009 V3011 V3013 V3015 V3017 V3019 V3021 V3023 V3025 V3027 V3029 V3031 V3033 V3035 V3037 V3039 V3041 V3043 V3045 V3047 V3049 V3051 V3053 V3055 V3057 V3059 V3061 V3063 V3065 V3067 V3069 V3071 V3073 V3075 V3077 V3079 V3081 V3083 V3085 V3087 V3089 V3091 V3093 V3095 V3097 V3099 V3001 V3003 V3005 V3007 V3009 V3011 V3013 V3015 V3017 V3019 V3021 V3023 V3025 V3027 V3029 V3031 V3033 V3035 V3037 V3039 V3041 V3043 V3045 V3047 V3049 V3051 V3053 V3055 V3057 V3059 V3061 V3063 V3065 V3067 V3069 V3071 V3073 V3075 V3077 V3079 V3081 V3083 V3085 V3087 V3089 V3091 V3093 V3095 V3097 V3099 V3001 V3003 V3005 V3007 V3009 V3011 V3013 V3015 V3017 V3019 V3021 V3023 V3025 V3027 V3029 V3031 V3033 V3035 V3037 V3039 V3041 V3043 V3045 V3047 V3049 V3051 V3053 V3055 V3057 V3059 V3061 V3063 V3065 V3067 V3069 V3071 V3073 V3075 V3077 V3079 V3081 V3083 V3085 V3087 V3089 V3091 V3093 V3095 V3097 V3099 V3001 V3003 V3005 V3007 V3009 V3011 V3013 V3015 V3017 V3019 V3021 V3023 V3025 V3027 V3029 V3031 V3033 V3035 V3037 V3039 V3



SpectroFlo® Software – Unmix





Evaluate Your Assay

- QC Controls before calculating unmixing
- Check accuracy after calculating unmixing
- Assess resolution of populations



Evaluate Unmixing

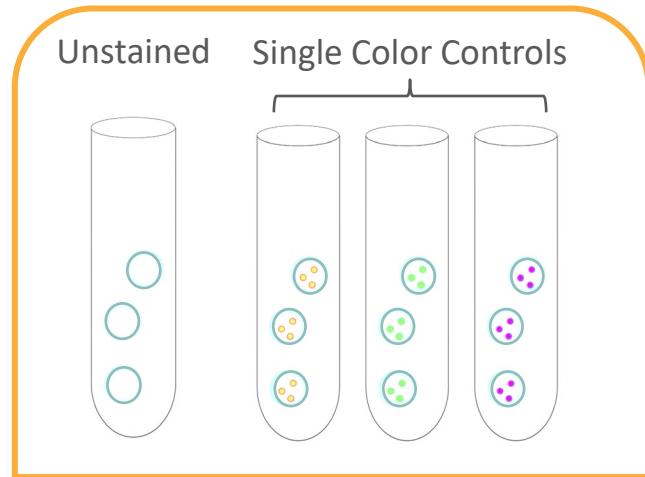
How to assess control quality *before* unmixing

How to assess data quality *after* unmixing



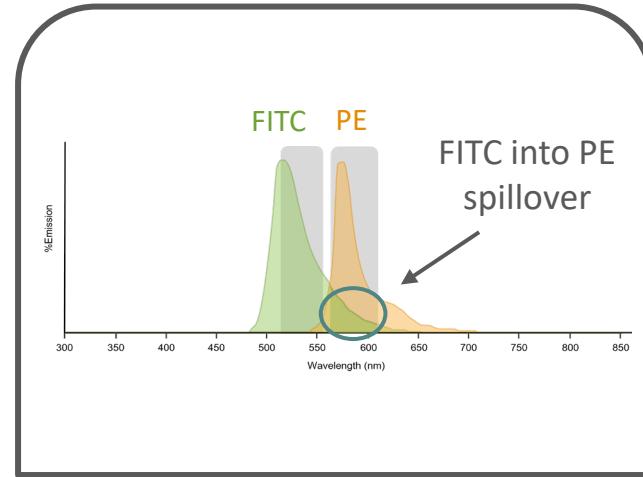
Unmixing Workflow

Run compensation/reference controls



Input

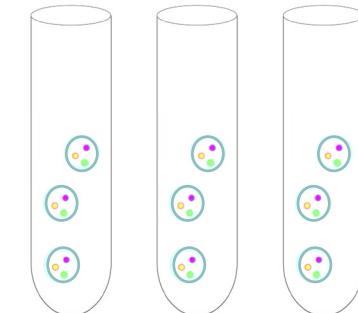
Calculate compensation/unmixing



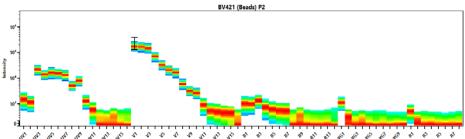
Output

Run multicolor samples with compensation/unmixing applied

Multicolor Assay



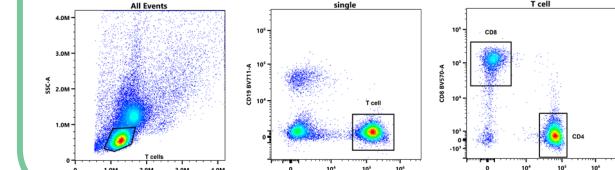
Raw Data



 Unmix

Check that the INPUT is good before calculating unmixing

Unmixed Data

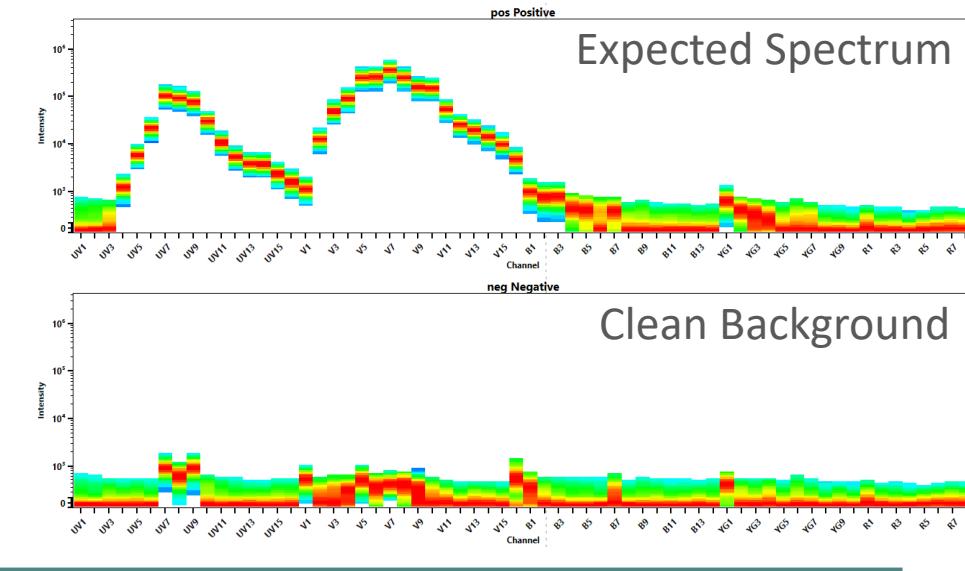
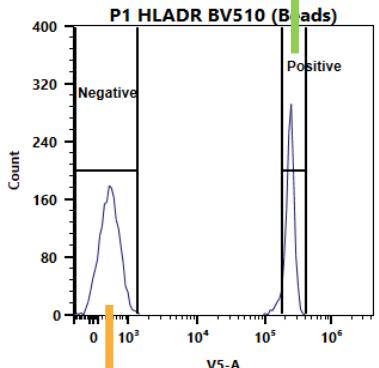
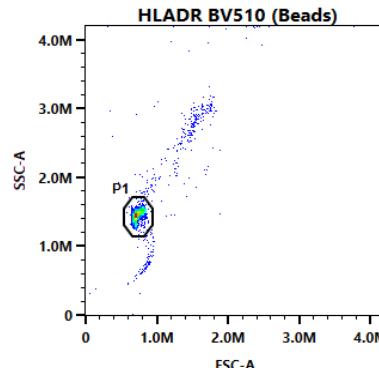


Check that the OUTPUT is good after calculating unmixing

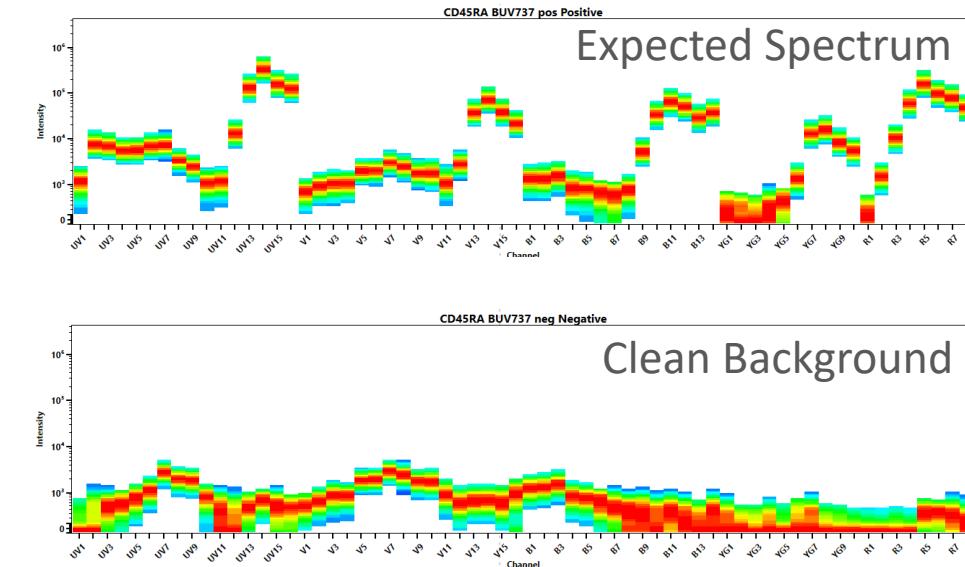
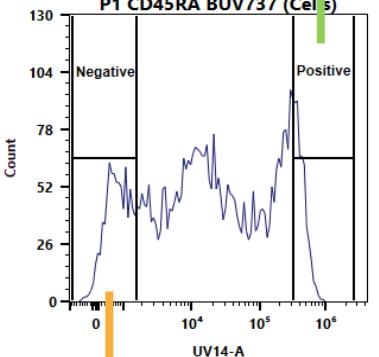
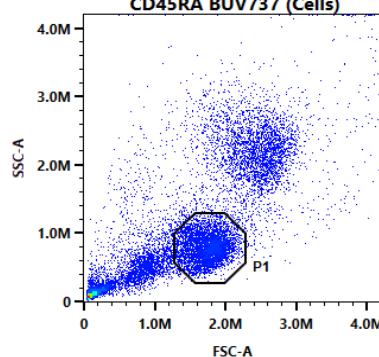


Examples of Good Reference Controls

Bead
Control



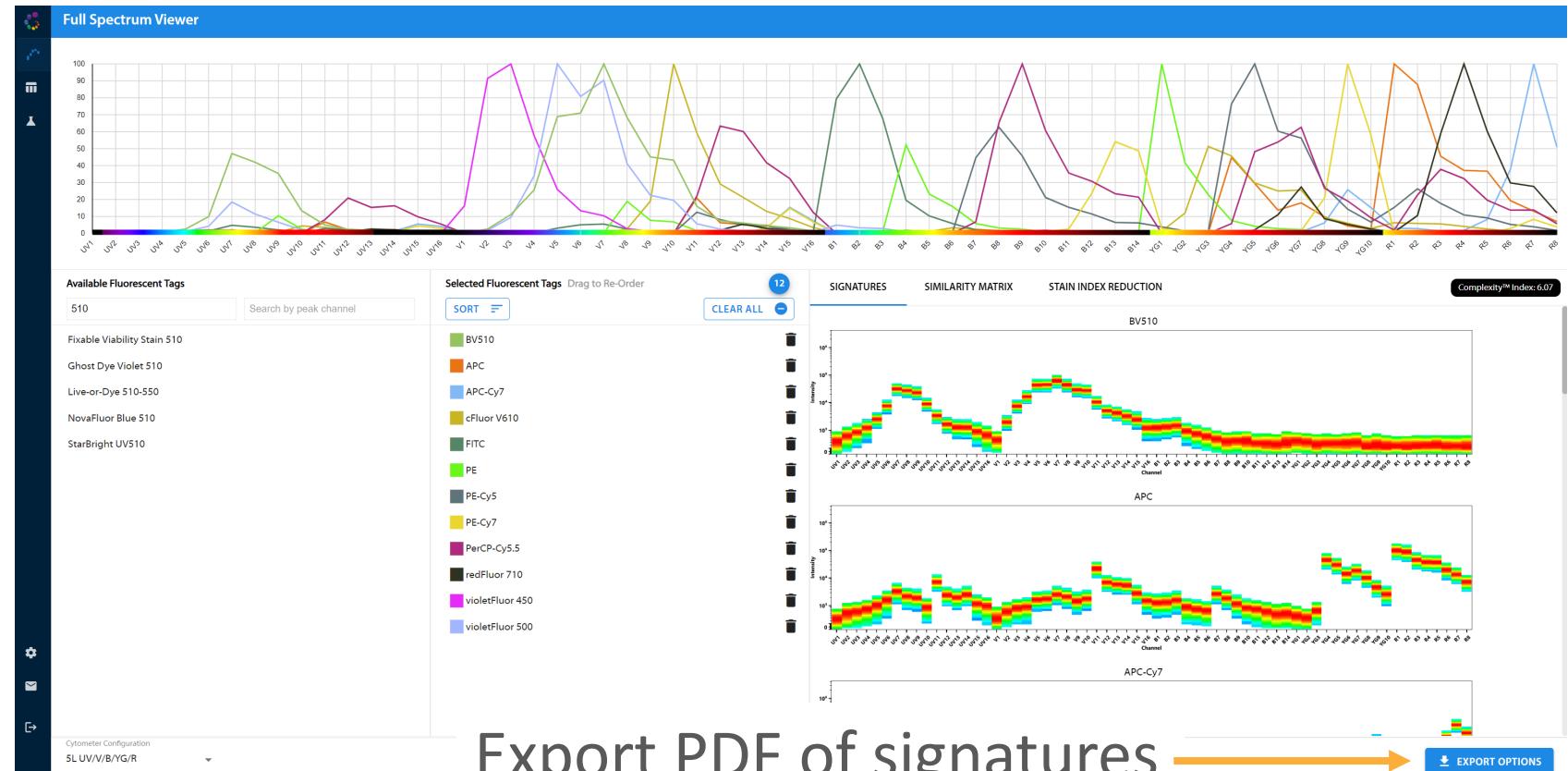
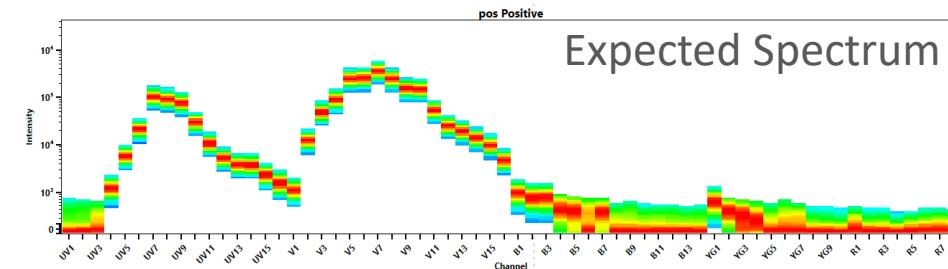
Cell
Control





Examples of Good Reference Controls

Use Cytek® Cloud to help determine if signatures are expected



Export PDF of signatures



Exercise 5: Reference Control QC

Goals

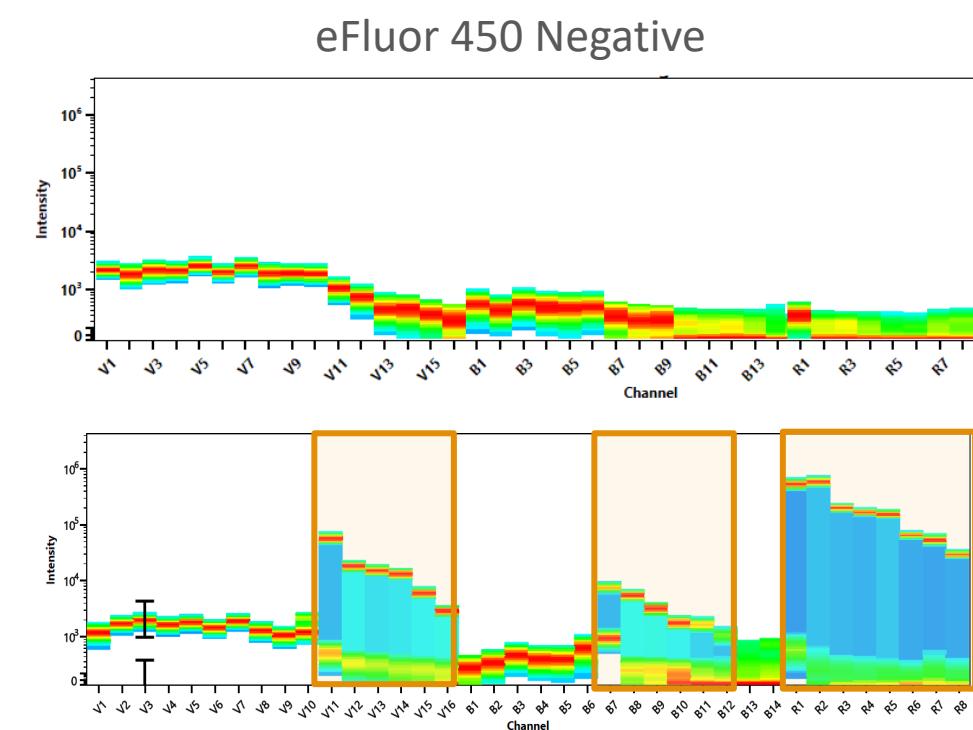
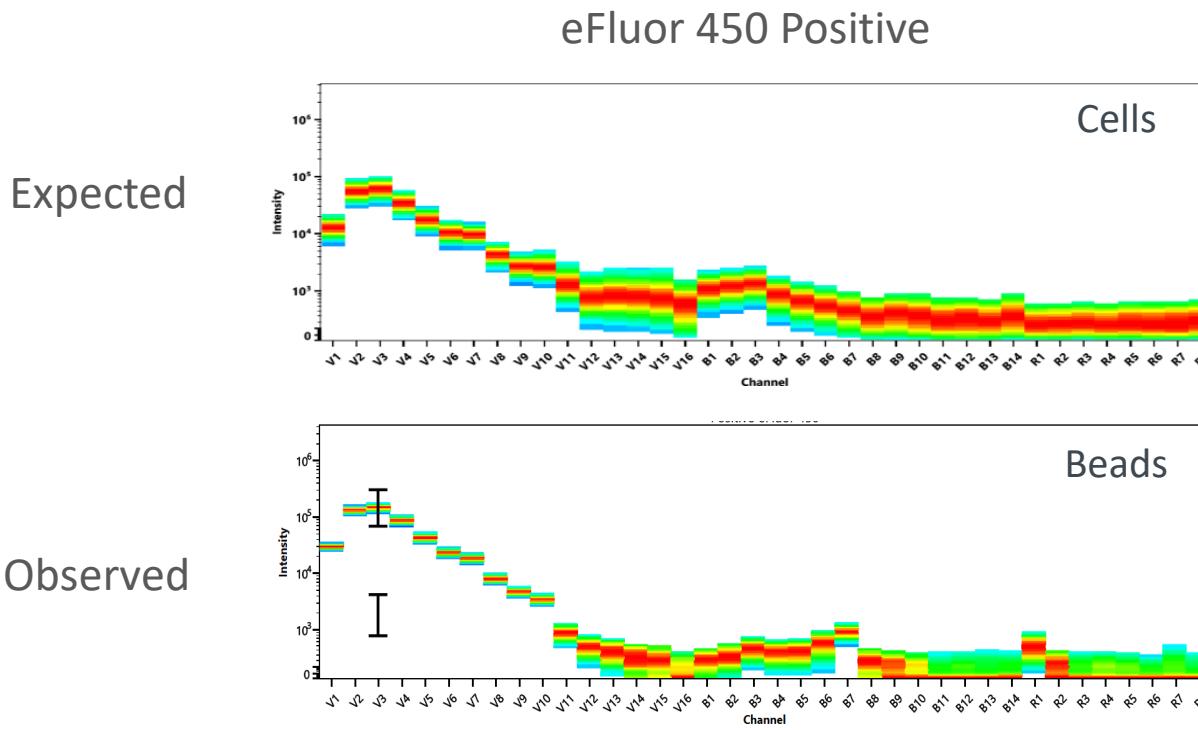
- Determine if the observed signature matches the expected signature



Exercise 5: Reference Control 1

Does the observed positive signature
match the expected positive signature?

Does the observed negative signature
match the expected negative signature?

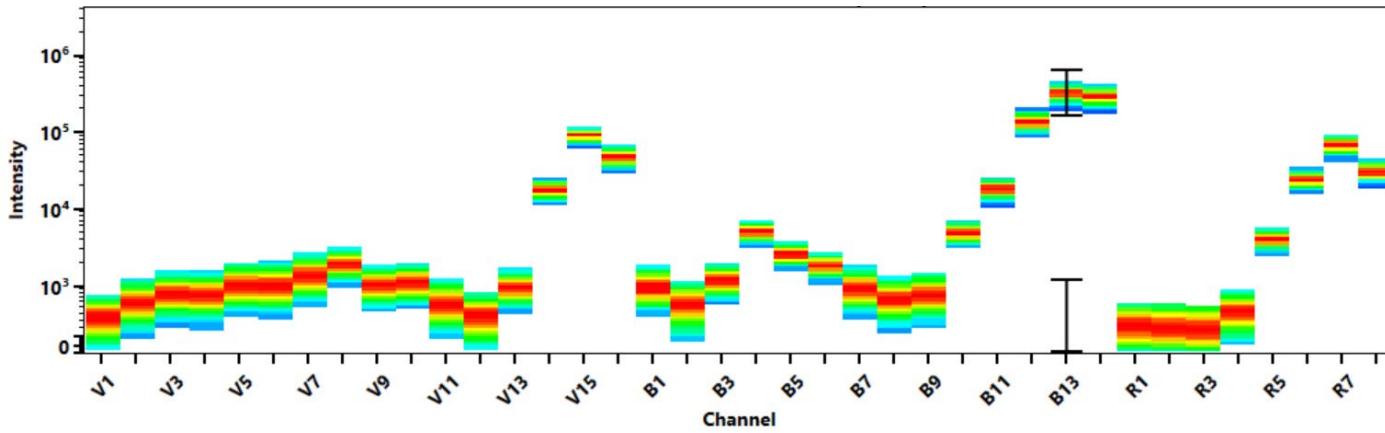




Exercise 5: Reference Control 2

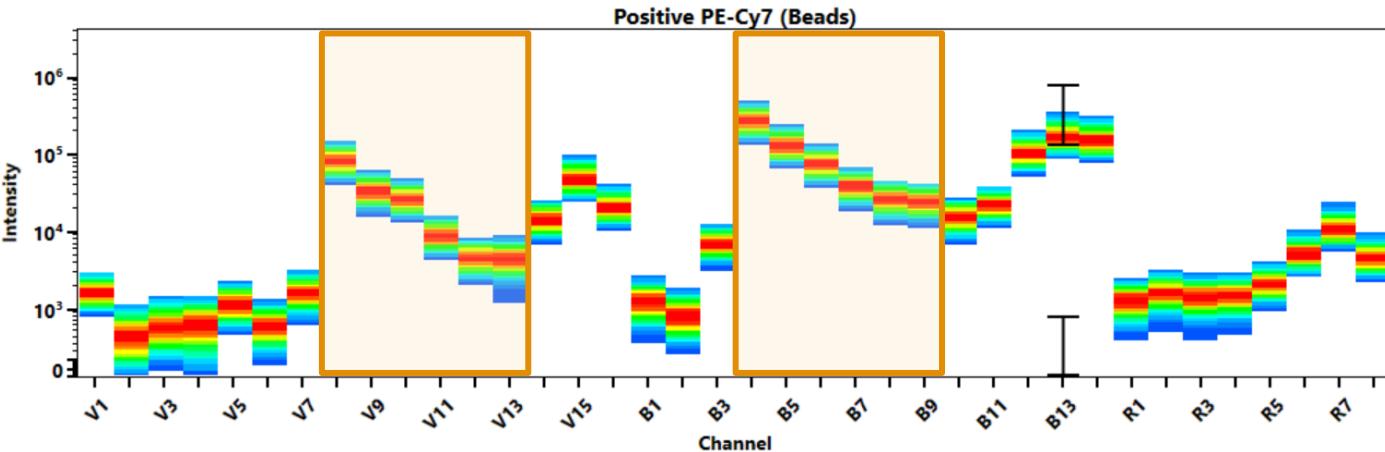
Does the observed PE-Cy7 signature
match the expected PE-Cy7 signature?

Expected



PE	1	
PE-Cy7	0.01	1
	PE	PE-Cy7

Observed



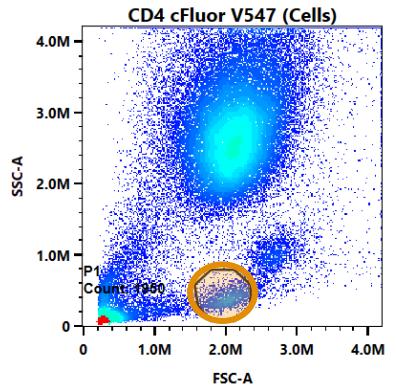
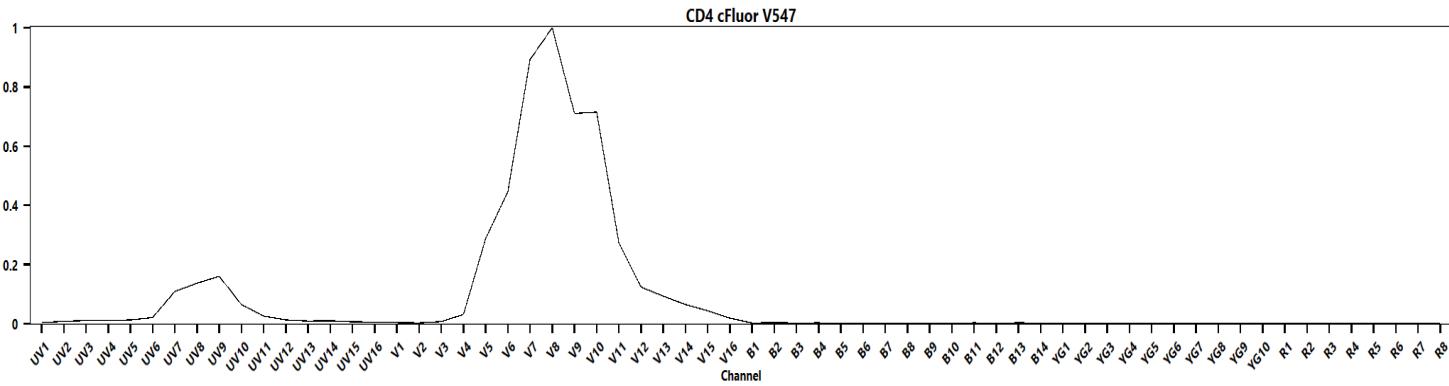
PE	1	
PE-Cy7	0.82	1
PE	PE-Cy7	



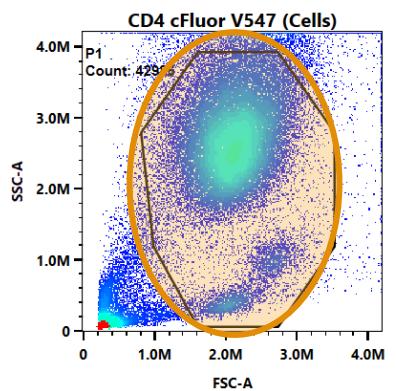
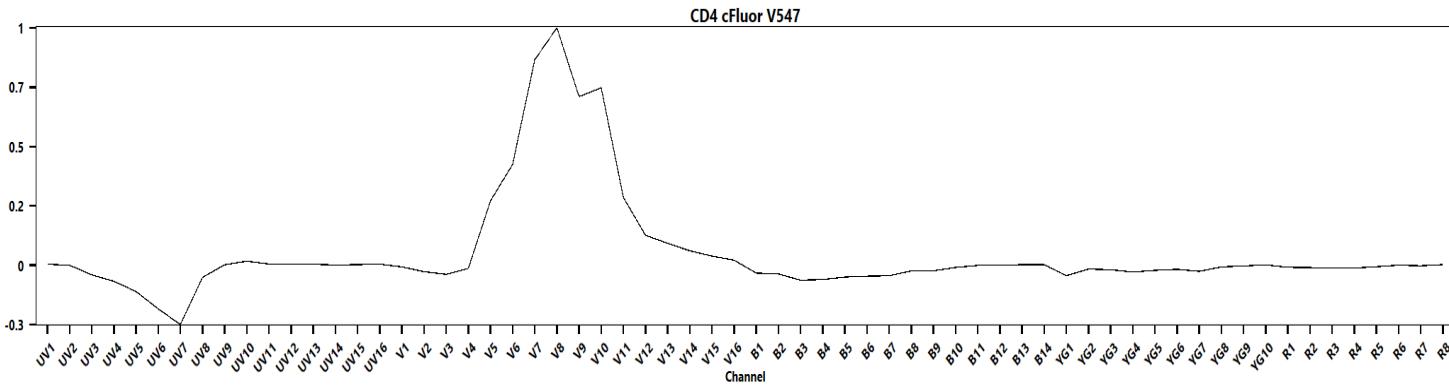
Exercise 5: Reference Control 3

Does the observed cFluor v547 signature
match the expected cFluor v547 signature?

Expected



Observed



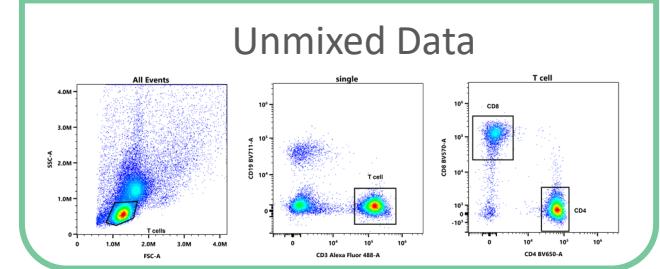


How to Determine if Unmixing Is Accurate

Check for accurate unmixing or compensation in three places:

- 1 Unstained cells
- 2 Single stained cells
- 3 Multicolor cells

Run multicolor samples with compensation/unmixing applied



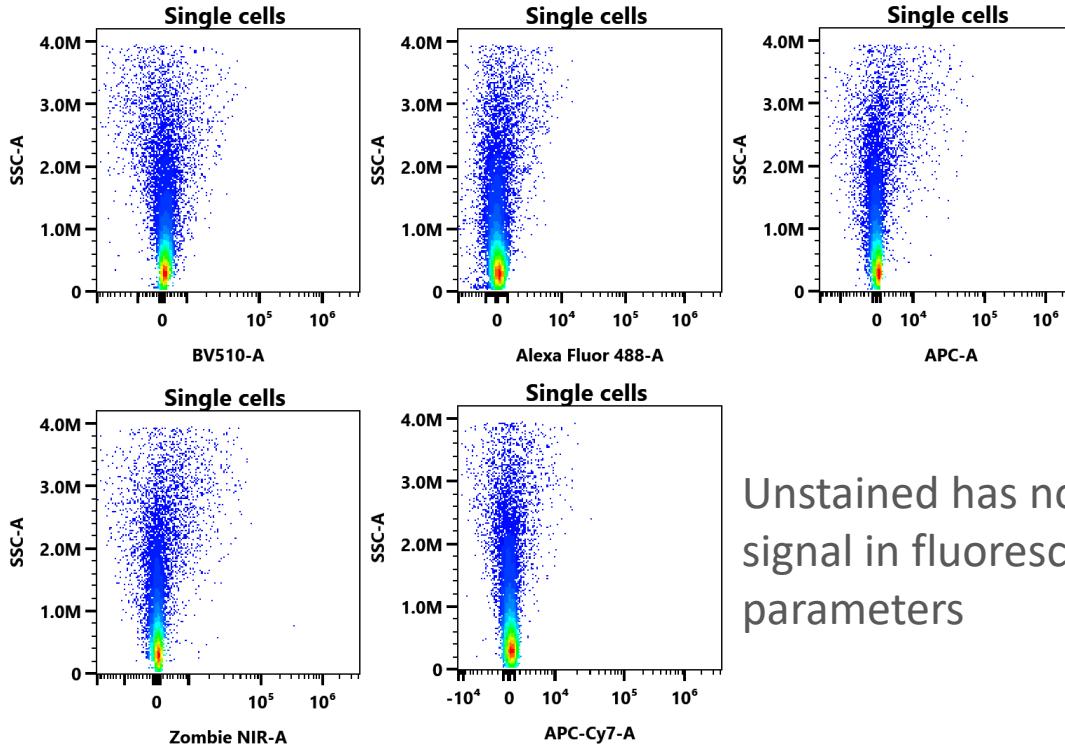
Check that the OUTPUT is good after calculating unmixing



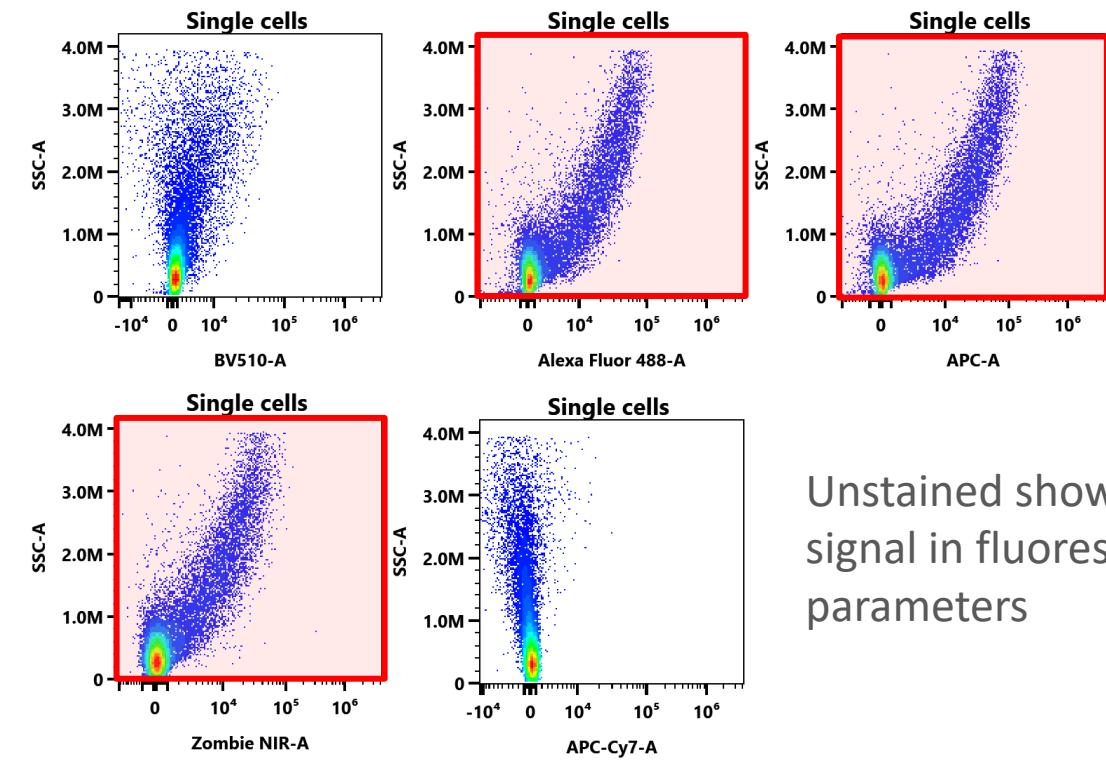
How to Determine if Unmixing Is Accurate

1

After unmixing, check if the unstained has signal in fluorescent parameters



Unstained has no signal in fluorescent parameters



Unstained shows signal in fluorescent parameters

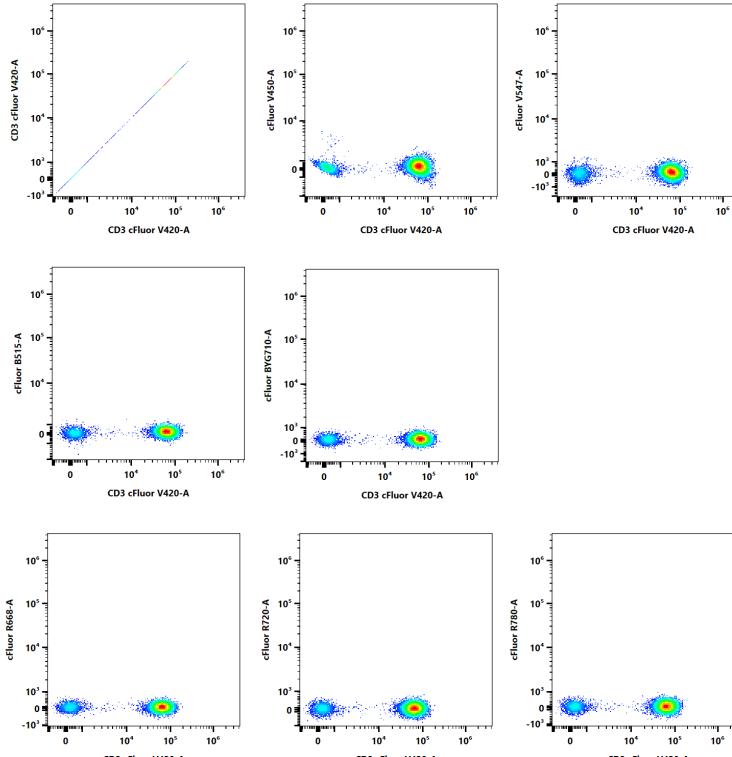


How to Determine if Unmixing Is Accurate

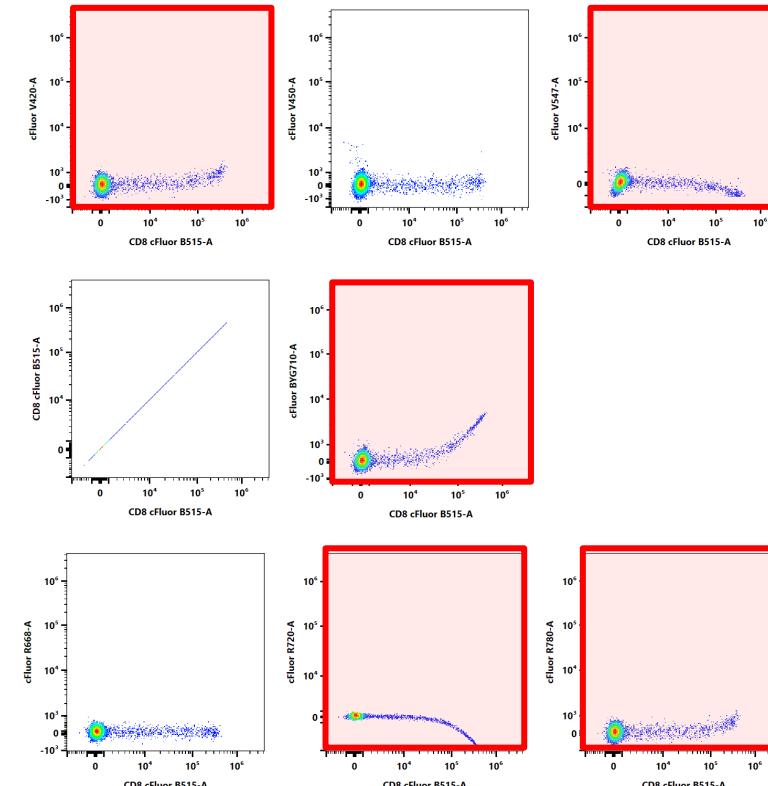
2

Check single stained cells against all other colors

Good Unmixing/Compensation



Bad Unmixing/Compensation





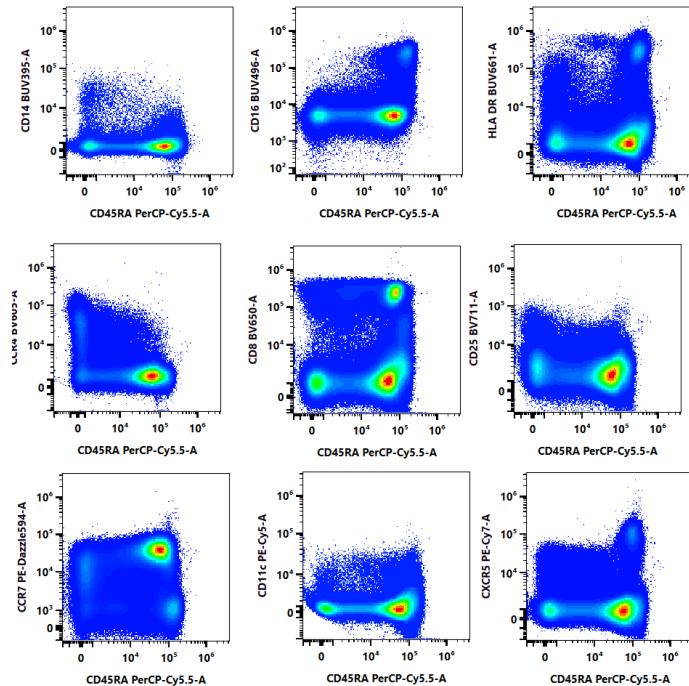
How to Determine if Unmixing Is Accurate

3

Check multicolor NxN permutations

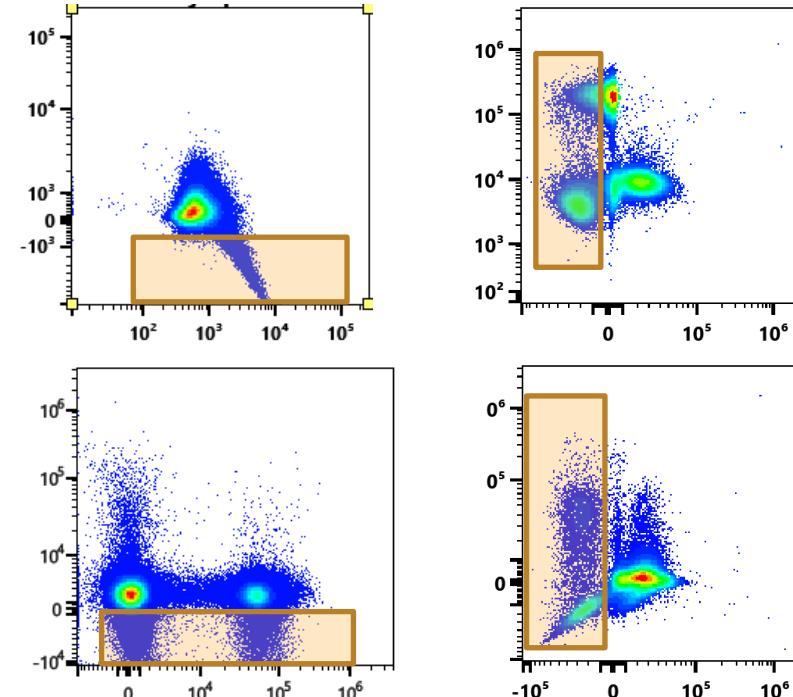
Good Unmixing/Compensation

No extreme negatives



Bad Unmixing/Compensation

Extremely negative populations



QC reference controls and ask your FAS/
technicalsupport@cytekbio.com for help



Assay Optimization

Plan Your Assay

- Design panel
- Titrate reagents
- Select reference controls

Run Your Assay

- Set up experiment
- Record reference controls
- Unmix

Evaluate Your Assay

- Optimize individual reagents
- Achieve accurate unmixing
- Resolve all populations

Is my assay ready to answer my scientific question?



NO

I CAN NOT resolve all populations of interest

Further assay optimization required



Assay Optimization

Plan Your Assay

- Design panel
- Titrate reagents
- Select reference controls

Run Your Assay

- Set up experiment
- Record reference controls
- Unmix

Evaluate Your Assay

- Optimize individual reagents
- Achieve accurate unmixing
- Resolve all populations

Is my assay ready to answer my scientific question?



YES

I CAN resolve all populations of interest

Proceed to optimized workflows



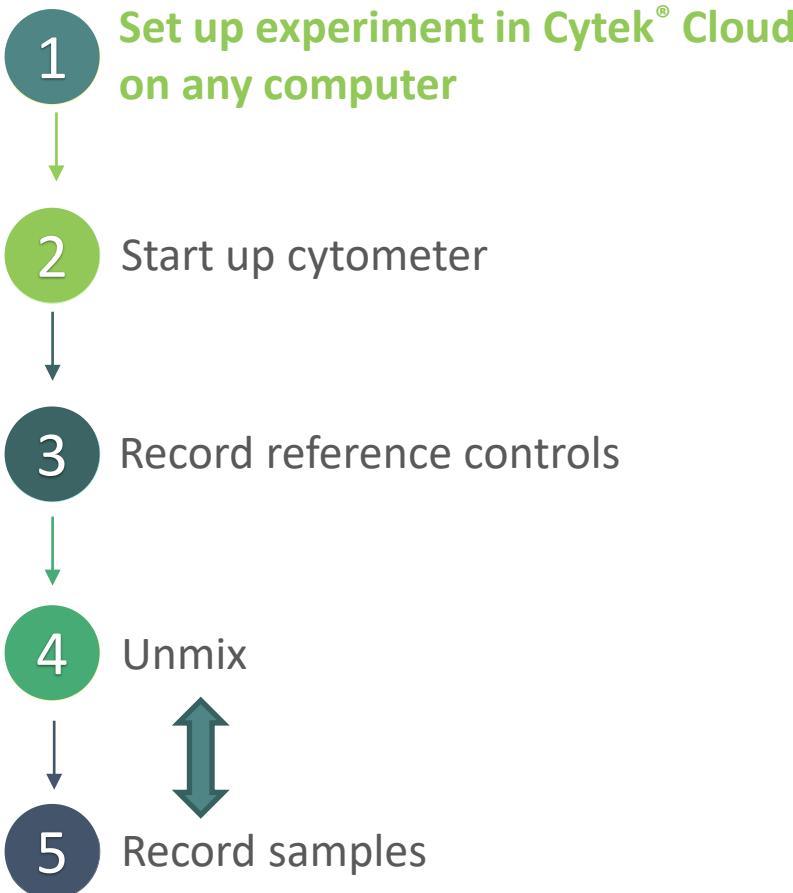
Working With Optimized Assays

- Acquisition Workflow for Optimized panels
- Storing and Reusing Reference Controls
- Pre-Optimized Kits

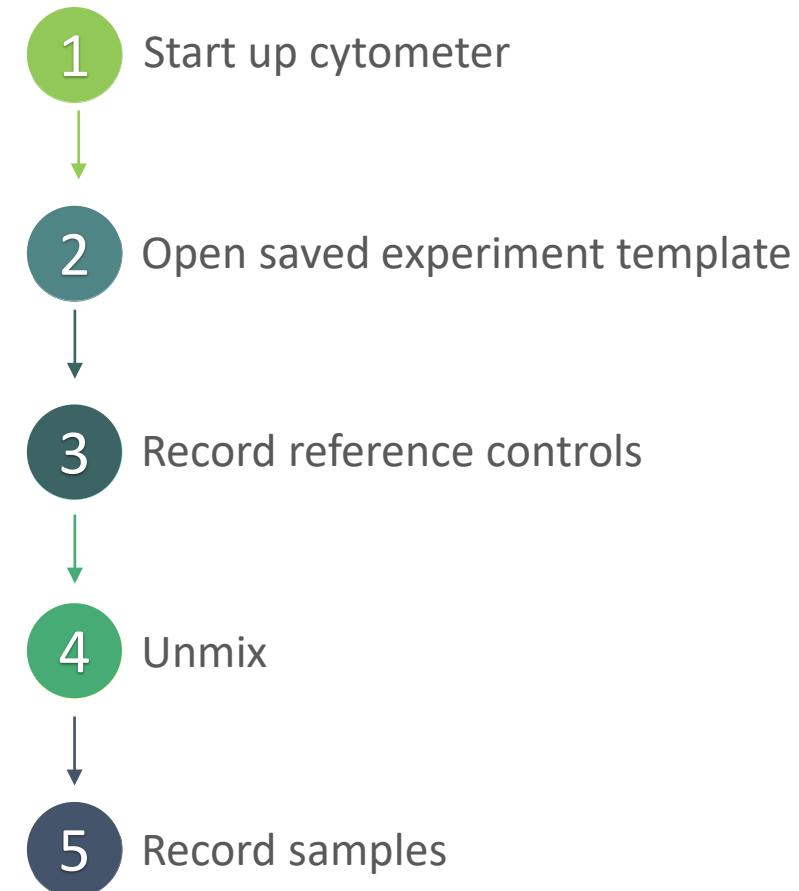


Acquisition Workflow – The Big Picture

Workflow for New Experiment



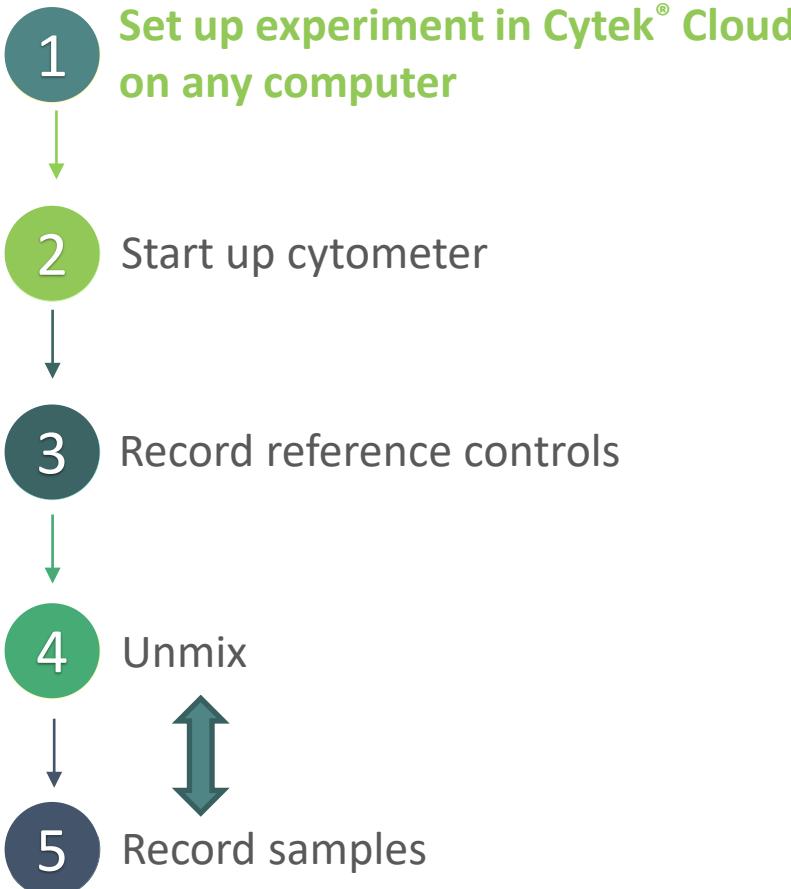
Workflow for Optimized Assays



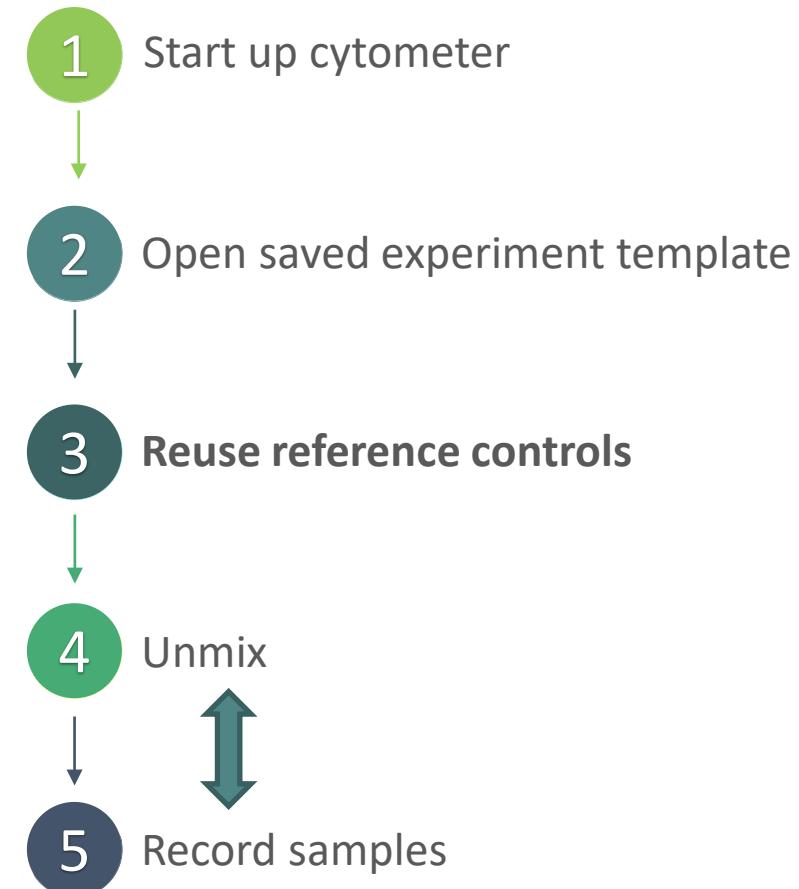


Acquisition Workflow – The Big Picture

Workflow for Initial Setup



Workflow for Optimized Assays





Running an Optimized Assay

Assay Development

Plan Your Assay

- Design panel
- Titrate reagents
- Select reference controls

Run Your Assay

- Set up experiment
- Record reference controls
- Unmix

Evaluate Your Assay

- Optimize individual reagents
- Achieve accurate unmixing
- Resolve all populations

Optimized Workflow

Run Your Assay

Can Reuse:

- Experiment templates
- Worksheet templates
- Reference controls and Unmixing

Evaluate Your Assay

Is my assay performing as expected?

- Is unmixing accurate?
- Are populations resolved?
- Are populations biologically appropriate?

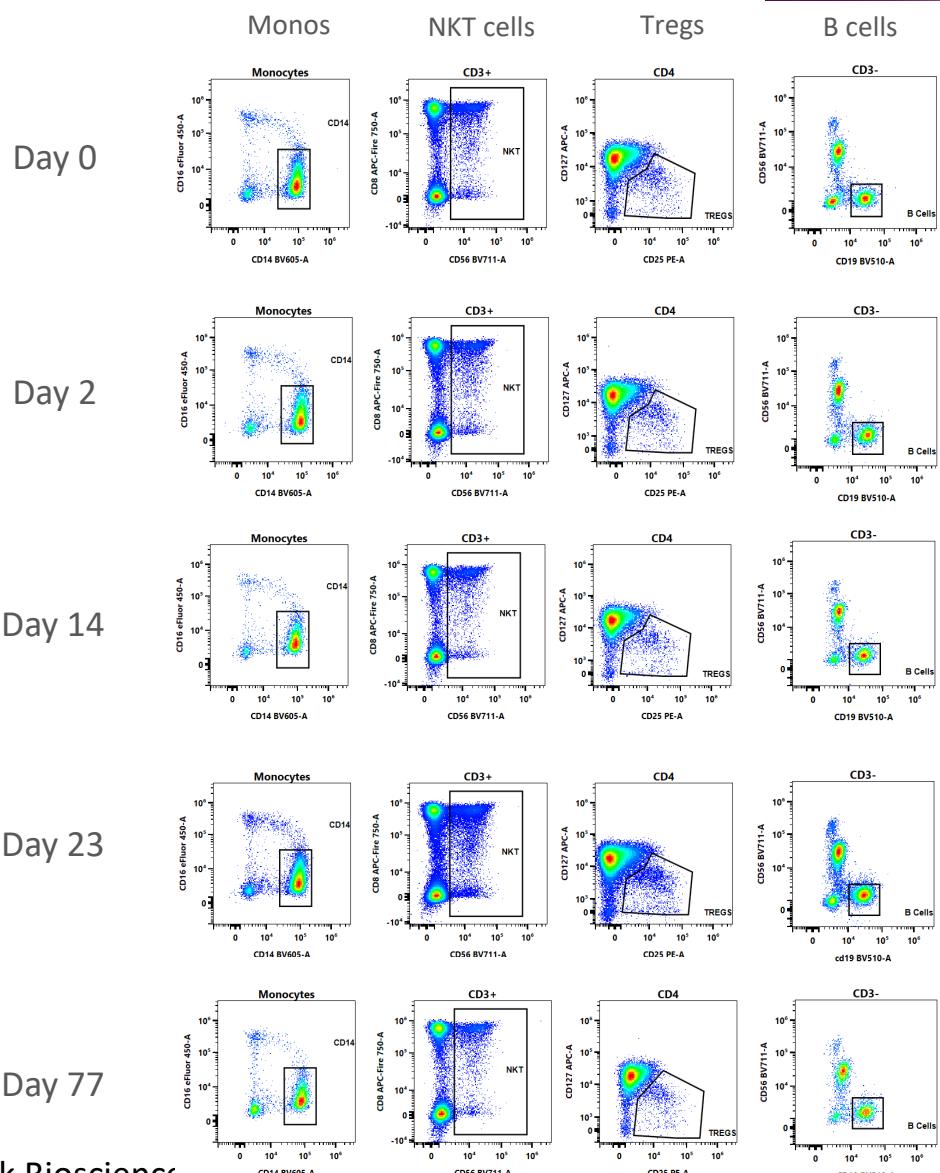


Storing and Reusing Reference Controls

Why and when can we reuse reference controls?



Reusing Reference Controls Across Time at CAS



Unmixing is successful because every FCS file is linked to the most recent QC

Benefits of reusing reference controls

- Useful when high level of consistency in the unmixing results is needed
- Save sample
- Save time



Criteria to Reuse Reference Controls

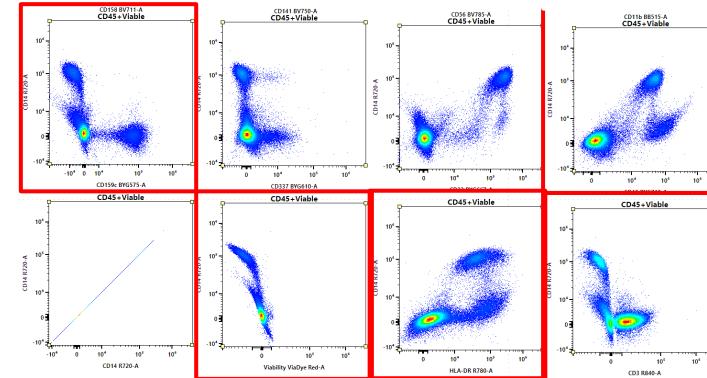
Hardware perspective

- Daily QC completed and passing

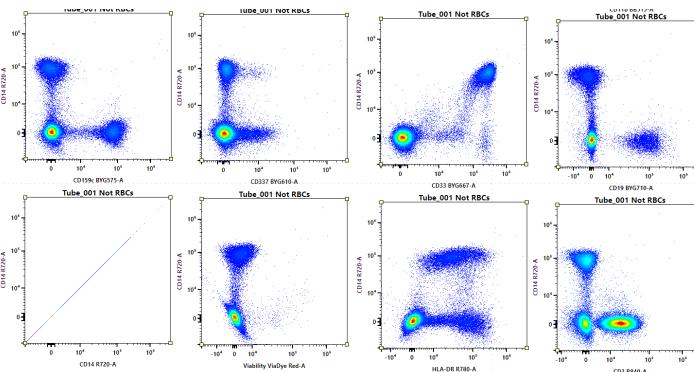
Assay Perspective

- Confirm stored reference controls follow best practices and accurately unmix samples
- Staining is consistent for multiple experiments

Incorrectly reusing controls



Correctly reusing controls





Which Workflow Is Best for You?

	Option 1:	Option 2:
Module used to record FCS file	Acquisition	
Each FCS file normalized to daily QC	Yes	
Can adjust gate setup for calculating unmixing	Flexible – change anytime	
QC Reference Control to confirm it is high quality	Can be performed AFTER storage	

To Store:

The screenshot shows the 'Experiment' tab of the Acquisition software. At the top, there's a toolbar with icons for Save, Save As, Edit, and Unmix. Below the toolbar, a table lists various tubes under a 'Reference Group': Unstained (Cells), BV421 (Cells), Super Bright 436 (Cells), Pacific Blue (Cells), BV480 (Cells), and BV650 (Cells). Each tube entry includes a small thumbnail icon and a 'Delete' button.

To Reuse:

The screenshot shows the 'My Experiments' interface. At the top, there are 'Import' and 'Export' buttons. Below them is a table with columns for 'Experiment' and 'Date Created'. The table lists several entries: 'Experiment_001' (created 5, 2023 - 11:10 AM), 'Experiment_002' (created 5, 2023 - 10:48 AM), 'Experiment_003' (highlighted with an orange box, created 5, 2023 - 10:33 AM), 'Experiment_004' (highlighted with an orange box, created 5, 2023 - 18:51 PM), 'InstrumentAssessment_TrainingReport' (created April 13, 2023 - 18:34 PM), and 'Experiment_005' (created April 12, 2023 - 16:01 PM). Each experiment entry has a 'Duplicate' button next to it, which is also highlighted with an orange box. Other buttons include 'Import', 'Export', 'Delete', and 'Duplicate without Data'.



Which Workflow Is Best for You?

	Option 1:	Option 2:
Module used to record FCS file		QC & Setup
Each FCS file normalized to daily QC		Yes
Can adjust gate setup for calculating unmixing		Locked – set when file is recorded
QC Reference Control to confirm it is high quality		Must be performed BEFORE storage

To Store:

QC & Setup Reference Controls

- Cytometer QC
- Reference Controls**
- Cytometer

New Reference Controls

- Import
- Export
- Benchmark

To Reuse:

Unmix Experiment

Select Controls Identify Positive/Negative Populations QC Controls

UNSTAINED CONTROLS

- Use Control from Library
- Use Control from Experiment** Reference Group - Unstained (Cells)

Name	Control Type
Reference Group - Unstained (Cells)	Cells

STAINED CONTROLS

From Library	Fluorescent Tag	Control	Unstained	Generic
<input type="checkbox"/>	cFluor BYG667	cFluor BYG667 (Cells) ▾	▼	<input checked="" type="checkbox"/>
<input type="checkbox"/>	cFluor B690	cFluor B690 (Cells) ▾	▼	<input checked="" type="checkbox"/>
<input type="checkbox"/>	cFluor R659	cFluor R659 (Cells) ▾	▼	<input checked="" type="checkbox"/>
<input checked="" type="checkbox"/>	cFluor V420	cFluor V420 (Cells) ▾	▼	<input checked="" type="checkbox"/>
<input checked="" type="checkbox"/>	cFluor B515	cFluor B515 (Cells) ▾	▼	<input checked="" type="checkbox"/>



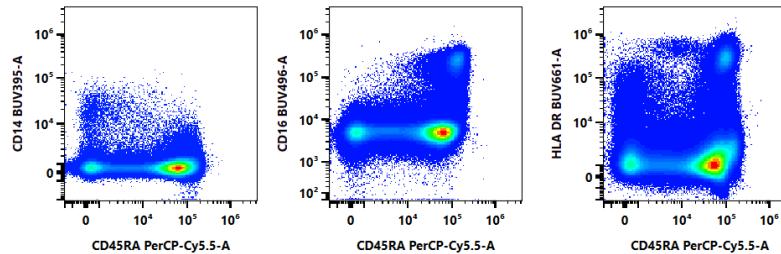
How to Determine if Stored Controls Are Good

3

Check multicolor NxN permutations

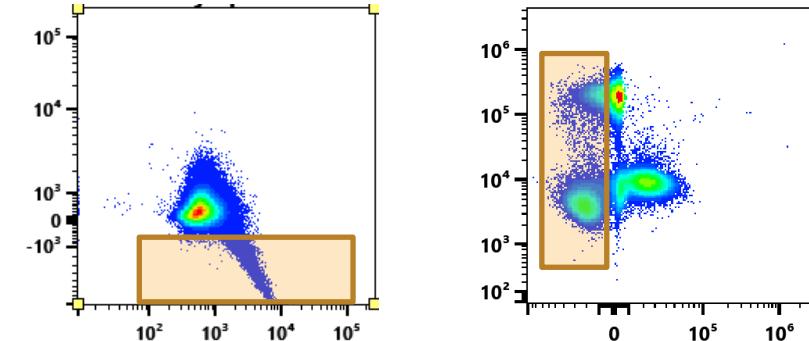
Good Unmixing/Compensation

No extreme negatives



Bad Unmixing/Compensation

Extremely negative populations



Factors that can impact accuracy of stored controls:

- Repair by Field Service Engineer
- New lot of tandem dye
- Storage and handling of reagents
- Time

If you are unsure, ask your FAS



Interactive Poll #7

When is it appropriate to reuse controls?



Cytek® Pre-Optimized Kits

- Pre-optimized kits streamline:
 - Panel design and optimization
 - Reagent titration
 - Selection of optimal reference controls
 - SpectroFlo® software setup with pre-made experiment and analysis templates



[Reagents for Full Spectrum Cytometry](#)

Optimized Workflow

Plan Your Assay

Run Your Assay

Evaluate Your Assay

Set Up:

- Import pre-made templates
- Record reference controls and unmix

Is my assay performing as expected?

- Is my unmixing accurate?
- Can I resolve my populations?
- Do the results make biological sense?



Summary

Full spectrum Signatures

- Use Cytek® tools to identify and QC

Full Spectrum Experiment Workflow

- Same as conventional cytometers

Plan, Run and Evaluate Your Assay

- Be thoughtful in selecting reference controls
- CytekAssaySetting is useful for most applications
- Check unmixing accuracy after each experiment
- Optimize your assay, then answer the scientific question

Working With Optimized Assays

- Evaluate that the assay is performing as expected
- Can use stored reference controls
- Cytek® pre-optimized kits are an easy place to start



Cytek® Resources

Plan Your Assay

- Cytek® Cloud
- SpectroLearn™ Educational Portal
- Webinars on CytekBio.com
- Cytek® Pre-Optimized Kits

Run Your Assay

- Cytek® Cloud
- SpectroFlo® User Guide
- SpectroFlo® Software Tutorials

Evaluate Your Assay

- Webinars on CytekBio.com
- Publications on assay optimization

TechnicalSupport@cytekbio.com



Follow-up Email

Check your email after this session for:

- Five-minute post-training survey
- Links to resources
- Recording of the lecture



FCS Express Complimentary License

Cytek® offers a complimentary 6-month FCS Express license with the purchase of any Aurora or Northern Lights™ system

After hands-on training, follow email instructions to claim license

Create Cytek® Cloud account using institutional email

Click on **Software Partners**

Click **Redeem License**

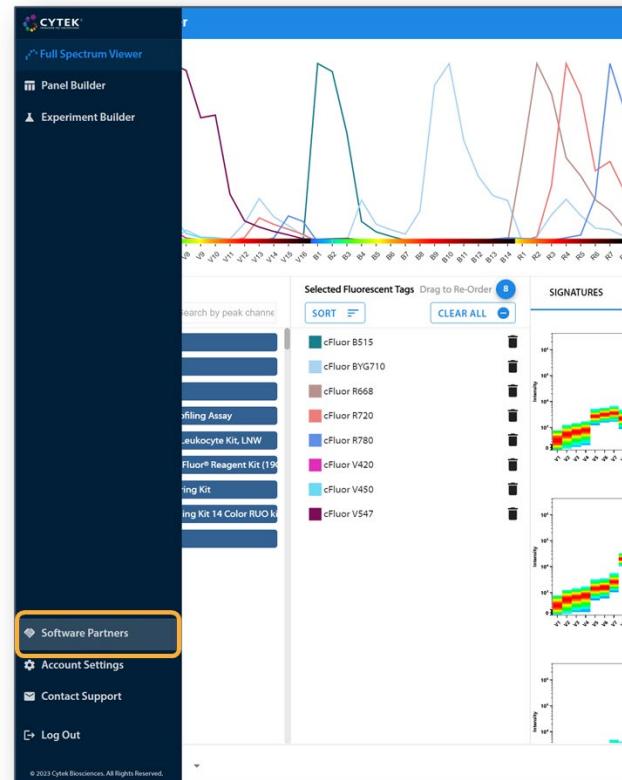
Enter cytometer serial number

Copy **FCS Express** Claim Code and Serial Number

Create **FCS Express** account at www.denovosoftware.com

Activate license on **De Novo Software** website using Claim Code

<https://cloud.cytekbio.com/>

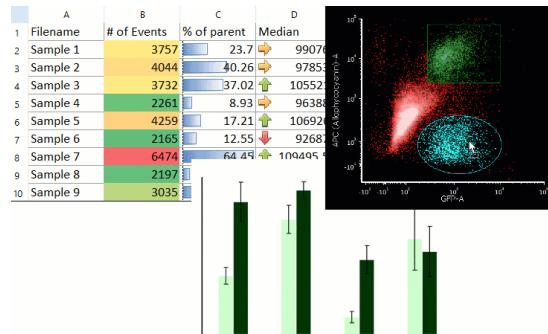


NOTE: Must redeem license within 2 months



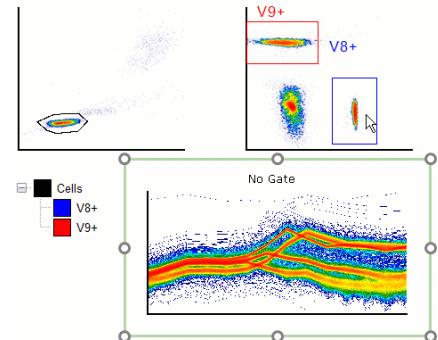
FCS Express Key Features for Cytek Aurora Users

Integrated Spreadsheets and Graphing



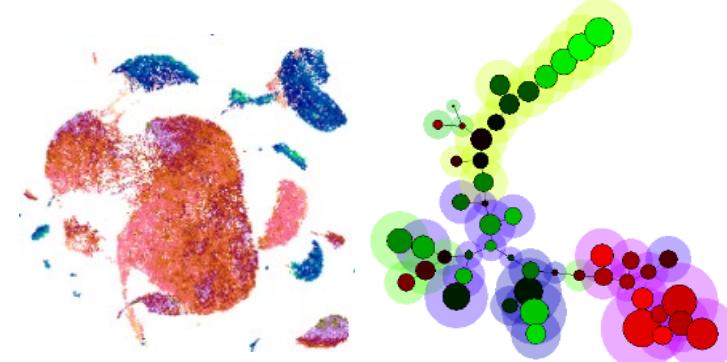
Integrated spreadsheets and graphing tools that link directly to gates. Real-time updating

Gate and Visualize on Spectral Graphs



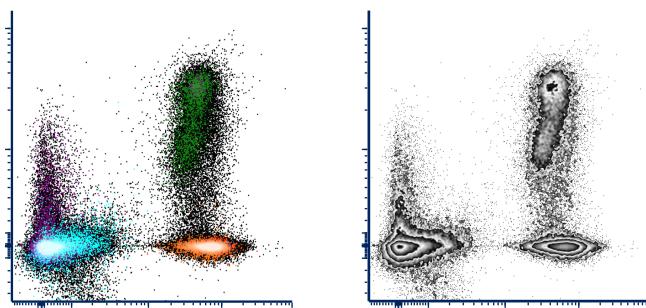
Analyze raw and unmixed data in the same layout. Apply individual gates to spectral plots.

High-Dimensional Data Analysis



Easy to use Pipelines to perform HDDR directly in the software – no plugins needed

Presentation & Publication-ready Graphics



FCS Express

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Additional FCS Express Resources

Free one-on-one training

- Contact support@denovosoftware.com to schedule a free intro training session
- Get help getting started or making the switch to FCS Express today!

Cytek and FCS Express resources page

- Visit denovosoftware.com/cytek
- Access tips/tricks, applications examples, short videos, tutorials, and more...

Additional features/versions available for GxP, CFR Part 11 Compliance, and IVD

- Visit denovosoftware.com/cfrpart11
- Contact support@denovosoftware.com for more information or a special trial



Questions?

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