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CYTEK®
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INTRODUCTION TO SPECTRAL CYTOMETRY

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

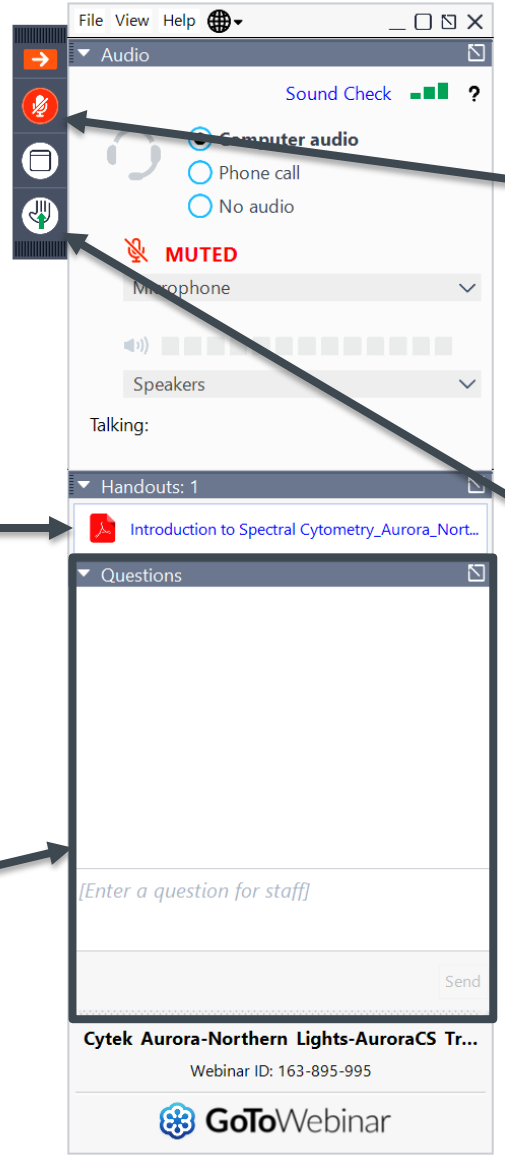
Cytek® Biosciences, Inc.
47215 Lakeview Blvd
Fremont, CA 94538

GoToWebinar Instructions

You can answer polls

What is your favorite color?

- Red
- Blue
- Green



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

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

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

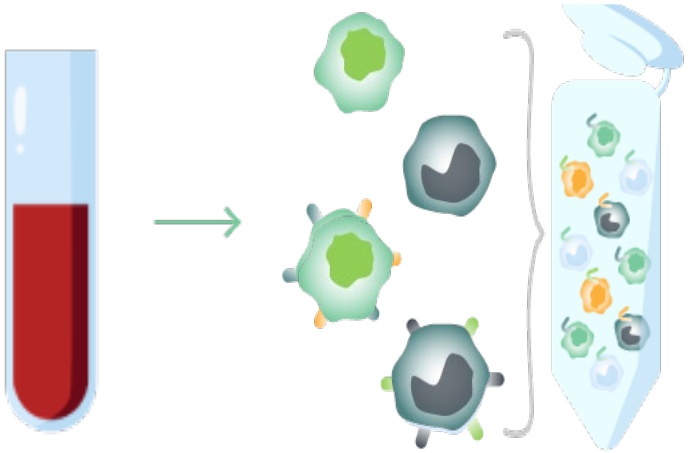
Full Spectrum Cytometry Basics

- 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

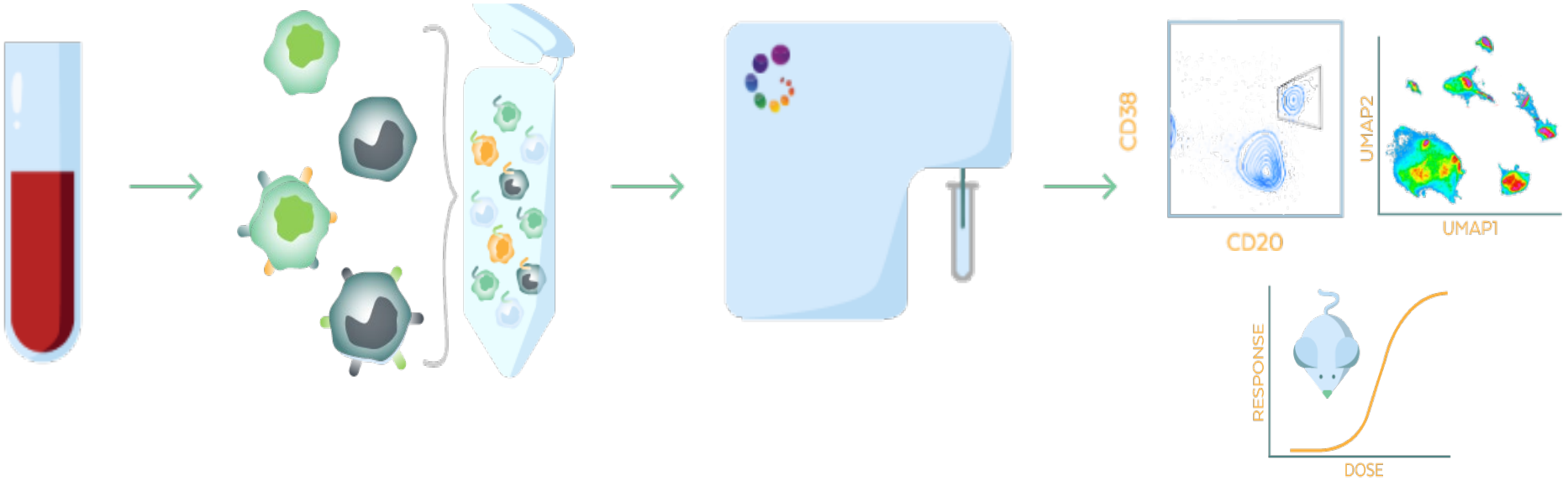
Flow Cytometry: Basics

Collect and prepare samples

Stain cells with labeled antibodies

Acquire samples on a cytometer

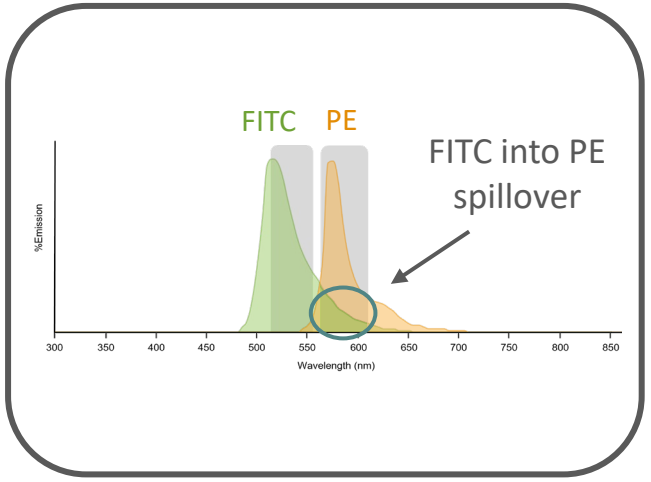
Analyze and interpret results



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

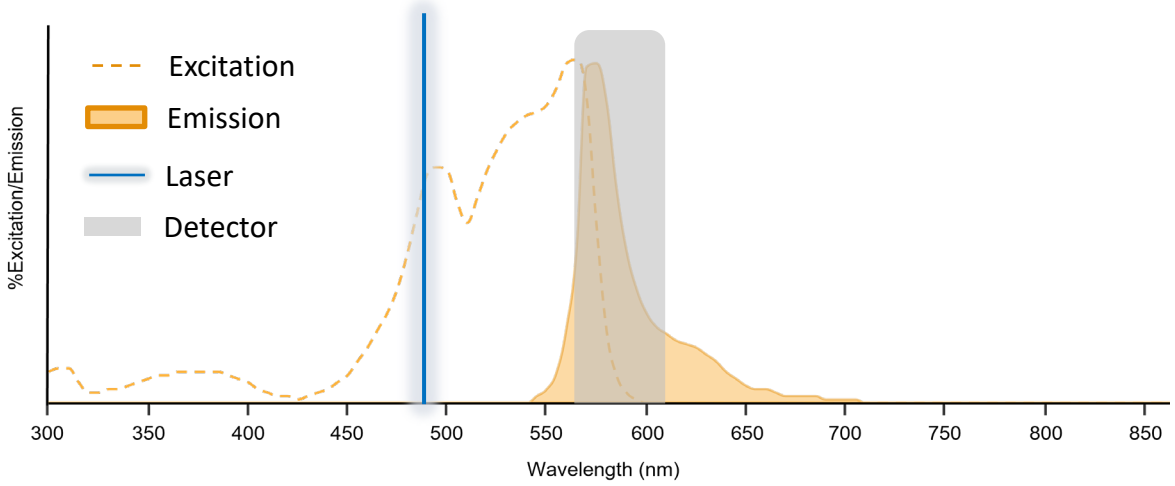
Conventional Flow Cytometry Terminology

Compensation Controls → Input → Compensation → Output → Compensated data

Full Spectrum Flow Cytometry Terminology

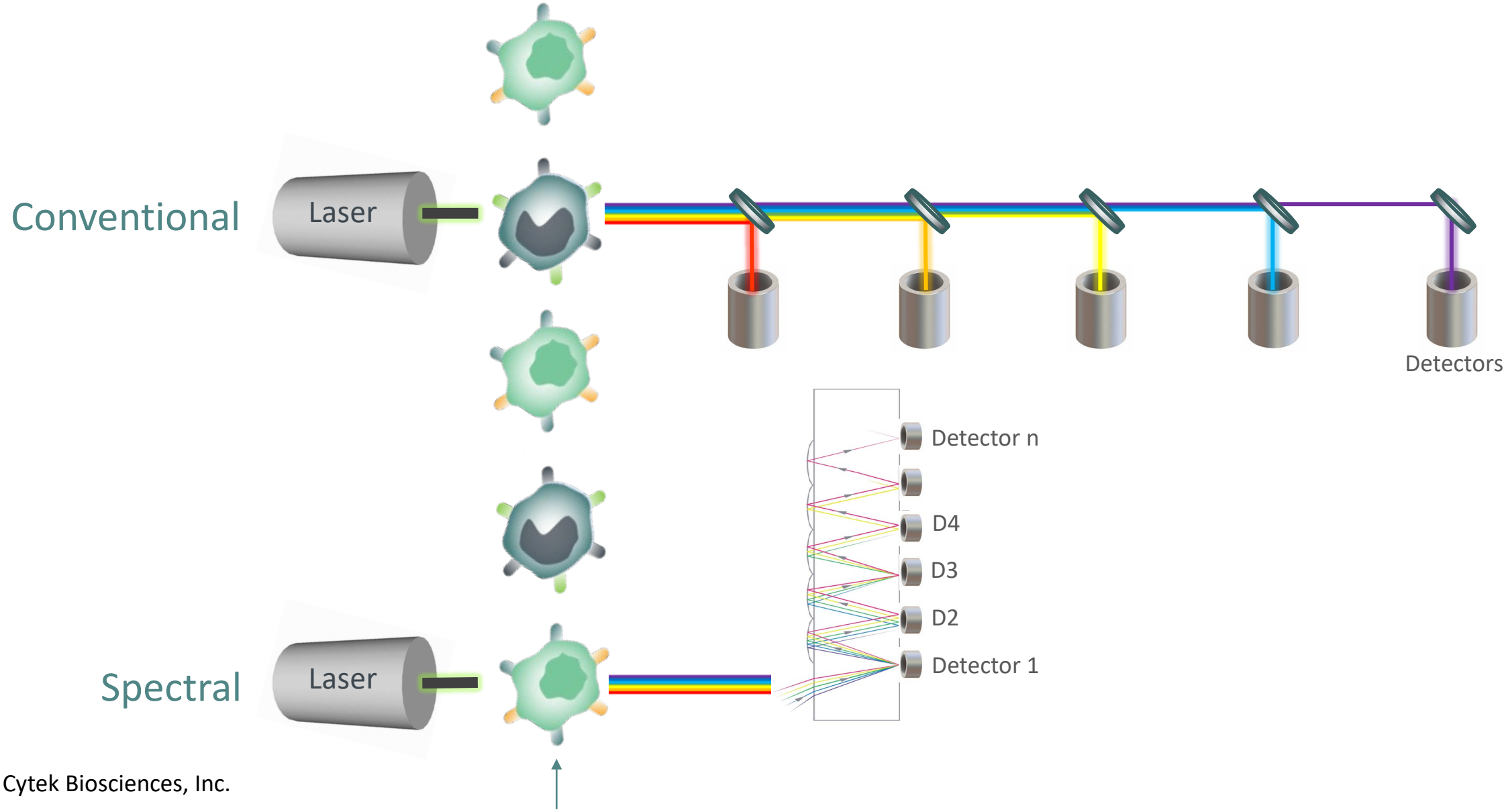
Reference Controls → Input → Spectral Unmixing → Output → Unmixed data

What Are We Capturing?

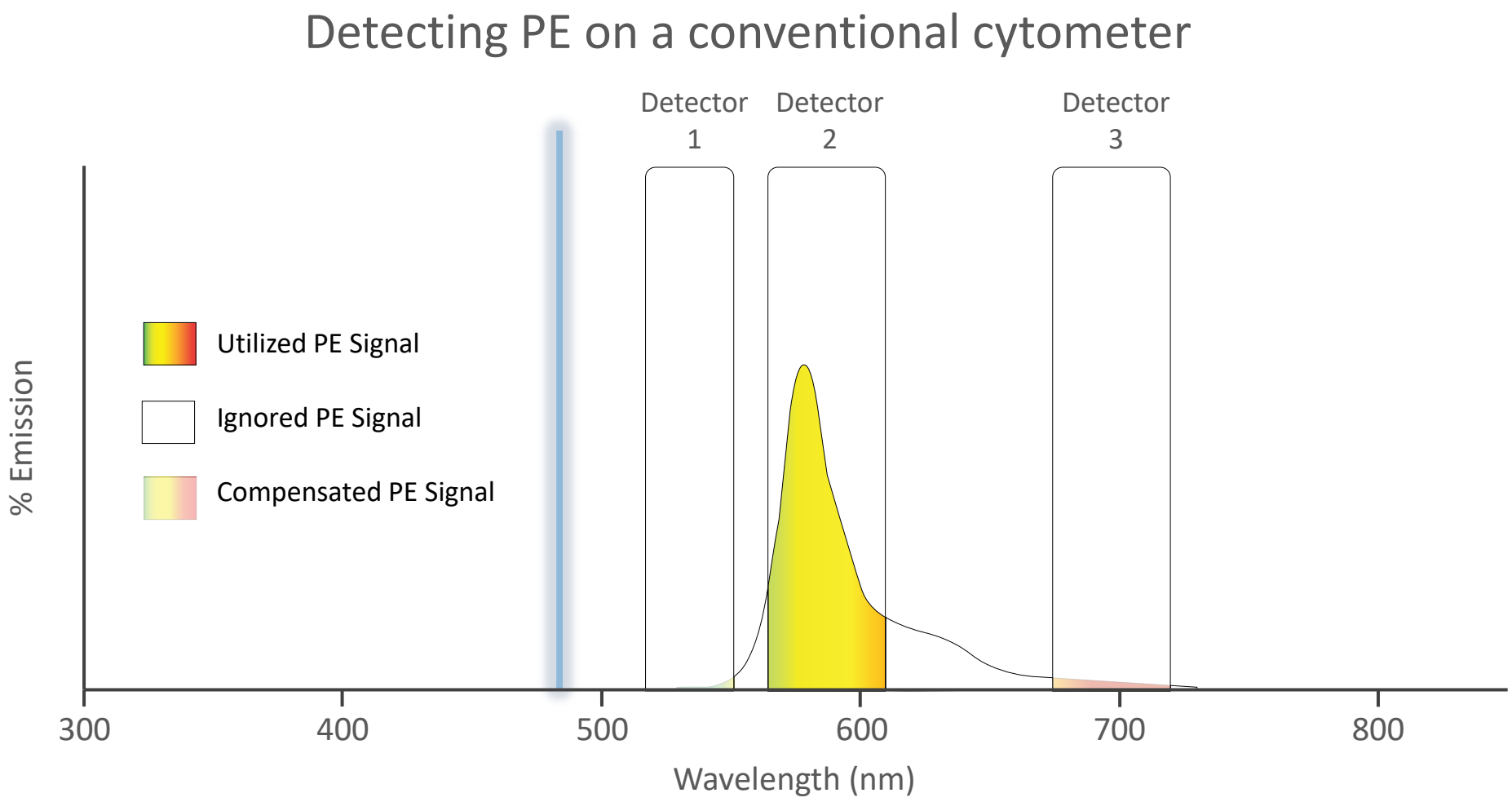


Is this the whole picture?

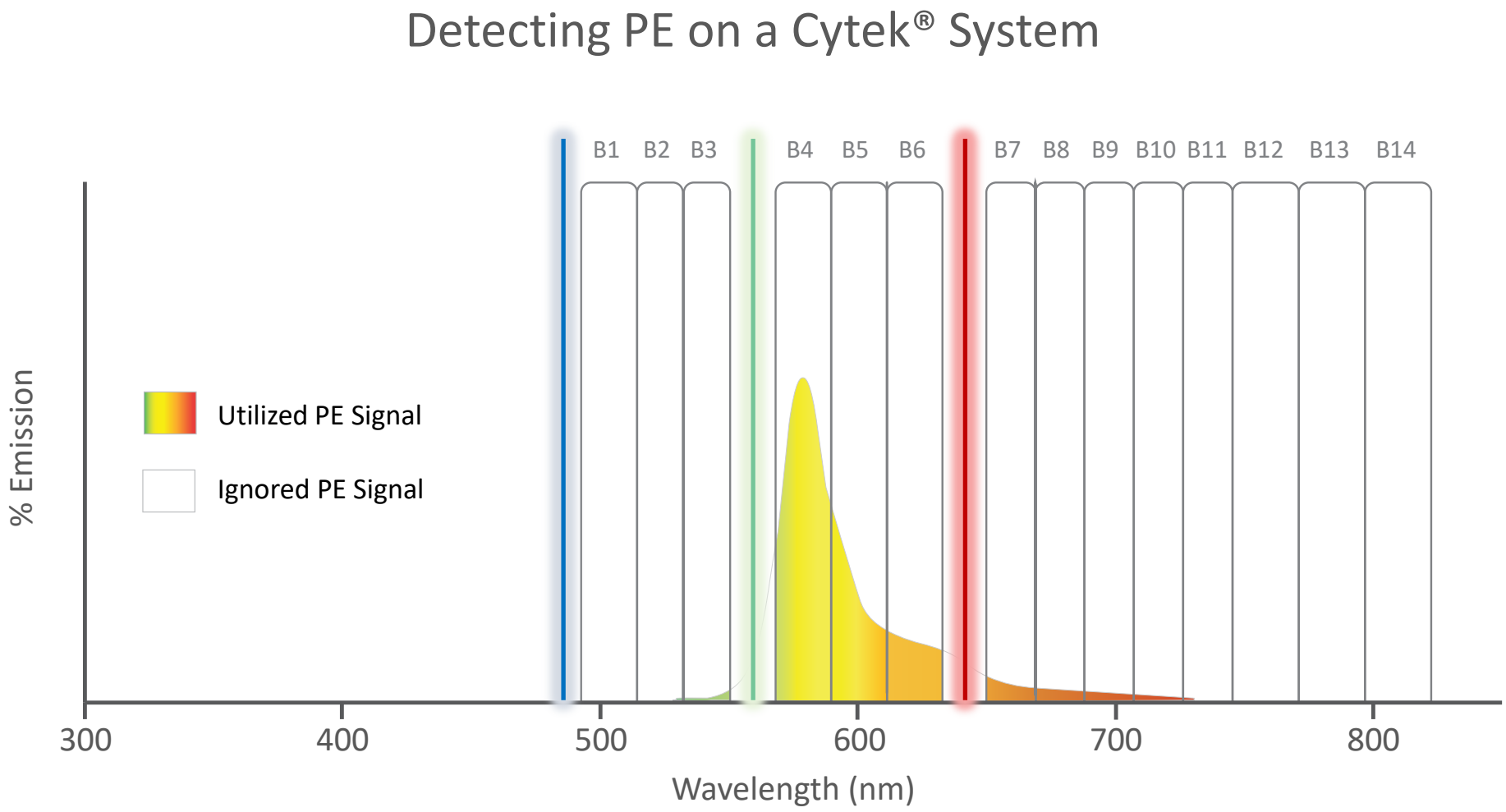
Flow Cytometry Optics



Conventional vs. Full Spectrum: Differences



Conventional vs. Full Spectrum: Differences



Interactive Poll #1

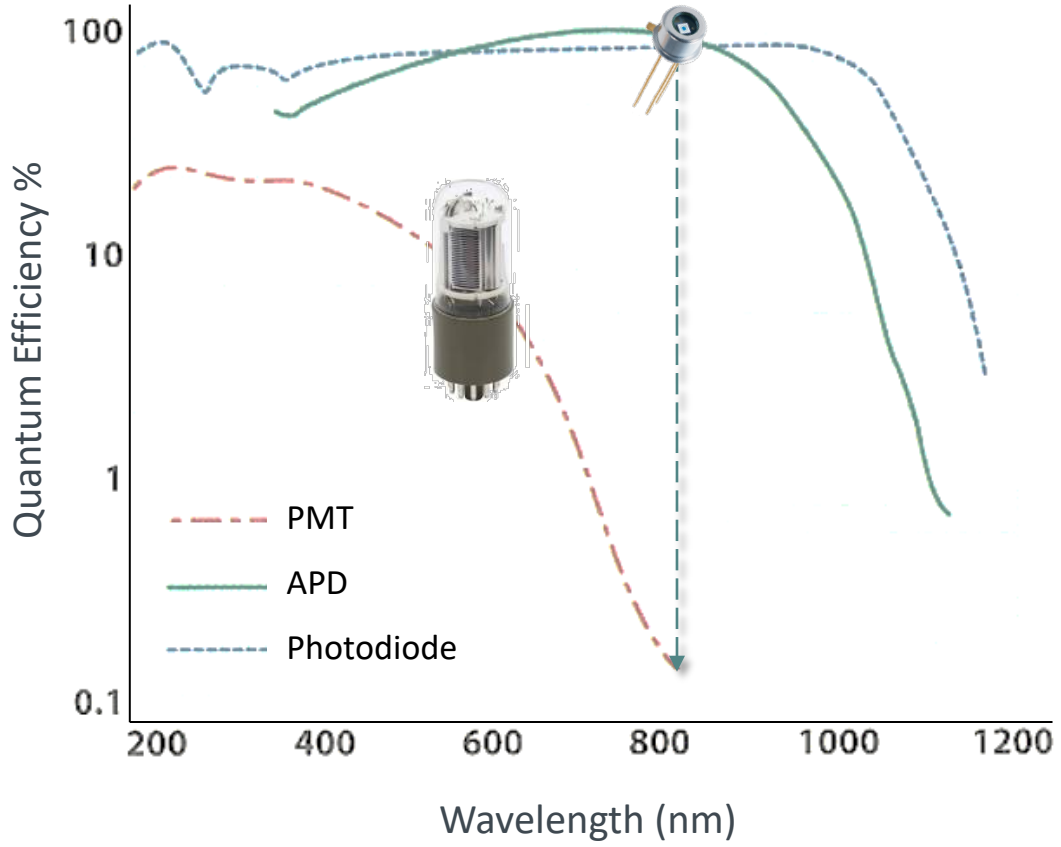
What is different about full spectrum cytometry?

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



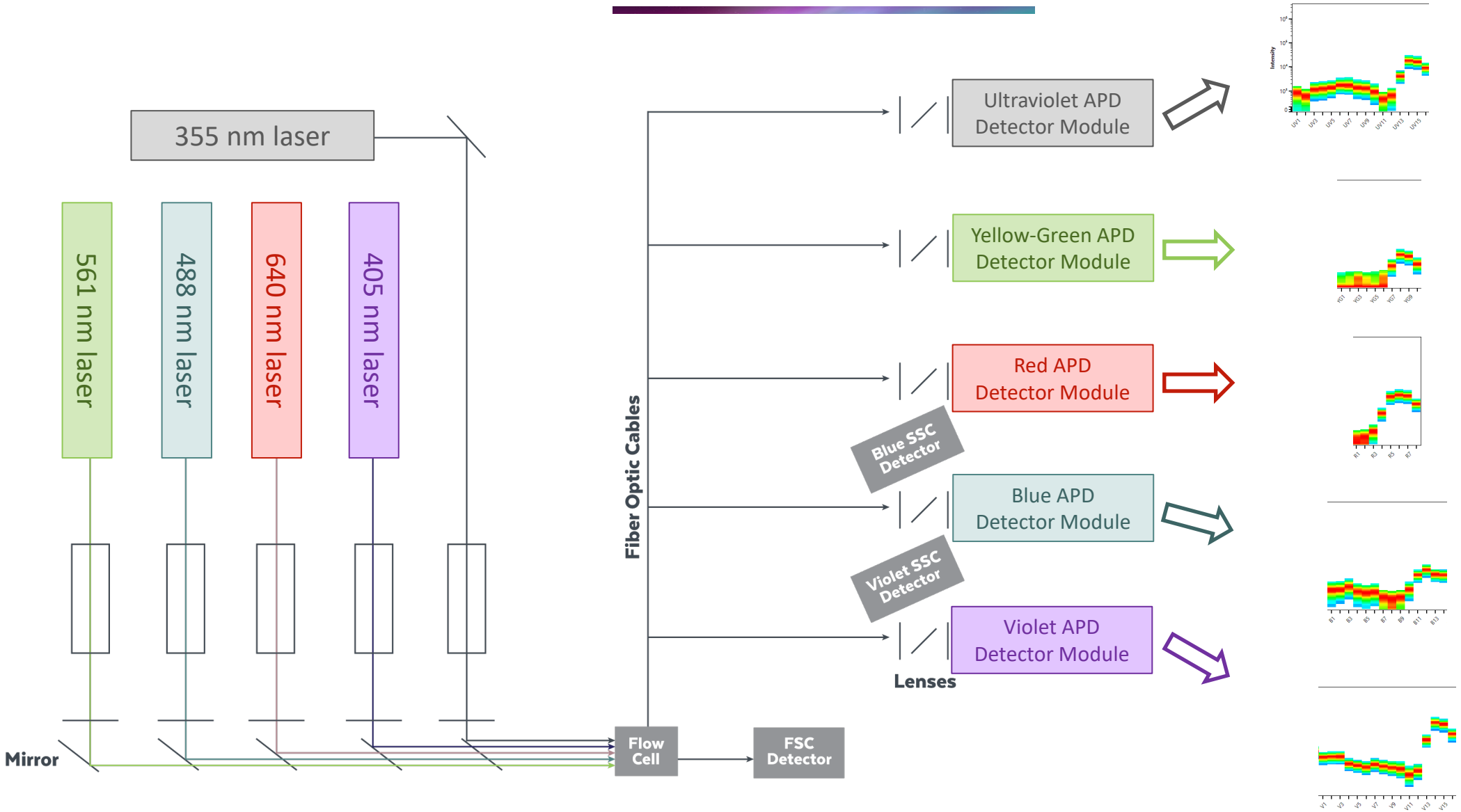
Data from Hamamatsu Photonics

Generating Full Spectrum Signatures

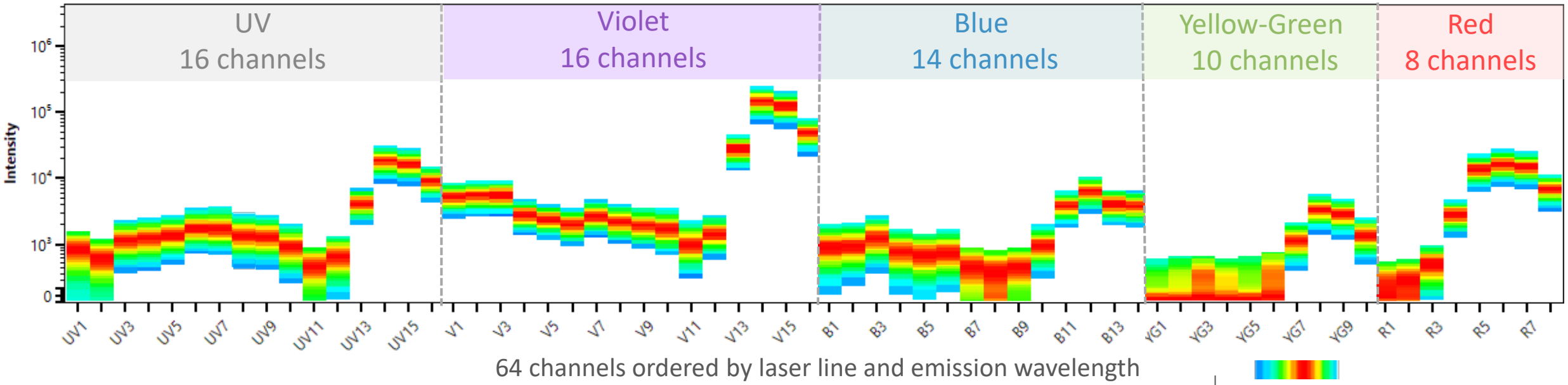
Components of a full spectrum signature

Options for viewing signatures

Generating a Full Spectrum Signature

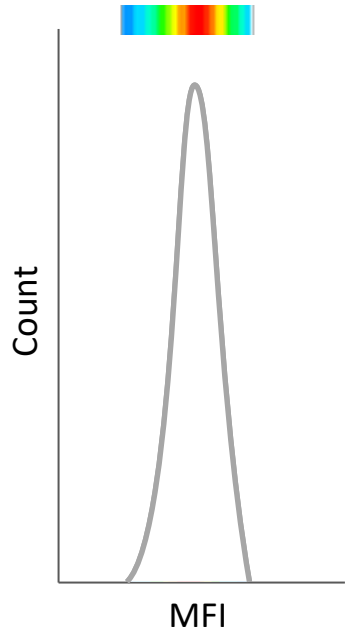


Building a Full Spectrum Signature

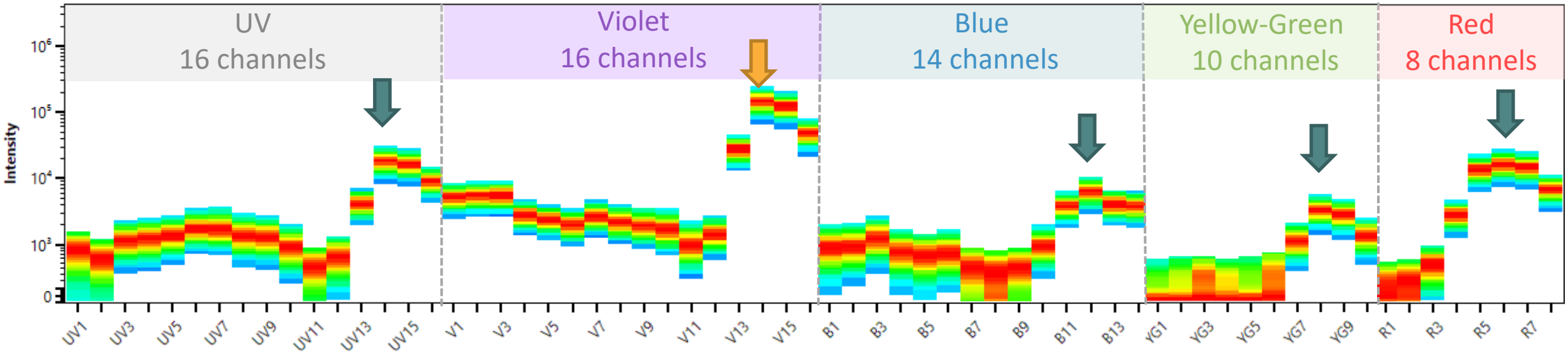


64 channels ordered by laser line and emission wavelength

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



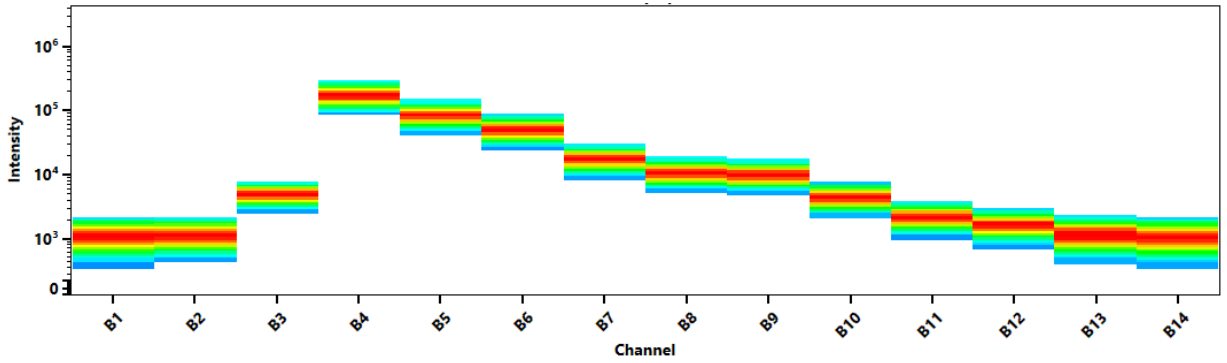
Building a Full Spectrum Signature



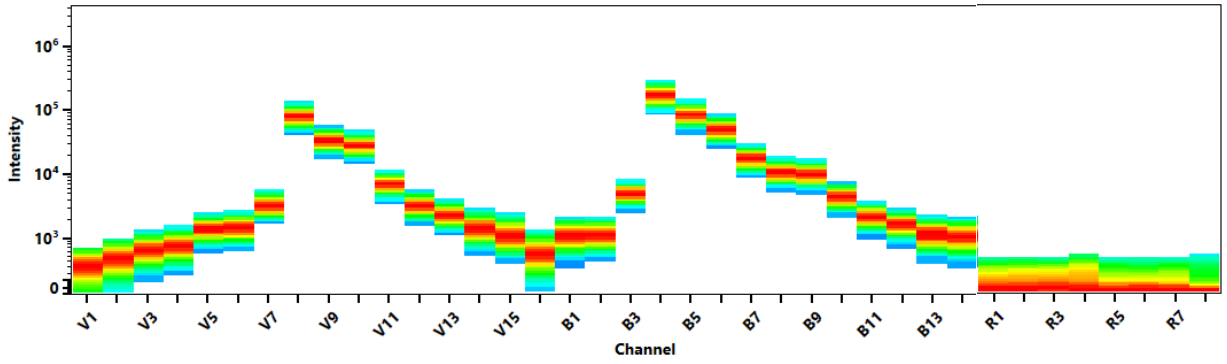
Primary Emission Channel: Captures the overall emission maxima. Occurs in detector array matching the primary excitation laser.

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

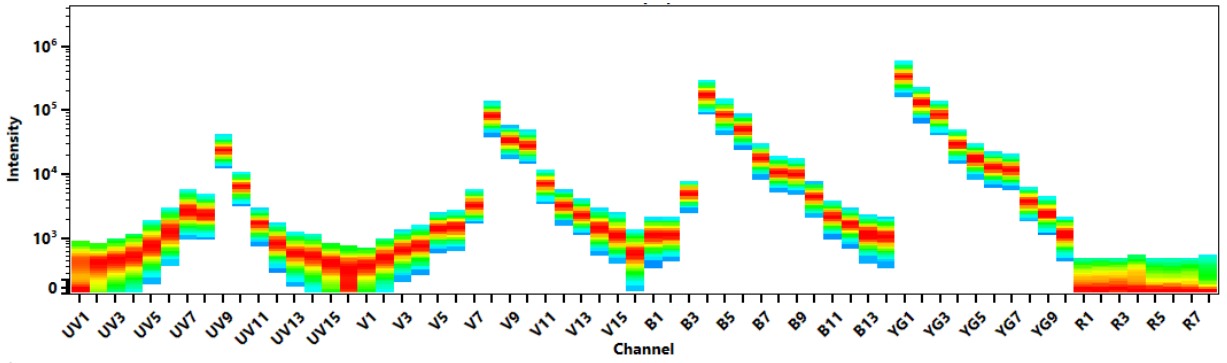
Signatures From Different Configurations



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

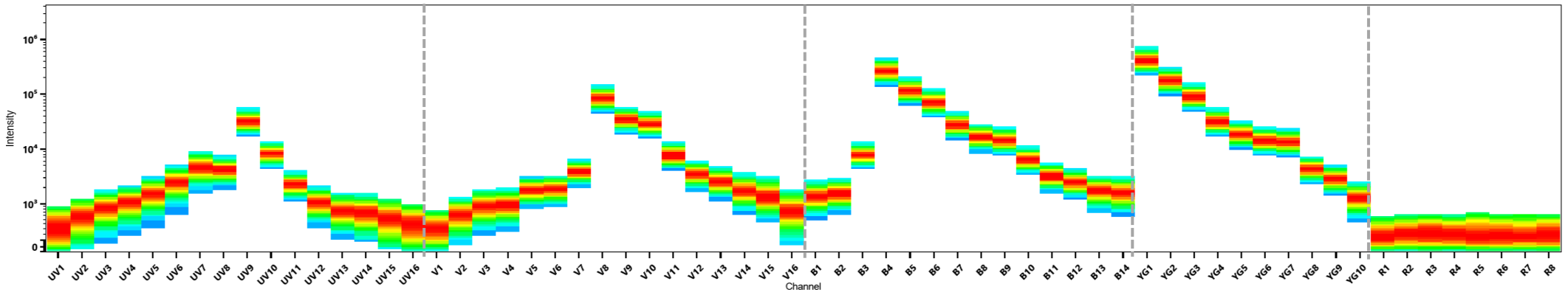
Goals

- Identify peak emission channel
- Identify secondary peak emission channels

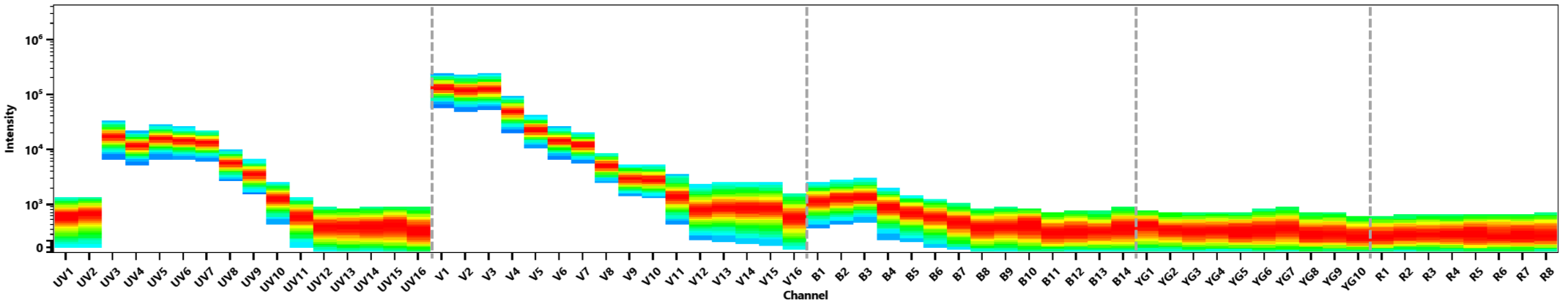
Exercise 1: Full Spectrum Signatures

UV	Violet	Blue	Yellow-Green	Red

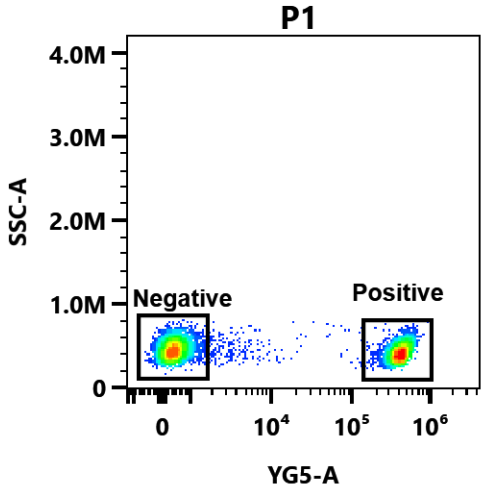
cFluor® BYG575 (PE)

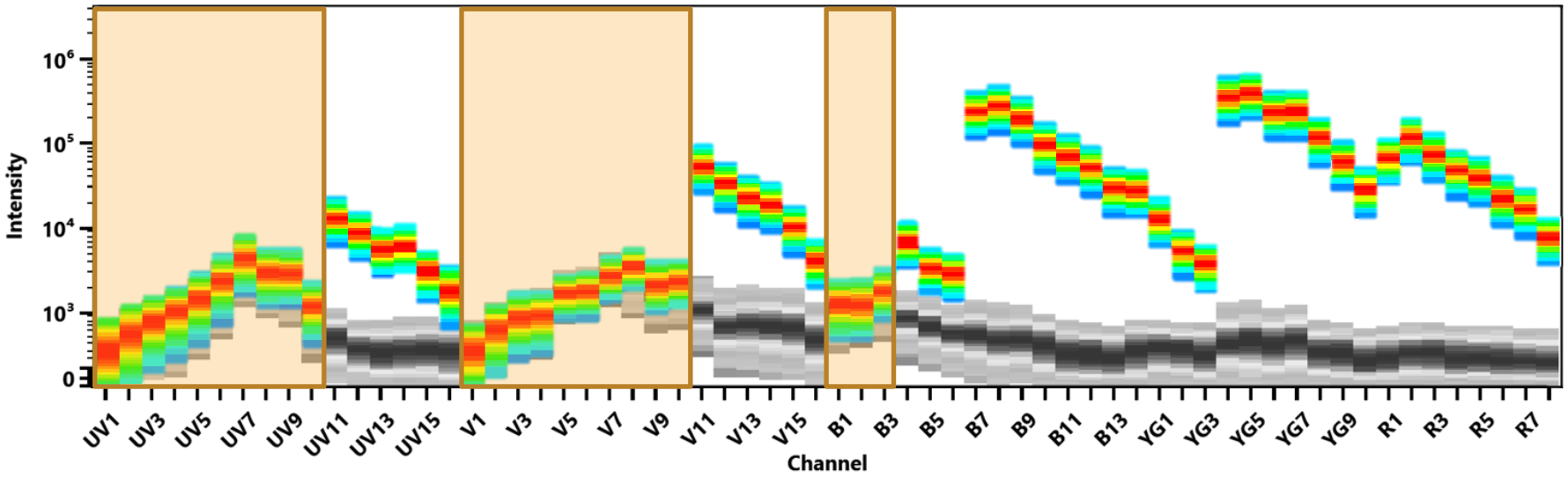


BV421



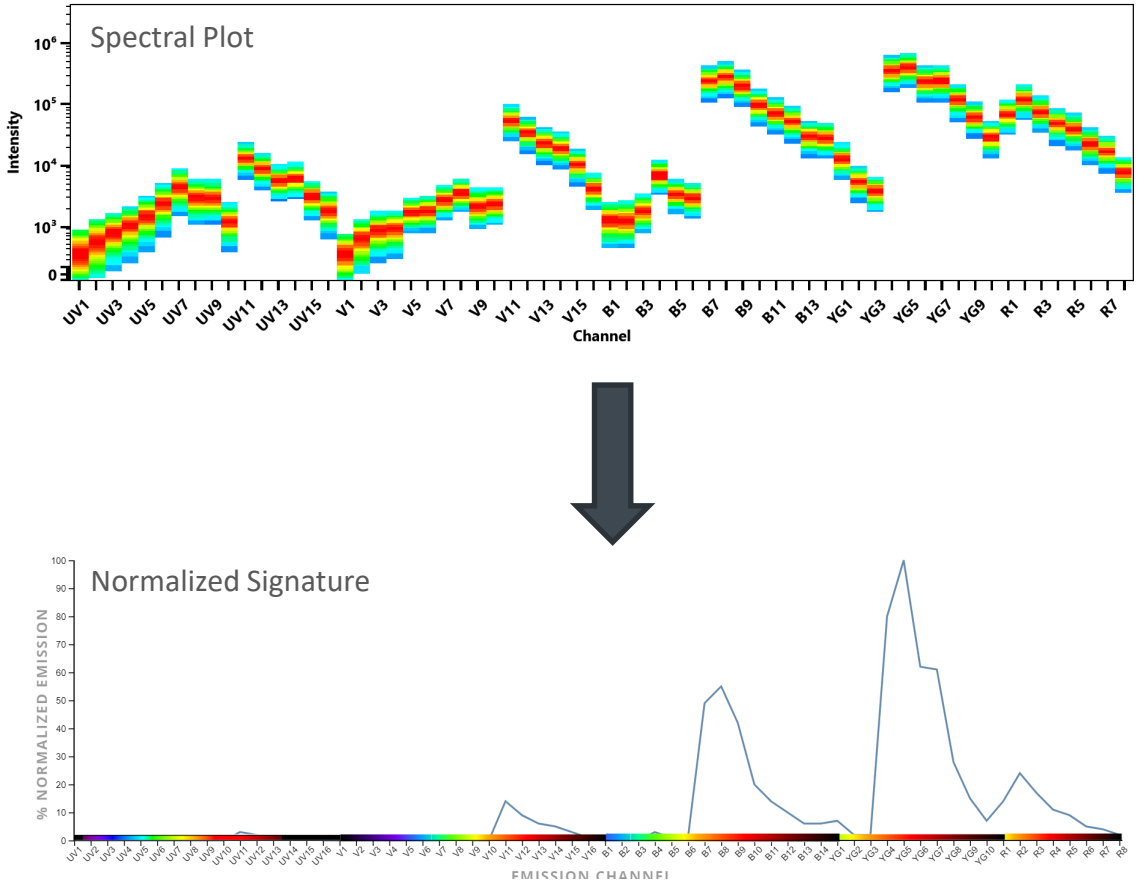
Spectral Plots





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%

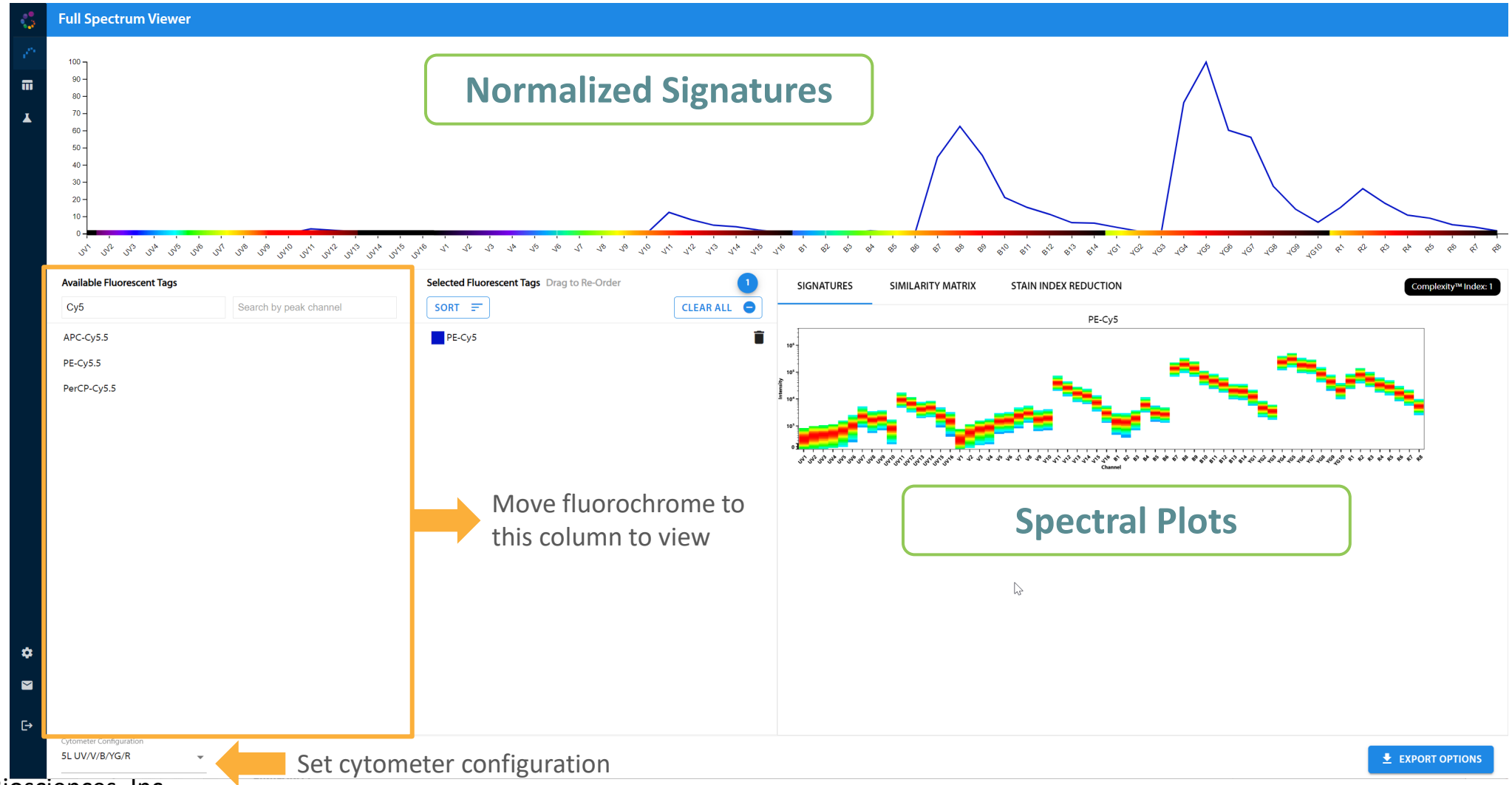
Access All Tools in Cytek® Cloud

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

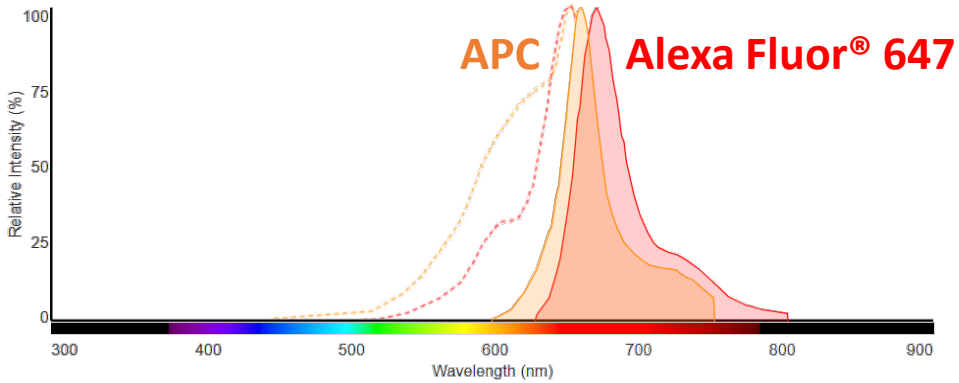
Sign up for a free account at https://cloud.cytekbio.com/

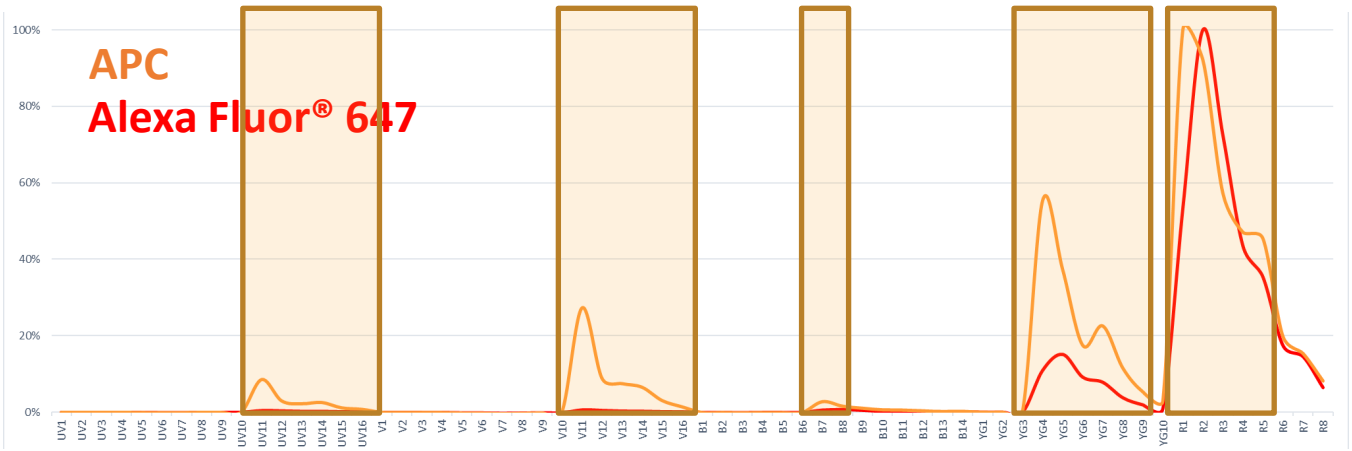
Cytek® Cloud – Full Spectrum Viewer

Signatures can be found in the Full Spectrum Viewer

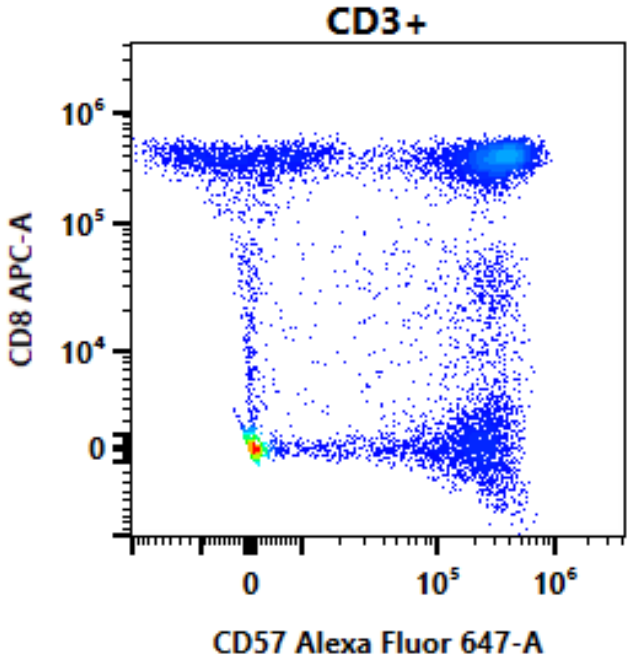


Use of Highly Overlapping Dyes in Panels





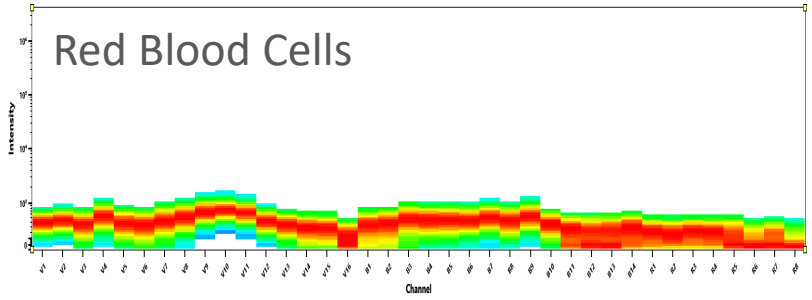
Plot gated on singlet lymphocytes

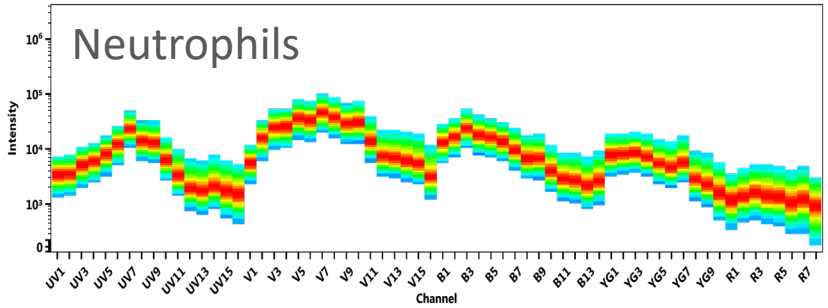


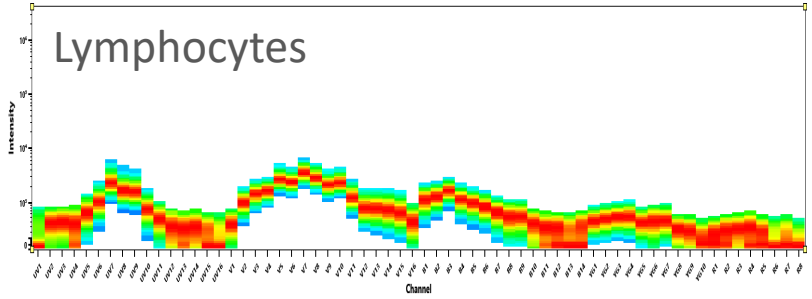
Fluorochromes with highly overlapping emission spectra can be used effectively on co-expressed markers

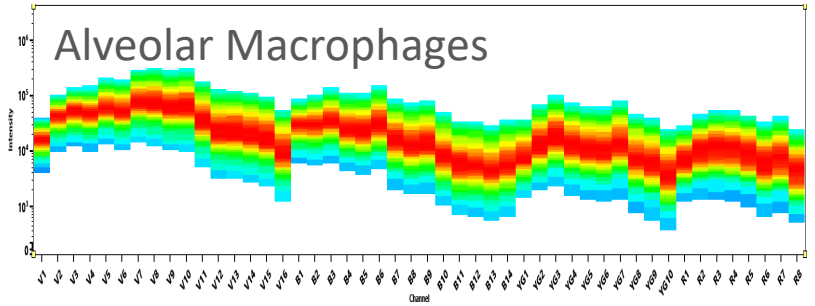
FSP™ Technology Easily Defines Autofluorescence

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





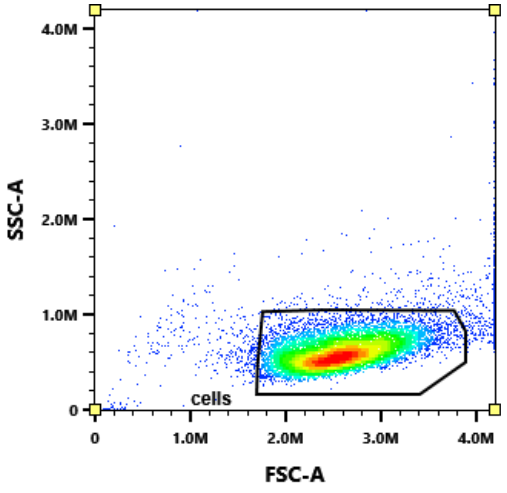


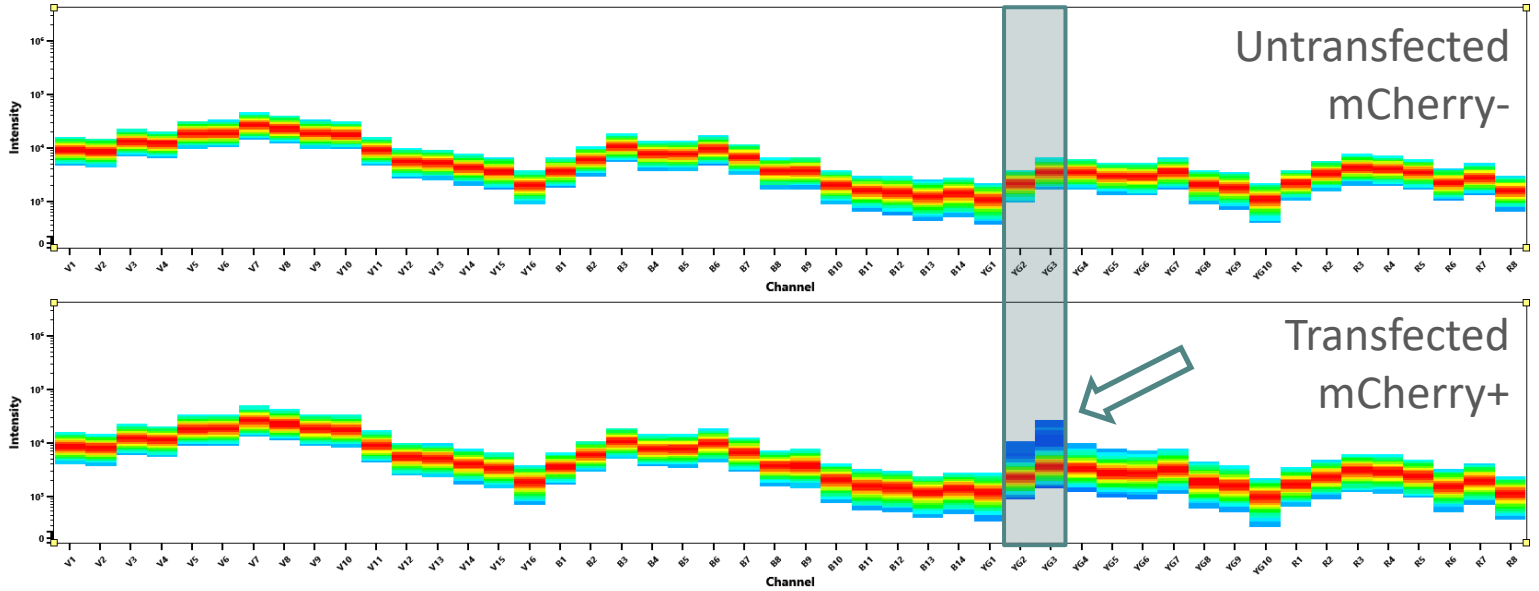


Cytek® Full Spectrum Profiling™ Technology can extract autofluorescence and potentially improve marker resolution

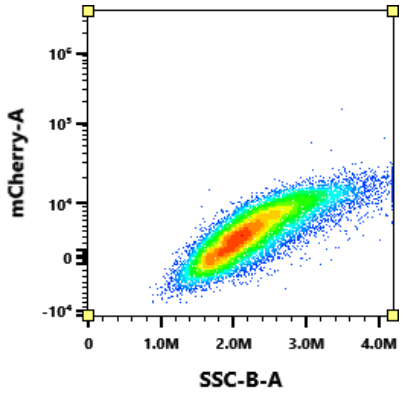
Benefits of Autofluorescence Extraction

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

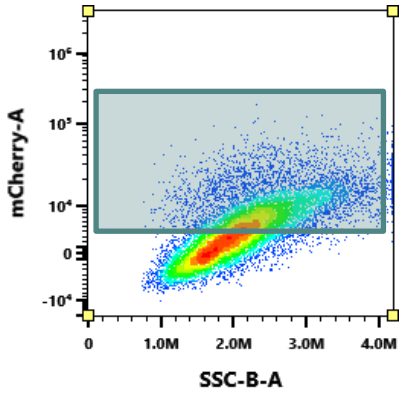




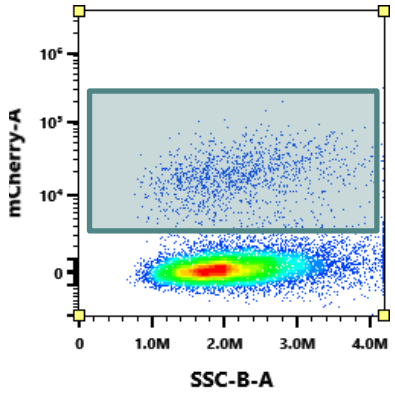
Untransfected Without Autofluorescence Extraction



Transfected Without Autofluorescence Extraction



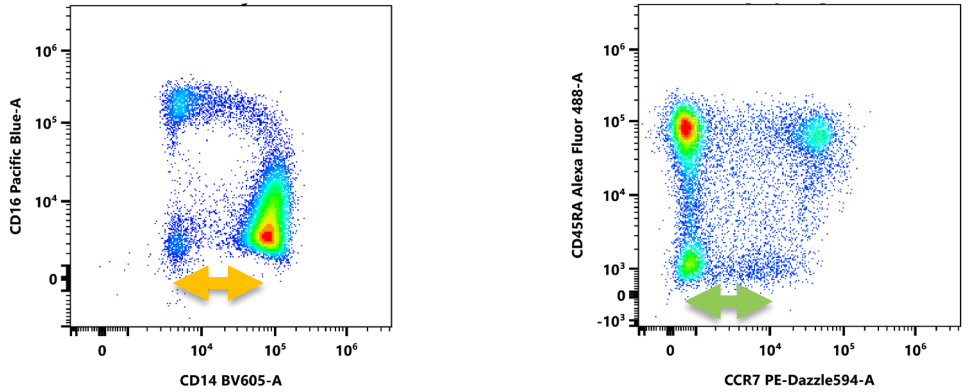
Transfected With Autofluorescence Extraction



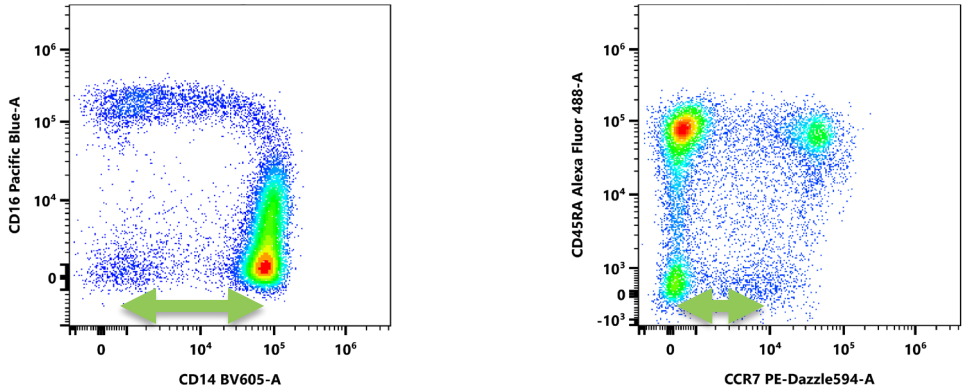
Autofluorescence Extraction Benefits Are Assay-Dependent

PBMCs stained with a 16-color assay

Without Autofluorescence Extraction



With Autofluorescence Extraction

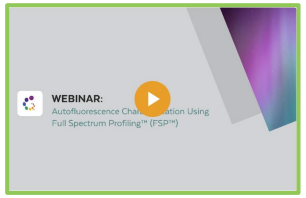


Software setup:

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



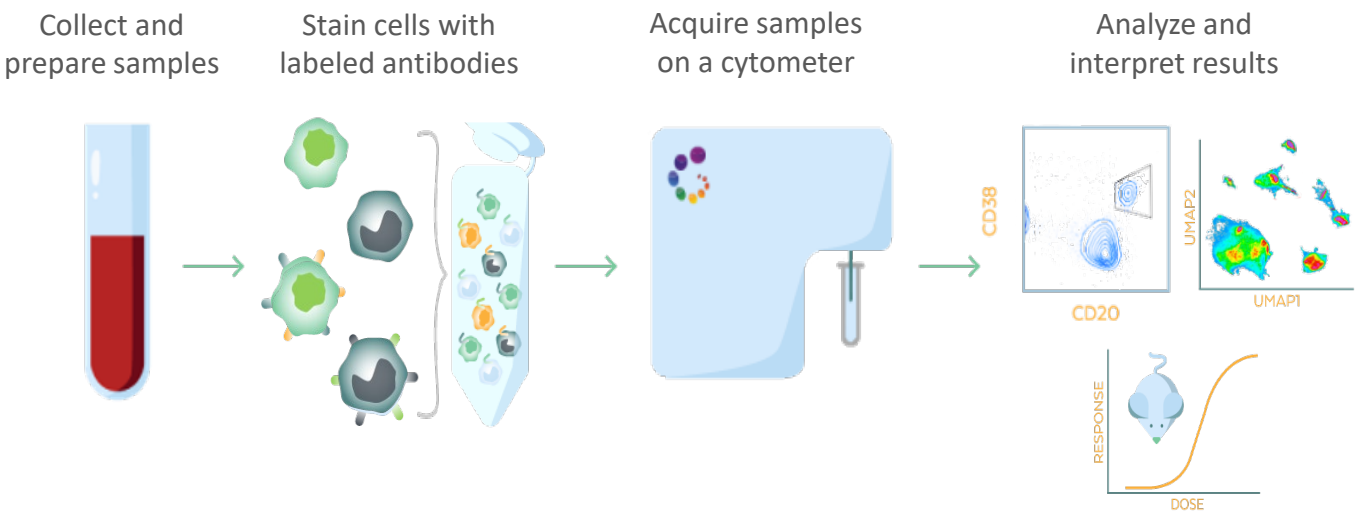
Cytek Webinars on Autofluorescence

Interactive Poll #2

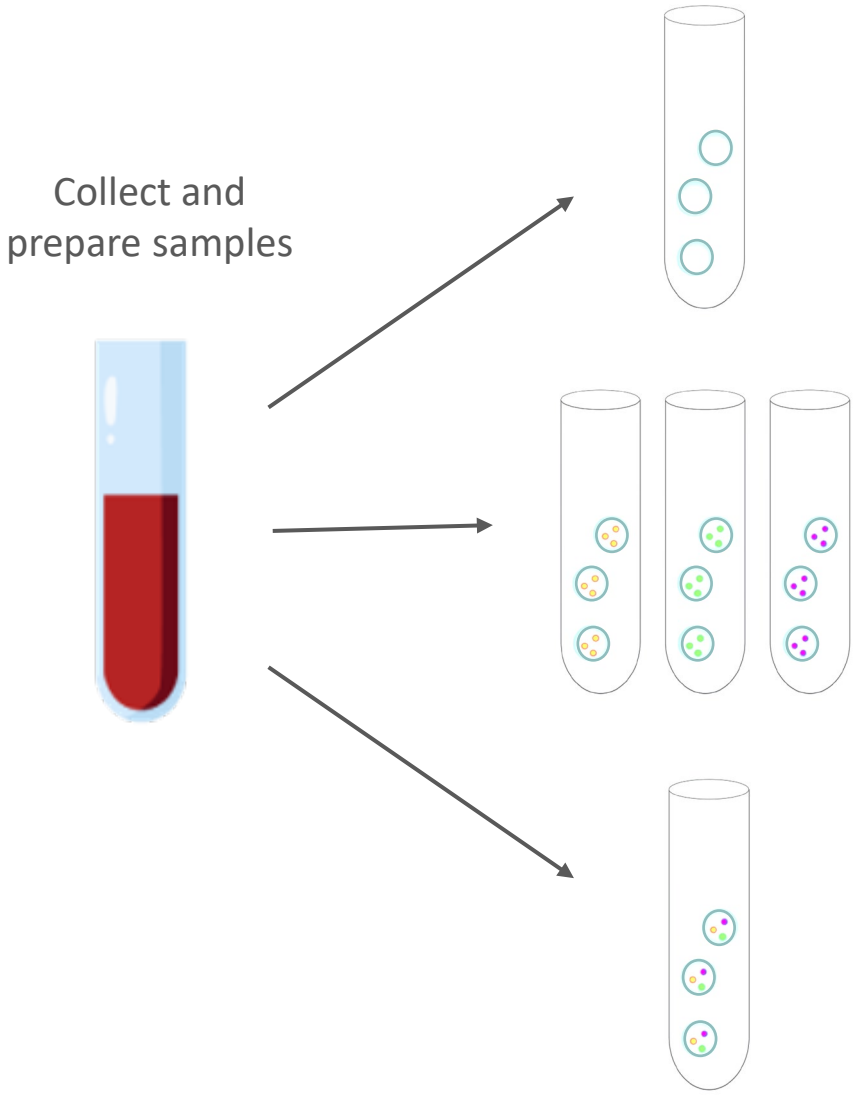
Benefits of Full Spectrum Profiling™ Technology (FSP™) include:

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

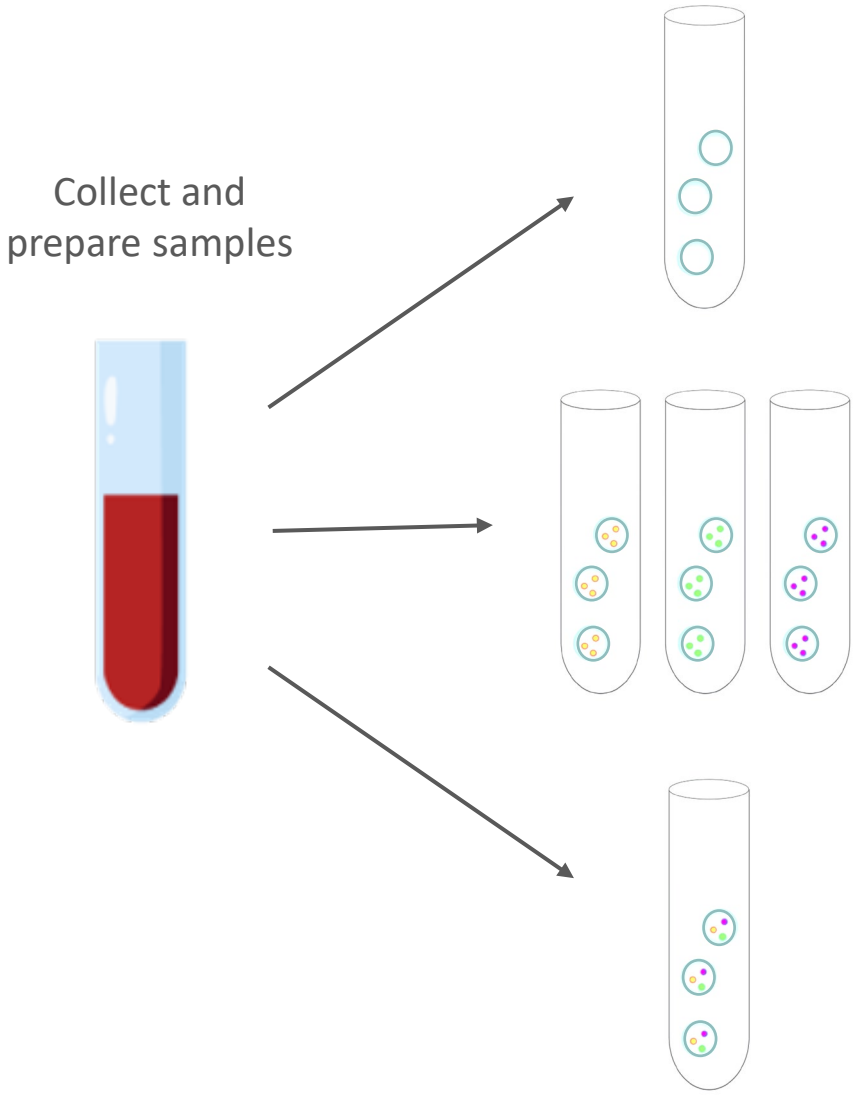
- Need one control for each fluorochrome present in the multicolor assay
- Defines a ***single*** fluorophore
- Not required to match cell type with multicolor

Multicolor Assay

- Contains all fluorophores in one tube/well

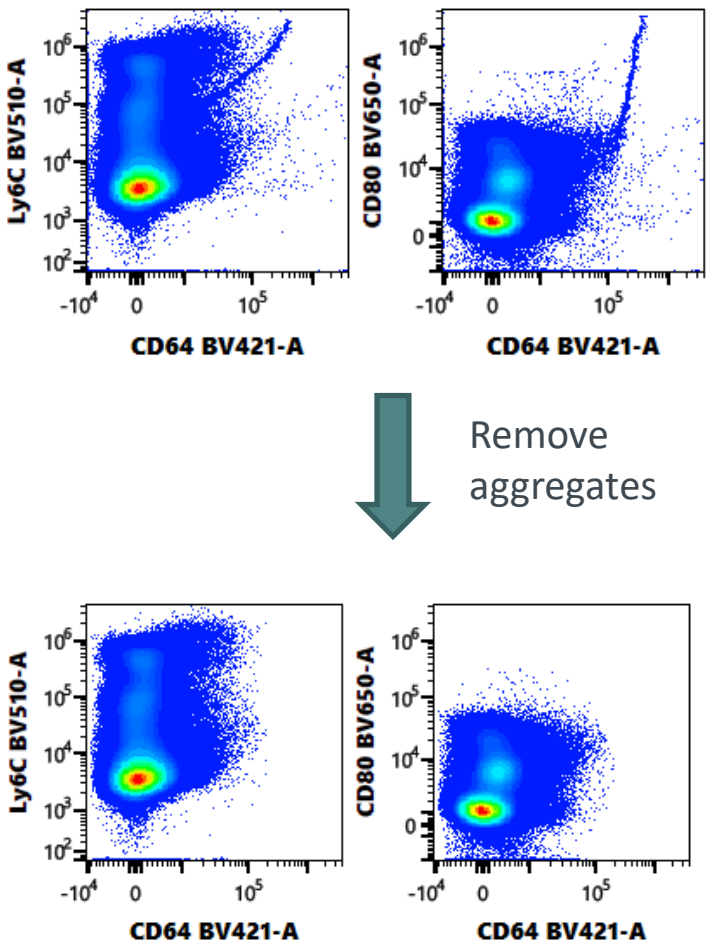
Additional experimental controls may be prepared depending on the assay

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.)*

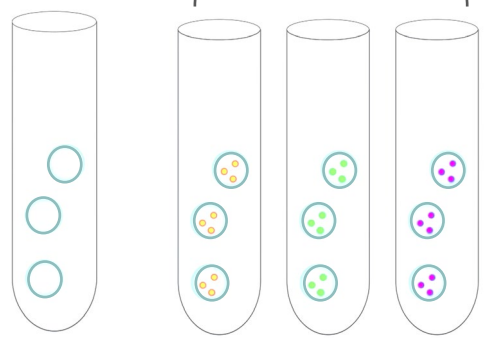
   **do not add buffer to beads or unstained cells, only antibody cocktail*
3. Protect fluorophores from light exposure

Optimize protocol for your own assay

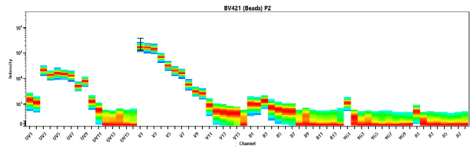
Acquisition Overview

Run compensation/reference controls

Unstained | Single Color Controls

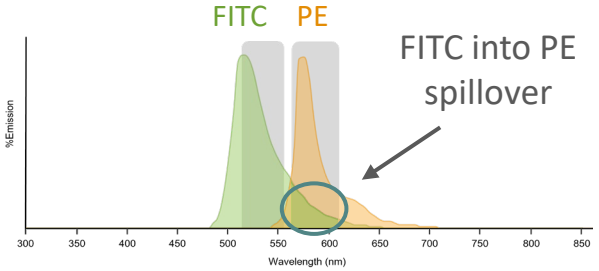


Raw Data



Input

Calculate compensation/unmixing

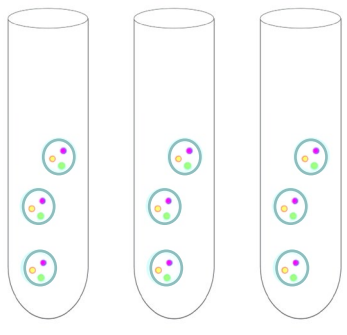


Unmix

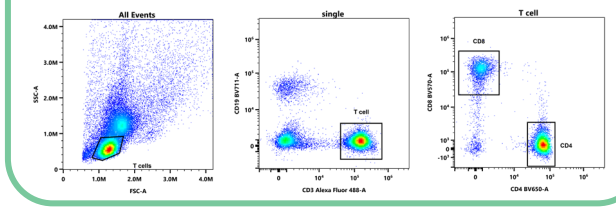
Output

Run multicolor samples with compensation/unmixing applied

Multicolor Assay

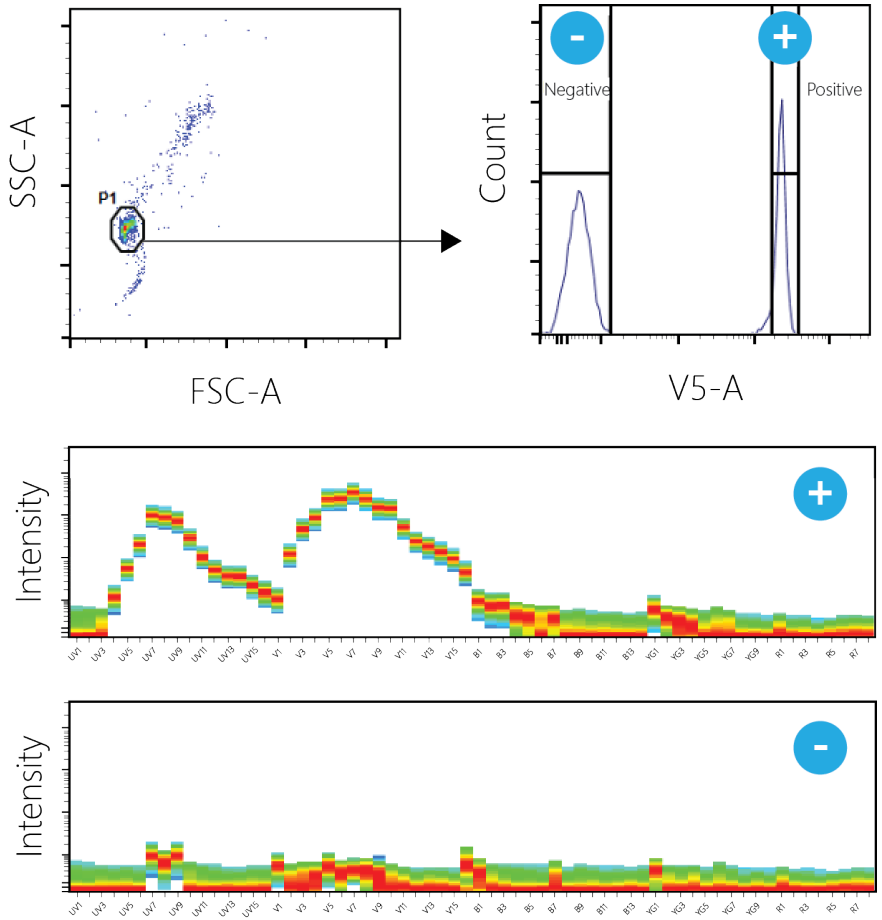


Unmixed Data



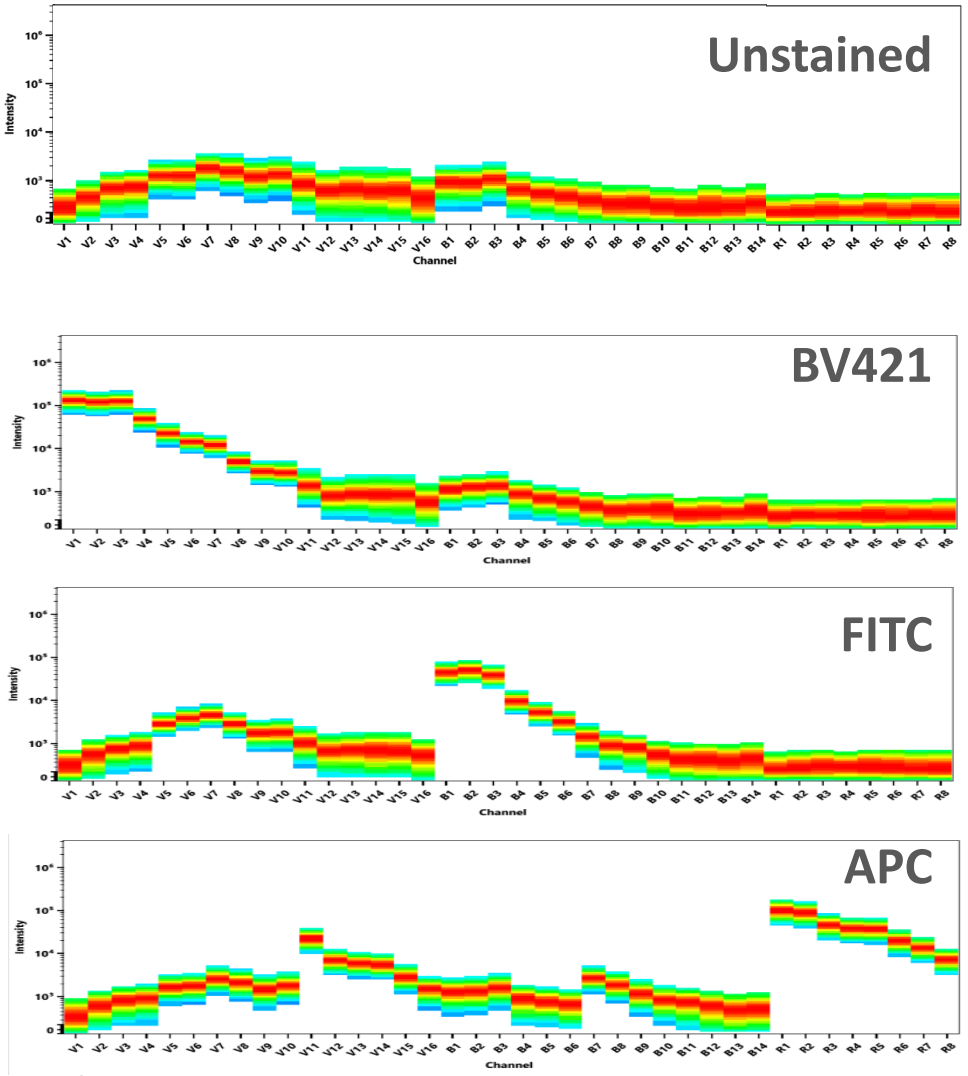
Unmixing converts raw data to unmixed data

Setting Up Spectral Unmixing in SpectroFlo® Software



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

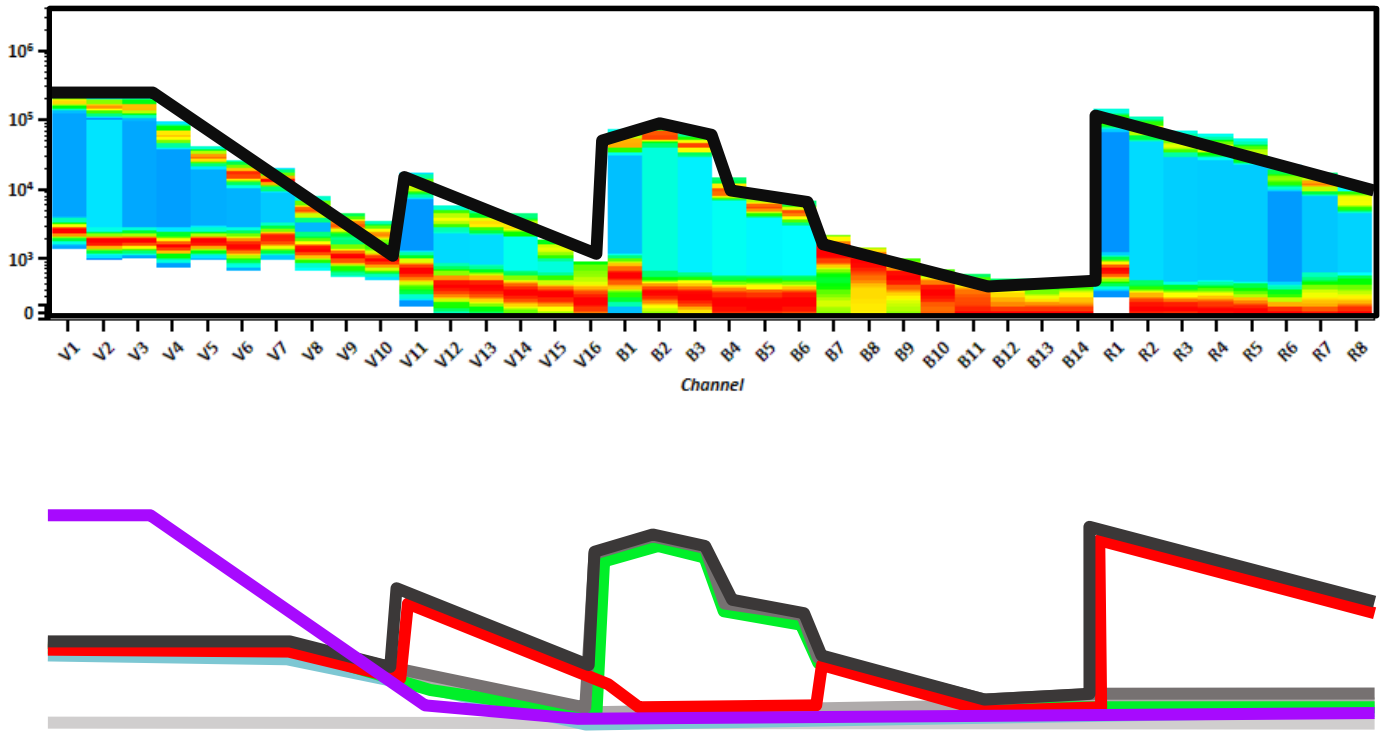
Setting Up Spectral Unmixing in SpectroFlo® Software



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

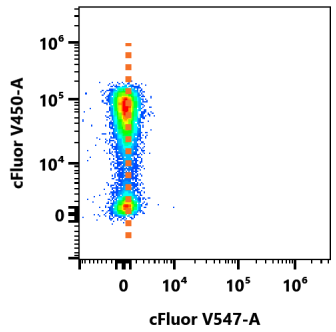
Spectral Unmixing - Ordinary Least Squares (OLS)

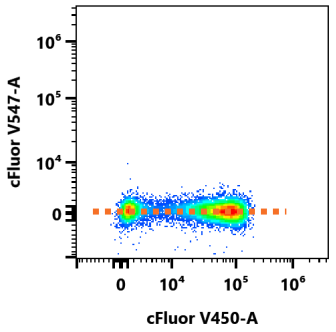
The spectral unmixing algorithm uses the provided controls to calculate the contribution of each fluorophore in the multicolor assay.



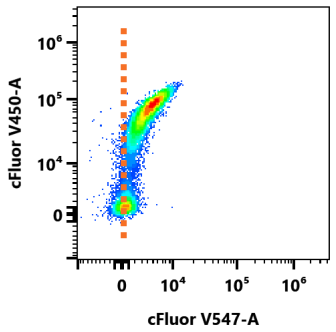
We can think of this as extracting or deconvoluting each component until we have nothing left.

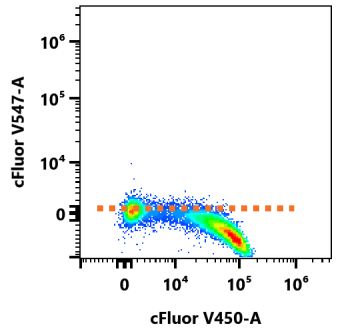
Spectral Unmixing Applied to Data





✓ MFI of the positive matches the MFI of the negative





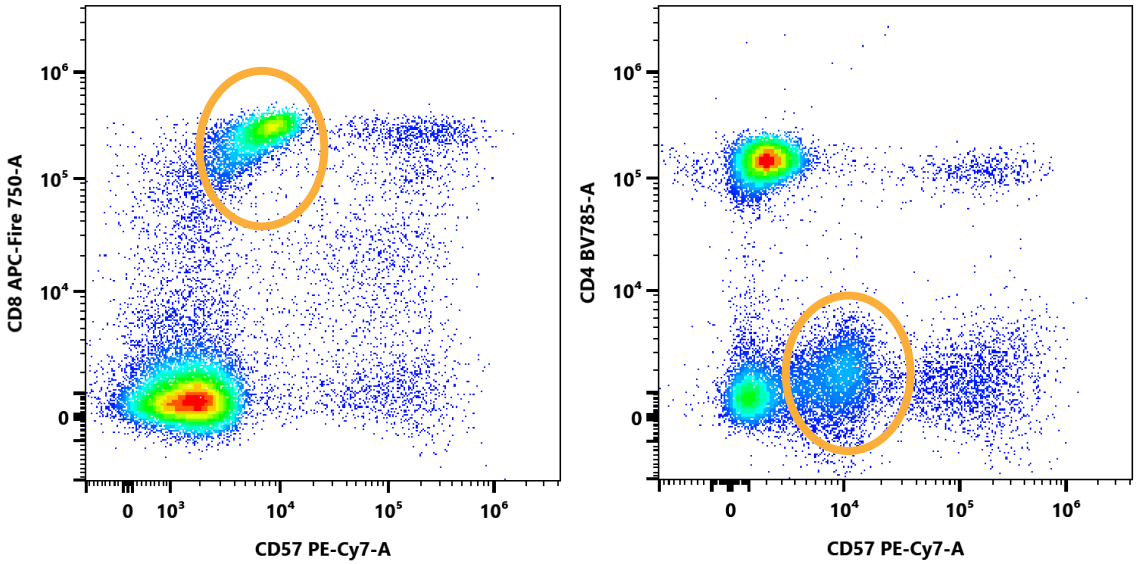
✗ MFI of the positive DOES NOT match the MFI of the negative

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

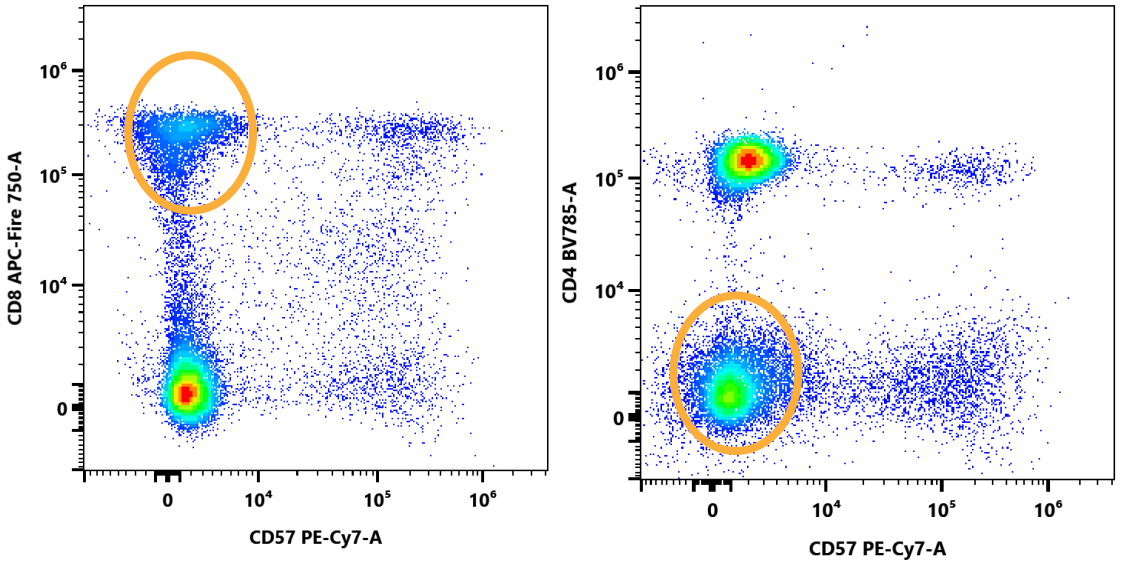
Unmixing/Compensation Errors Lead to Wrong Conclusions

Impacts data accuracy and result interpretation: false populations!

Data WITH Unmixing/Compensation Error

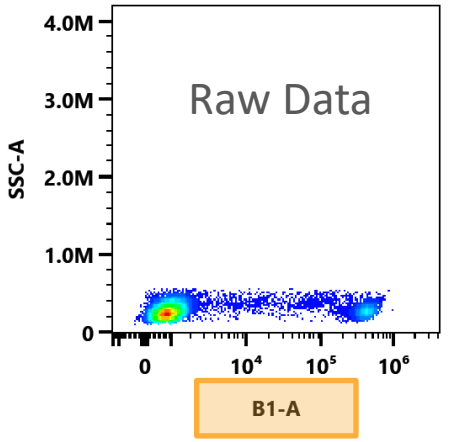


Correct Unmixing/Compensation

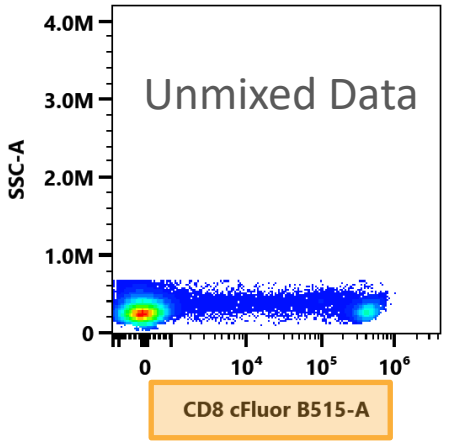


Options for Analyzing Data

- Can analyze **raw data** when:
  - Only one fluorophore analyzed
  - Fluorophores in panel have no spectral overlap



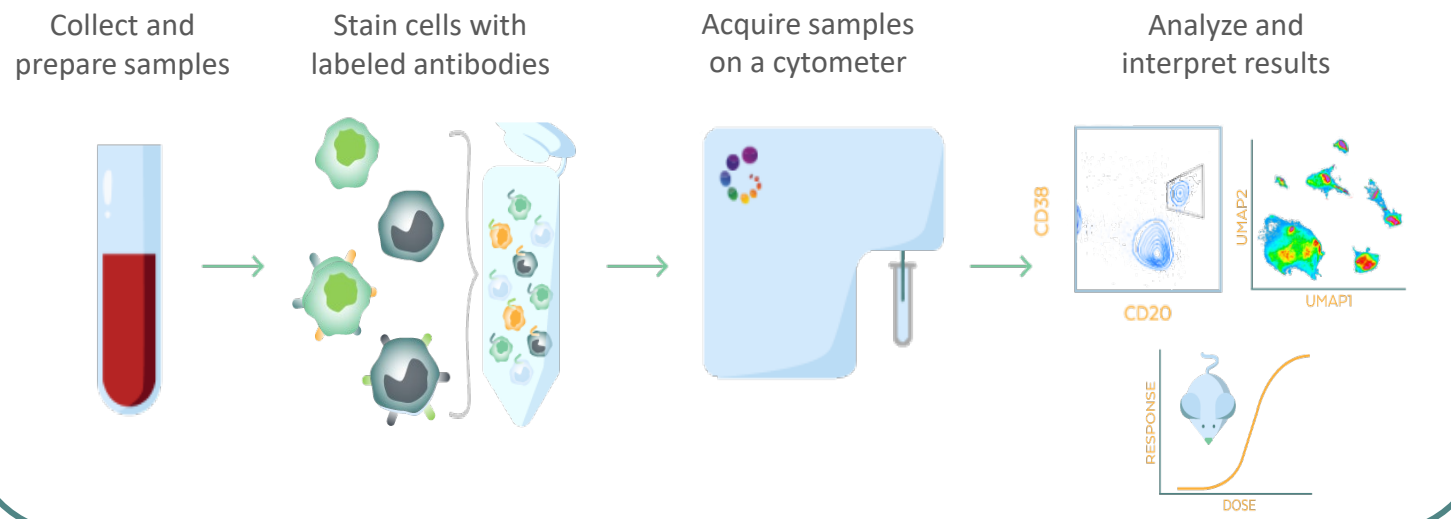
- Analyze **unmixed data** when:
  - Multiple fluorophores in panel with spectral overlap



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

Single Experiment



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

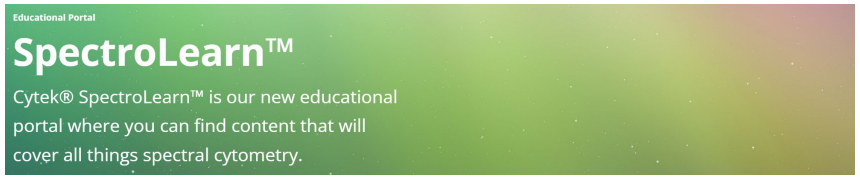
- Cytek® Tools for panel design

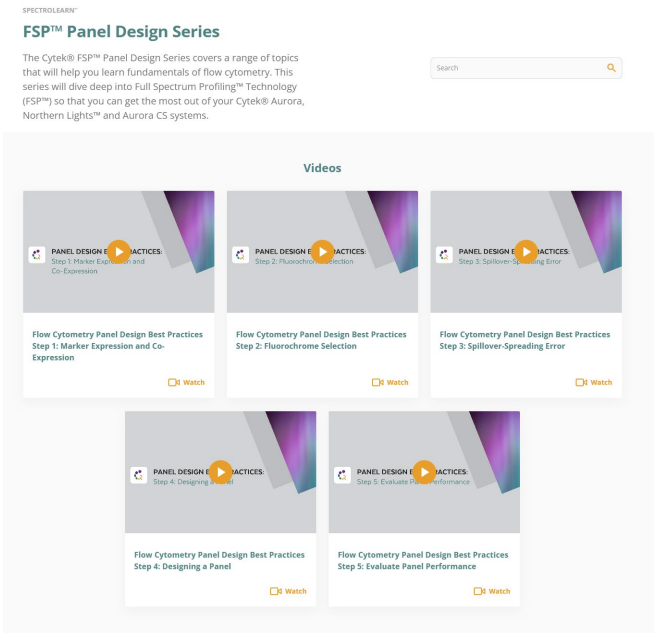
Select Appropriate Reference Controls

- How controls impact unmixing quality

Cytek® Panel Design Videos

SpectroLearn
https://cytekbio.com/blogs/spectrolearn





Webinars
https://cytekbio.com/blogs/videos

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...

Watch

Webinar: Debunking Antibody Titration Myths

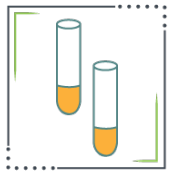
Click here to watch this webinar, led by Diana Bonilla Escobar, PhD as she covers the fundamentals of antibody titrations, a critical step in the development and optimization of multicolor flow cytometry assays.

Watch

Panel Design Methods Apply to All Cytometers

Panel Design Should be An Informed Process

1 The Biology



2 The Fluorochromes

3 The Design

4 The Optimization

Tools for Panel Design

The Fluorochromes

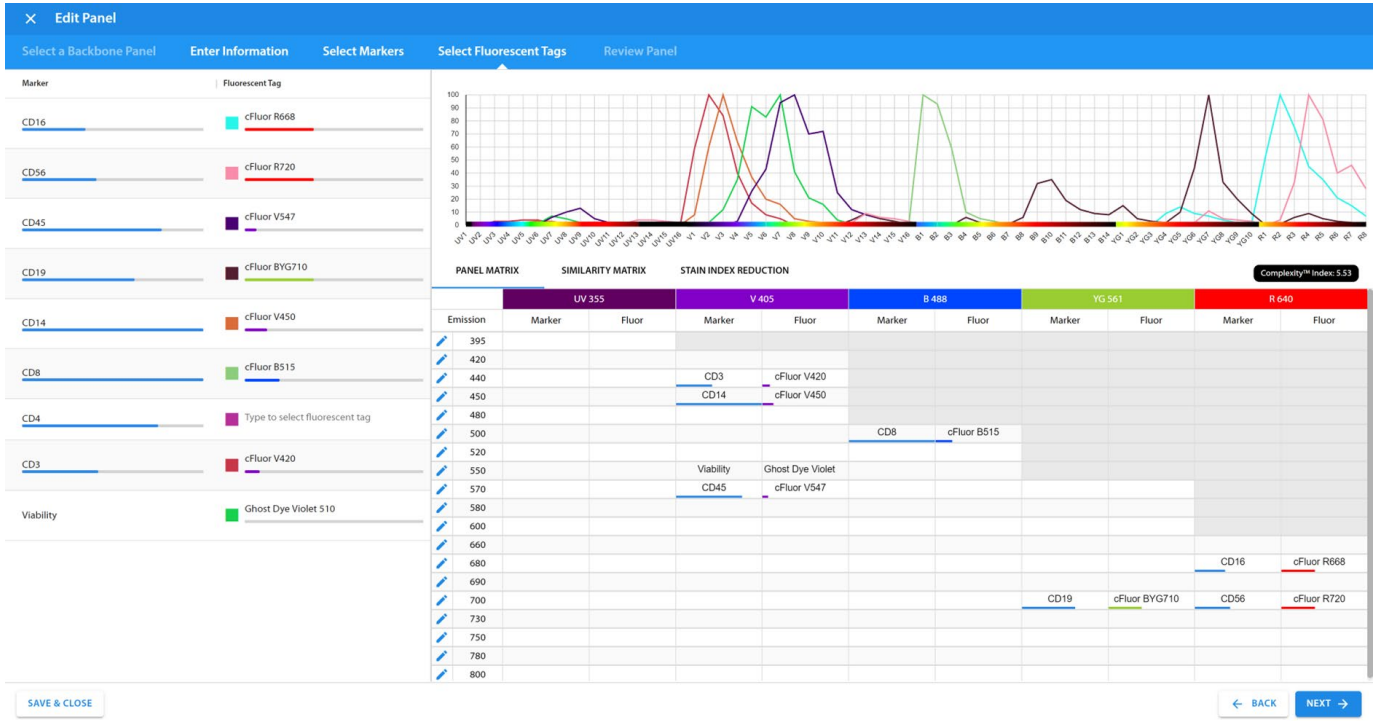
2

3

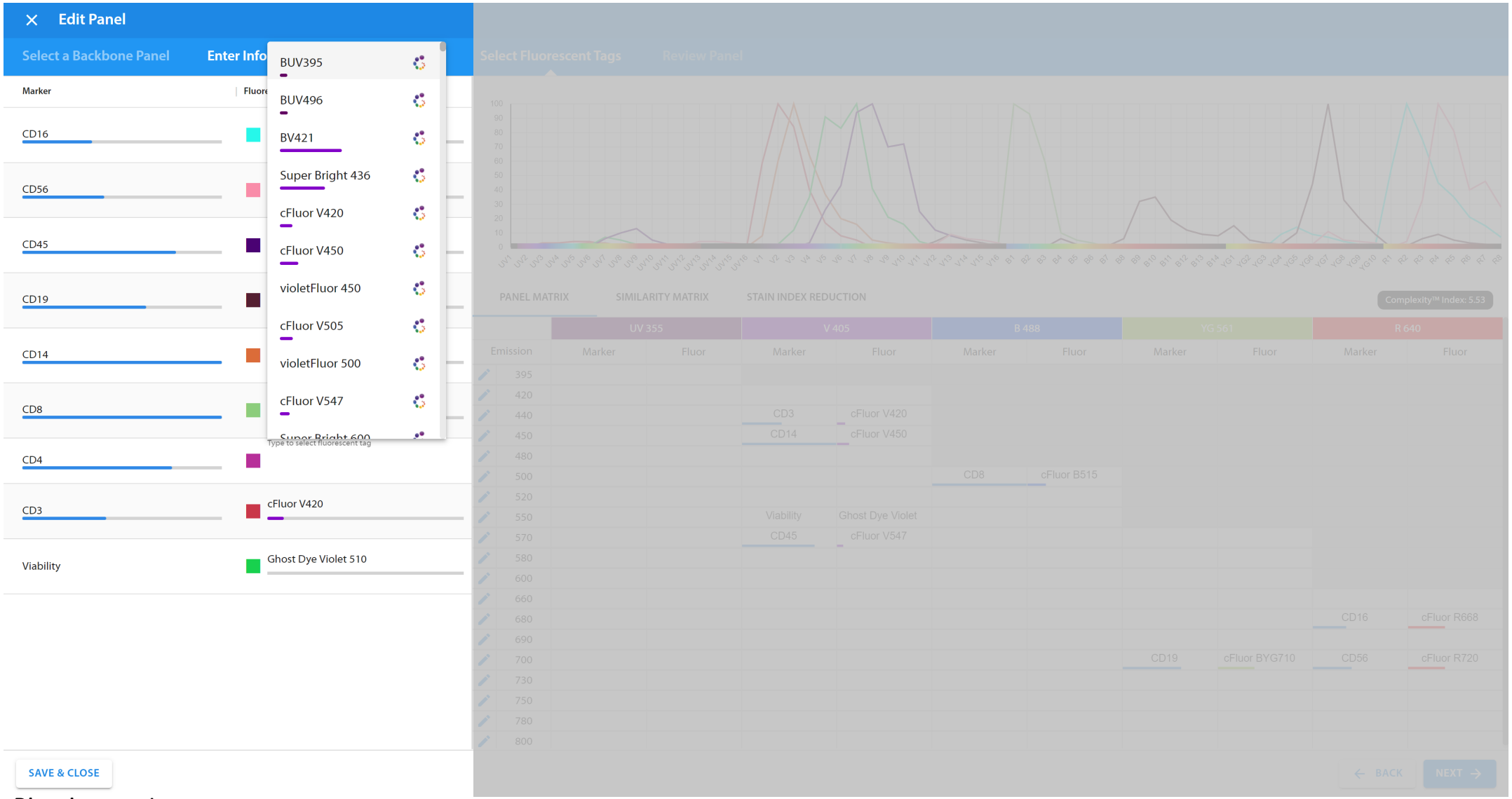
The Design

Cytek® Cloud – Panel Builder

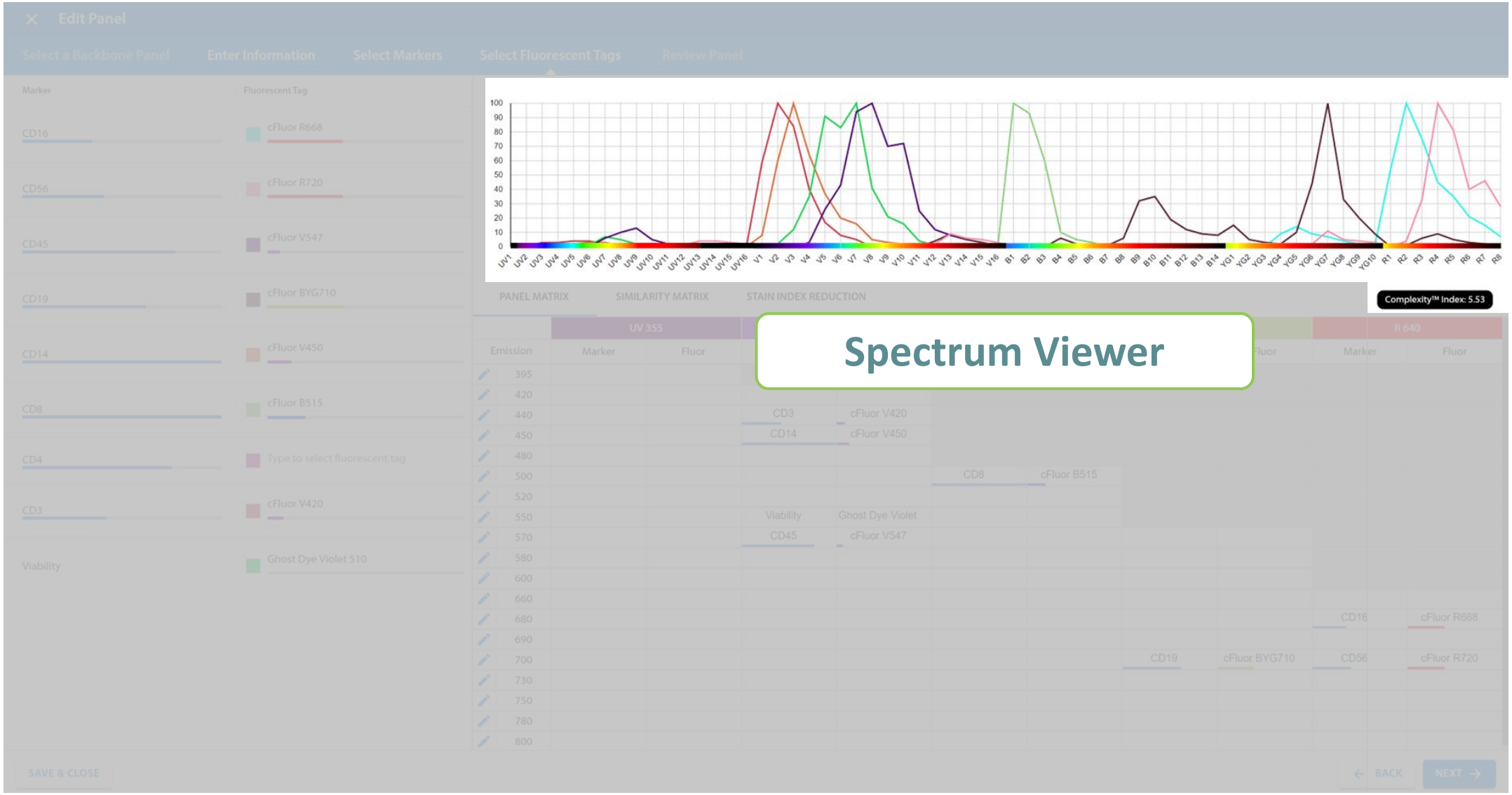
- Combines all full spectrum design tools in one place
- Options to:
  - Build custom panels from scratch
  - Modify pre-designed panels
  - Enter a previous panel from another cytometer



Cytek® Cloud – Panel Builder

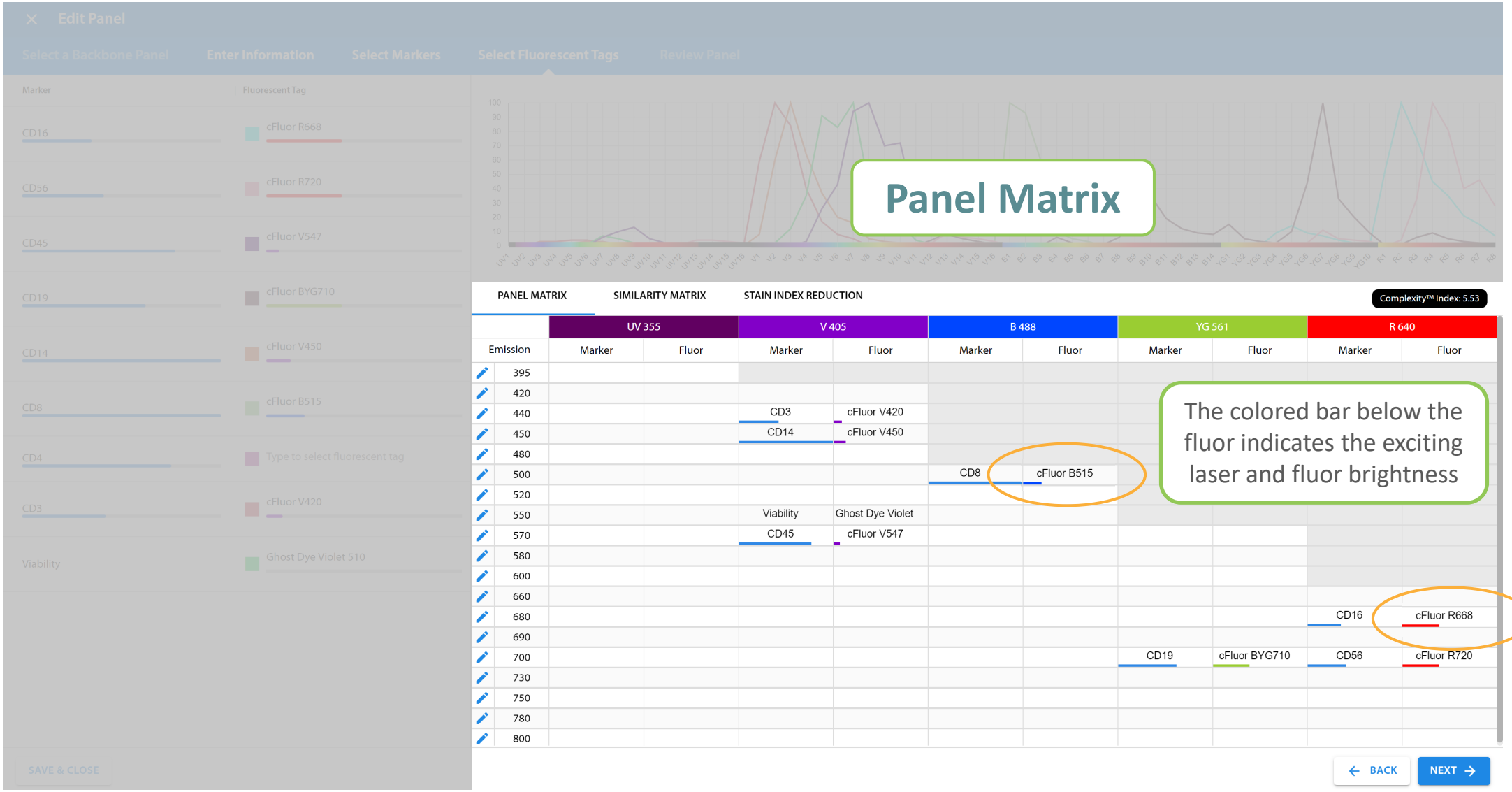


Cytek® Cloud – Panel Builder



Spectrum Viewer

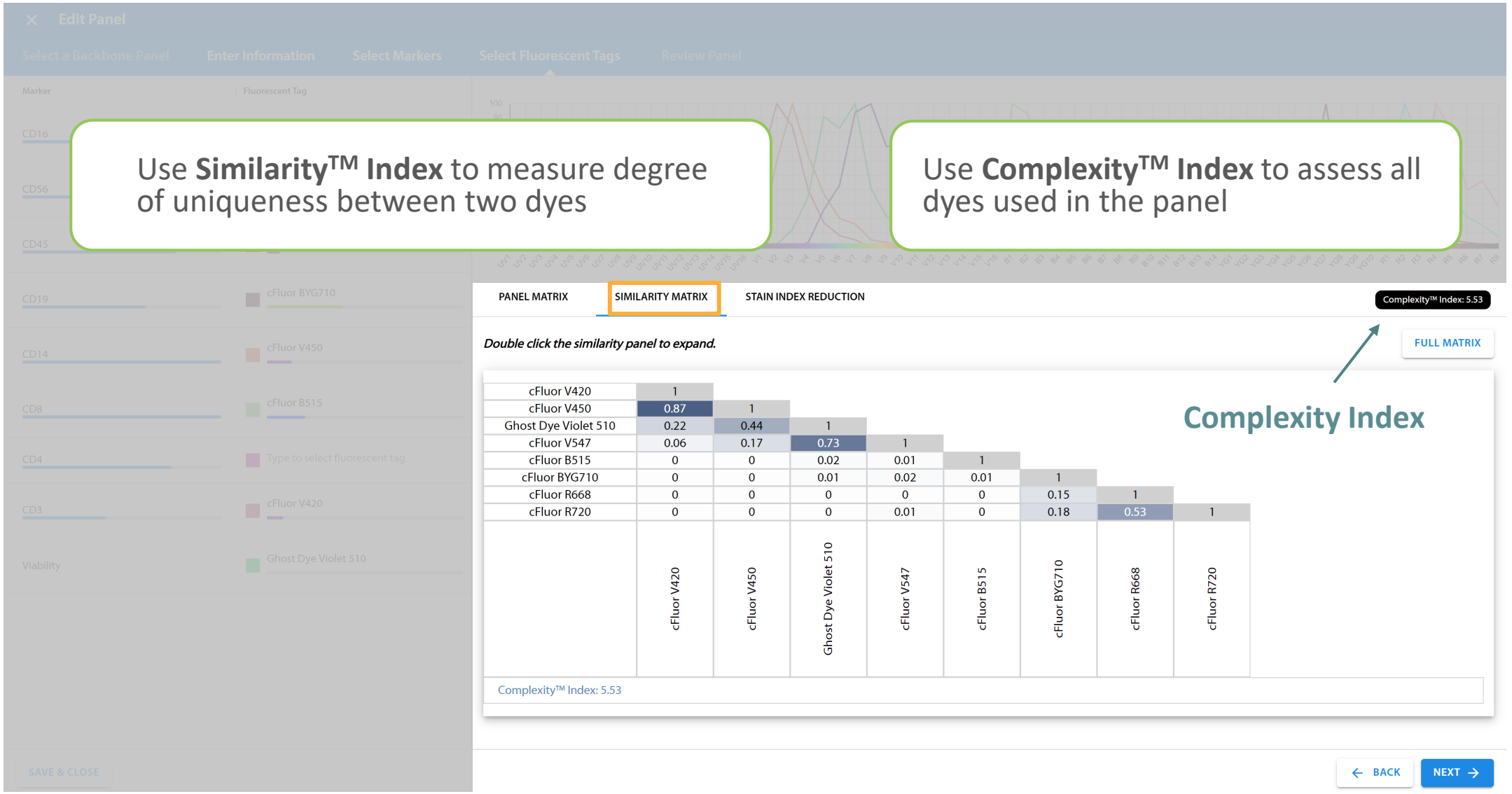
Cytek® Cloud – Panel Builder



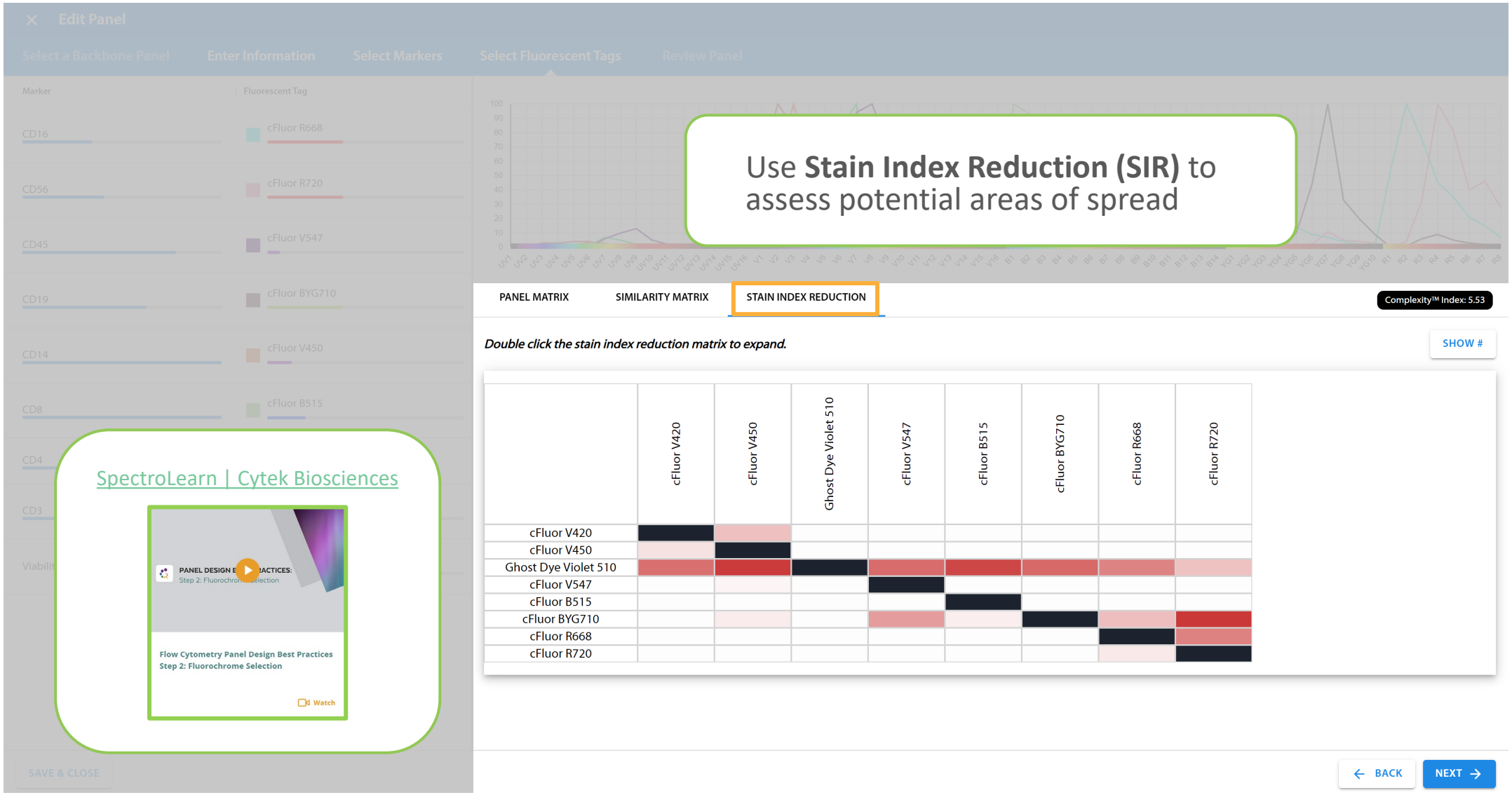
Cytek® Cloud – Panel Builder

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

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



Cytek® Cloud – Panel Builder



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

The Optimization

4

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

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

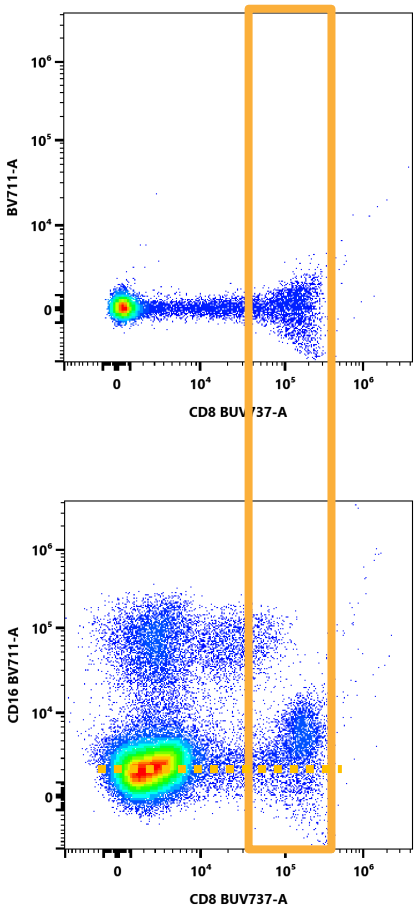
Guidelines for Best Controls

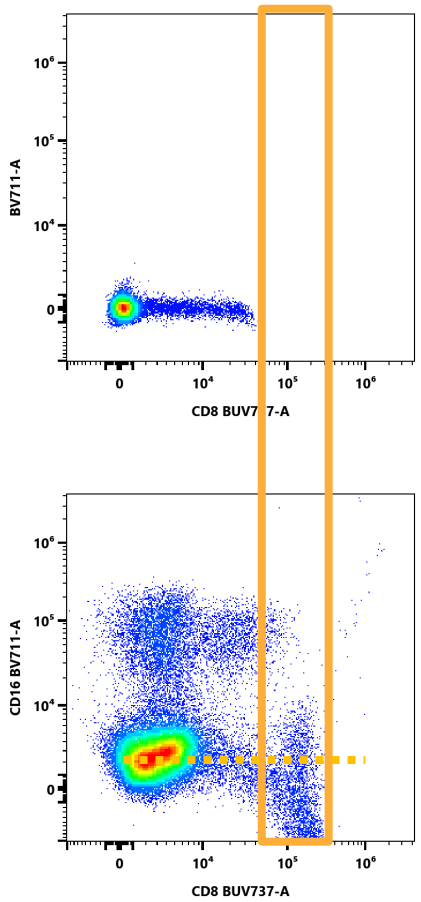
1. Must be as-bright or brighter than the multicolor sample with positive and negative particles clearly separated

1 Fluorescence Intensity Affects Unmixing

Reference Control

Multicolor Sample





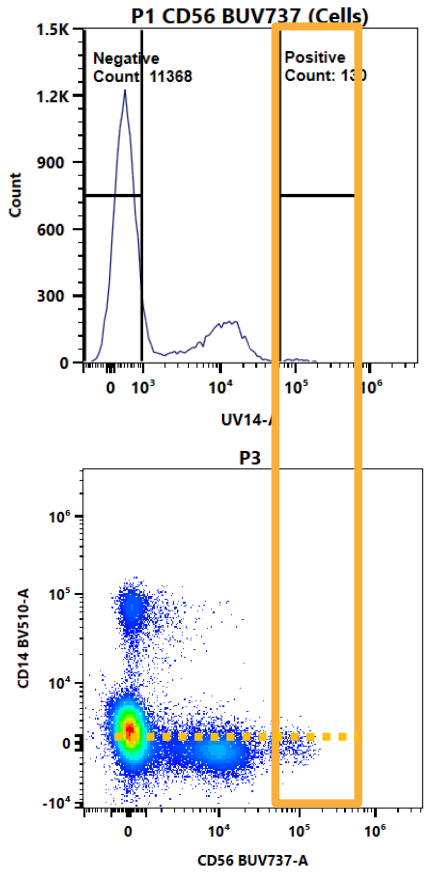
Reference has **same** fluorescence intensity as multicolor

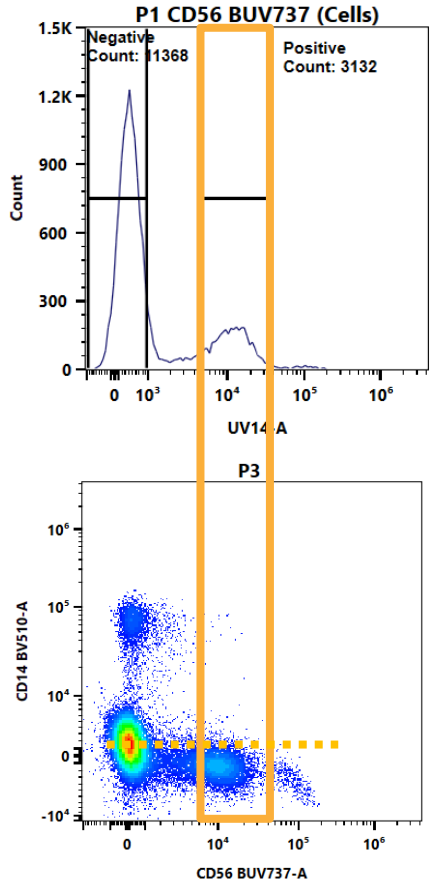
Reference has **lower** fluorescence intensity than multicolor

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

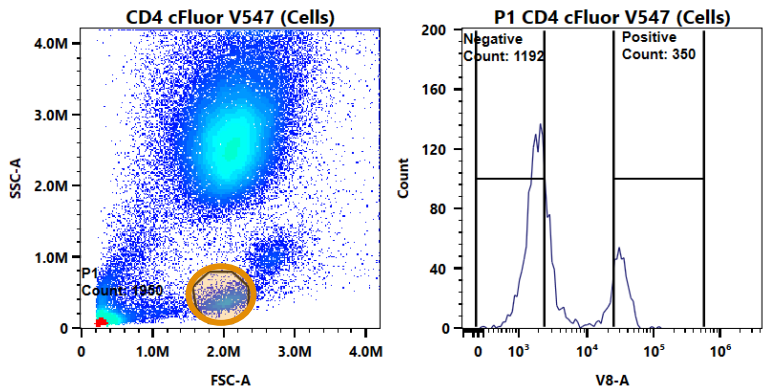
Compensation and Reference Controls should account for:

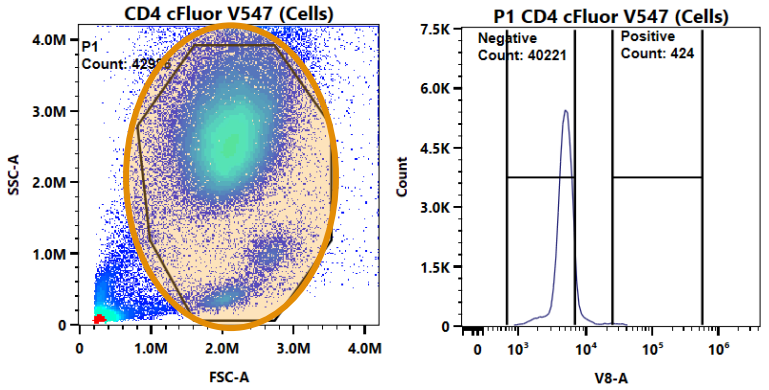
- Fluorescence intensity
- Accuracy of fluorophore signature – must match multicolor
- Collecting enough events to appropriately define the fluorophore

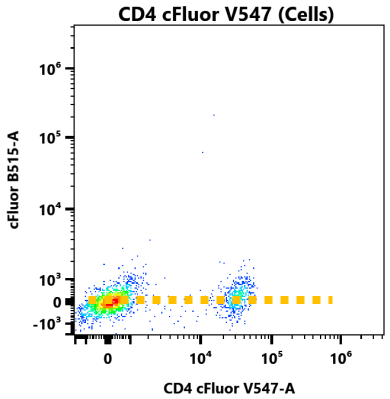
Guidelines for Best Controls

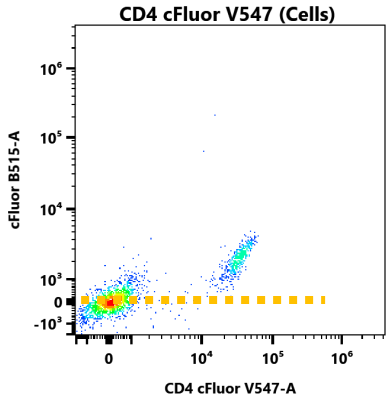
1. Must be as-bright or brighter than the multicolor sample with positive and negative particles clearly separated
2. Negative and positive particles must have IDENTICAL autofluorescence characteristics
3. Fluorescence spectrum of reference control needs to be accurate and IDENTICAL to the one in the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

2 Negative Signatures Affect Unmixing







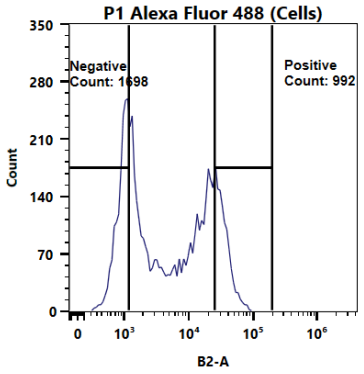


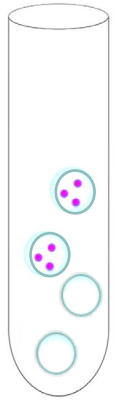
✓ Positive and negative autofluorescence match

✗ Positive and negative autofluorescence do not match

What Are Internal vs. Universal Negative Controls?

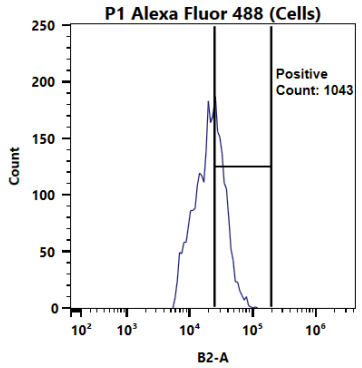
Internal negative is found in the same tube as the positive population

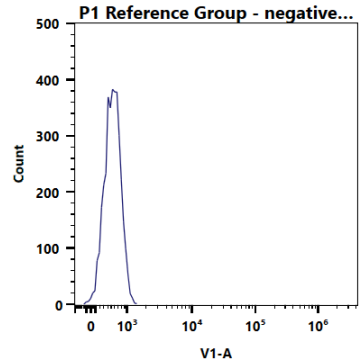


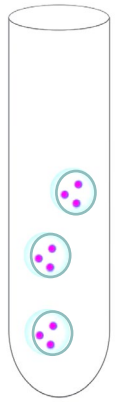


Internal negative in reference control

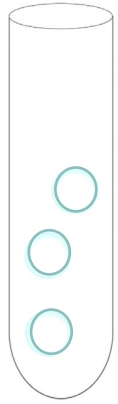
Universal negative is needed when reference control does not contain a negative







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

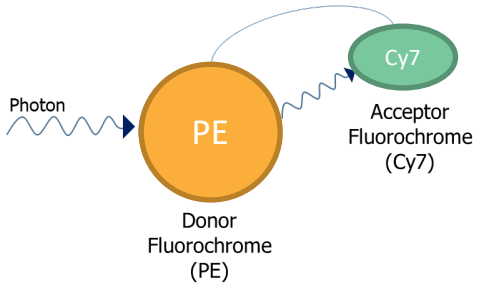
Define Fluorescence Signature		
Reference Control	**Particle type**	**Universal or Internal Negative?**
CD4 BV421	PBMCs	
CD45 FITC (all cells +)	PBMCs	
CD19 PE-Cy7	PBMCs	

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

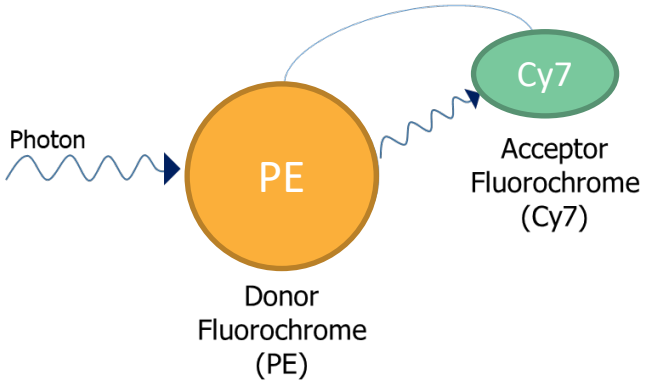
Staining Considerations

- Stain Buffers
- Fixatives
- Temperature
- Time

Guideline

Prepare all controls and samples using the same protocol

What Is a Tandem Dye?



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	

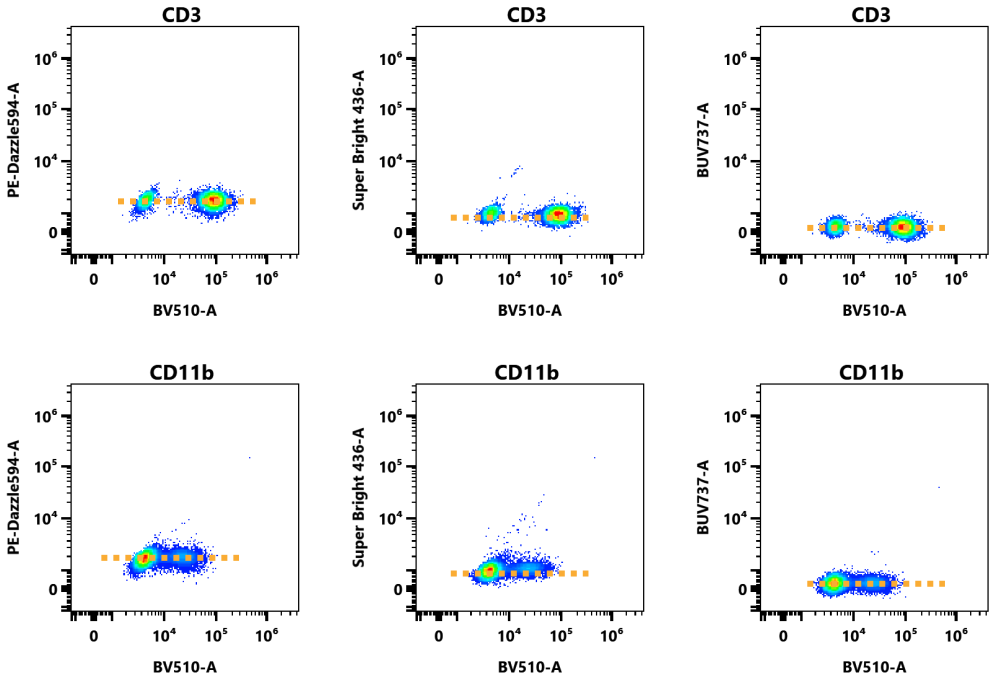
Tandem Dyes

- 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

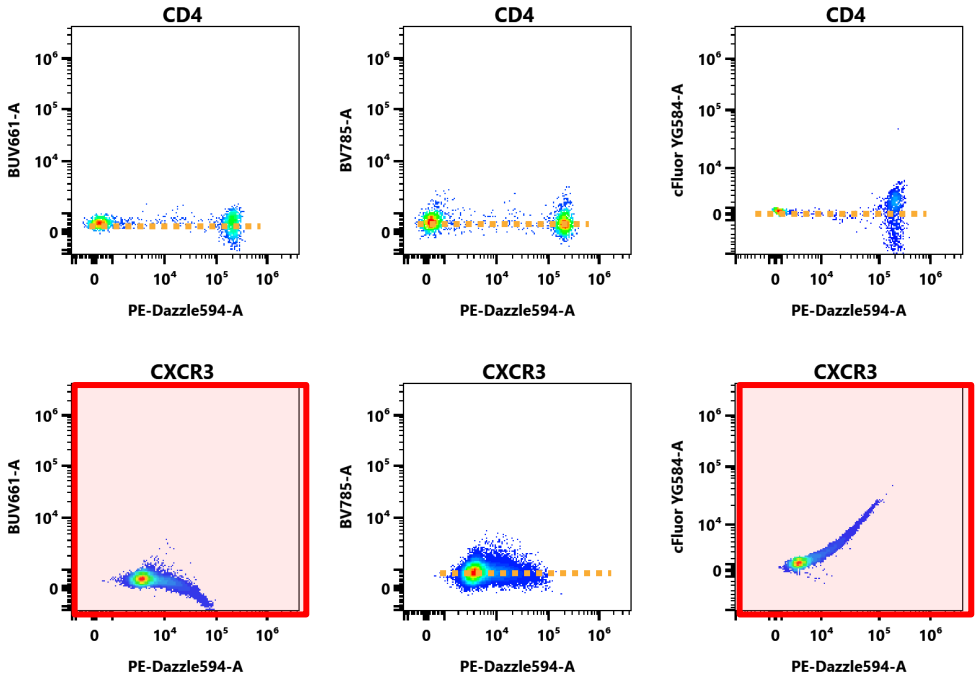
Reference Control Signatures and Tandem Dye Variation

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

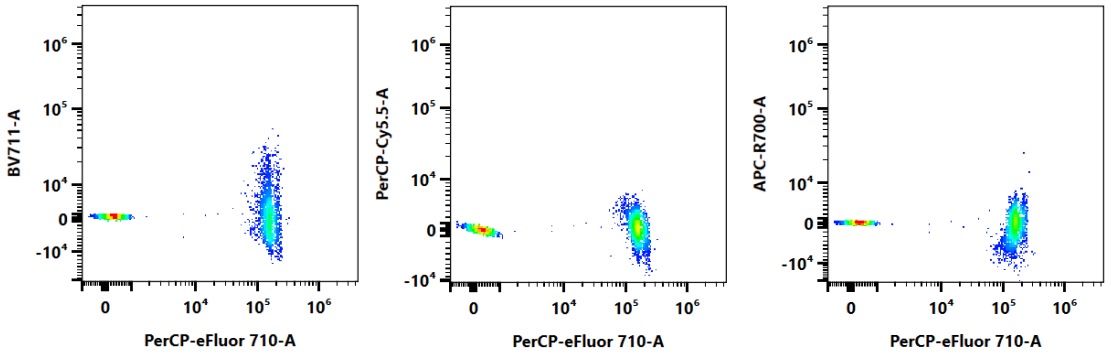
CD4 PE-Dazzle594 cannot unmix CXCR3 PE-Dazzle594 because of spectral mismatch

Comparison of Beads vs. Cells As Reference Controls

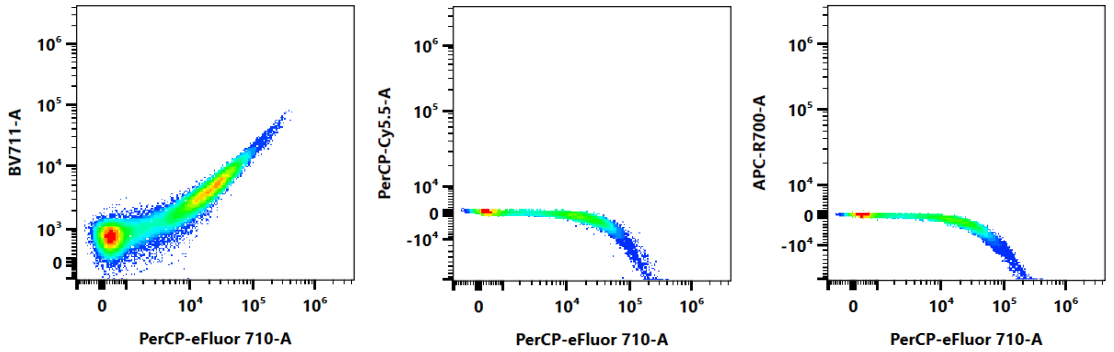
Inaccurate Unmixing Results When Signatures Do Not Match

Beads used for unmixing

Unmixed beads



Unmixed cells



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:

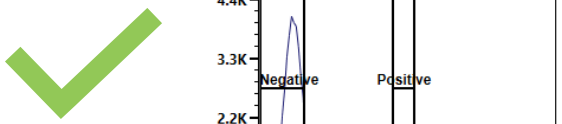
- Fluorescence intensity
- Accuracy of fluorophore signature – must match multicolor
- Collecting enough events to appropriately define the fluorophore

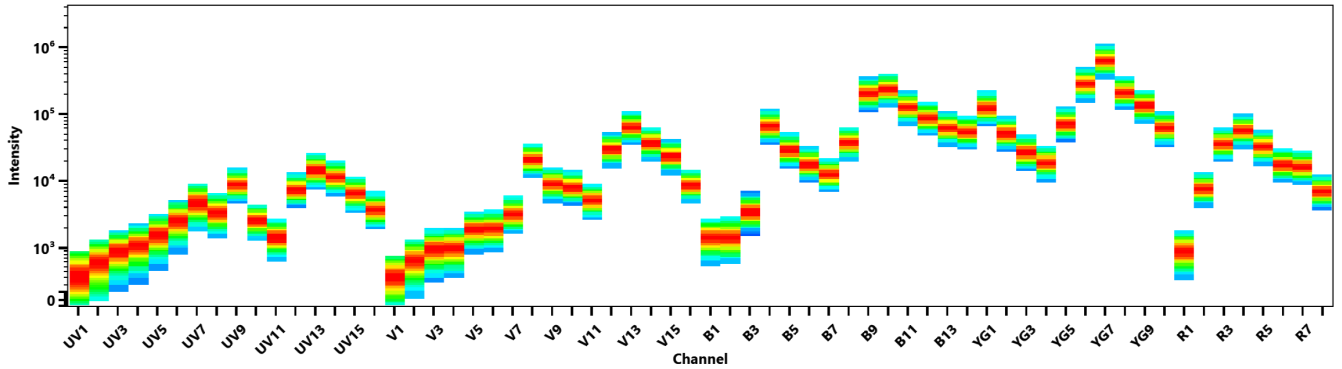
Guidelines for Best Controls

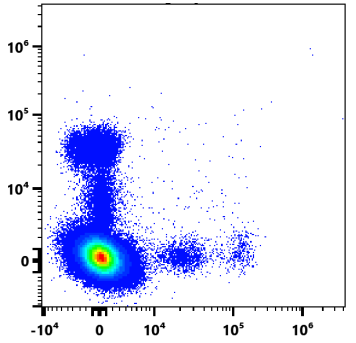
1. Must be as-bright or brighter than the multicolor sample with positive and negative particles clearly separated
2. Negative and positive particles must have IDENTICAL autofluorescence characteristics
3. Fluorescence spectrum of reference control needs to be accurate and IDENTICAL to the one in the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

4 Event Count Affects Unmixing

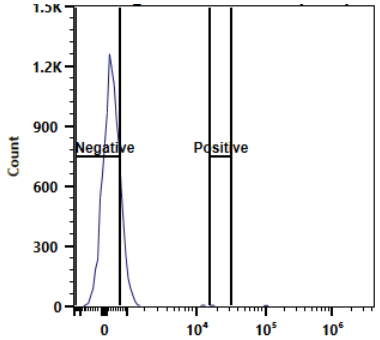
Sufficient Events

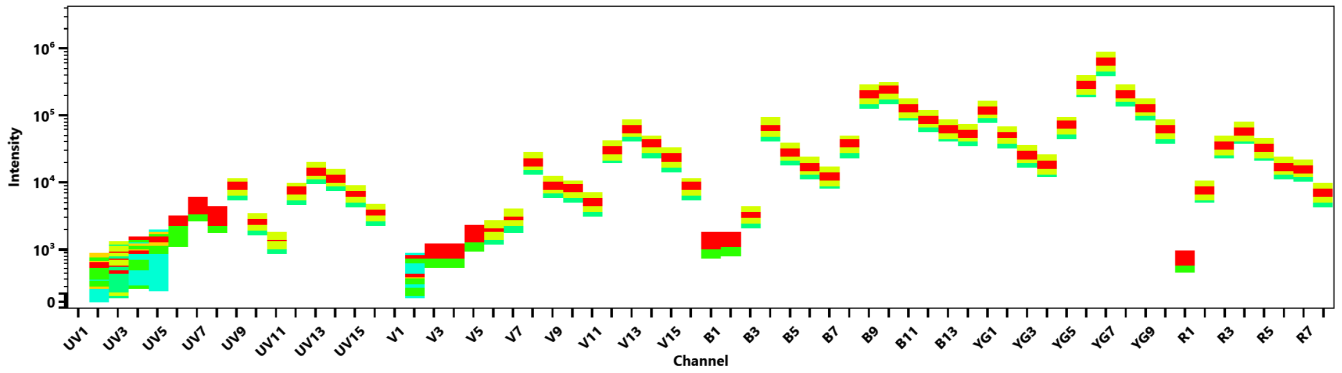


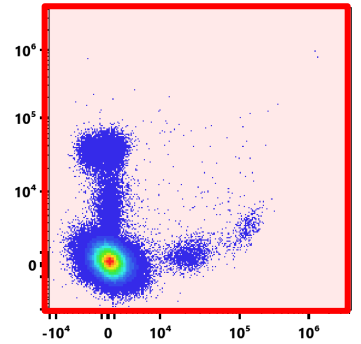




Insufficient Events







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.

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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			
IFNγ	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

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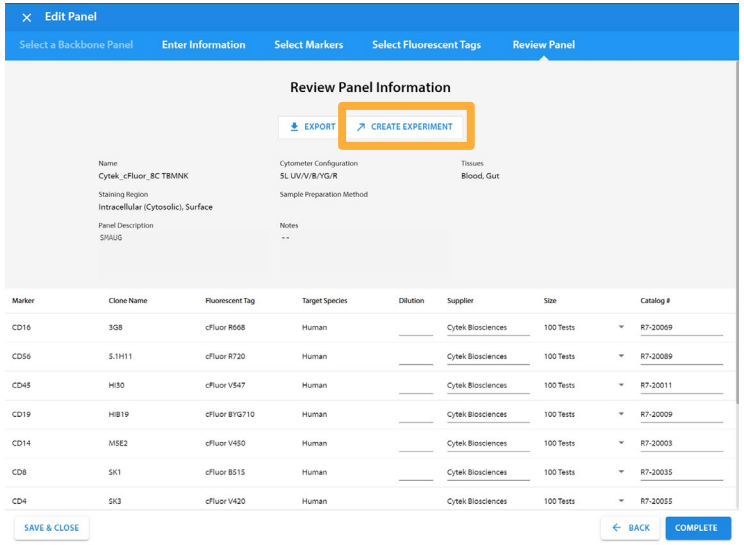
Interactive Poll #5

At what point in the workflow can unmixing be calculated?

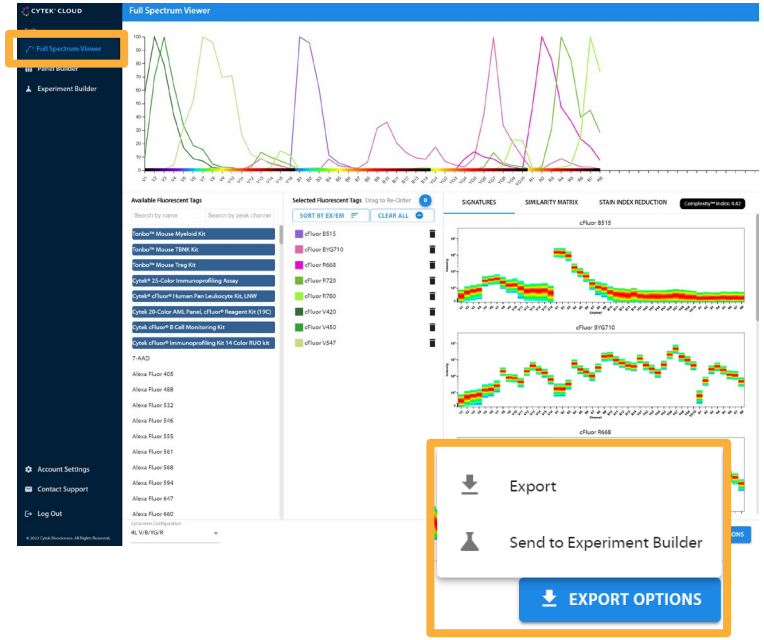
Cytek® Cloud – Experiment Builder

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



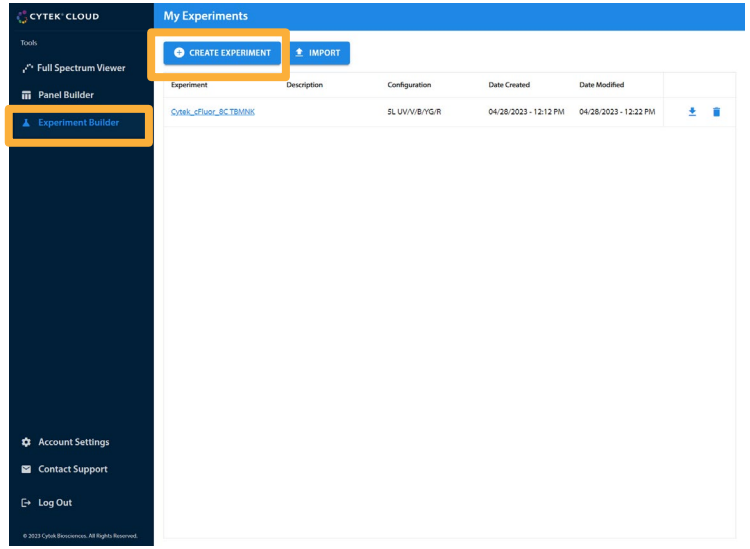
Fluorophores and markers will be transferred to Experiment Builder

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



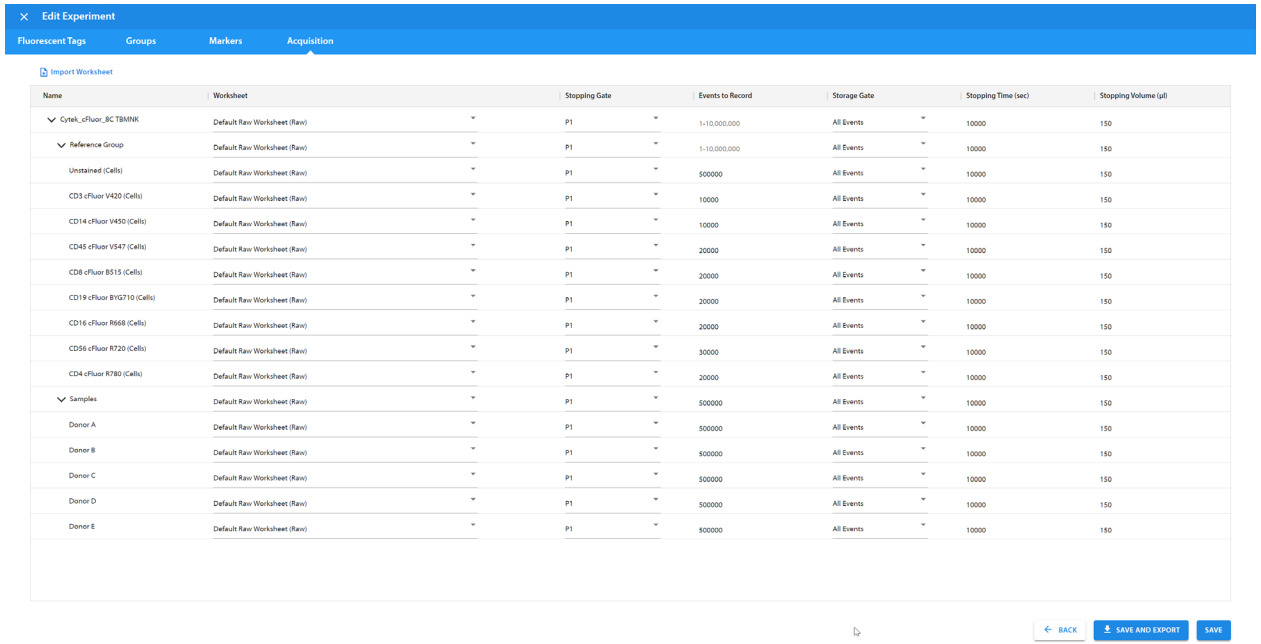
Fluorophores will be transferred to Experiment Builder

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



Cytek® Cloud – Experiment Builder

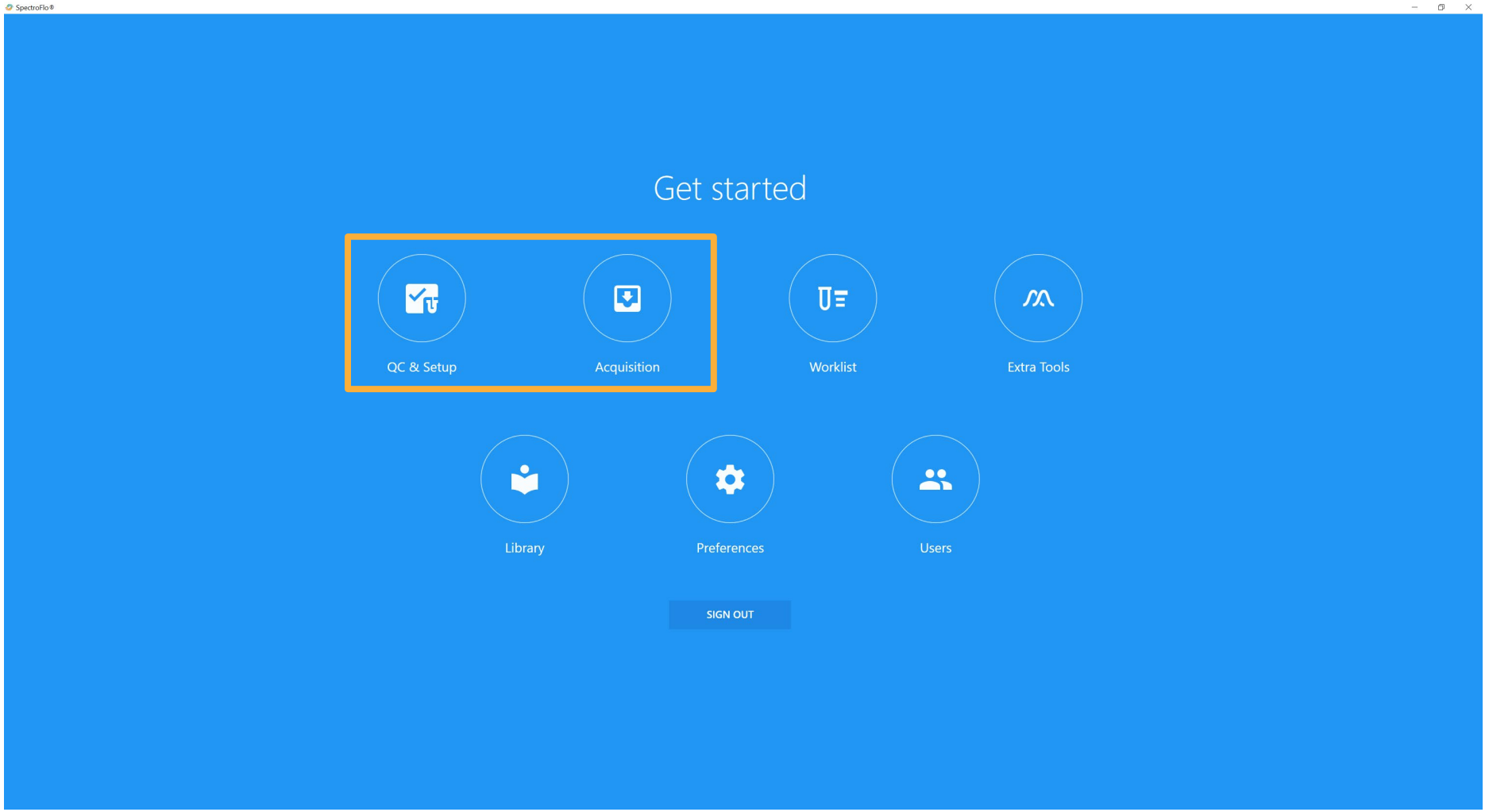
1. **Fluorescent Tags**
   Add fluorophores to the experiment
2. **Groups**
   Add and organize tubes/plate(s)
3. **Markers**
   Add labels to fluorophores
4. **Acquisition**
   Add stopping parameters for recording files



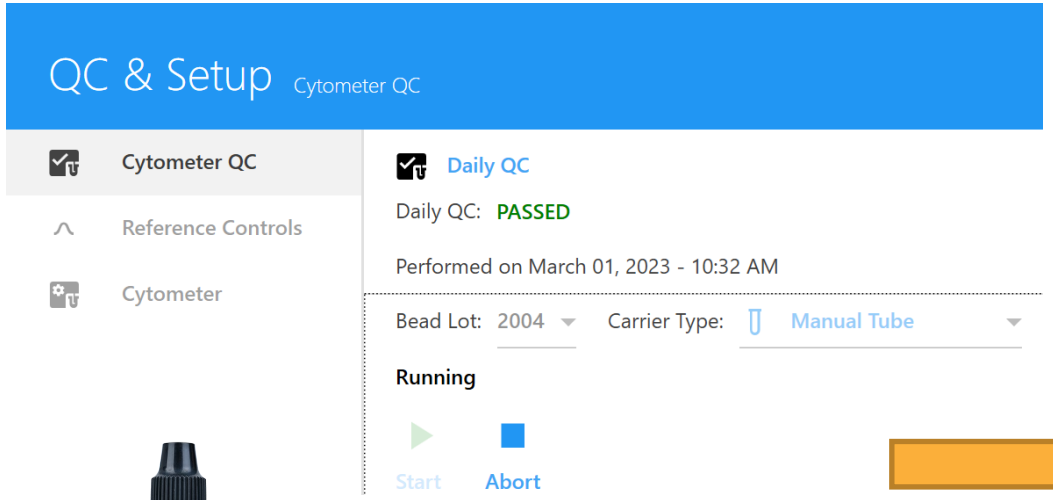
Any information entered in the Experiment Builder can be changed anytime, even after importing to the cytometer workstation

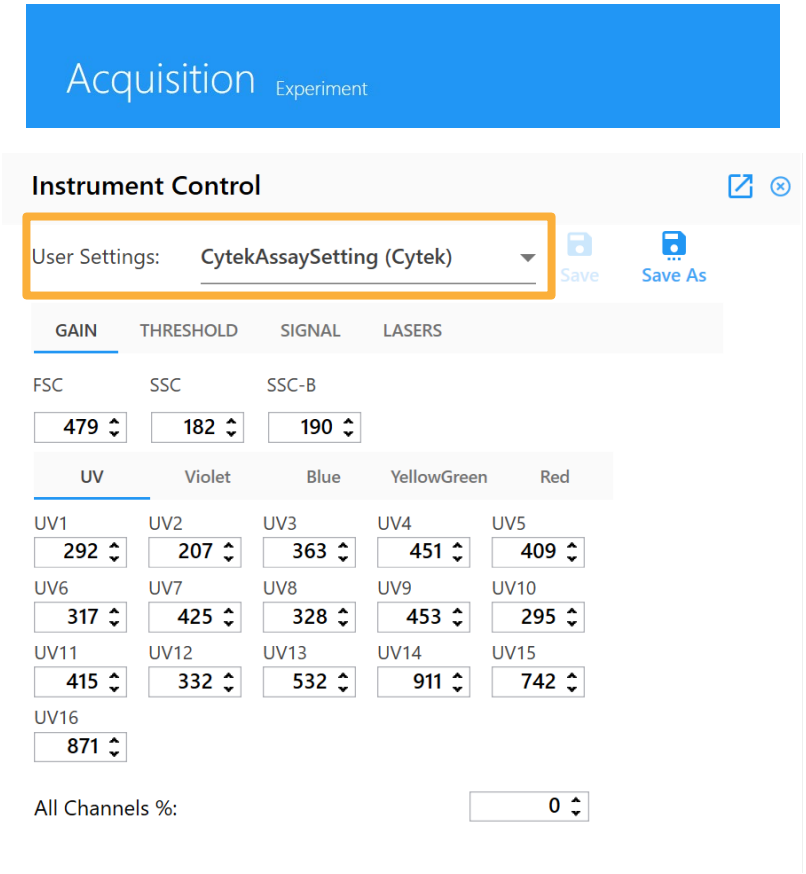
SpectroFlo® Software

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



SpectroFlo® Software – Run Daily QC





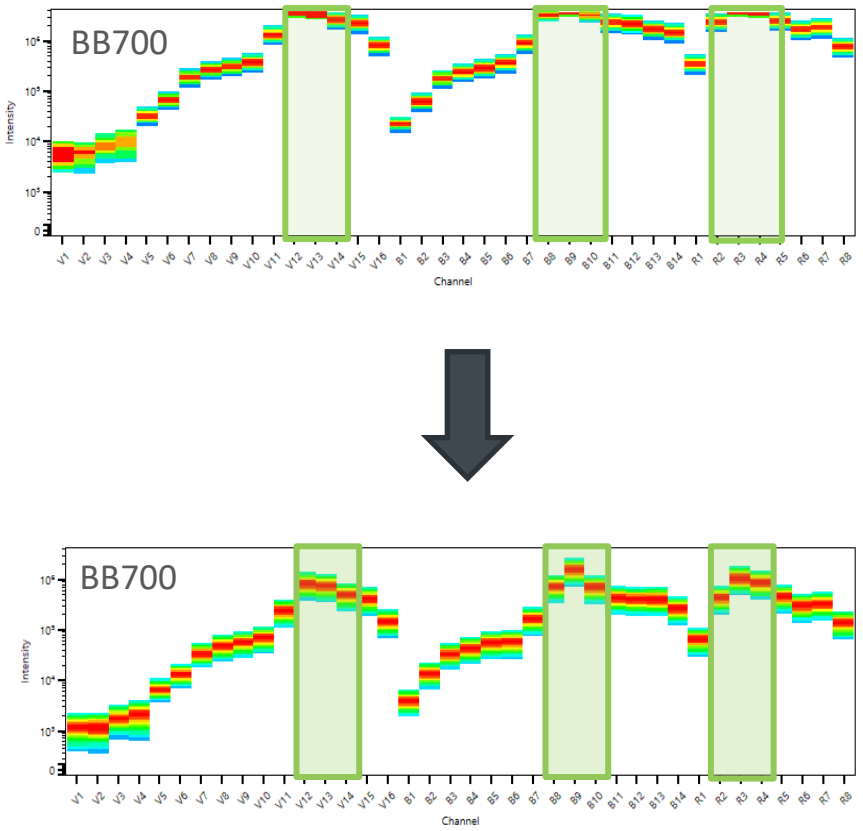
- Run Daily QC to track cytometer performance
- Gains in CytekAssaySetting (CAS) will be automatically updated
- Complete every day the instrument is used

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



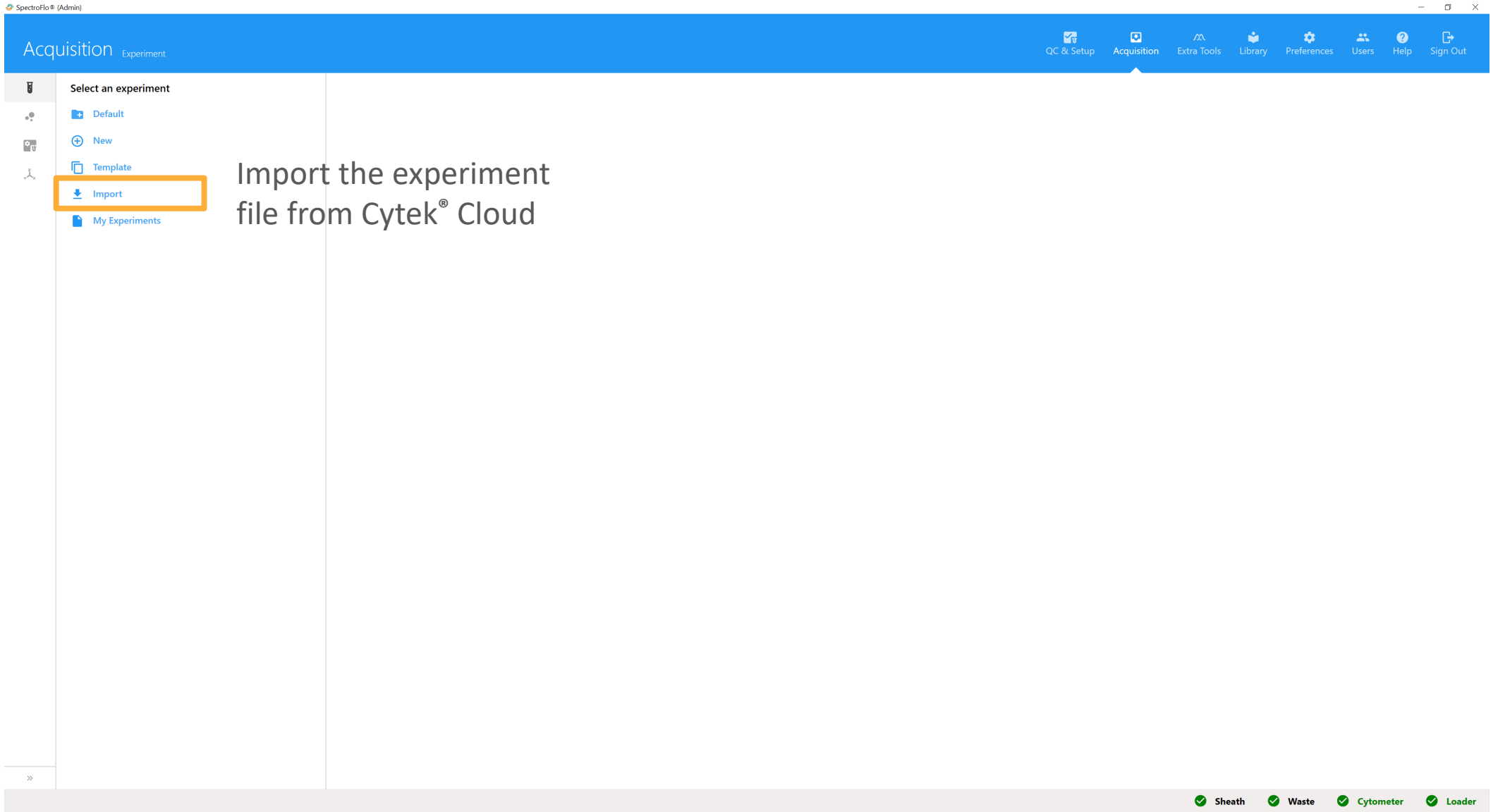
- When would CAS not be used?
  - If signals are off scale using CAS - lower all gains proportionally*

**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

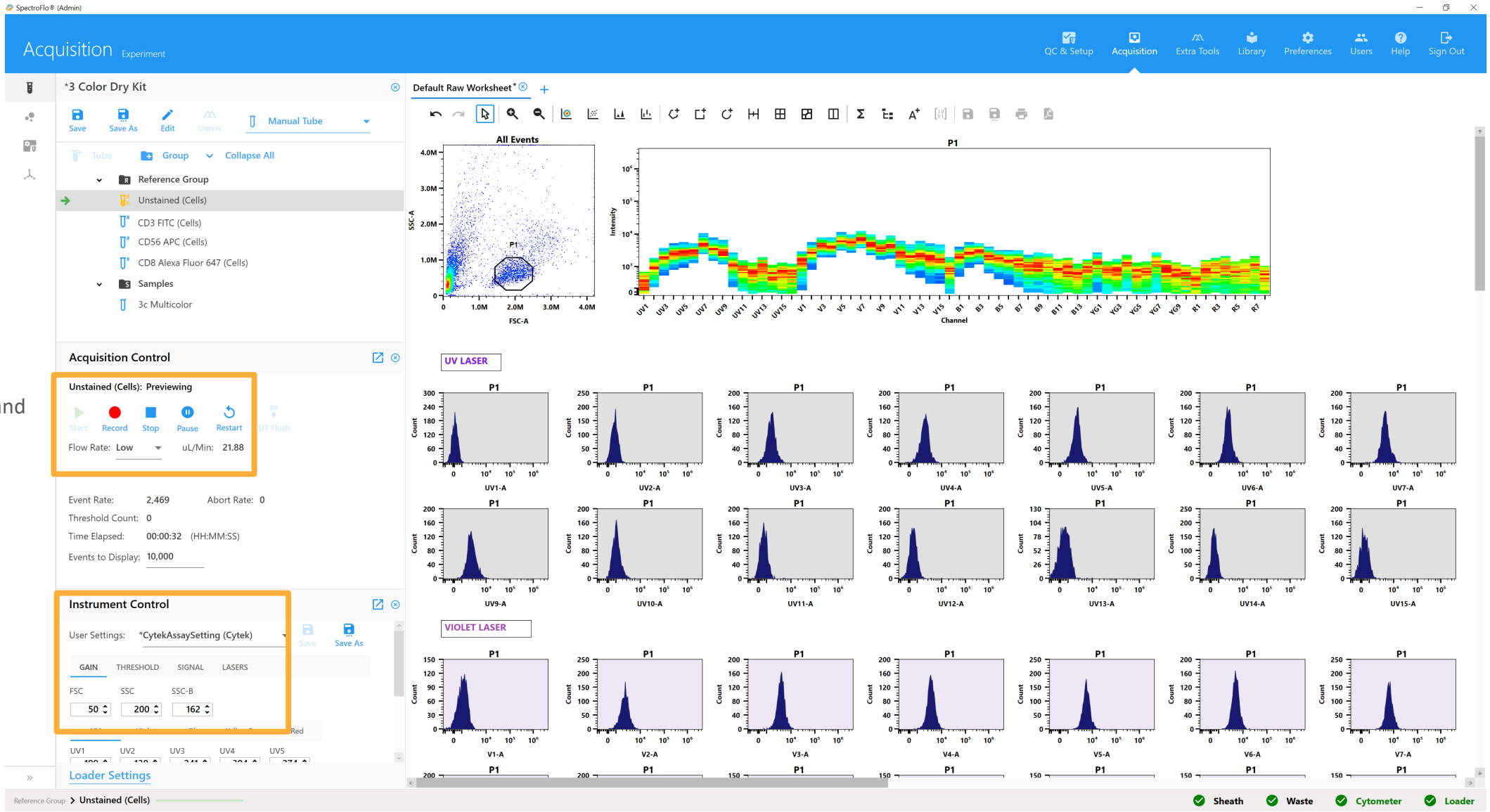


Import the experiment file from Cytek® Cloud

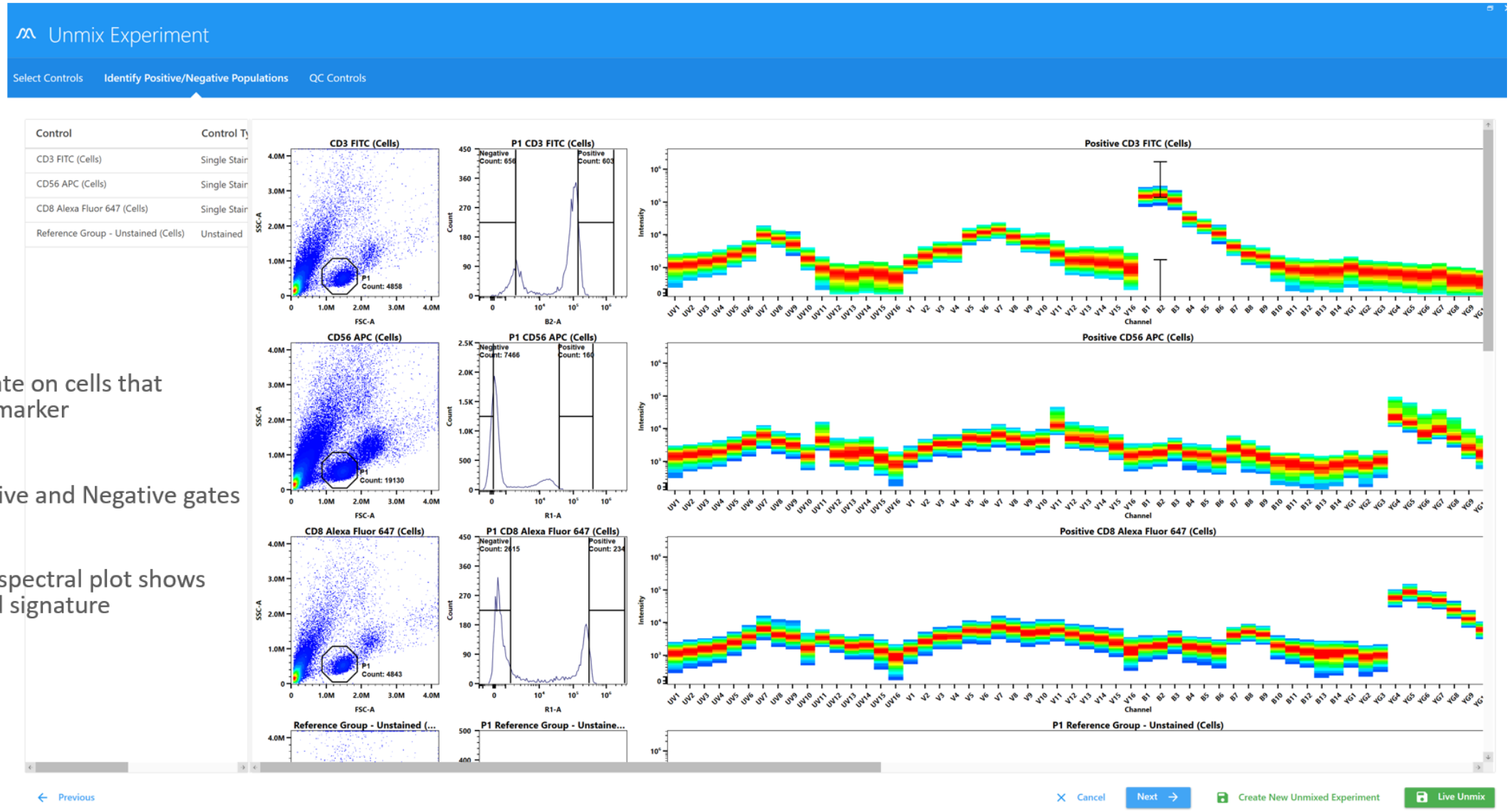
SpectroFlo® Software – Record Reference Controls

Set flow rate and start/record

Adjust FSC and SSC or select saved settings



SpectroFlo® Software – Unmix



1. Set P1 gate on cells that express marker
2. Set Positive and Negative gates
3. Confirm spectral plot shows expected signature

Evaluate Your Assay

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

Evaluate Unmixing

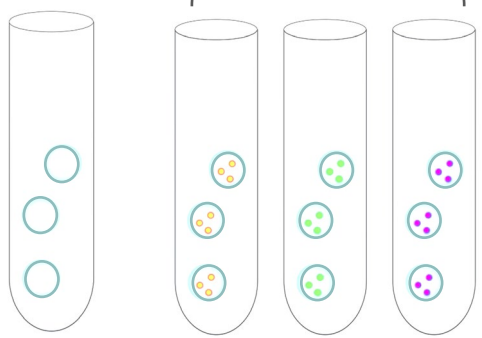
How to assess control quality ***before*** unmixing

How to assess data quality ***after*** unmixing

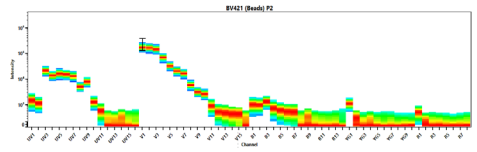
Unmixing Workflow

Run compensation/reference controls

Unstained | Single Color Controls



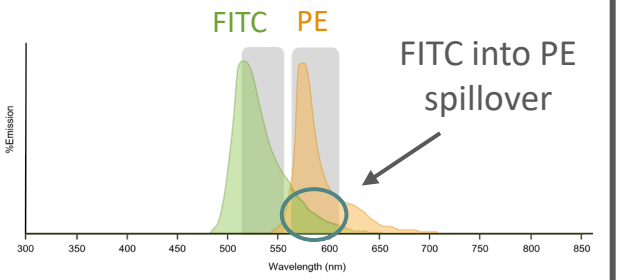
Raw Data



Check that the INPUT is good before calculating unmixing

Input

Calculate compensation/unmixing

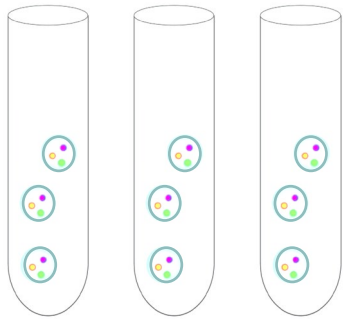


Unmix

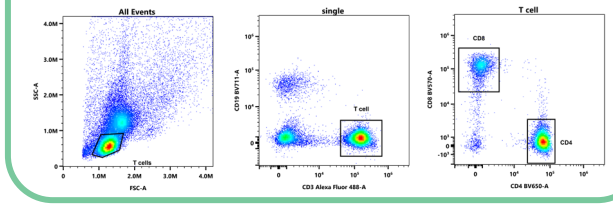
Output

Run multicolor samples with compensation/unmixing applied

Multicolor Assay



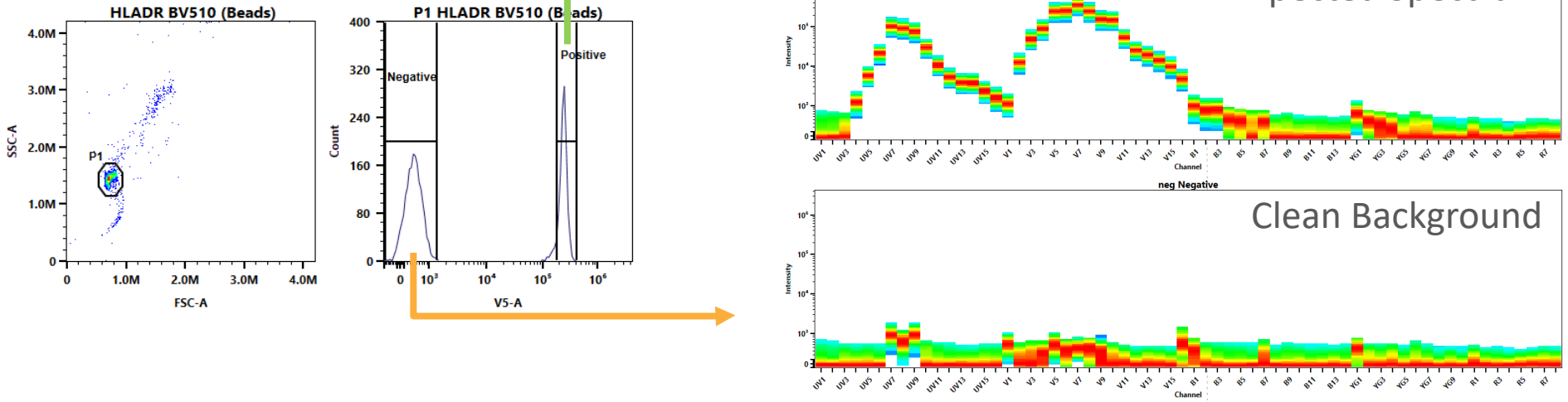
Unmixed Data



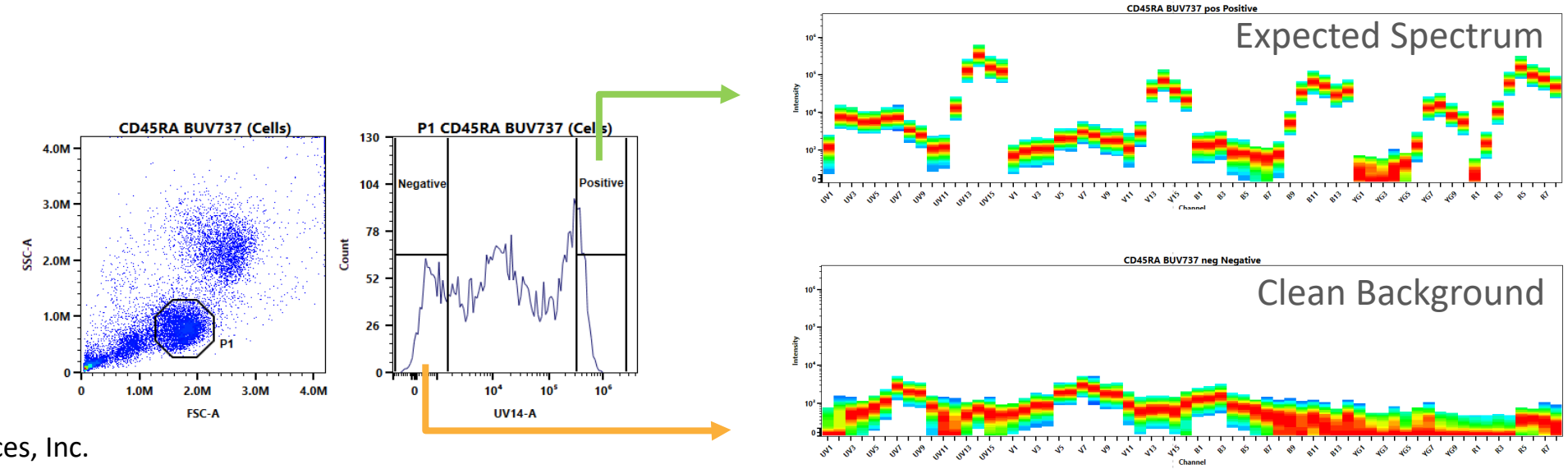
Check that the OUTPUT is good after calculating unmixing

Examples of Good Reference Controls

Bead Control

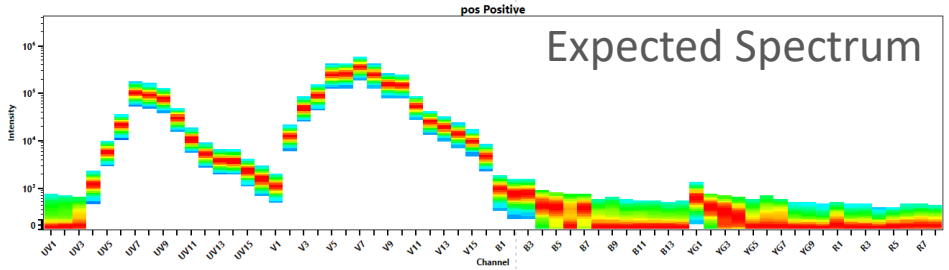


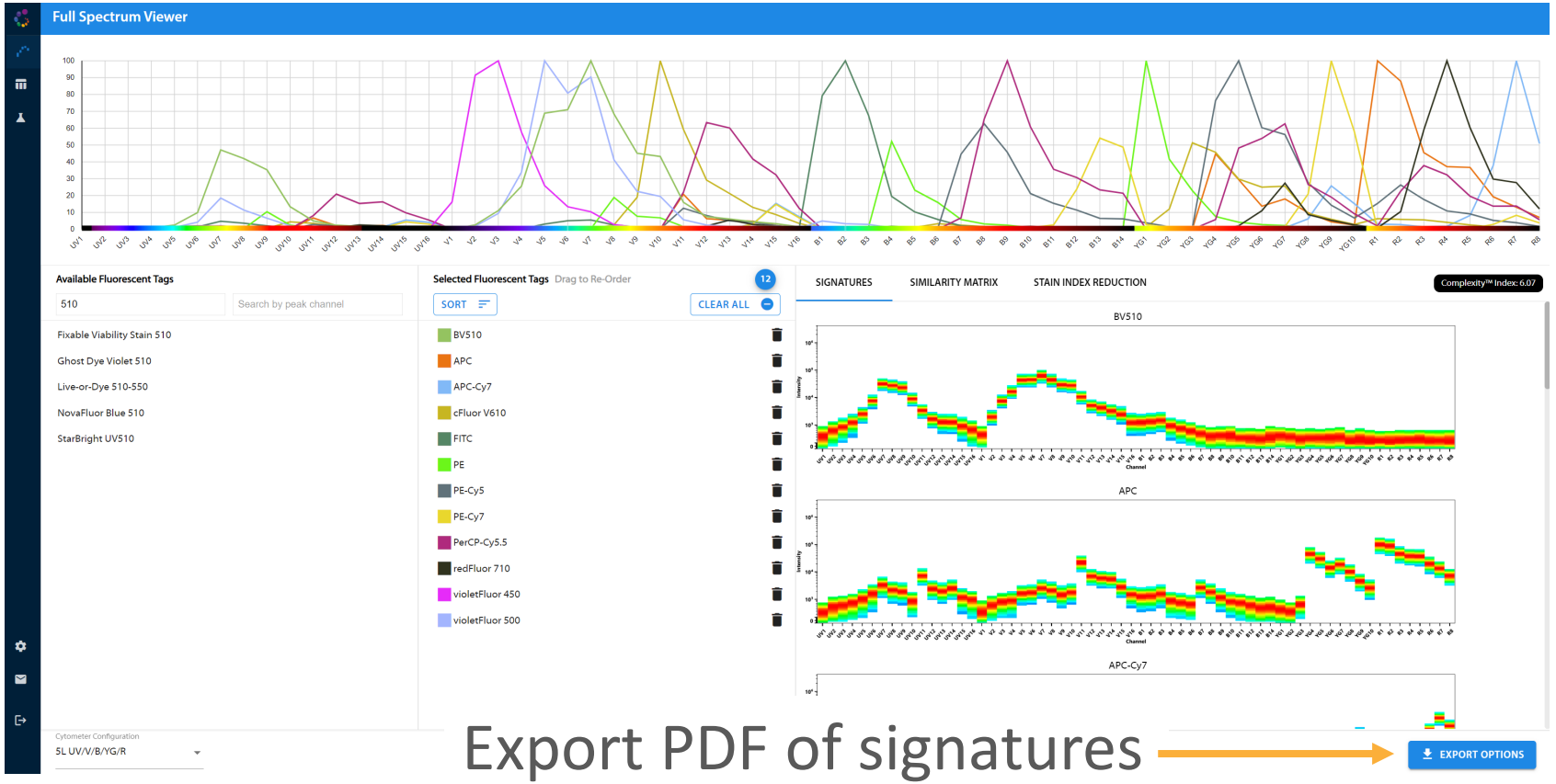
Cell Control



Examples of Good Reference Controls

Use Cytek® Cloud to help determine if signatures are expected





Export PDF of signatures → EXPORT OPTIONS

Exercise 5: Reference Control QC

Goals

- Determine if the observed signature matches the expected signature

Exercise 5: Reference Control 1

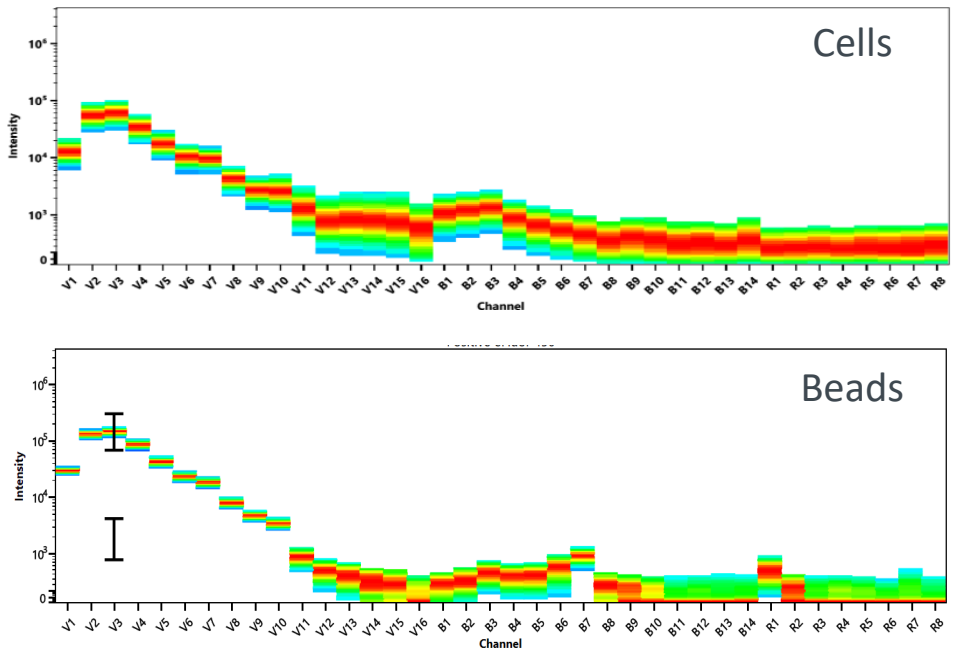
Does the observed positive signature match the expected positive signature?

Does the observed negative signature match the expected negative signature?

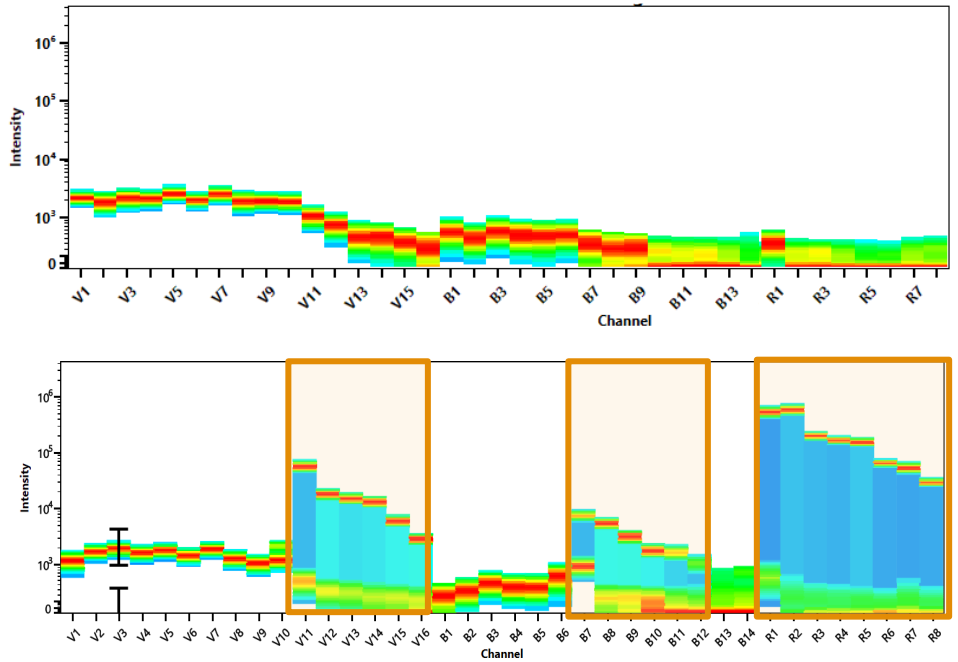
Expected

Observed

eFluor 450 Positive



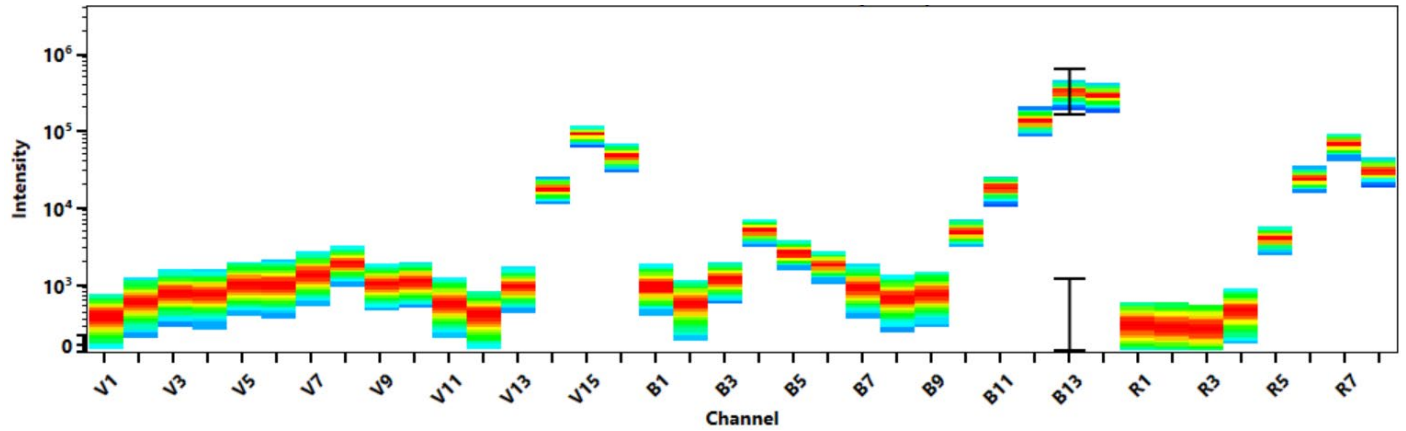
eFluor 450 Negative



Exercise 5: Reference Control 2

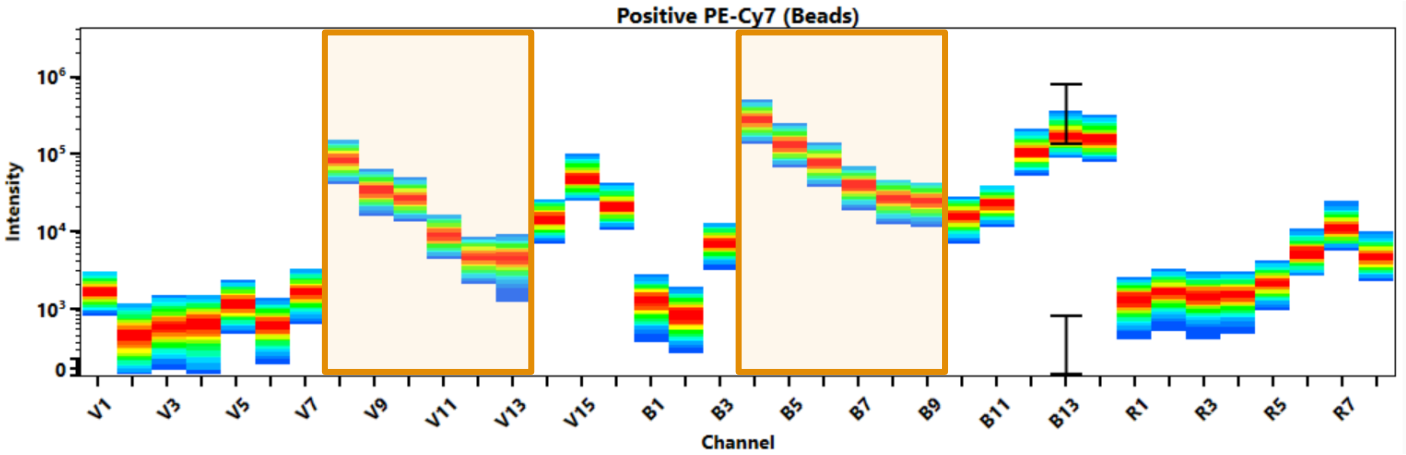
Does the observed PE-Cy7 signature match the expected PE-Cy7 signature?

Expected



PE	1	
PE-Cy7	0.01	1
	PE	PE-Cy7

Observed

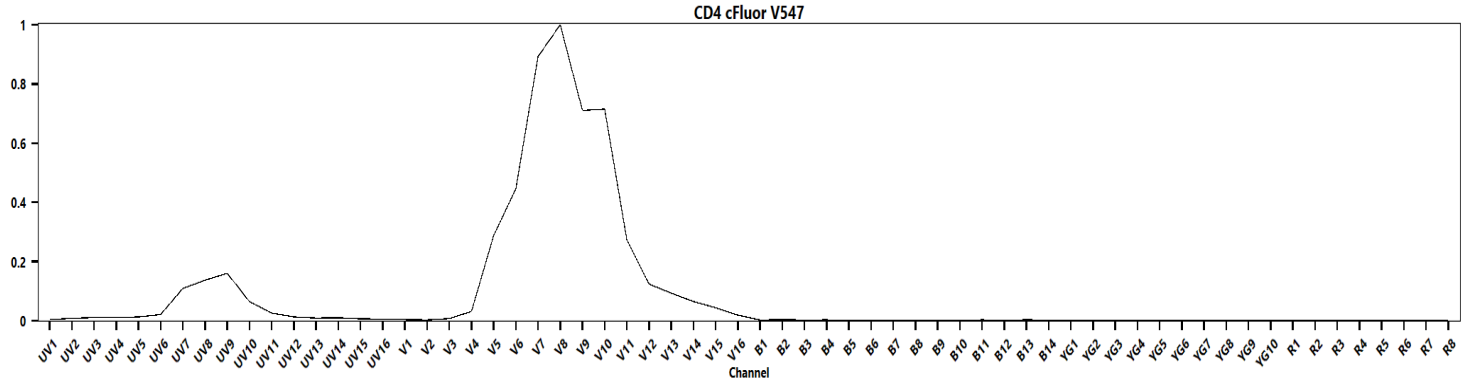


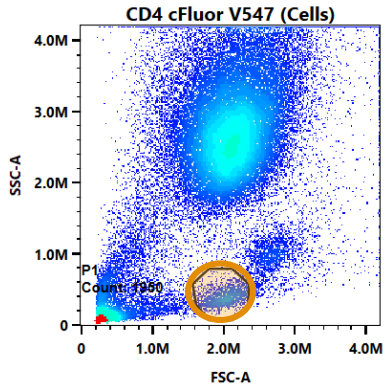
PE	1	
PE-Cy7	0.82	1
	PE	PE-Cy7

Exercise 5: Reference Control 3

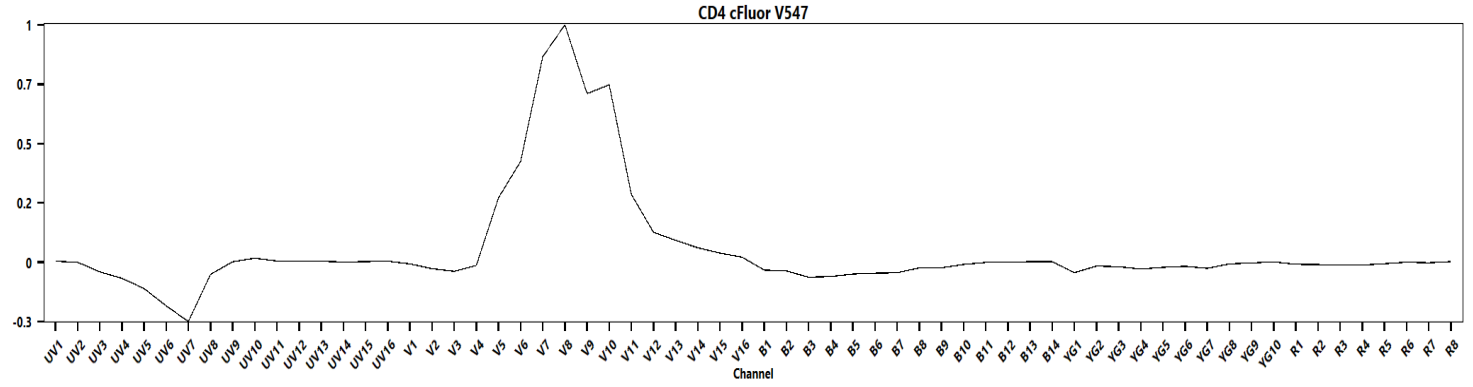
Does the observed cFluor v547 signature match the expected cFluor v547 signature?

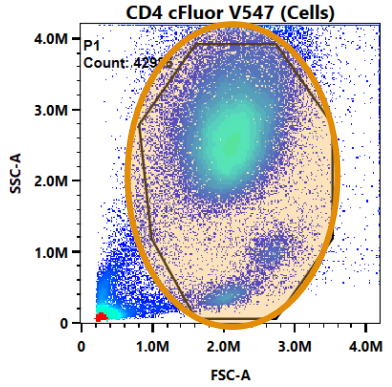
Expected





Observed





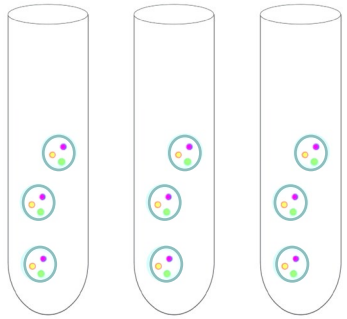
How to Determine if Unmixing Is Accurate

Check for accurate unmixing or compensation in three places:

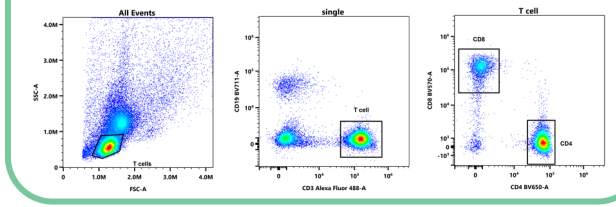
1. Unstained cells
2. Single stained cells
3. Multicolor cells

Run multicolor samples with compensation/unmixing applied

Multicolor Assay



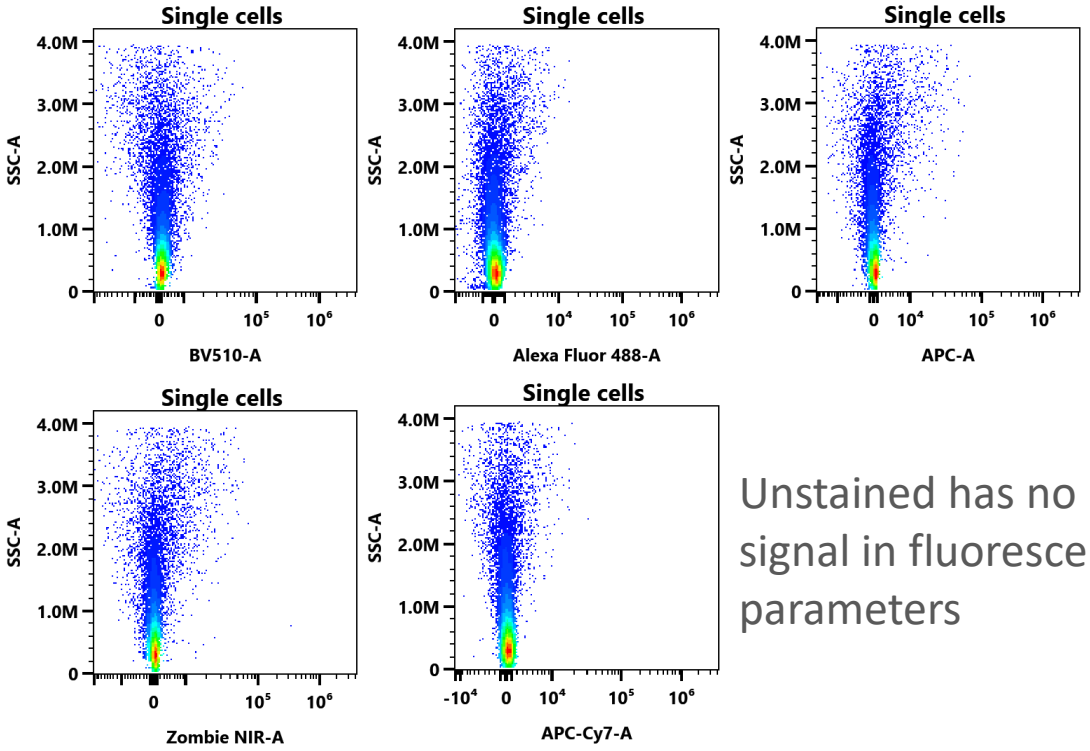
Unmixed Data



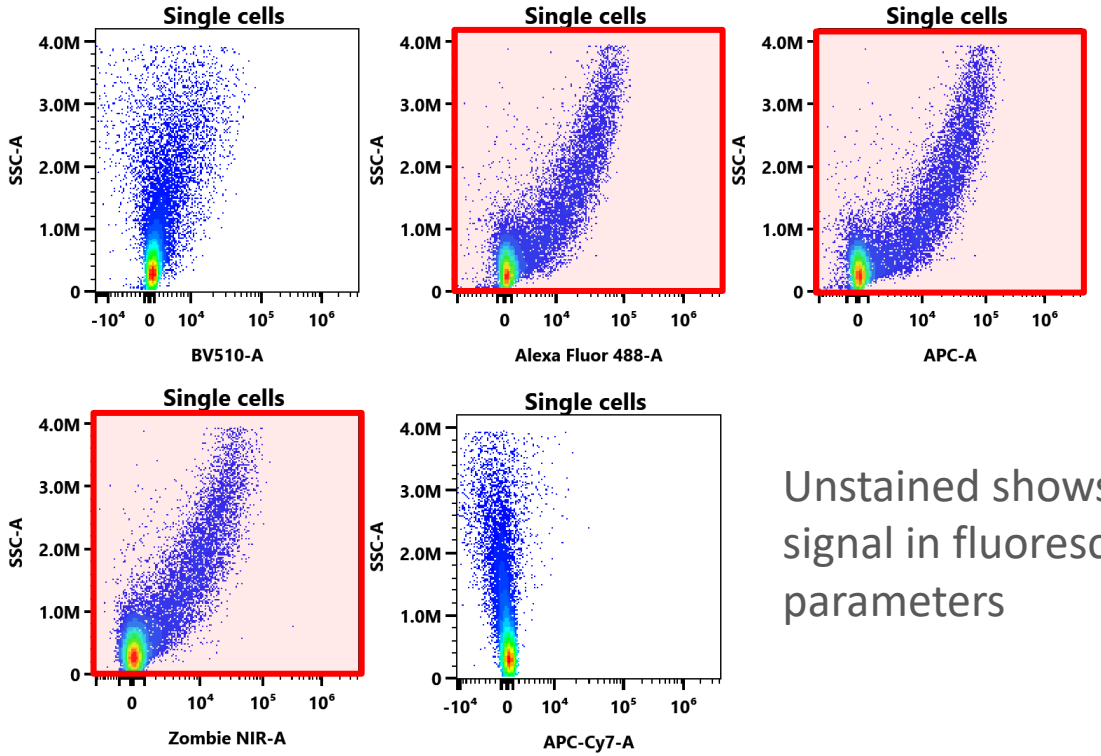
Check that the OUTPUT is good after calculating unmixing

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

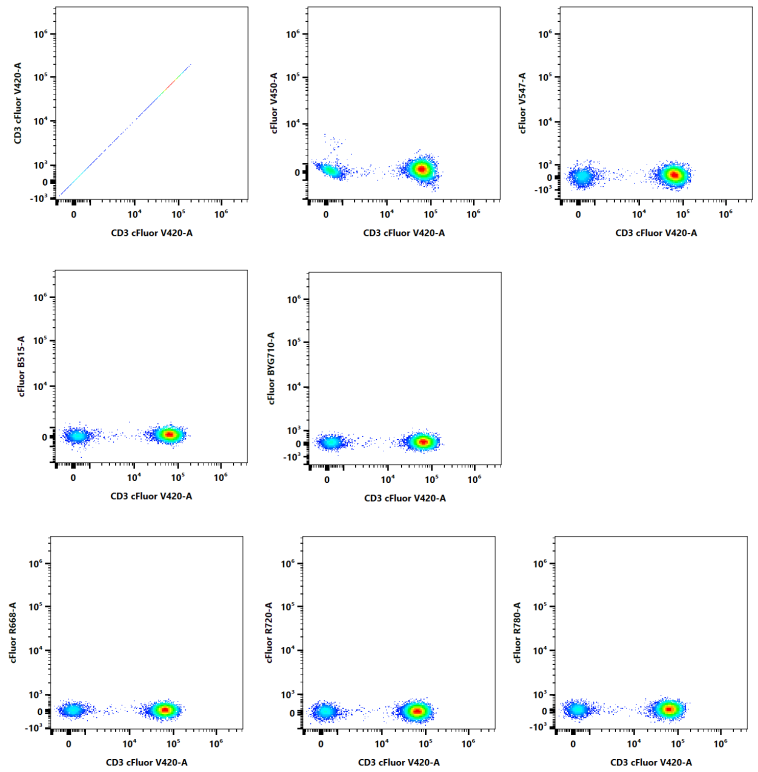


Unstained shows signal in fluorescent parameters

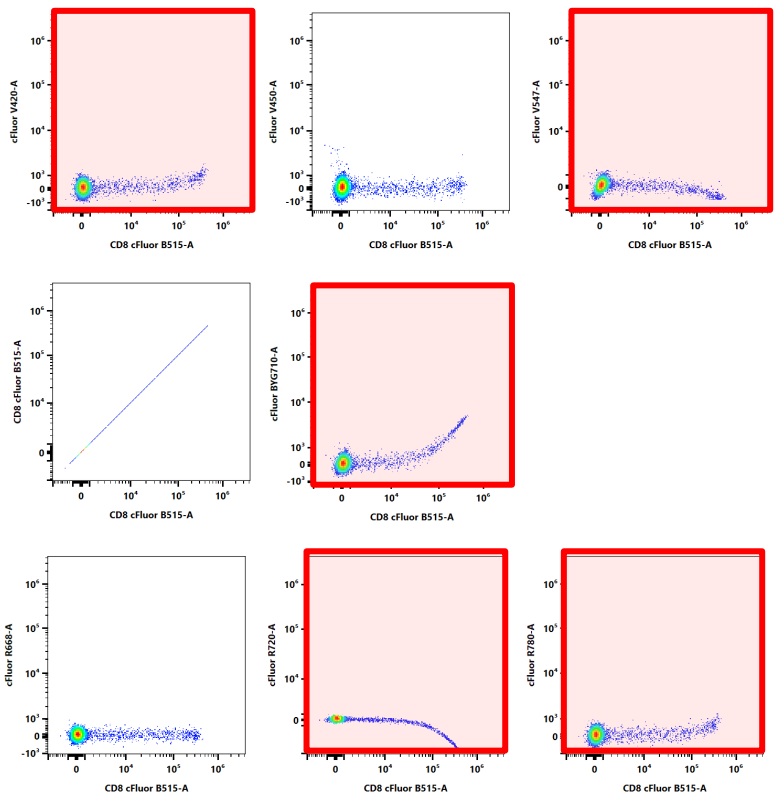
How to Determine if Unmixing Is Accurate

2 Check single stained cells against all other colors

Good Unmixing/Compensation



Bad Unmixing/Compensation

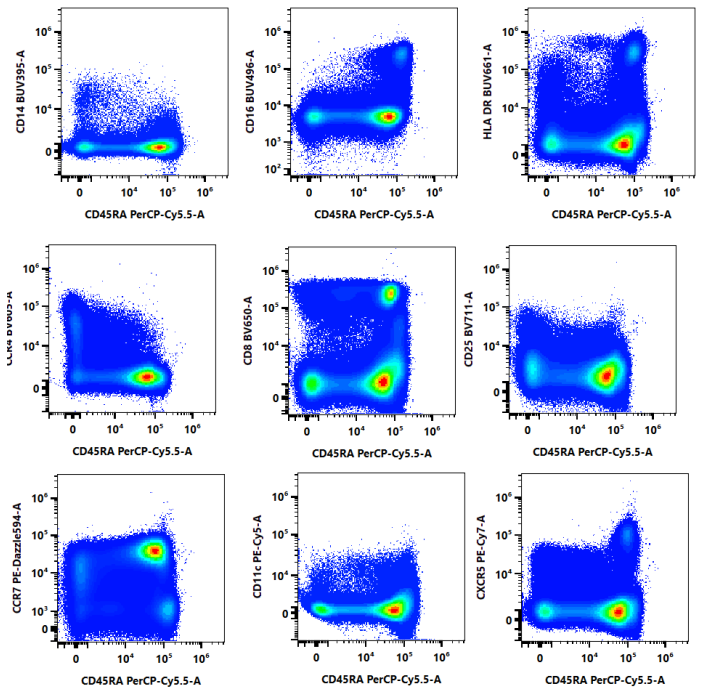


How to Determine if Unmixing Is Accurate

3 Check multicolor NxN permutations

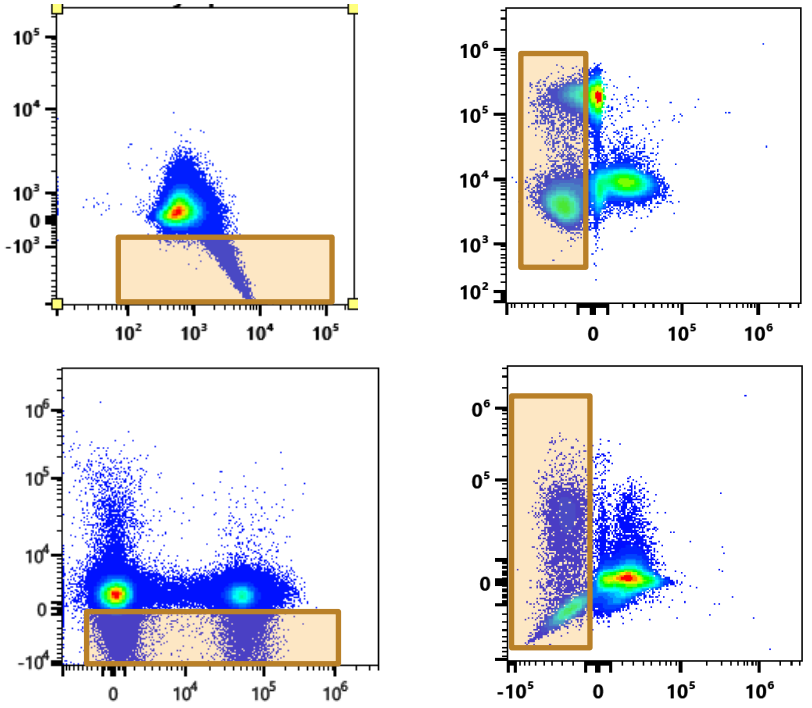
Good Unmixing/Compensation

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

1. **Set up experiment in Cytek® Cloud on any computer**
2. Start up cytometer
3. Record reference controls
4. Unmix
5. Record samples

Workflow for Optimized Assays

1. Start up cytometer
2. Open saved experiment template
3. Record reference controls
4. Unmix
5. Record samples

Acquisition Workflow – The Big Picture

Workflow for Initial Setup

1. **Set up experiment in Cytek® Cloud on any computer**
2. Start up cytometer
3. Record reference controls
4. Unmix
5. Record samples

Workflow for Optimized Assays

1. Start up cytometer
2. Open saved experiment template
3. **Reuse reference controls**
4. Unmix
5. Record samples

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

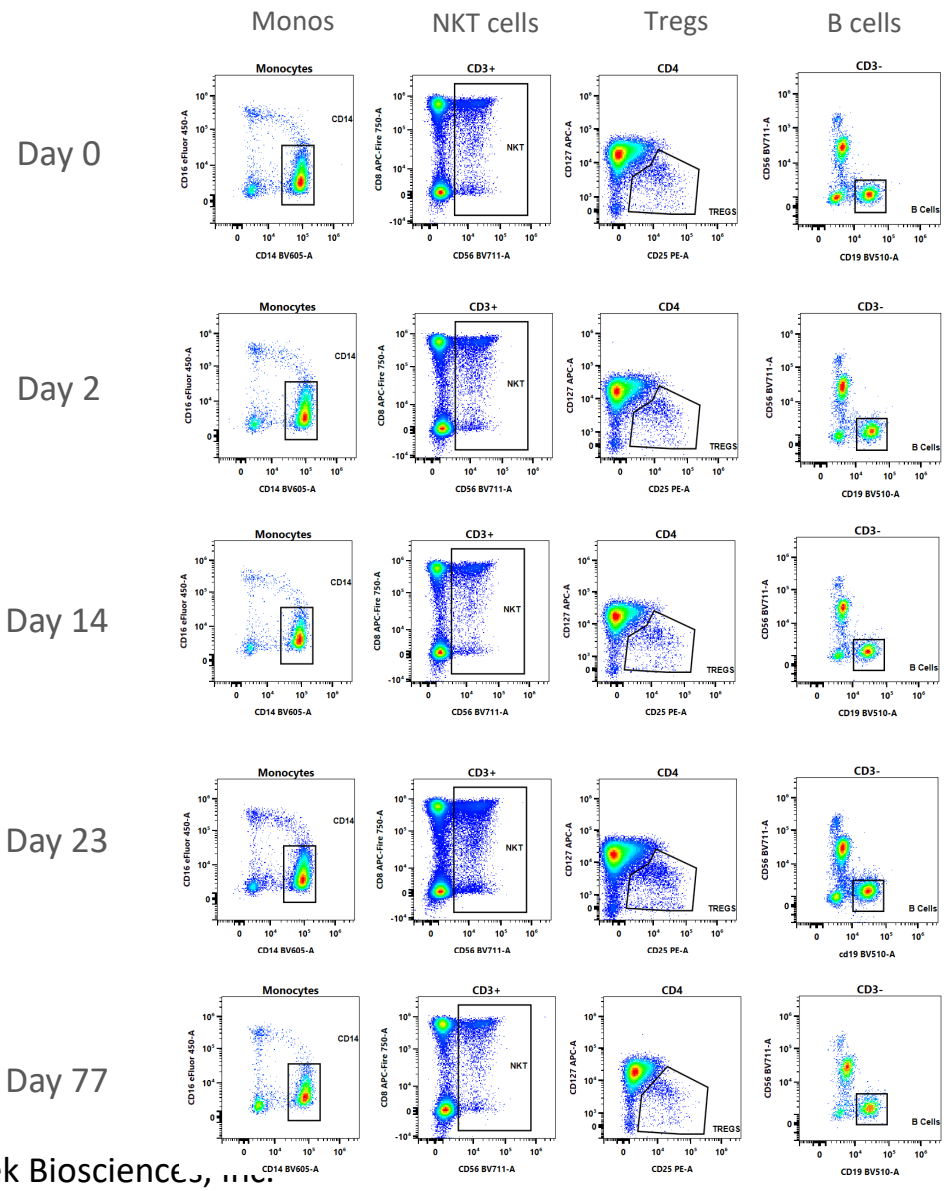
Is my assay performing as expected?

- 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

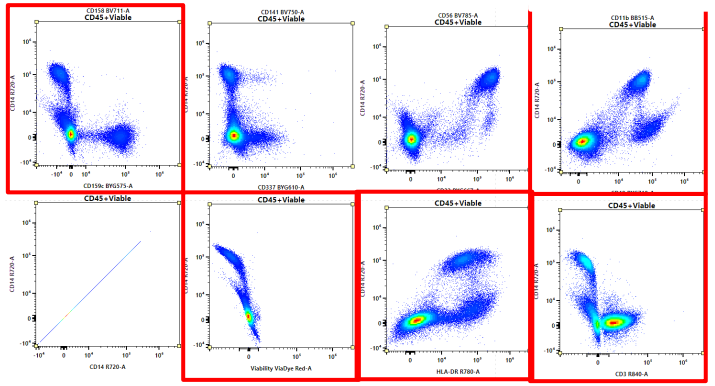
Hardware perspective

- Daily QC completed and passing

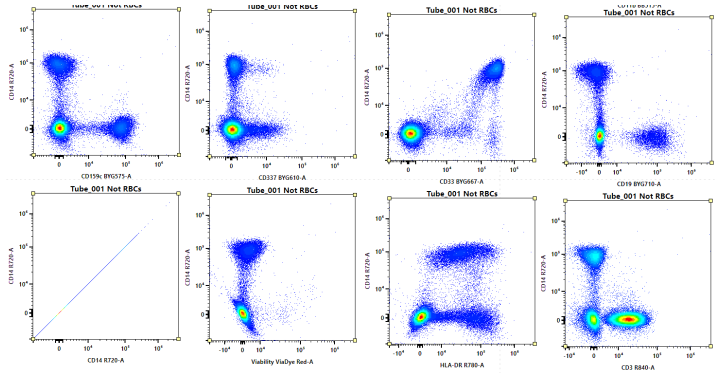
Assay Perspective

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

Incorrectly reusing controls



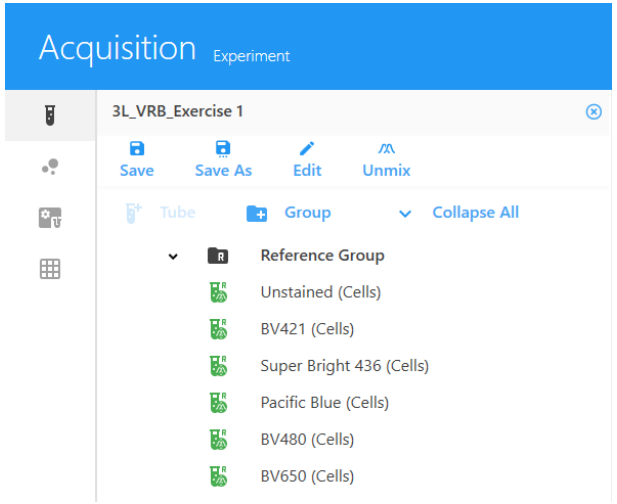
Correctly reusing controls



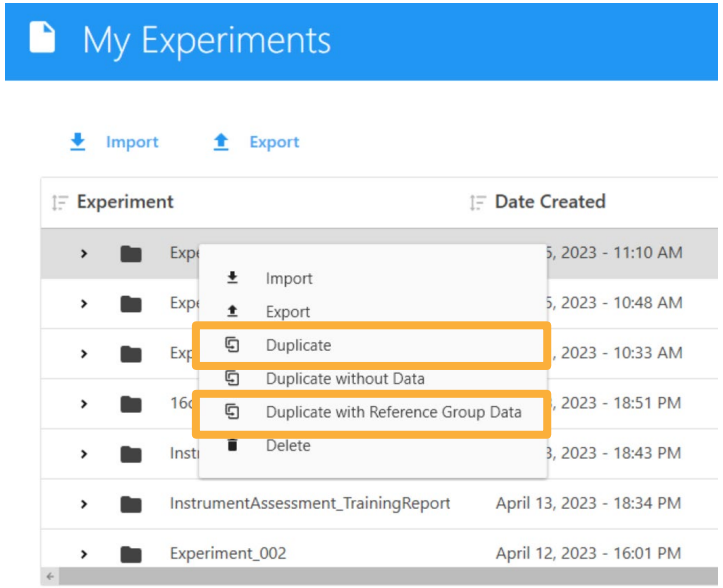
Which Workflow Is Best for You?

	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:



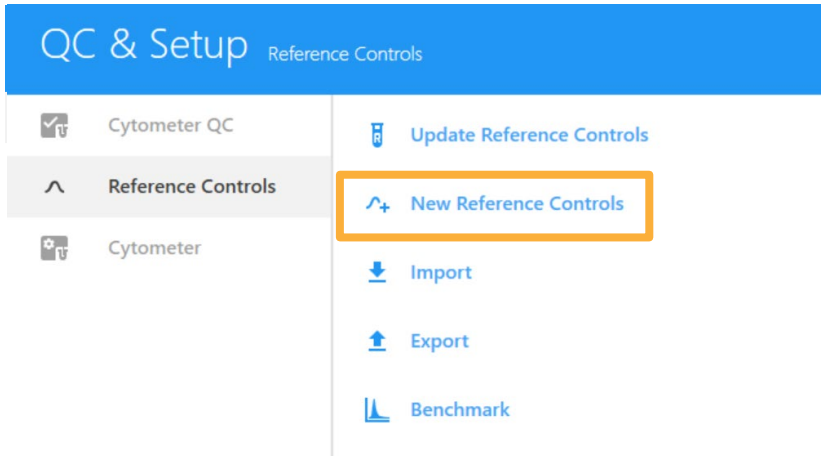
To Reuse:



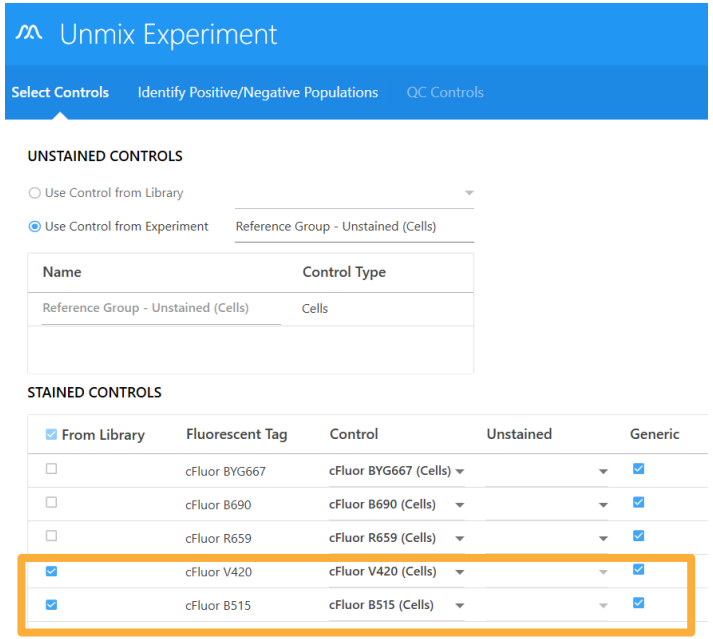
Which Workflow Is Best for You?

	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:



To Reuse:

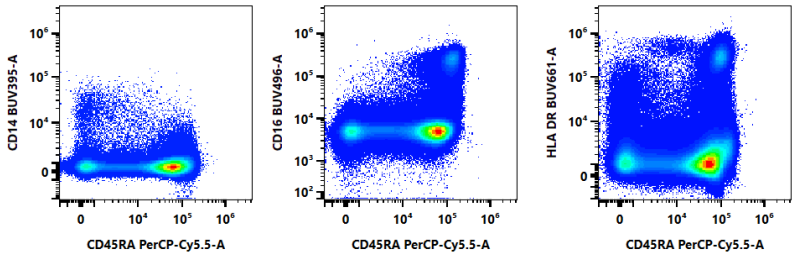


How to Determine if Stored Controls Are Good

3 Check multicolor NxN permutations

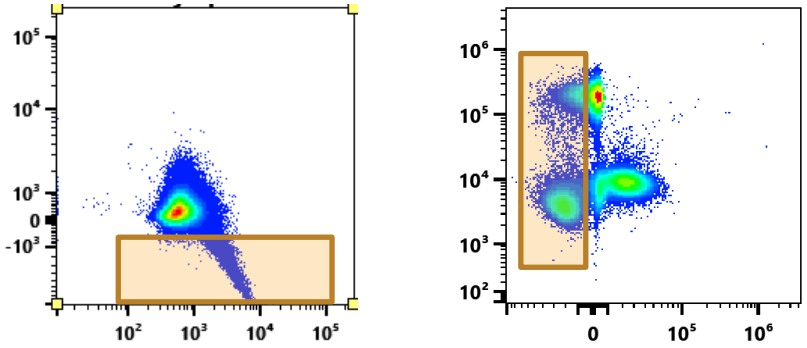
Good Unmixing/Compensation

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

Set Up:

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

Evaluate Your Assay

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

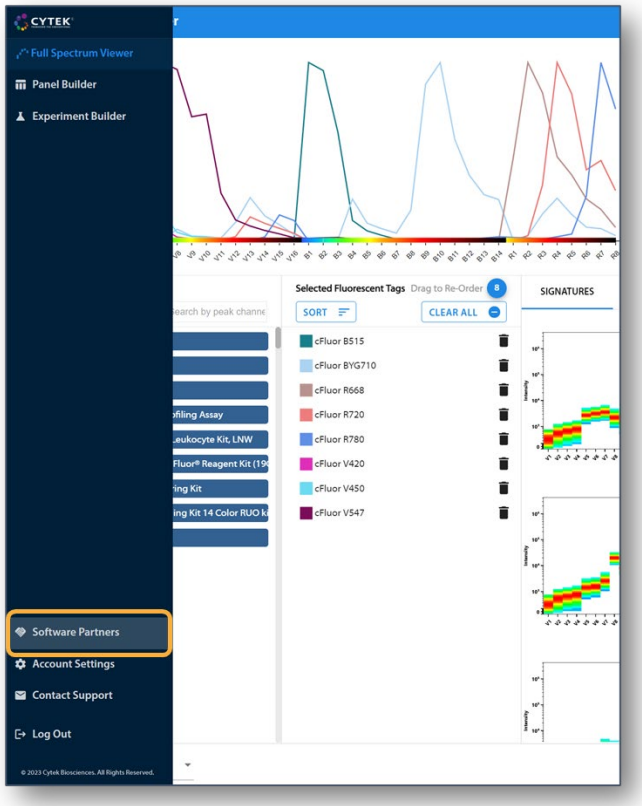
Copy **FCS Express** Claim Code and Serial Number

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

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

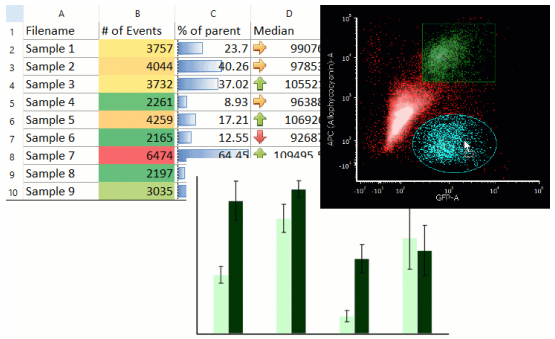
NOTE: Must redeem license within 2 months

https://cloud.cytekbio.com/



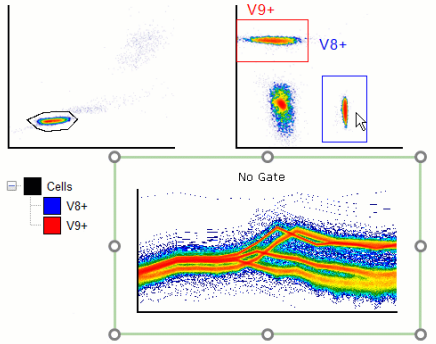
FCS Express Key Features for Cytek Aurora Users

Integrated Spreadsheets and Graphing



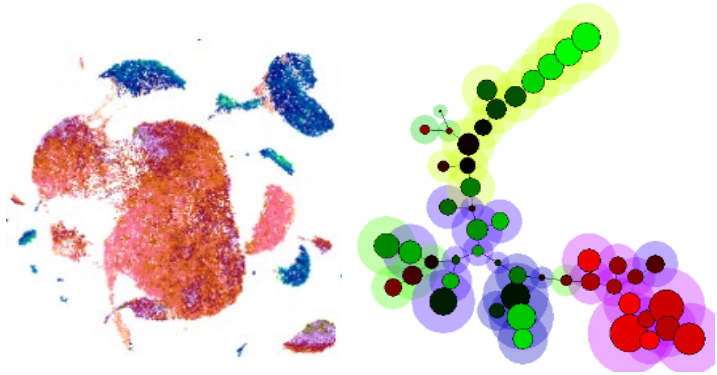
Integrated spreadsheets and graphing tools that link directly to gates. Real-time updating

Gate and Visualize on Spectral Graphs



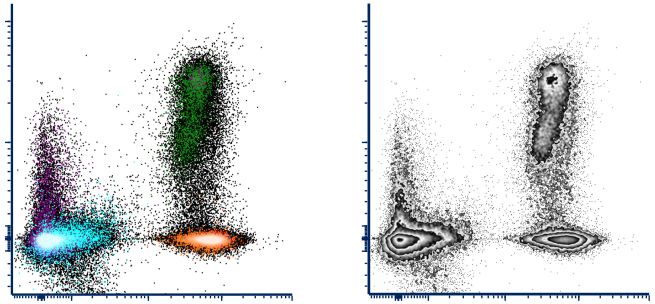
Analyze raw and unmixed data in the same layout. Apply individual gates to spectral plots.

High-Dimensional Data Analysis



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

Presentation & Publication-ready Graphics



Dotmatics FCS Express

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?

Contact Support

technicalsupport@cytekbio.com

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