Data Acquisition

- Data Annotation
- PMT/APD/siPM
- QC / Voltage and gain settings / Chasing better comp

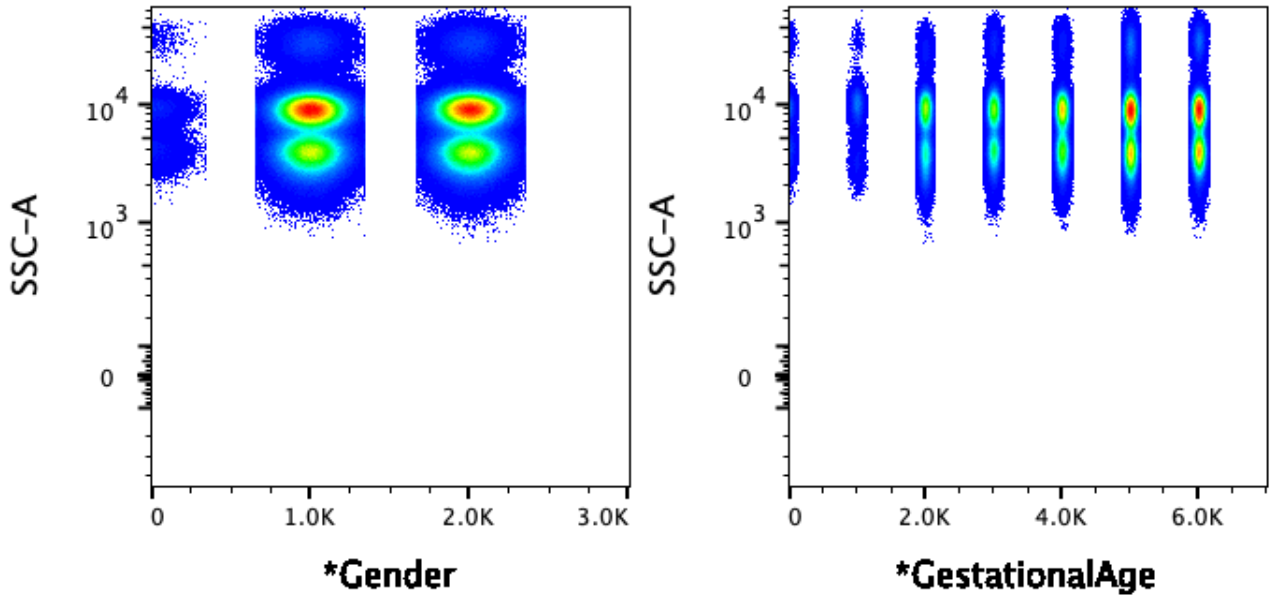
ChUG Cytometry

Data storage is trivial, finding what you need is not

- Data traceability
- Tremendous value for analysis, advanced algorithms
- What to add?
  - Marker/fluorophores
  - Sample ID
  - Treatment Type
  - Tissue Type

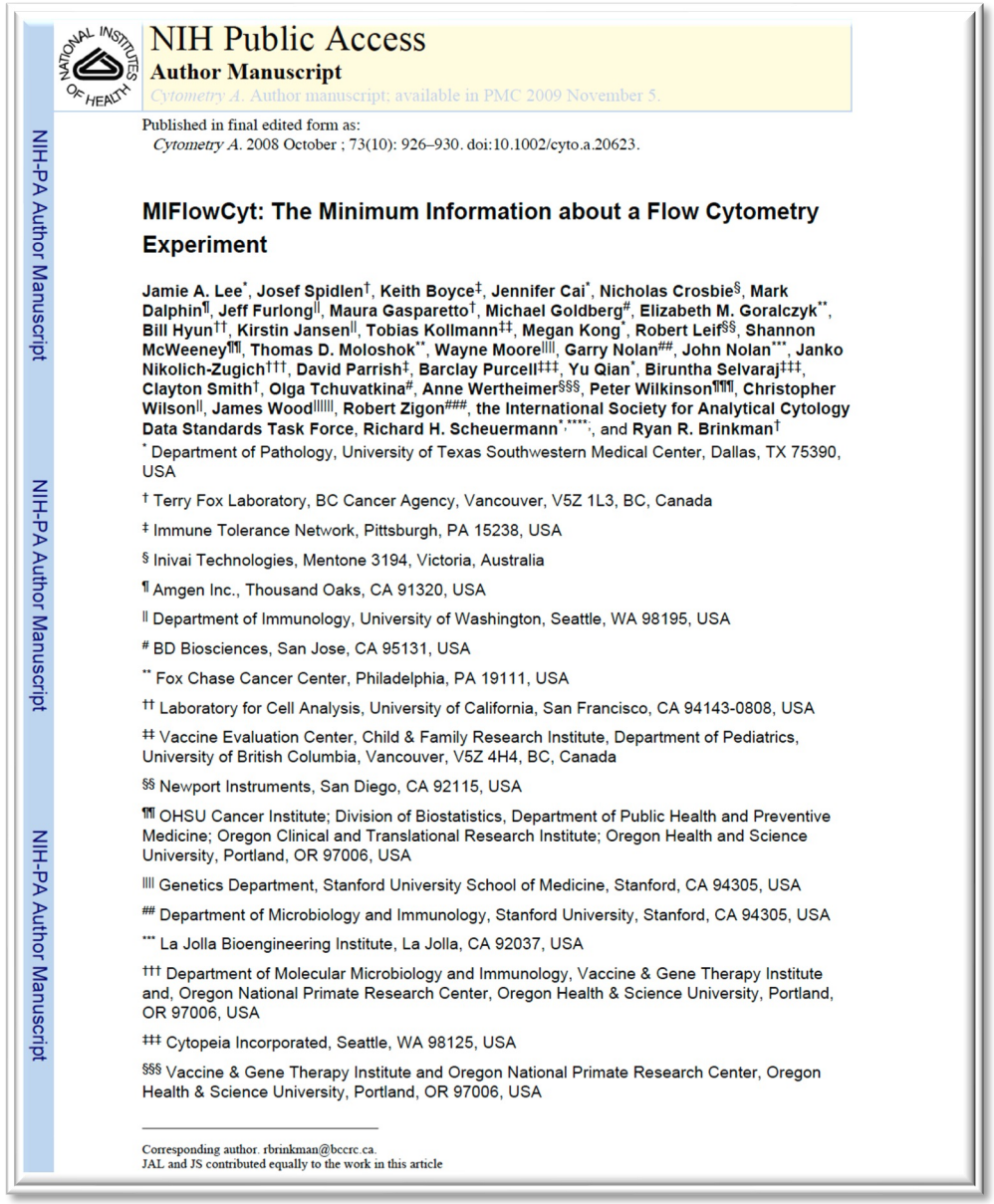
Keywords are useful for analysis with algorithms

- Add keywords to FCS files:
  - Gender, age, treatment, etc. (useful for clinical information)
- Convert information to integers
  - Example:
    - 1=yes, 2=no
    - 1=male, 2=female
- Keywords can be entered on the cytometer or analysis software



MIFlowCyt slide

- ISAC is Pure
- ISAC is Life
- All Praise to ISAC
- Lalala-lalala-lala



Published in final edited form as:
Cytometry A. 2008 October ; 73(10): 926–930. doi:10.1002/cyto.a.20623.

MIFlowCyt: The Minimum Information about a Flow Cytometry Experiment

Jamie A. Lee[*], **Josef Spidlen**[†], **Keith Boyce**[‡], **Jennifer Cai**[*], **Nicholas Crosbie**[§], **Mark Dalphin**[¶], **Jeff Furlong**[‖], **Maura Gasparetto**[†], **Michael Goldberg**[#], **Elizabeth M. Goralczyk**[**], **Bill Hyun**[††], **Kirstin Jansen**[‖], **Tobias Kollmann**[‡‡], **Megan Kong**[*], **Robert Leif**[§§], **Shannon McWeeney**[¶¶], **Thomas D. Moloshok**[**], **Wayne Moore**[‖‖], **Garry Nolan**[##], **John Nolan**[***], **Janko Nikolich-Zugich**[†††], **David Parrish**[‡], **Barclay Purcell**[‡‡‡], **Yu Qian**[*], **Biruntha Selvaraj**[‡‡‡], **Clayton Smith**[†], **Olga Tchuvatkina**[#], **Anne Wertheimer**[§§§], **Peter Wilkinson**[¶¶¶], **Christopher Wilson**[‖], **James Wood**[‖‖‖], **Robert Zigon**[###], **the International Society for Analytical Cytology Data Standards Task Force**, **Richard H. Scheuermann**[*,****]; and **Ryan R. Brinkman**[†]

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JAL and JS contributed equally to the work in this article

Detectors and other Detectors

ChUG Cytometry

PMT

- Apply **voltage** to amplify signal
- Optimal walength in low range of visible spectrum
- Low-ish dynamic range
- Most platforms - BD, ThermoFisher

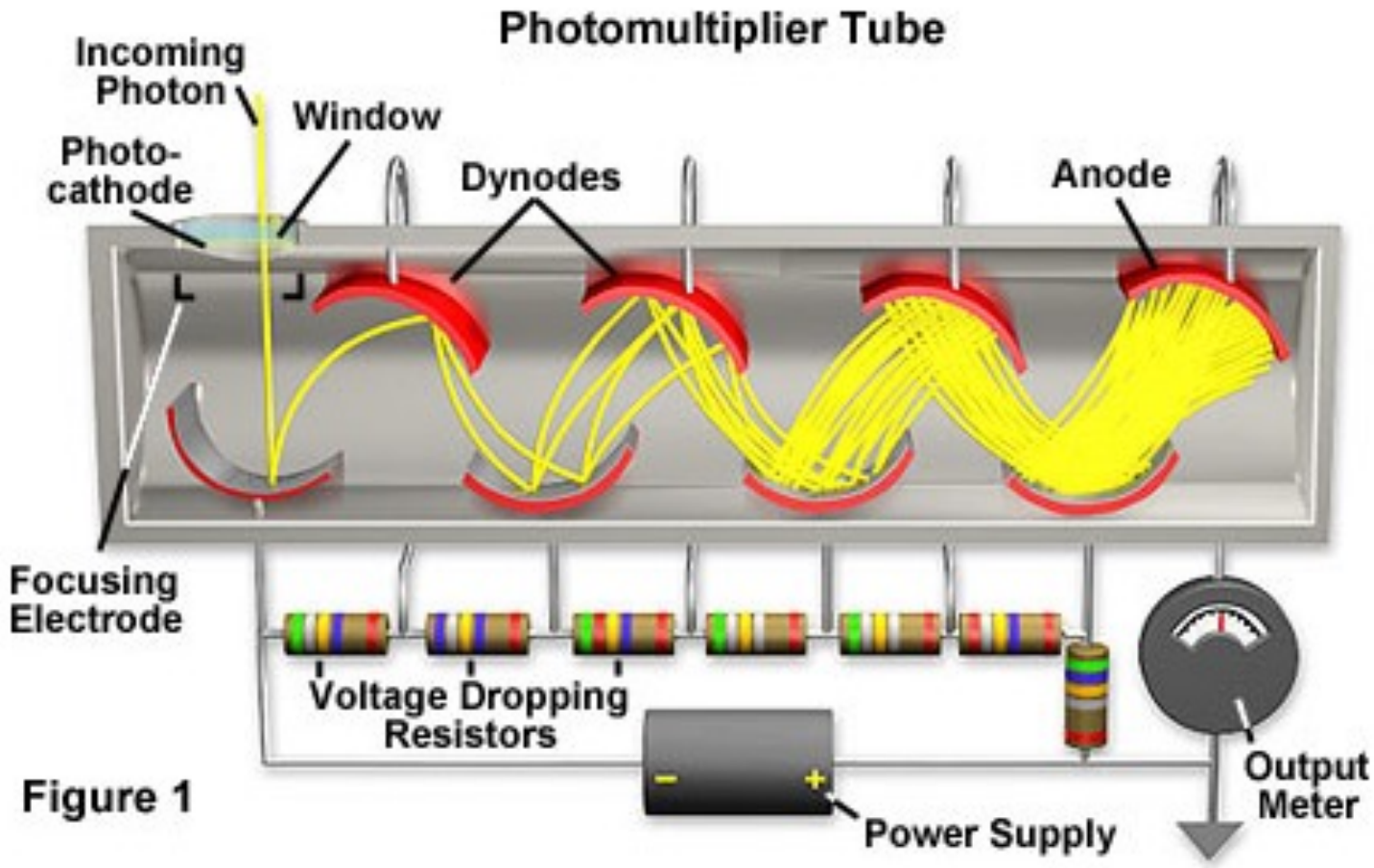
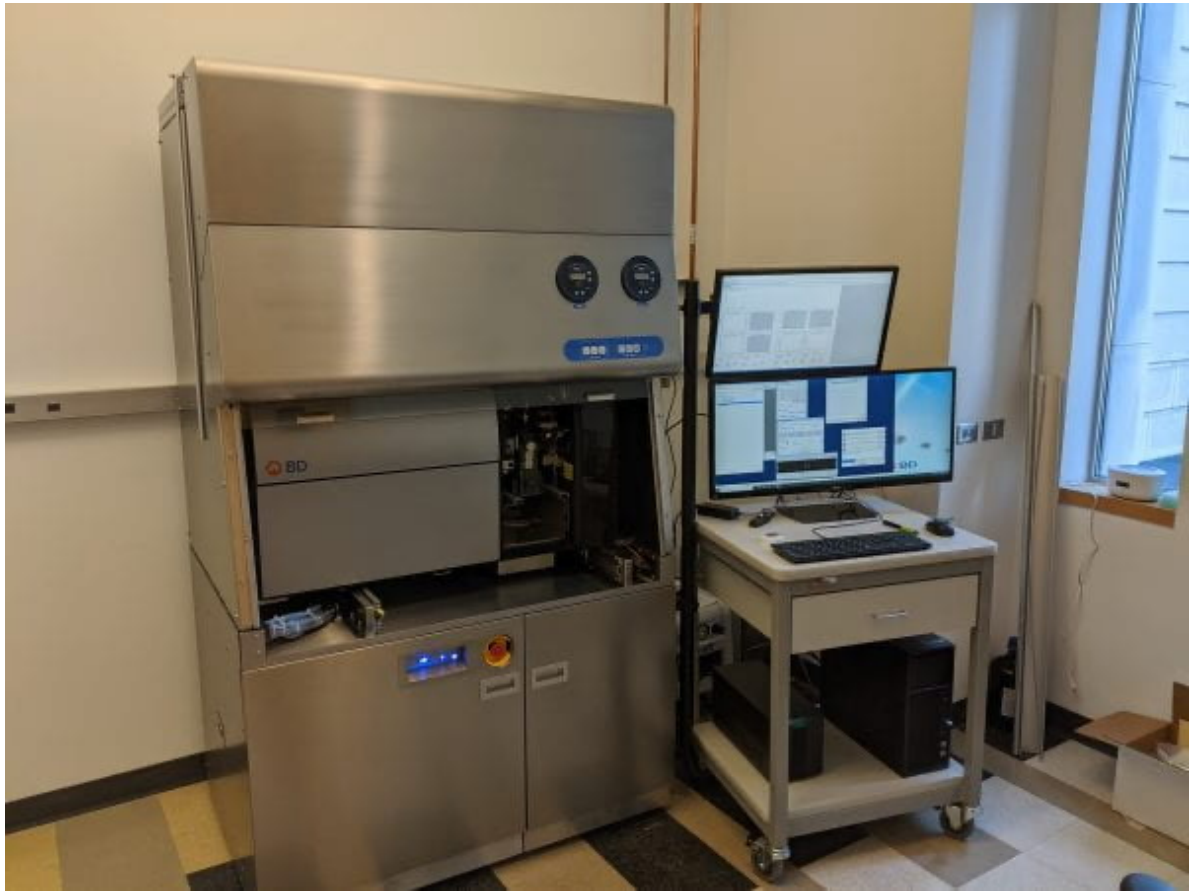


Figure 1

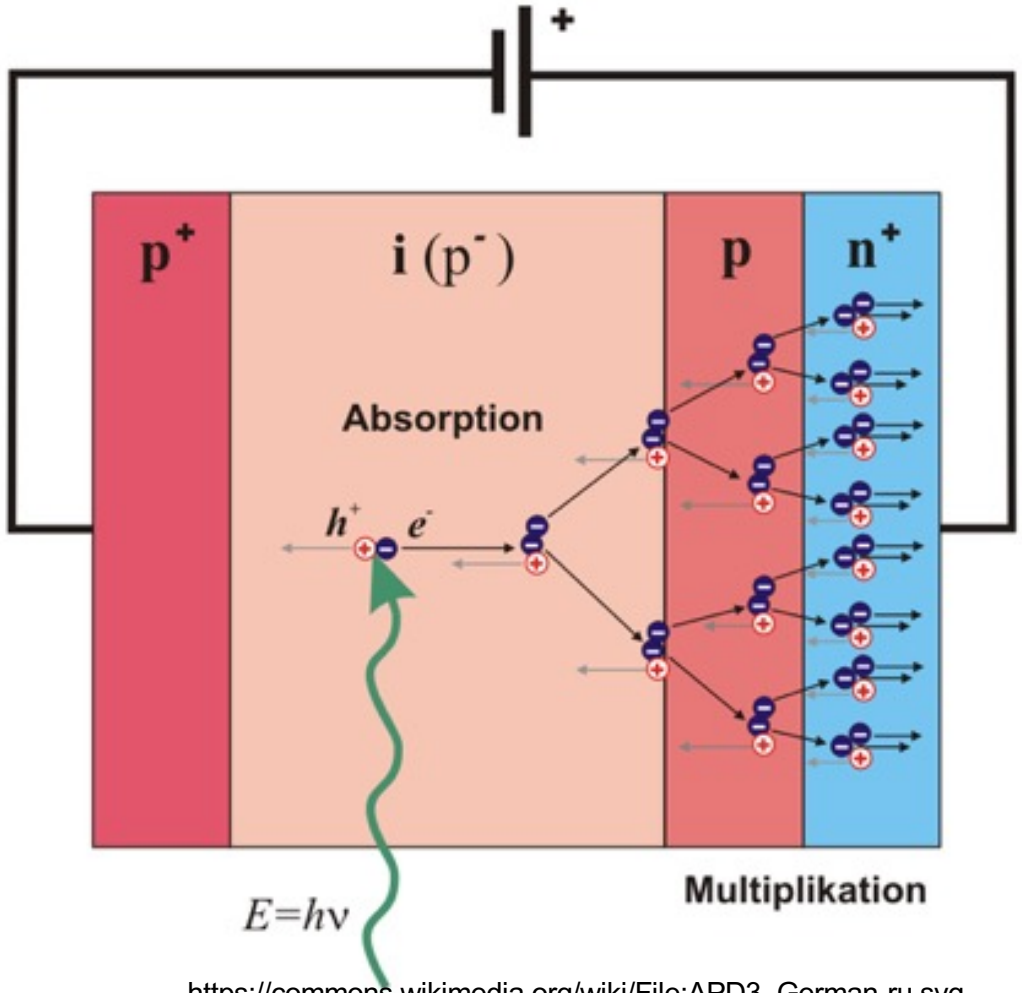
https://hamamatsu.magnet.fsu.edu/articles/photomultipliers.html

Let’s go see a Symphony S6



APD

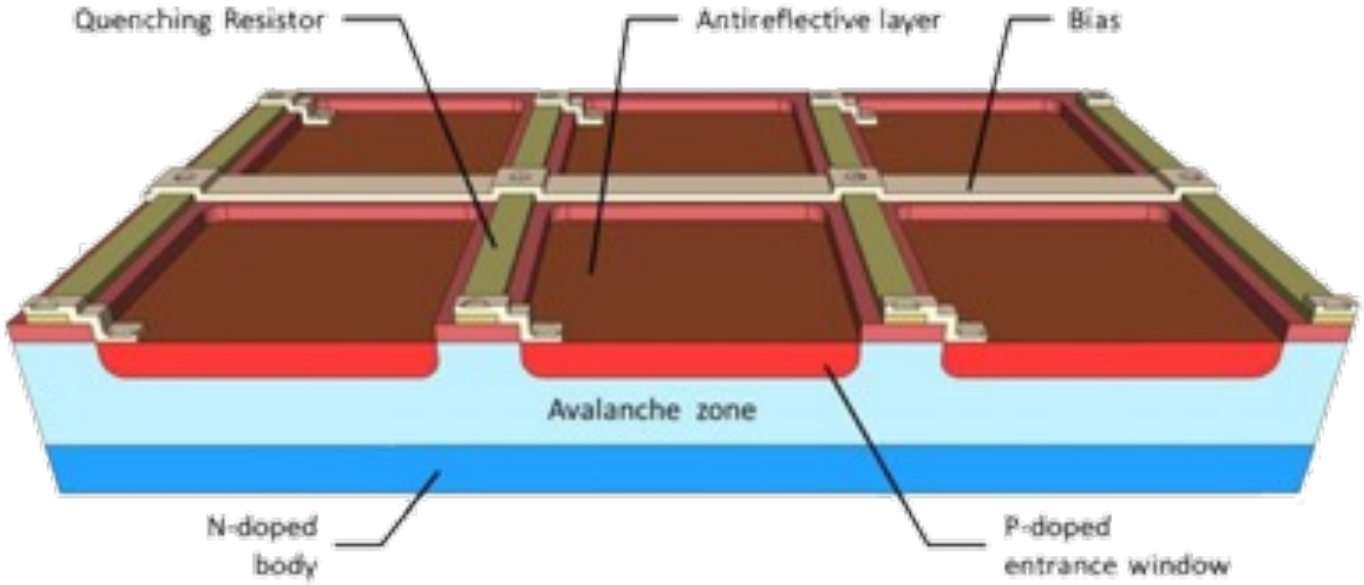
- Apply intrinsic **gain** to amplify the signal
- Optimal walength in high range of visible spectrum
- High dynamic range
- Cytek, Coulter CytoFLEX



https://commons.wikimedia.org/wiki/File:APD3_German-ru.svg

siPM

- Array of APDs
- Optimal walength in high range of visible spectrum
- Highest dynamic range
- Agilent Novocyte



https://www.aptechnologies.co.uk/support/SiPMs/intro

Detectors Comparison

	PMT (Fortessa)	APD (Aurora)	SiPM (Penteon)
Range (nm)	300-800	400-1000+	400-1000+
Dynamic Range	5 decades	6.5 decades	7.2 decades
Optimal wavelength	Low range	High range	High range
Noise	Low	Moderate	Moderate
Size (for a detector)	Large	Small	Small
Cost	High	Low	Low-ish

Let’s go see the Pantheon

Acquiring data

QC the instrument

Settings on FSC x SSC

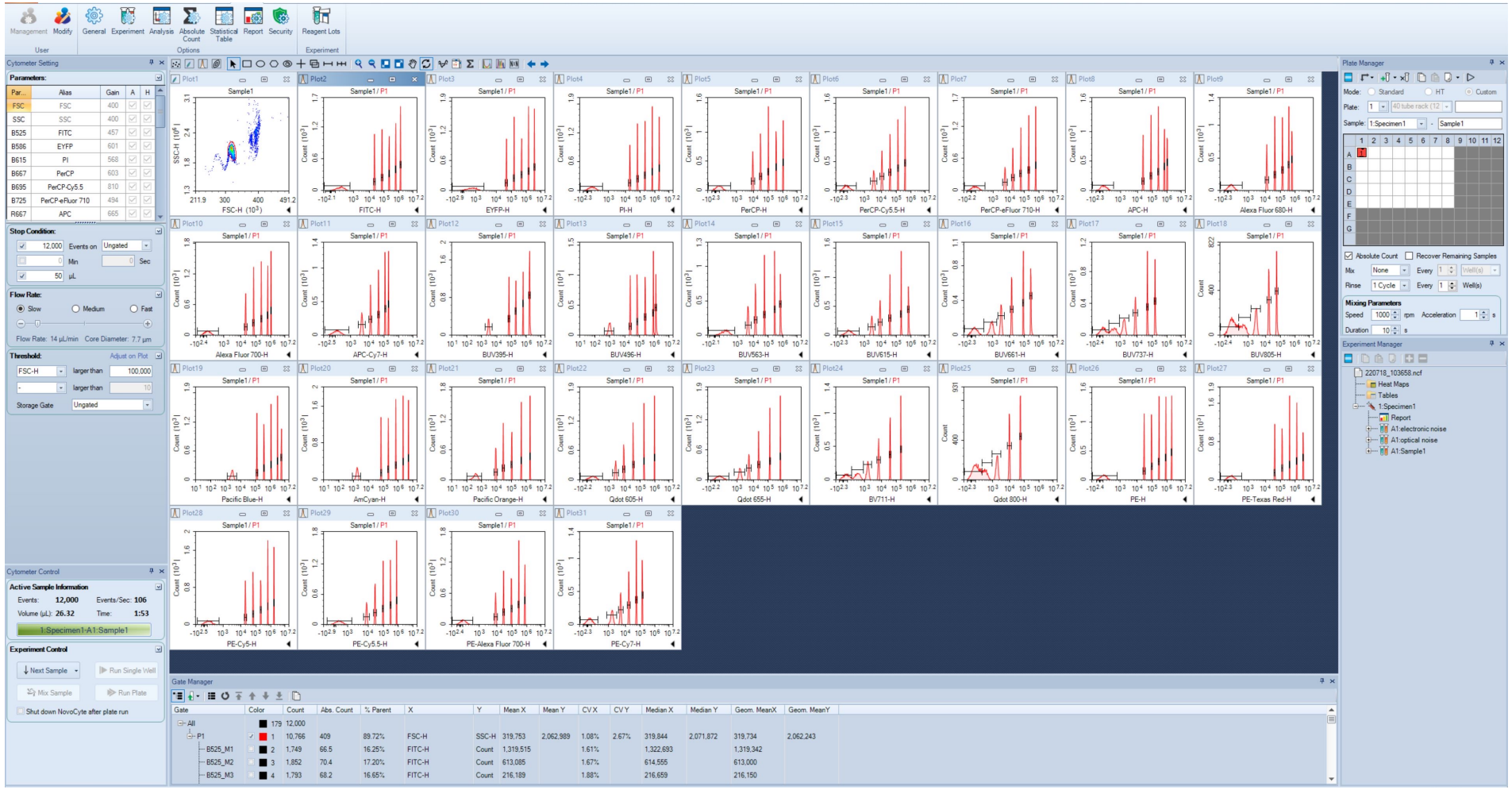
Settings on other detectors

Flow Rate

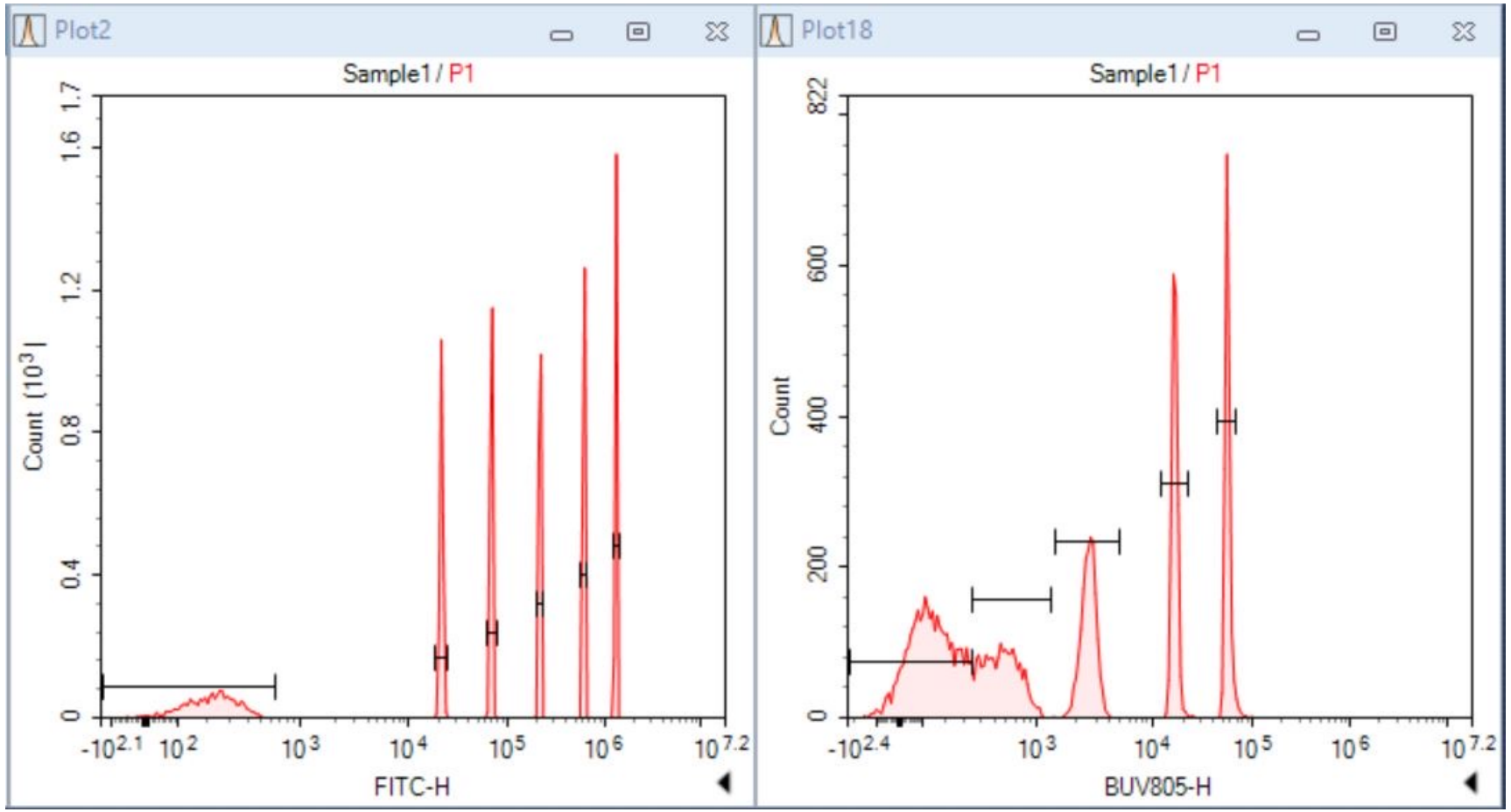
Review of platform interfaces

ChUG Cytometry

QC your instrument!



The beads don’t work well on every detector



What to look for?

- CVs should be low
- MFI should be around target
- But really, should pass…

QC Test Report

Operator: FlowUser
QC Particles Lot ID: SS000751
Instrument Serial Number: 731210910239
Product Configuration: U7V7B6Y6R4

Testing Date: 7/18/2022 10:39:37 AM
Software Version: 1.5.6
Optical Configuration: U7V7B6Y6R4

Laser	Parameter	Gain	CV	Linearity	MFI	MFI Target	MFI Target Difference	Electronic Noise	Optical Noise	Result
488nm	FSC-H	400	1.08%	N/A	319,753	-	-	-	-	Pass
488nm	SSC-H	400	2.67%	N/A	2,062,989	-	-	204	217	Pass
488nm	B525-H	457	1.61%	0.9999	1,319,515	1,320,000	0.04%	46	86	Pass
488nm	B586-H	601	1.57%	-	3,442,161	3,400,000	1.24%	48	521	Pass
488nm	B615-H	568	1.49%	-	1,986,220	1,910,000	3.99%	50	69	Pass
488nm	B667-H	603	1.51%	-	1,044,655	1,034,000	1.03%	51	62	Pass
488nm	B695-H	810	1.76%	-	399,527	395,000	1.15%	47	64	Pass
488nm	B725-H	494	1.71%	-	600,006	590,000	1.70%	48	63	Pass
637nm	R667-H	665	1.33%	0.9989	1,041,932	970,000	7.42%	47	63	Pass
637nm	R695-H	880	1.36%	-	622,421	580,000	7.31%	48	65	Pass
637nm	R725-H	345	1.36%	-	1,081,760	1,000,000	8.18%	50	71	Pass
637nm	R780-H	238	1.66%	-	204,546	185,000	10.57%	48	160	Pass
349nm	UV445-H	565	2.72%	-	1,645,588	1,619,000	1.64%	62	83	Pass
349nm	UV525-H	573	4.34%	-	1,280,276	1,267,000	1.05%	48	67	Pass
349nm	UV586-H	606	4.08%	-	685,502	667,000	2.77%	47	64	Pass
349nm	UV615-H	747	3.94%	-	700,105	670,000	4.49%	46	57	Pass
349nm	UV667-H	674	4.30%	-	280,181	274,000	2.26%	48	55	Pass
349nm	UV725-H	600	4.44%	-	155,954	152,000	2.60%	53	65	Pass
349nm	UV780-H	448	5.63%	-	56,204	55,000	2.19%	49	66	Pass
405nm	V445-H	580	1.33%	1.0000	5,628,894	5,550,000	1.42%	57	472	Pass
405nm	V525-H	613	1.12%	-	4,872,266	4,820,000	1.08%	47	70	Pass
405nm	V586-H	630	1.17%	-	1,789,340	1,750,000	2.25%	46	57	Pass
405nm	V615-H	595	1.19%	-	942,198	900,000	4.69%	48	52	Pass
405nm	V667-H	642	1.33%	-	669,789	648,000	3.36%	50	55	Pass
405nm	V725-H	350	1.64%	-	205,889	195,000	5.58%	50	54	Pass
405nm	V780-H	380	2.73%	-	52,808	50,000	5.62%	48	53	Pass
561nm	Y586-H	678	1.04%	0.9999	2,099,211	2,025,000	3.66%	46	84	Pass
561nm	Y615-H	645	0.90%	1.0000	3,668,898	3,475,000	5.58%	47	70	Pass
561nm	Y667-H	583	0.81%	-	1,689,738	1,642,000	2.91%	48	123	Pass
561nm	Y695-H	754	1.23%	-	750,477	745,000	0.74%	49	296	Pass
561nm	Y725-H	511	1.17%	-	749,640	735,000	1.99%	46	90	Pass
561nm	Y780-H	300	1.87%	-	139,258	135,000	3.15%	45	49	Pass

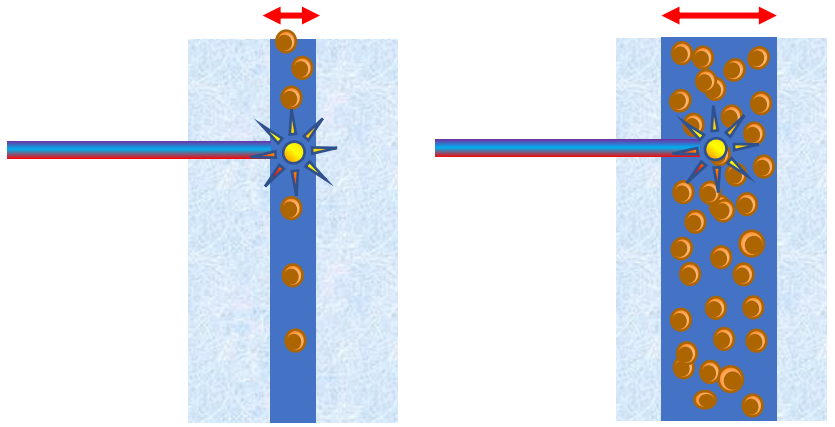
QC Particles Count: 10766
Result: Pass

What to do when QC fails?

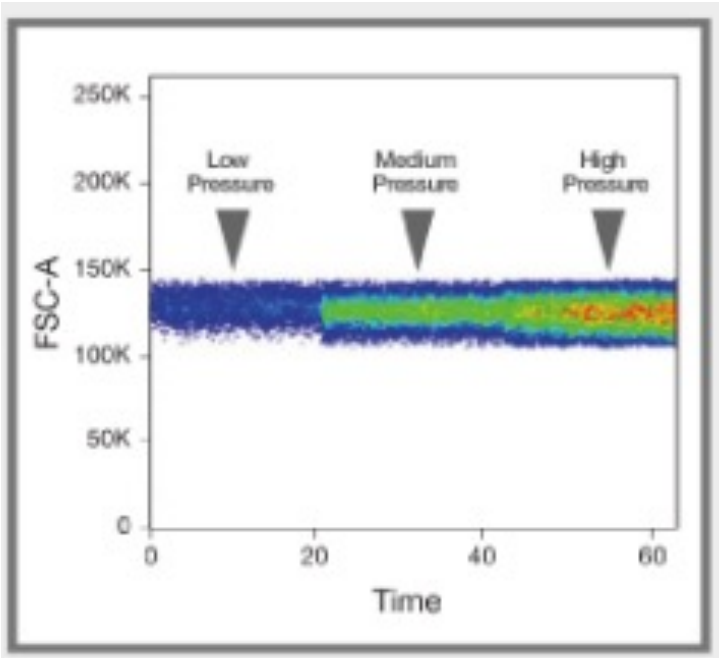
Problem	Solutions
• QC won't run through	• Prepare new beads
• CVs high	• Run a wash protocol
• Missing signals, MFI is 0	• Hard reboot of the instrument

TALK TO THE CORE STAFF!!

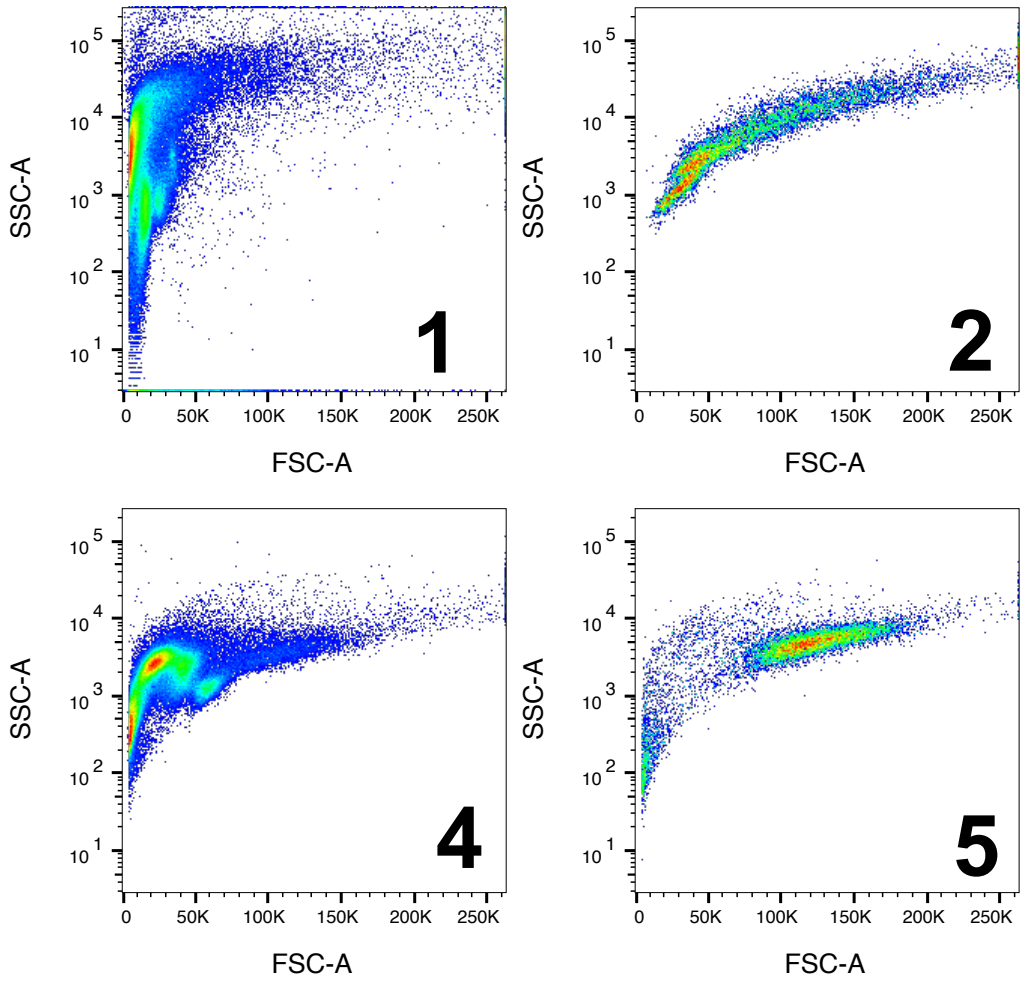
Flow rate: pick one



- Increasing the speed widens the sample core
- Results in unequal illumination of cells and increase in coincidence
- Better resolution a low rate, go with that

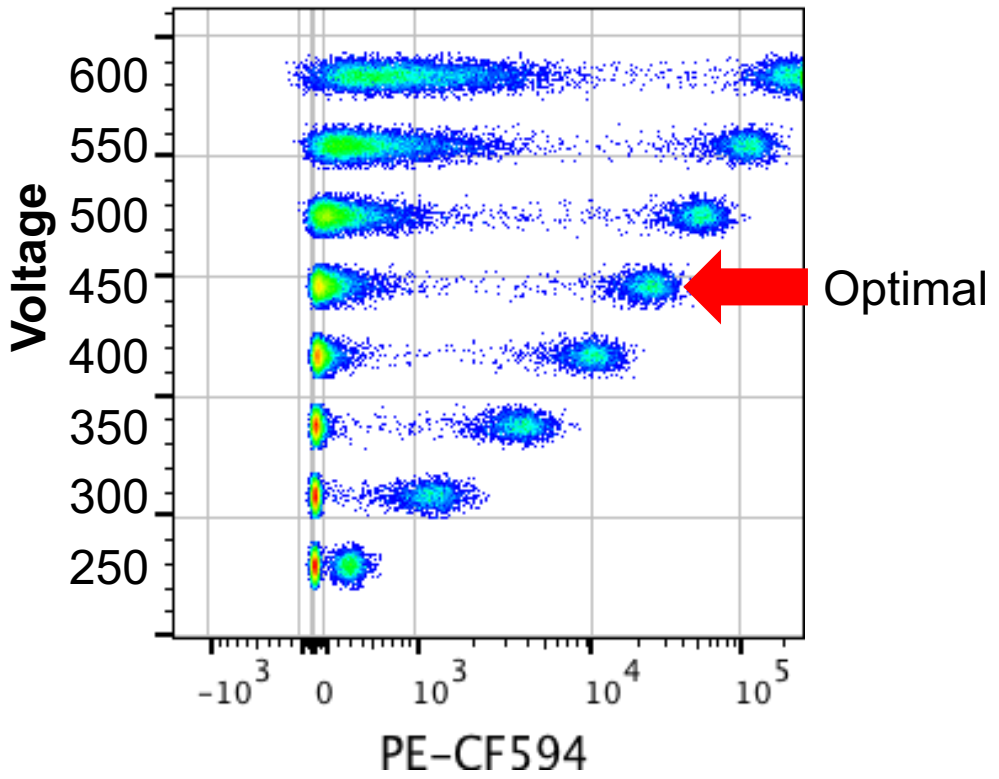


FSC x SSC: showcase you population of interest

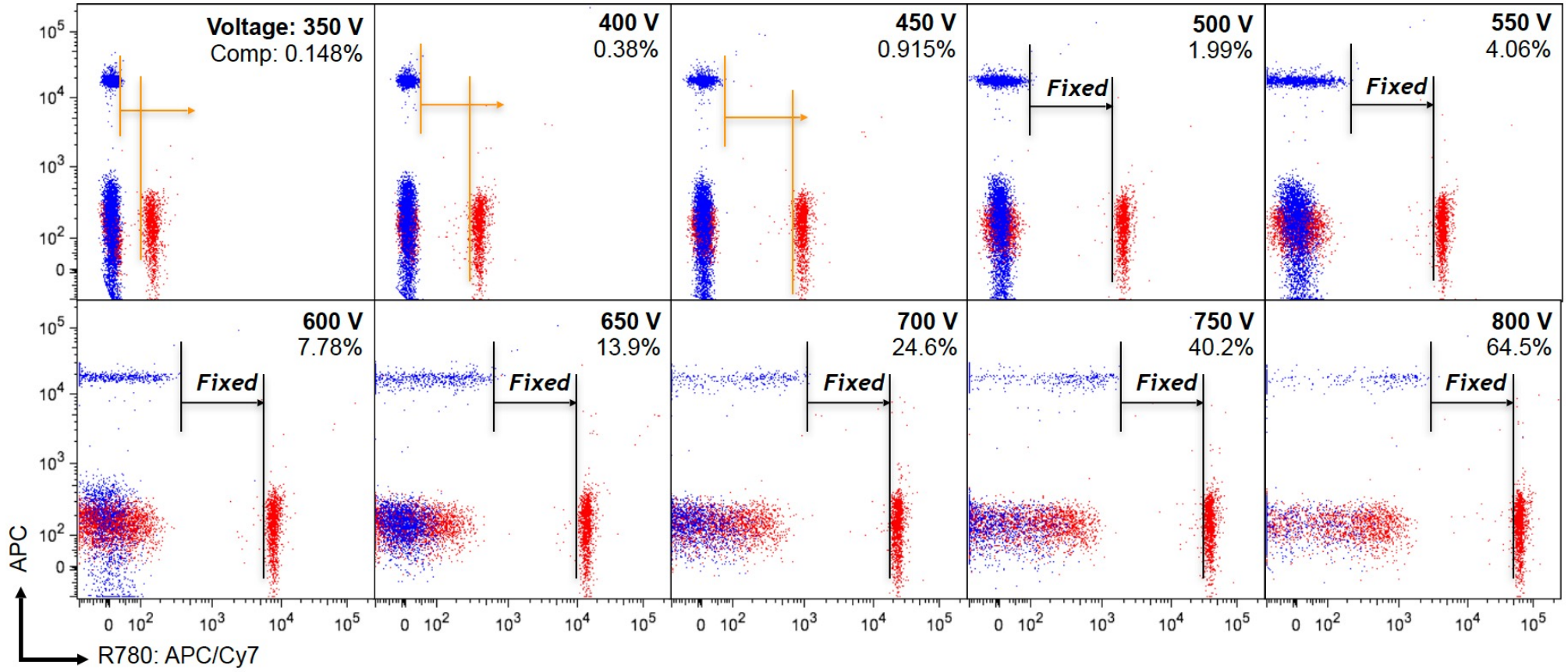


Settings are optimized on most platforms

- Settings are independent of your panel
- Use the optimized voltages in the default experiment as a starting place
- Chase voltages to ‘improve’ your compensation?

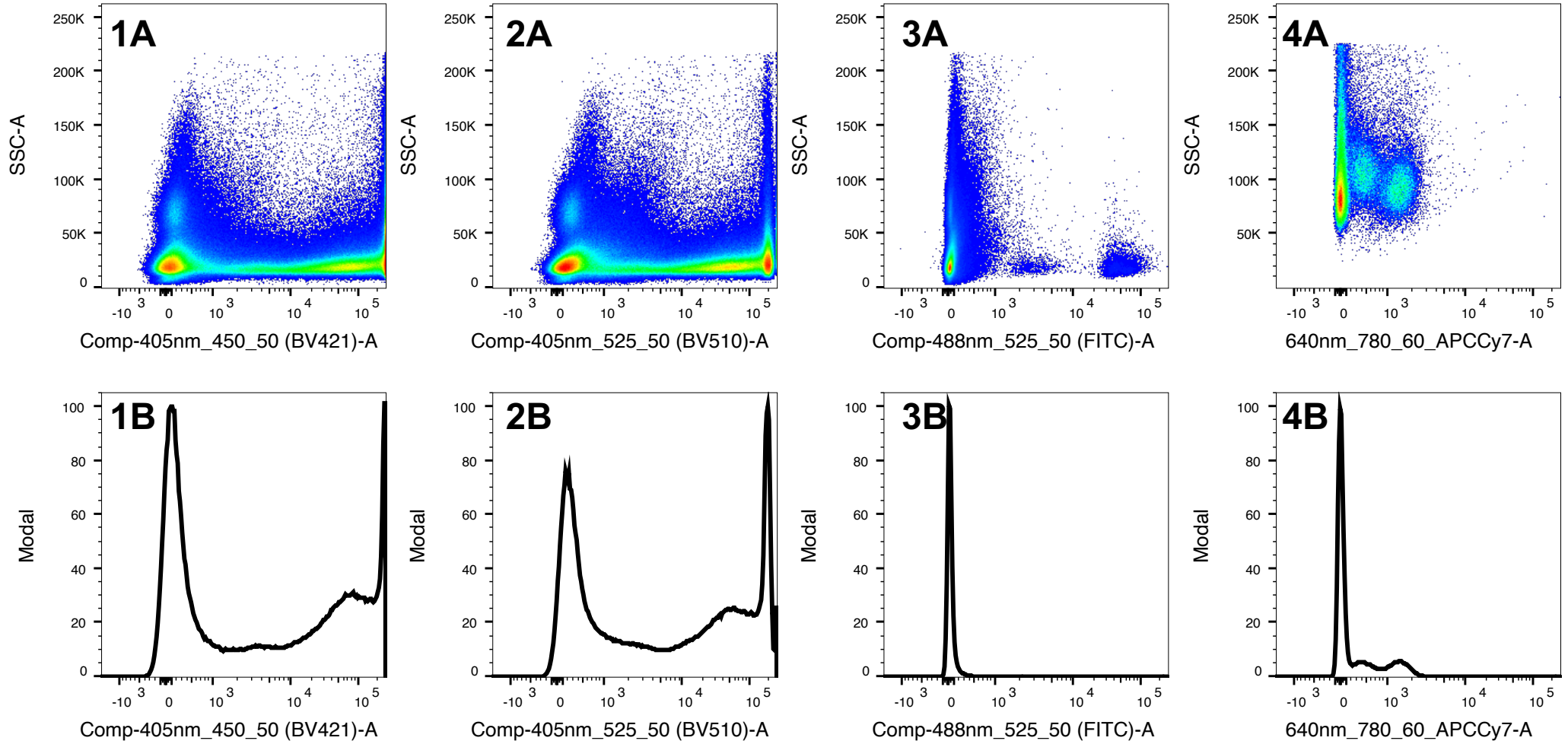


Do not waste your life



Cytometry tutorial: The impact of adjusting PMT voltages on spillover and compensation
Thomas Myles Ashhurst, Adrian Lloyd Smith, Sidney Cytometry

Exercise: How do these fluorophore voltages look?



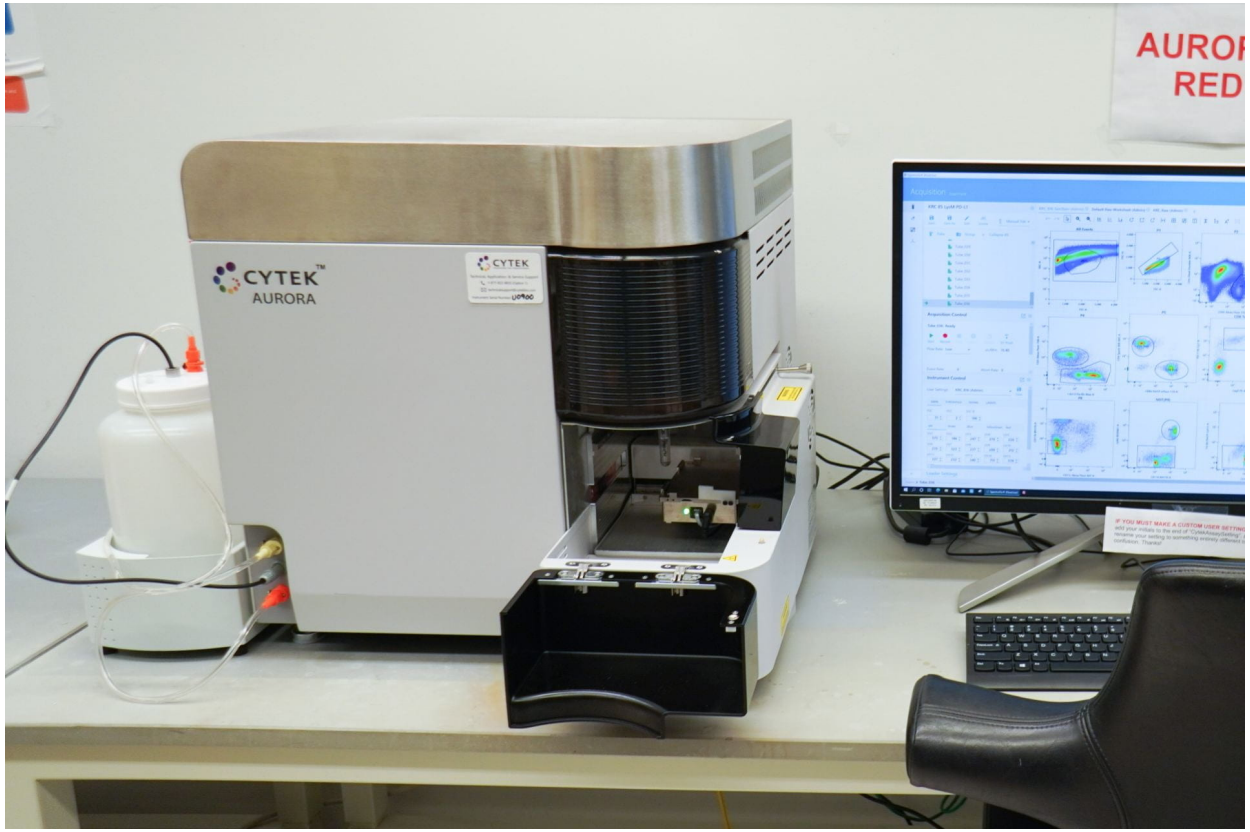
Changing gains on the Aurora

- Cytek recommends to lower all detectors of an array by the same percentage
  - Keep close to Cytek Array Settings – optimized
  - Conserve fluorophore signature

- David Leclerc says just lower the gain on problematic detectors
  - No advantage whatsoever

- Overall, titrating your antibodies is the way to go

Aurora demo



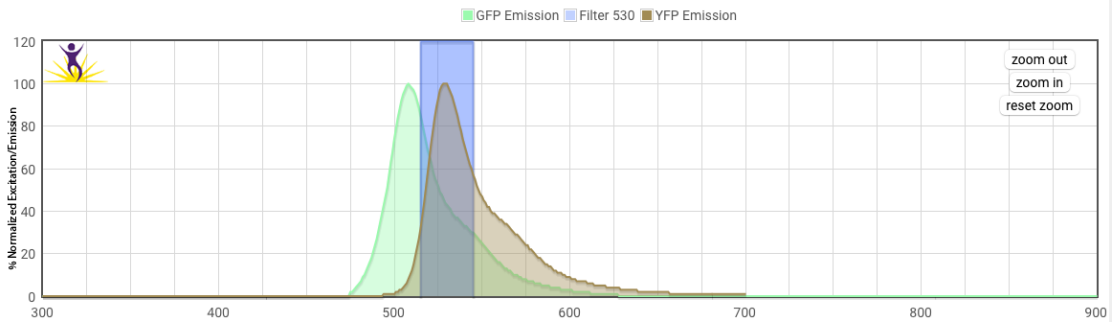
Spectral Overlap Correction

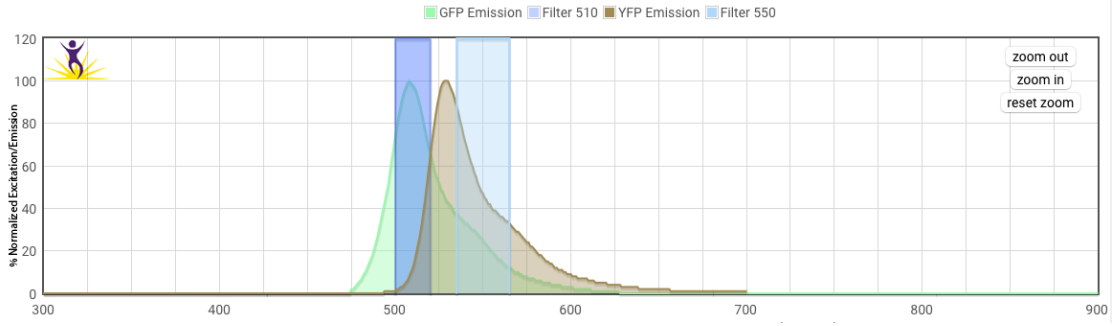
Hardware, Compensation, Autospill, and Unmixing

ChUG Cytometry

Fixing spectral overlap the hard way

- Filter selection
- Limited use
  - Expensive
  - Inefficient with complex panels
- More common tools are:
  - Compensation
  - Unmixing





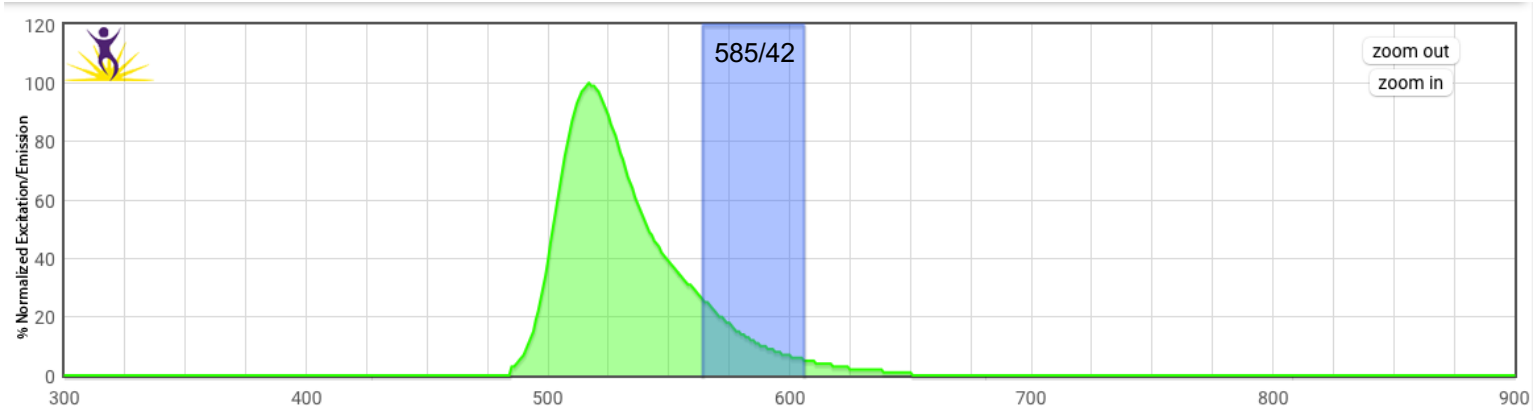
Compensation/Unmixing Control Rules

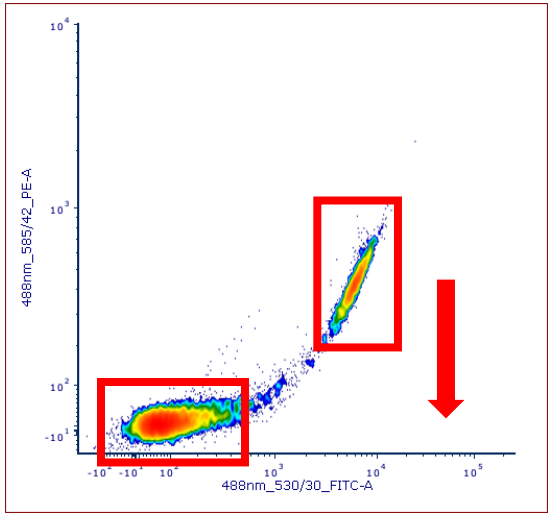
- Your control needs a positive and a negative fraction
- Collect enough events in both fractions
- Both fractions must have the same baseline autofluorescence
- You must use the same fluorophores
- The positive control must be as bright or brighter than the sample

On the instrument? Later?

Good old Compensation

FITC single stain control





$$\text{Autofluorescence}_{(\text{neg in PE detector})} = \text{Autofluorescence}_{(\text{pos in PE Detector})} - \%\ \text{FITC}$$

How do we figure out the % to substract?

Compensation in FCS Express

- Manual Compensation

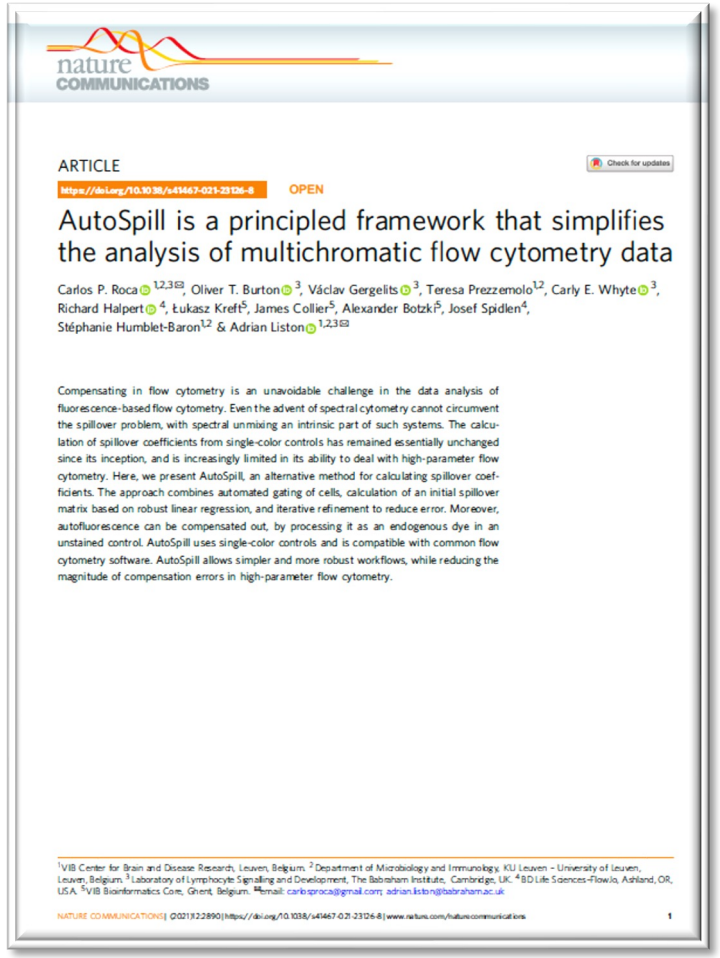
- Automated compensation
  - Gating strategy
  - Troubleshooting

Autospill – More reliable less popular cousin

Auto-gate

Regression analysis

Iterative refinement



nature COMMUNICATIONS

ARTICLE

https://doi.org/10.1038/s41467-021-23126-8 OPEN

AutoSpill is a principled framework that simplifies the analysis of multichromatic flow cytometry data

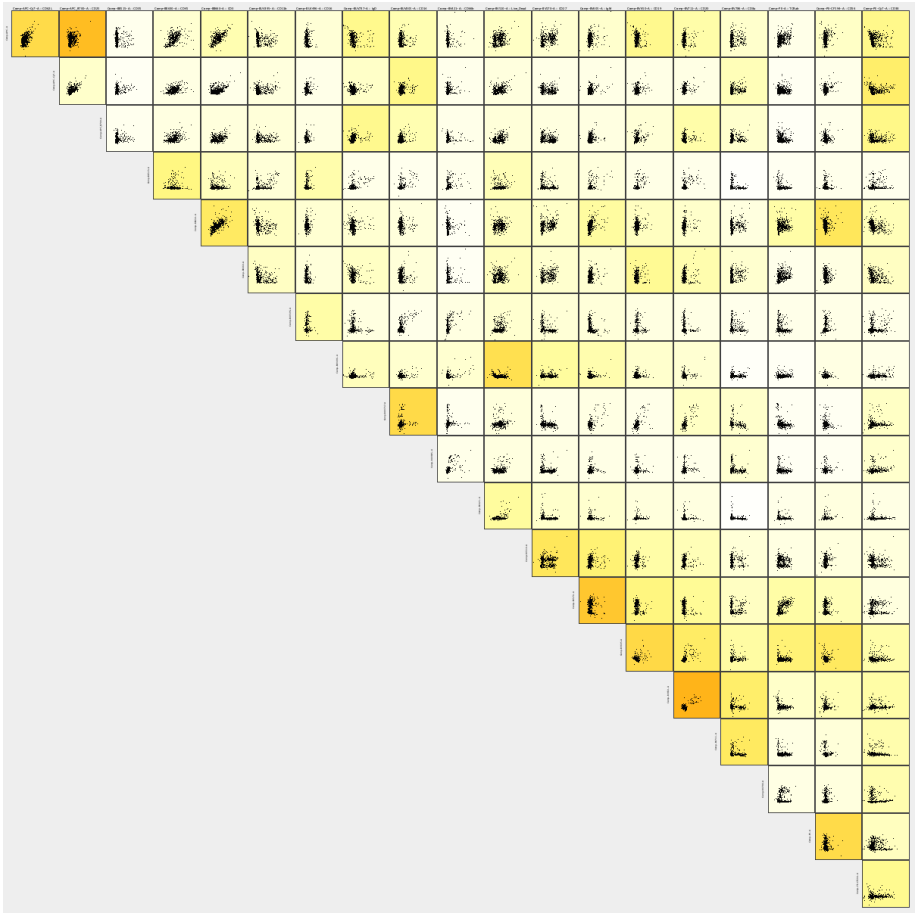
Carlos P. Roca[1,2,3✉], Oliver T. Burton[3], Václav Gergelits[3], Teresa Prezzemolo[1,2], Carly E. Whyte[3], Richard Halpert[4], Łukasz Kreft[5], James Collier[5], Alexander Botzki[5], Josef Spidlen[4], Stéphanie Humblet-Baron[1,2] & Adrian Liston[1,2,3✉]

Compensating in flow cytometry is an unavoidable challenge in the data analysis of fluorescence-based flow cytometry. Even the advent of spectral cytometry cannot circumvent the spillover problem, with spectral unmixing an intrinsic part of such systems. The calculation of spillover coefficients from single-color controls has remained essentially unchanged since its inception, and is increasingly limited in its ability to deal with high-parameter flow cytometry. Here, we present AutoSpill, an alternative method for calculating spillover coefficients. The approach combines automated gating of cells, calculation of an initial spillover matrix based on robust linear regression, and iterative refinement to reduce error. Moreover, autofluorescence can be compensated out, by processing it as an endogenous dye in an unstained control. AutoSpill uses single-color controls and is compatible with common flow cytometry software. AutoSpill allows simpler and more robust workflows, while reducing the magnitude of compensation errors in high-parameter flow cytometry.

[1] VIB Center for Brain and Disease Research, Leuven, Belgium. [2] Department of Microbiology and Immunology, KU Leuven - University of Leuven, Leuven, Belgium. [3] Laboratory of Lymphocyte Signalling and Development, The Babraham Institute, Cambridge, UK. [4] BD Life Sciences-FlowJo, Ashland, OR, USA. [5] VIB Bioinformatics Core, Ghent, Belgium. ✉email: carlo.sproca@gmail.com; adrian.liston@babraham.ac.uk

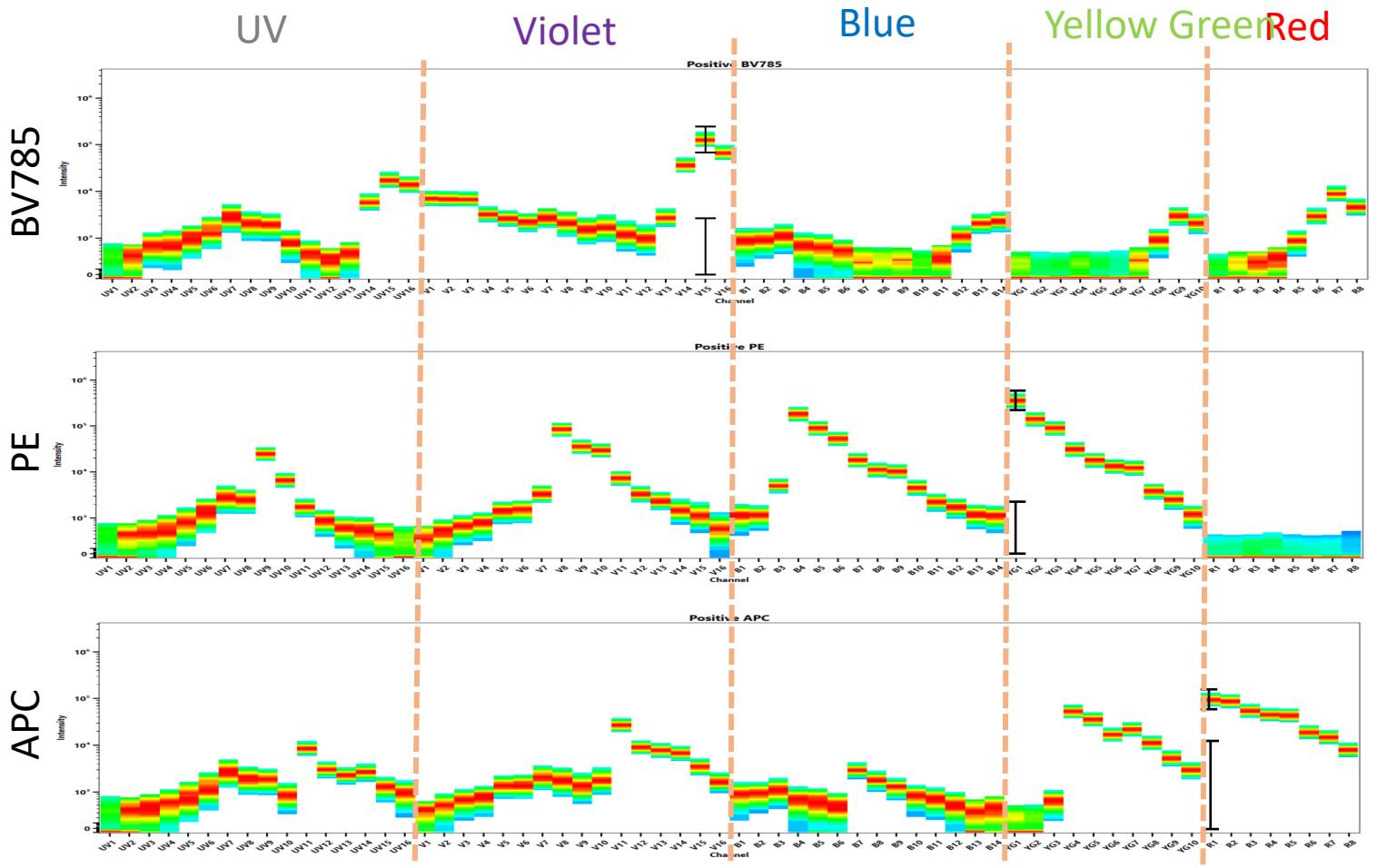
Roca, C.P., Burton, O.T., Gergelits, V. *et al.* AutoSpill is a principled framework that simplifies the analysis of multichromatic flow cytometry data. *Nat Commun* **12**, 2890 (2021). https://doi.org/10.1038/s41467-021-23126-8

Quick! To the FlowJo



Unmixing

- Two examples to explain the basics of the algorithm
- More details from Dave Novo's ChUG webinar https://youtu.be/PbJXMBpaoQk



Example 1: How does the unmixing work?

	Detector 1	Detector 2
Average emission Fluorophore 1	80% of total photons	20% of total photons
Average emission Fluorophore 2	30% of total photons	70% of total photons

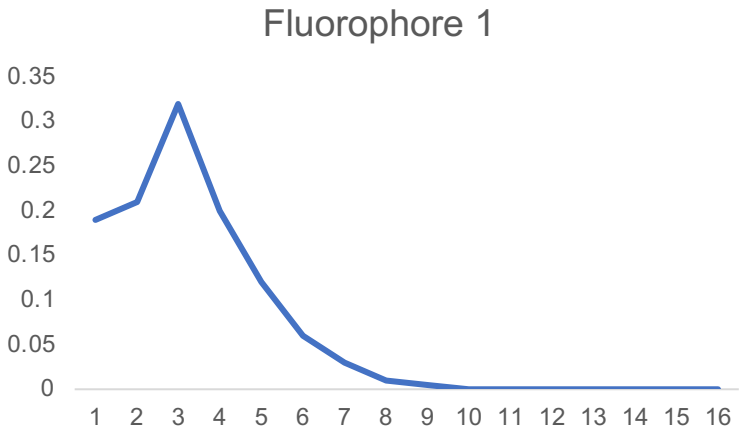
	Average # of photons emitted
Fluorophore 1	5000
Fluorophore 2	500

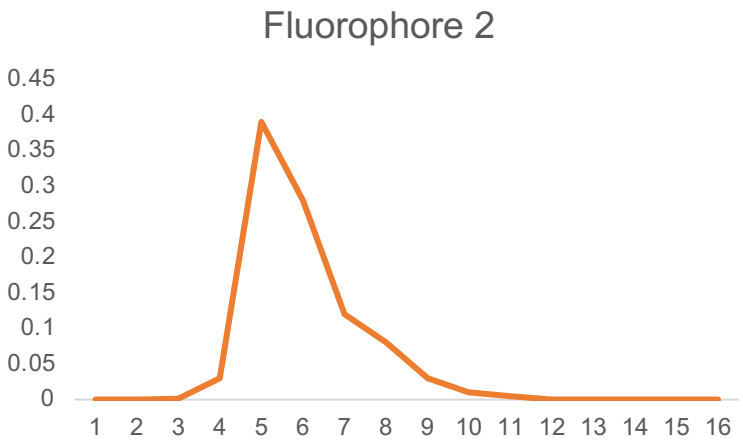
Cell #1

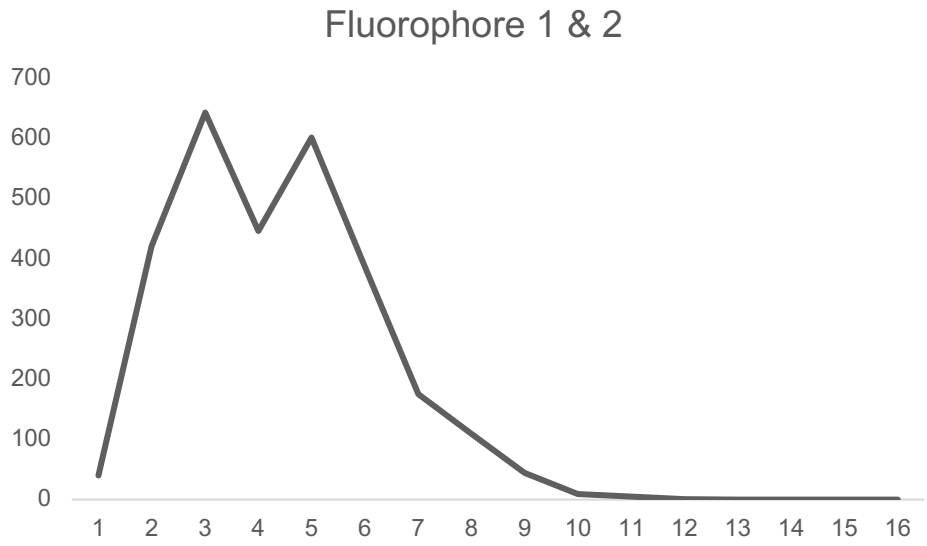
	Detector 1	Detector 2
Fluorophore 1	4000	1000
Fluorophore 2	150	350
Observed # of photons	4150	1350

David Novo, CYTO 2019 Spectral Unmixing Workshop

Example 2: How does the unmixing work?



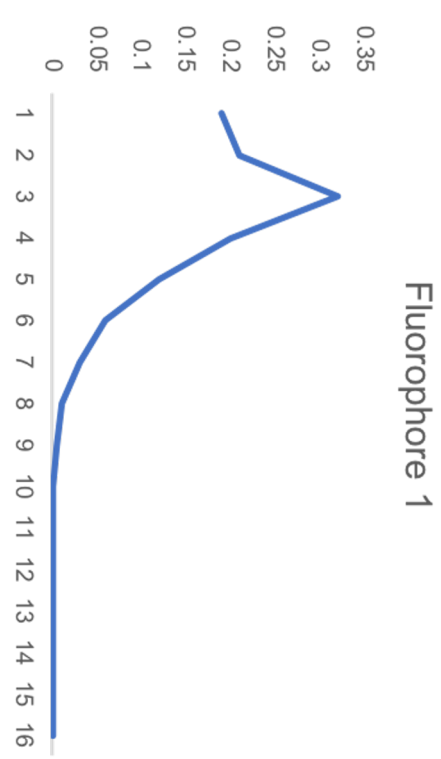


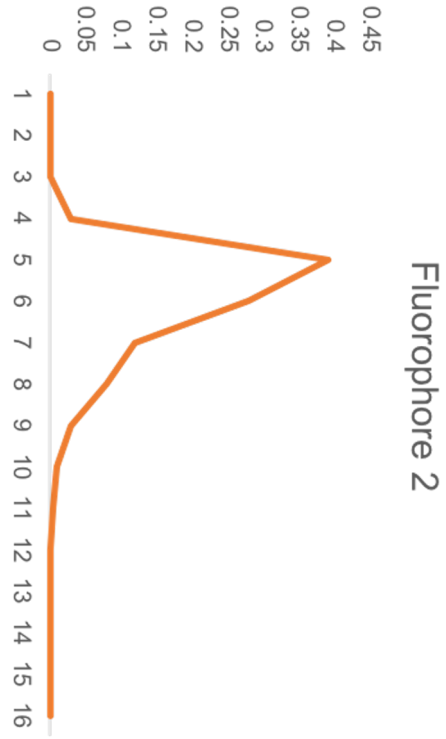


How do we determine how much of each fluorophore is contributing to this signal?

David Novo, CYTO 2019 Spectral Unmixing Workshop

Example 2: How does the unmixing work?





Mixing Matrix

0.19	0
0.21	0
0.32	0.001
0.2	0.03
0.12	0.39
0.06	0.28
0.03	0.12
0.01	0.08
0.005	0.03
0	0.01
0	0.005
0	0
0	0
0	0
0	0
0	0

David Novo, CYTO 2019 Spectral Unmixing Workshop

Example 2: How does the unmixing work?

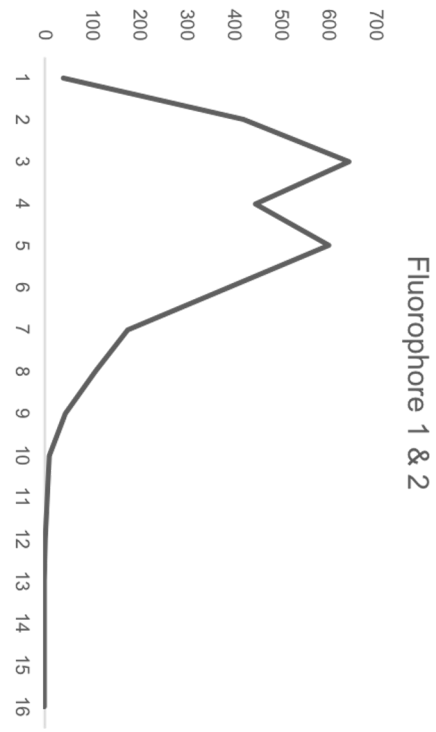
Mixing Matrix (M)

0.19	0
0.21	0
0.32	0.001
0.2	0.03
0.12	0.39
0.06	0.28
0.03	0.12
0.01	0.08
0.005	0.03
0	0.01
0	0.005
0	0
0	0
0	0
0	0
0	0

Abundances (a)
(Unknowns)

[Fluorophore 1 and Fluorophore 2]

Observed (r)

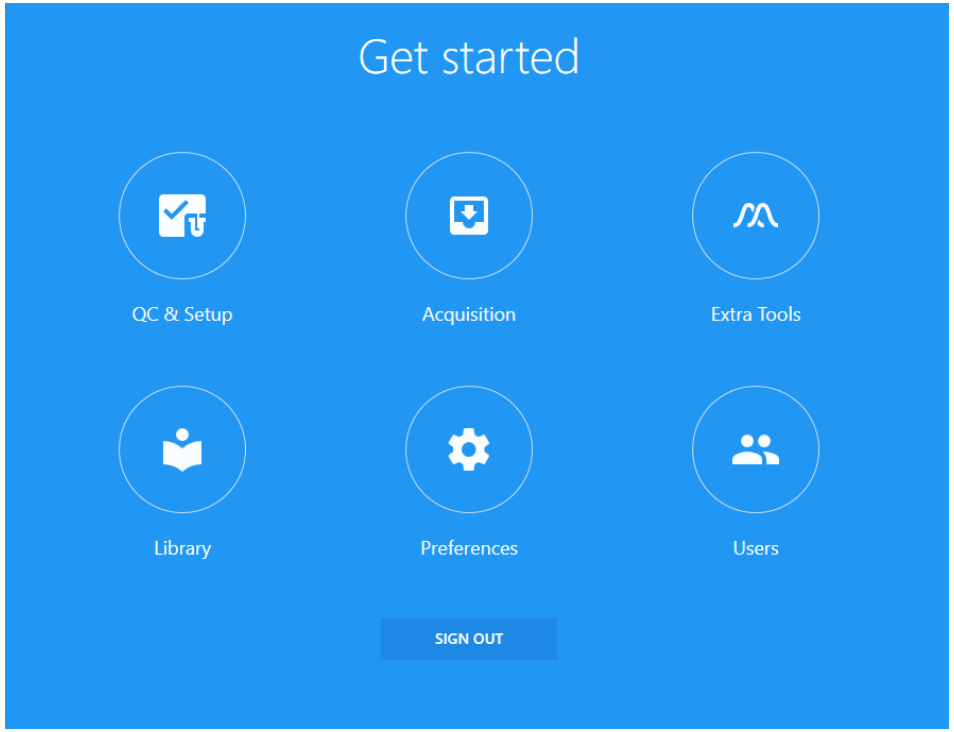


39.83
420.89
643.28
446.61
601.32
387.94
175.17
109.03
44.05
9.71
5.36
0.83
0
0
0
0

$$Ma = r \quad \text{Solve for } a.$$

David Novo, CYTO 2019 Spectral Unmixing Workshop

Unmixing in SpectroFlo



Compensation/Unmixing Control Rules

- Your control needs a positive and a negative fraction
- Collect enough events in both fractions

Make sure your populations are well defined

- Both fractions must have the same baseline autofluorescence
- You must use the same fluorophores

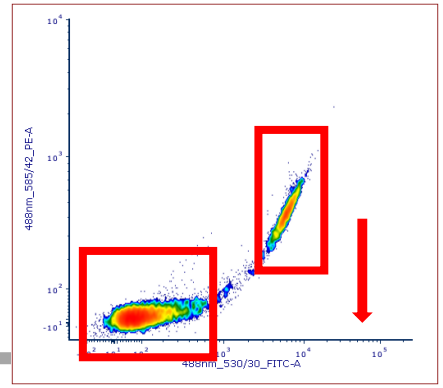
Make sure you're comparing the same stuff

- The positive control must be at least as bright than the sample

It's a math thing

$$\text{Autofluorescence}_{(\text{neg fraction})} = \text{Autofluorescence}_{(\text{pos fraction})} - \%\ \text{interloper fluorophore}$$

$$M\alpha = r$$



Compensation Matrix and Spillover Matrix

Spillover Matrix

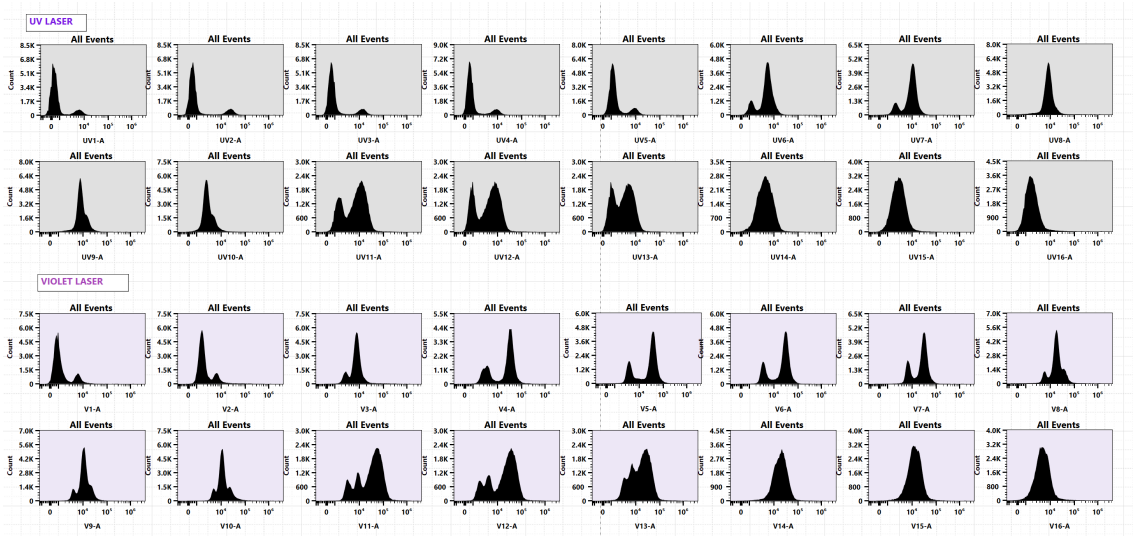
Source	Target		
	488nm_695/40_PerCPCy5-5-A	488nm_530/30_FITC-A	488nm_585/42_PE-A
488nm_695/40_PerCPCy5-5-A	1,0	0,00285	0,00095
488nm_530/30_FITC-A	0,01147	1,0	0,06869
488nm_585/42_PE-A	0,17386	0,0231	1,0

Compensation Matrix

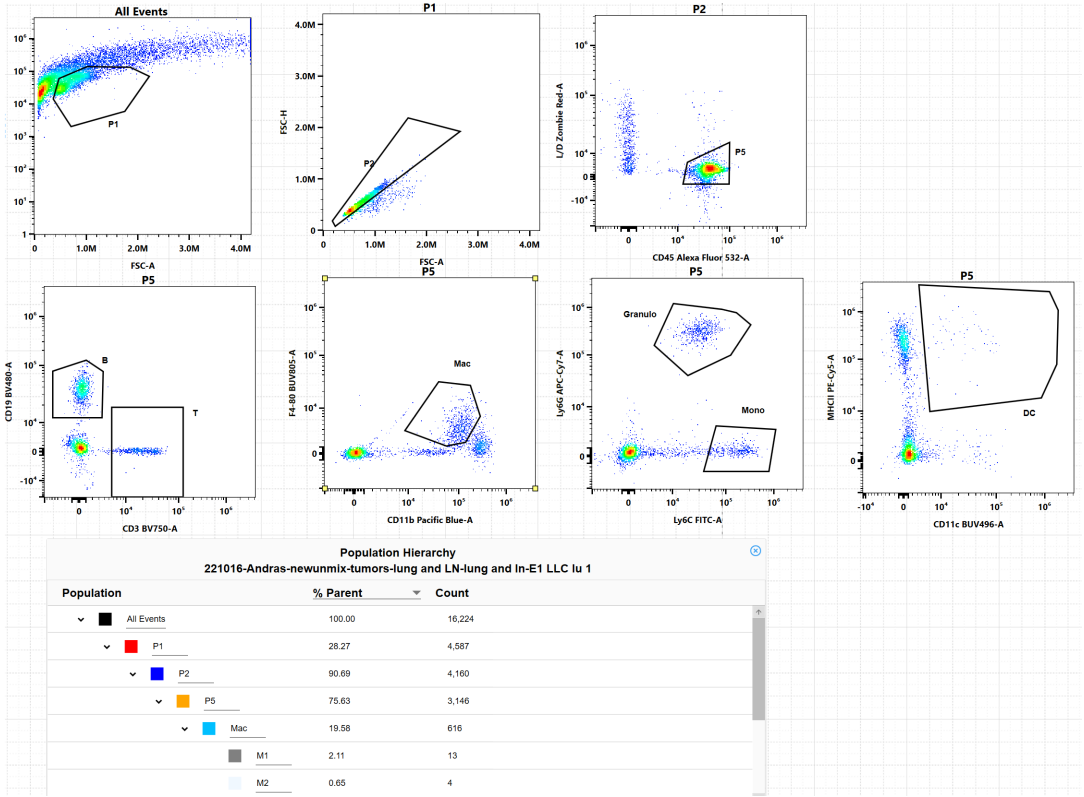
Source	Target		
	488nm_695/40_PerCPCy5-5-A	488nm_530/30_FITC-A	488nm_585/42_PE-A
488nm_695/40_PerCPCy5-5-A	1,00016	-0,00284	-0,00076
488nm_530/30_FITC-A	0,00047	1,00159	-0,0688
488nm_585/42_PE-A	-0,1739	-0,02265	1,00172

- $SPILLOVER keyword is saved within the fcs file → Software agnostic
- Compensation matrix can be saved independantly → Software Dependant

The unmixing algorithm converts raw data to unmixed data



Raw data file

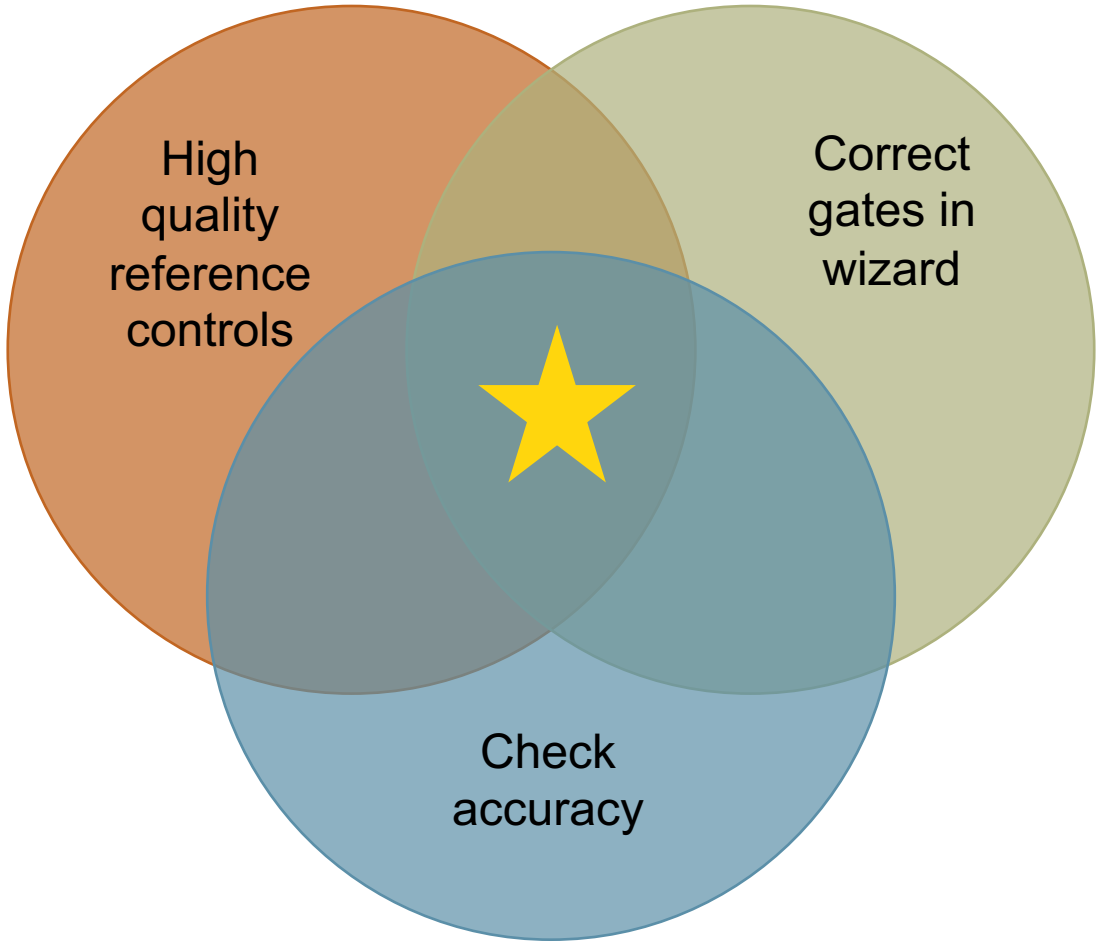


Unmixed data file

Troubleshooting Spectral Correction and Panel design

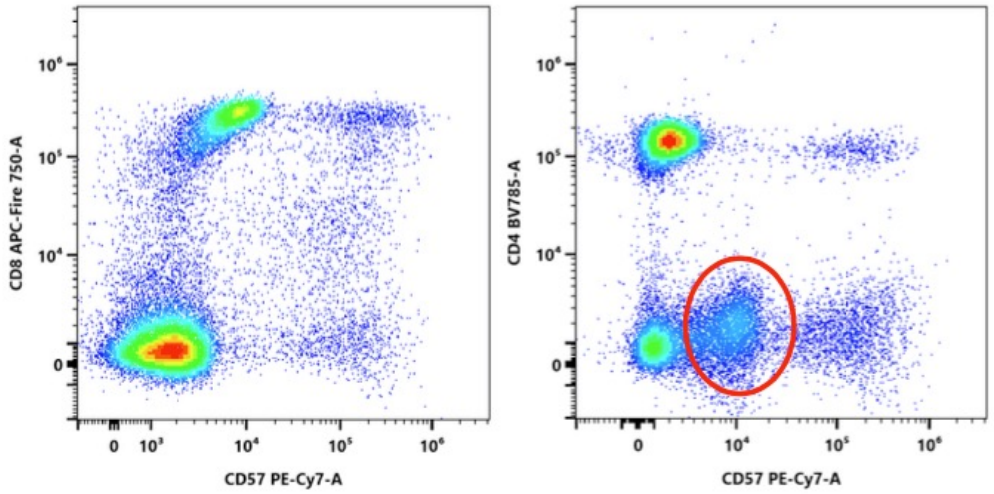
ChUG Cytometry

How to get accurate correction

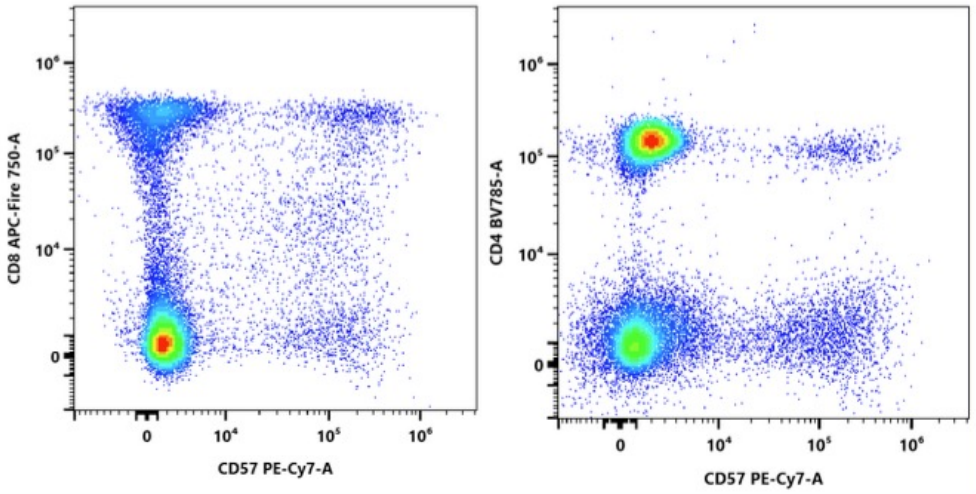


Check the correction every time!

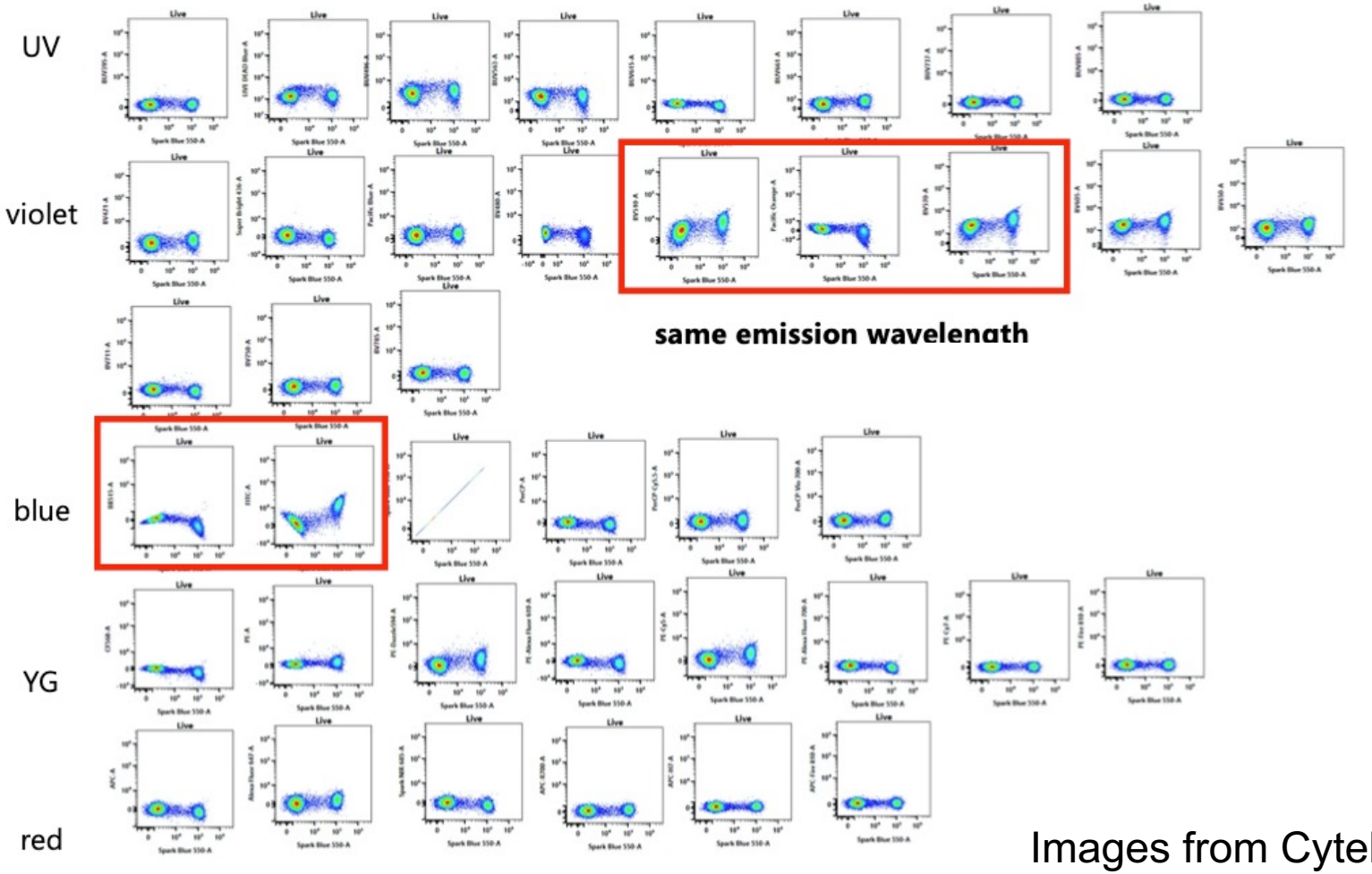
Data WITH Unmixing Error



Correct Unmixing



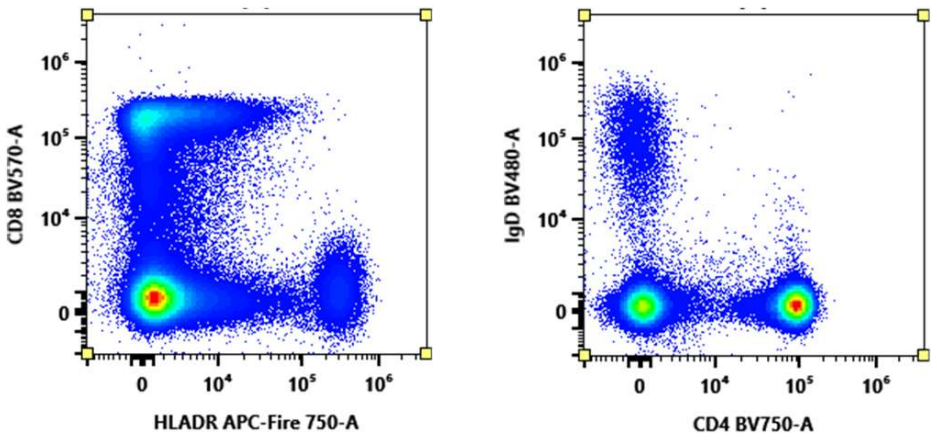
Always check correction with single stain data



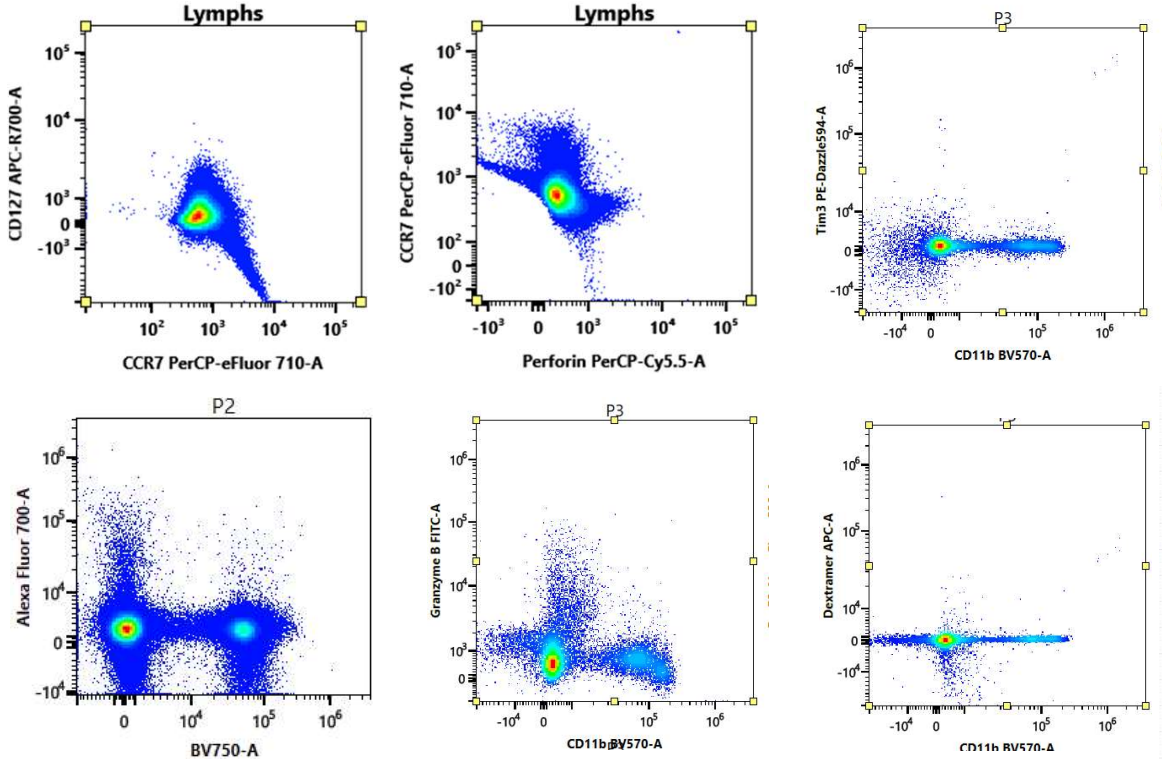
Images from Cytek

Unmixing errors can be seen in fully stained samples by looking at negative populations

Examples of correct unmixing:

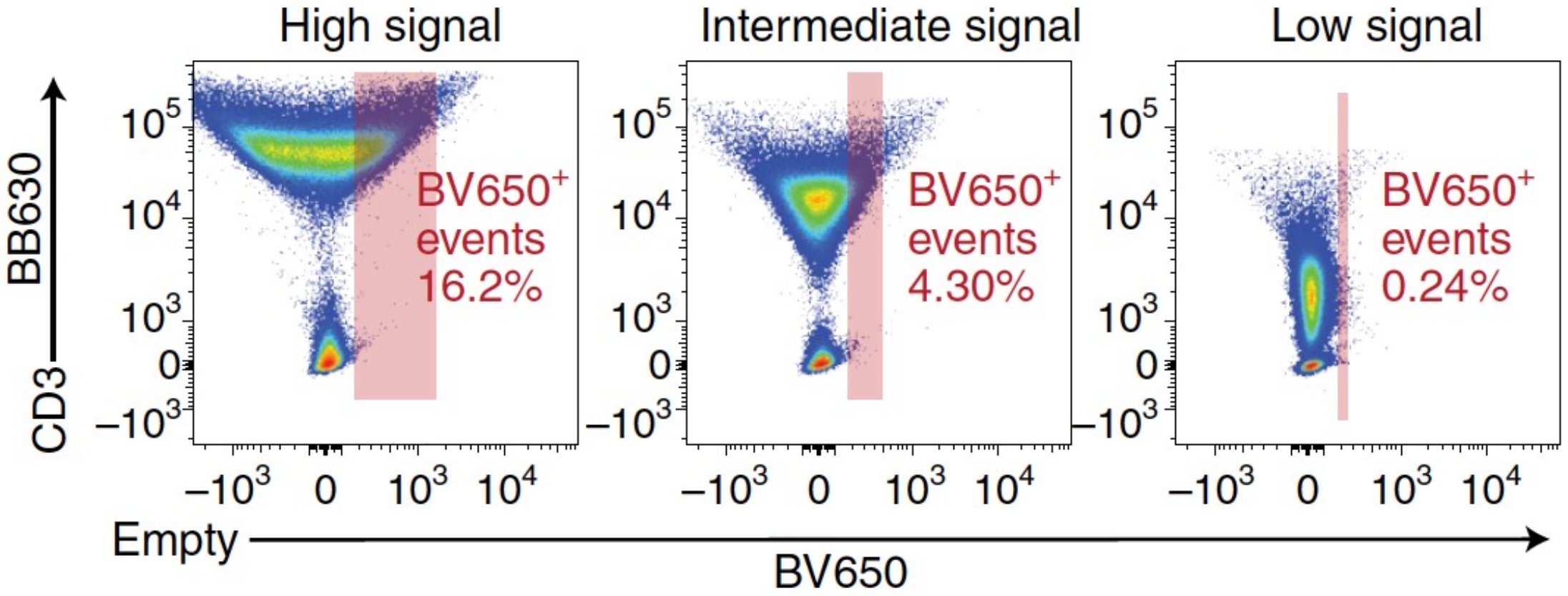


Examples of unmixing errors:



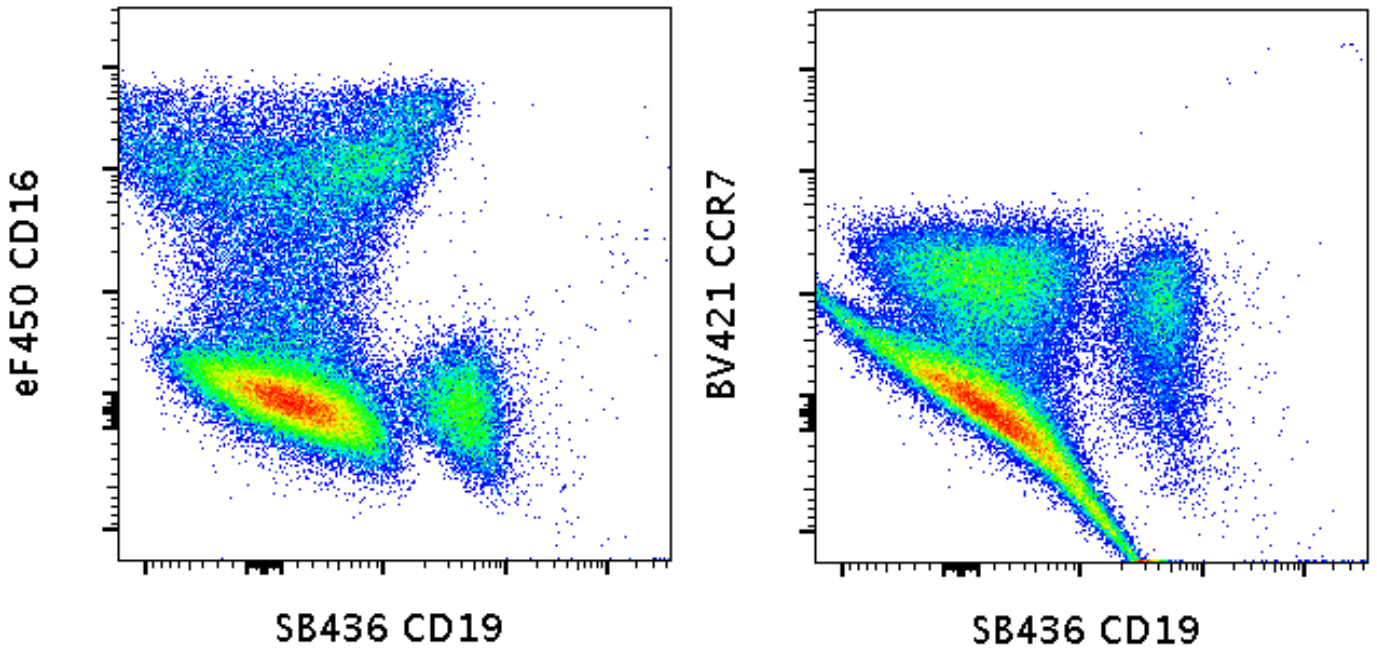
Images from Cytek

How should your data look like?



Liechti T et al. Nat. Immunol. 2021

Spectral flow weirdness



- Are these plots pretty? No.
- Am I going to make this a critically important figure in my paper? No.
- Can I gate on CD19^{+} cells? Yes!

What to do about unmixing errors

	Unmixing Errors?	
Single Stained Controls	YES	NO
Full Stained Sample	YES	YES

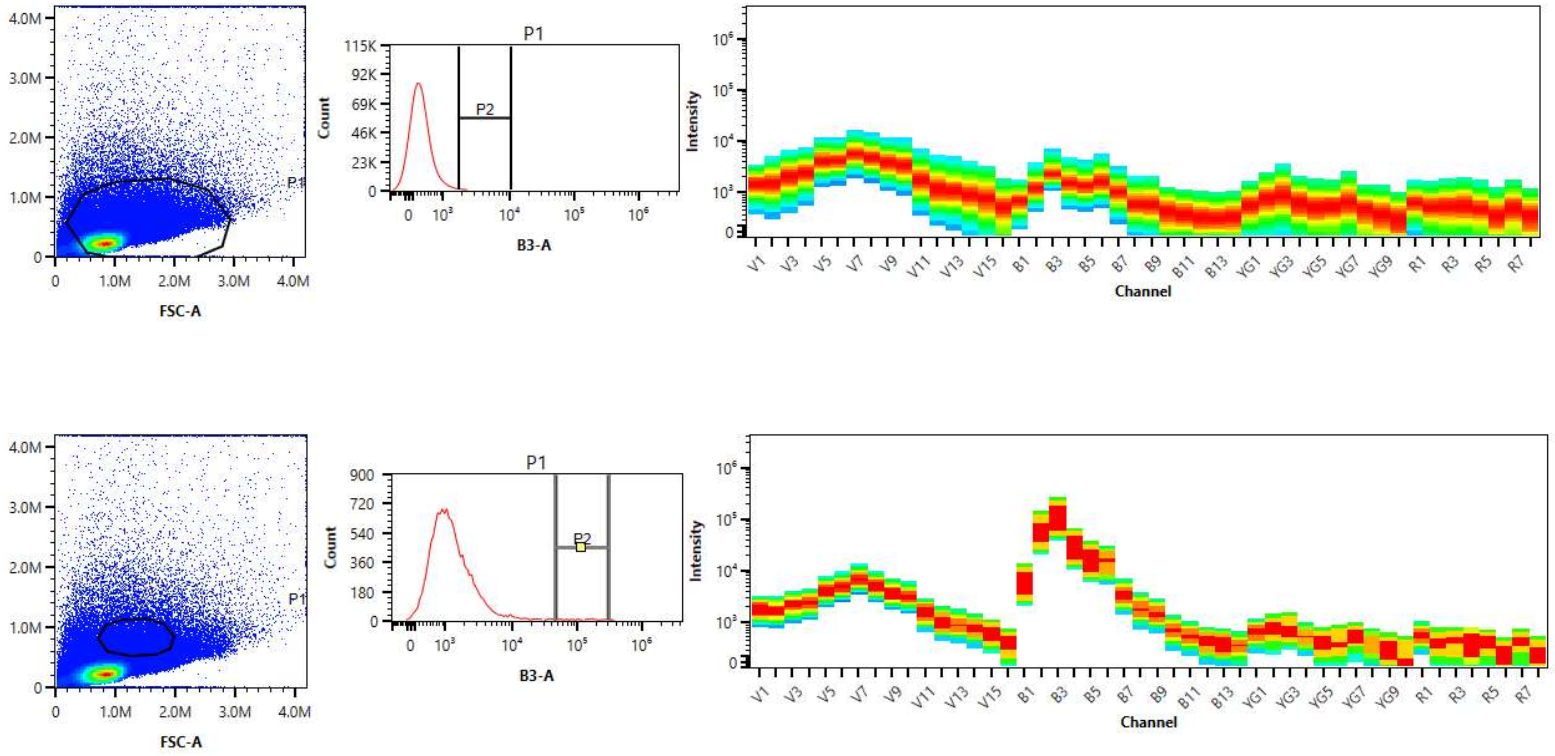
Revisit your gating in the wizard

- Gate only the cells of interest in FSCxSSC
  - Cells expressing your marker
  - Exclude irrelevant populations

- Tippytop

Reference Controls Troubleshooting Examples (5)

Where are the YFP positive cells ?

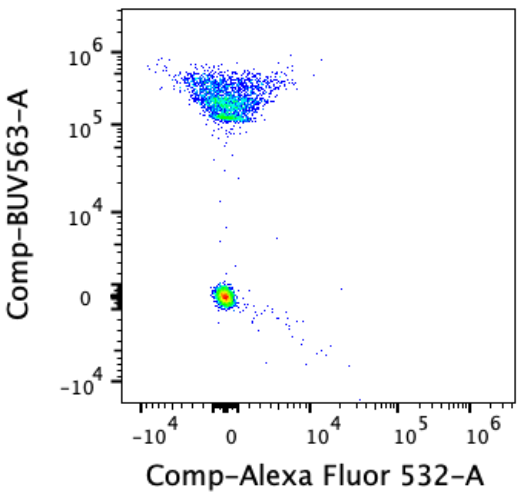


Slides from Cytek

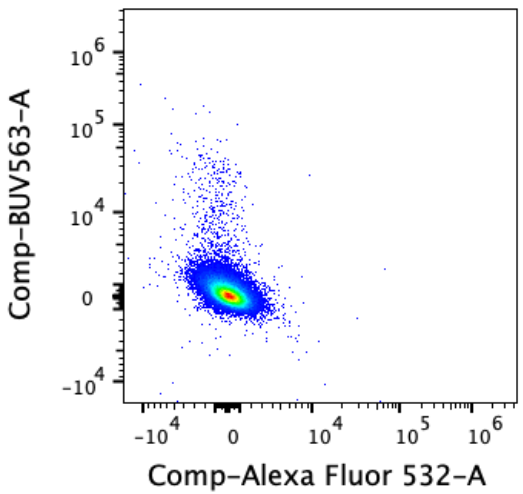
Revisit your controls

- If single stained controls are correctly unmixed but full stained is not, look deeper at your controls

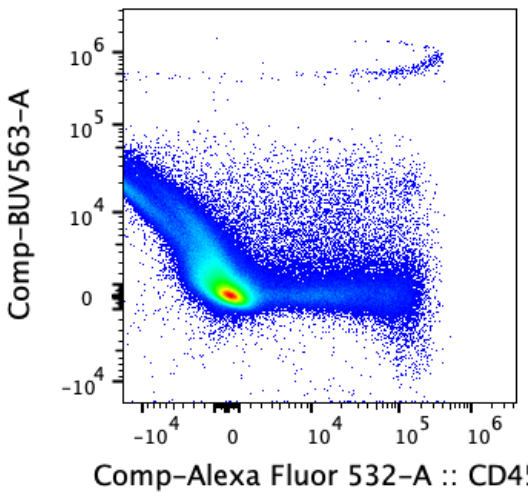
Single Stained Beads



Single Stained Cells

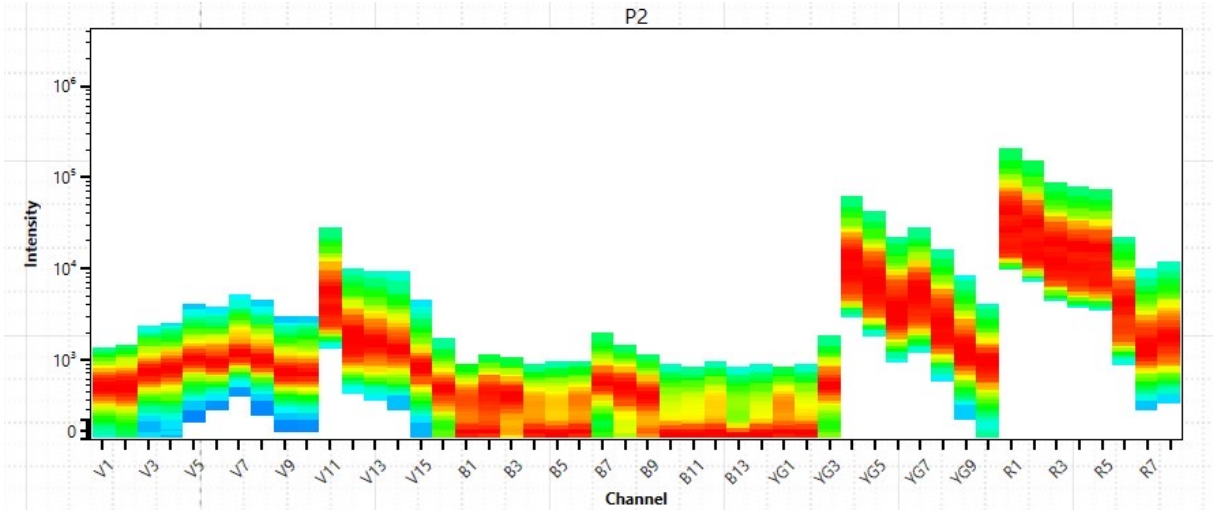


Full Stained Cells

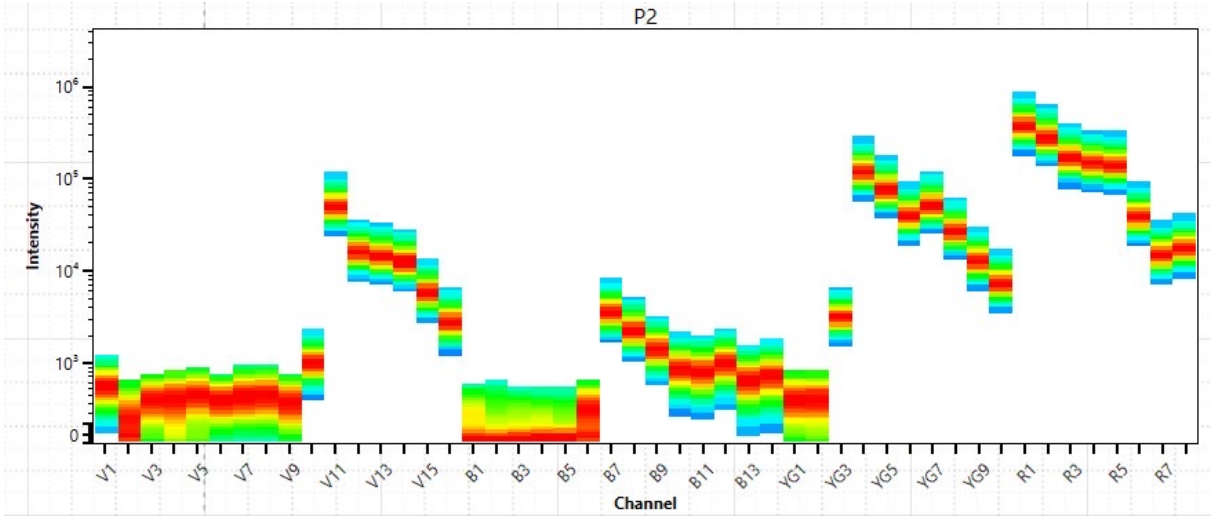


Should you use compensation beads or cells?

CD62L APC on cells

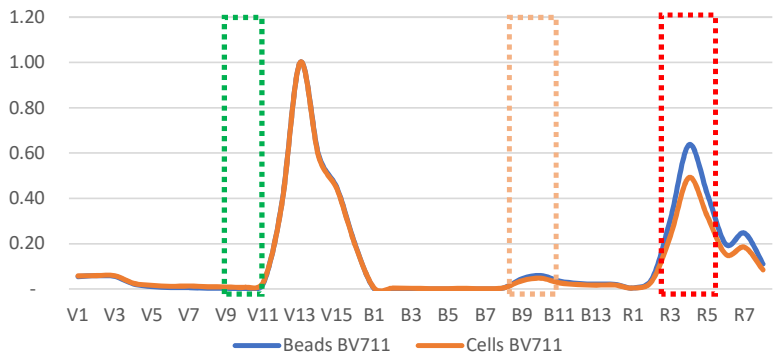


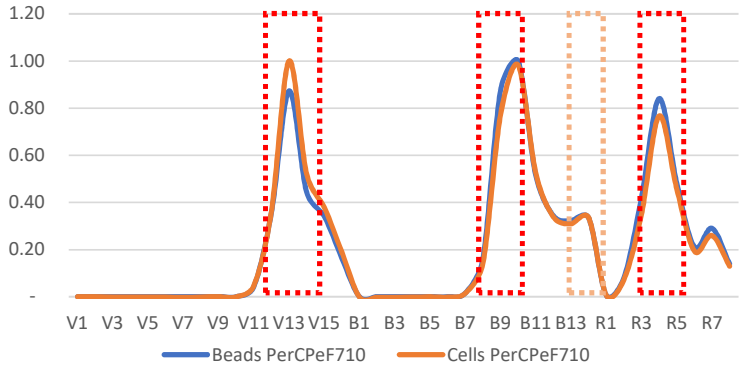
CD62L APC on beads



Compensation beads can give a cleaner signature than cells for some markers.
BUT beads are not cells, and sometimes beads do not provide accurate unmixing.

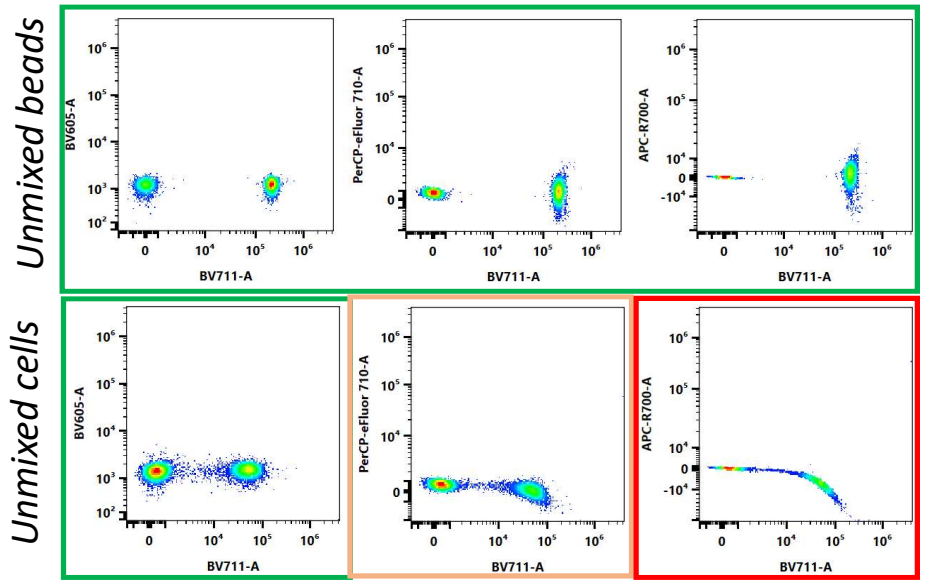
Spectrum Mismatch = Unmixing Inaccuracy

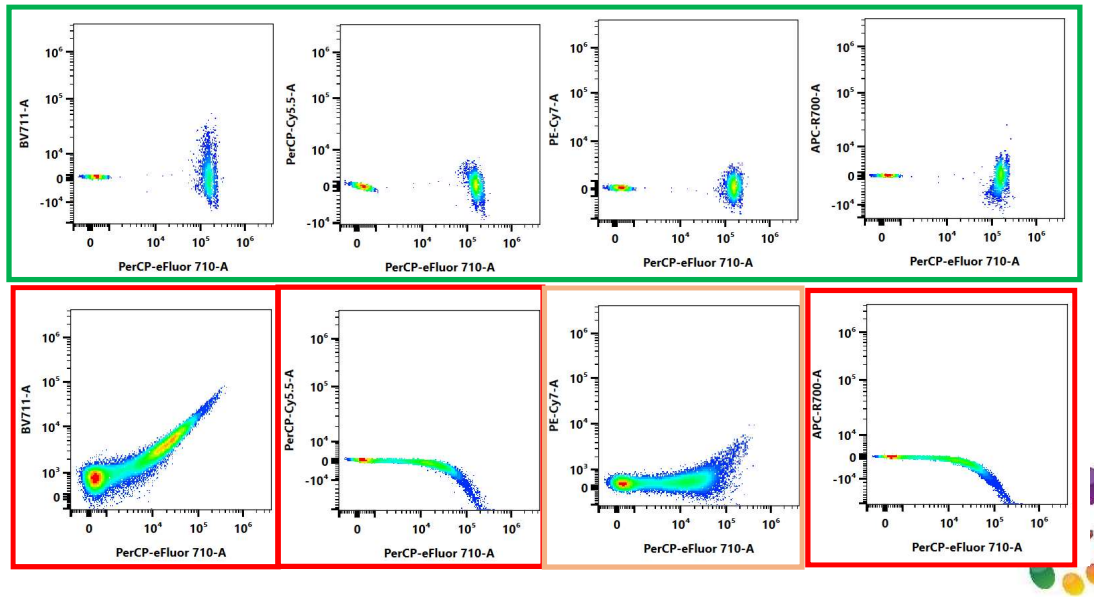




Use BV711 beads as control

Use PerCP-eFluor 710 beads as control





Slides from Cytek

Recommended Assay Procedure:

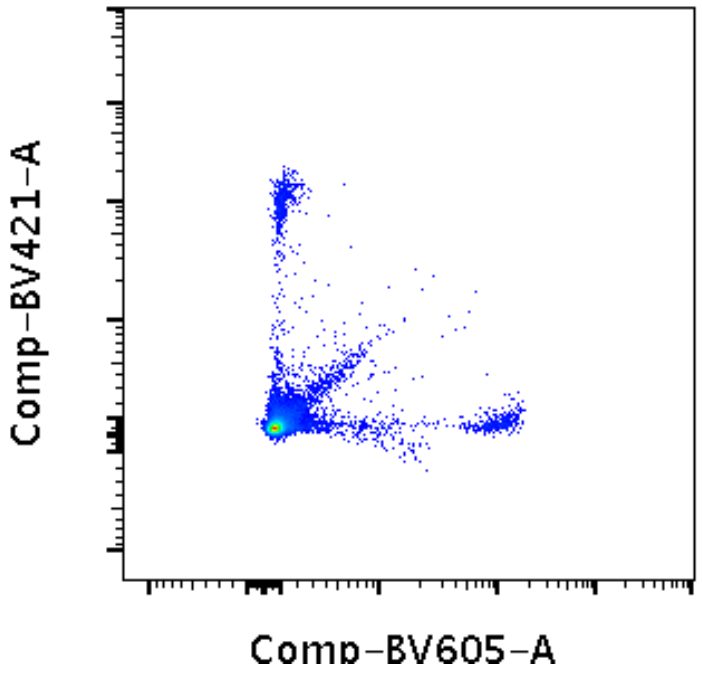
BD™ CompBeads can be used as surrogates to assess fluorescence spillover (Compensation). When fluorochrome conjugated antibodies are bound to BD CompBeads, they have spectral properties very similar to cells. However, for some fluorochromes there can be small differences in spectral emissions compared to cells, resulting in spillover values that differ when compared to biological controls. It is strongly recommended that when using a reagent for the first time, users compare the spillover on cells and BD CompBead to ensure that BD CompBeads are appropriate for your specific cellular application.

When compensating dyes in this spectral range (such as Horizon™ V500 and AmCyan), the most accurate compensation can be obtained using single stained cellular controls. Due to spectral differences between cells and beads in this channel, using BD CompBeads can result in spillover errors for V500 and AmCyan reagents. Therefore, the use of BD CompBeads or BD CompBeads Plus to determine spillover values for these reagents is not recommended. Different V500 reagents (e.g. CD4 vs. CD45) can have slightly different fluorescence spillover therefore, it may also be necessary to use clone specific compensation controls when using these reagents.

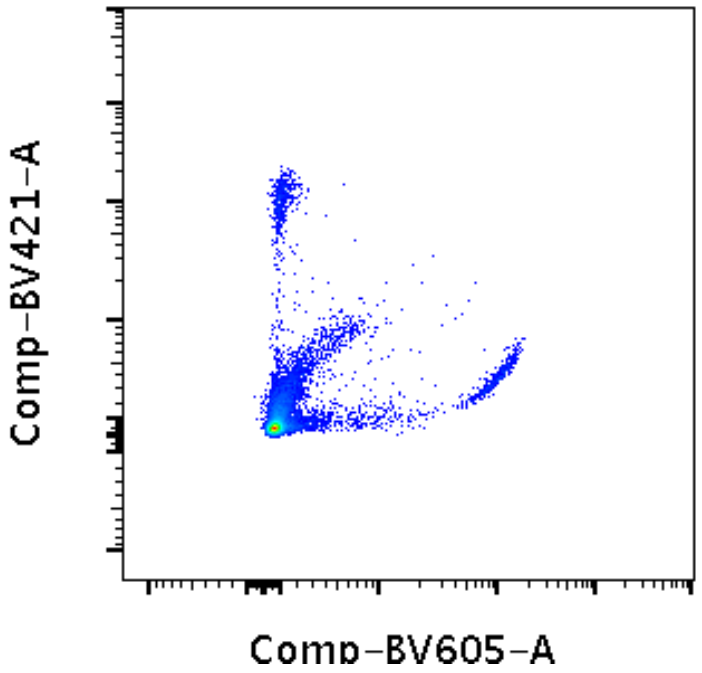
Due to spectral differences between labeled cells and beads, using BD™ CompBeads can result in incorrect spillover values when used with BD Horizon BUV661 reagents. Therefore, the use of BD CompBeads or BD CompBeads Plus to determine spillover values for these reagents is not recommended. Different BUV661 reagents (eg, CD4 vs. CD45) can have slightly different fluorescence spillover therefore, it may also be necessary to use clone-specific compensation controls when using these reagents.

Slides from Cytek

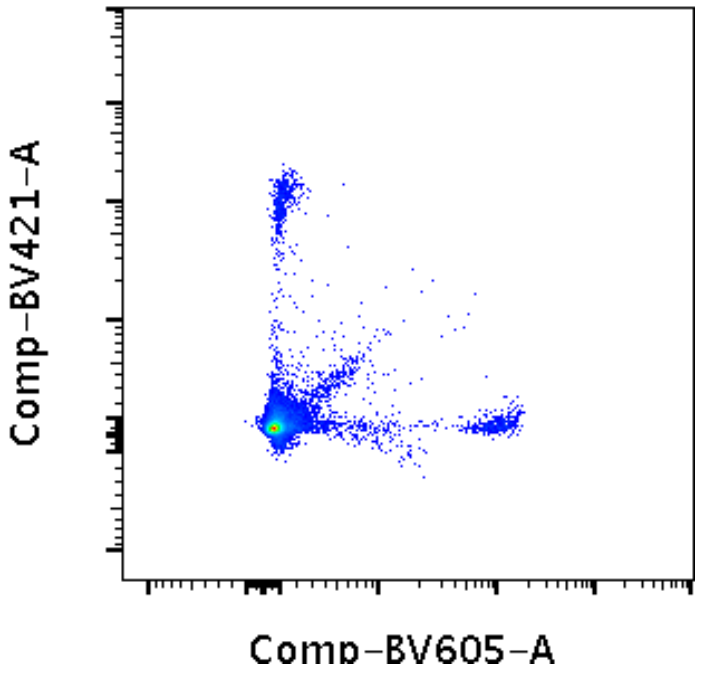
Unmixing a “fully stained” sample with different reference controls (mixed multiple CD4 single stains)



Cells



AbC Total



UltraComp

”Full stain” cells were unmixed with:

New Product Improved Comp Beads (Summer 2020)

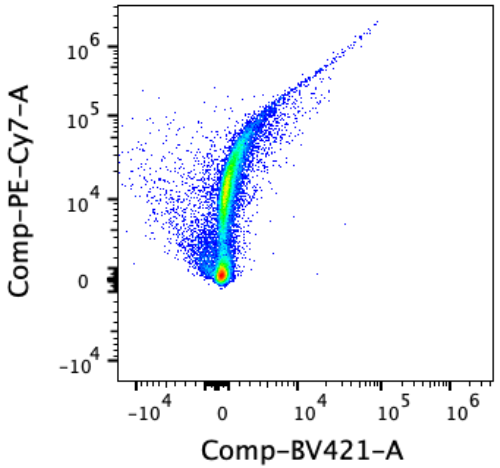
- UltraComp eBeads™ Plus Compensation Beads
  - ThermoFisher 01-3333-41, 01-3333-42
  - “Unlike the first generation beads, UltraComp eBeads Plus compensate in a manner similar to cells when used with Brilliant Violet 785/786-, Brilliant Violet 711-, Super Bright 780- and Super Bright 702-conjugated antibodies.”

Slingshot!

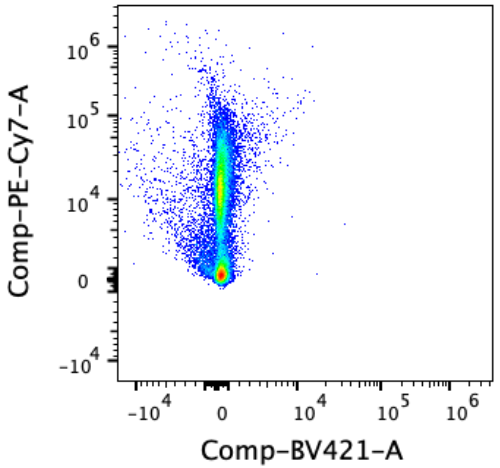
- Synthetic cells
- ViaComp
- SpectraComp

Apply Manual Compensation on top of unmixed data?

Single Stained Cells

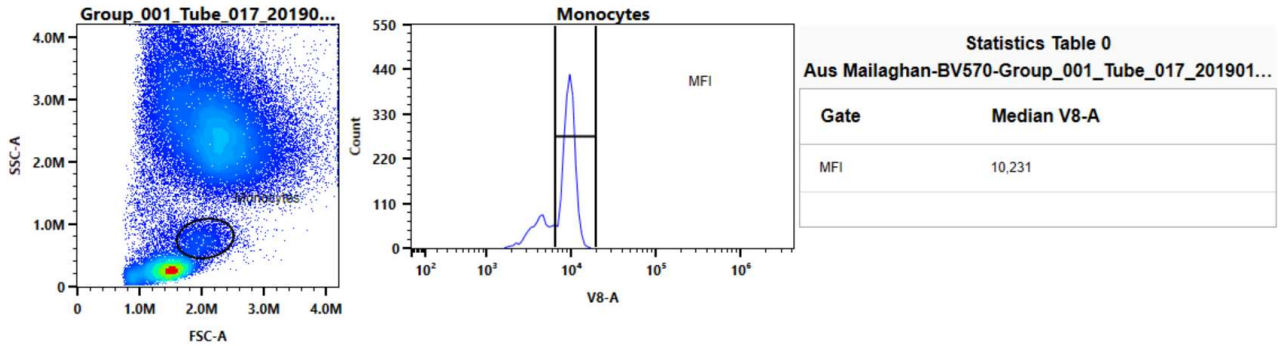


Single Stained Cells

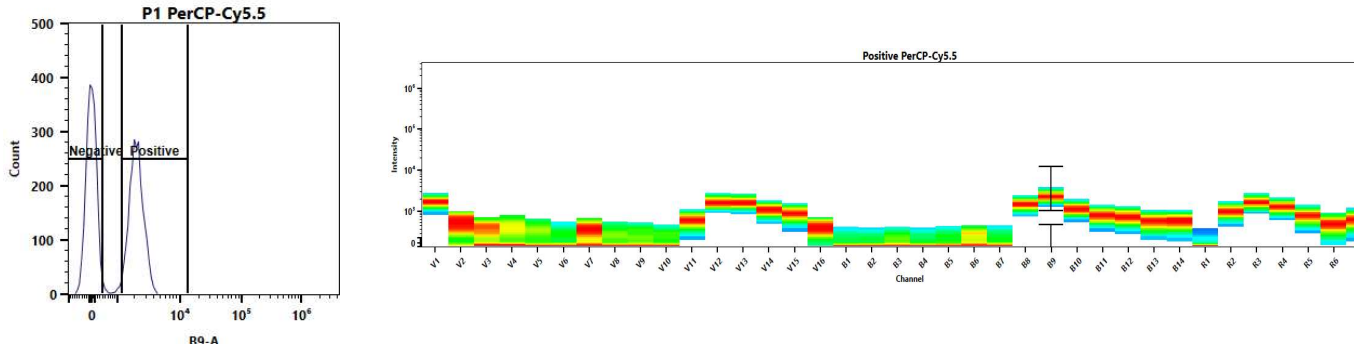


Reference Controls Troubleshooting Examples (1)

Case 1



Case 2

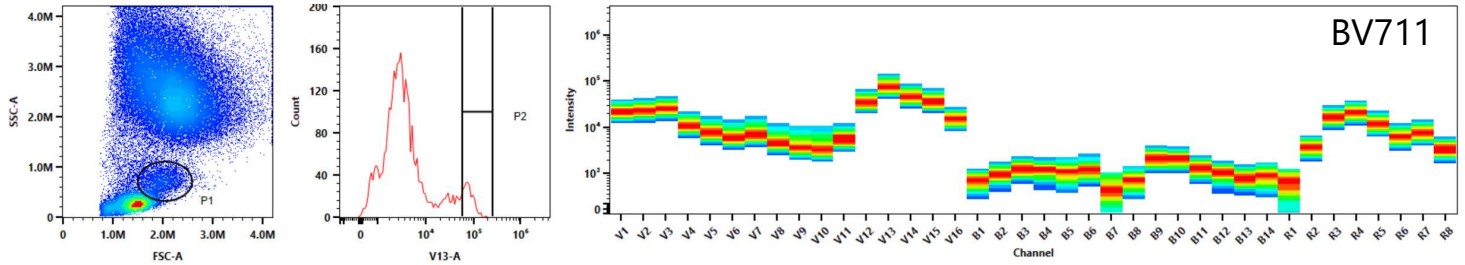


These controls are too dim, not meeting Rule 1 of what a good control should be

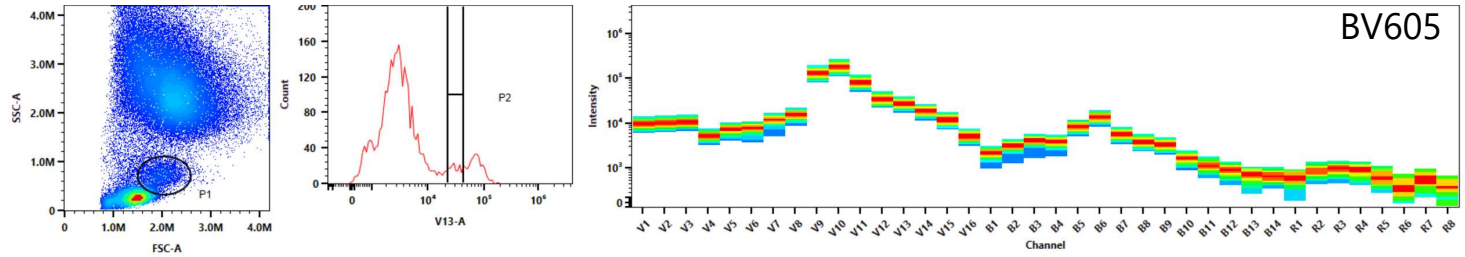
Slides from Cytek

Reference Controls Troubleshooting Examples (3)

Gated on Brightest Positive Peak



Gated on Dimmer Positive Peak

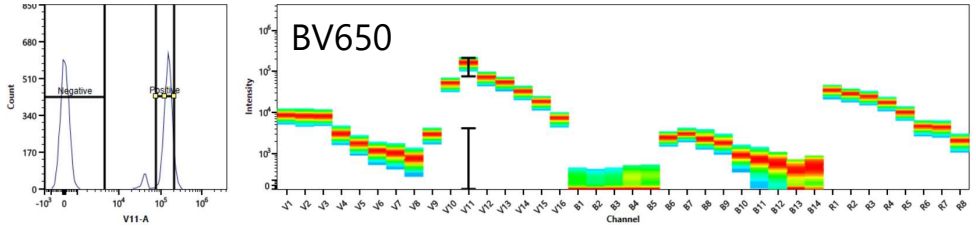


Carry over?

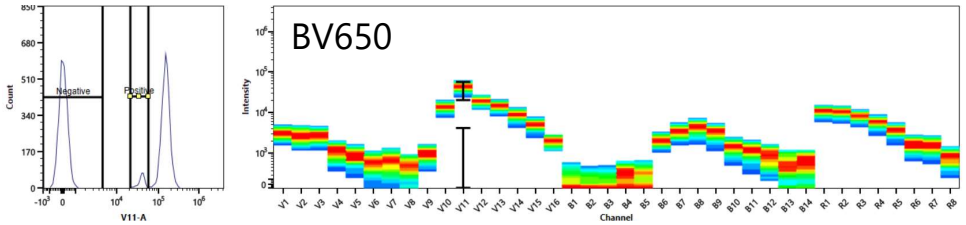
Slides from Cytek

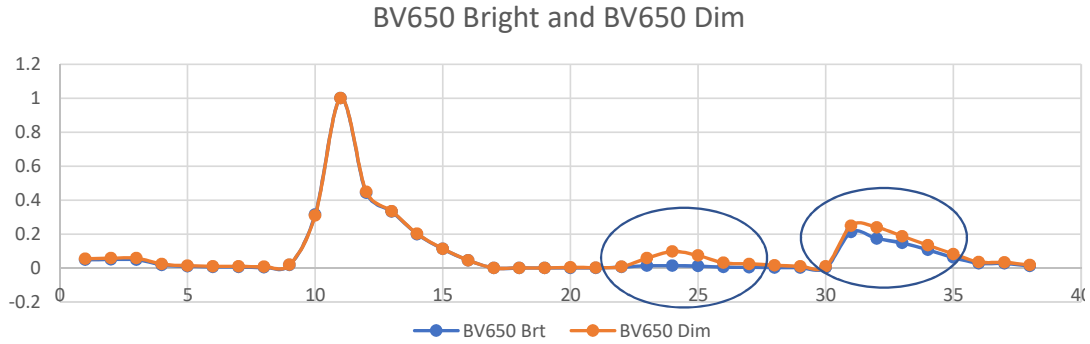
Reference Controls Troubleshooting Examples (4)

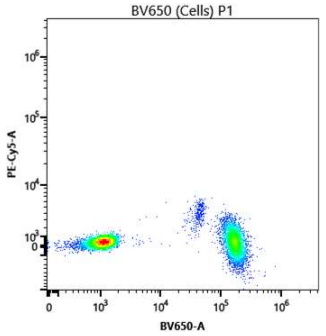
Gated on Brightest Positive Peak



Gated on Dimmer Positive Peak







The intermediate population appears uncompensated when looking at BV650 vs PE-Cy5

Slides from Cytek

Testing your pannel

Unlikely to work form the get-go

Don’t try your full pannel on your first try!!

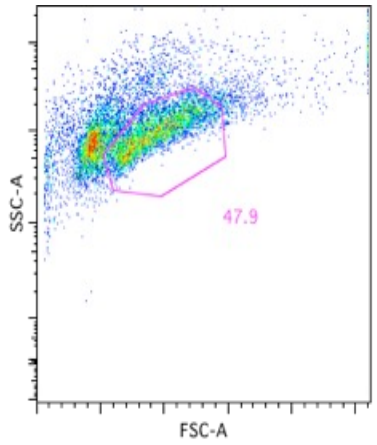
FMO to the rescue – bad panel experiment

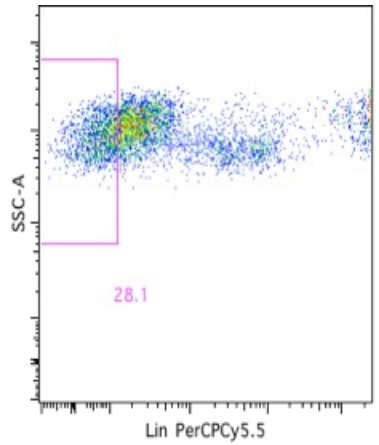
- Untitrated everything
- Heavy in the far red

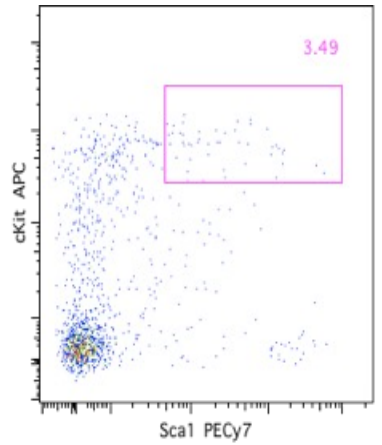
Fluorophore	Marker
BV510	KLRG1
FITC	CD3
PE	CD25
PerCP-Cy5.5	CD4
PE-Dazzle594	CD24
PE-Cy5	ICOS
PE-Cy7	CD62L
APC	Thy1.2
AF700	TCRb
APC-eFluor780	CD8
Zombie NIR	Live/dead

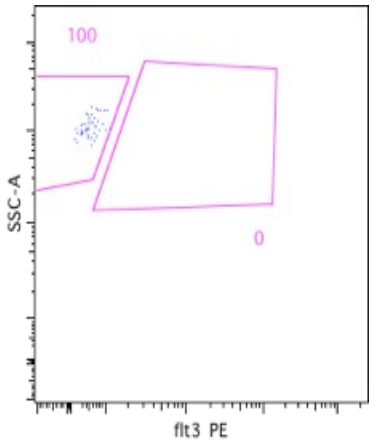
Deciding on gates: FMO Controls

FMO PE

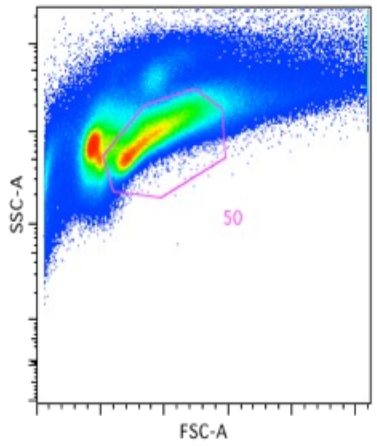


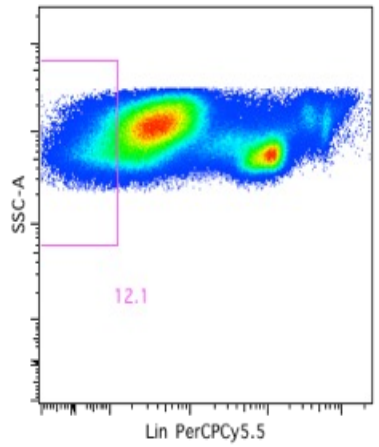


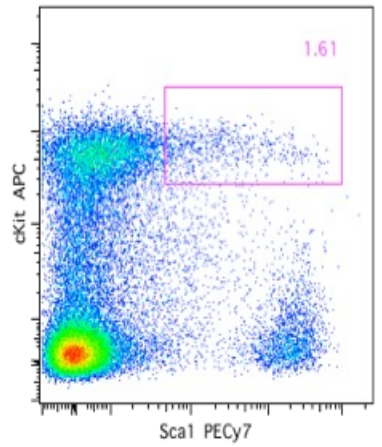


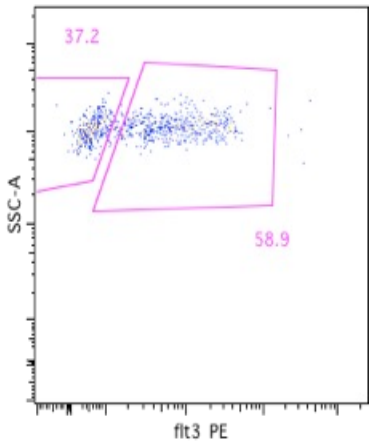


Sample

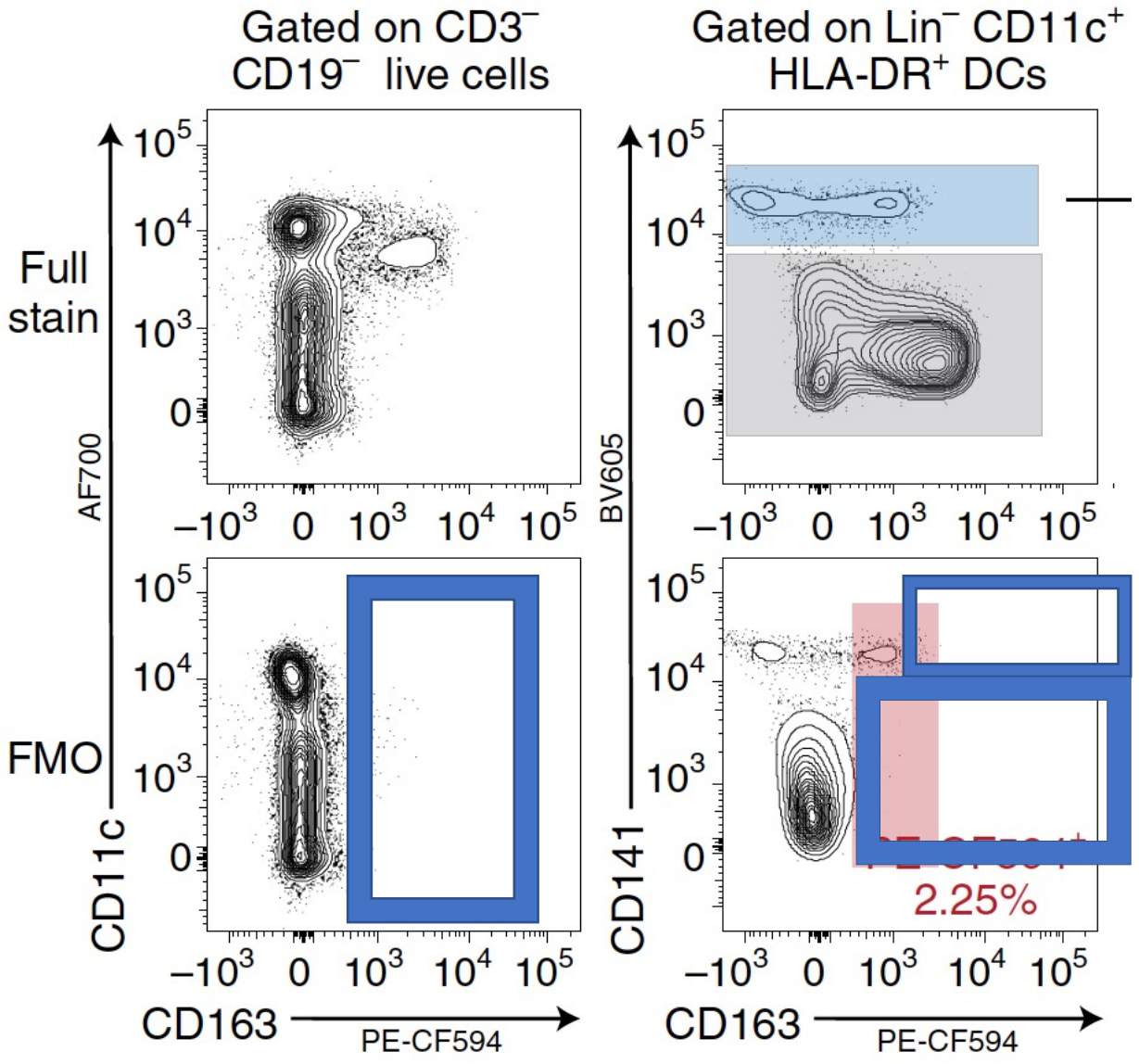






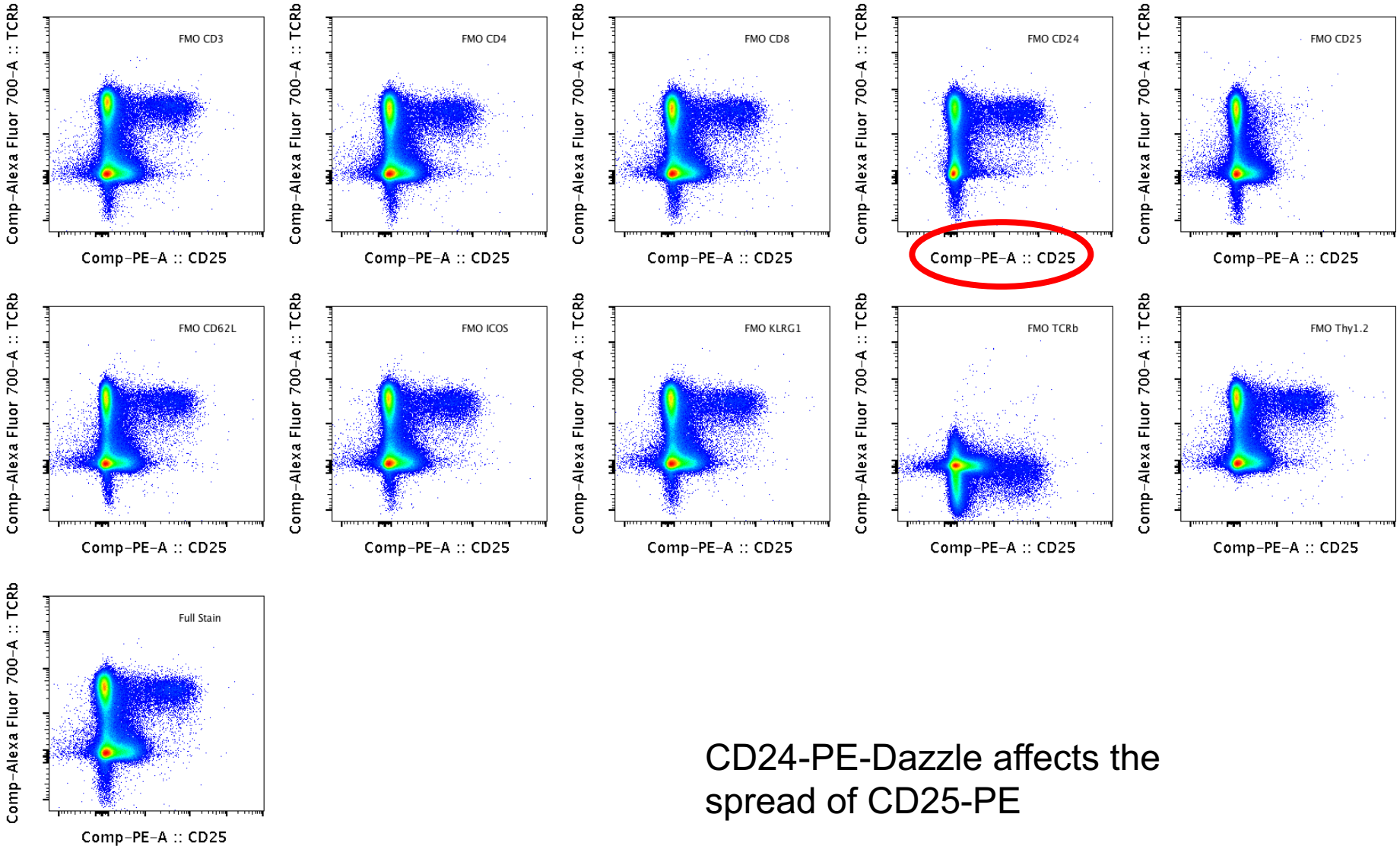


FMOs and brightness



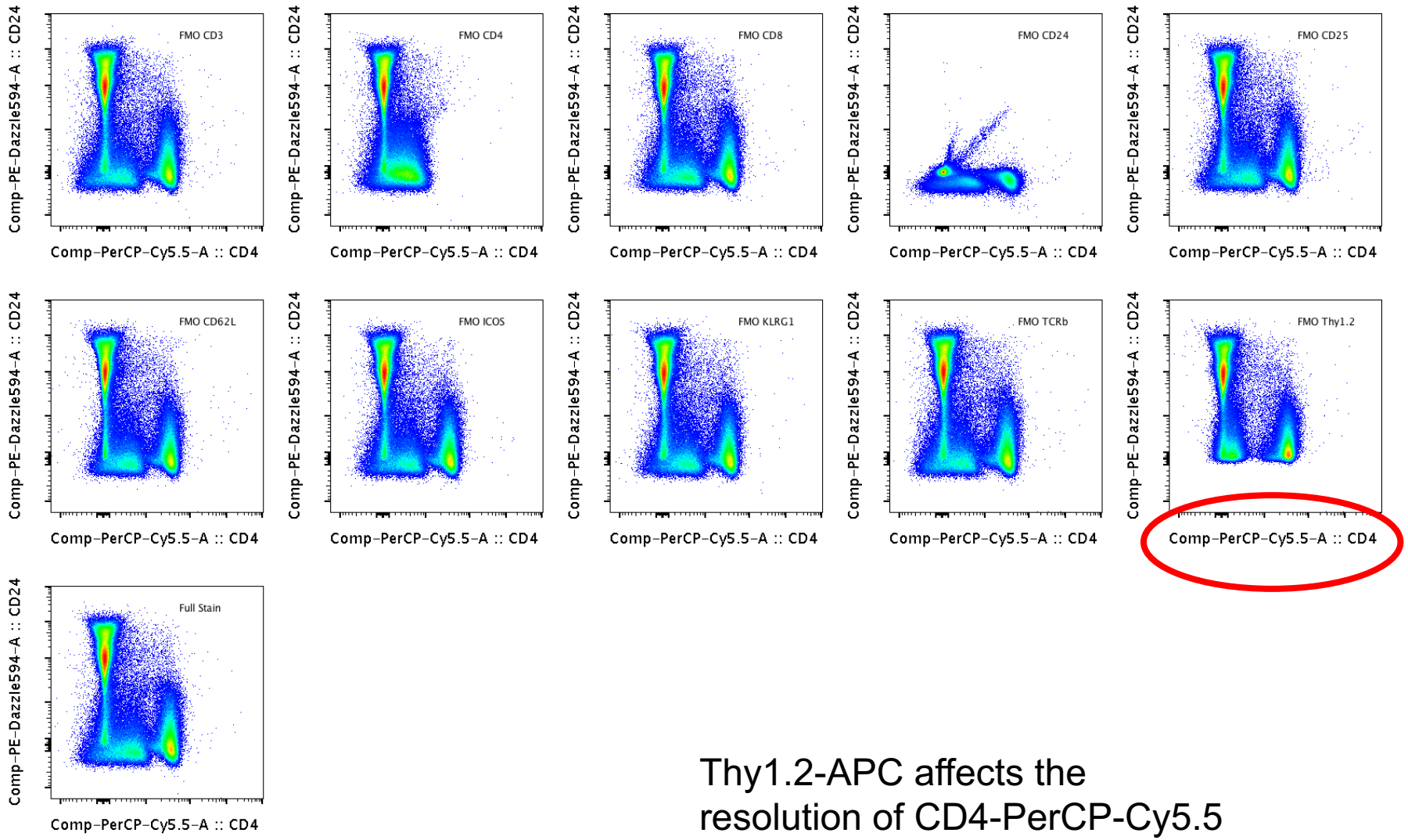
Liechti T et al. Nat. Immunol. 2021

Using FMOs to determine issues with the panel



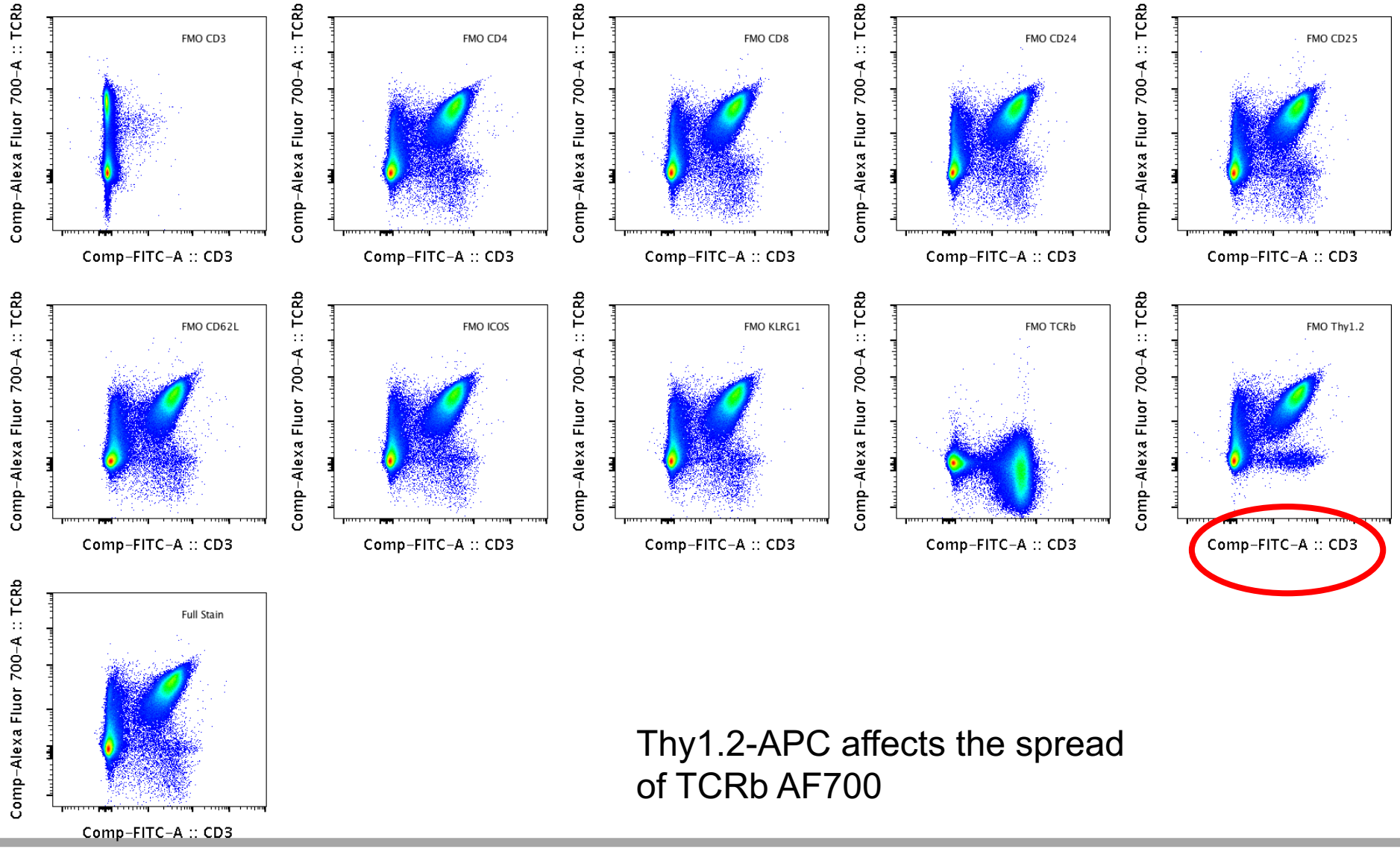
CD24-PE-Dazzle affects the spread of CD25-PE

Using FMOs to determine issues with the panel



Thy1.2-APC affects the resolution of CD4-PerCP-Cy5.5

Using FMOs to determine issues with the panel

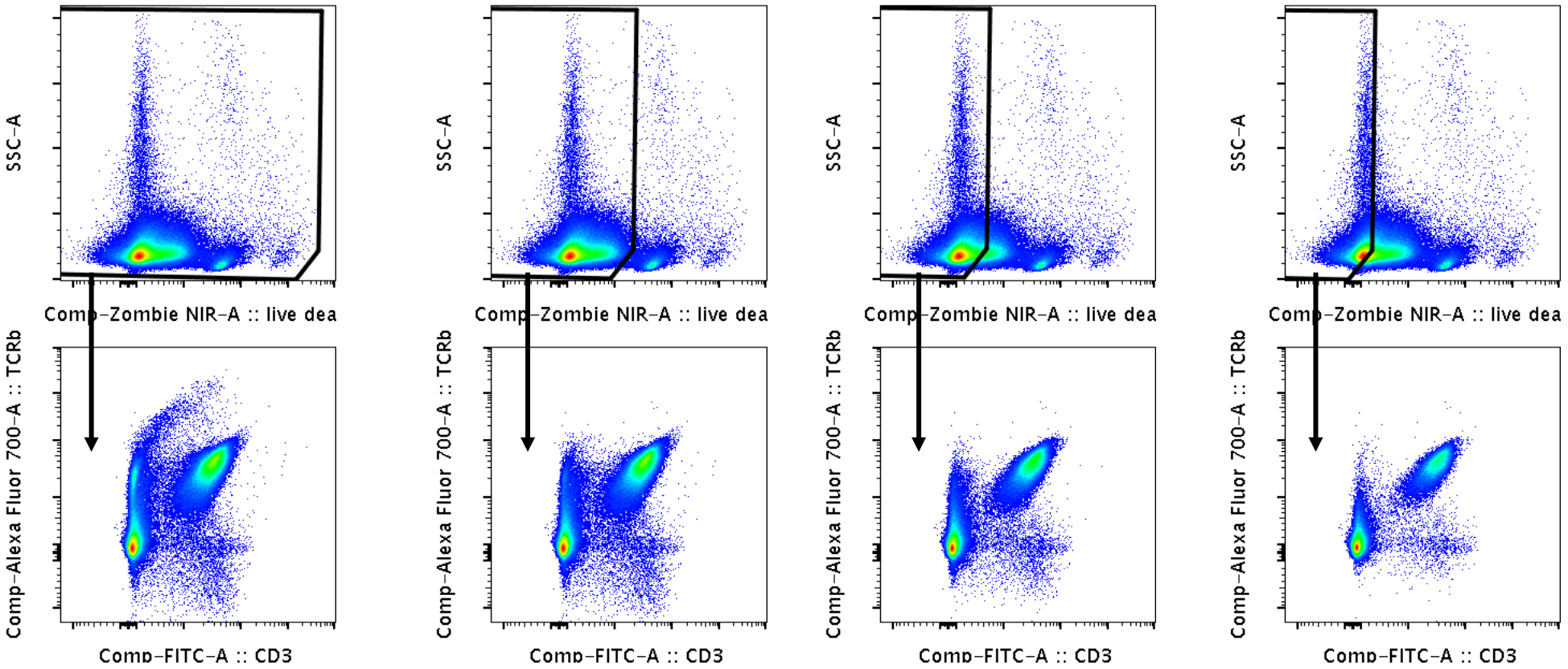


Thy1.2-APC affects the spread of TCRb AF700

Zombie NIR seems to affect AF700

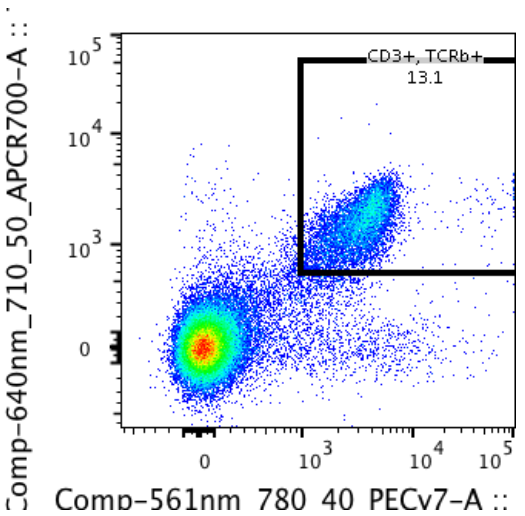
Decreasing live/dead gate

TCRb^{+}CD3^{-} cells should not exist!!

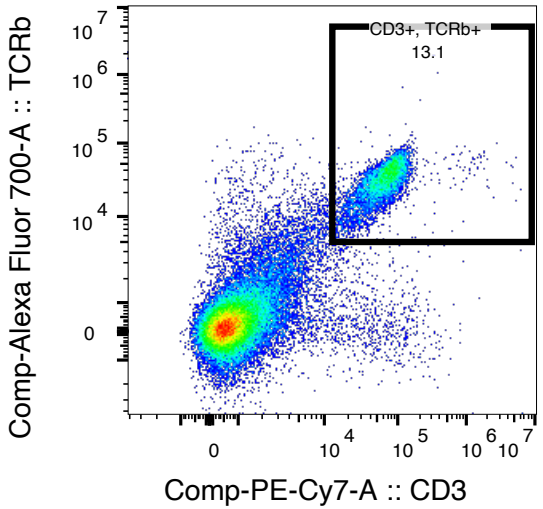


Mystery TCRb+CD3- population disappears with switch to Zombie Aqua, but TCRb FMO is not as helpful

Fortessa 4-15

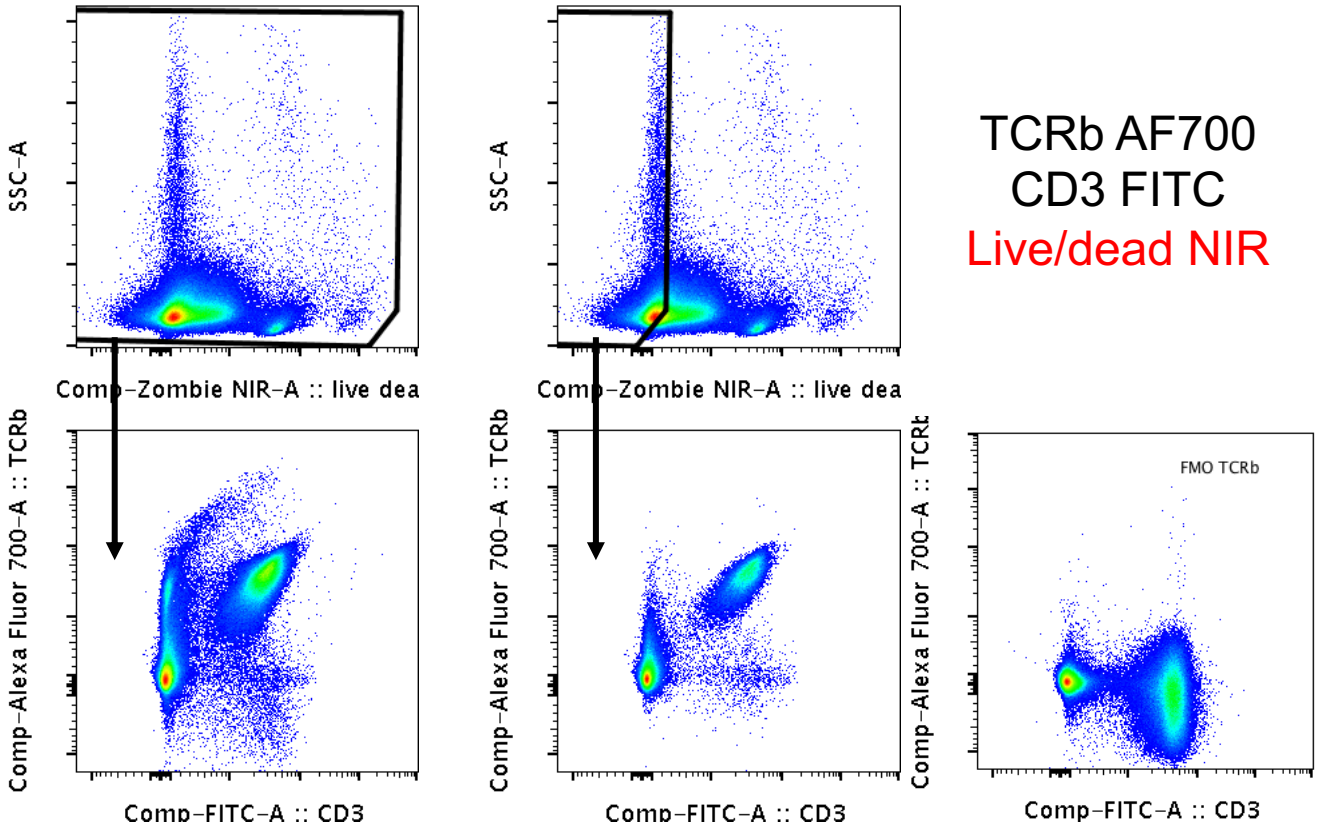


Aurora



TCRb AF700
CD3 PE-Cy7
Live/dead Aqua

Aurora



TCRb AF700
CD3 FITC
Live/dead NIR

This FMO is not helpful, but other FMOs work as expected