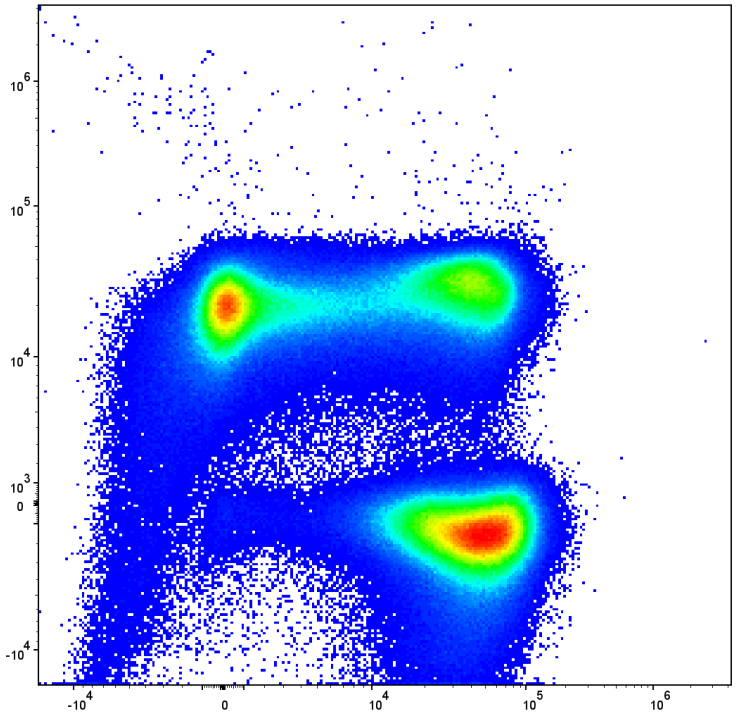
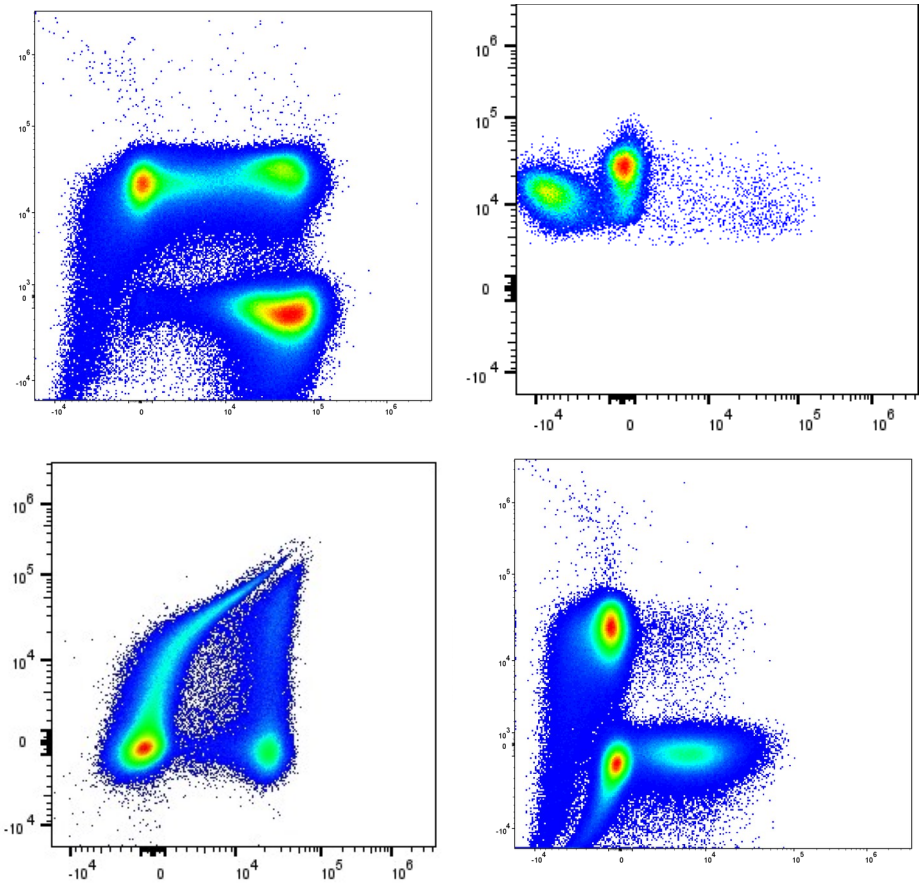


Troubleshooting unmixing controls (both single-color and unstained)

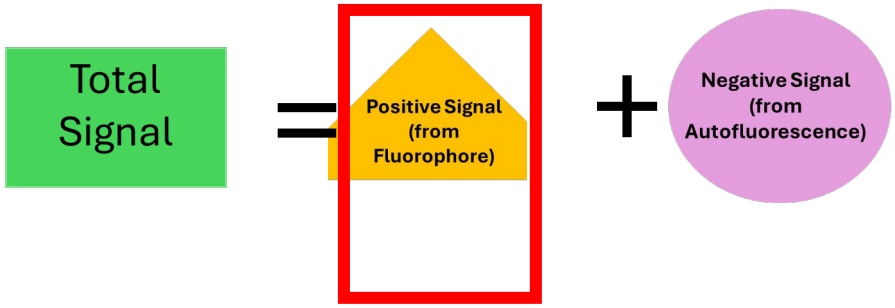
David Rach
University of Maryland, Baltimore
September 17th, 2026

You have encountered an unmixing error (s)



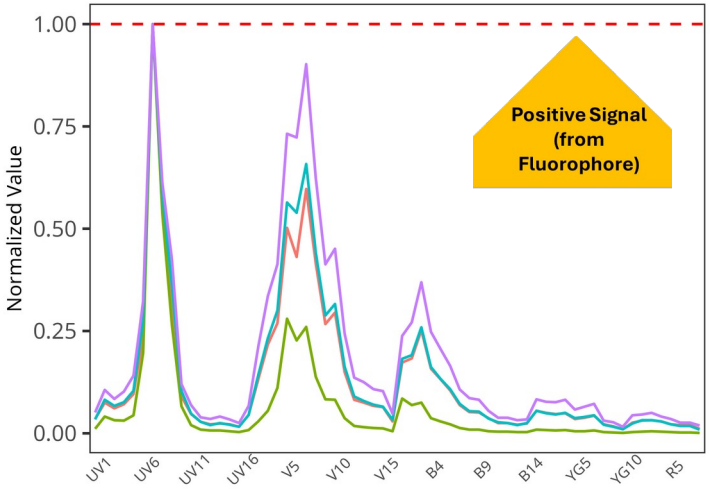
What do you do next?

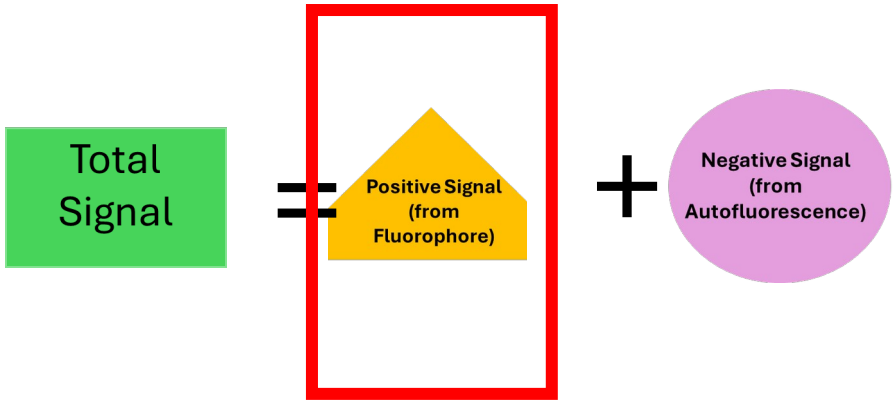
Unmixing errors occur when:



Fluorescent signature of your unmixing control doesn't match the fluorescent signature of that same fluorophore on your full-stained sample

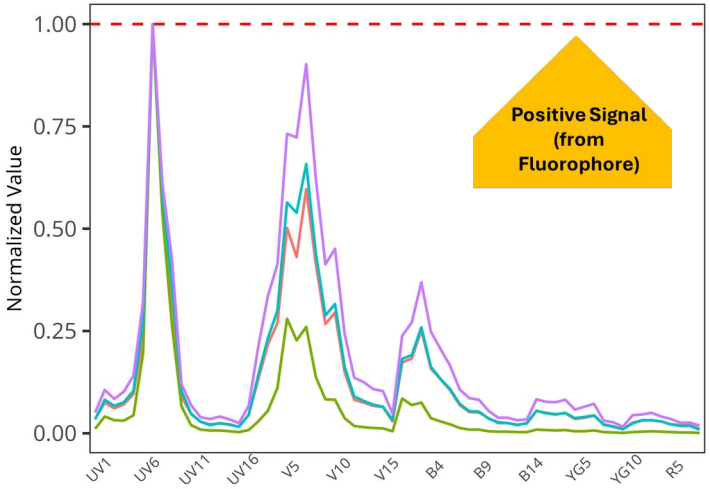
Unmixing errors occur when:

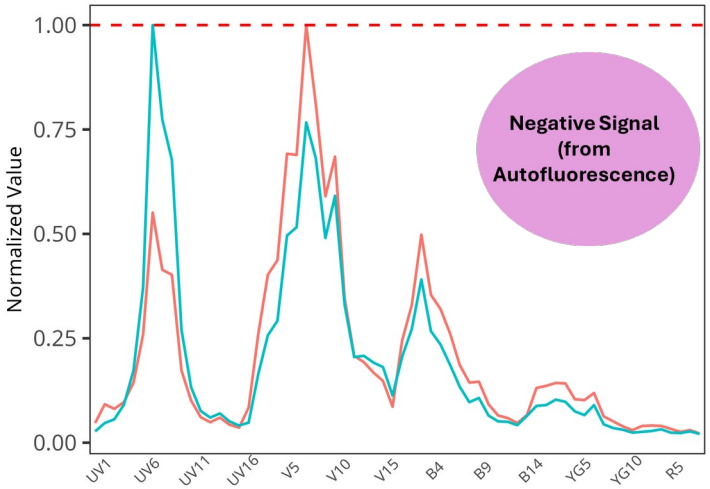


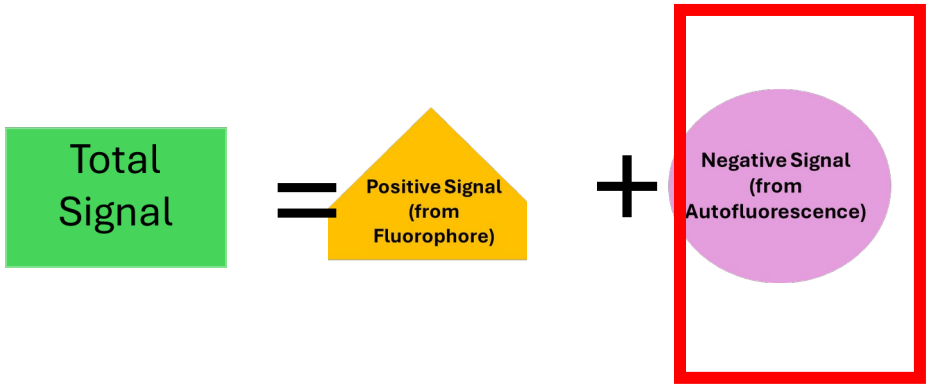


Fluorescent signature of your unmixing control doesn't match the fluorescent signature of that same fluorophore on your full-stained sample

Unmixing errors occur when:

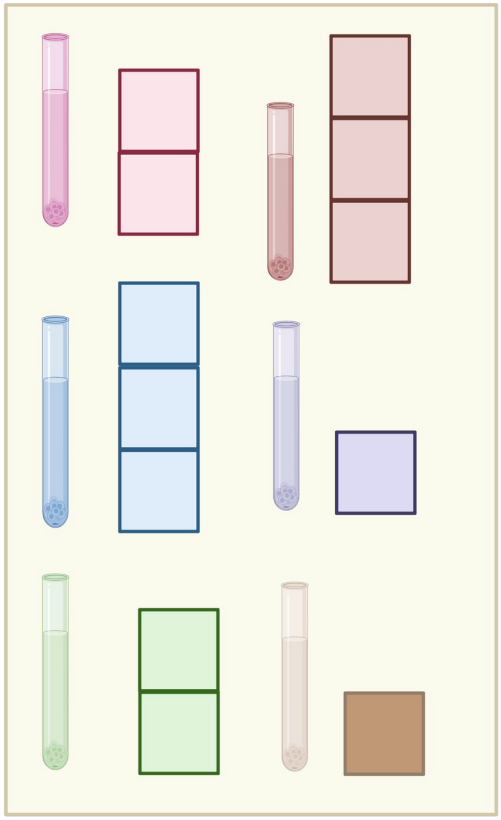






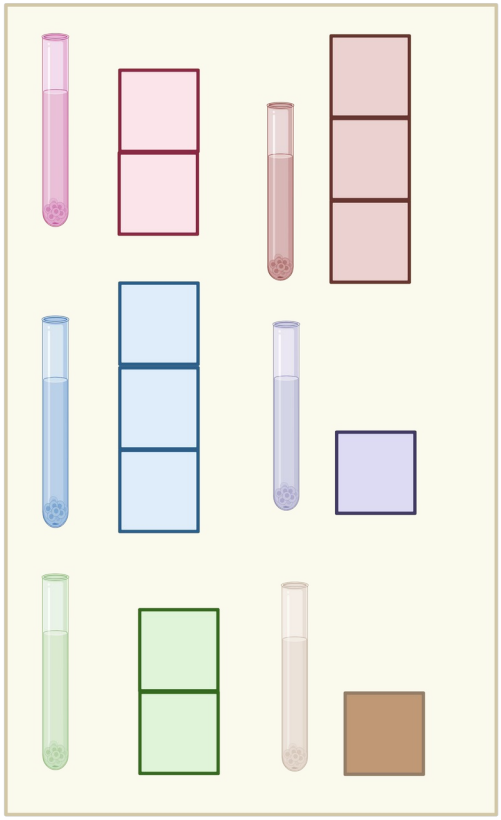
Fluorescent signature of your unmixing control doesn't match the fluorescent signature of that same fluorophore on your full-stained sample

With a successful unmixing

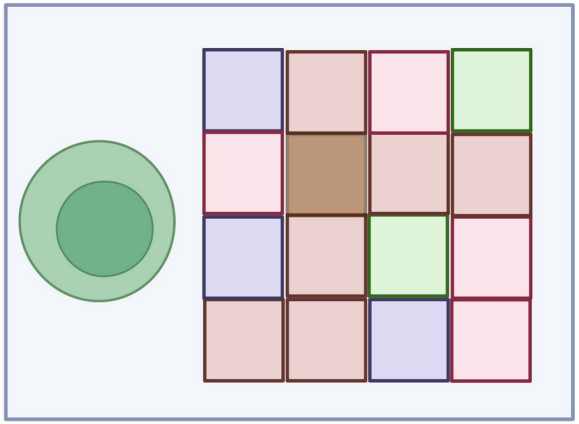


Unmixing Controls (Single Color and Unstained)

With a successful unmixing

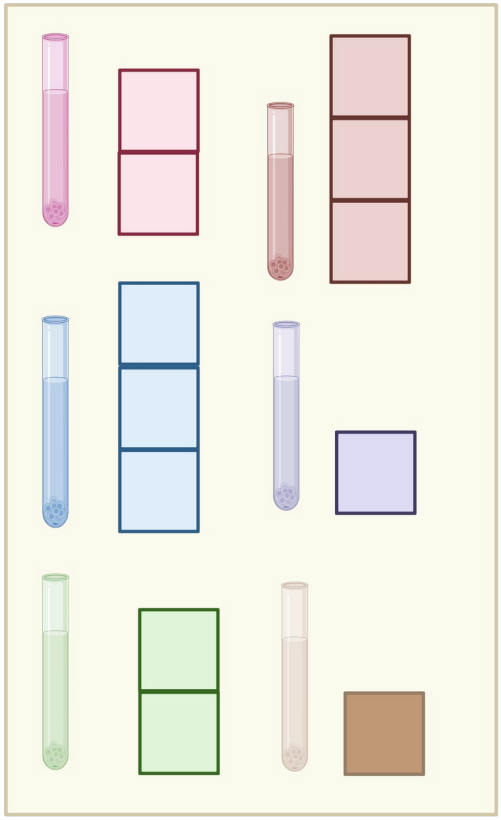


Unmixing Controls
(Single Color and
Unstained)

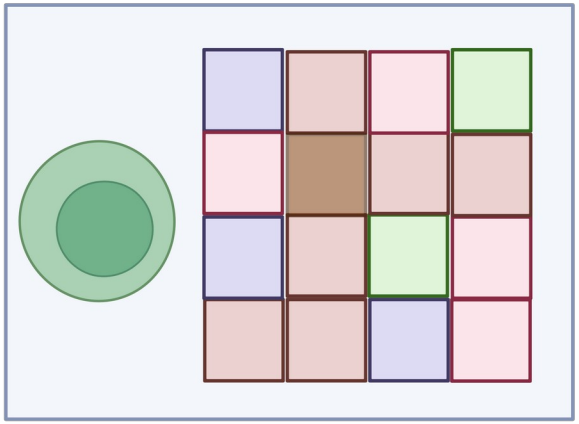


Single cell from
full-stained sample

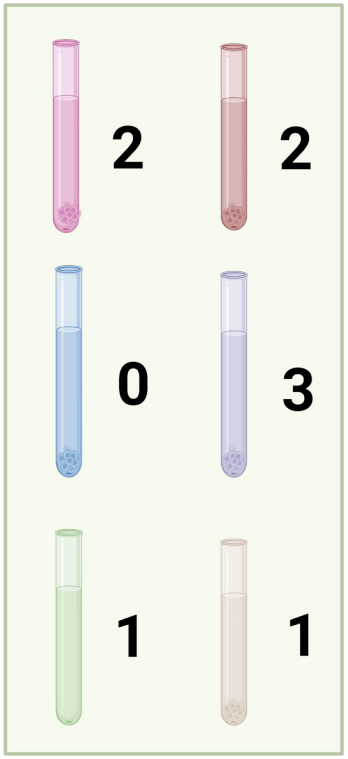
With a successful unmixing



Unmixing Controls
(Single Color and
Unstained)

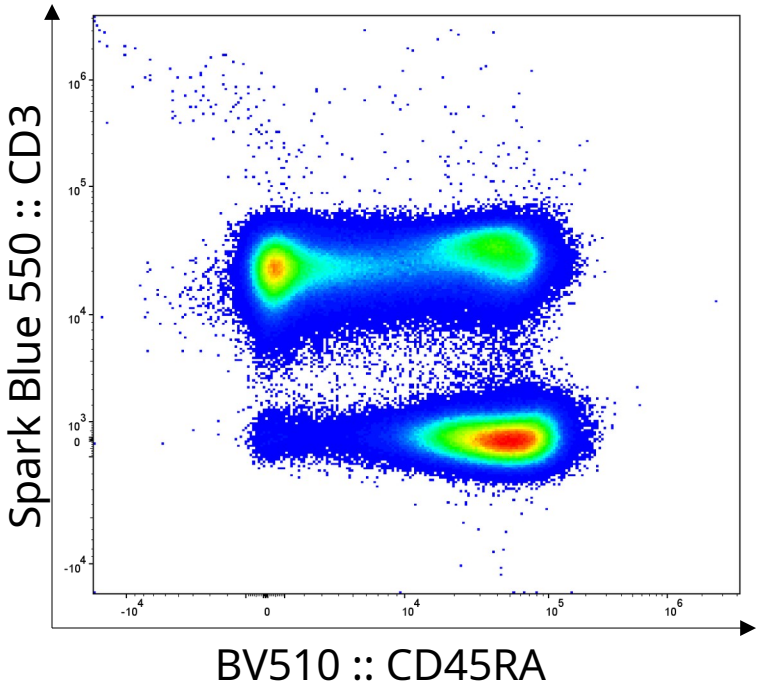


Single cell from
full-stained sample

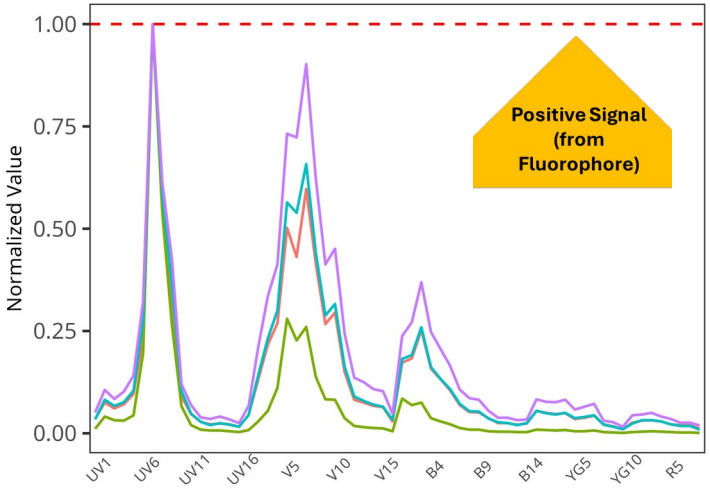


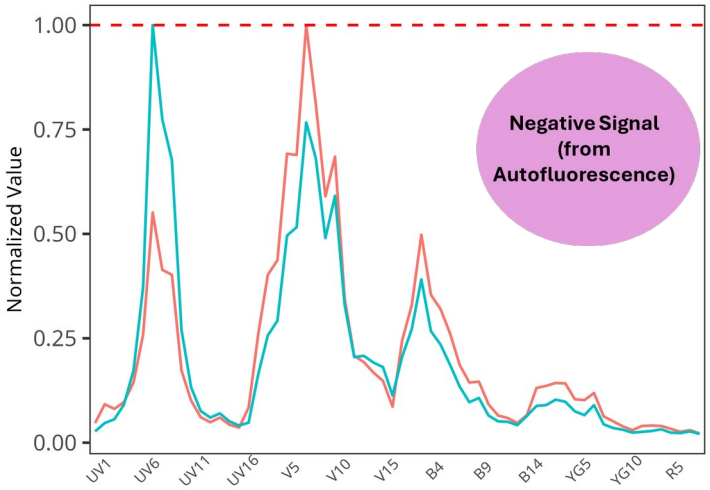
What fluorophores
are present, and
roughly how much

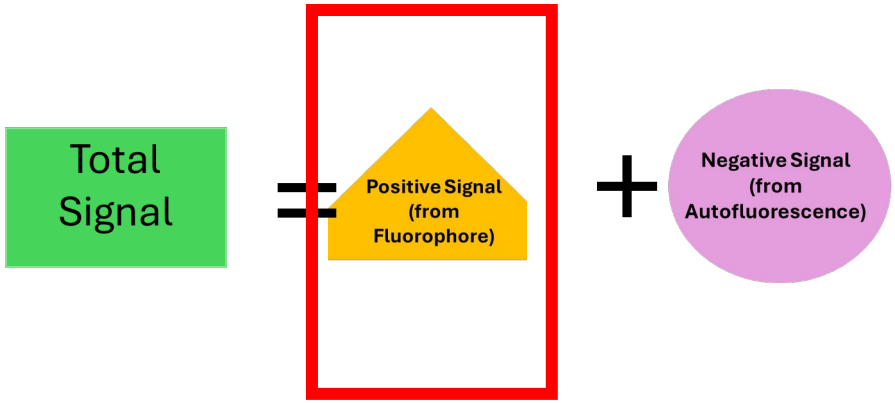
With a successful unmixing



Unmixing errors occur when:

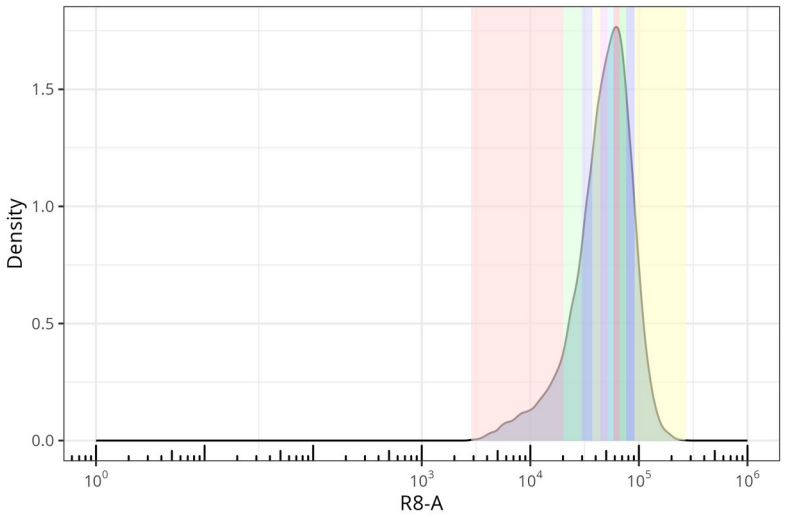


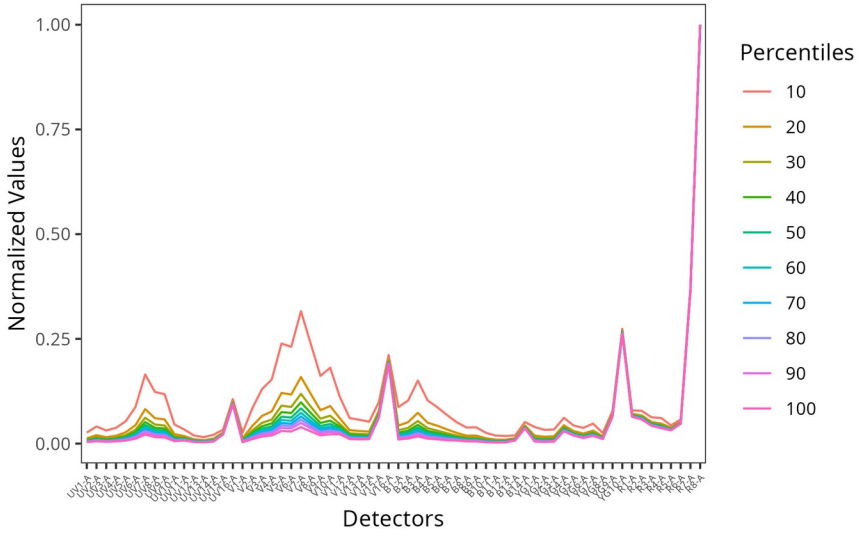




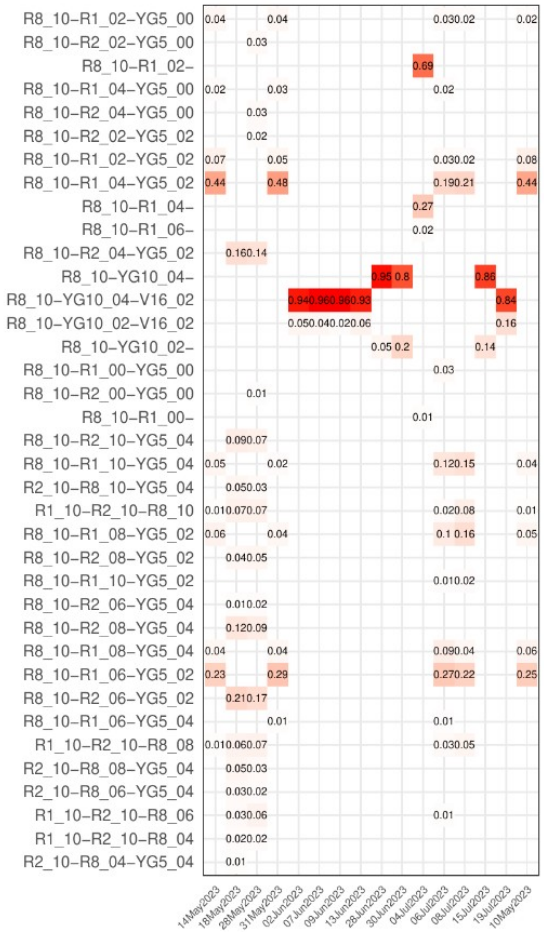
Fluorescent signature of your unmixing control doesn't match the fluorescent signature of that same fluorophore on your full-stained sample

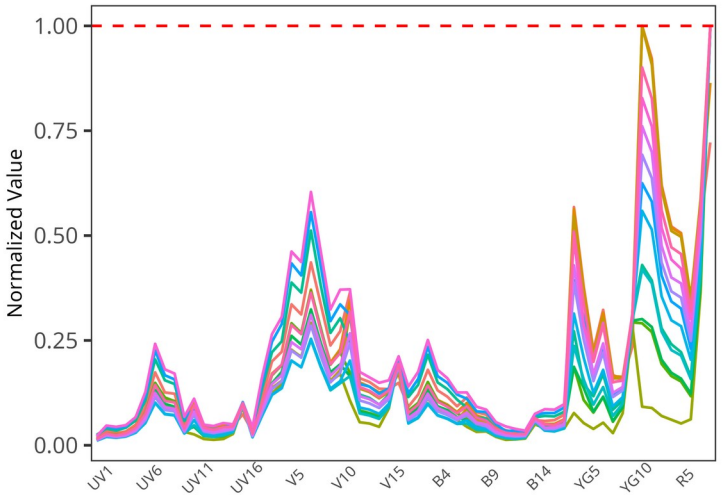
If you set a gate incorrectly



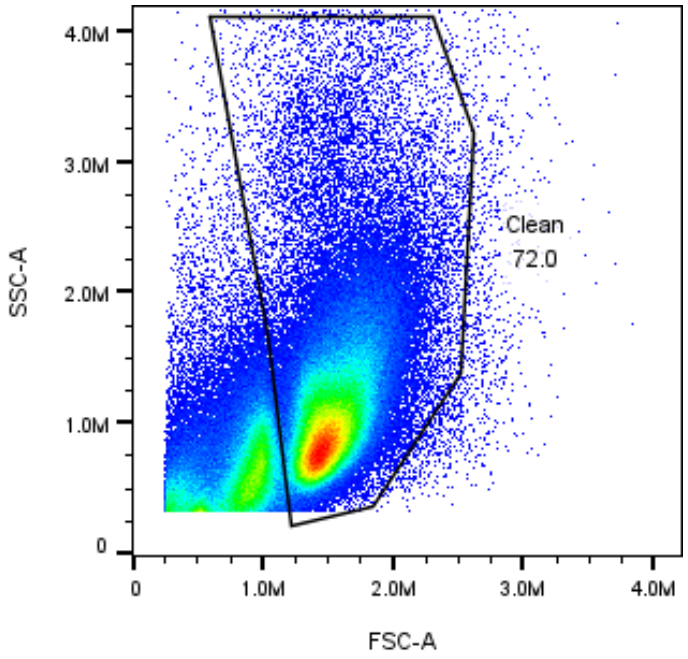


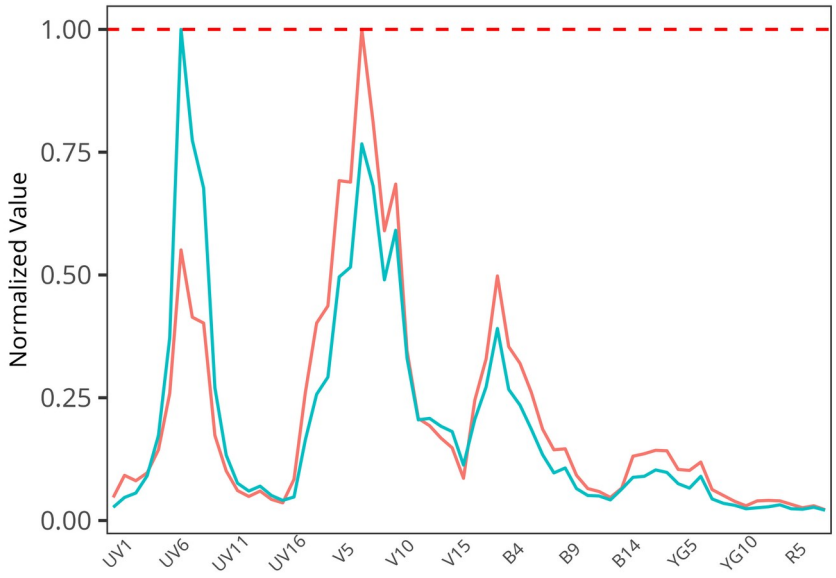
Or have a tandem-fluorophore degrade



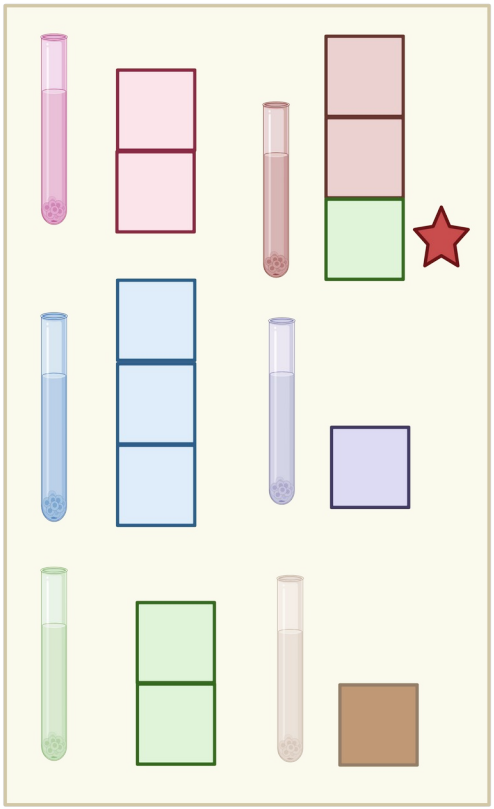


Or set our unstained on the wrong cell population



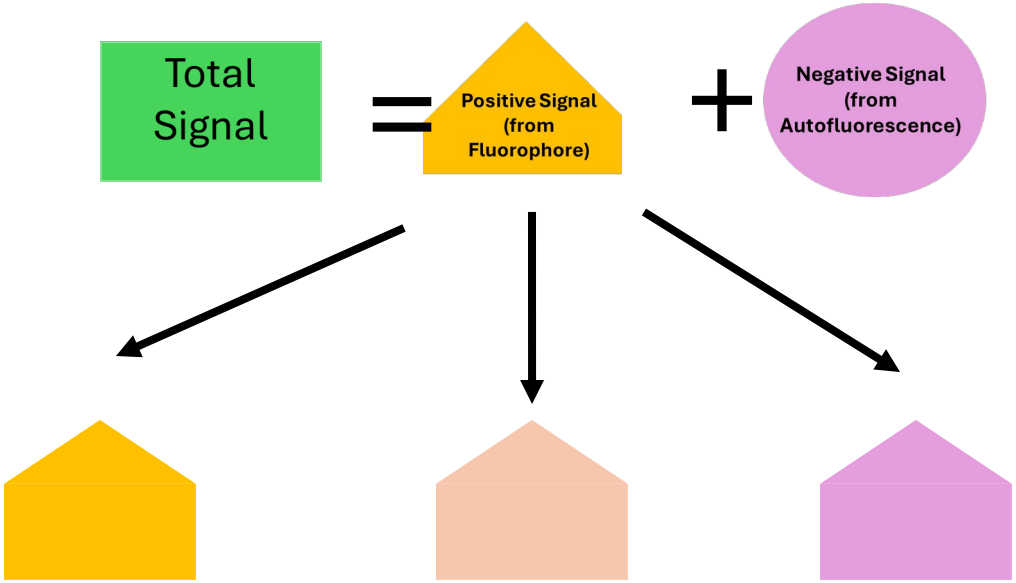


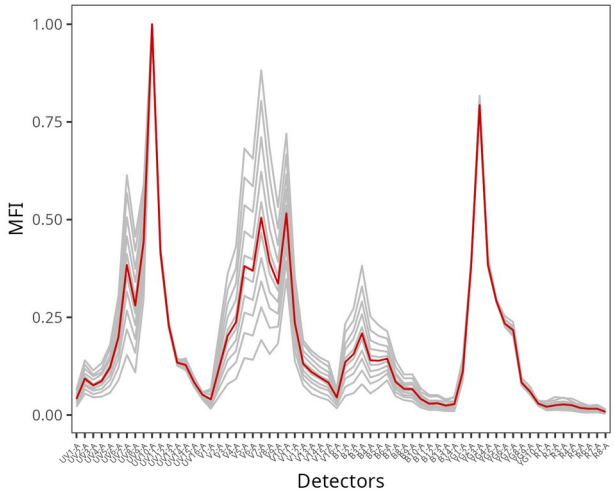
With an unsuccessful unmixing



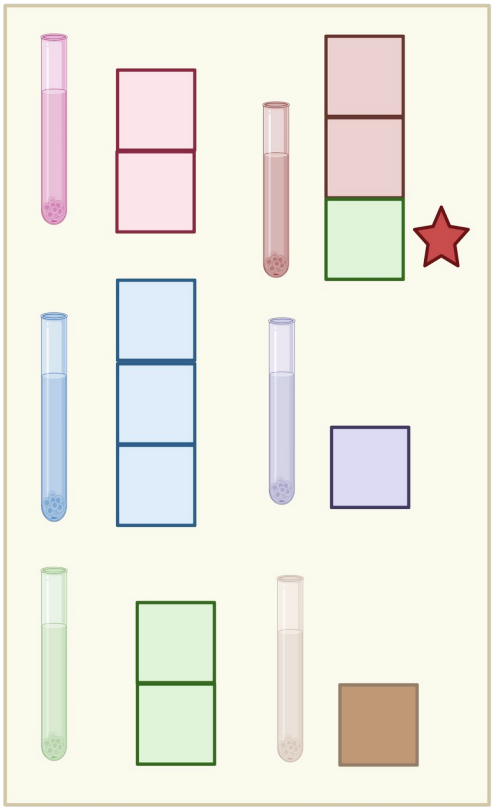
Unmixing Controls (Single Color and Unstained)

We introduce uncertainty into the calculation



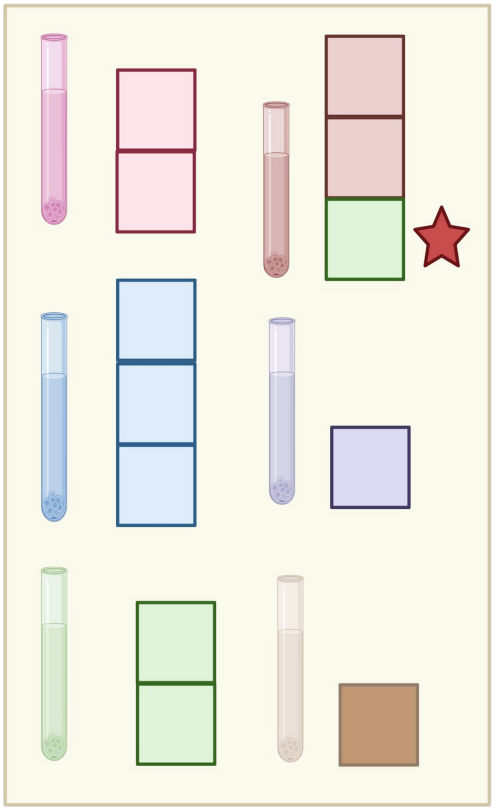


With an unsuccessful unmixing

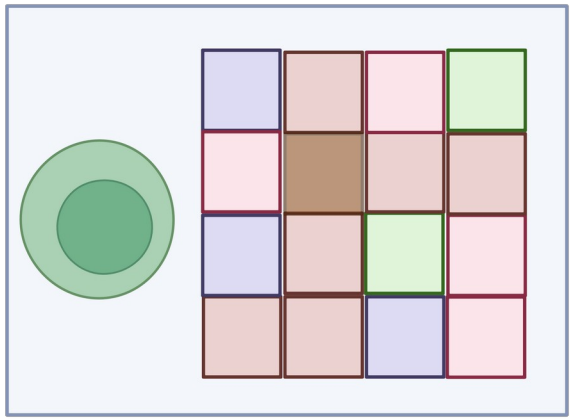


Unmixing Controls (Single Color and Unstained)

With an unsuccessful unmixing

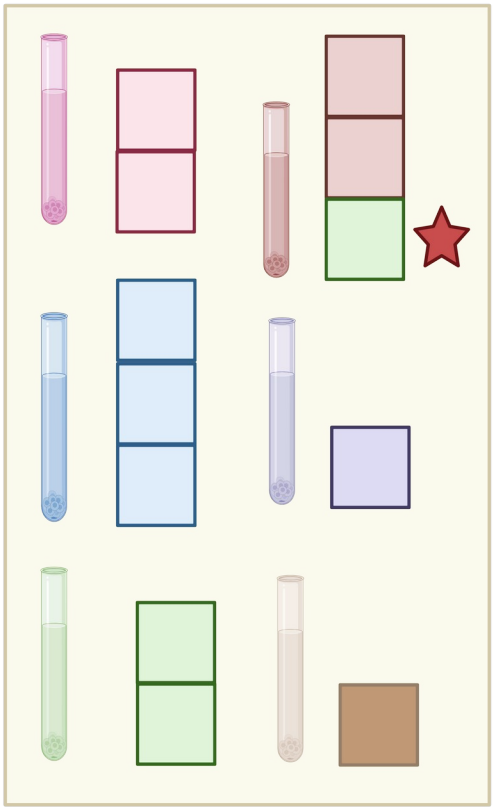


Unmixing Controls
(Single Color and
Unstained)

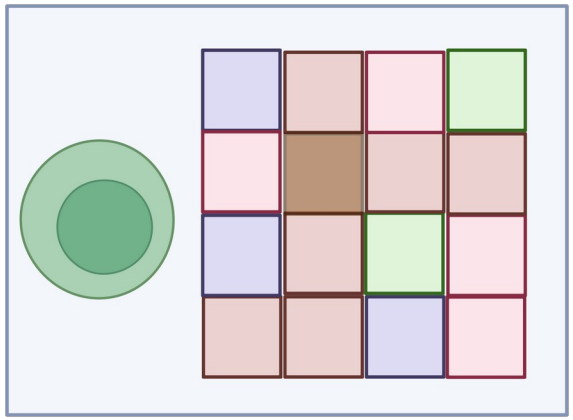


Single cell from
full-stained sample

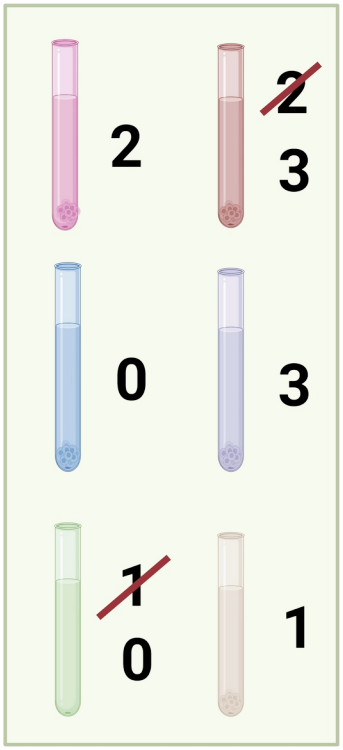
With an unsuccessful unmixing



Unmixing Controls
(Single Color and
Unstained)

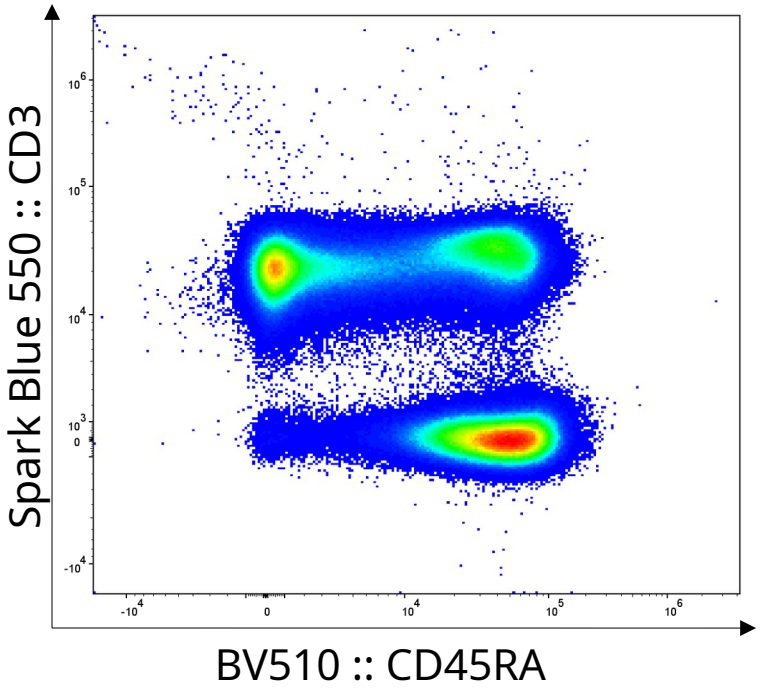


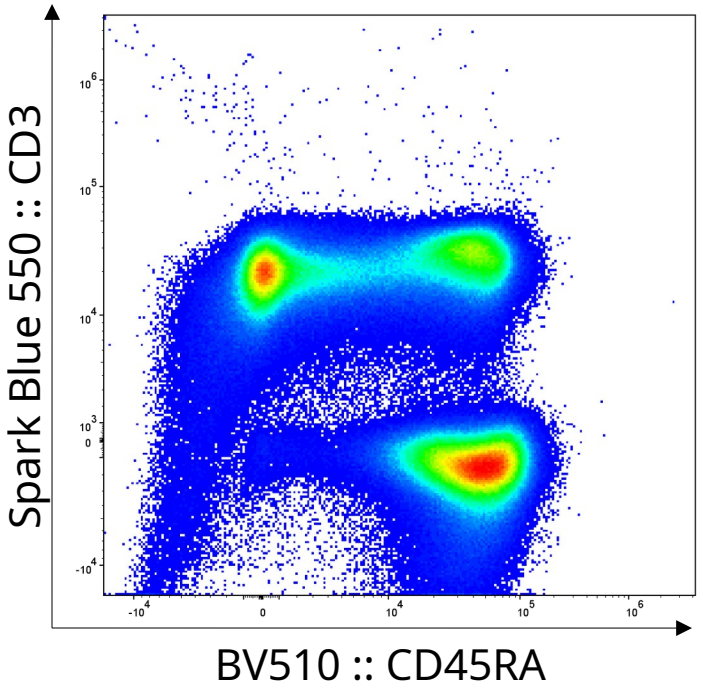
Single cell from
full-stained sample

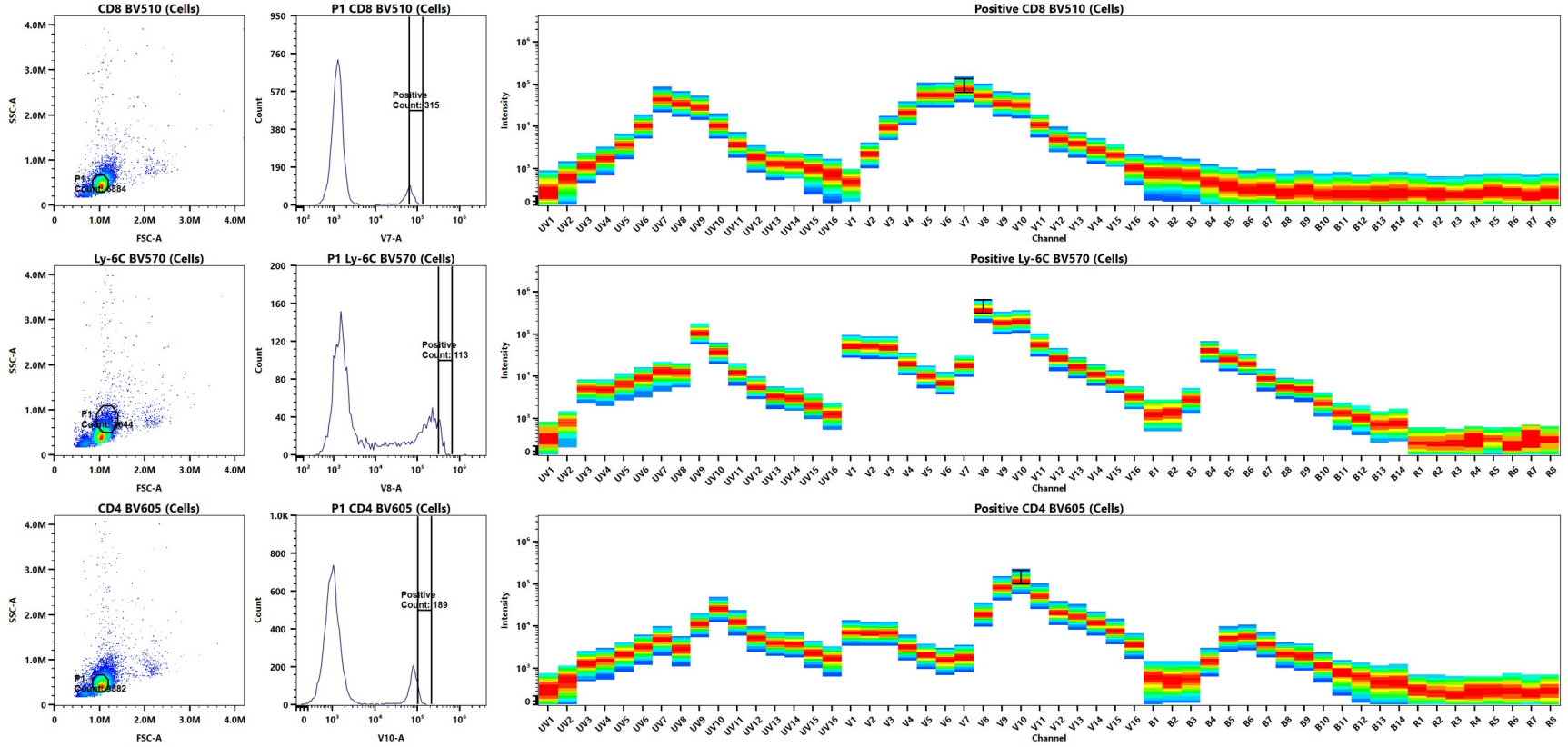


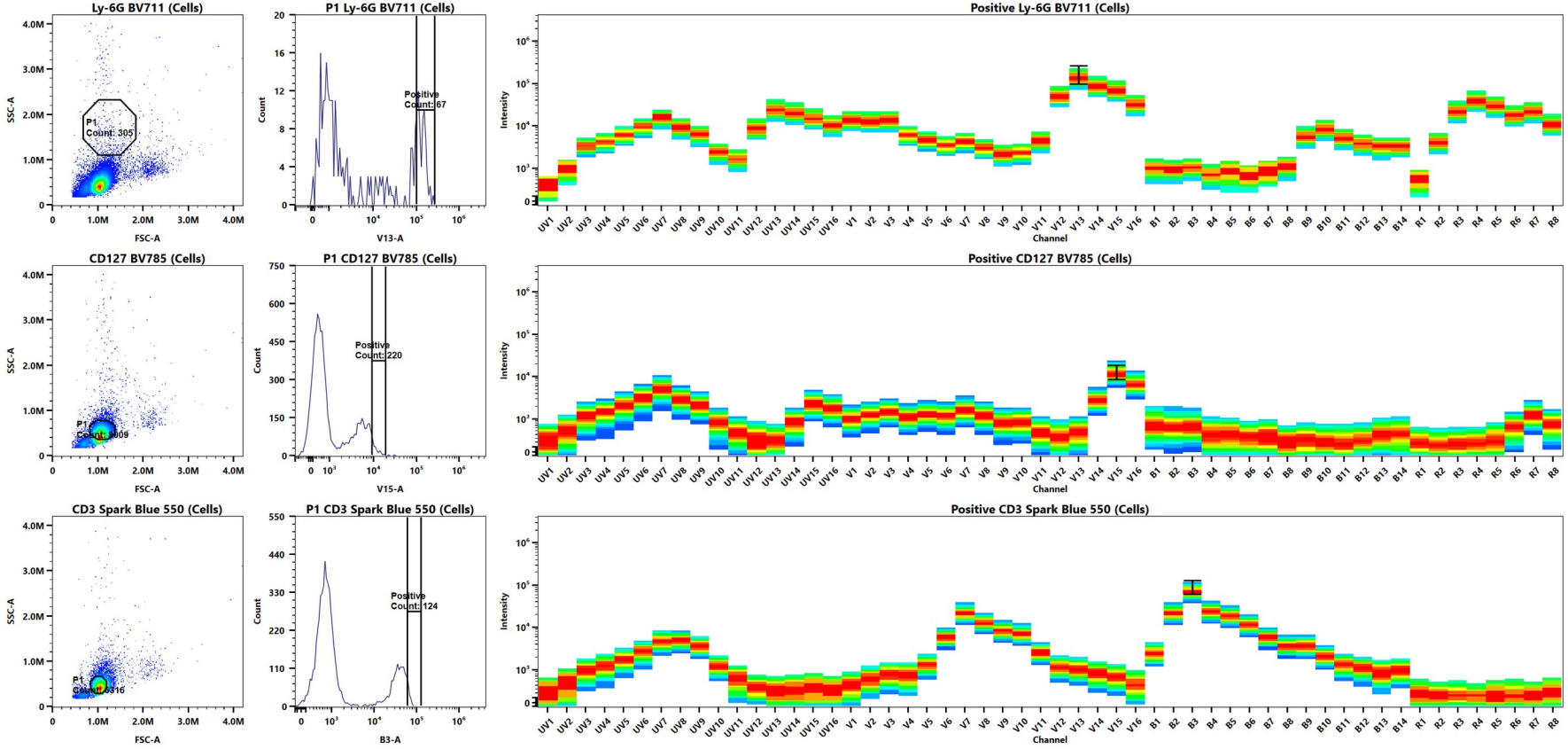
What fluorophores
are present, and
roughly how much

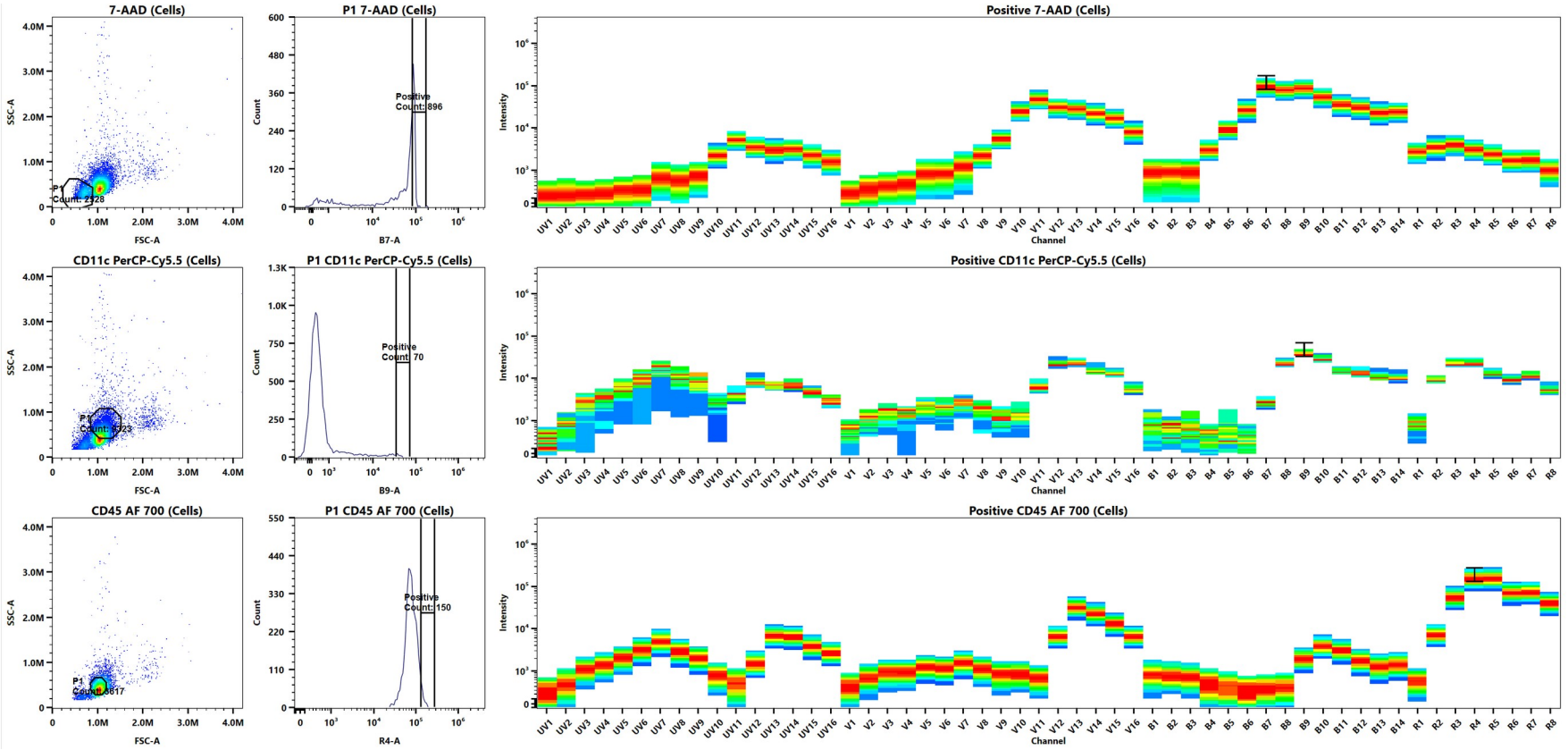
With a unsuccessful unmixing

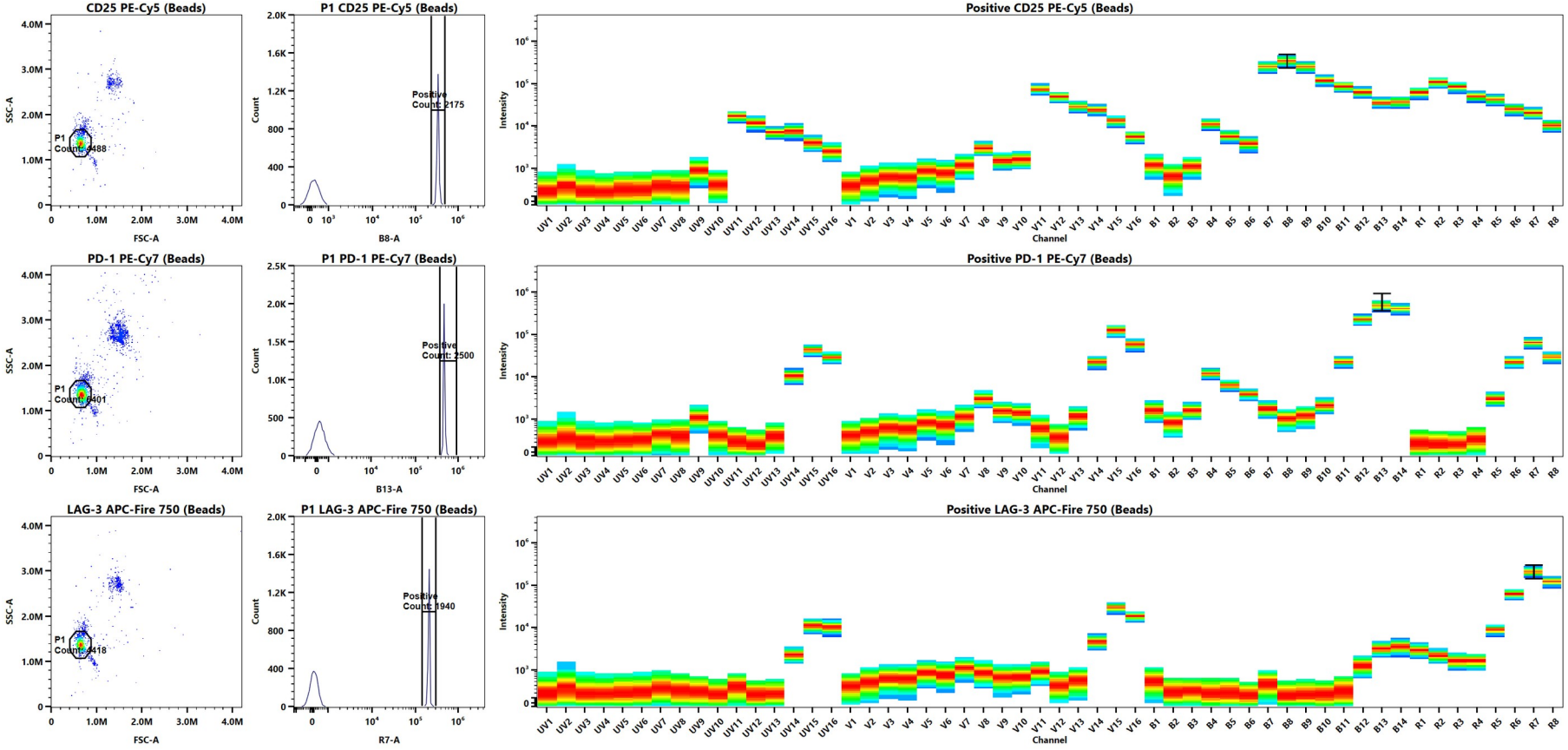


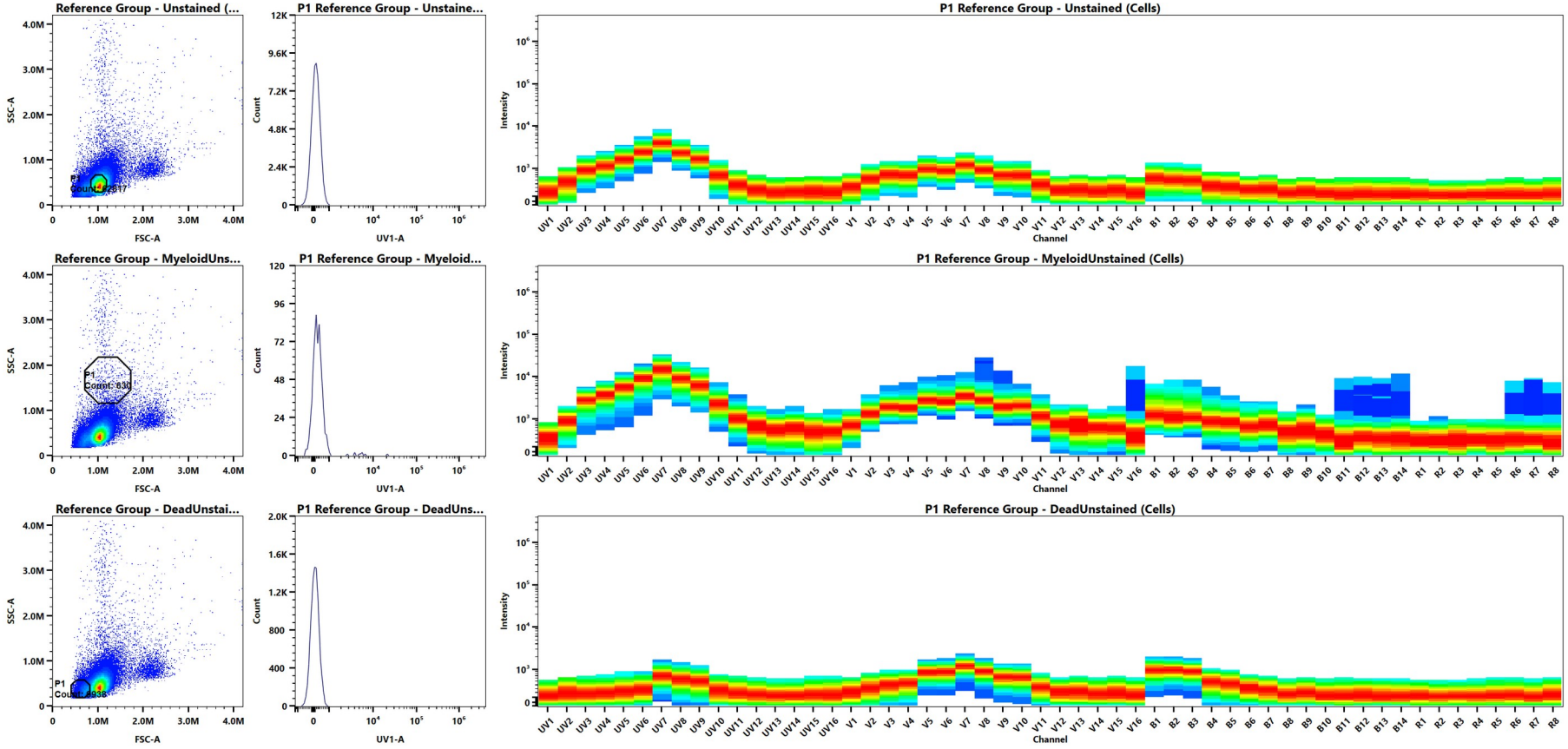


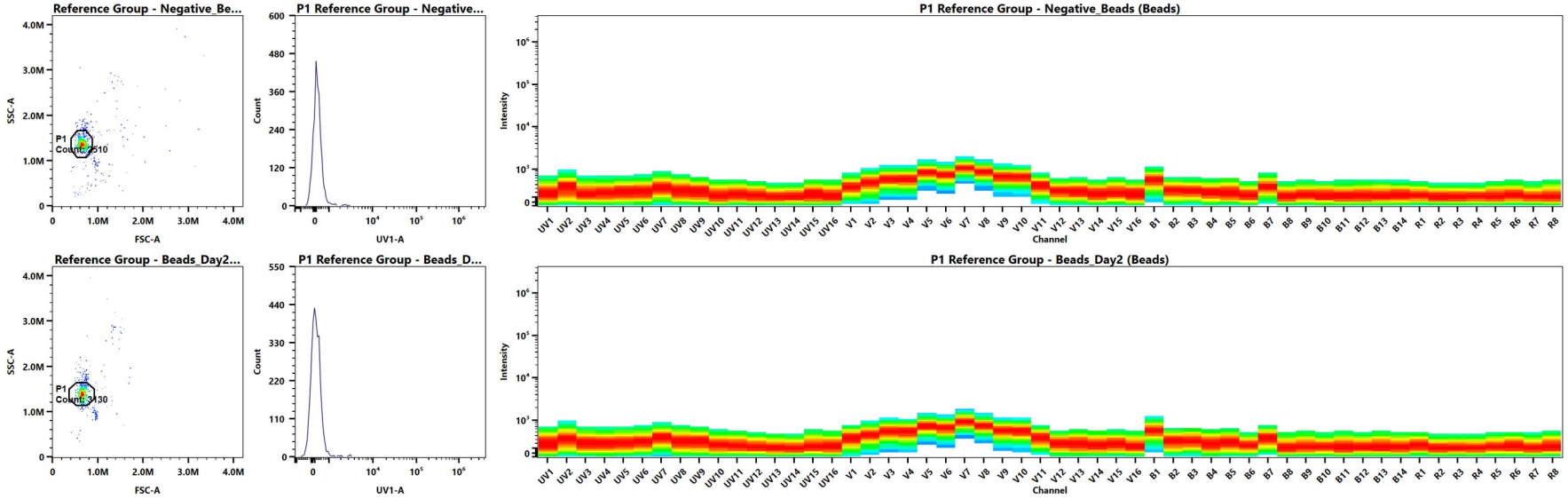

CD8 BV510 (Cells)
P1
Count: 6884
SSC-A
FSC-A
P1 CD8 BV510 (Cells)
Positive
Count: 315
Count
V7-A
Positive CD8 BV510 (Cells)
Intensity
Channel
Ly-6C BV570 (Cells)
P1
Count: 7044
SSC-A
FSC-A
P1 Ly-6C BV570 (Cells)
Positive
Count: 113
Count
V8-A
Positive Ly-6C BV570 (Cells)
Intensity
Channel
CD4 BV605 (Cells)
P1
Count: 6882
SSC-A
FSC-A
P1 CD4 BV605 (Cells)
Positive
Count: 189
Count
V10-A
Positive CD4 BV605 (Cells)
Intensity
Channel

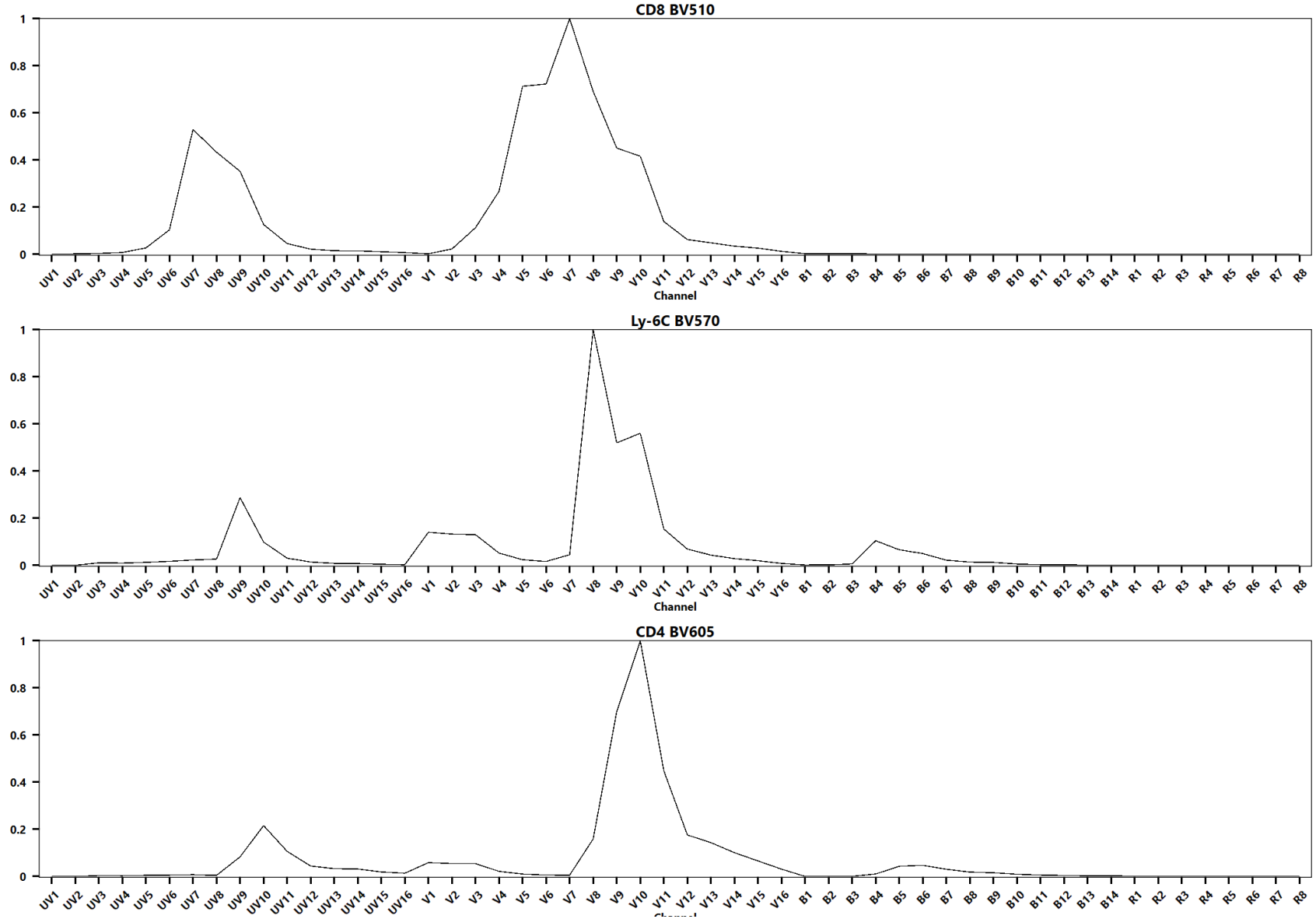

Ly-6G BV711 (Cells)
P1
Count: 305
SSC-A
FSC-A
P1 Ly-6G BV711 (Cells)
Positive
Count: 67
Count
V13-A
Positive Ly-6G BV711 (Cells)
Intensity
Channel
CD127 BV785 (Cells)
Count: 3009
P1 CD127 BV785 (Cells)
Positive
Count: 220
V15-A
Positive CD127 BV785 (Cells)
CD3 Spark Blue 550 (Cells)
Count: 6316
P1 CD3 Spark Blue 550 (Cells)
Positive
Count: 124
B3-A
Positive CD3 Spark Blue 550 (Cells)

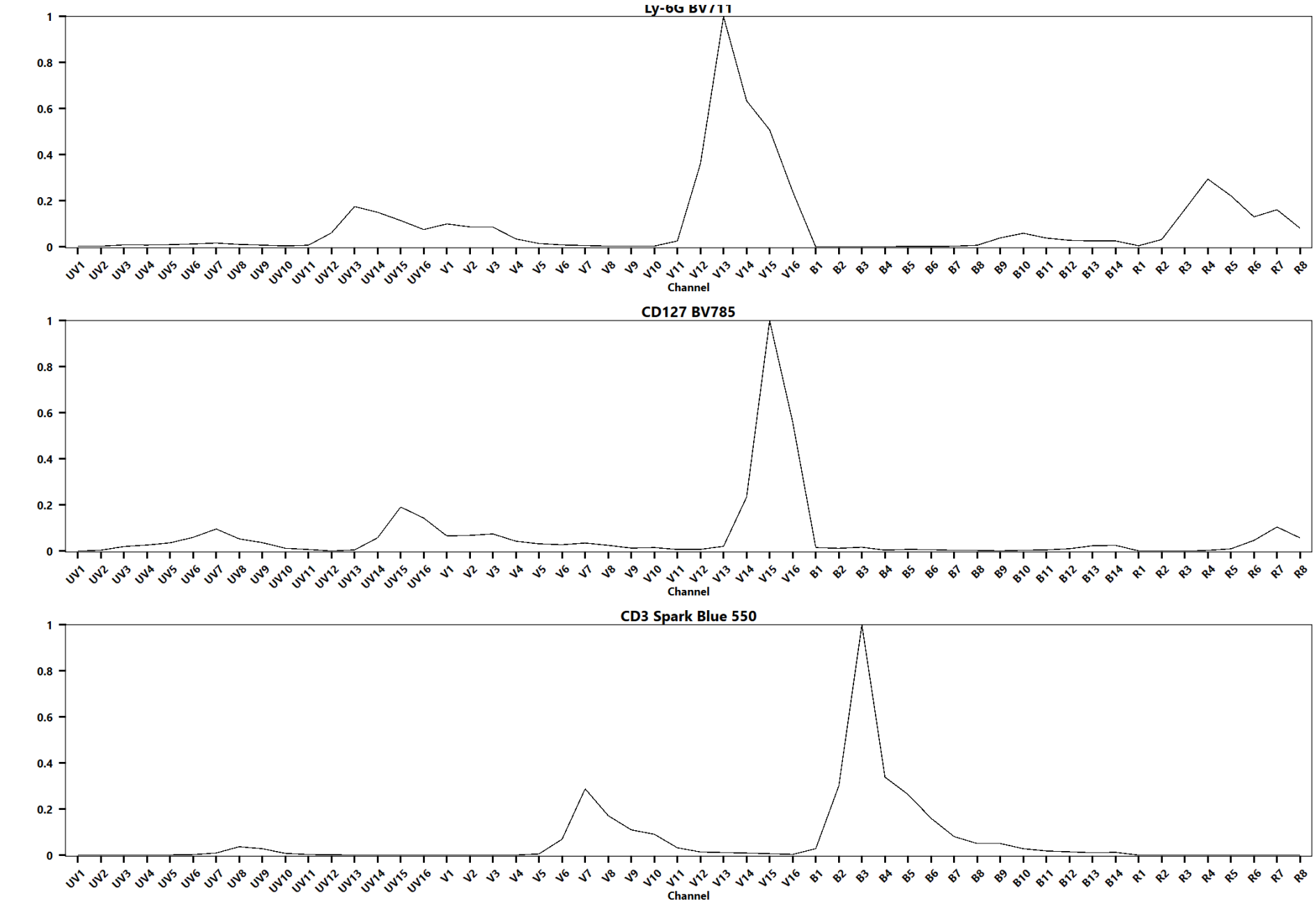

7-AAD (Cells)
SSC-A
FSC-A
P1
Count: 2328
P1 7-AAD (Cells)
Count
B7-A
Positive
Count: 896
Positive 7-AAD (Cells)
Intensity
Channel
CD11c PerCP-Cy5.5 (Cells)
P1
Count: 1023
B9-A
P1 CD11c PerCP-Cy5.5 (Cells)
Positive
Count: 70
Positive CD11c PerCP-Cy5.5 (Cells)
CD45 AF 700 (Cells)
P1
Count: 3617
R4-A
P1 CD45 AF 700 (Cells)
Positive
Count: 150
Positive CD45 AF 700 (Cells)

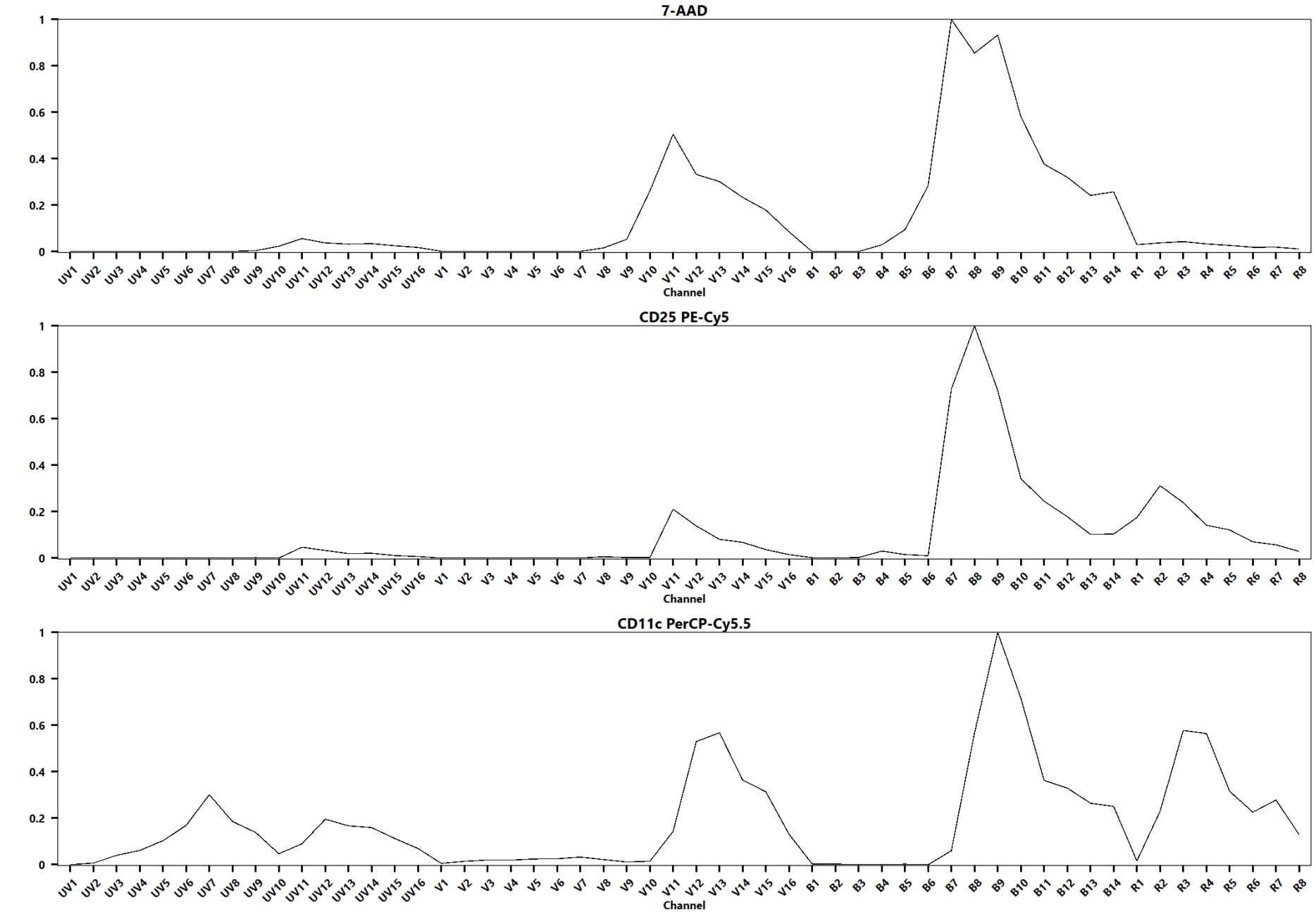

CD25 PE-Cy5 (Beads)
SSC-A
FSC-A
P1
Count: 4488
P1 CD25 PE-Cy5 (Beads)
Count
B8-A
Positive
Count: 2175
Positive CD25 PE-Cy5 (Beads)
Intensity
Channel
PD-1 PE-Cy7 (Beads)
P1
Count: 6401
B13-A
Positive
Count: 2500
Positive PD-1 PE-Cy7 (Beads)
LAG-3 APC-Fire 750 (Beads)
P1
Count: 4418
R7-A
Positive
Count: 1940
Positive LAG-3 APC-Fire 750 (Beads)

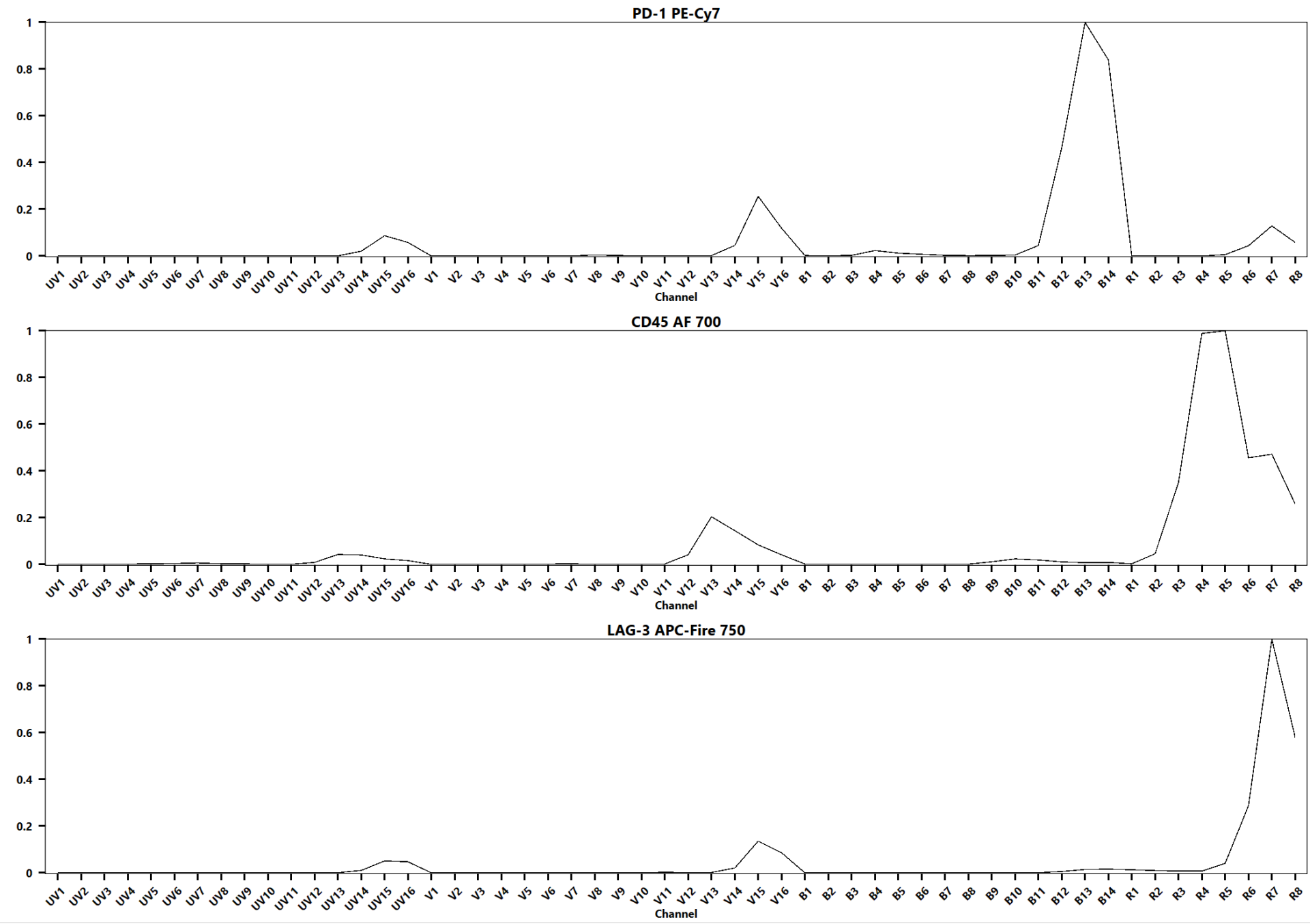

Reference Group - Unstained (...
P1 Reference Group - Unstaine...
P1 Reference Group - Unstained (Cells)
Reference Group - MyeloidUns...
P1 Reference Group - Myeloid...
P1 Reference Group - MyeloidUnstained (Cells)
Reference Group - DeadUnstai...
P1 Reference Group - DeadUns...
P1 Reference Group - DeadUnstained (Cells)
SSC-A
FSC-A
Count
UV1-A
Intensity
Channel
P1
Count: 630
Count: 4938

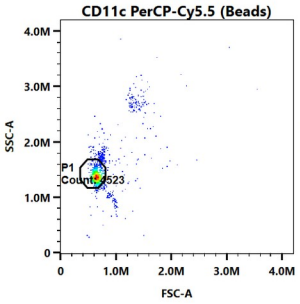

Reference Group - Negative_Be...
P1 Reference Group - Negative...
P1 Reference Group - Negative_Beads (Beads)
Reference Group - Beads_Day2...
P1 Reference Group - Beads_D...
P1 Reference Group - Beads_Day2 (Beads)
SSC-A
FSC-A
Count
UV1-A
Intensity
Channel
P1
Count: 2510
Count: 3130

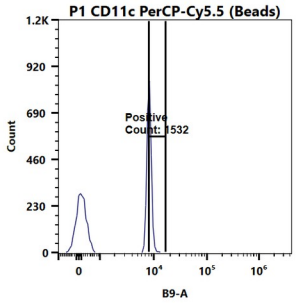

CD8 BV510
Ly-6C BV570
CD4 BV605
1
0.8
0.6
0.4
0.2
0
UV1 UV2 UV3 UV4 UV5 UV6 UV7 UV8 UV9 UV10 UV11 UV12 UV13 UV14 UV15 UV16 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 R1 R2 R3 R4 R5 R6 R7 R8
Channel

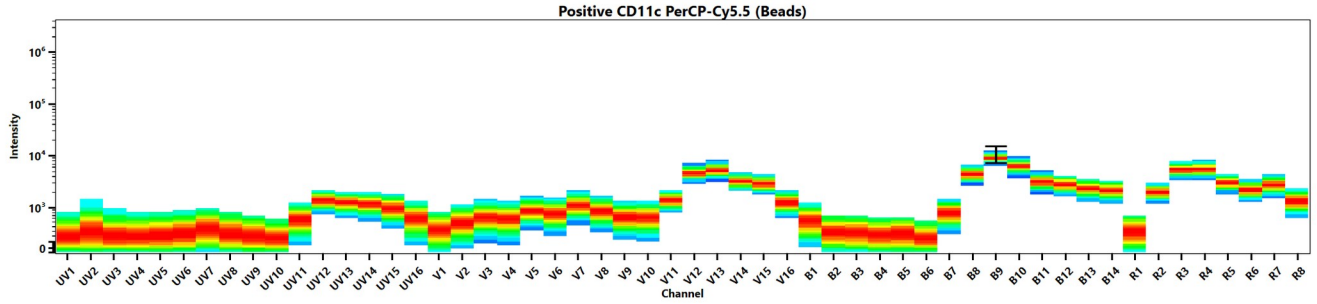

Ly-6G BV711
CD127 BV785
CD3 Spark Blue 550
1
0.8
0.6
0.4
0.2
0
UV1 UV2 UV3 UV4 UV5 UV6 UV7 UV8 UV9 UV10 UV11 UV12 UV13 UV14 UV15 UV16 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 R1 R2 R3 R4 R5 R6 R7 R8
Channel

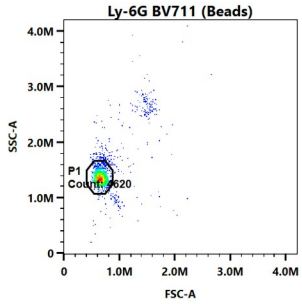

7-AAD
1
0.8
0.6
0.4
0.2
0
UV1 UV2 UV3 UV4 UV5 UV6 UV7 UV8 UV9 UV10 UV11 UV12 UV13 UV14 UV15 UV16 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 R1 R2 R3 R4 R5 R6 R7 R8
Channel
CD25 PE-Cy5
1
0.8
0.6
0.4
0.2
0
UV1 UV2 UV3 UV4 UV5 UV6 UV7 UV8 UV9 UV10 UV11 UV12 UV13 UV14 UV15 UV16 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 R1 R2 R3 R4 R5 R6 R7 R8
Channel
CD11c PerCP-Cy5.5
1
0.8
0.6
0.4
0.2
0
UV1 UV2 UV3 UV4 UV5 UV6 UV7 UV8 UV9 UV10 UV11 UV12 UV13 UV14 UV15 UV16 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 R1 R2 R3 R4 R5 R6 R7 R8
Channel

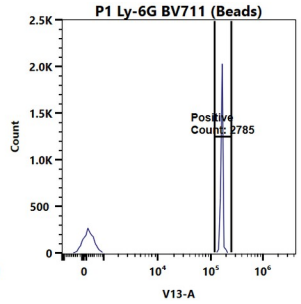

PD-1 PE-Cy7
CD45 AF 700
LAG-3 APC-Fire 750
1
0.8
0.6
0.4
0.2
0
UV1 UV2 UV3 UV4 UV5 UV6 UV7 UV8 UV9 UV10 UV11 UV12 UV13 UV14 UV15 UV16 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 R1 R2 R3 R4 R5 R6 R7 R8
Channel

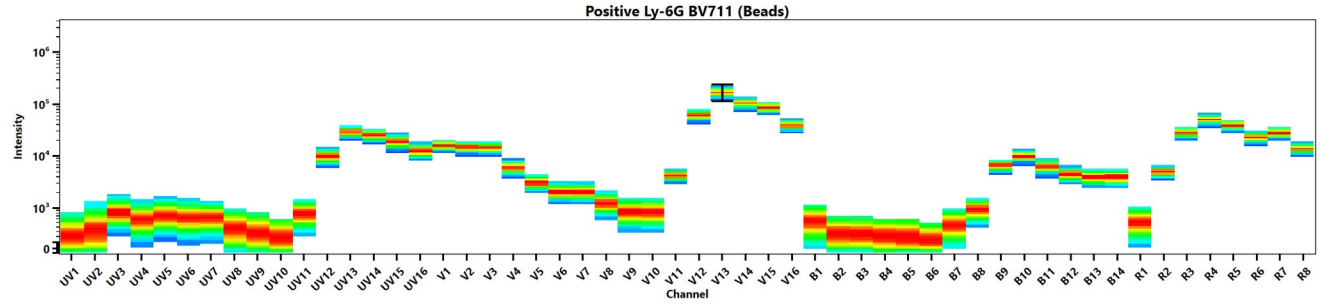

CD11c PerCP-Cy5.5 (Beads)
SSC-A
FSC-A
P1
Count: 4523

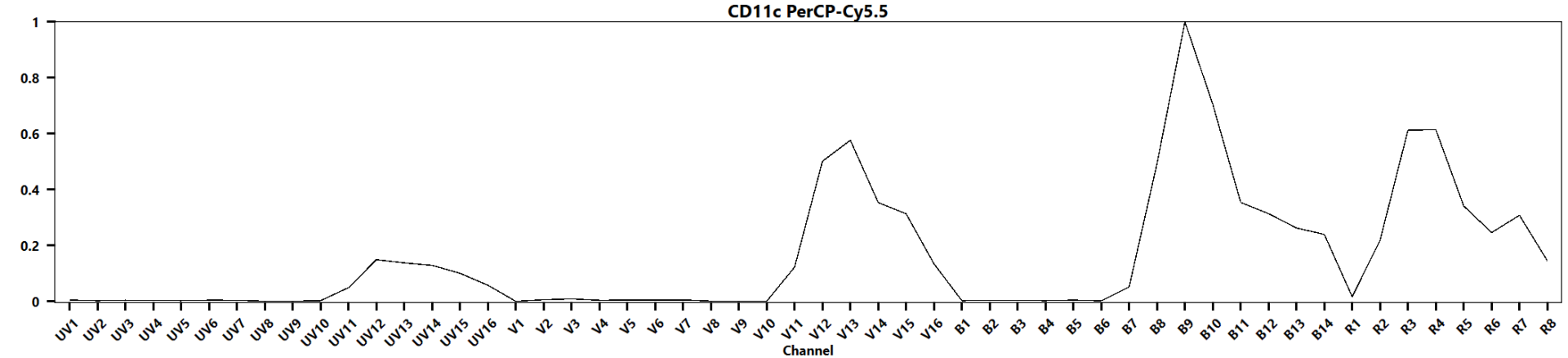

P1 CD11c PerCP-Cy5.5 (Beads)
Count
B9-A
Positive
Count: 1532

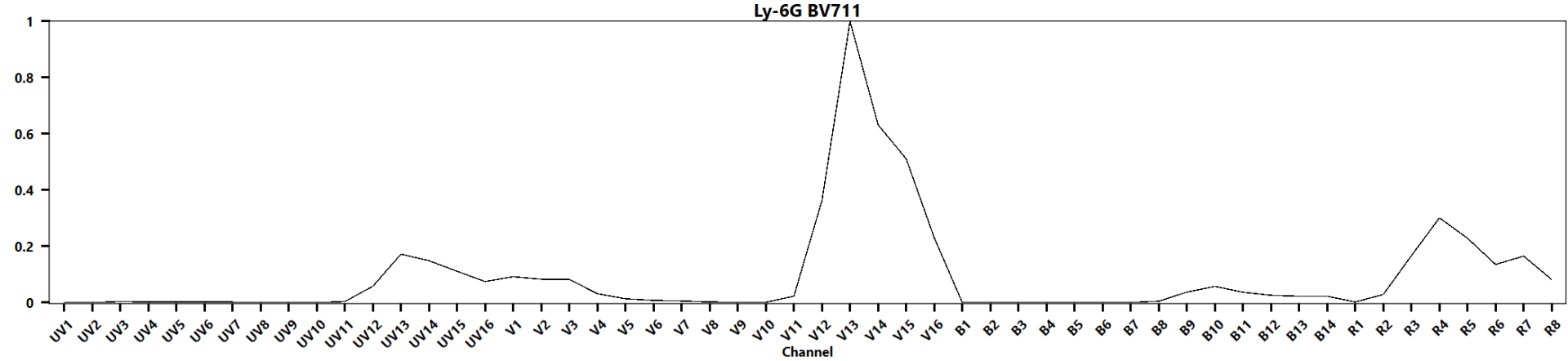

Positive CD11c PerCP-Cy5.5 (Beads)
Intensity
Channel

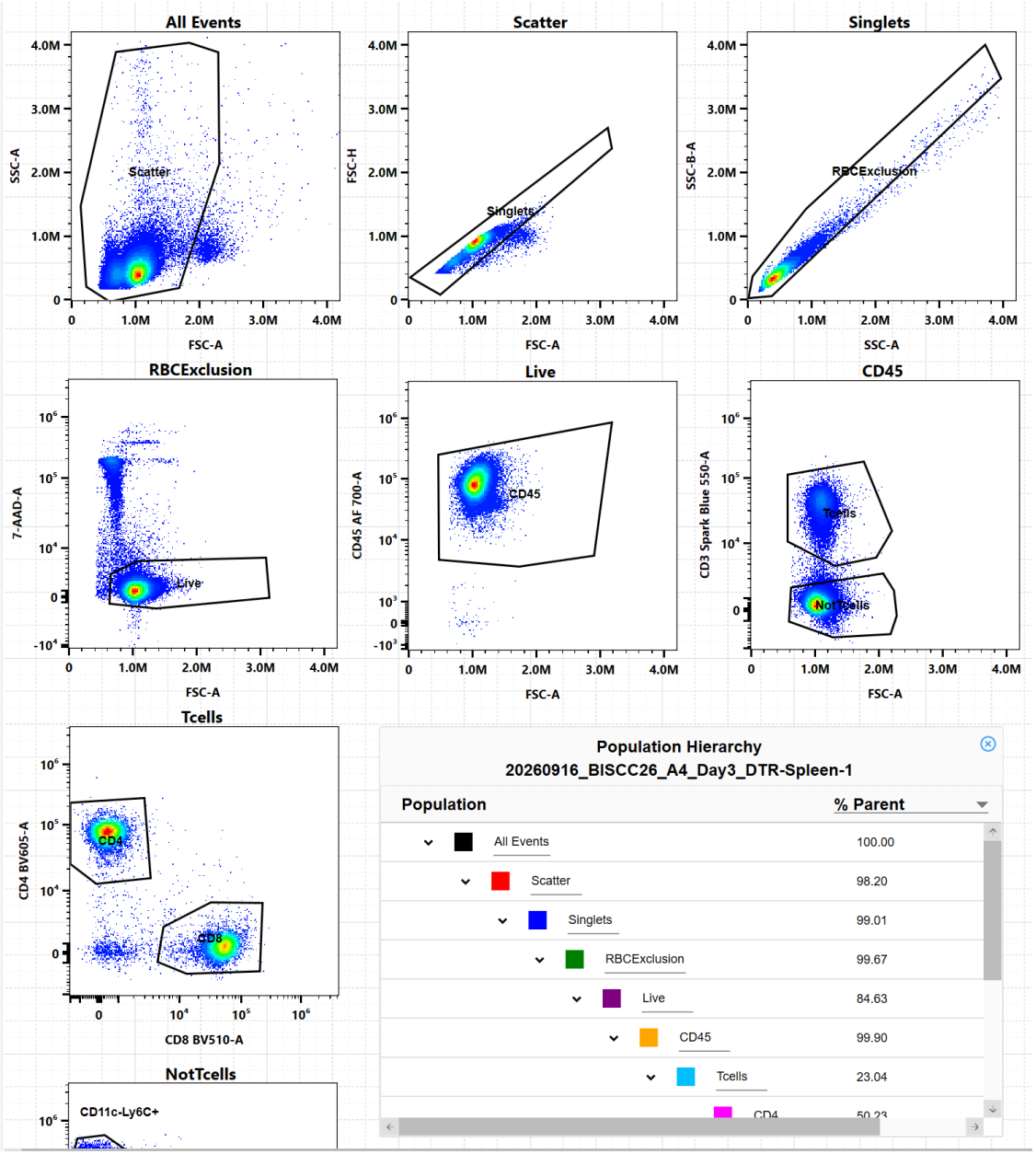

Ly-6G BV711 (Beads)
SSC-A
FSC-A
P1
Count: 3620

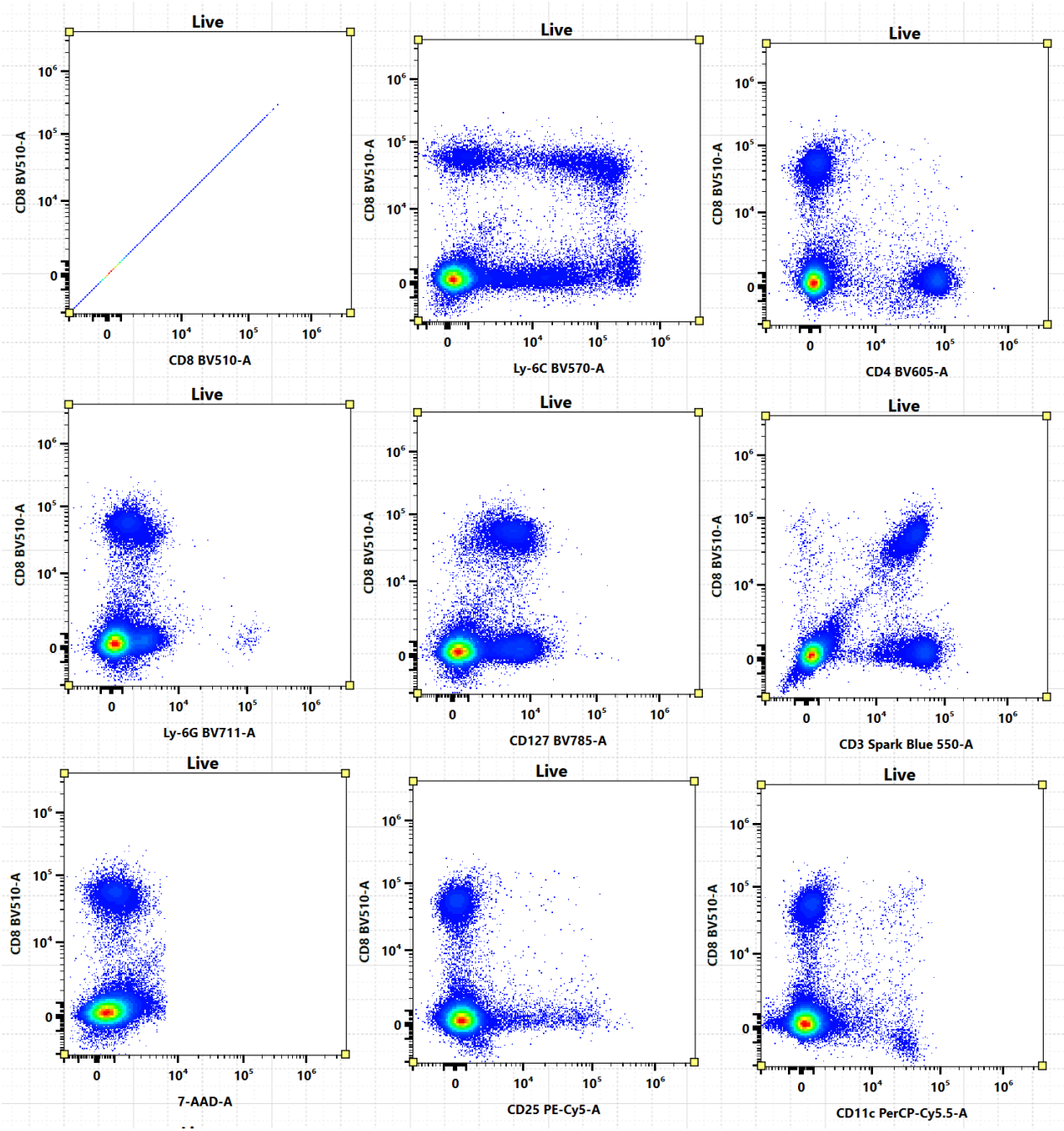

P1 Ly-6G BV711 (Beads)
Count
V13-A
Positive
Count: 2785

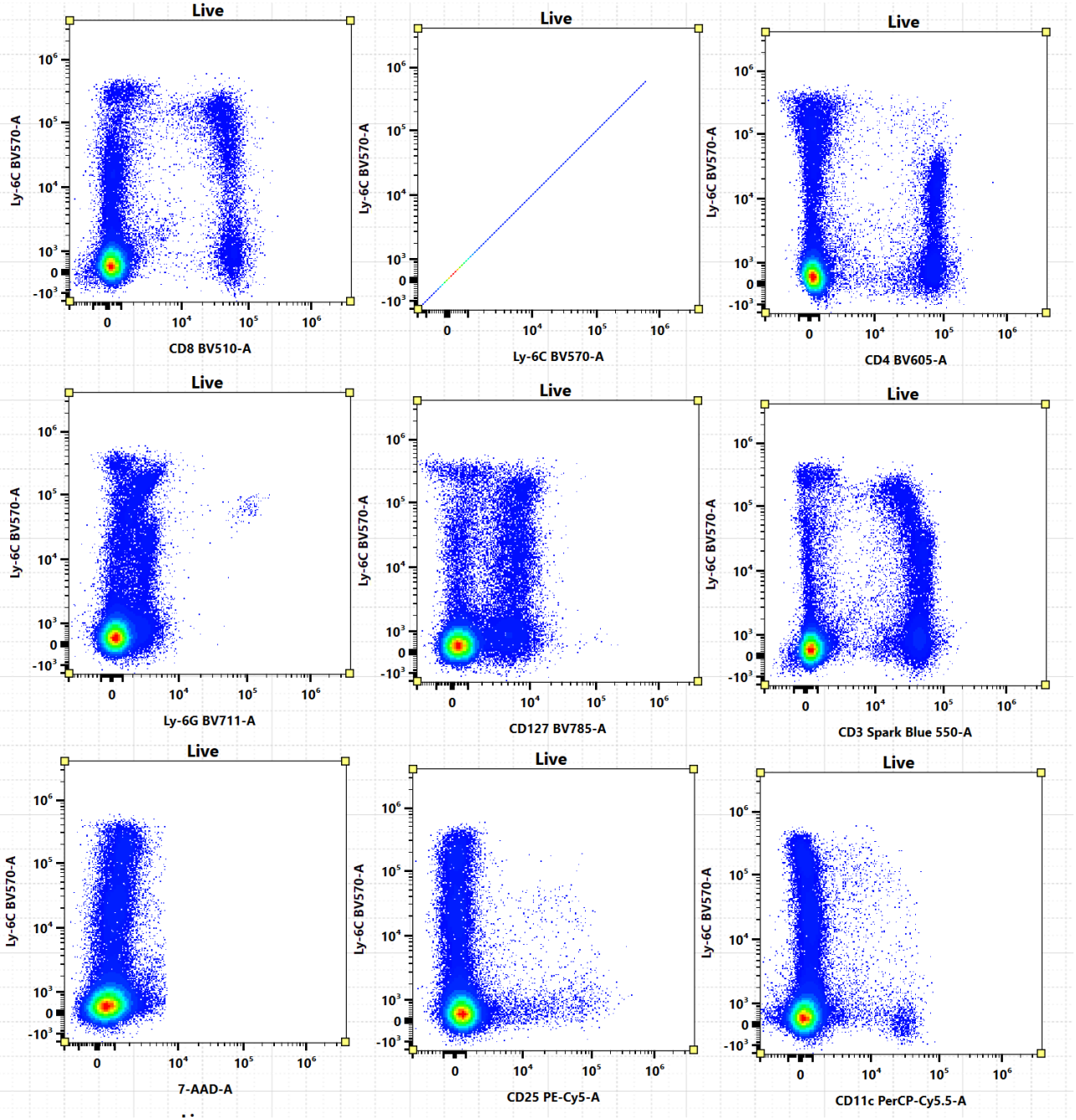

Positive Ly-6G BV711 (Beads)
Intensity
Channel

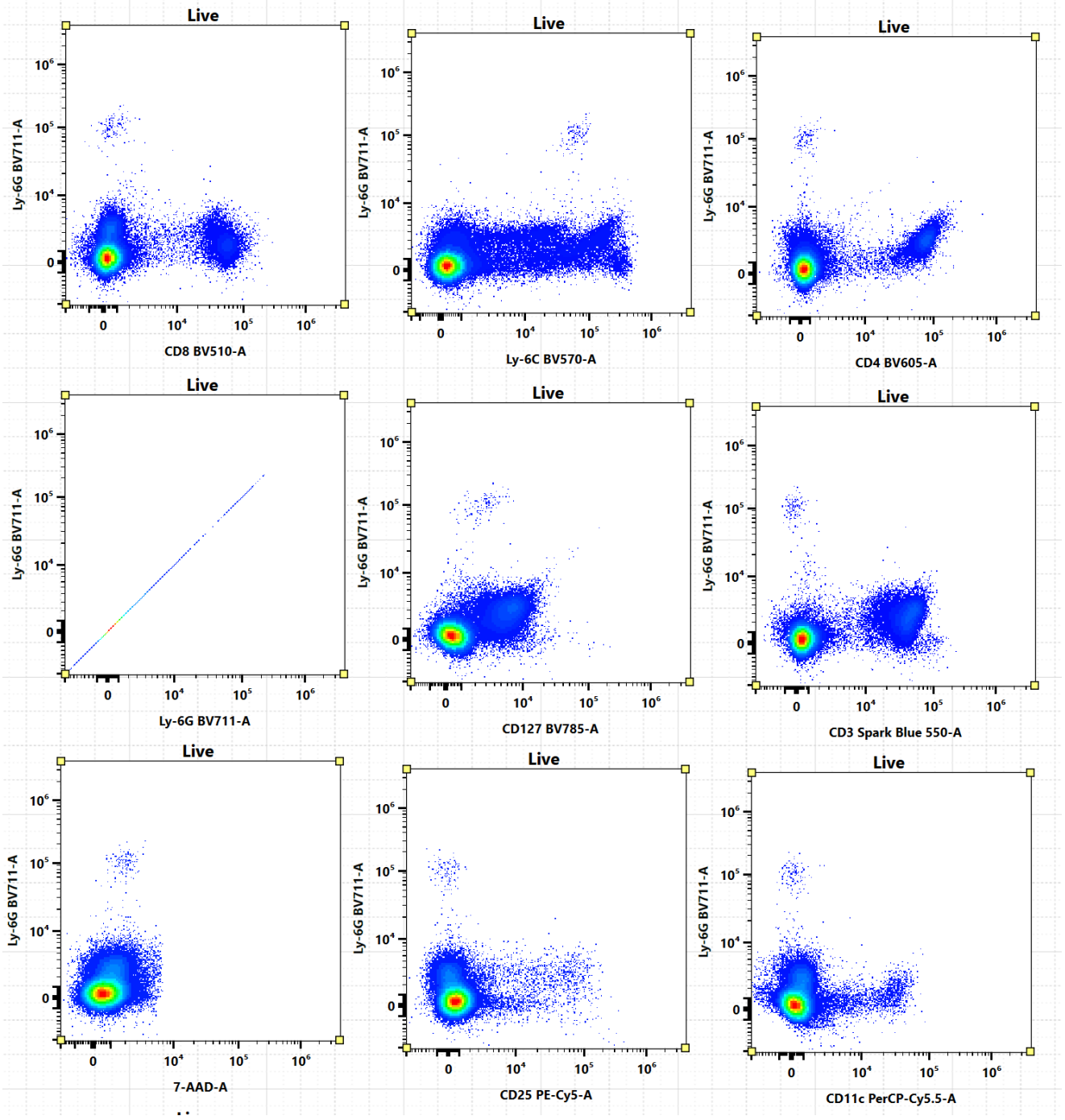

CD11c PerCP-Cy5.5
1
0.8
0.6
0.4
0.2
0
UV1 UV2 UV3 UV4 UV5 UV6 UV7 UV8 UV9 UV10 UV11 UV12 UV13 UV14 UV15 UV16 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 R1 R2 R3 R4 R5 R6 R7 R8
Channel

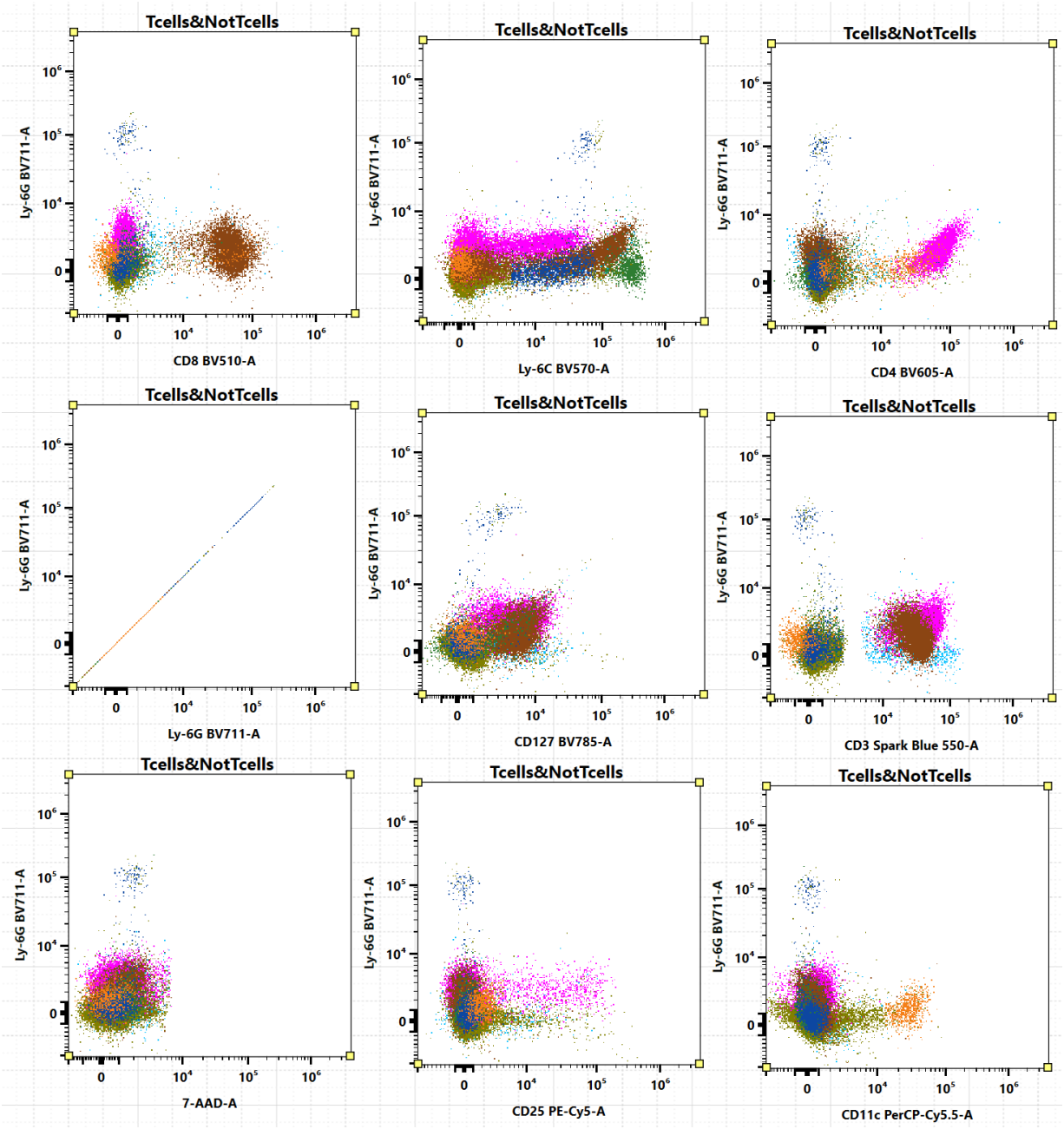

Ly-6G BV711
1
0.8
0.6
0.4
0.2
0
UV1 UV2 UV3 UV4 UV5 UV6 UV7 UV8 UV9 UV10 UV11 UV12 UV13 UV14 UV15 UV16 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 R1 R2 R3 R4 R5 R6 R7 R8
Channel

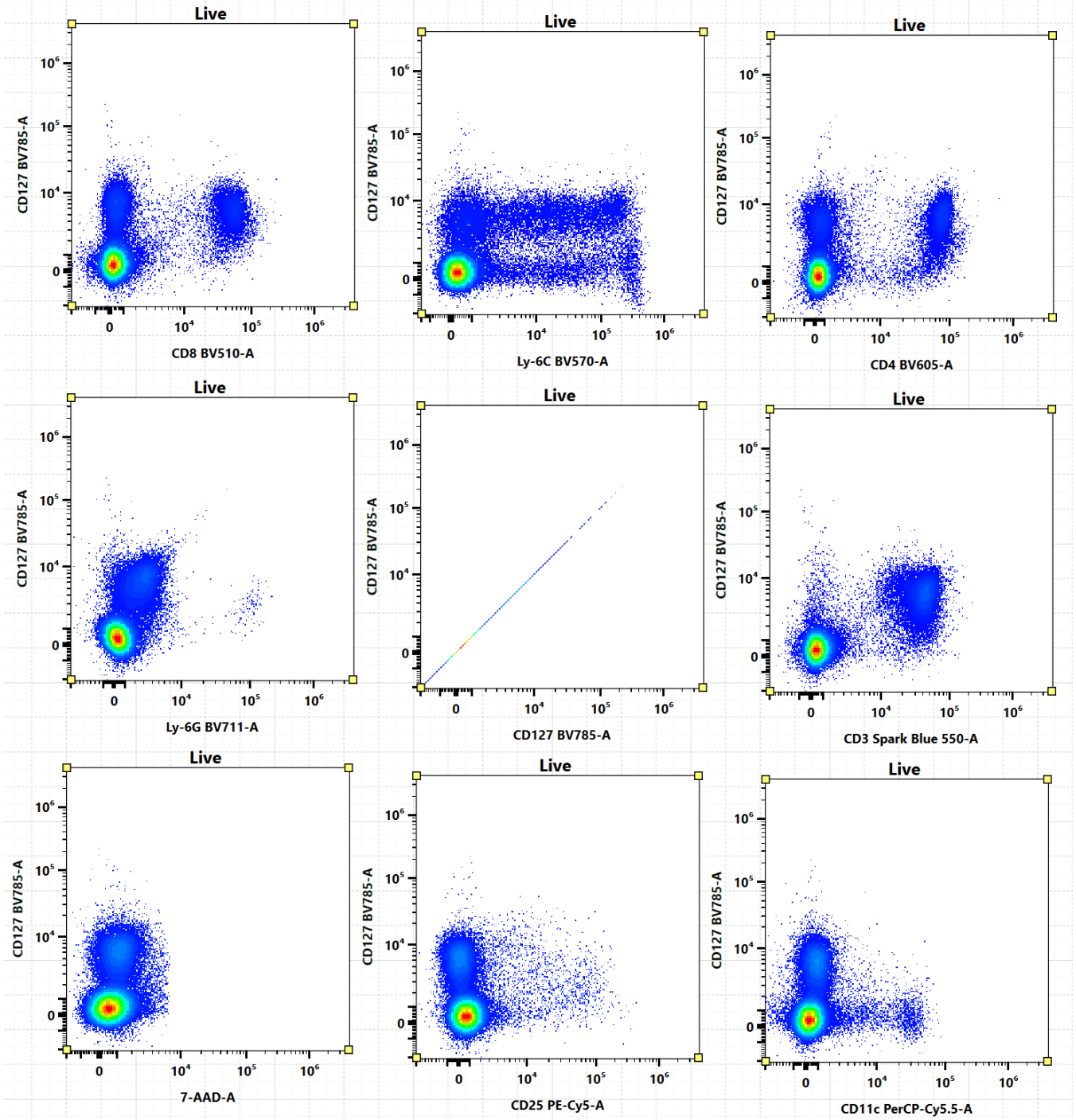

All Events
Scatter
SSC-A
FSC-A
Scatter
Singlets
FSC-H
FSC-A
Singlets
RBCExclusion
SSC-B-A
SSC-A
RBCExclusion
7-AAD-A
FSC-A
Live
Live
CD45 AF 700-A
FSC-A
CD45
CD45
CD3 Spark Blue 550-A
FSC-A
Tcells
NotTcells
Tcells
CD4 BV605-A
CD8 BV510-A
CD4
CD8
NotTcells
CD11c-Ly6C+
Population Hierarchy
20260916_BISCC26_A4_Day3_DTR-Spleen-1
Population
% Parent
All Events 100.00
Scatter 98.20
Singlets 99.01
RBCExclusion 99.67
Live 84.63
CD45 99.90
Tcells 23.04
CD4 50.23

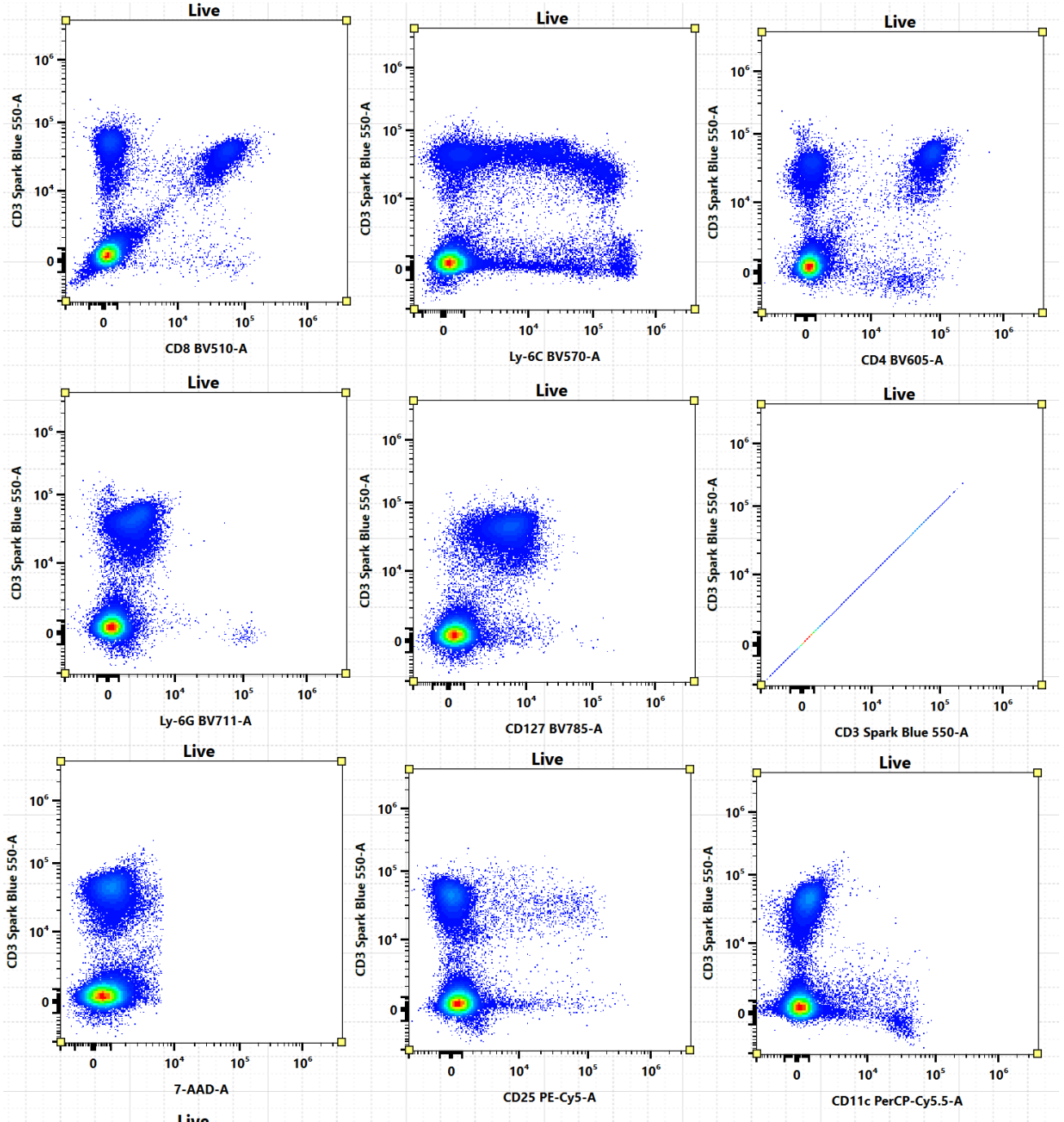

Live
CD8 BV510-A
CD8 BV510-A
Live
CD8 BV510-A
Ly-6C BV570-A
Live
CD8 BV510-A
CD4 BV605-A
Live
CD8 BV510-A
Ly-6G BV711-A
Live
CD8 BV510-A
CD127 BV785-A
Live
CD8 BV510-A
CD3 Spark Blue 550-A
Live
CD8 BV510-A
7-AAD-A
Live
CD8 BV510-A
CD25 PE-Cy5-A
Live
CD8 BV510-A
CD11c PerCP-Cy5.5-A

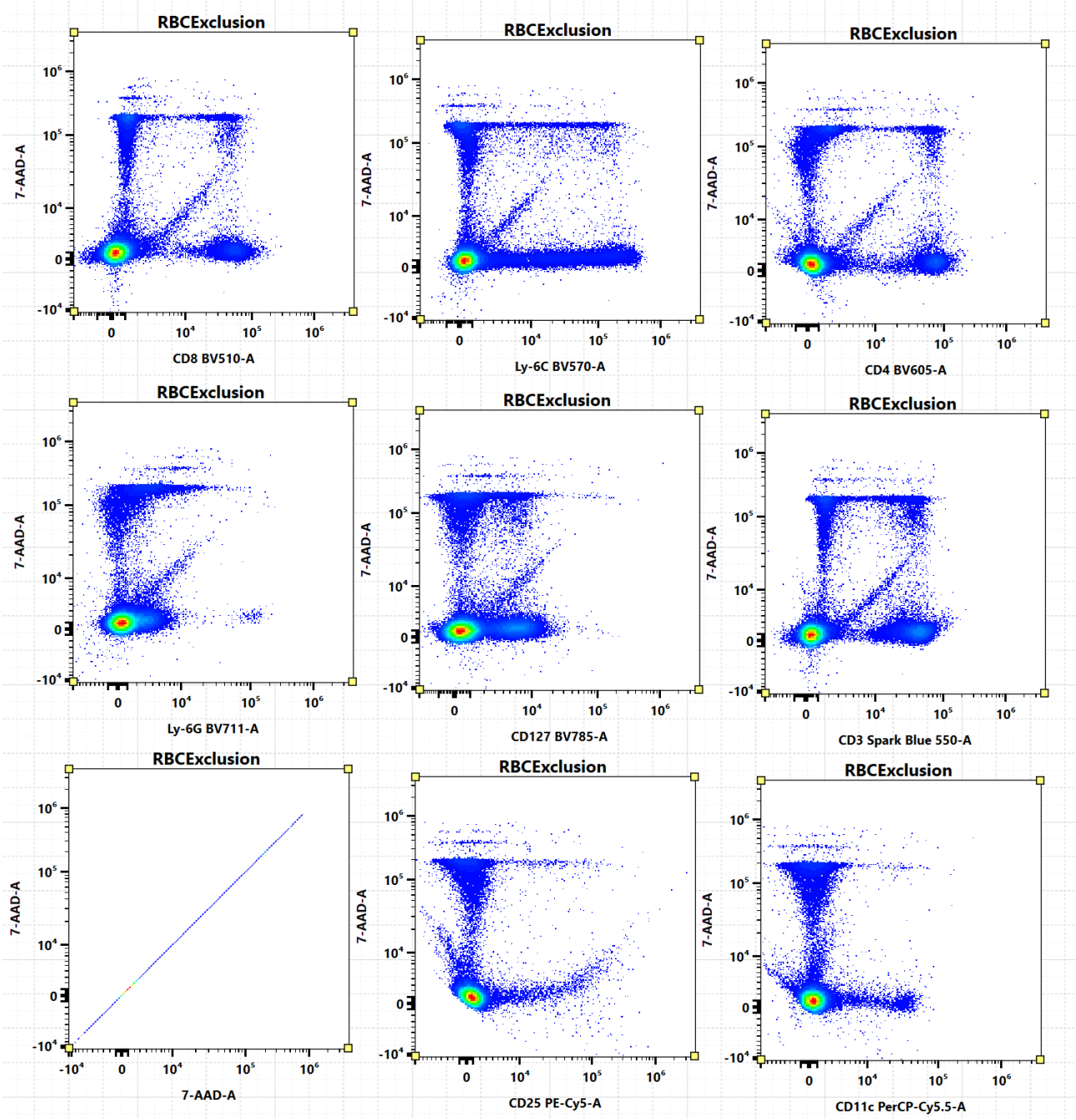

Live
Ly-6C BV570-A
CD8 BV510-A
Live
Ly-6C BV570-A
Ly-6C BV570-A
Live
Ly-6C BV570-A
CD4 BV605-A
Live
Ly-6C BV570-A
Ly-6G BV711-A
Live
Ly-6C BV570-A
CD127 BV785-A
Live
Ly-6C BV570-A
CD3 Spark Blue 550-A
Live
Ly-6C BV570-A
7-AAD-A
Live
Ly-6C BV570-A
CD25 PE-Cy5-A
Live
Ly-6C BV570-A
CD11c PerCP-Cy5.5-A

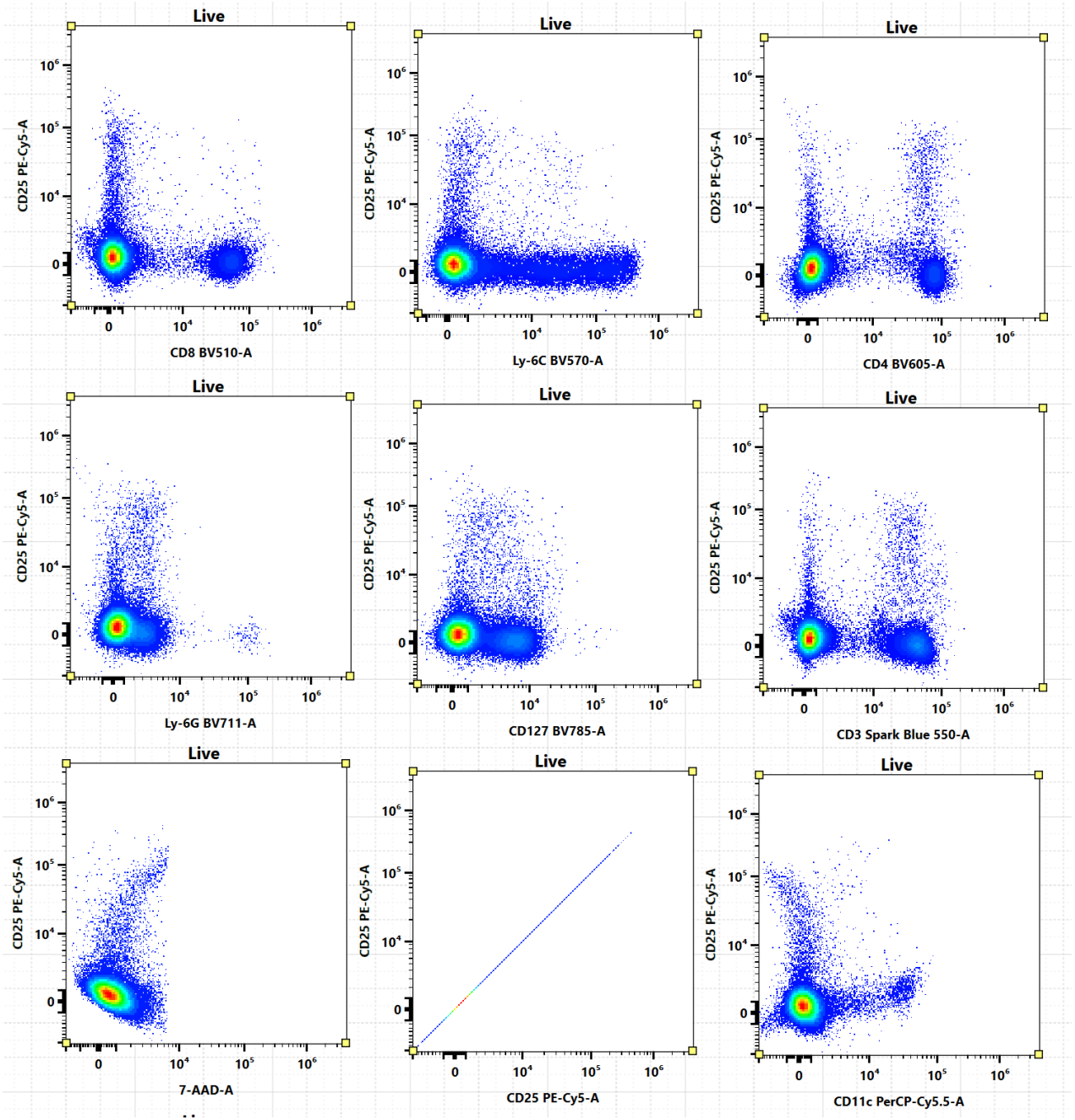

Live
Ly-6G BV711-A
CD8 BV510-A
Live
Ly-6G BV711-A
Ly-6C BV570-A
Live
Ly-6G BV711-A
CD4 BV605-A
Live
Ly-6G BV711-A
Ly-6G BV711-A
Live
Ly-6G BV711-A
CD127 BV785-A
Live
Ly-6G BV711-A
CD3 Spark Blue 550-A
Live
Ly-6G BV711-A
7-AAD-A
Live
Ly-6G BV711-A
CD25 PE-Cy5-A
Live
Ly-6G BV711-A
CD11c PerCP-Cy5.5-A

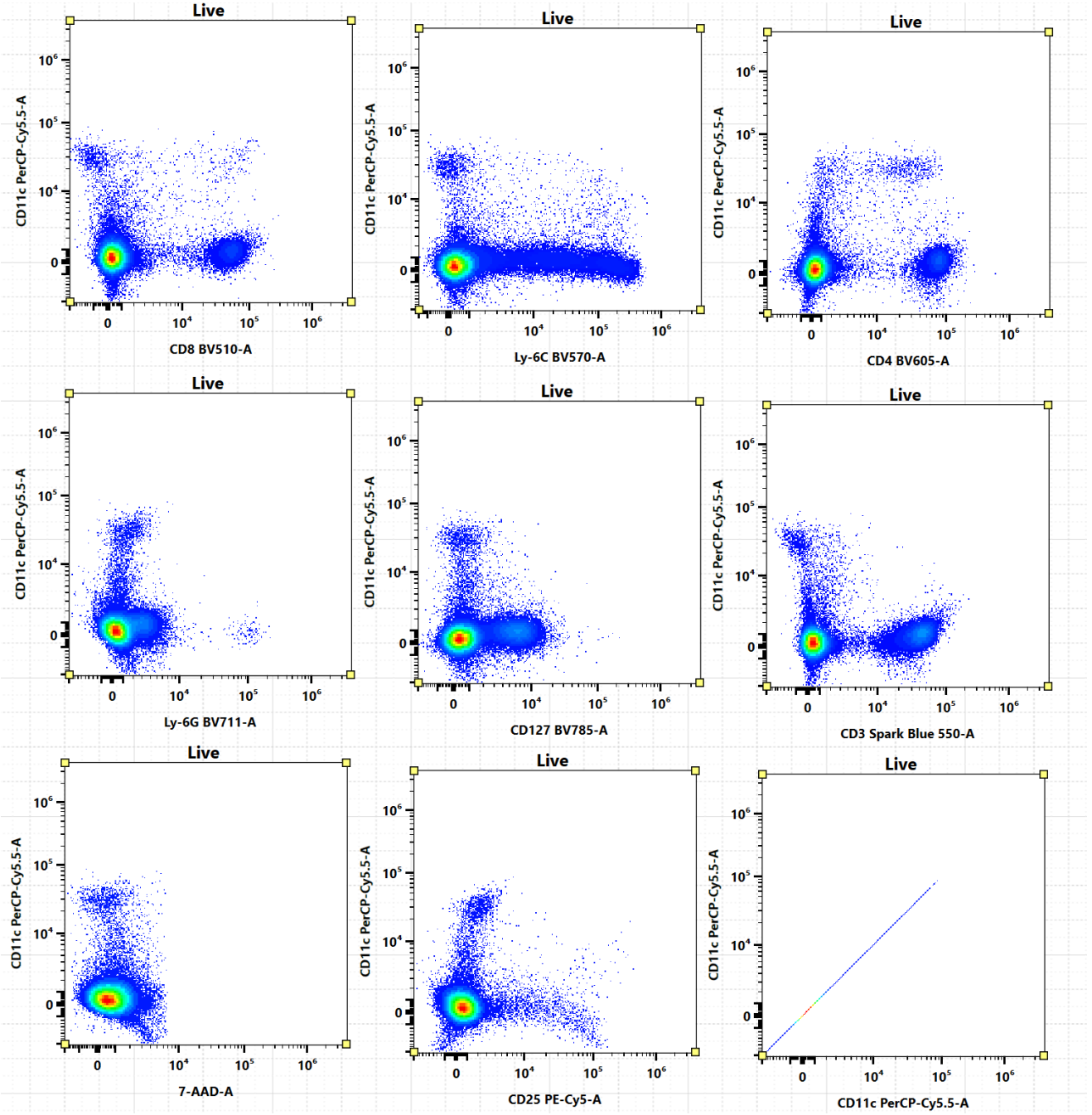

Tcells&NotTcells
Ly-6G BV711-A
CD8 BV510-A
Tcells&NotTcells
Ly-6G BV711-A
Ly-6C BV570-A
Tcells&NotTcells
Ly-6G BV711-A
CD4 BV605-A
Tcells&NotTcells
Ly-6G BV711-A
Ly-6G BV711-A
Tcells&NotTcells
Ly-6G BV711-A
CD127 BV785-A
Tcells&NotTcells
Ly-6G BV711-A
CD3 Spark Blue 550-A
Tcells&NotTcells
Ly-6G BV711-A
7-AAD-A
Tcells&NotTcells
Ly-6G BV711-A
CD25 PE-Cy5-A
Tcells&NotTcells
Ly-6G BV711-A
CD11c PerCP-Cy5.5-A


Live
CD127 BV785-A
CD8 BV510-A
Live
CD127 BV785-A
Ly-6C BV570-A
Live
CD127 BV785-A
CD4 BV605-A
Live
CD127 BV785-A
Ly-6G BV711-A
Live
CD127 BV785-A
CD127 BV785-A
Live
CD127 BV785-A
CD3 Spark Blue 550-A
Live
CD127 BV785-A
7-AAD-A
Live
CD127 BV785-A
CD25 PE-Cy5-A
Live
CD127 BV785-A
CD11c PerCP-Cy5.5-A

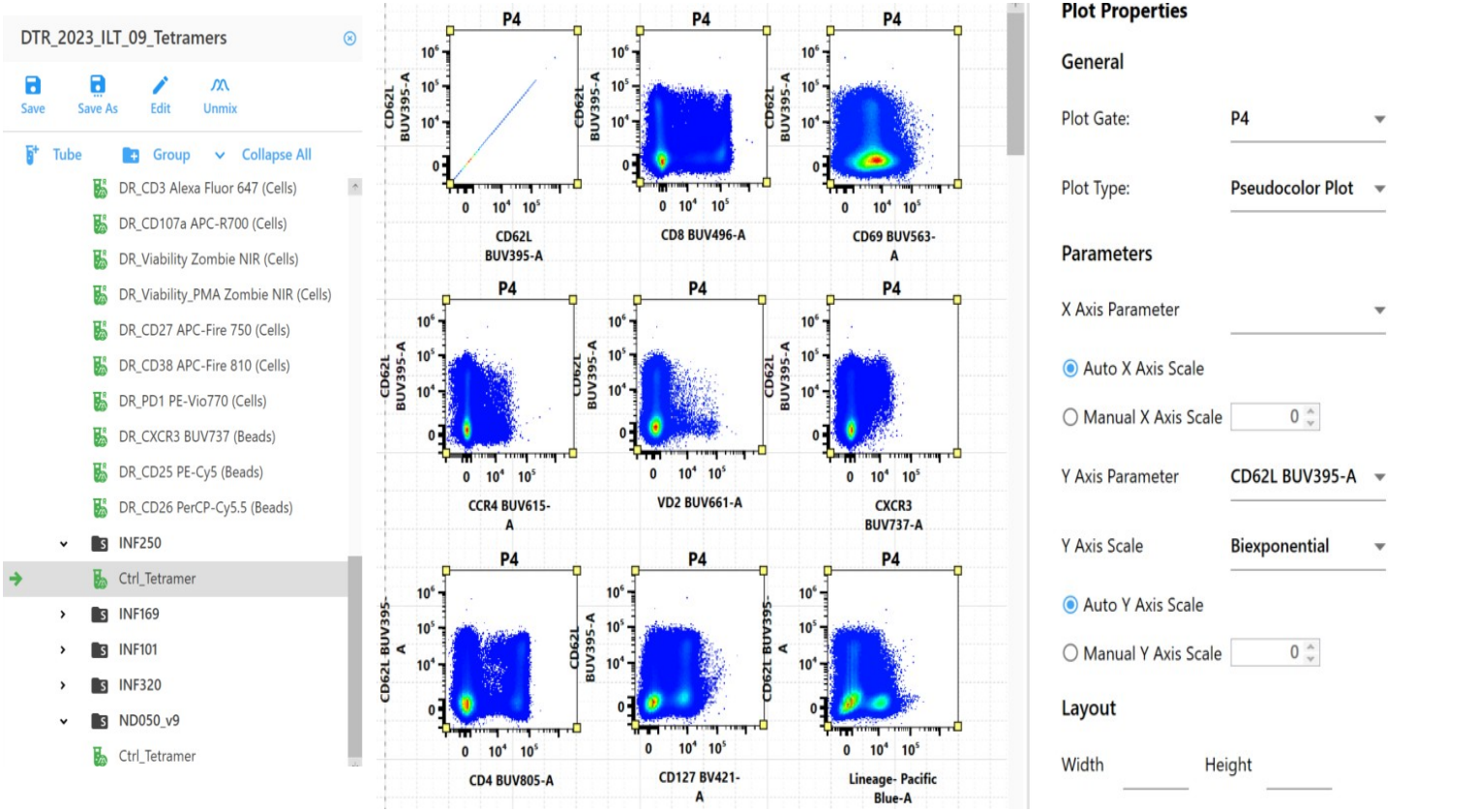

Live
CD3 Spark Blue 550-A
CD8 BV510-A
Live
CD3 Spark Blue 550-A
Ly-6C BV570-A
Live
CD3 Spark Blue 550-A
CD4 BV605-A
Live
CD3 Spark Blue 550-A
Ly-6G BV711-A
Live
CD3 Spark Blue 550-A
CD127 BV785-A
Live
CD3 Spark Blue 550-A
CD3 Spark Blue 550-A
Live
CD3 Spark Blue 550-A
7-AAD-A
Live
CD3 Spark Blue 550-A
CD25 PE-Cy5-A
Live
CD3 Spark Blue 550-A
CD11c PerCP-Cy5.5-A


RBCExclusion
7-AAD-A
CD8 BV510-A
RBCExclusion
7-AAD-A
Ly-6C BV570-A
RBCExclusion
7-AAD-A
CD4 BV605-A
RBCExclusion
7-AAD-A
Ly-6G BV711-A
RBCExclusion
7-AAD-A
CD127 BV785-A
RBCExclusion
7-AAD-A
CD3 Spark Blue 550-A
RBCExclusion
7-AAD-A
7-AAD-A
RBCExclusion
7-AAD-A
CD25 PE-Cy5-A
RBCExclusion
7-AAD-A
CD11c PerCP-Cy5.5-A


Live
CD25 PE-Cy5-A
CD8 BV510-A
Live
CD25 PE-Cy5-A
Ly-6C BV570-A
Live
CD25 PE-Cy5-A
CD4 BV605-A
Live
CD25 PE-Cy5-A
Ly-6G BV711-A
Live
CD25 PE-Cy5-A
CD127 BV785-A
Live
CD25 PE-Cy5-A
CD3 Spark Blue 550-A
Live
CD25 PE-Cy5-A
7-AAD-A
Live
CD25 PE-Cy5-A
CD25 PE-Cy5-A
Live
CD25 PE-Cy5-A
CD11c PerCP-Cy5.5-A

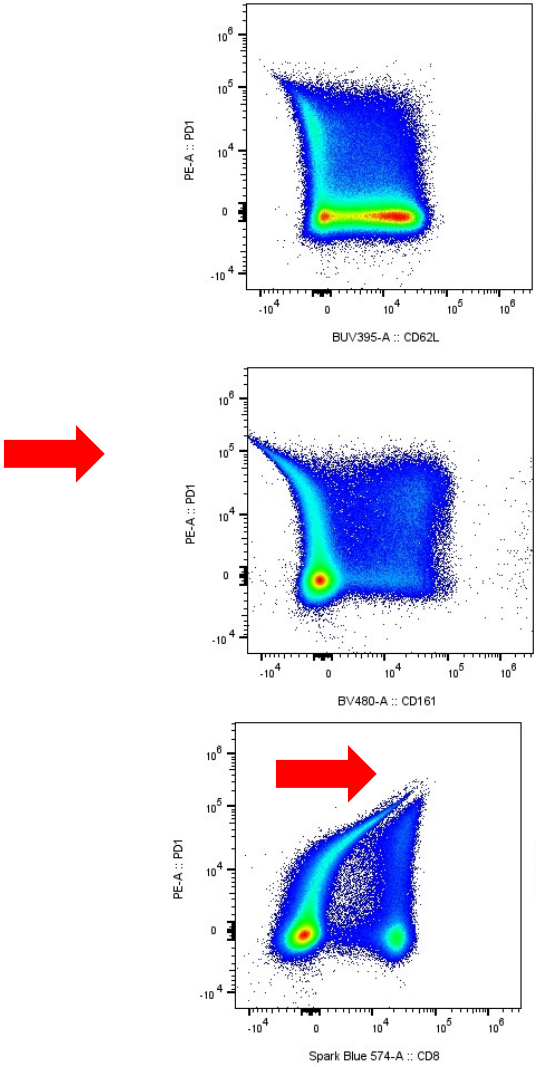

Live
CD11c PerCP-Cy5.5-A
CD8 BV510-A
Live
CD11c PerCP-Cy5.5-A
Ly-6C BV570-A
Live
CD11c PerCP-Cy5.5-A
CD4 BV605-A
Live
CD11c PerCP-Cy5.5-A
Ly-6G BV711-A
Live
CD11c PerCP-Cy5.5-A
CD127 BV785-A
Live
CD11c PerCP-Cy5.5-A
CD3 Spark Blue 550-A
Live
CD11c PerCP-Cy5.5-A
7-AAD-A
Live
CD11c PerCP-Cy5.5-A
CD25 PE-Cy5-A
Live
CD11c PerCP-Cy5.5-A
CD11c PerCP-Cy5.5-A

Check the full-stained samples 1xN plots



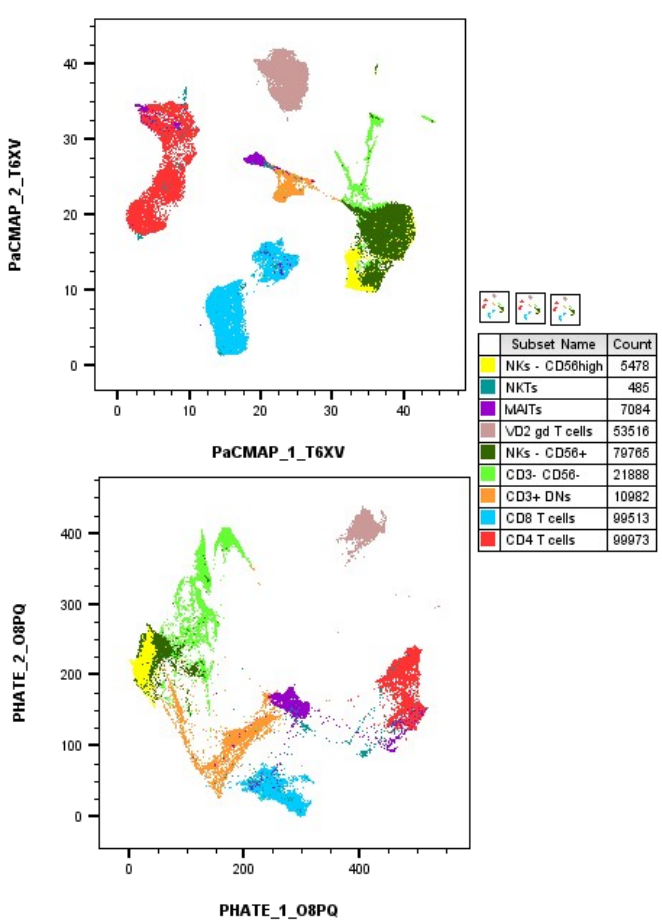
What fluorophores are having issues?
What cell subsets are affected?
How prevalent are they?

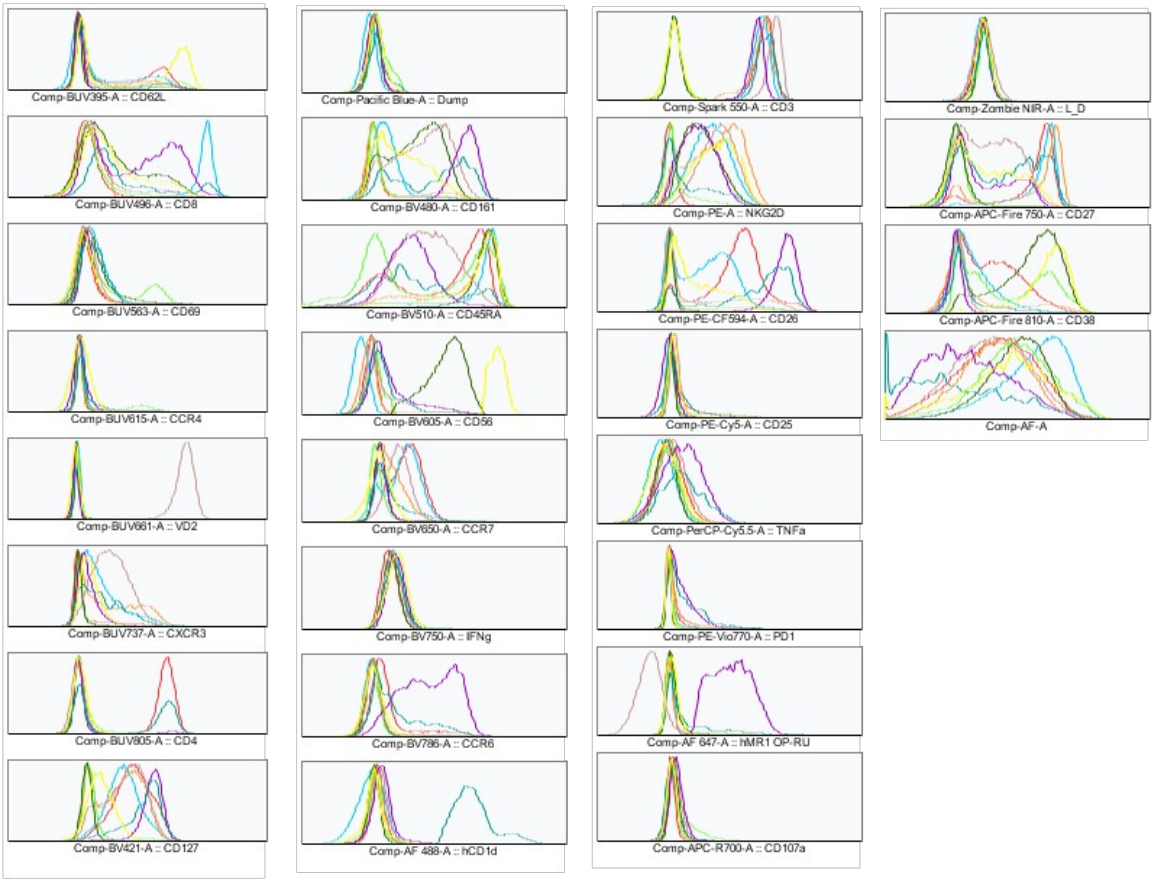
Check the full-stained samples 1xN plots



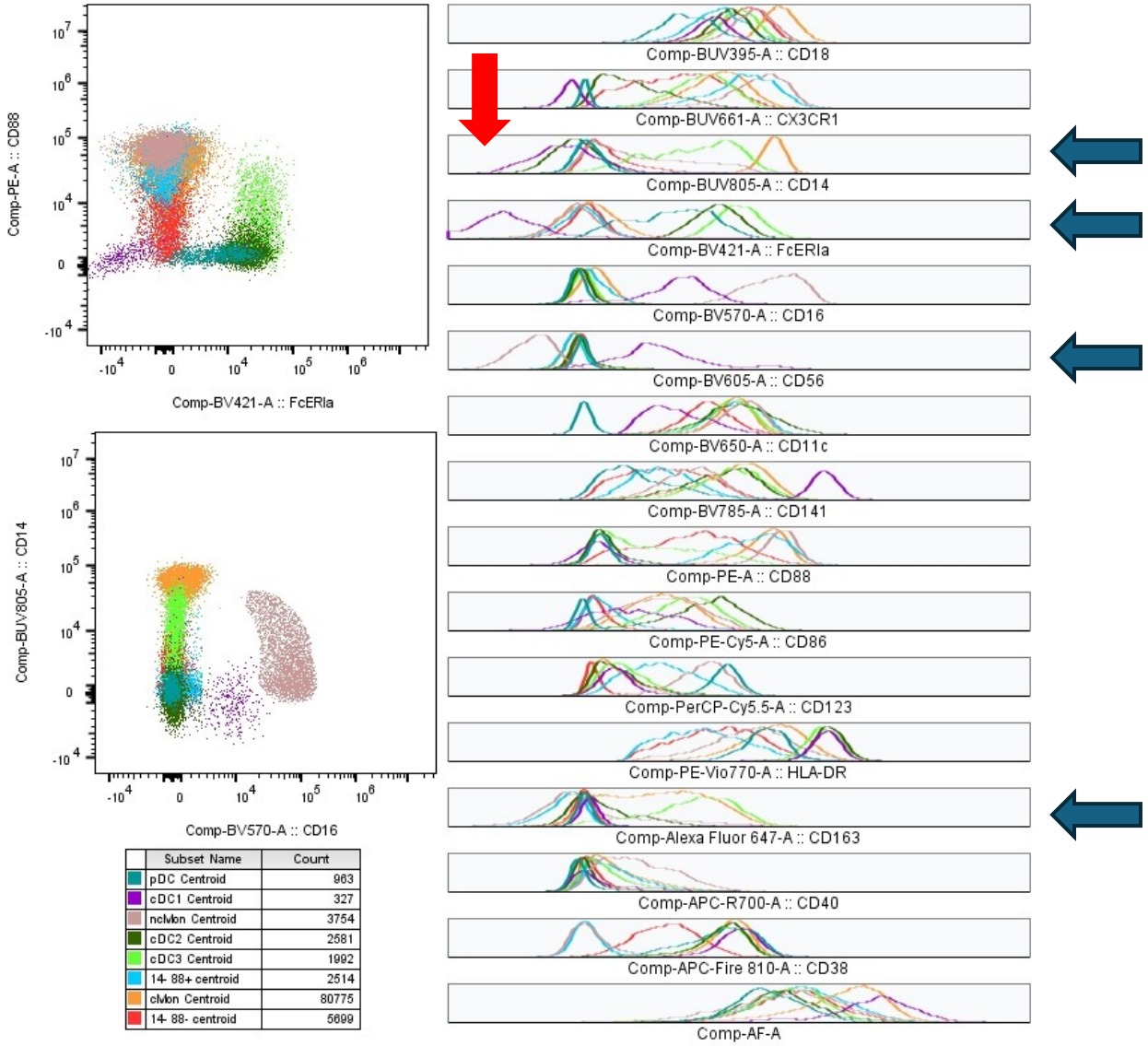
What fluorophores are having issues?
What cell subsets are affected?
How prevalent are they?

Alternatively: Gate cell population subsets, and visualize marker distributions



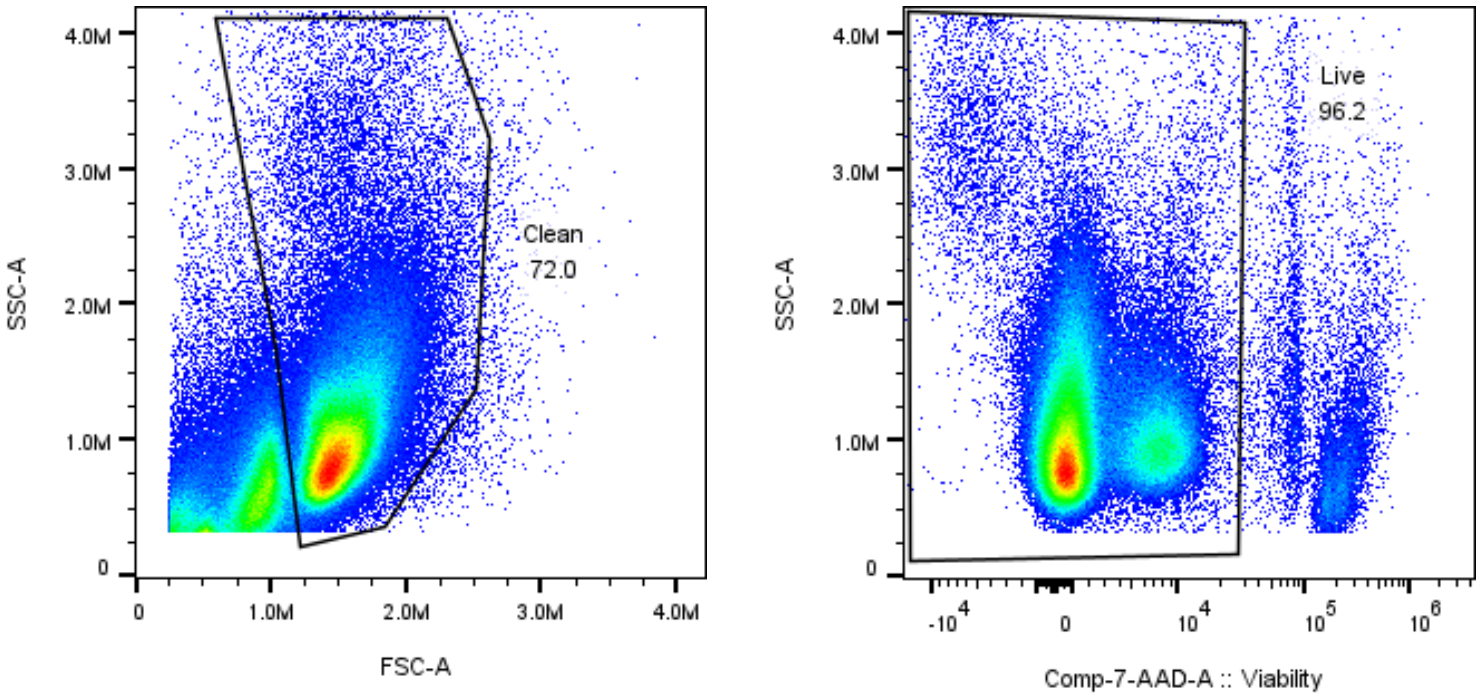


Highly-negative populations

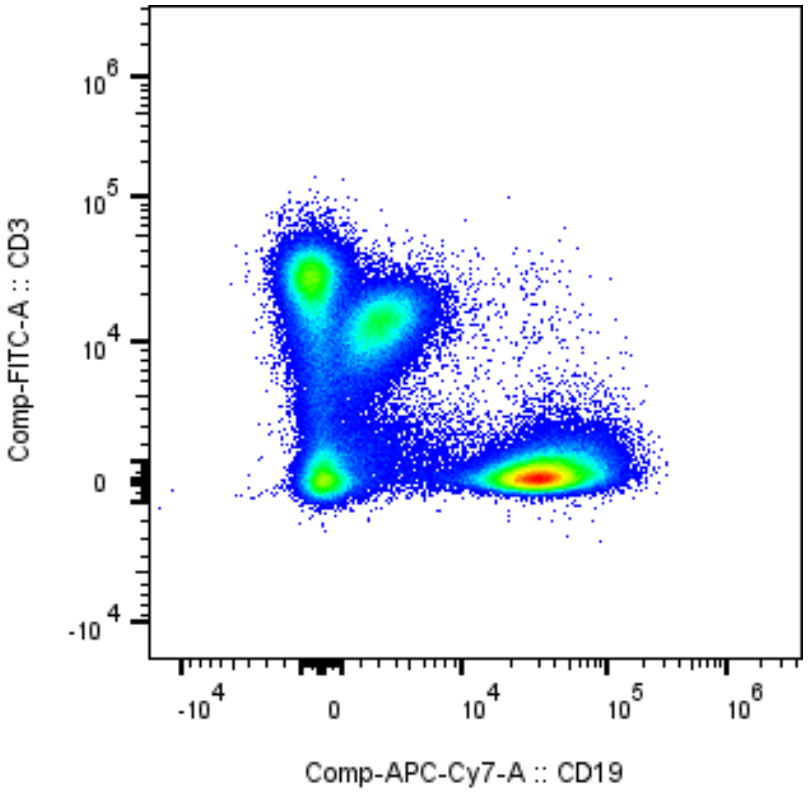


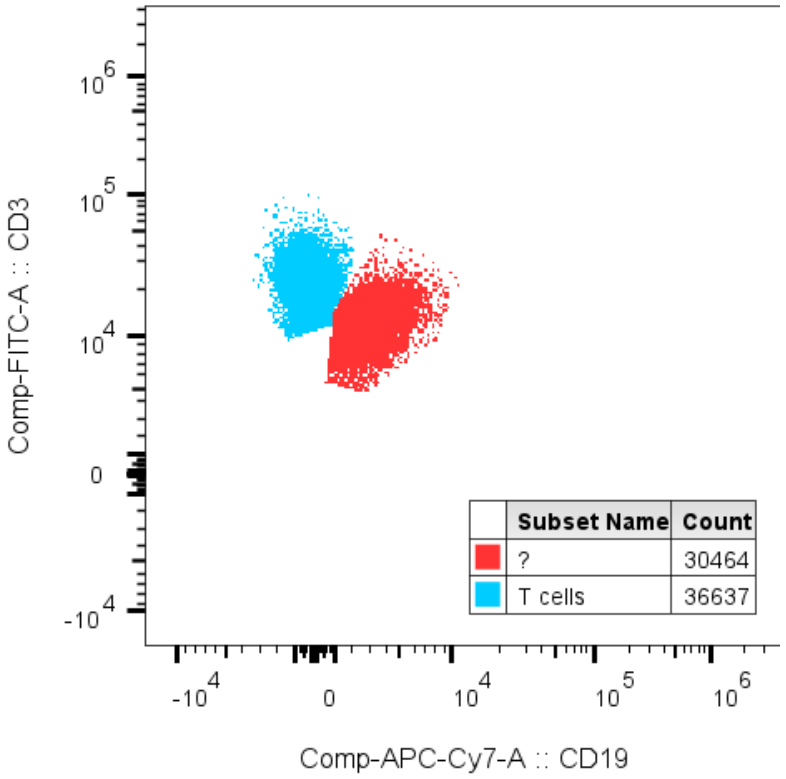
	Subset Name	Count
	pDC Centroid	963
	cDC1 Centroid	327
	nclMon Centroid	3754
	cDC2 Centroid	2581
	cDC3 Centroid	1992
	14- 88+ centroid	2514
	clMon Centroid	80775
	14- 88- centroid	5699

After gating out dead cells and debris

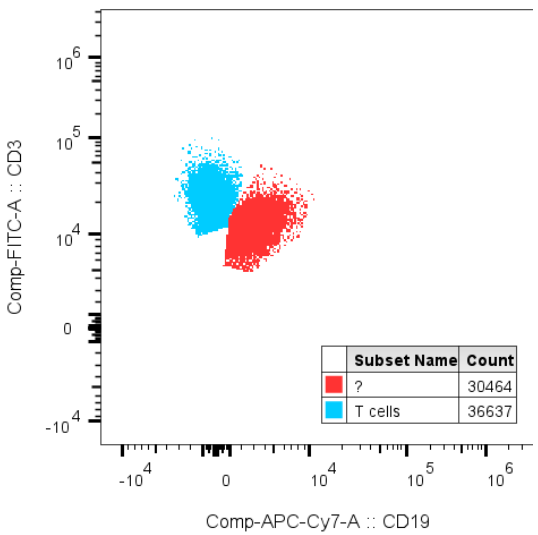


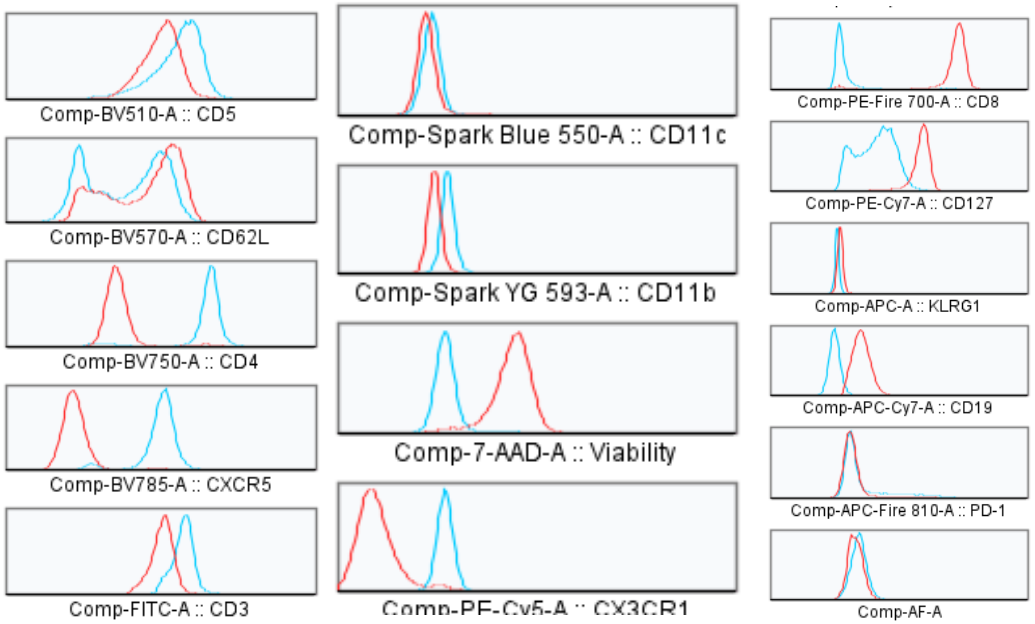
Ending up with two CD3+ populations



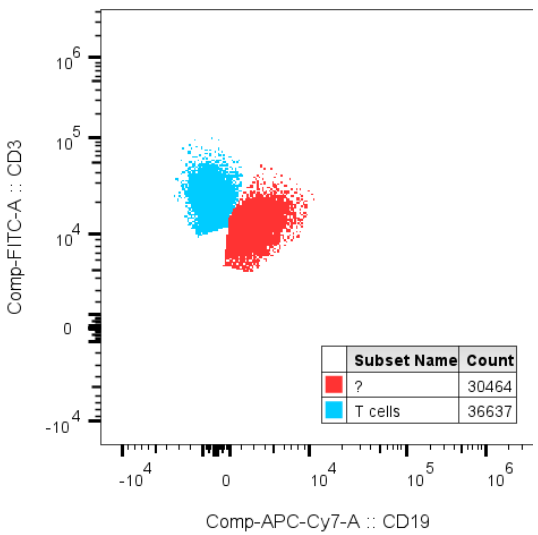


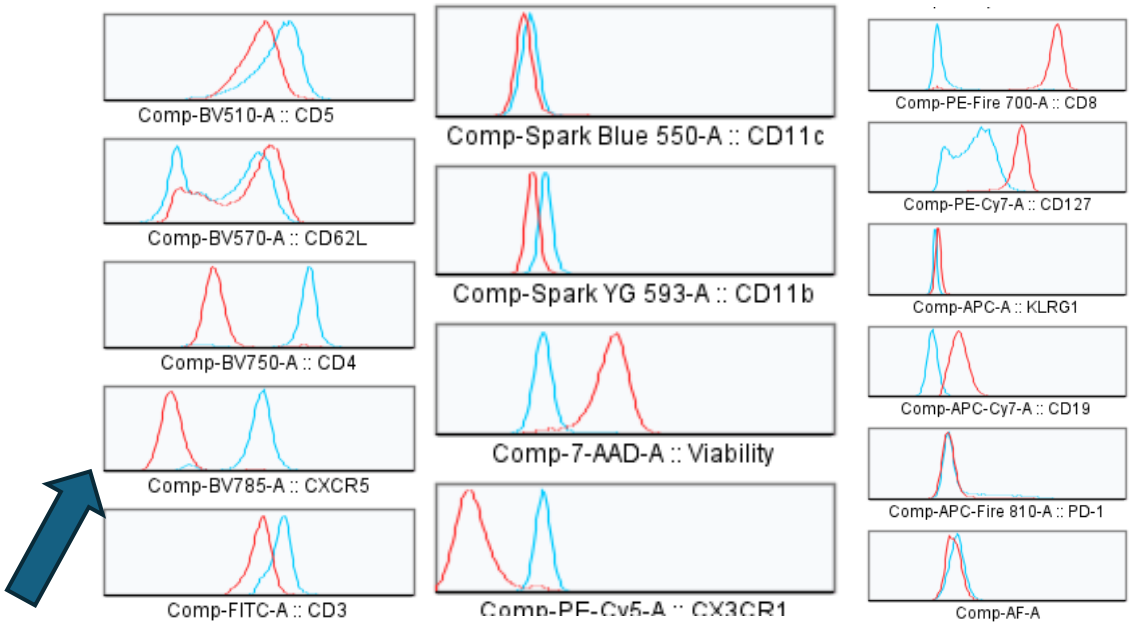
Visualizing both by Histograms





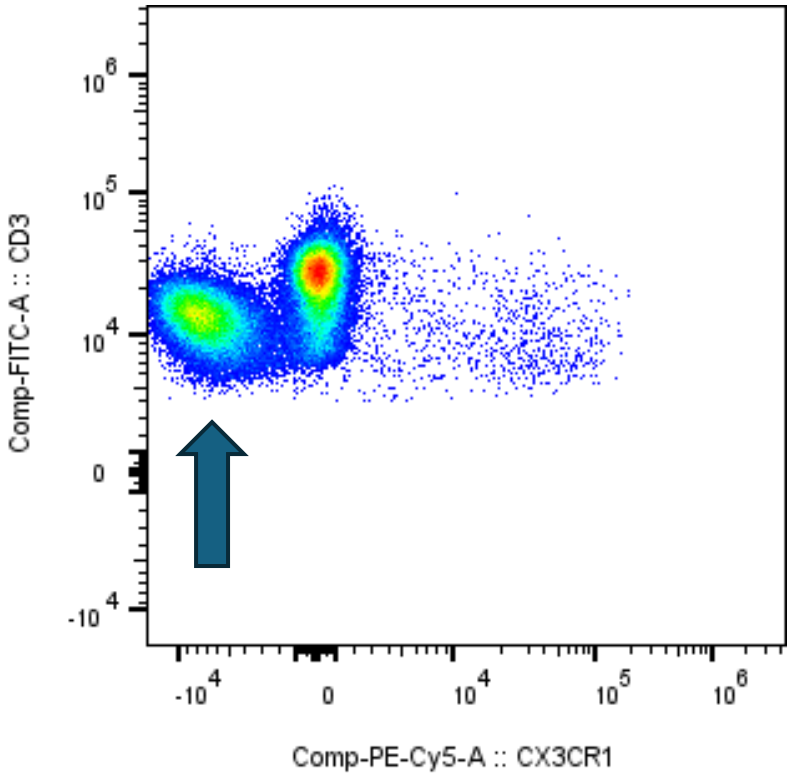
Note super-negative events



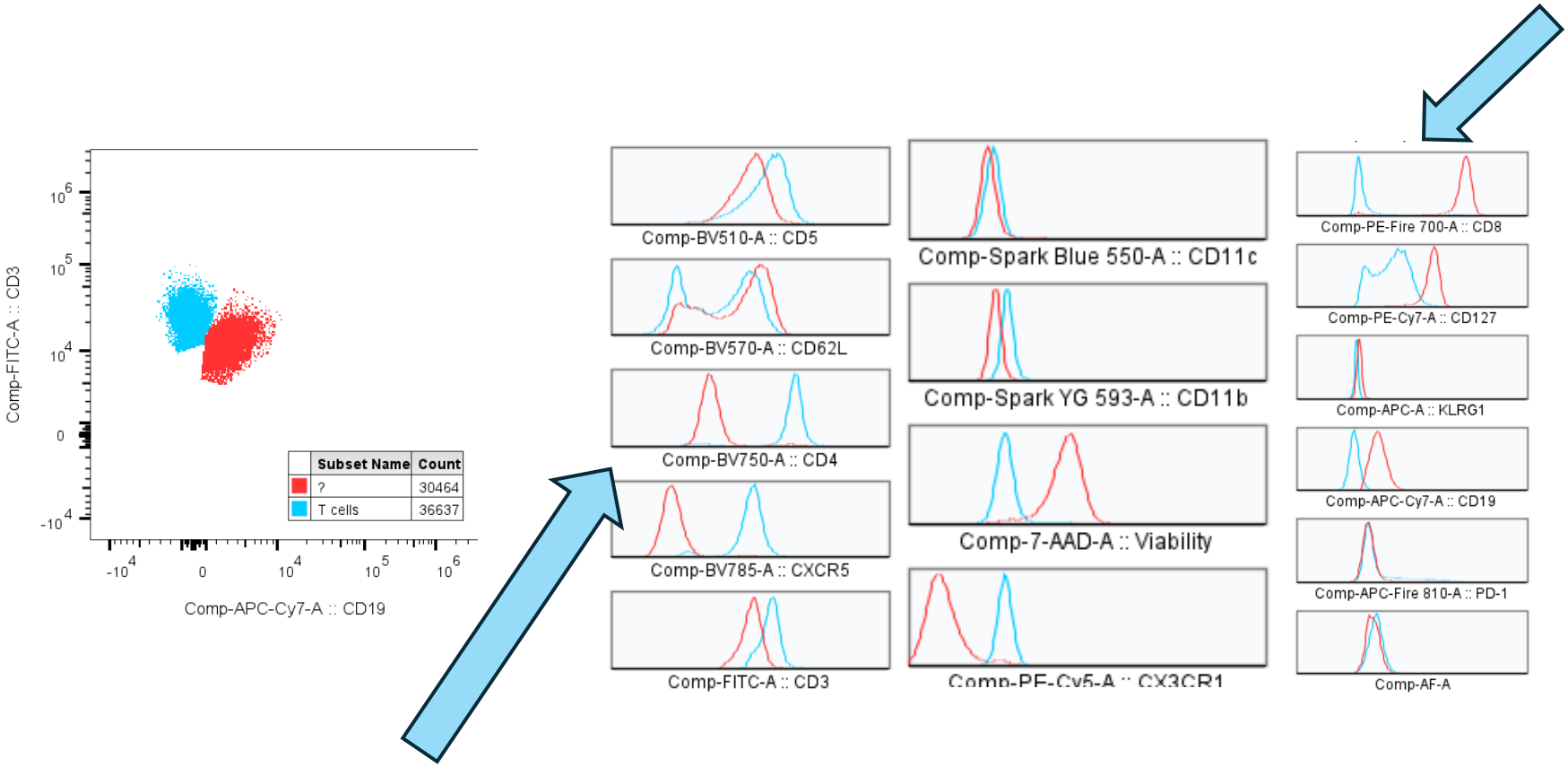


Super-negative events

Visualizing these markers by pseudocolor

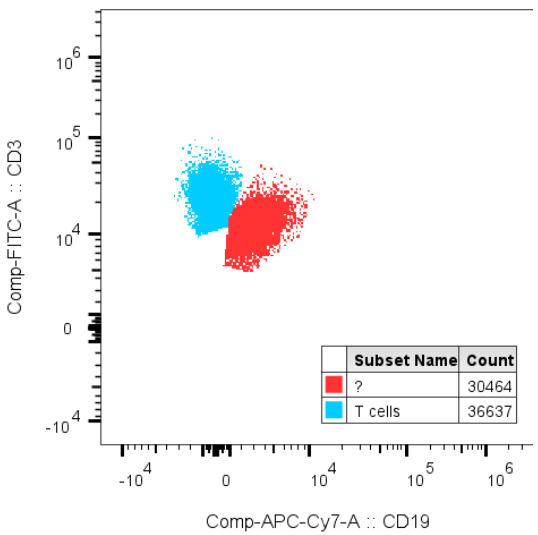


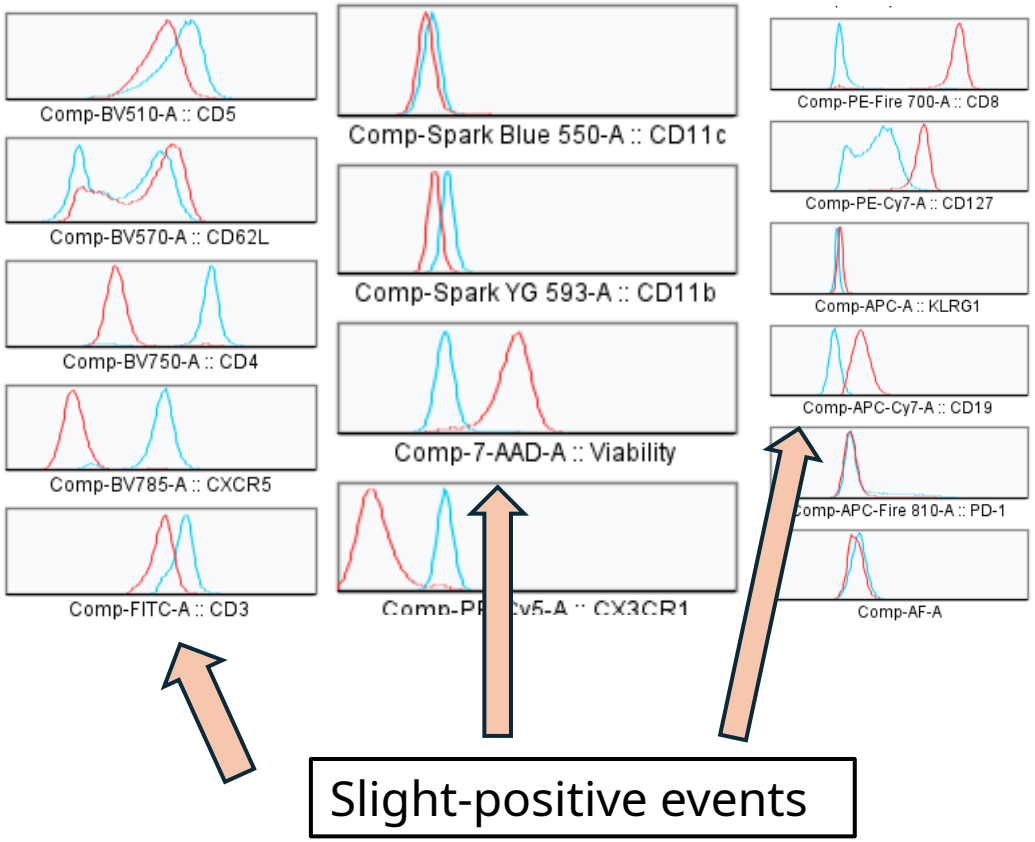
Note subset specific primary markers



Note which primary markers with good staining (negative at 0, positive 10^4) fully separate between the populations

Note slight-positive events





Our markers of interest

- The populations seem to be split between CD4+ and CD8+ T cells.

The super-negative fluorophores are:

- BV785 and PE-Cy5

The slightly positive markers are:

- FITC, 7-AAD, APC-Cy7

Which fluorophore is actually driving the unmixing issue?

Cytek Aurora 5L **4/25/2024**

Spectrum	UV			Violet			Blue			Yellow-Green			Red		
373	UV1														
388	UV2														
428	UV3			V1											
443	UV4			V2											
458	UV5			V3											
473	UV6			V4											
508				V5			B1								
514	UV7														
525				V6			B2	FITC	CD3						
542	UV8			V7	BV510	CD5	B3	Spark Blue 550	CD11c						
582	UV9			V8	BV570	CD62L	B4			YG1					
598				V9			B5			YG2	Spark YG 593	CD11b			
613	UV10			V10			B6			YG3					
664	UV11			V11			B7			YG4	7-AAD	Viability	R1	APC	KLRG1
679							B8			YG5	PE-Cy5	CXC3CR1	R2		
697	UV12			V12			B9			YG6			R3		
717	UV13			V13			B10			YG7	PE-Fire 700	CD8	R4		
738							B11						R5		
750	UV14			V14	BV750	CD4				YG8					
760							B12						R6		
783	UV15			V15	BV785	CXCR5	B13			YG9	PE-Cy7	CD127	R7	APC-Cy7	CD19
812	UV16			V16			B14			YG10			R8	APC-Fire 810	PD-1

Three main types of fluorophore interactions

Cytek Aurora 5L — 4/25/2024

Spectrum	UV		Violet			Blue			Yellow-Green			Red		
373	UV1													
388	UV2													
428	UV3		V1											
443	UV4		V2											
458	UV5		V3											
473	UV6		V4											
508			V5			B1								
514	UV7													
525			V6			B2	FITC	CD3						
542	UV8		V7	BV510	CD5	B3	Spark Blue 550	CD11c						
582	UV9		V8	BV570	CD62L	B4			YG1					
598			V9			B5			YG2	Spark YG 593	CD11b			
613	UV10		V10			B6			YG3					
664	UV11		V11			B7			YG4	7-AAD	Viability	R1	APC	KLRG1
679						B8			YG5	PE-Cy5	CXC3CR1	R2		
697	UV12		V12			B9			YG6			R3		
717	UV13		V13			B10			YG7	PE-Fire 700	CD8	R4		
738						B11						R5		
750	UV14		V14	BV750	CD4				YG8					
760						B12						R6		
783	UV15		V15	BV785	CXCR5	B13			YG9	PE-Cy7	CD127	R7	APC-Cy7	CD19
812	UV16		V16			B14			YG10			R8	APC-Fire 810	PD-1

1. Intra-Laser 2. Inter-Laser 3. Tandem

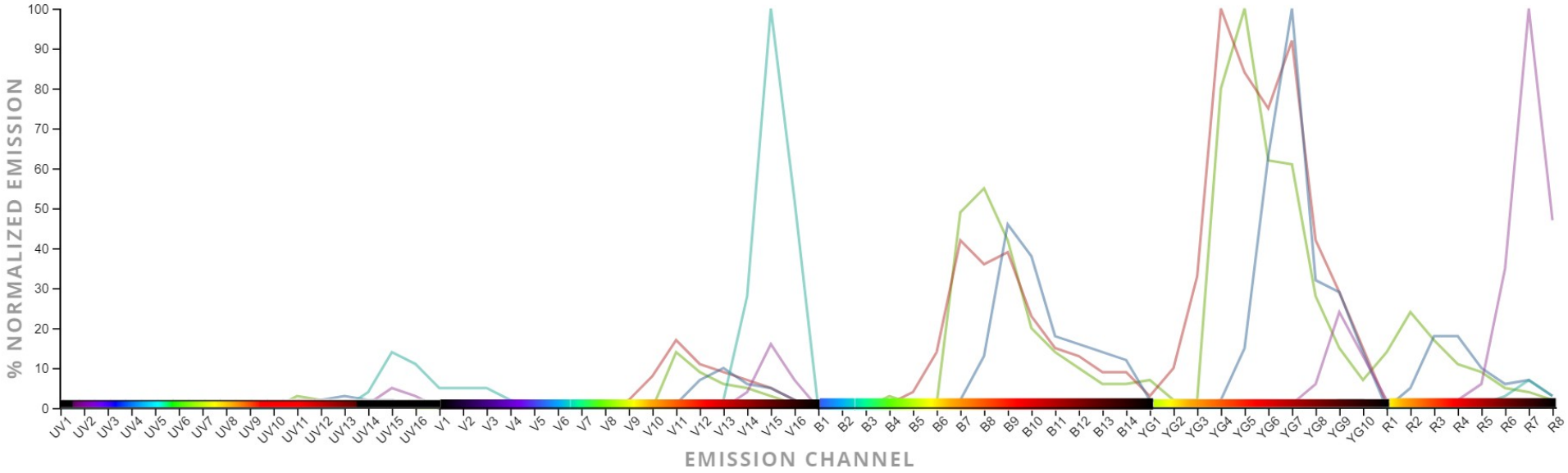
Intra-Laser

Cytek Aurora 5L — 4/25/2024

Spectrum	UV		Violet			Blue			Yellow-Green			Red		
373	UV1													
388	UV2													
428	UV3		V1											
443	UV4		V2											
458	UV5		V3											
473	UV6		V4											
508			V5			B1								
514	UV7													
525			V6			B2	FITC	CD3						
542	UV8		V7	BV510	CD5	B3	Spark Blue 550	CD11c						
582	UV9		V8	BV570	CD62L	B4			YG1					
598			V9			B5			YG2	Spark YG 593	CD11b			
613	UV10		V10			B6			YG3					
664	UV11		V11			B7			YG4	7-AAD	Viability	R1	APC	KLRG1
679						B8			YG5	PE-Cy5	CXC3CR1	R2		
697	UV12		V12			B9			YG6			R3		
717	UV13		V13			B10			YG7	PE-Fire 700	CD8	R4		
738						B11						R5		
750	UV14		V14	BV750	CD4				YG8					
760						B12						R6		
783	UV15		V15	BV785	CXCR5	B13			YG9	PE-Cy7	CD127	R7	APC-Cy7	CD19
812	UV16		V16			B14			YG10			R8	APC-Fire 810	PD-1

- Fluorescent signature plots and Cosine (Similarity) matrix are good proxies to identify fluorophores with extensive overlap.

Same information viewed differently



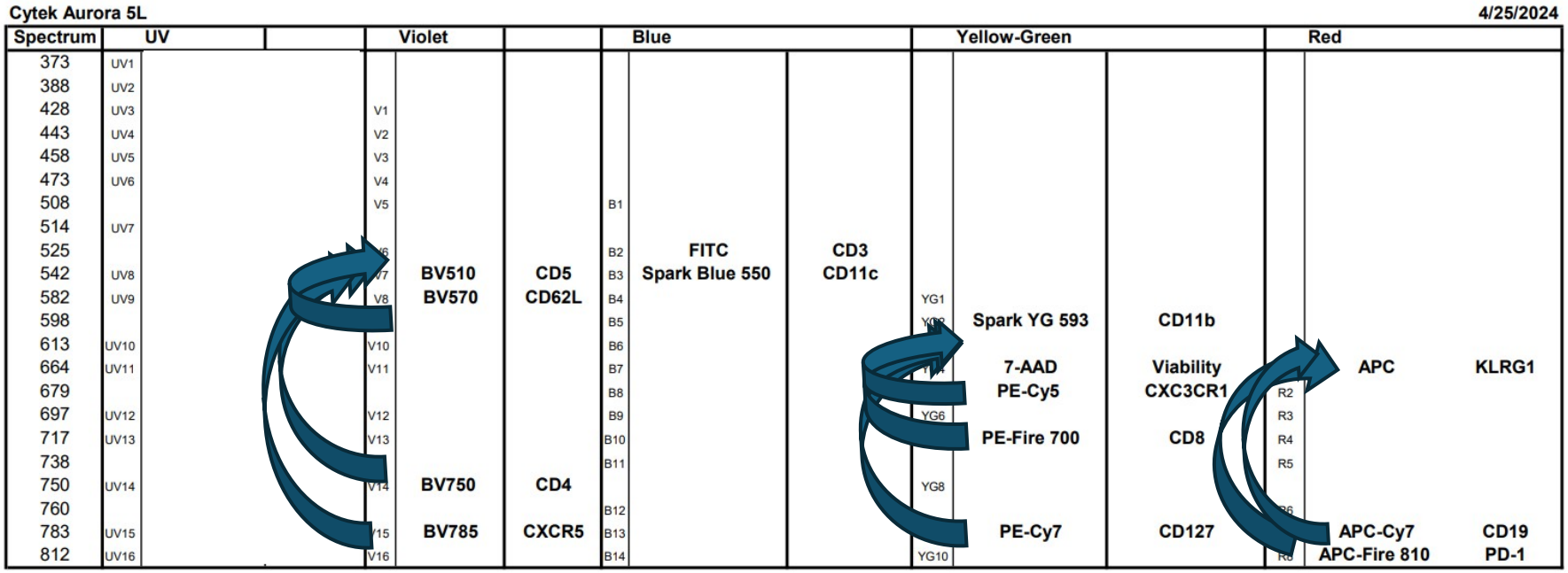
Inter-Laser

Cytek Aurora 5L 4/25/2024

Spectrum	UV		Violet			Blue			Yellow-Green			Red		
373	UV1													
388	UV2													
428	UV3		V1											
443	UV4		V2											
458	UV5		V3											
473	UV6		V4											
508			V5											
514	UV7													
525			V6			B2	FITC	CD3						
542	UV8		V7	BV510	CD5	B3	Spark Blue 550	CD11c						
582	UV9		V8	BV570	CD62L	B4			YG1					
598			V9			B5			YG2	Spark YG 593	CD11b			
613	UV10		V10			B6			YG3					
664	UV11		V11			B7			YG4	7-AAD	Viability	R1	APC	KLRG1
679						B8			YG5	PE-Cy5	CXC3CR1	R2		
697	UV12		V12			B9			YG6			R3		
717	UV13		V13			B10			YG7	PE-Fire 700	CD8	R4		
738						B11						R5		
750	UV14		V14	BV750	CD4				YG8					
760						B12						R6		
783	UV15		V15	BV785	CXCR5	B13			YG9	PE-Cy7	CD127	R7	APC-Cy7	CD19
812	UV16		V16			B14			YG10			R8	APC-Fire 810	PD-1

- Fluorophores have emission peaks across multiple lasers. Check on a by laser basis the equivalent wavelength

Tandems



- Leaf-frogging
- Tandem dyes (especially if degrading) can cause issues on the parent fluorophore

Intra-Laser for our panel

Cytek Aurora 5L — 4/25/2024

Spectrum	UV		Violet			Blue			Yellow-Green			Red		
373	UV1													
388	UV2													
428	UV3		V1											
443	UV4		V2											
458	UV5		V3											
473	UV6		V4											
508			V5			B1								
514	UV7													
525			V6			B2	FITC	CD3						
542	UV8		V7	BV510	CD5	B3	Spark Blue 550	CD11c						
582	UV9		V8	BV570	CD62L	B4			YG1					
598			V9			B5			YG2	Spark YG 593	CD11b			
613	UV10		V10			B6			YG3					
664	UV11		V11			B7			YG4	7-AAD	Viability	R1	APC	KLRG1
679						B8			YG5	PE-Cy5	CXC3CR1	R2		
697	UV12		V12			B9			YG6			R3		
717	UV13		V13			B10			YG7	PE-Fire 700	CD8	R4		
738						B11						R5		
750	UV14		V14	BV750	CD4				YG8					
760						B12						R6		
783	UV15		V15	BV785	CXCR5	B13			YG9	PE-Cy7	CD127	R7	APC-Cy7	CD19
812	UV16		V16			B14			YG10			R8	APC-Fire 810	PD-1

- Spectral Overlap and Cosine Similarity Matrix are good proxies to identify the extent

Inter-Laser

Cytek Aurora 5L — 4/25/2024

Spectrum	UV		Violet			Blue			Yellow-Green			Red		
373	UV1													
388	UV2													
428	UV3		V1											
443	UV4		V2											
458	UV5		V3											
473	UV6		V4											
508			V5			B1								
514	UV7													
525			V6			B2	FITC	CD3						
542	UV8		V7	BV510	CD5	B3	Spark Blue 550	CD11c						
582	UV9		V8	BV570	CD62L	B4			YG1					
598			V9			B5			YG2	Spark YG 593	CD11b			
613	UV10		V10			B6			YG3					
664	UV11		V11			B7			YG4	7-AAD	Viability	R1	APC	KLRG1
679						B8			YG5	PE-Cy5	CXC3CR1	R2		
697	UV12		V12			B9			YG6			R3		
717	UV13		V13			B10			YG7	PE-Fire 700	CD8	R4		
738						B11						R5		
750	UV14		V14	BV750	CD4				YG8					
760														
783	UV15		V15	BV785	CXCR5	B13			YG9	PE-Cy7	CD127	R7	APC-Cy7	CD19
812	UV16		V16			B14			YG10			R8	APC-Fire 810	PD-1



- Fluorophores have emission peaks across multiple lasers. Check on a by laser basis the equivalent wavelength

If there were a Tandem Issue

Cytek Aurora 5L — 4/25/2024

Spectrum	UV		Violet			Blue			Yellow-Green			Red		
373	UV1													
388	UV2													
428	UV3		V1											
443	UV4		V2											
458	UV5		V3											
473	UV6		V4											
508			V5			B1								
514	UV7													
525			V6			B2	FITC	CD3						
542	UV8		V7	BV510	CD5	B3	Spark Blue 550	CD11c						
582	UV9		V8	BV570	CD62L	B4			YG1					
598			V9			B5			YG2	Spark YG 593	CD11b			
613	UV10		V10			B6								
664	UV11		V11			B7			YG4	7-AAD	Viability		APC	KLRG1
679						B8				PE-Cy5	CXC3CR1	R2		
697	UV12		V12			B9			YG6			R3		
717	UV13		V13			B10			YG7	PE-Fire 700	CD8	R4		
738						B11						R5		
750	UV14		V14	BV750	CD4				YG8					
760						B12						R6		
783	UV15		V15	BV785	CXCR5	B13			YG9	PE-Cy7	CD127	R7	APC-Cy7	CD19
812	UV16		V16			B14			YG10			R8	APC-Fire 810	PD-1

- Leaf-frogging
- Tandem dyes (especially if degrading) can cause issues on the parent fluorophore

What are you doing with your data?

Automated gating

Manual gating

Unsupervised Analysis

Supervised Analysis

Model Organisms

Clinical Samples

Blood

Tissue

Generalized Panel
(few co-expressed markers)

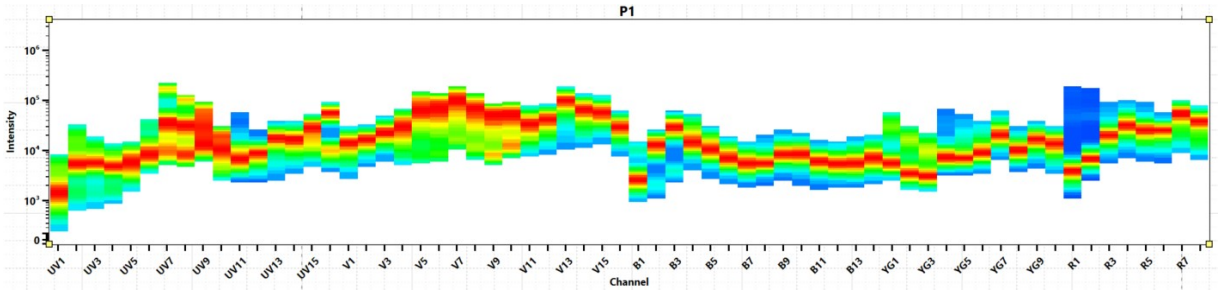
Targeted Panel
(many co-expressed markers)

A single cell population of interest

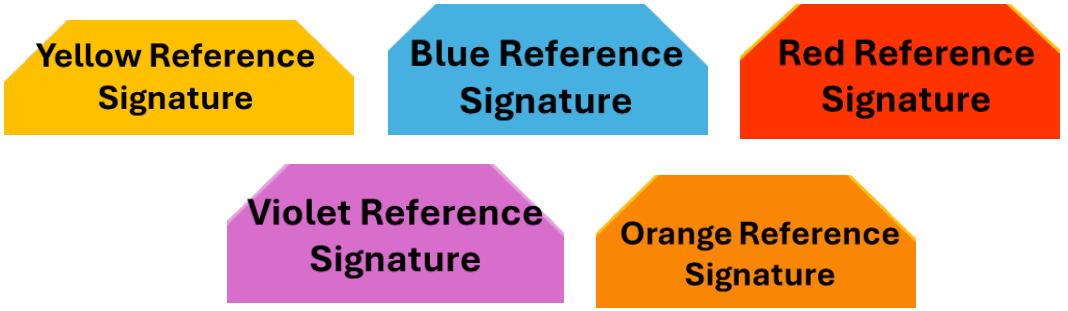
If the tiniest T cell subset sneezes, you want to know about it

A spectral cytometer needs three things for successful unmixing:

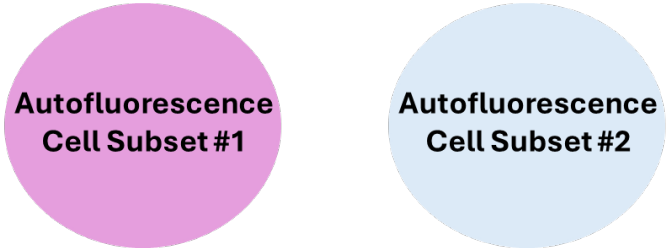
- **Full-stained Samples**



- **Single-color Reference Controls**



- **Unstained Reference Controls**



The four rules for good unmixing controls

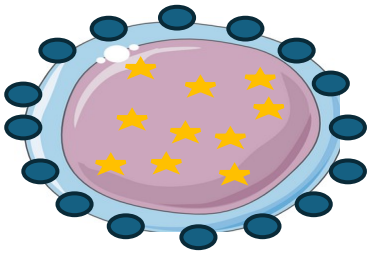
1. The single stained control must be as bright or brighter than the fully stained sample.
2. The exact same fluorophore must be used the stain the control and the fully stained sample.
3. Background autofluorescence should be the same for the positive and negative control
4. Enough Events

THE UNIVERSITY OF CHICAGO

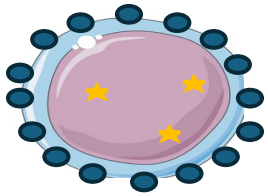
https://voices.uchicago.edu/ucflow/flow-cytometry-resources/

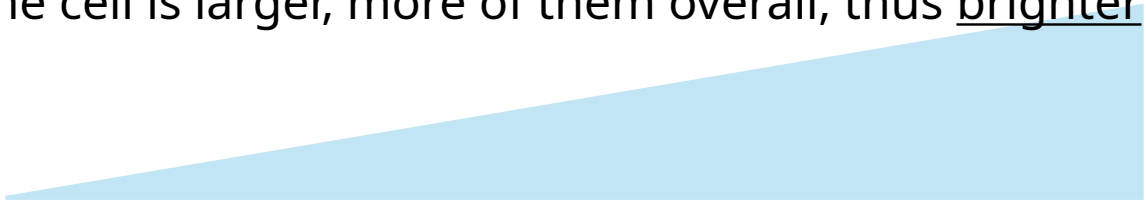
The issue: Variation in autofluorescence

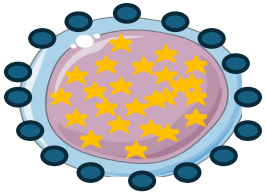




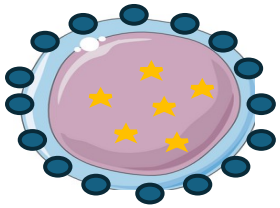
Difference in cell size:
Same proportion of autofluorescent molecules, but because one cell is larger, more of them overall, thus brighter



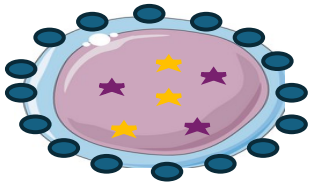




Difference in amount of autofluorescence:
Same cell size, just one has more autofluorescent molecules, thus brighter

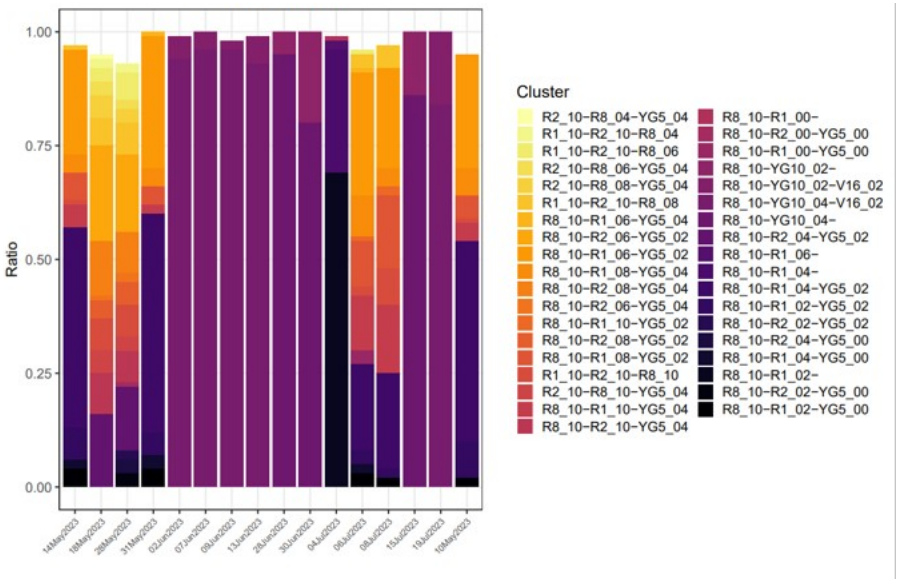






Variation in autofluorescent molecules:
Same cell size, but express different mixture of autofluorescent molecules, resulting in a different autofluorescent signature

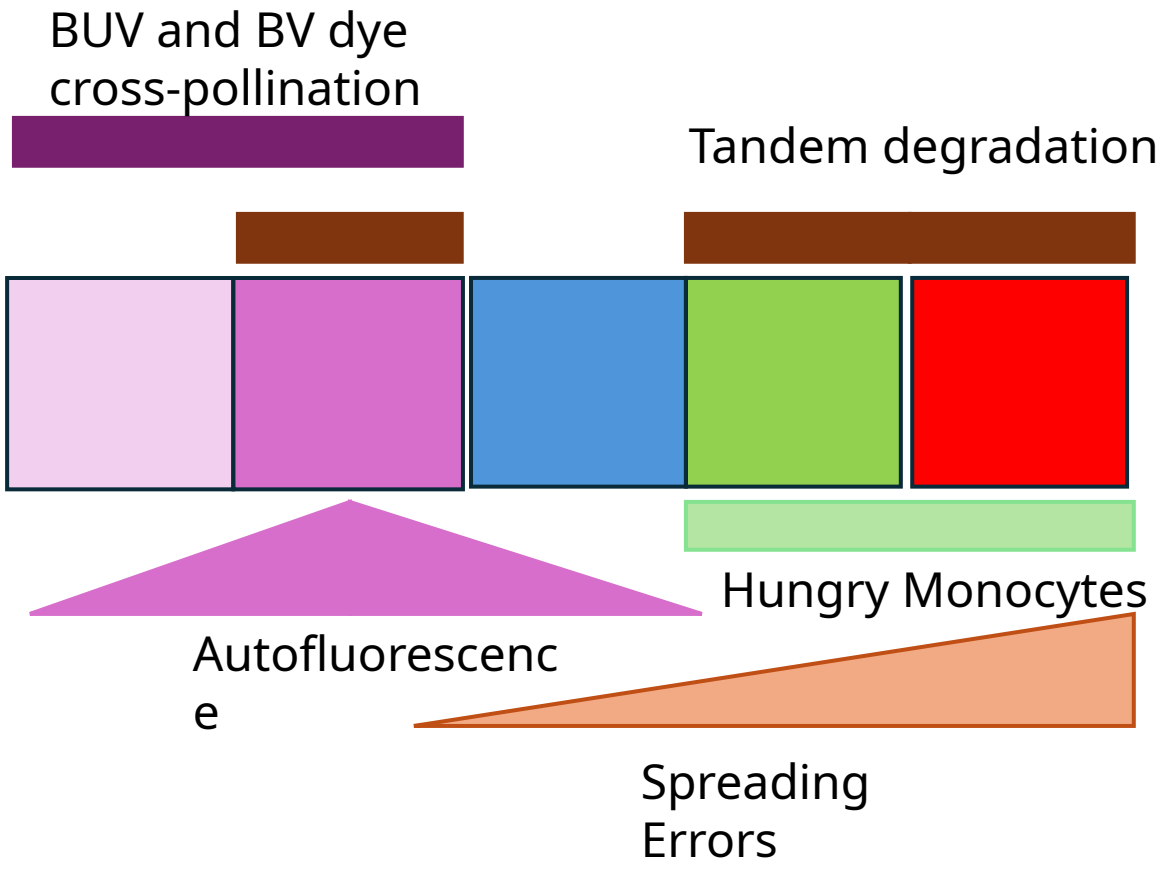
Your fluorophores can also vary in their signature!!!



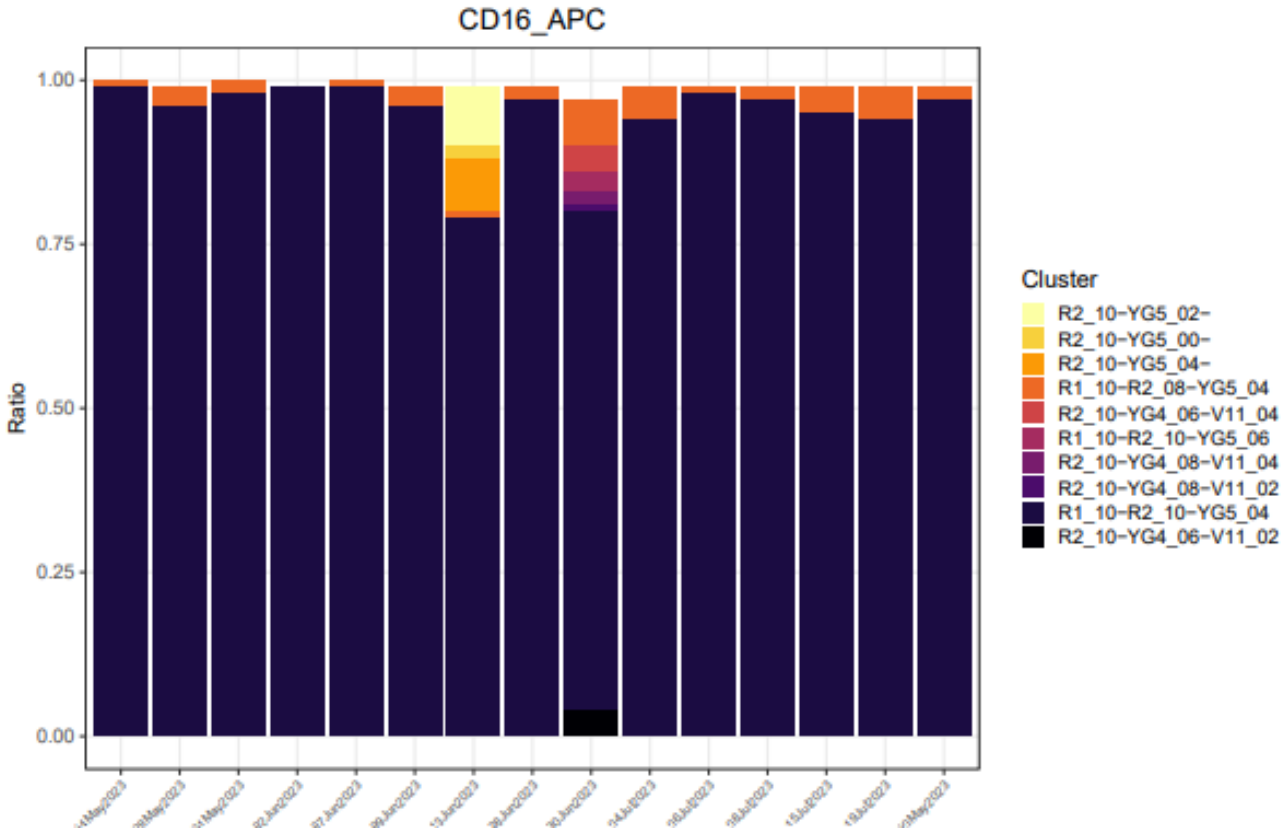
- ❑ Manufacturer Lot Variation
- ❑ Cross-pollinating BUV and BV fluorophores
- ❑ Monocytes eating PE and APC tandem dyes
- ❑ Tandem degradation
- ❑ FRET

And others!

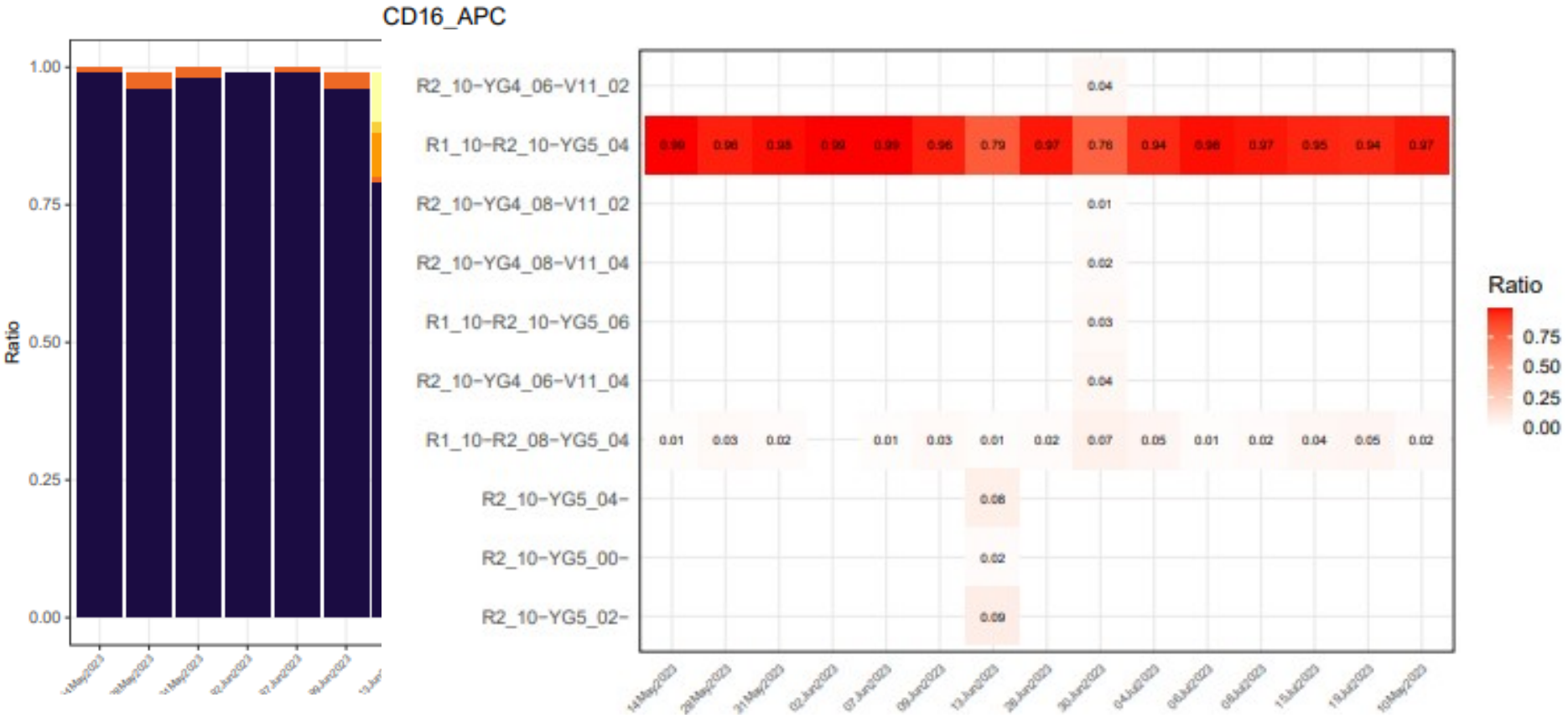
You encounter unmixing errors….



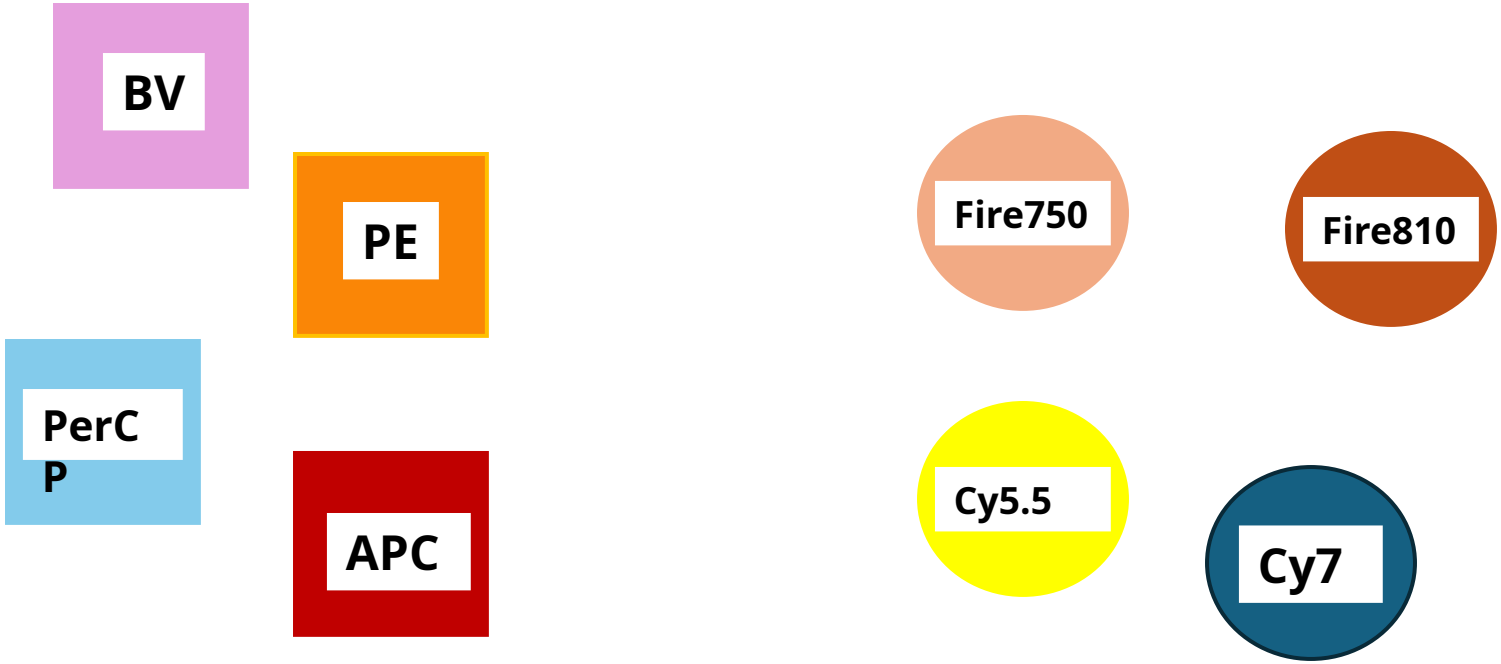
Many Fluorophores Quite Stable



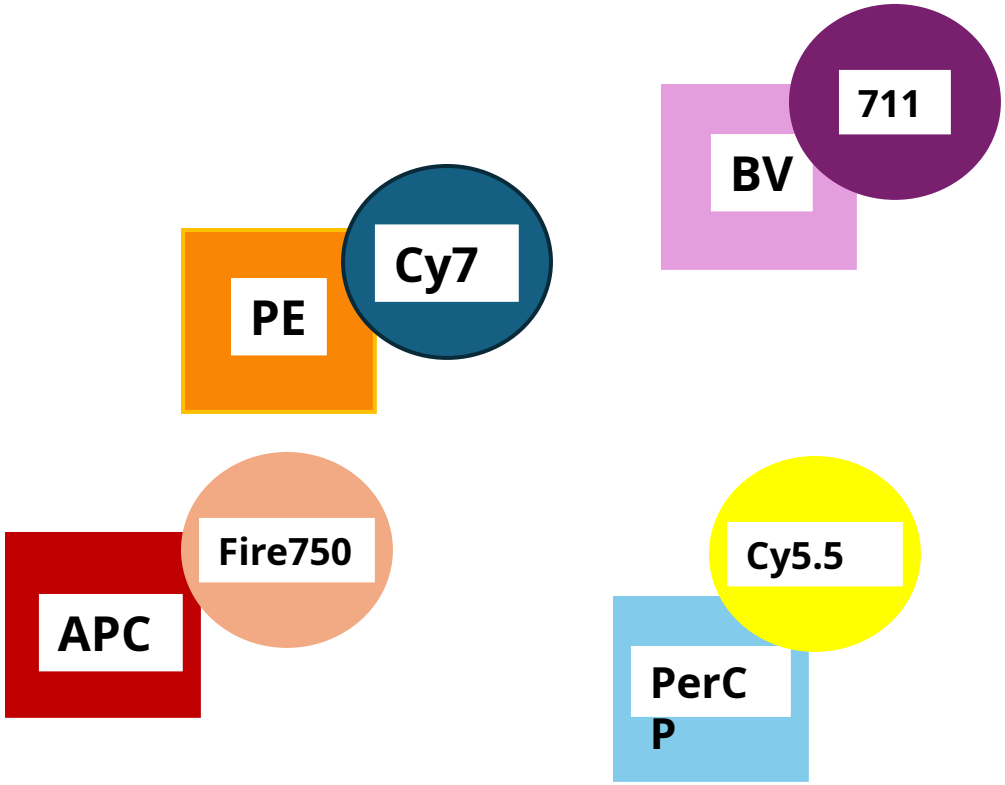
Many Fluorophores Quite Stable



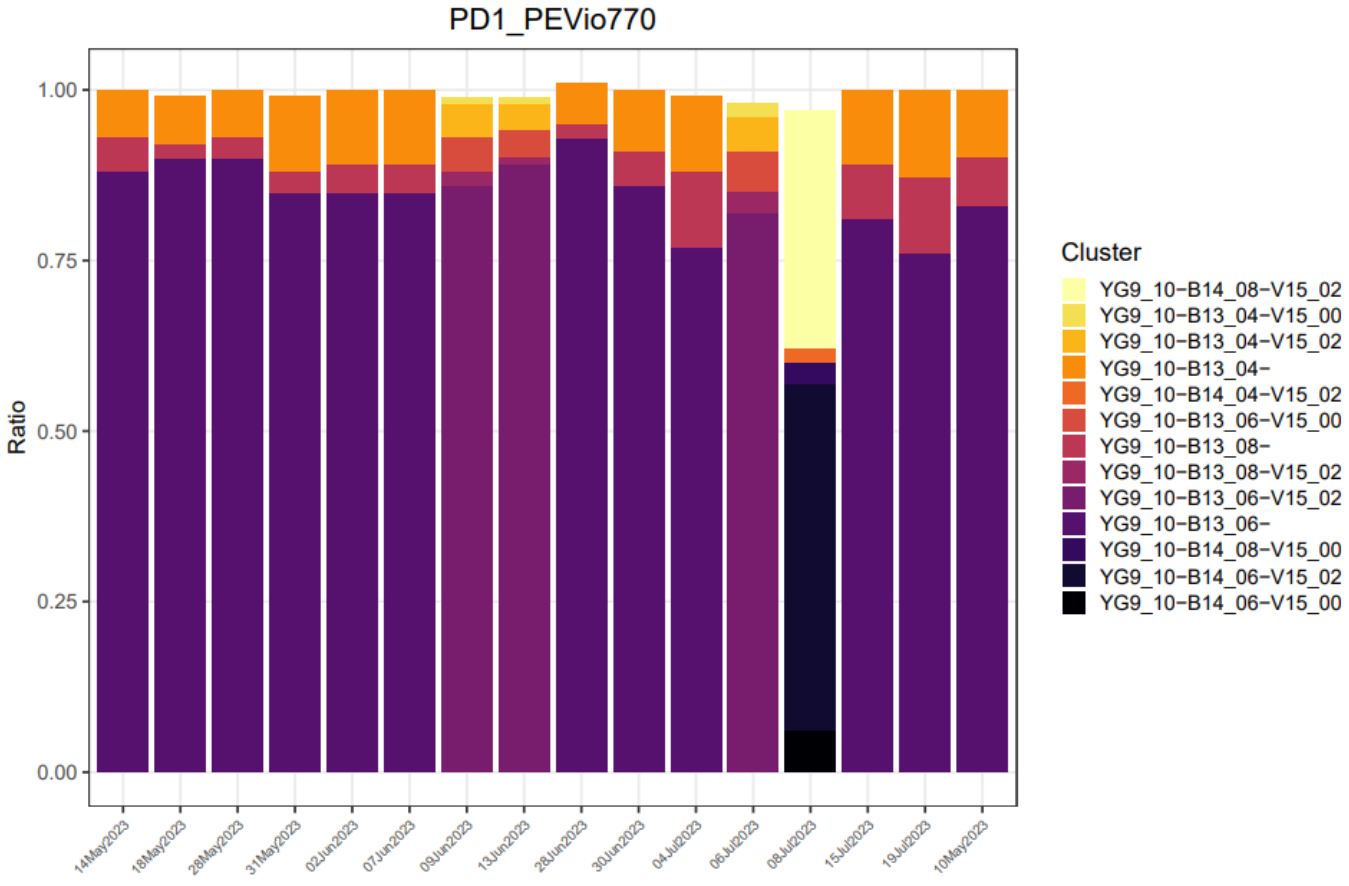
Tandem dyes



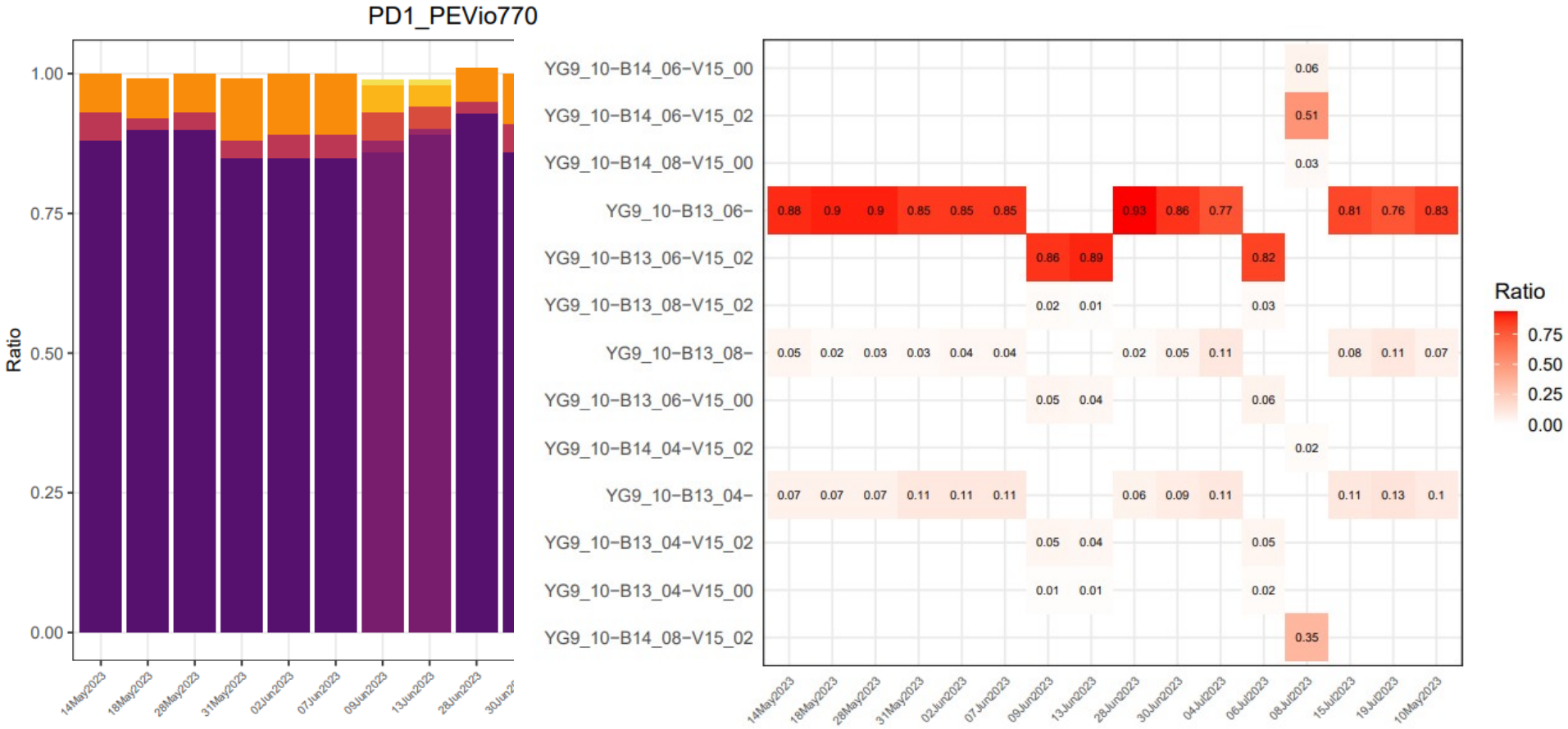
Tandem dyes



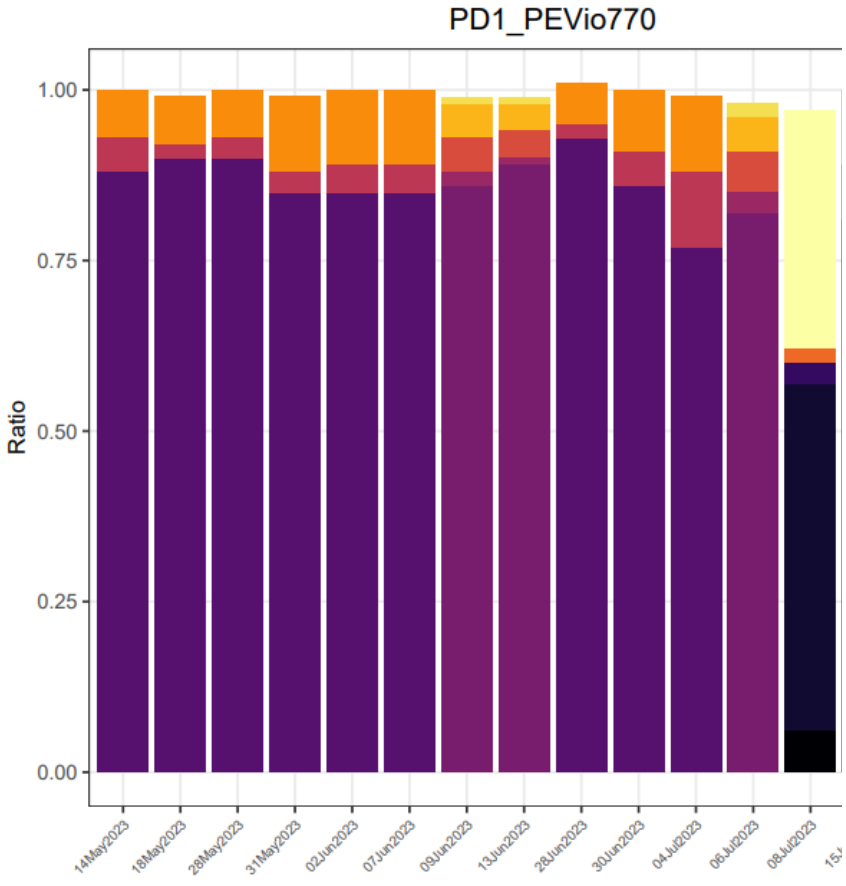
Tandem degradation

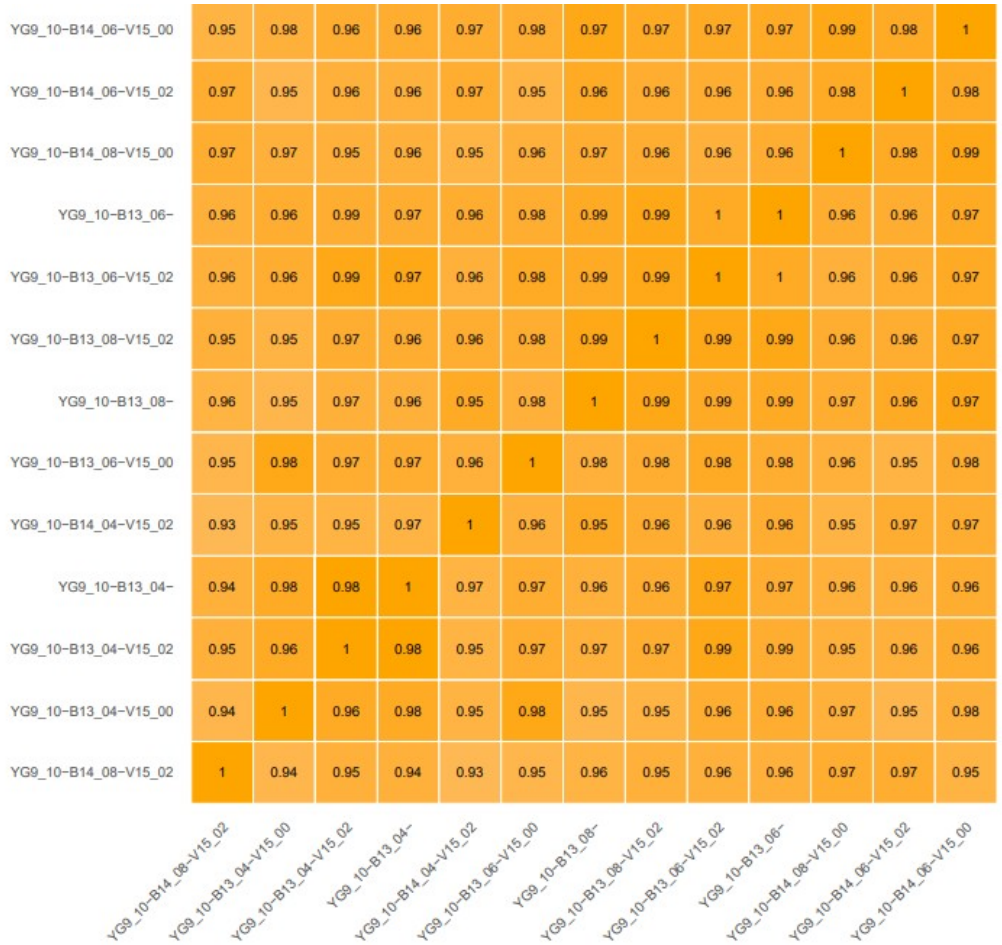


Tandem degradation

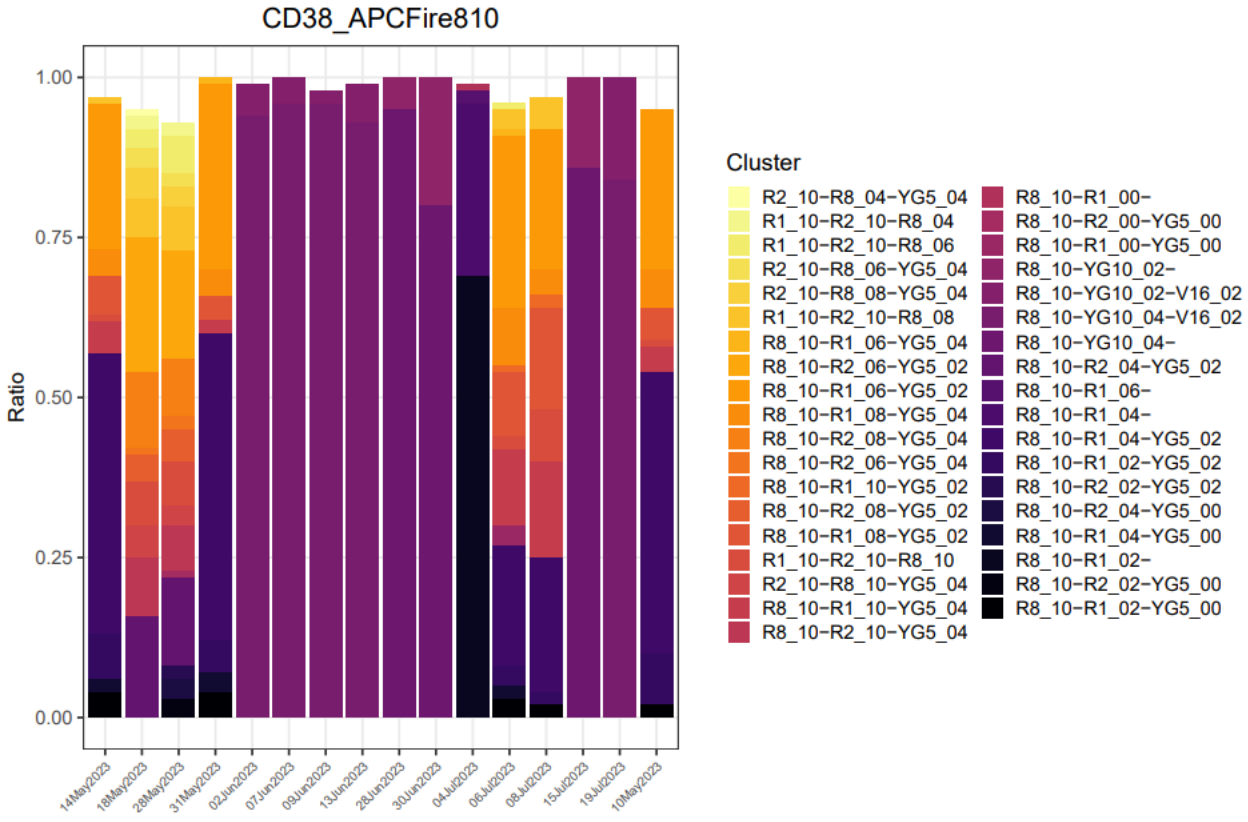


Tandem degradation

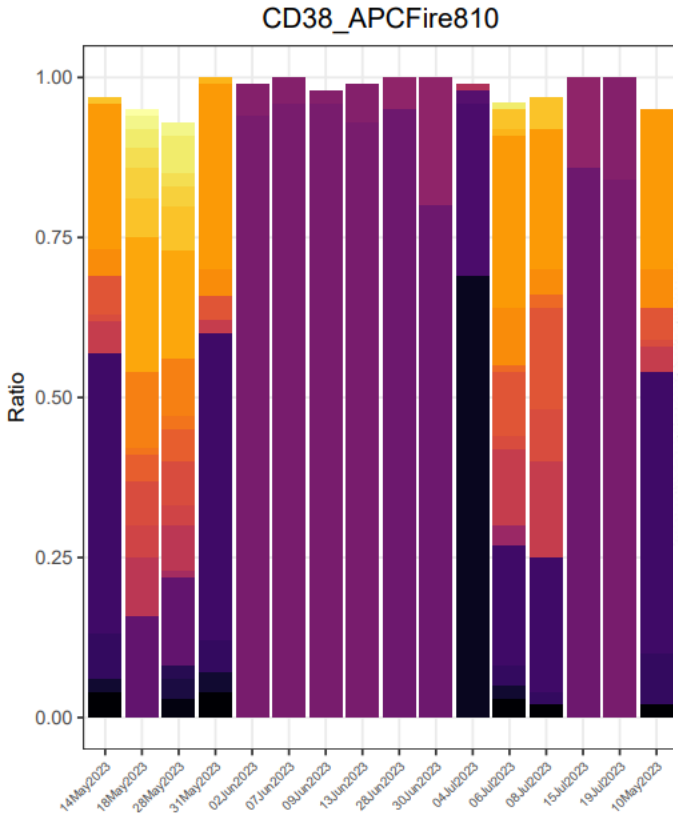


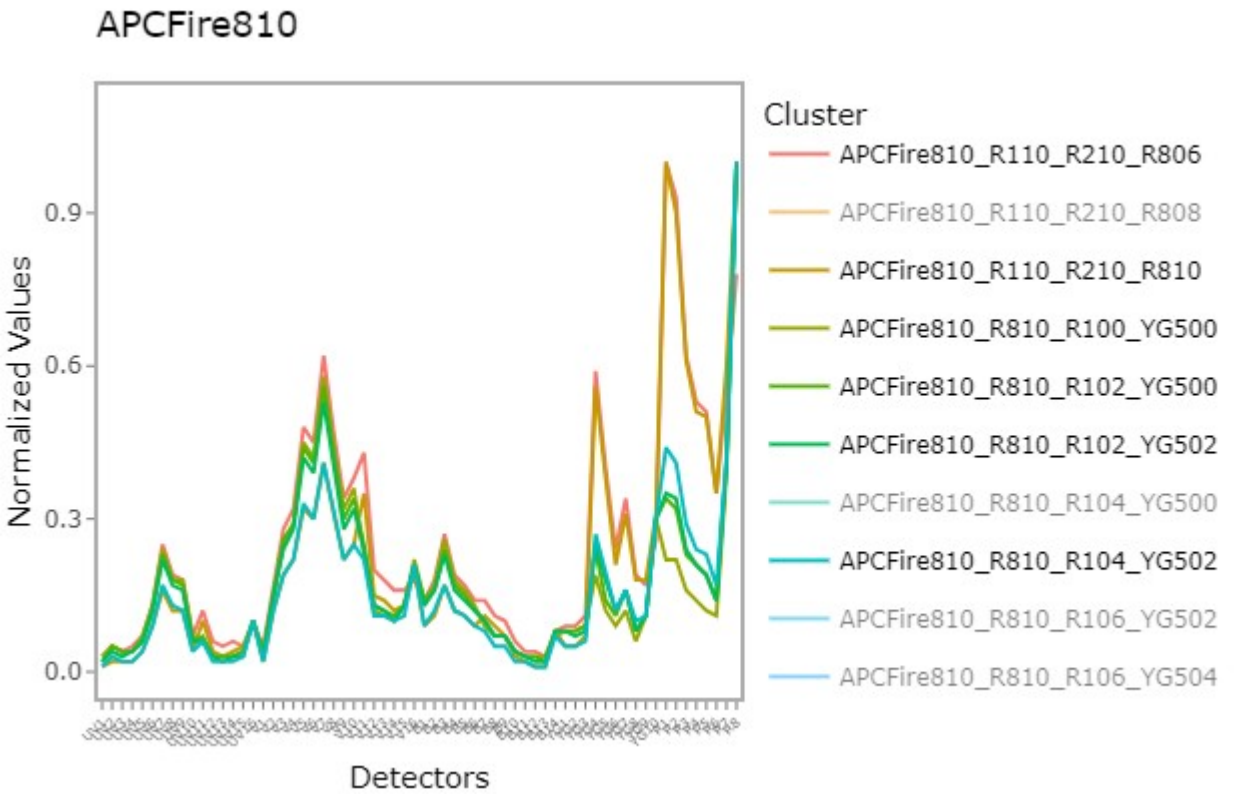


Tandem degradation

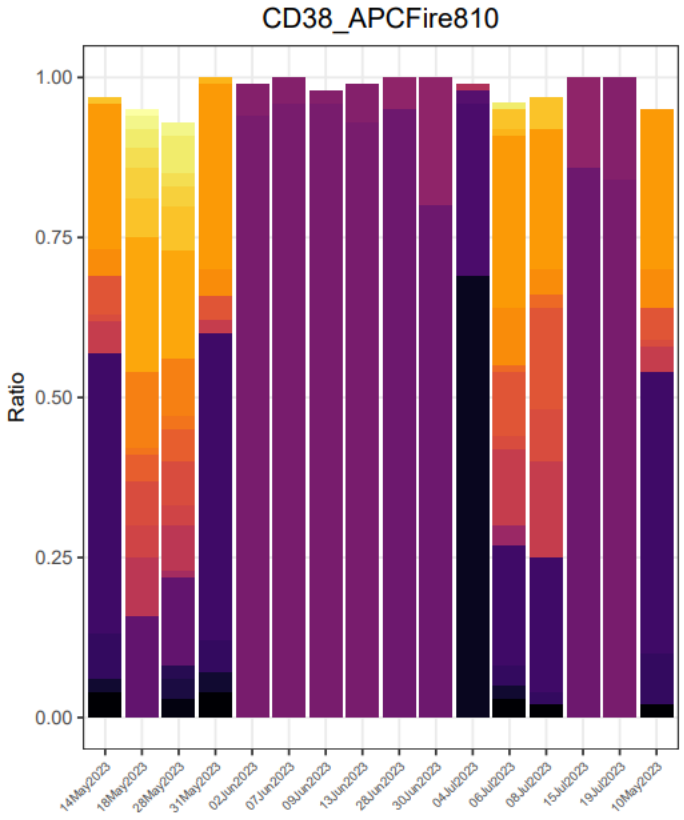


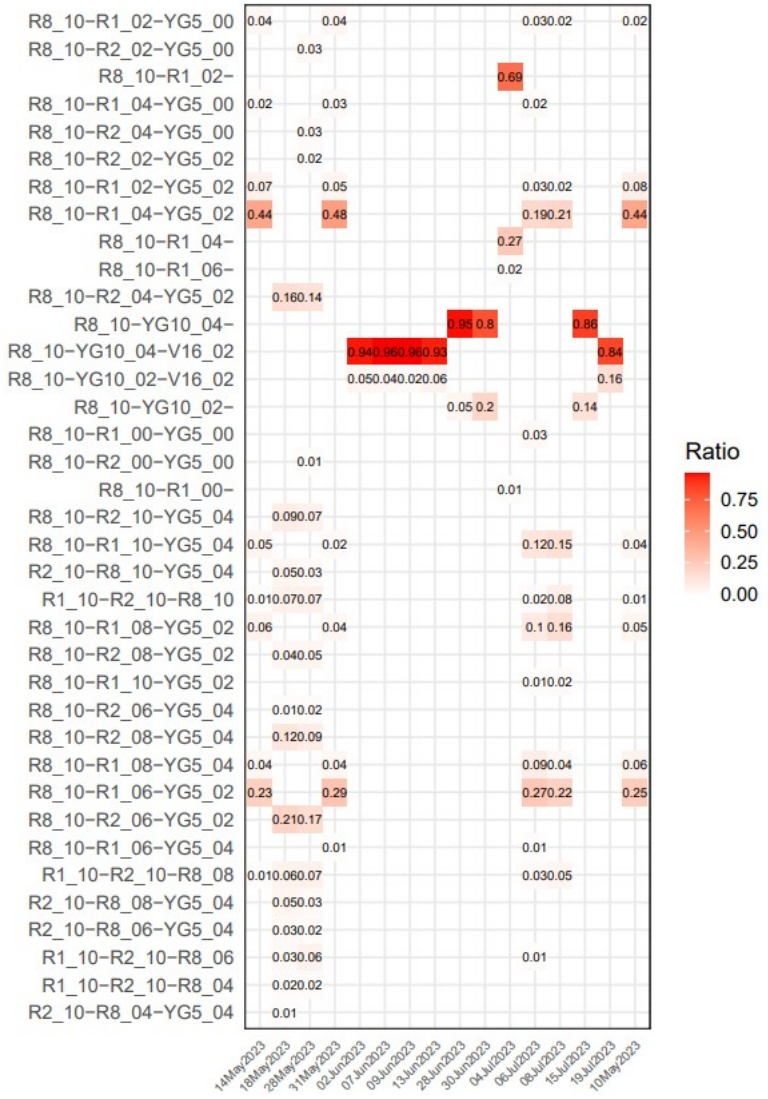
Tandem degradation





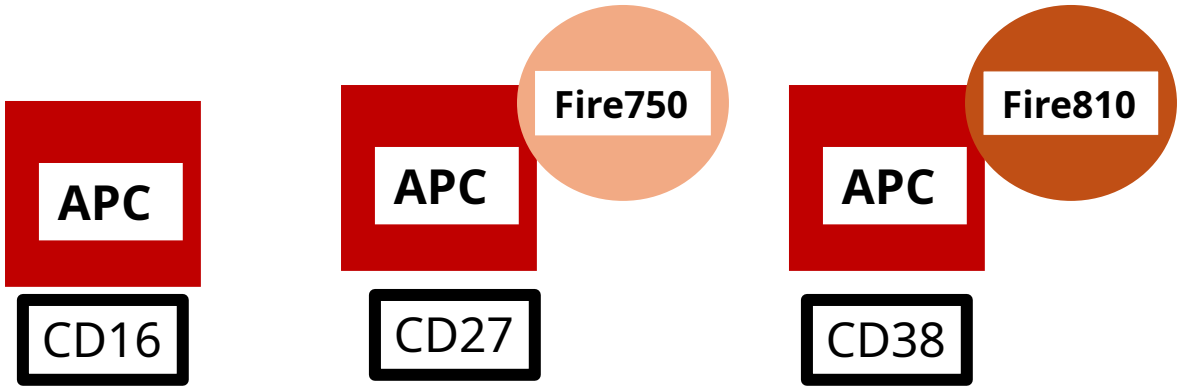
Tandem degradation



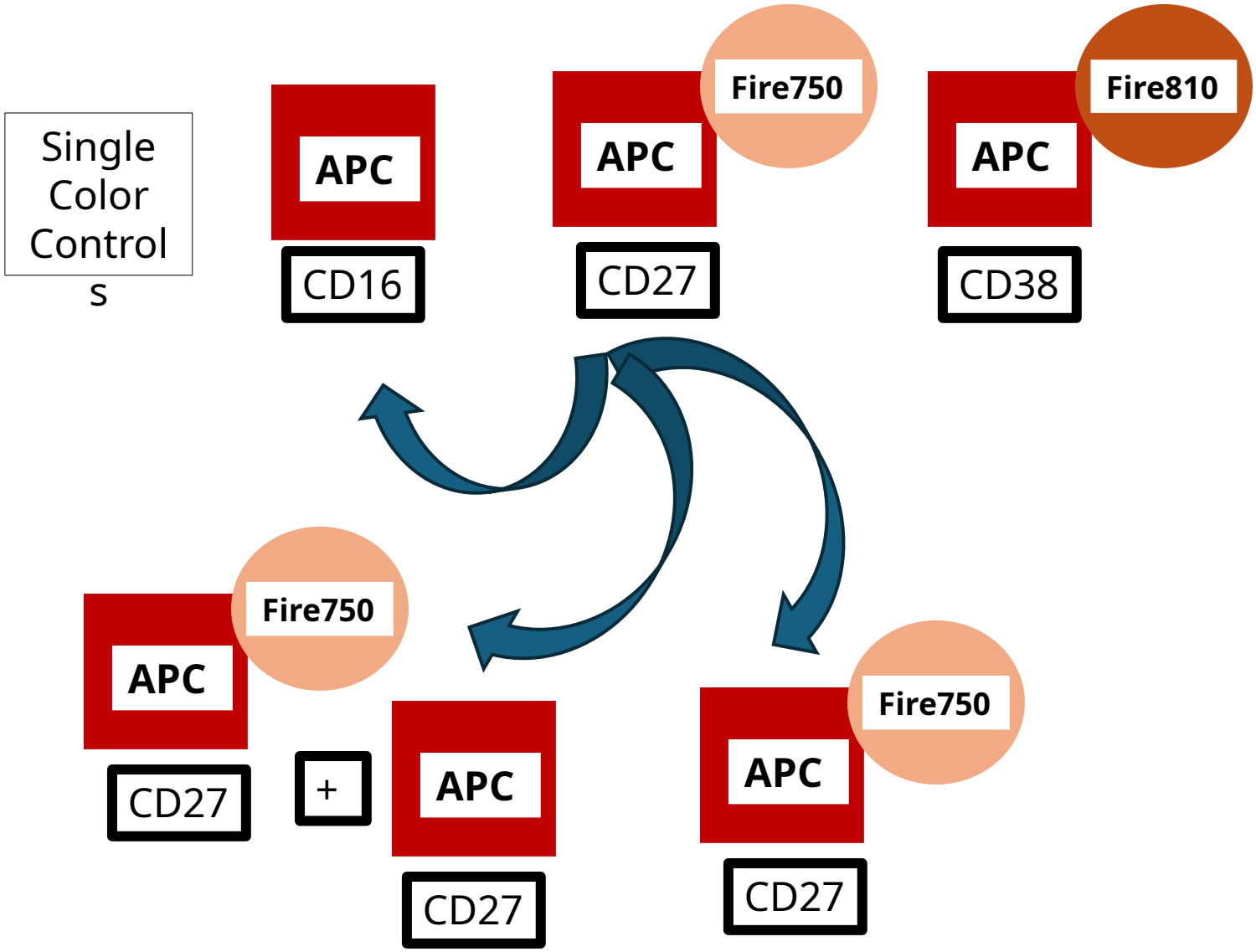


The main issue with tandem degradation:

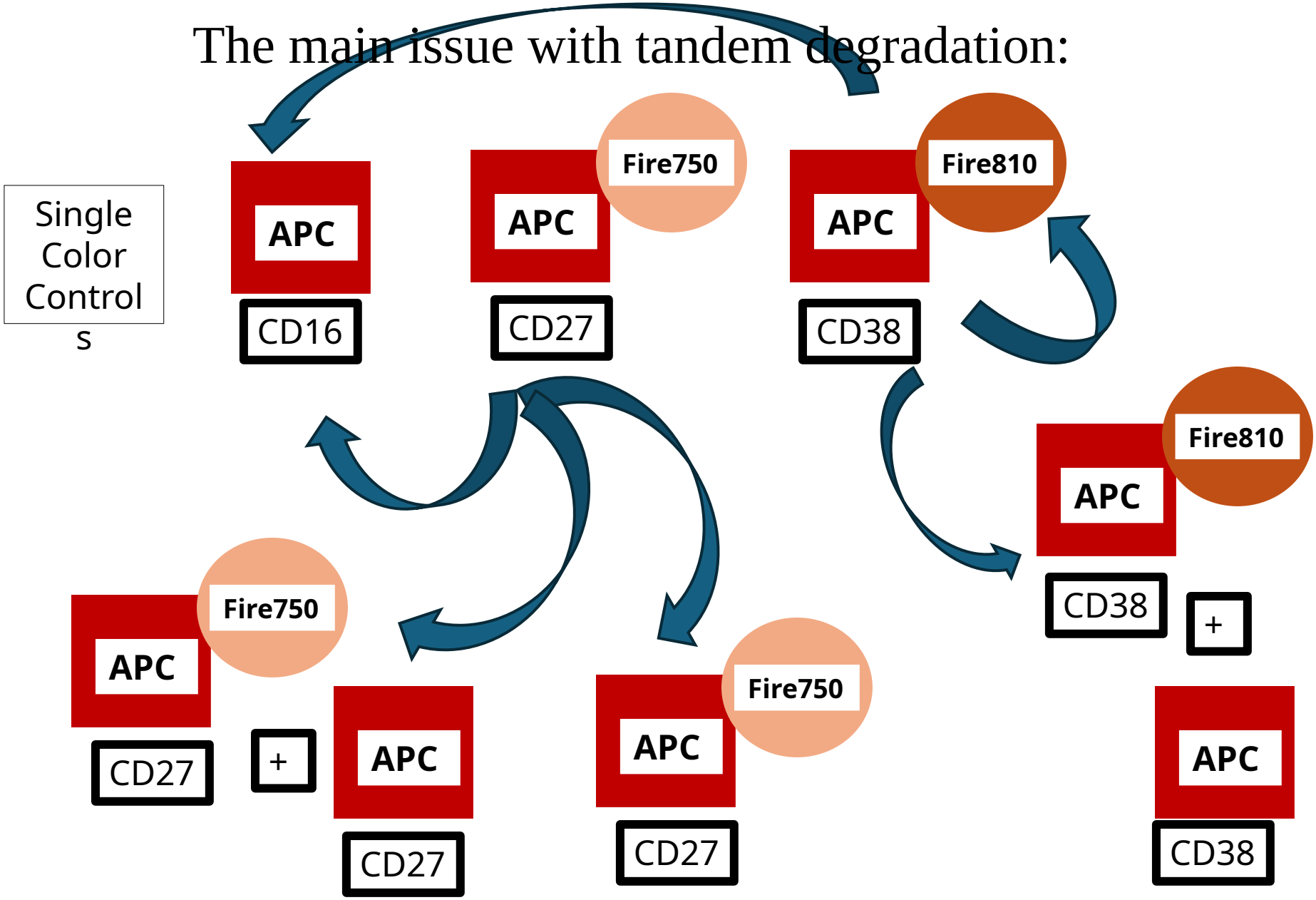
Single Color Controls



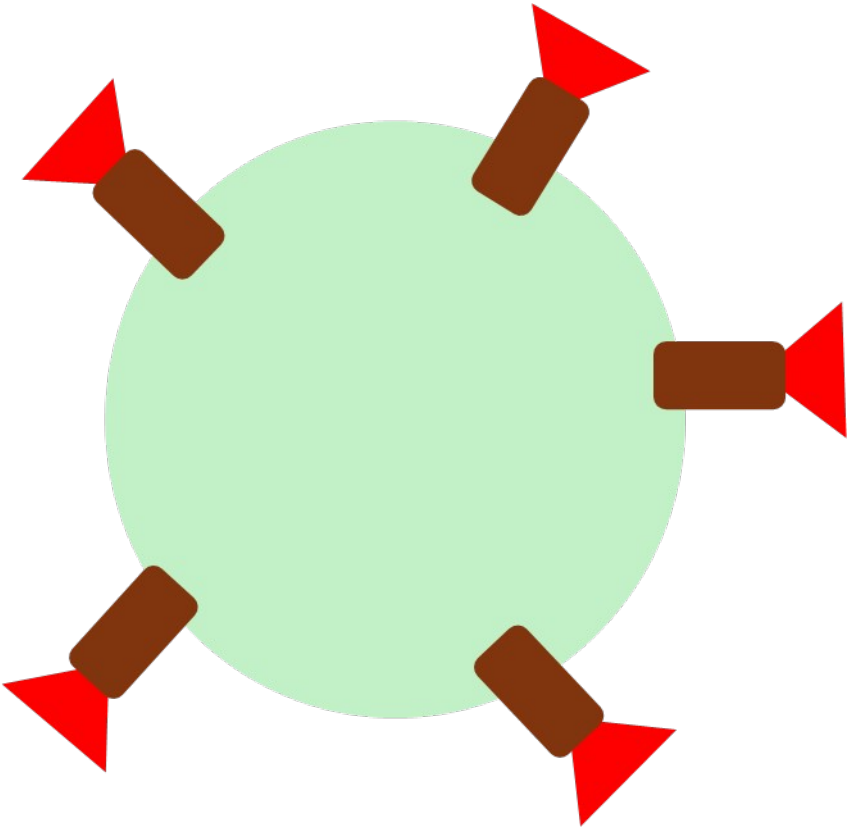
The main issue with tandem degradation:



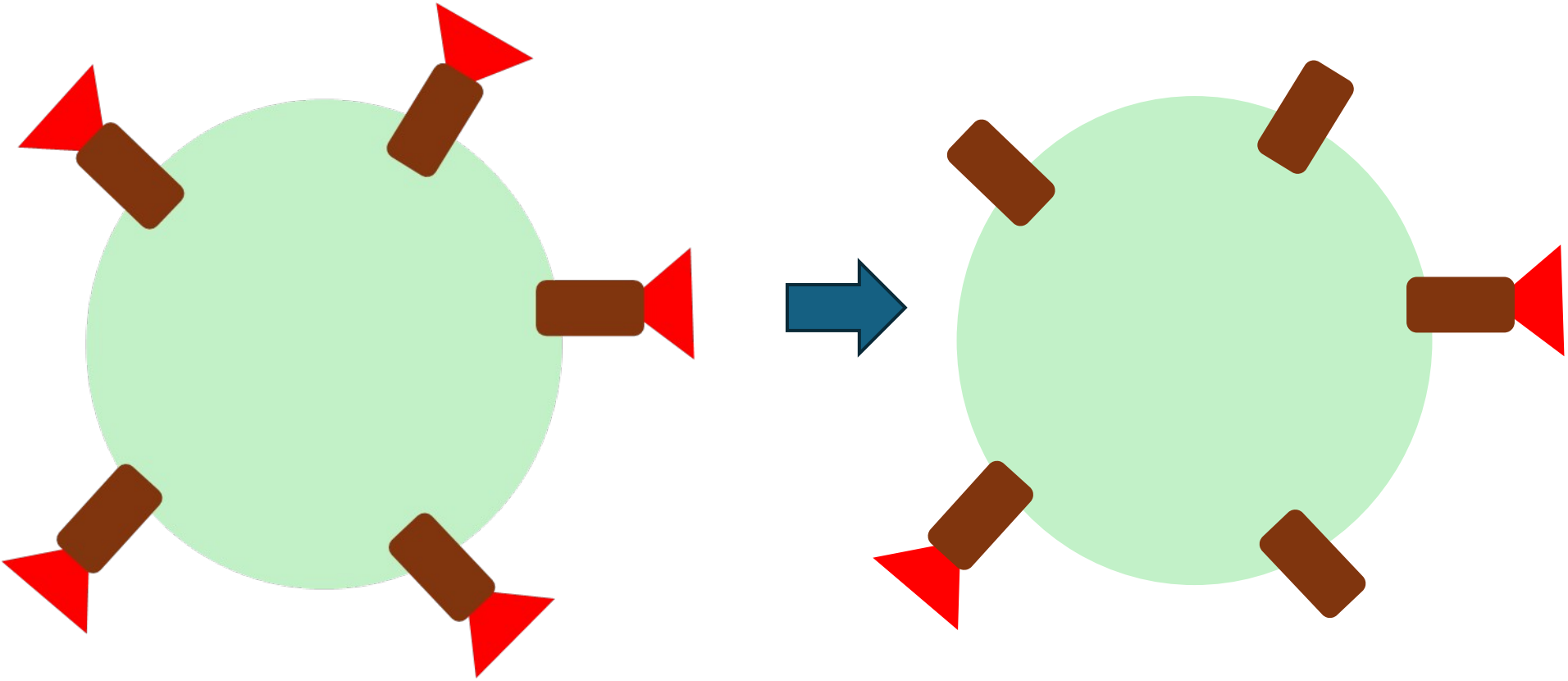
The main issue with tandem degradation:



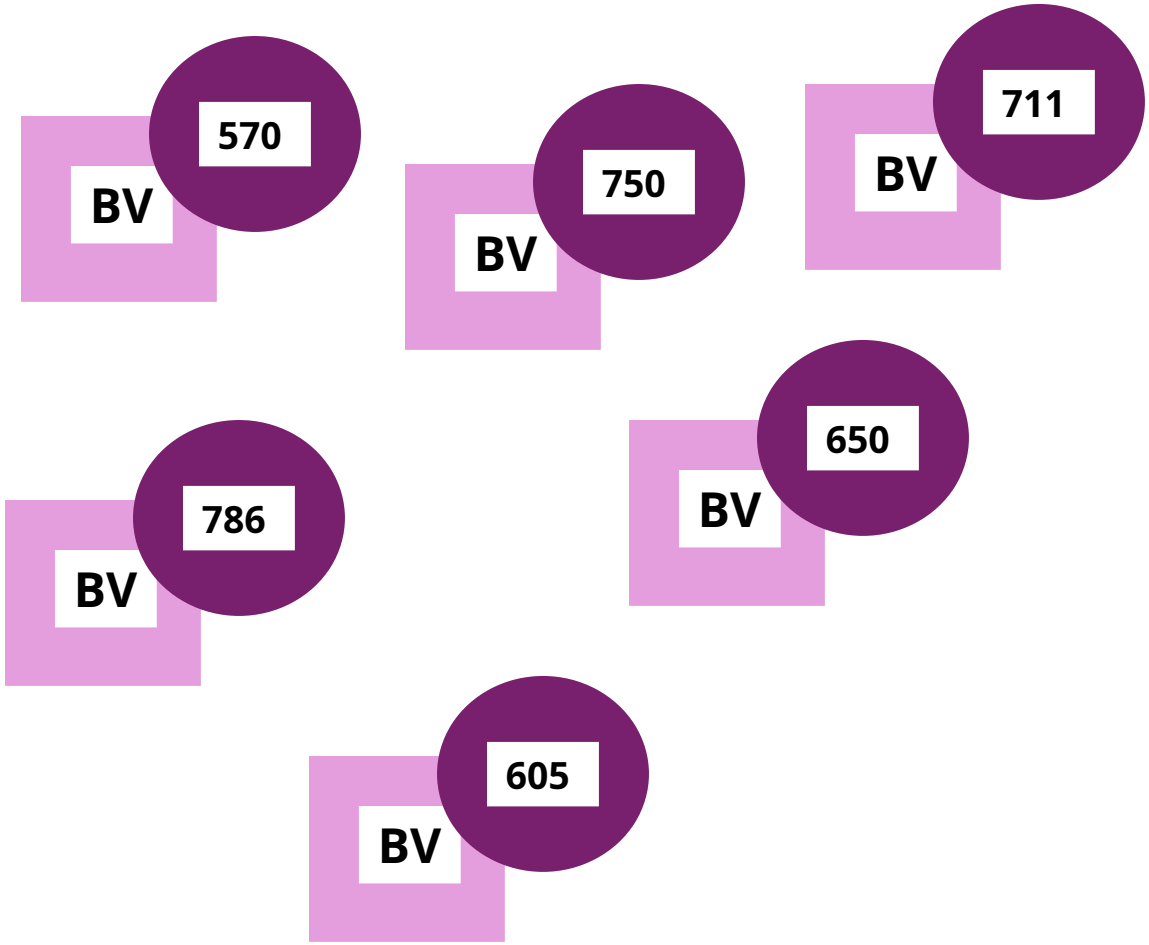
Tandem Degradation affecting your single-color stains



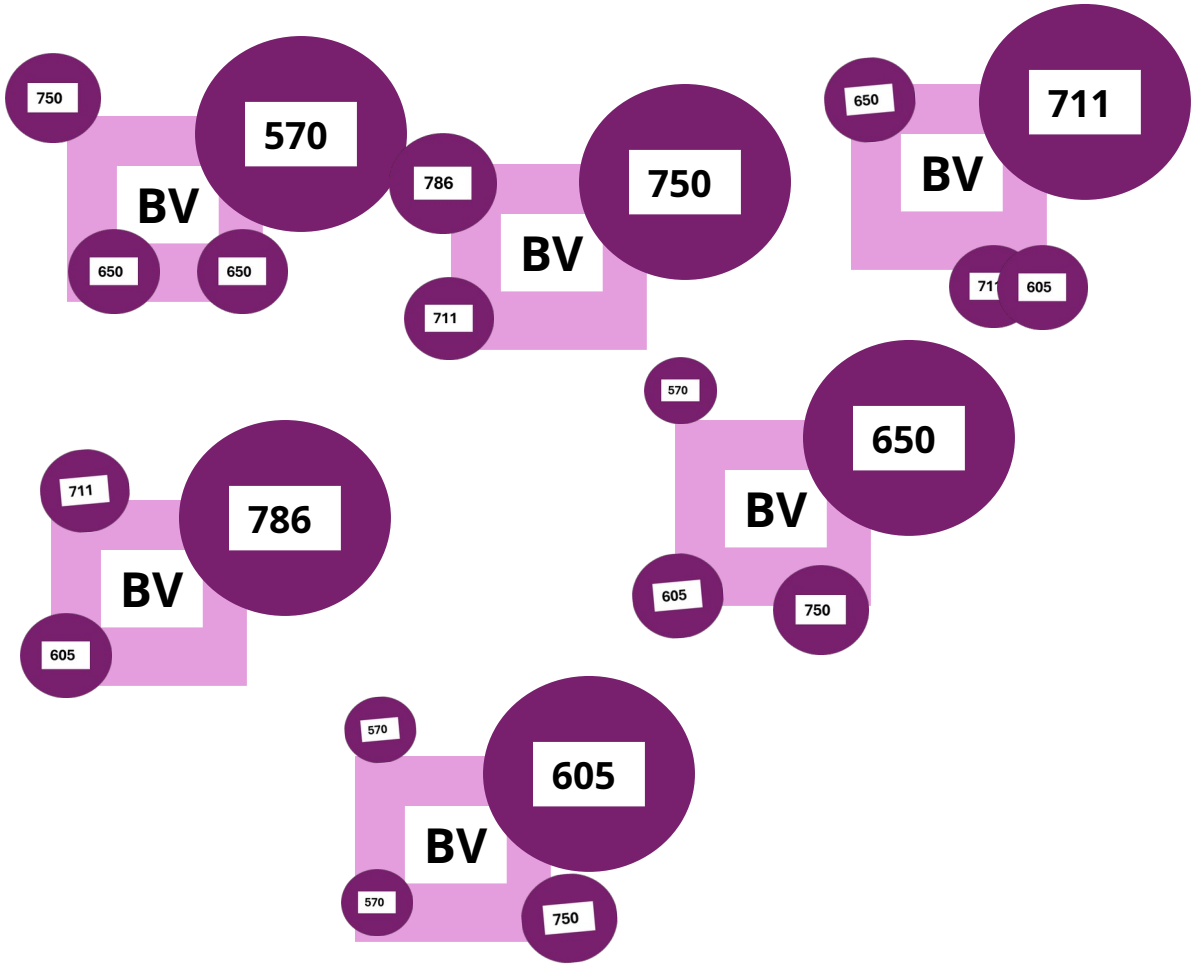
Tandem Degradation affecting your single-color stains



BUV & BV Dyes: Cross-pollinating

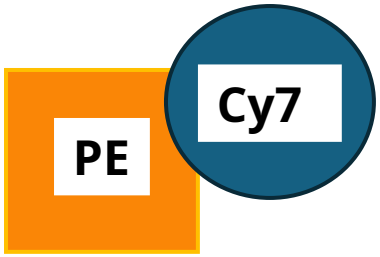


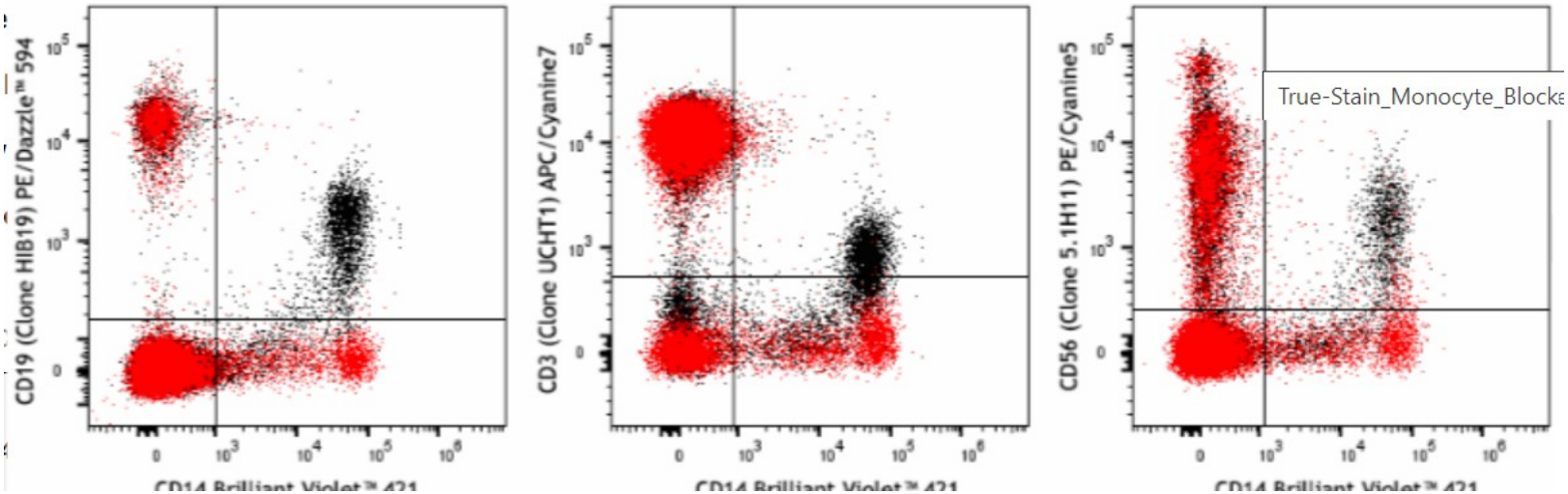
BUV & BV Dyes: Cross-pollinating



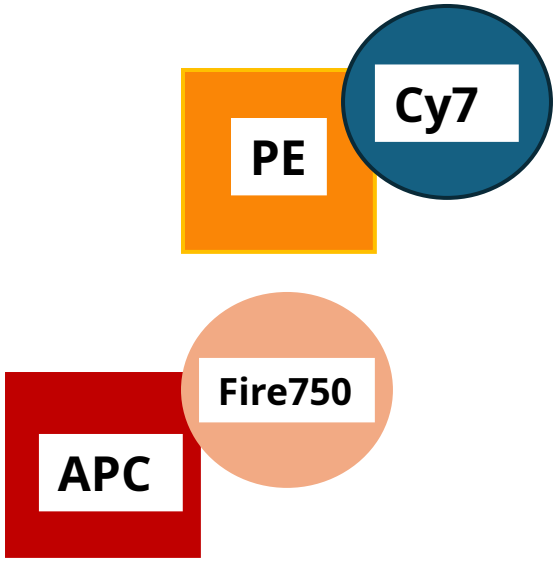
PE and APC Dyes: Hungry Monocytes

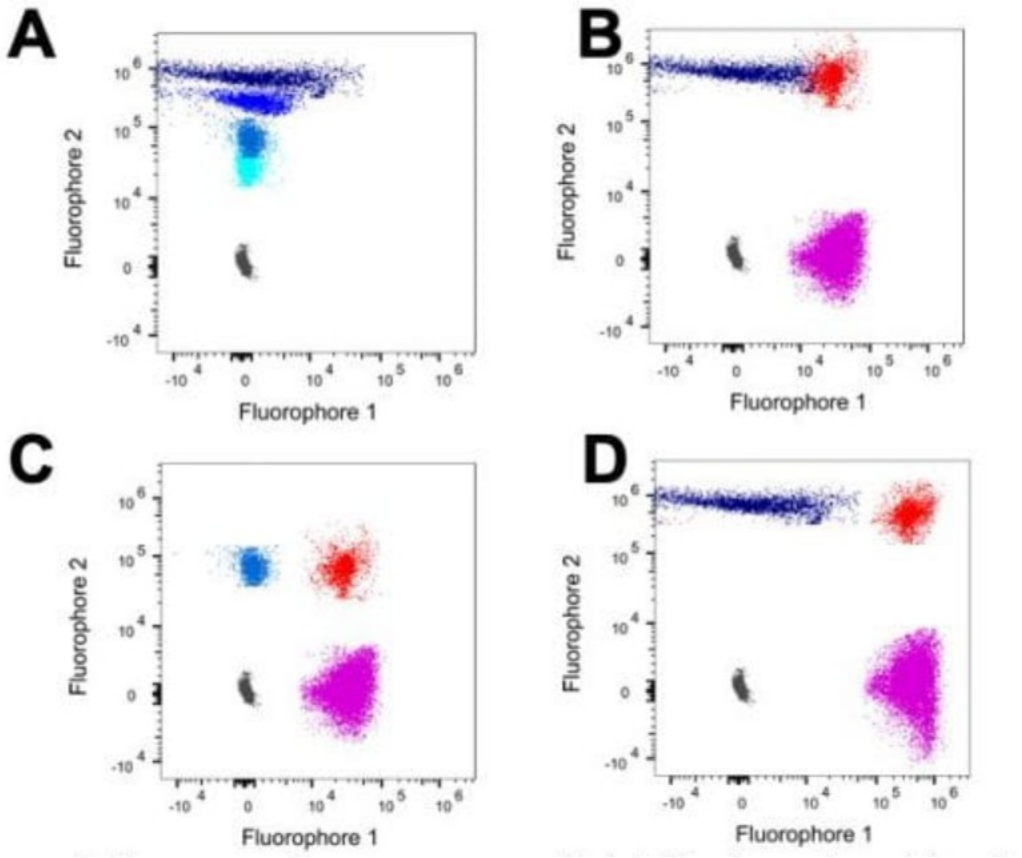






Yellow and Red Fluors: Bad spreading errors





THE UNIVERSITY OF CHICAGO

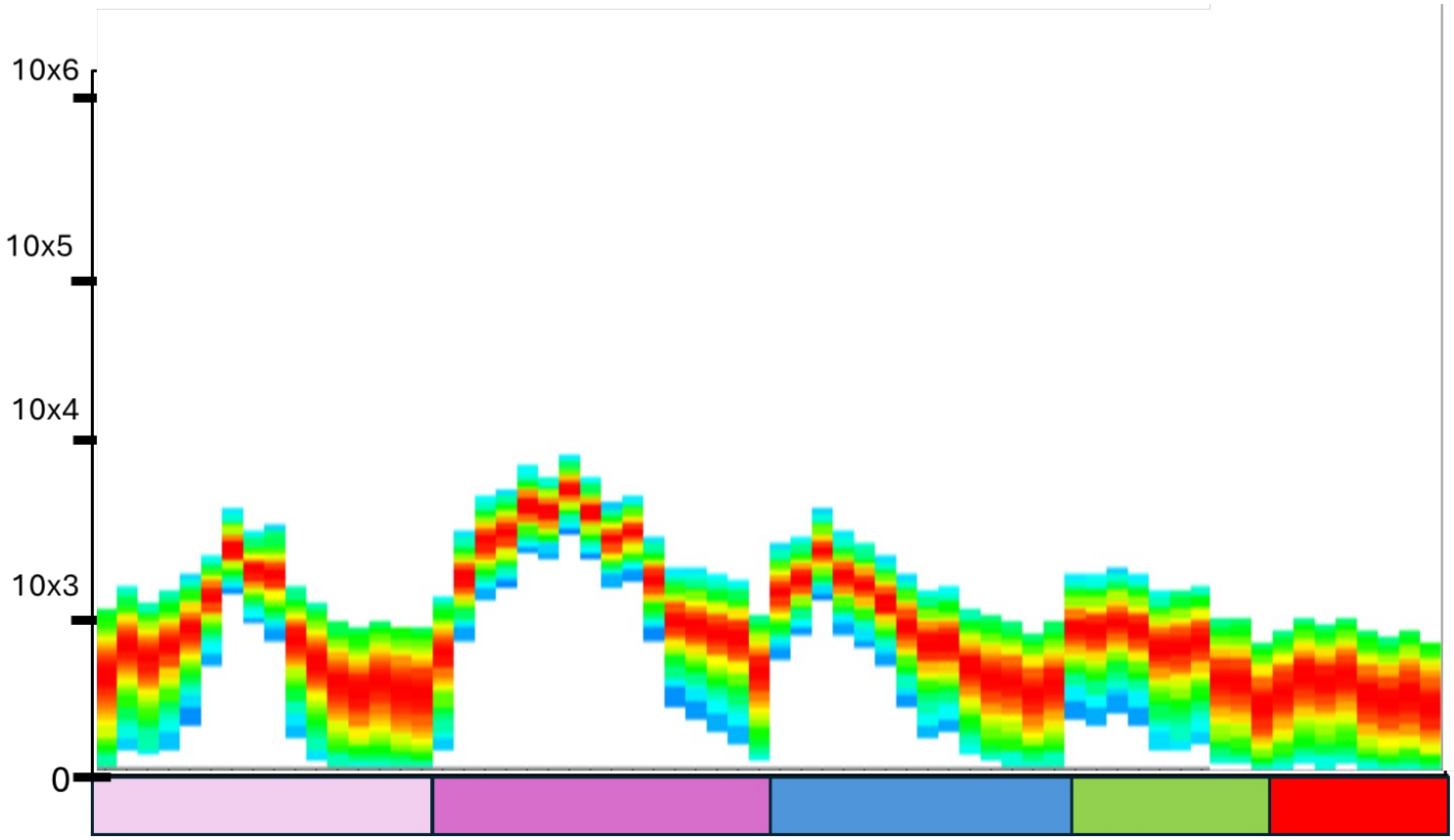
https://voices.uchicago.edu/ucflow/2020/03/04/understanding-the-trumpet-effect-how-to-design-aurora-panels-around-spreading-error/

Violet: Autofluorescence Issues

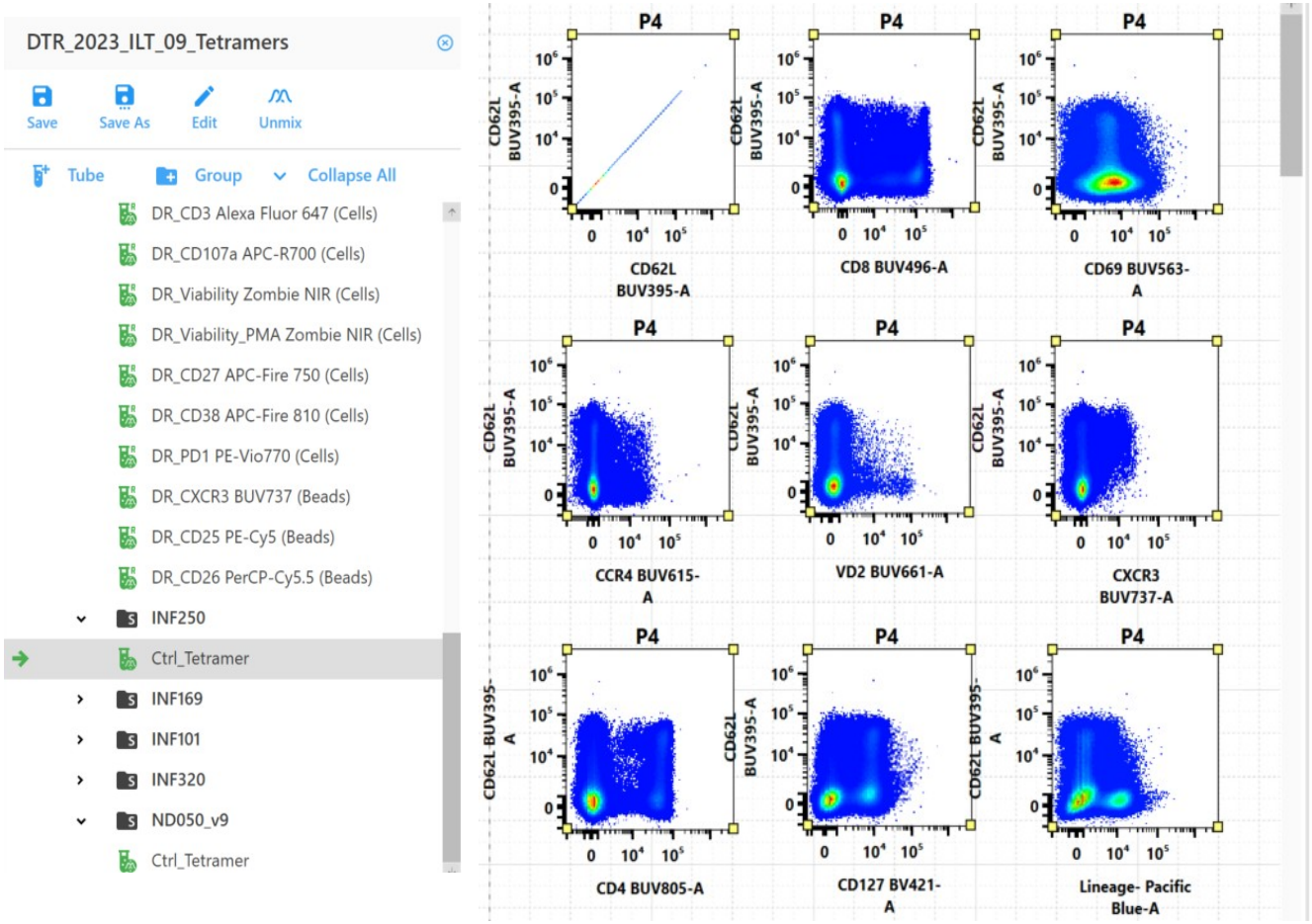
UV

V

B

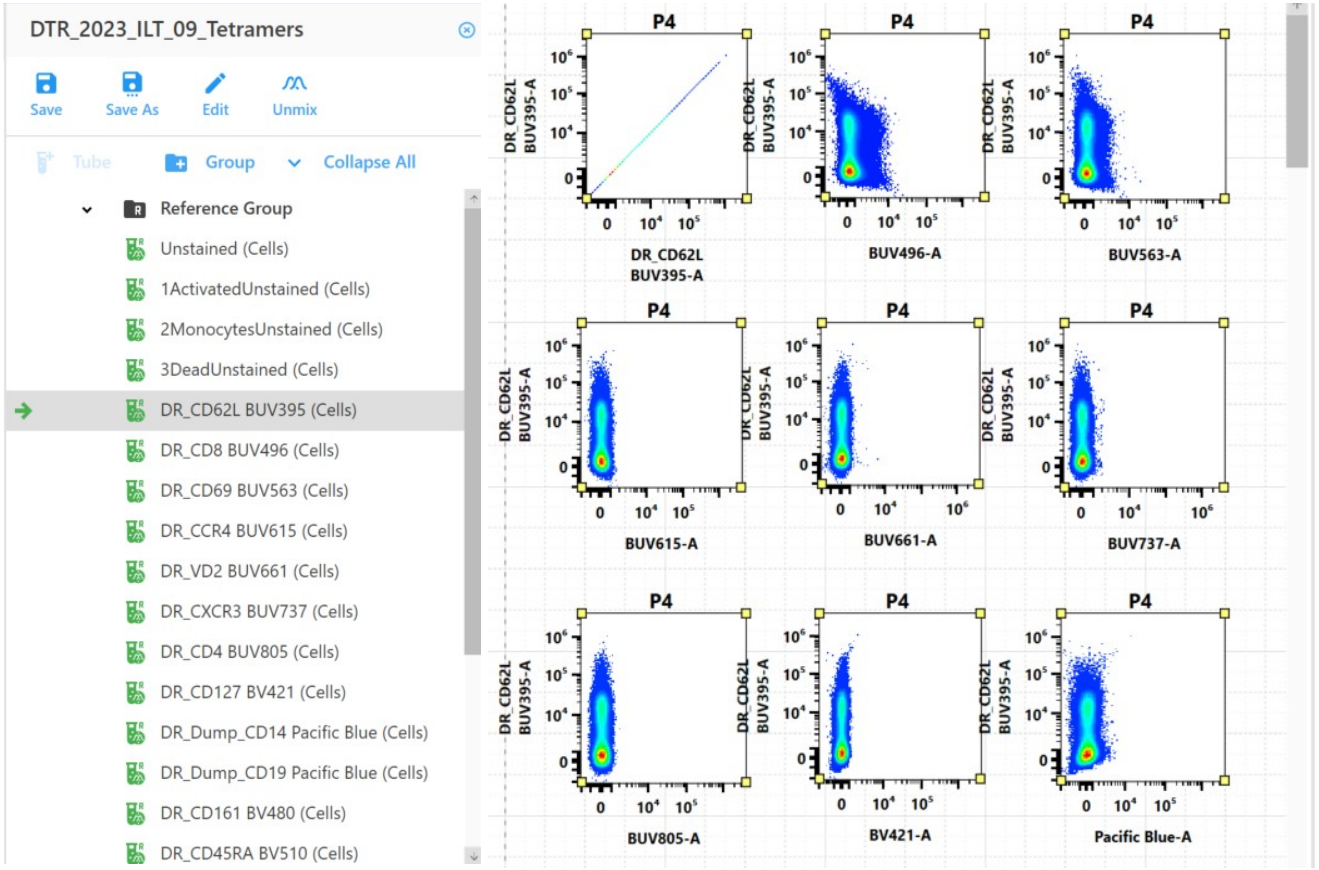


Check your NxN plots for full-stained samples!



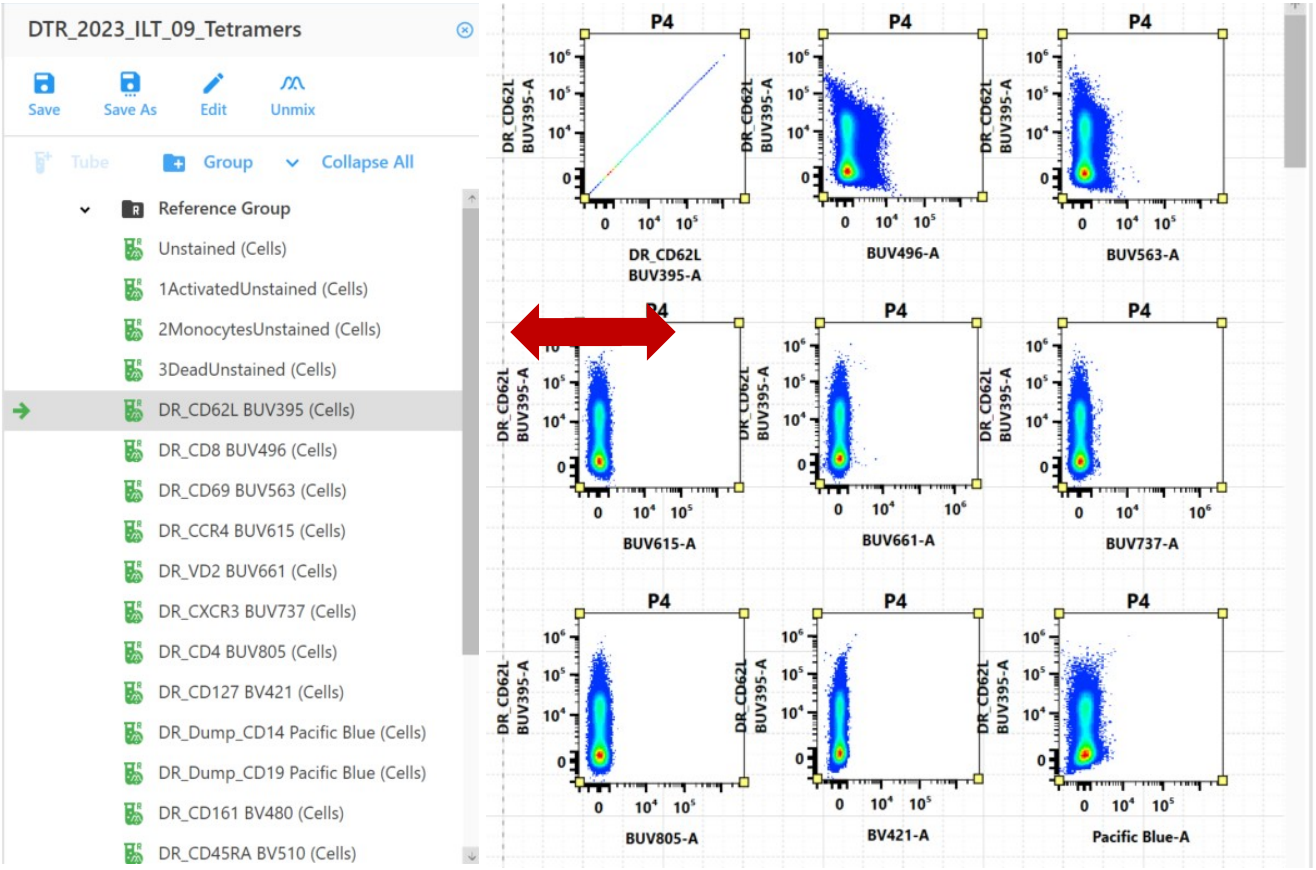
What fluorophores are having issues? What cell subsets are affected? How prevalent are they?

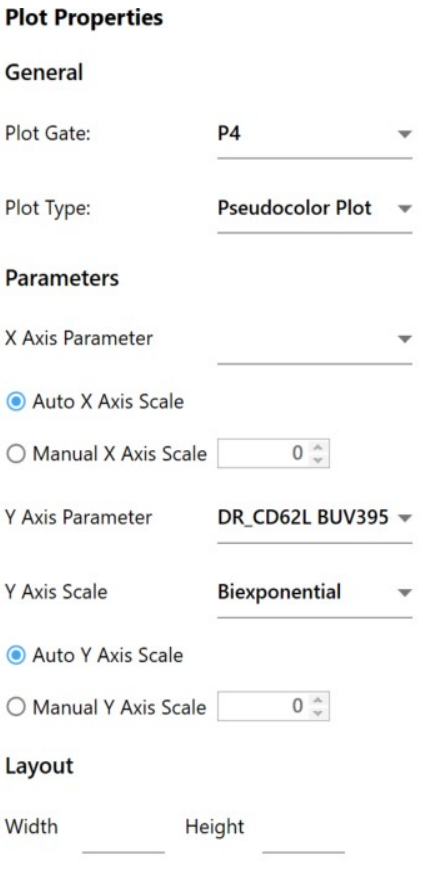
Check your single-color cell controls!



Are they unmixing correctly?

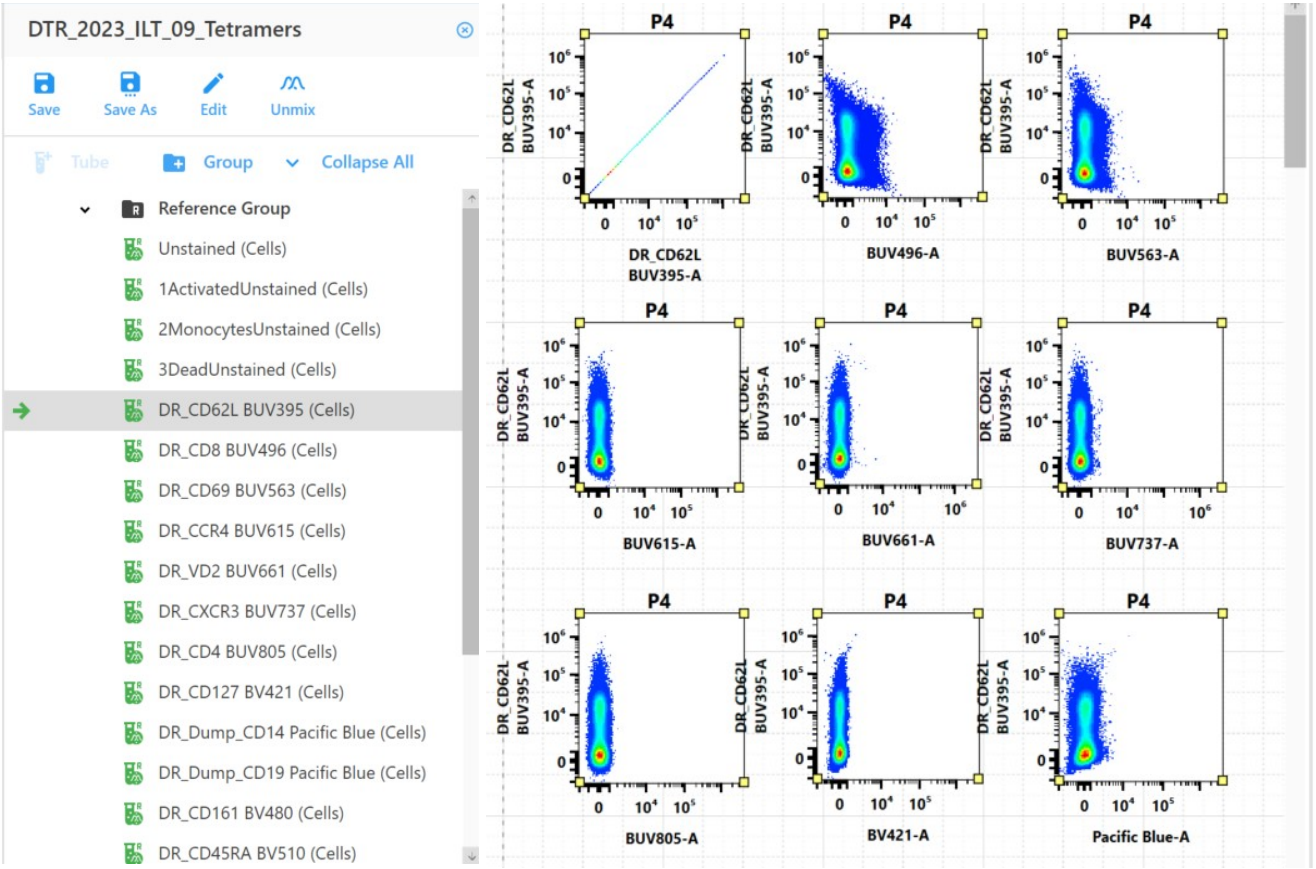
Check your single-color cell controls!





If the cell single-color controls are fish-tailing after unmixing with beads, using beads as the single-color controls won’t work for unmixing cells.

If all your cell single color controls are okay, But your full-stained samples are having issues:



Plot Properties

General

Plot Gate: P4

Plot Type: Pseudocolor Plot

Parameters

X Axis Parameter

Auto X Axis Scale

Manual X Axis Scale 0

Y Axis Parameter DR_CD62L BUV395

Y Axis Scale Biexponential

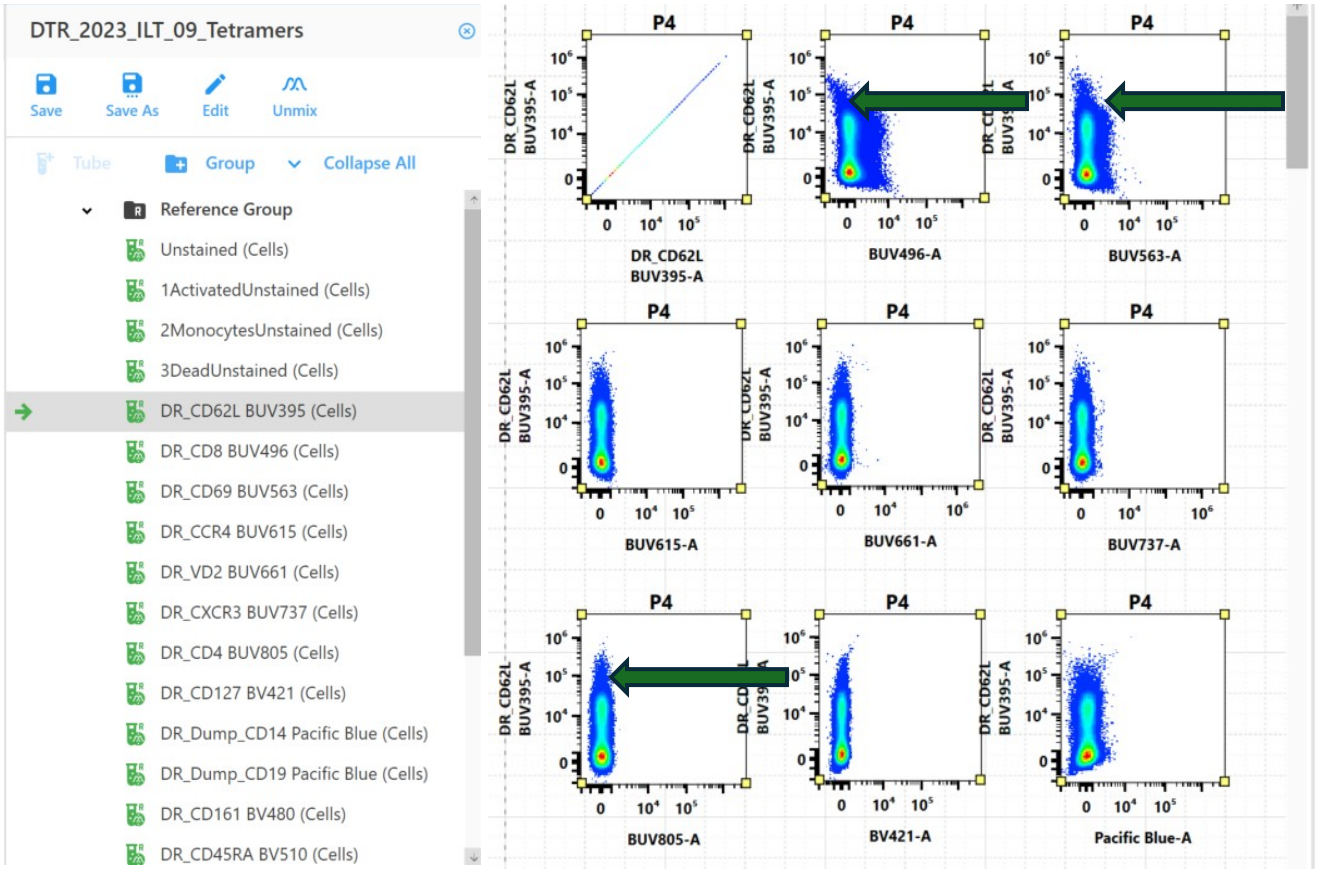
Auto Y Axis Scale

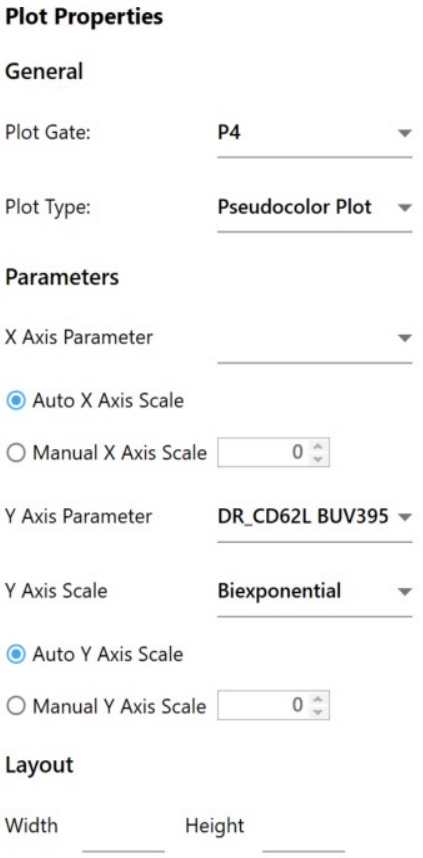
Manual Y Axis Scale 0

Layout

Width Height

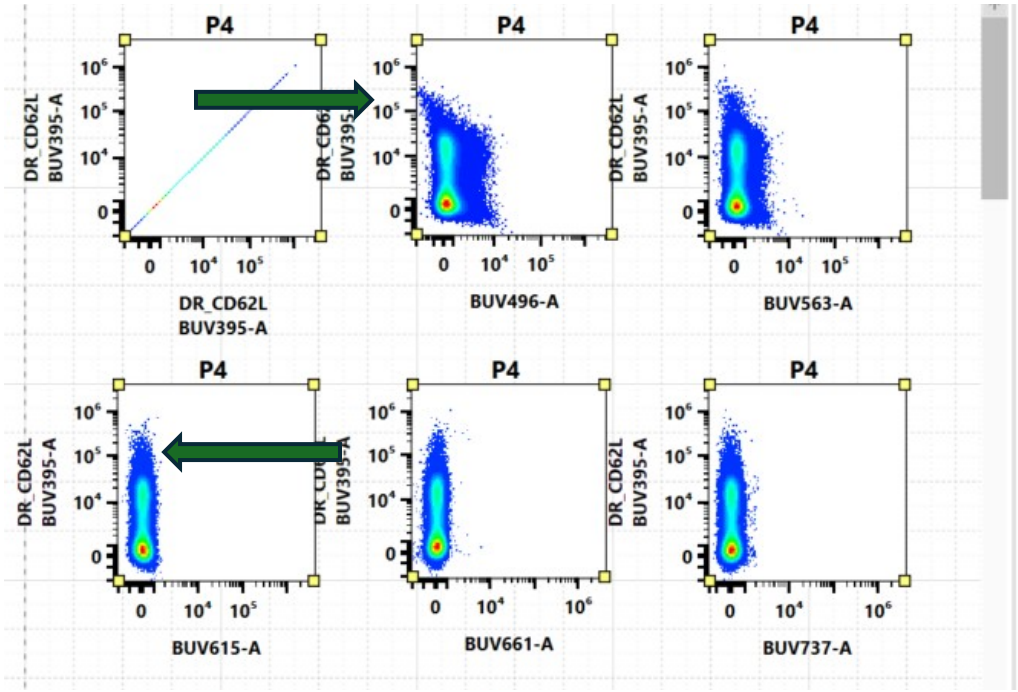
Check the brightness of your single-color control

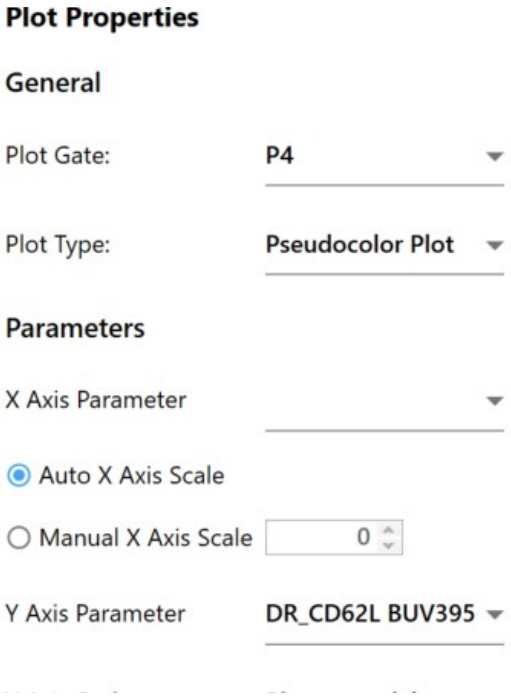




Locate what value on the axis the brightest cells in your single-color controls are located at.

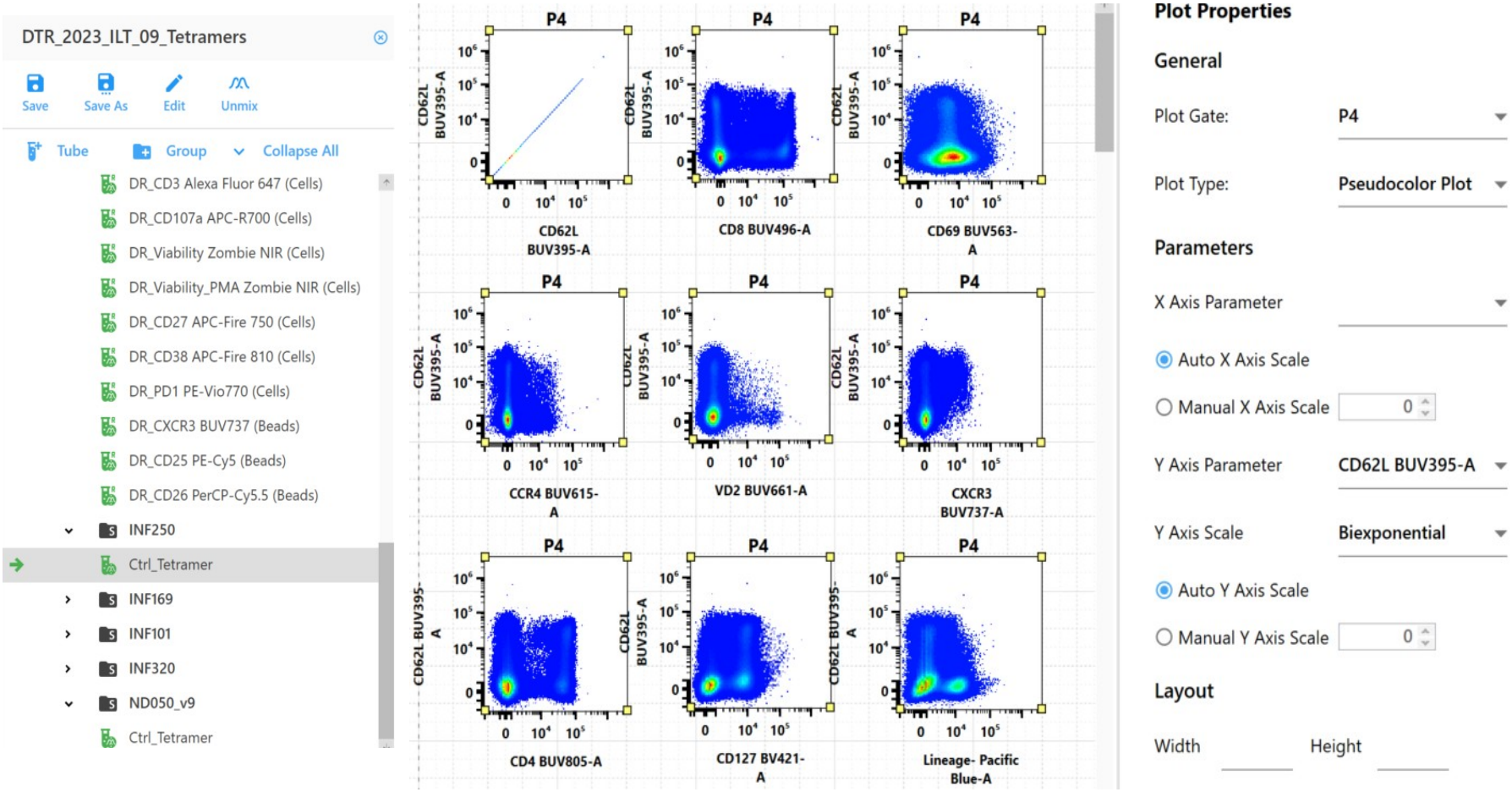
Then compare where the brightest cells in your full-stained sample are at for that fluorophore





Are they roughly similar, or is the full-stained sample brighter?

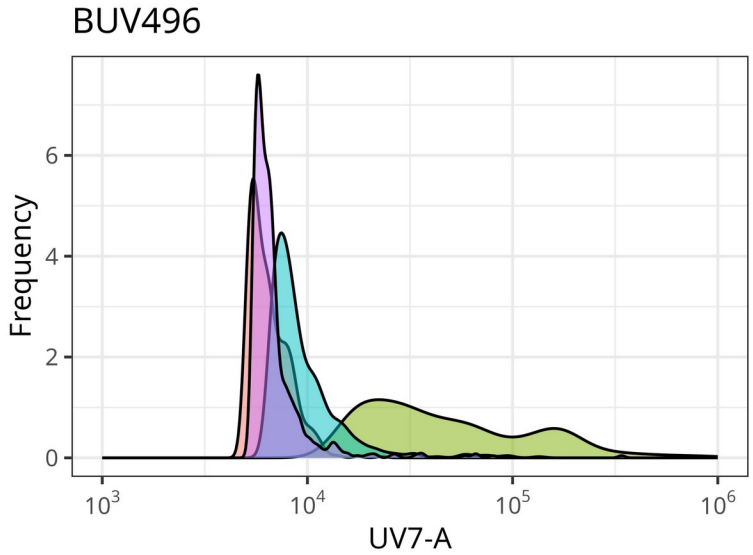
Why does the brightness of the full-stained sample matter?

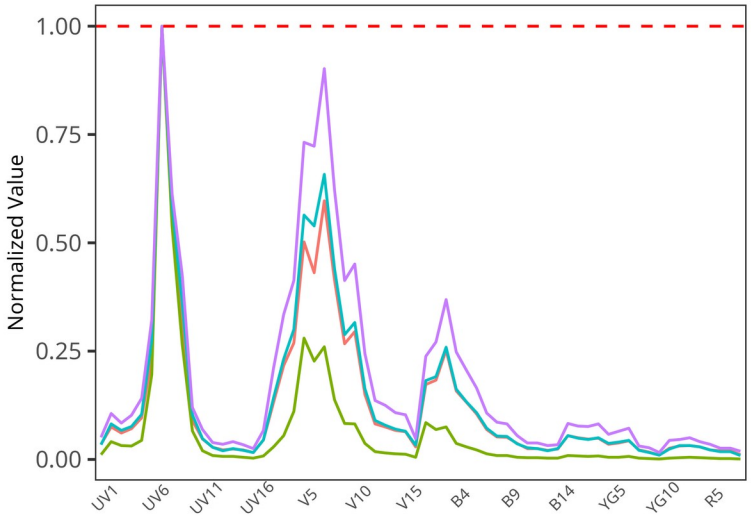


There are several hand-wavy theories

Possible explanations:

1) Brighter Single Colors -> Unique Signature





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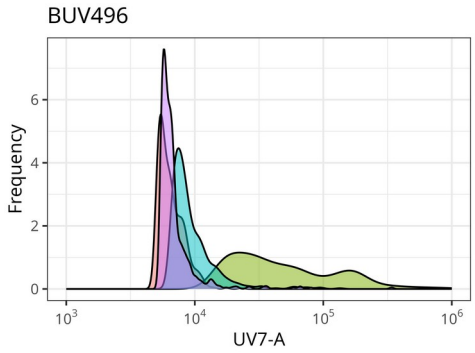
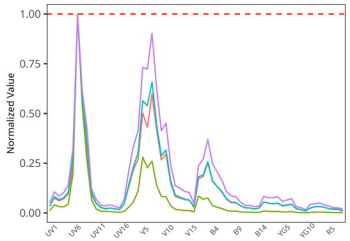
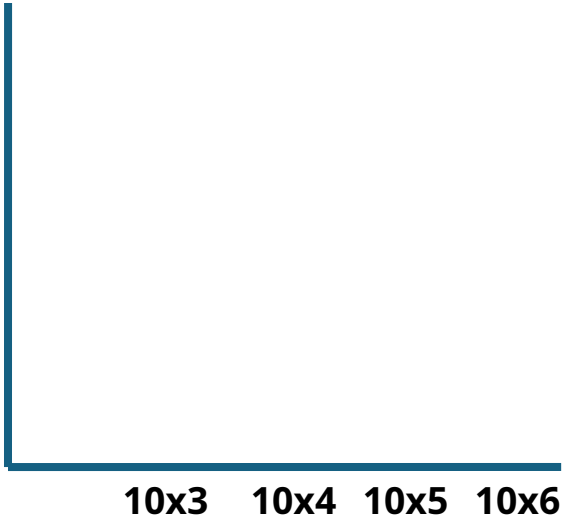




2) Extrapolation Issues



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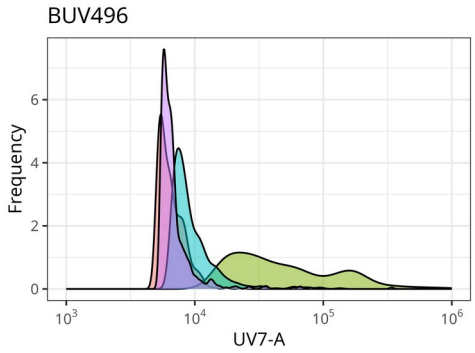

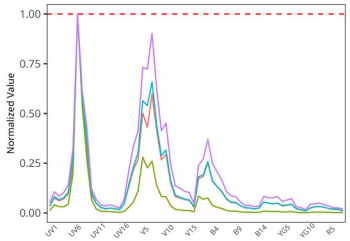

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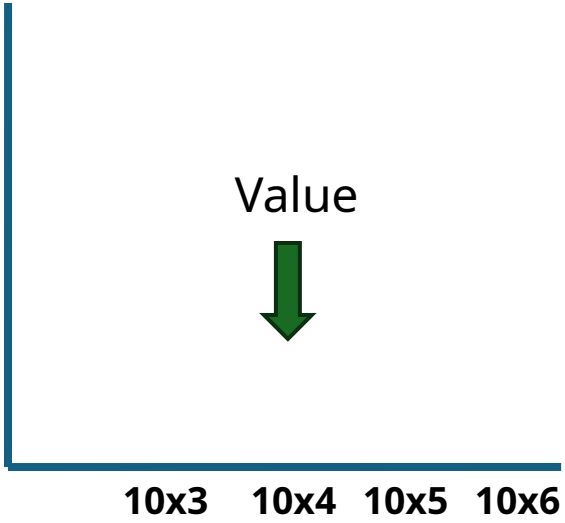
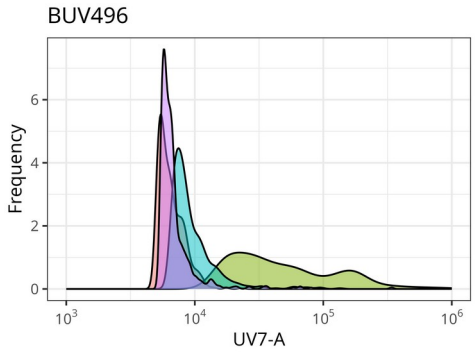
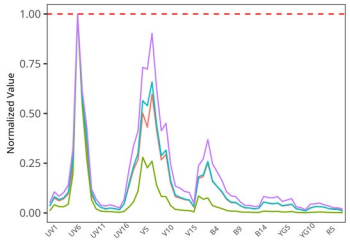


- Input corresponds to a certain amount of photons being detected from single-color control

Possible explanations:

1) Brighter Single Colors -> Unique





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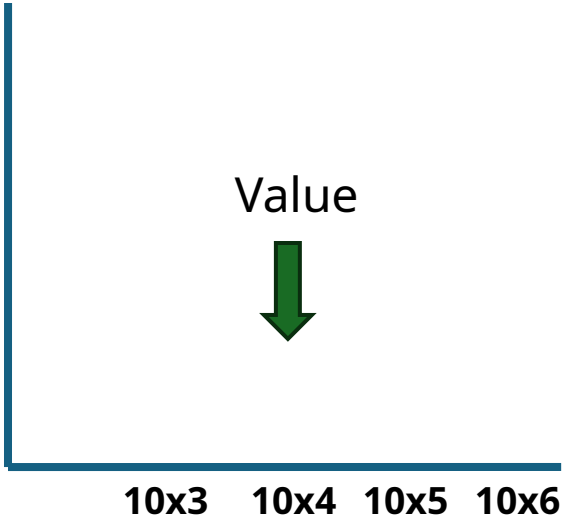

- Input corresponds to a certain amount of photons being detected from single-color control
- A similar amount of detected photons is a set-point for the full-stained sample

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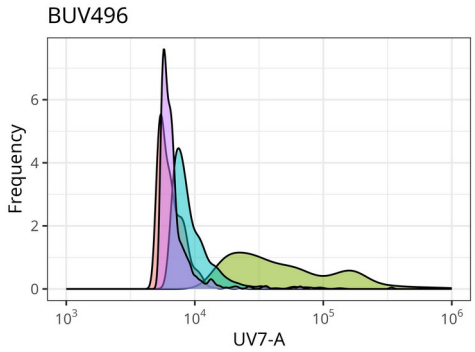

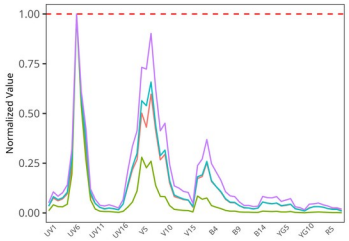

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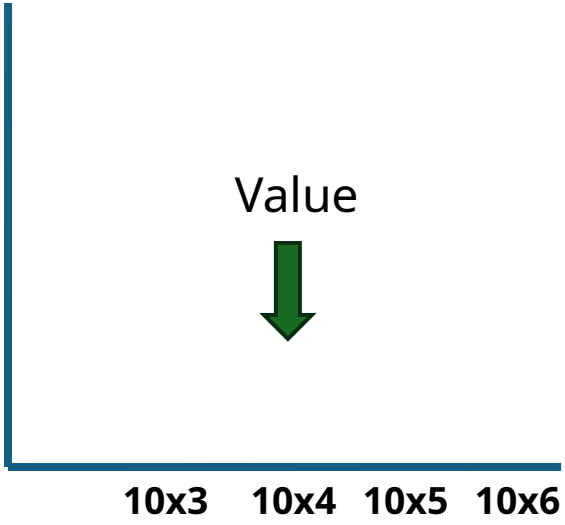

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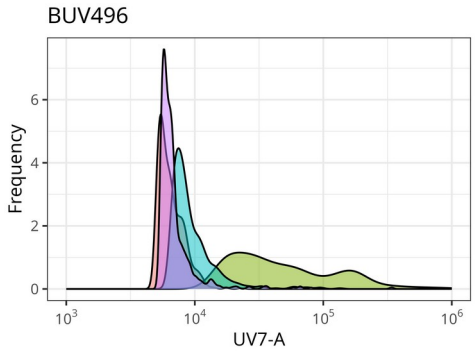
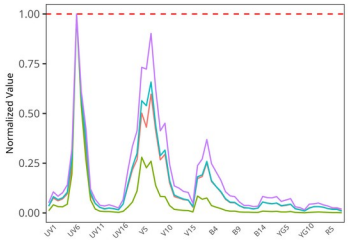
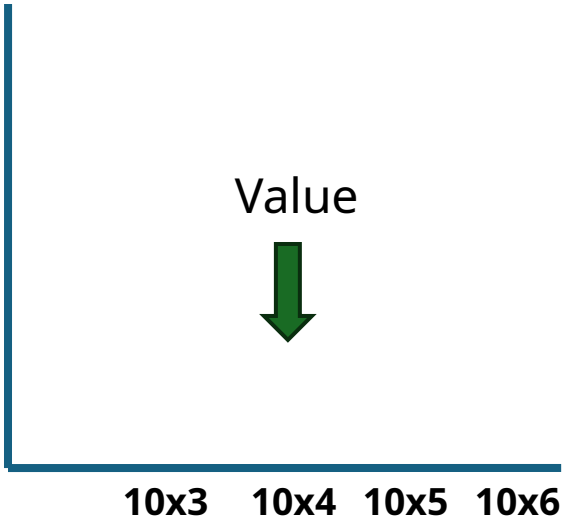




2) Extrapolation Issues

- Input corresponds to a certain amount of photons being detected from single-color control
- A similar amount of detected photons is a set-point for the full-stained sample
- Presence of larger values than expected throws off unmixing



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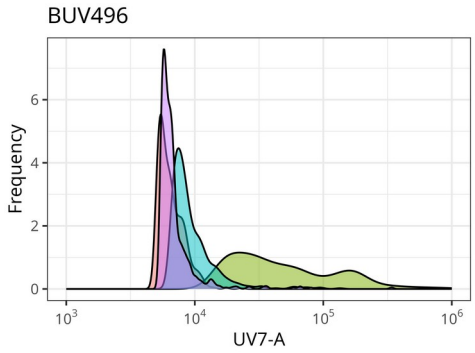

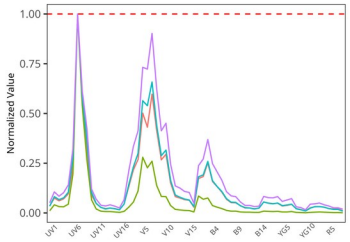

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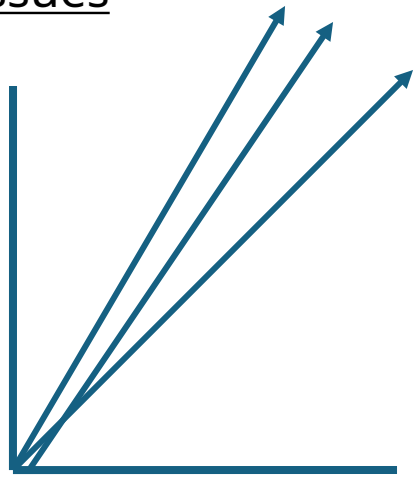
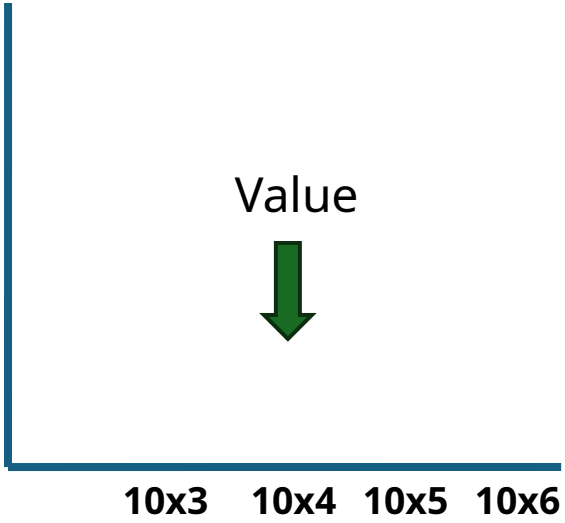
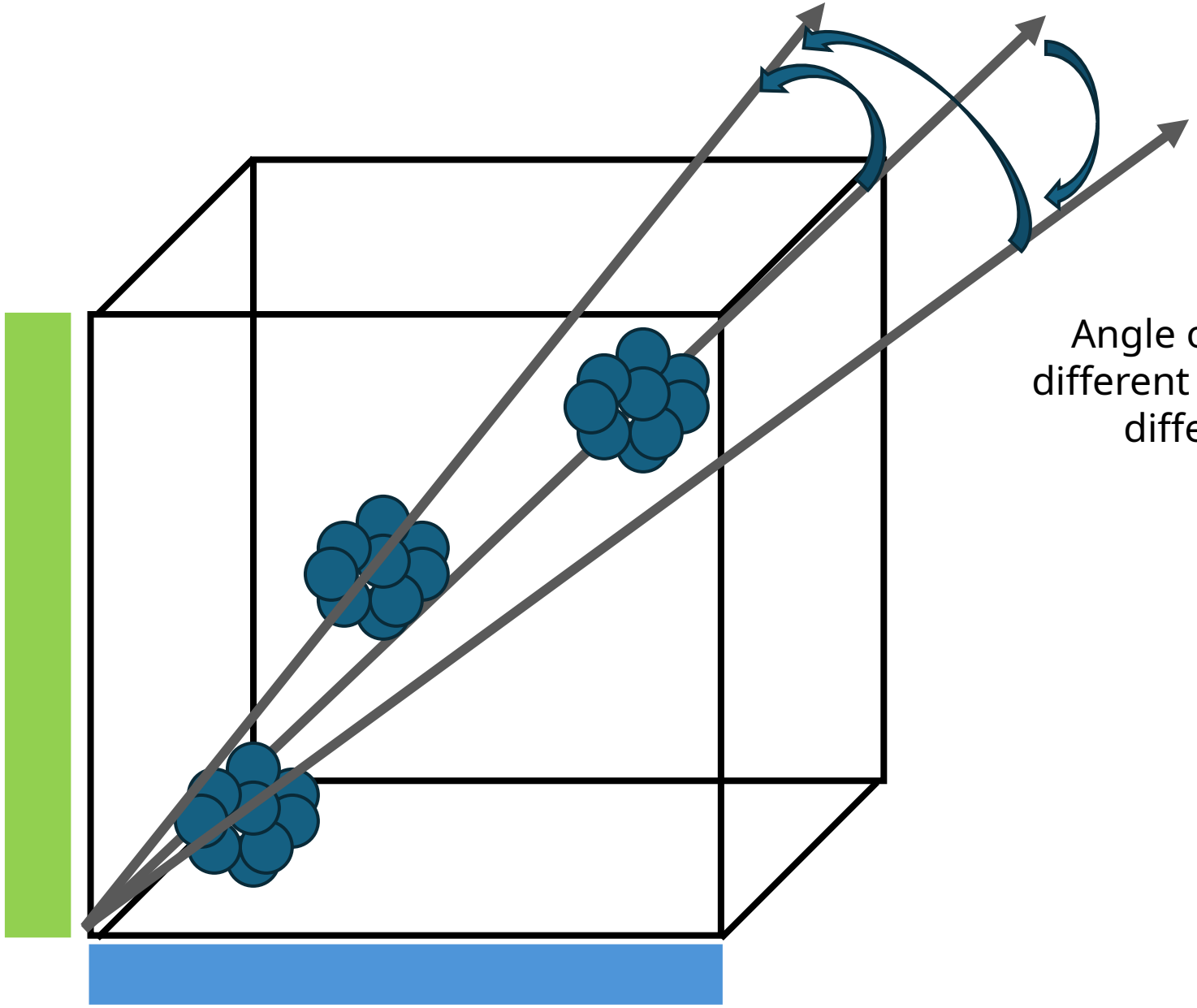




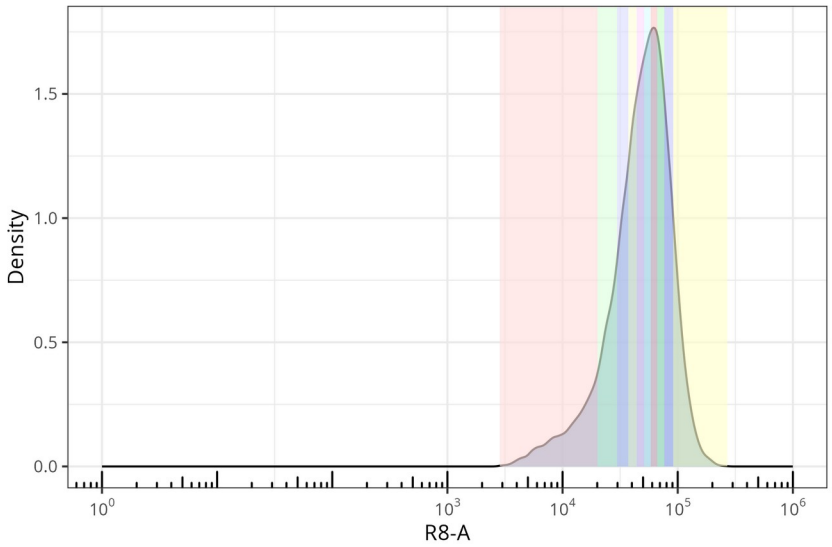
3) More representative

Brighter cells more representative on average



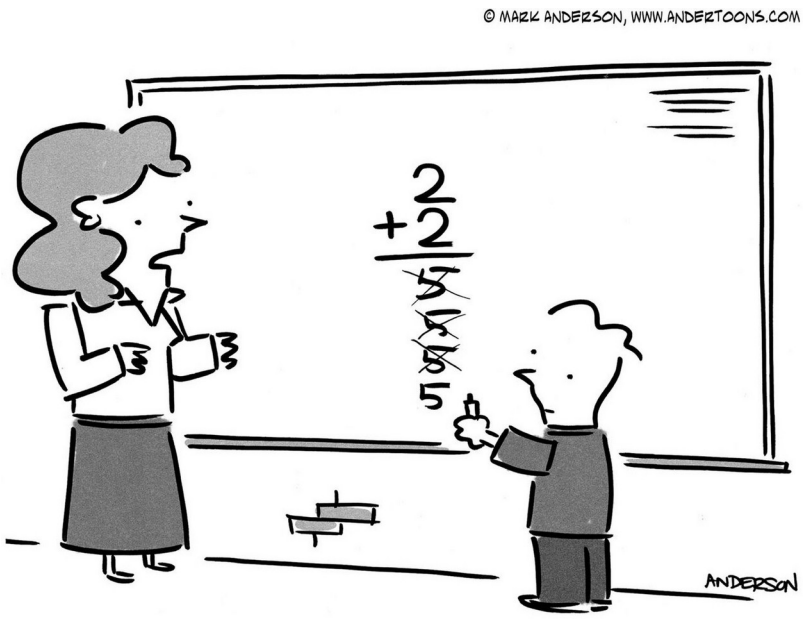
Angle of the different vectors differs

The tool of first resort: Troubleshooting your single-color controls



- ❑ Increase the MFI of your staining for the single colors
- ❑ Test the differences between using cells or beads
- ❑ Choices: Library; Duplicate with experiment; new single colors each time.
- ❑ Swap in CD3/CD4 for the same fluorophore (from same manufacturer).
- ❑ Once optimized, maintain consistent unmixing throughout an experiment run.

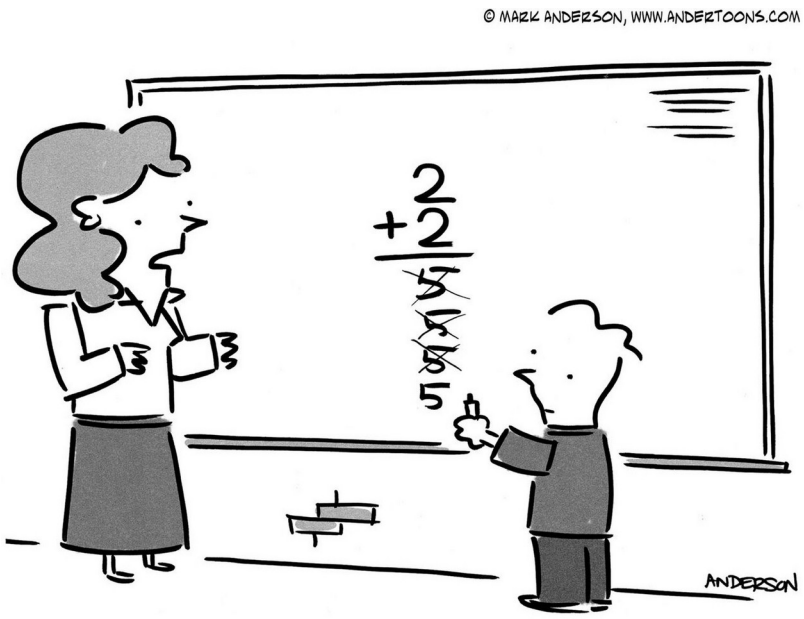
The tool of last resort: Compensation



"I appreciate your persistence, but that's never not going to be wrong."

- Spectral Flow Cytometry is not Conventional Flow Cytometry.
- Best practice papers actively discourage it (see math discussion)
- If your reference controls are bad from the get-go, it is not going to save your data.
- That being said: Sometimes is needed.

The tool of last resort: Compensation



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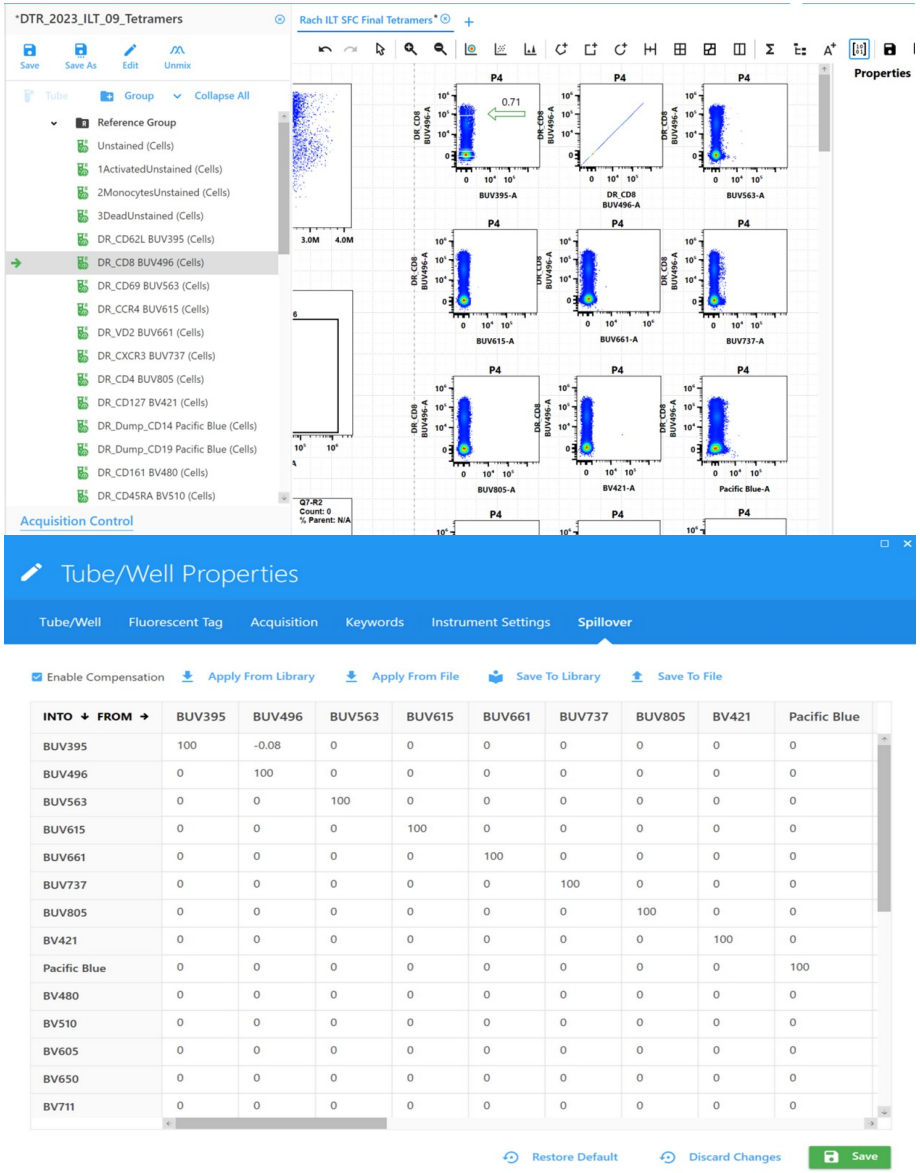
- Best practice papers actively discourage it (see math discussion)
- If your reference controls are bad from the get-go, it is not going to save your data.
- That being said: Sometimes is needed.

If you are going to do it, I advocate the build the compensation matrix from your cell single-color controls and then apply it to the full-stained samples

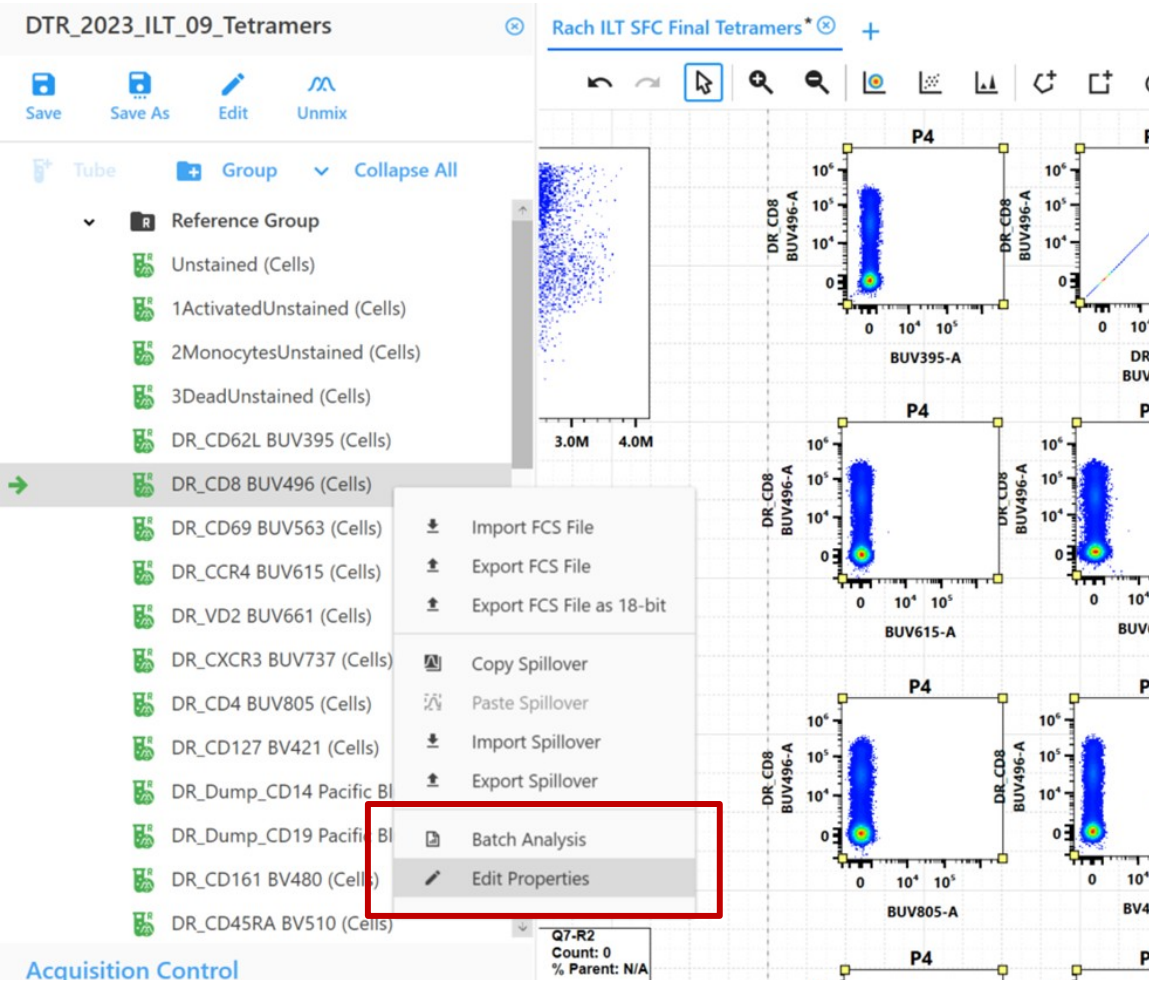
To what are you applying compensation?

- Adjust spillover on the individual cell single color controls after unmixing
- You see the unmixing error on a single-stained sample, and the effect of the adjustment as visualized on an NxN plot.
- By saving and importing spillover to each single color sequentially, you build a full compensation matrix for all combinations in your panel
- This can then be applied to the full-stained sample
- Can be saved to library for reuse to correct specific panel issues.
- Reproducible



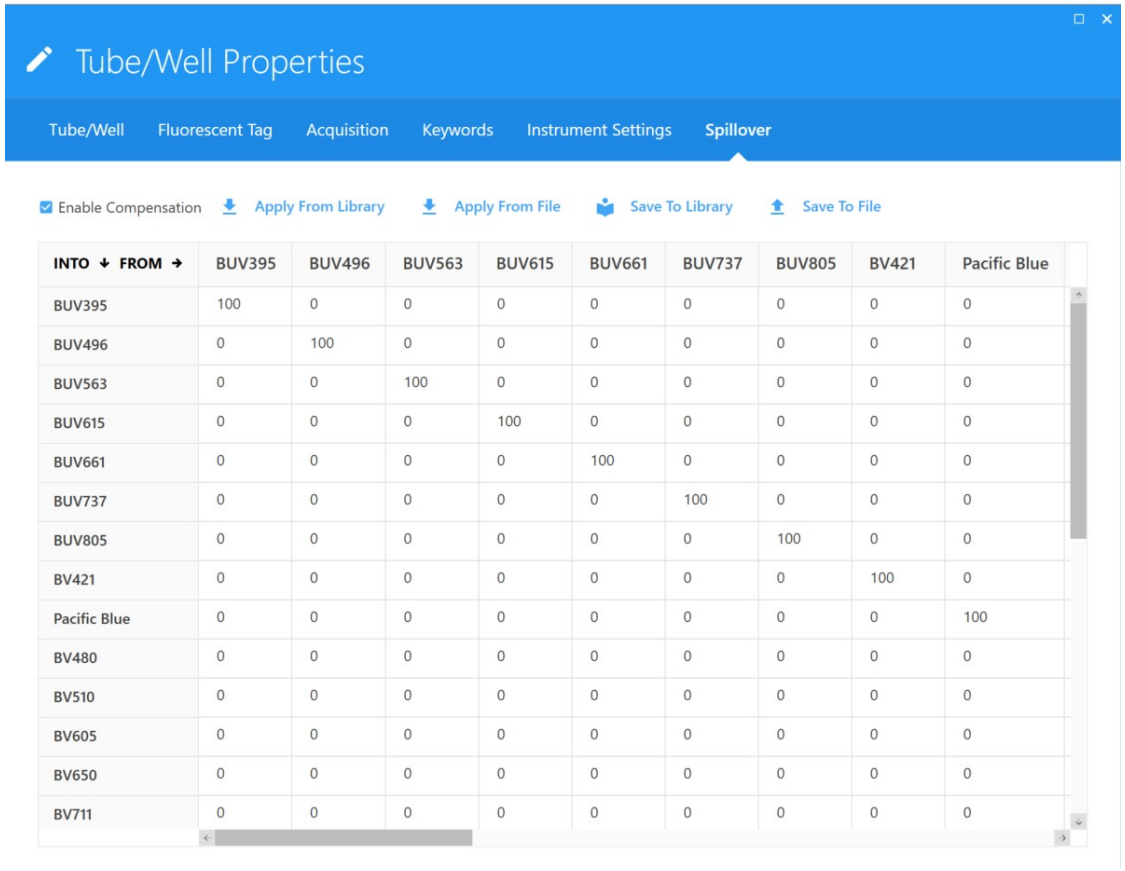
INTO ↓ FROM →	BUV395	BUV496	BUV563	BUV615	BUV661	BUV737	BUV805	BV421	Pacific Blue
BUV395	100	-0.08	0	0	0	0	0	0	0
BUV496	0	100	0	0	0	0	0	0	0
BUV563	0	0	100	0	0	0	0	0	0
BUV615	0	0	0	100	0	0	0	0	0
BUV661	0	0	0	0	100	0	0	0	0
BUV737	0	0	0	0	0	100	0	0	0
BUV805	0	0	0	0	0	0	100	0	0
BV421	0	0	0	0	0	0	0	100	0
Pacific Blue	0	0	0	0	0	0	0	0	100
BV480	0	0	0	0	0	0	0	0	0
BV510	0	0	0	0	0	0	0	0	0
BV605	0	0	0	0	0	0	0	0	0
BV650	0	0	0	0	0	0	0	0	0
BV711	0	0	0	0	0	0	0	0	0

How to do this:



- On your unmixed experiment, identify the cell single color control with unmixing issues
- Right click on the single color, select Edit Properties

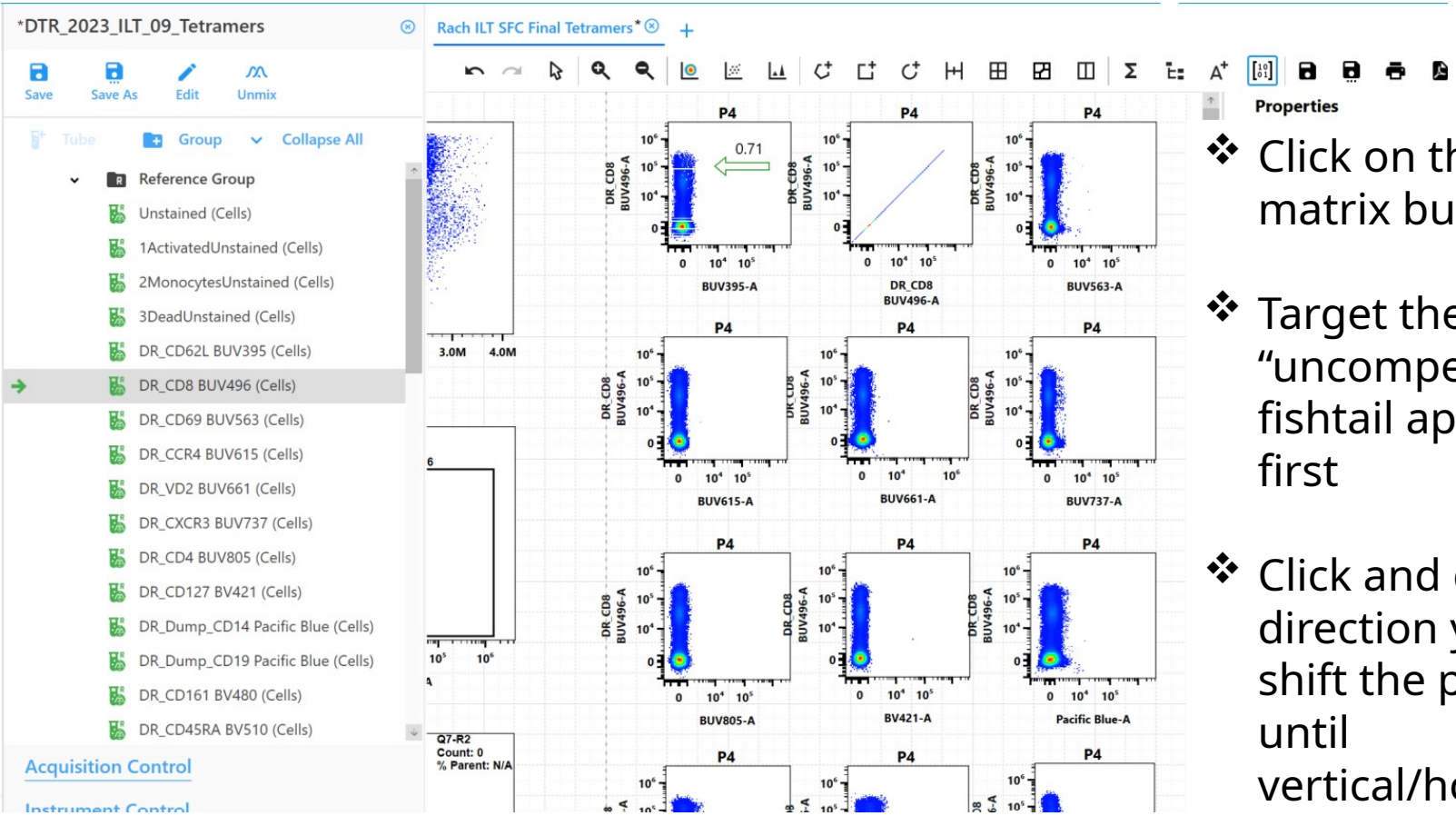
How to do this:



INTO ↓ FROM →	BUV395	BUV496	BUV563	BUV615	BUV661	BUV737	BUV805	BV421	Pacific Blue
BUV395	100	0	0	0	0	0	0	0	0
BUV496	0	100	0	0	0	0	0	0	0
BUV563	0	0	100	0	0	0	0	0	0
BUV615	0	0	0	100	0	0	0	0	0
BUV661	0	0	0	0	100	0	0	0	0
BUV737	0	0	0	0	0	100	0	0	0
BUV805	0	0	0	0	0	0	100	0	0
BV421	0	0	0	0	0	0	0	100	0
Pacific Blue	0	0	0	0	0	0	0	0	100
BV480	0	0	0	0	0	0	0	0	0
BV510	0	0	0	0	0	0	0	0	0
BV605	0	0	0	0	0	0	0	0	0
BV650	0	0	0	0	0	0	0	0	0
BV711	0	0	0	0	0	0	0	0	0

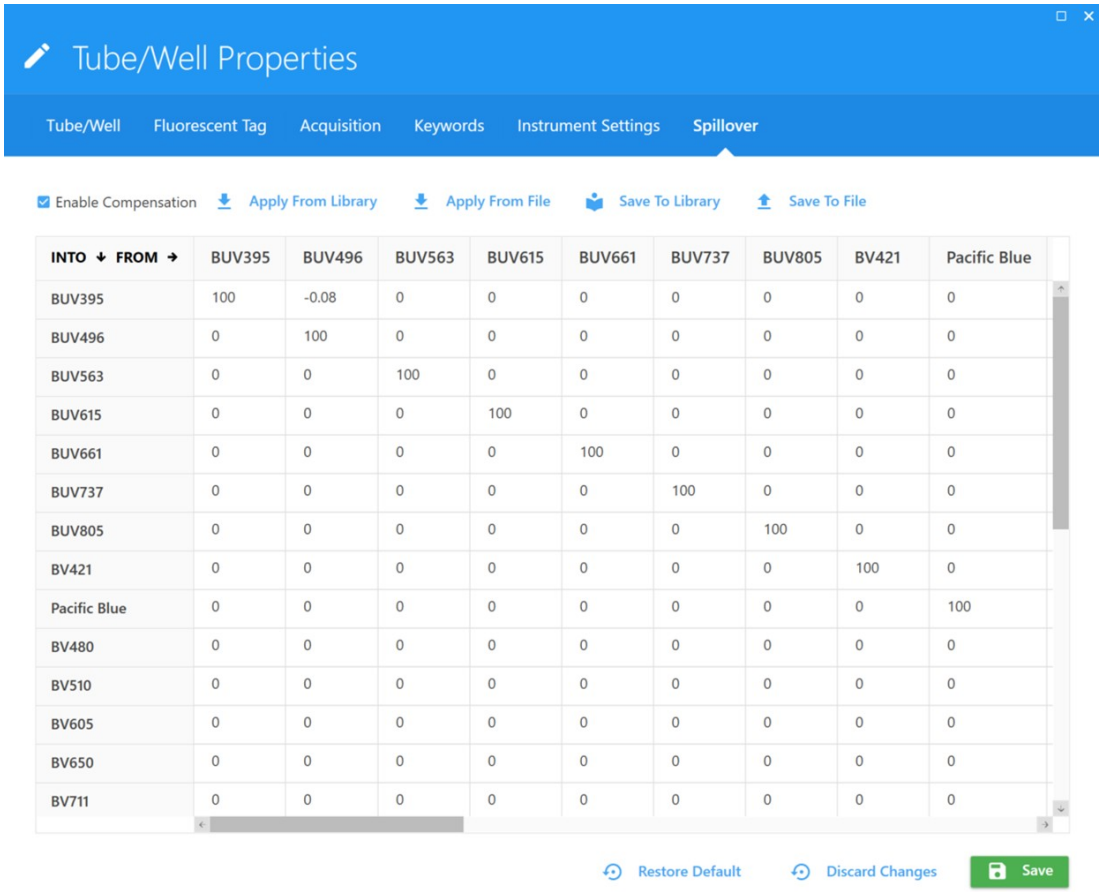
- Click on Spillover
- Click Enable Compensation
- Slide the window out of the way

How to do this:



- Click on the Spillover matrix button
- Target the most “uncompensated” fishtail appearances first
- Click and drag in the direction you want to shift the population until vertical/horizontal
- Observe effect of the intervention on other NxN plots

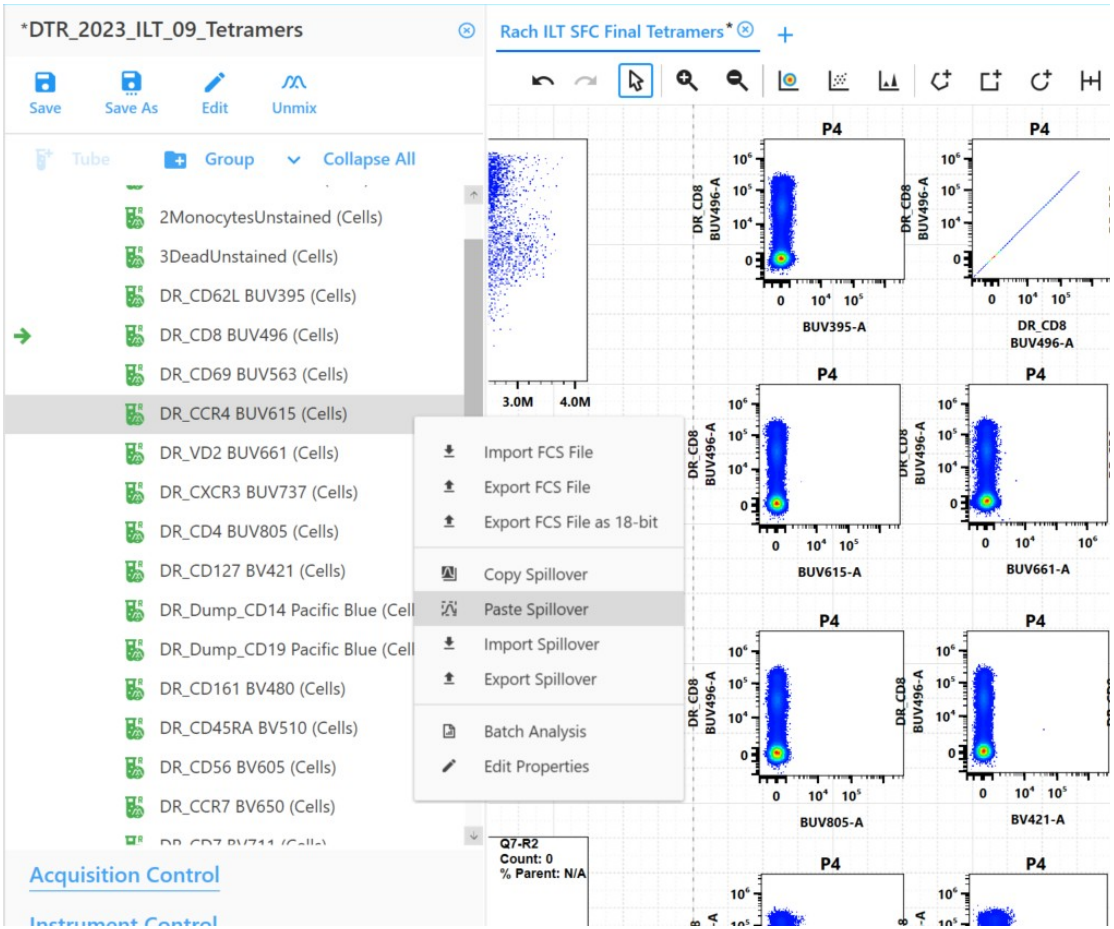
How to do this:



INTO ↓ FROM →	BUV395	BUV496	BUV563	BUV615	BUV661	BUV737	BUV805	BV421	Pacific Blue
BUV395	100	-0.08	0	0	0	0	0	0	0
BUV496	0	100	0	0	0	0	0	0	0
BUV563	0	0	100	0	0	0	0	0	0
BUV615	0	0	0	100	0	0	0	0	0
BUV661	0	0	0	0	100	0	0	0	0
BUV737	0	0	0	0	0	100	0	0	0
BUV805	0	0	0	0	0	0	100	0	0
BV421	0	0	0	0	0	0	0	100	0
Pacific Blue	0	0	0	0	0	0	0	0	100
BV480	0	0	0	0	0	0	0	0	0
BV510	0	0	0	0	0	0	0	0	0
BV605	0	0	0	0	0	0	0	0	0
BV650	0	0	0	0	0	0	0	0	0
BV711	0	0	0	0	0	0	0	0	0

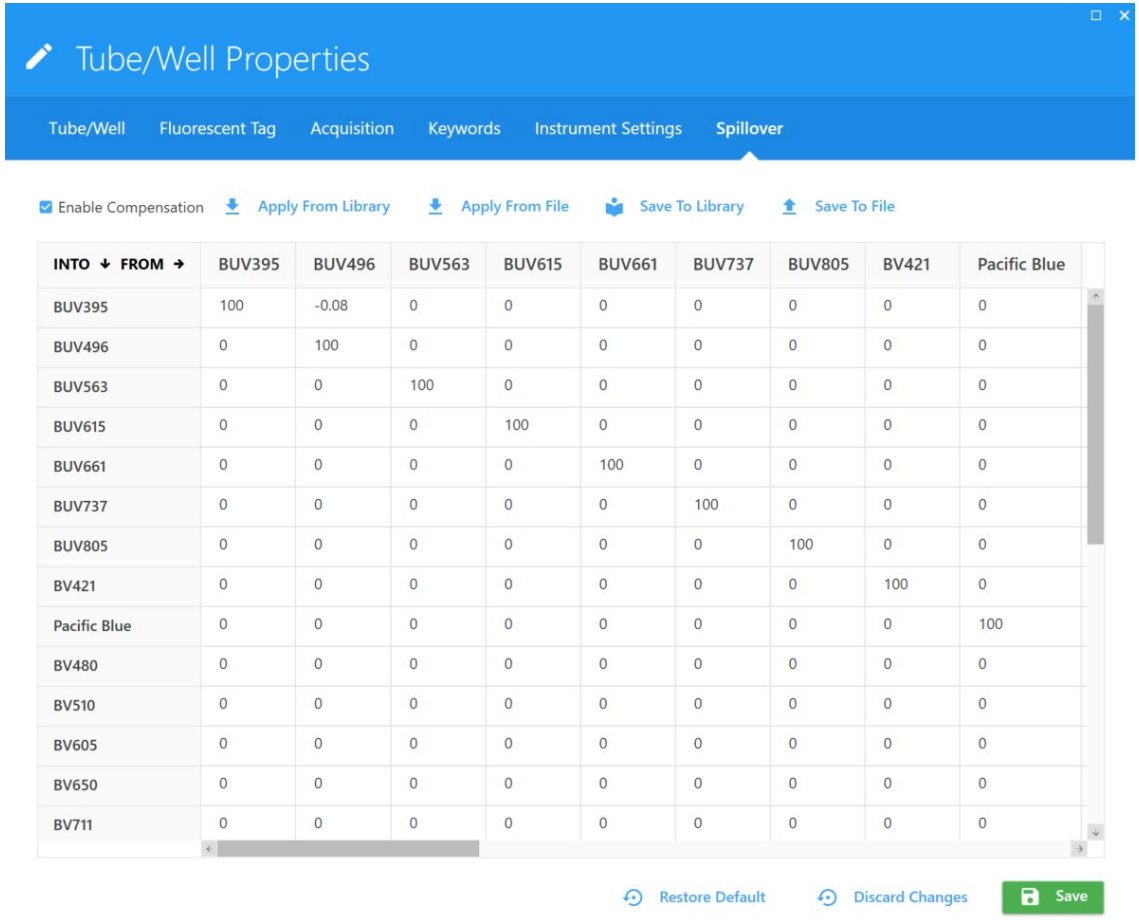
- Once done with adjustments to the individual single-color, click save on this screen.

How to do this:



- Right click and copy the saved spillover from the first single color.
- Navigate to the next single color with issues and paste the spillover.
- Repeat the process until full compensation matrix is assembled.

How to do this:



INTO ↓ FROM →	BUV395	BUV496	BUV563	BUV615	BUV661	BUV737	BUV805	BV421	Pacific Blue
BUV395	100	-0.08	0	0	0	0	0	0	0
BUV496	0	100	0	0	0	0	0	0	0
BUV563	0	0	100	0	0	0	0	0	0
BUV615	0	0	0	100	0	0	0	0	0
BUV661	0	0	0	0	100	0	0	0	0
BUV737	0	0	0	0	0	100	0	0	0
BUV805	0	0	0	0	0	0	100	0	0
BV421	0	0	0	0	0	0	0	100	0
Pacific Blue	0	0	0	0	0	0	0	0	100
BV480	0	0	0	0	0	0	0	0	0
BV510	0	0	0	0	0	0	0	0	0
BV605	0	0	0	0	0	0	0	0	0
BV650	0	0	0	0	0	0	0	0	0
BV711	0	0	0	0	0	0	0	0	0

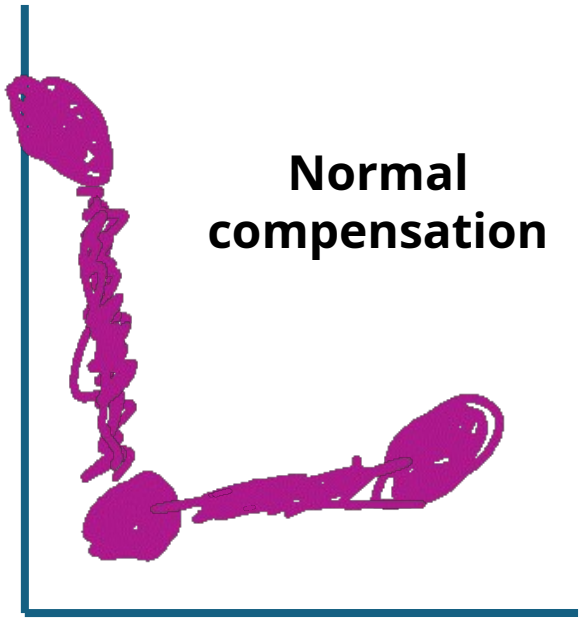
- Save the assembled compensation matrix specific to your single-color controls to either a file or the library.
- Apply this compensation matrix to your full-stained samples by right clicking on the sample and importing spillover .

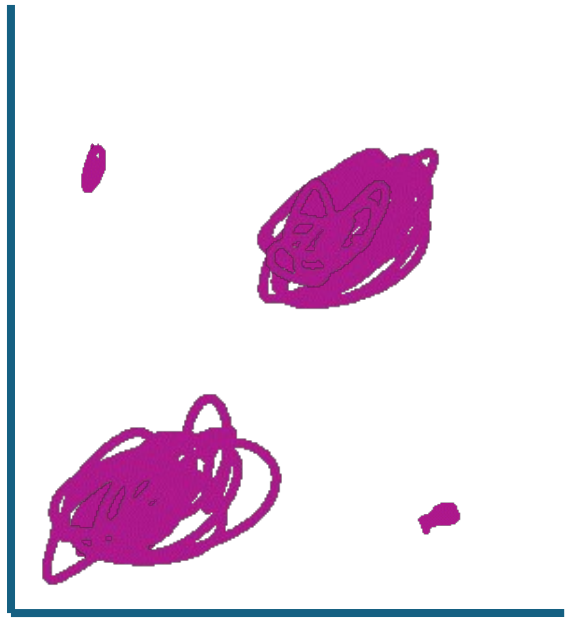
Why not just directly apply to the full-stained sample?

- Adjust spillover on the individual cell single color controls after unmixing
- You know exactly what needs adjustment, and can visually effect on an NxN plot.
- By importing spillover to each single single color sequentially, you build a compensation matrix that can be saved to library to correct specific panel issues.

- Adjust spillover on your full-stained sample after unmixing

30-color panel with high co-expression





NxN plot

30-color panel with high co-expression





30-color panel with high co-expression





30-color panel with high co-expression





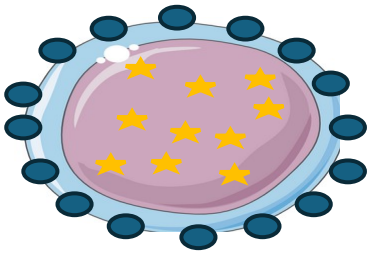
30-color panel with high co-expression



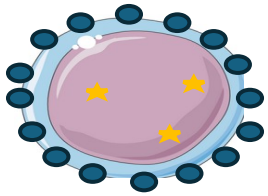


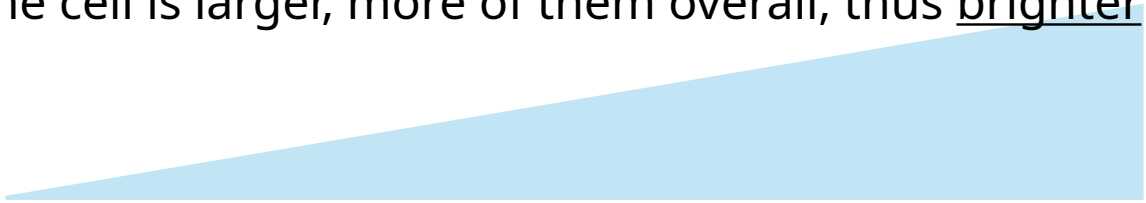
The issue: Variation in autofluorescence

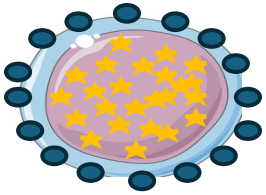




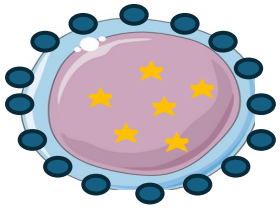
Difference in cell size:
Same proportion of autofluorescent molecules, but because one cell is larger, more of them overall, thus brighter



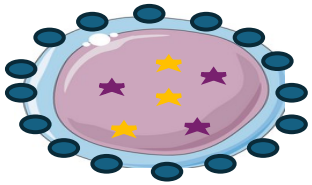




Difference in amount of autofluorescence:
Same cell size, just one has more autofluorescent molecules, thus brighter



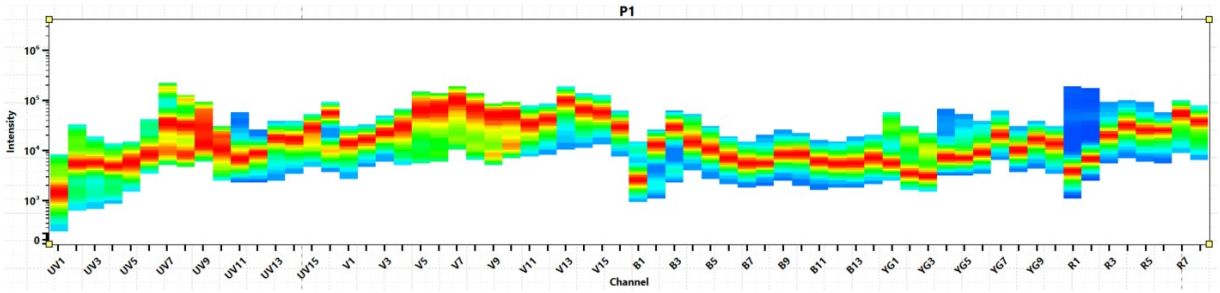




Variation in autofluorescent molecules:
Same cell size, but express different mixture of autofluorescent molecules, resulting in a different autofluorescent signature

Autofluorescence is both a concern for single-colors, but importantly also full-stained sample

Full-stained Samples



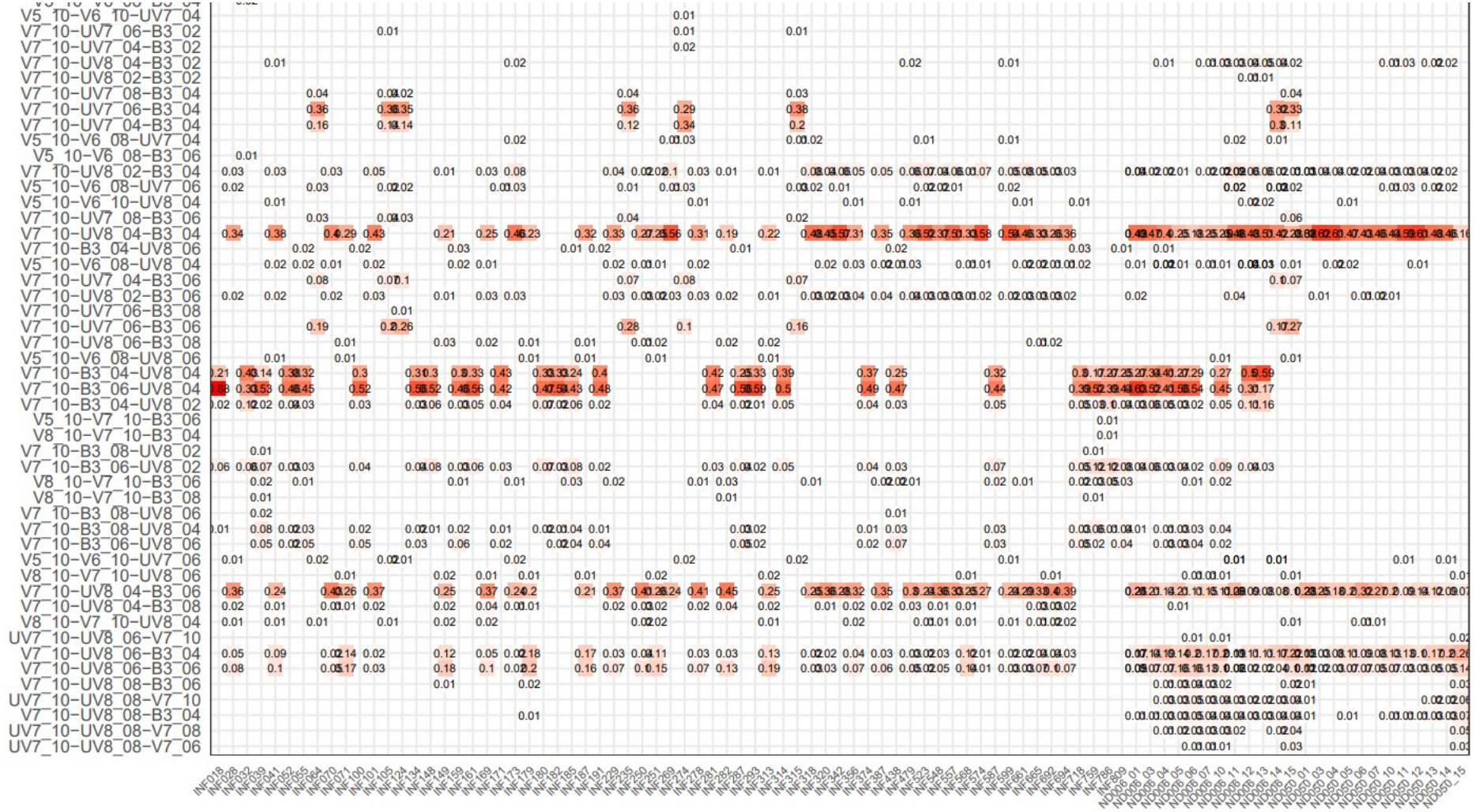
Single-color Reference Controls

- Yellow Reference Signature
- Blue Reference Signature
- Red Reference Signature
- Violet Reference Signature
- Orange Reference Signature

Unstained Reference Controls

- Autofluorescence Cell Subset #1
- Autofluorescence Cell Subset #2

Autofluorescence can differ between individuals and treatment conditions

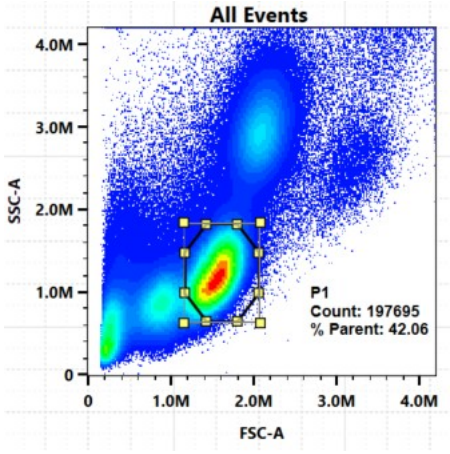


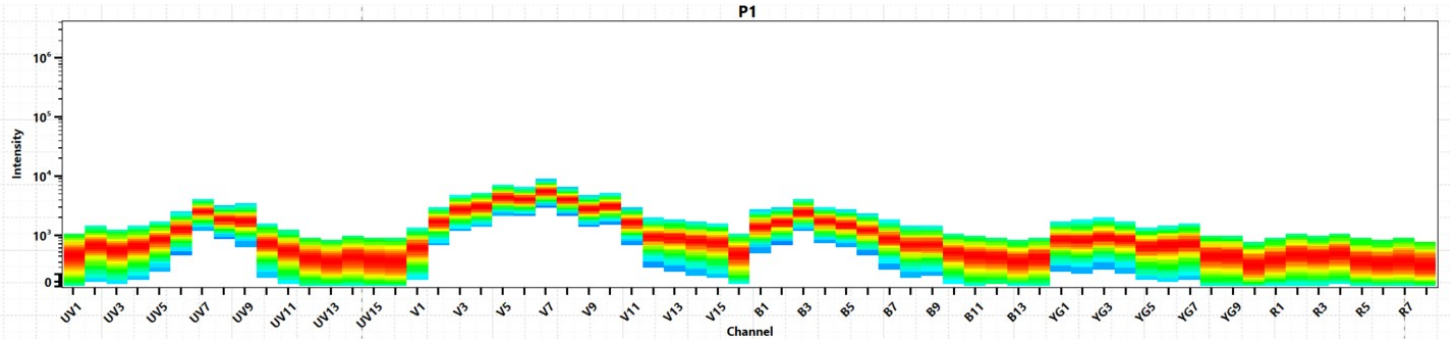
Types of Autofluorescence

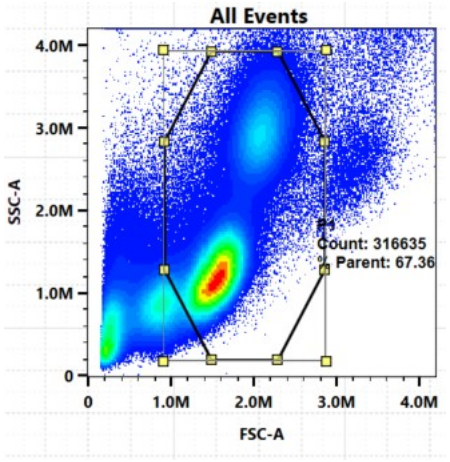
- Practically identical (PBMCs: B cells, T cells, NK cells): 0.99-1.00
- Difference in brightness: Monocytes and PBMCs (0.92-1.00)
- Difference in signatures: Granulocytes and PBMCs. Treat them differently.
- Complex Tissues: Tons of different autofluorescence when you run unstained samples

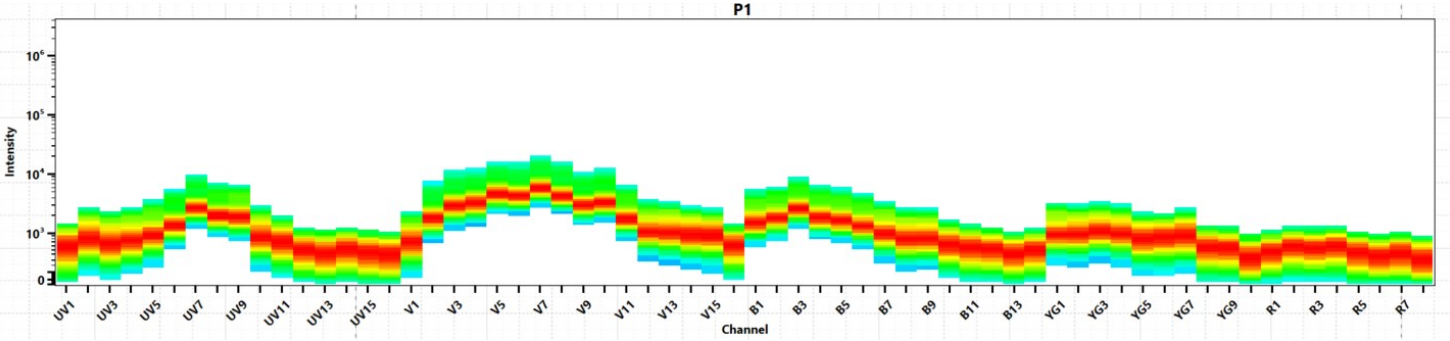
Identifying Multiple Autofluorescences

- Original Method

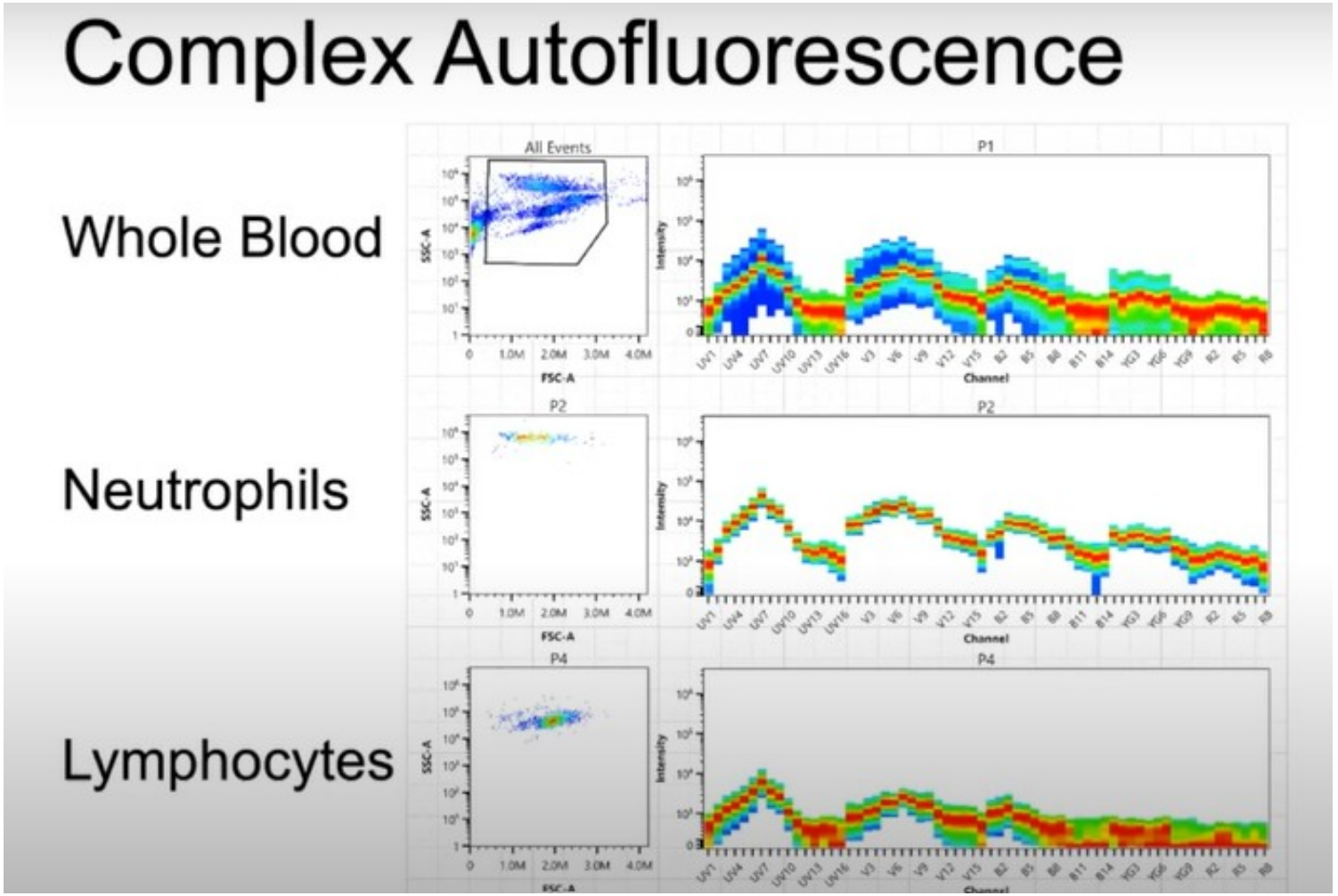








When dealing with many different autofluorescences



THE UNIVERSITY OF CHICAGO

https://youtu.be/vJsdM1B1uBU?si=BspdK3IjFj67MOX

2) The dimensionality reduction approach

Cytometry PART A
Journal of Quantitative Cell Science
ISAC

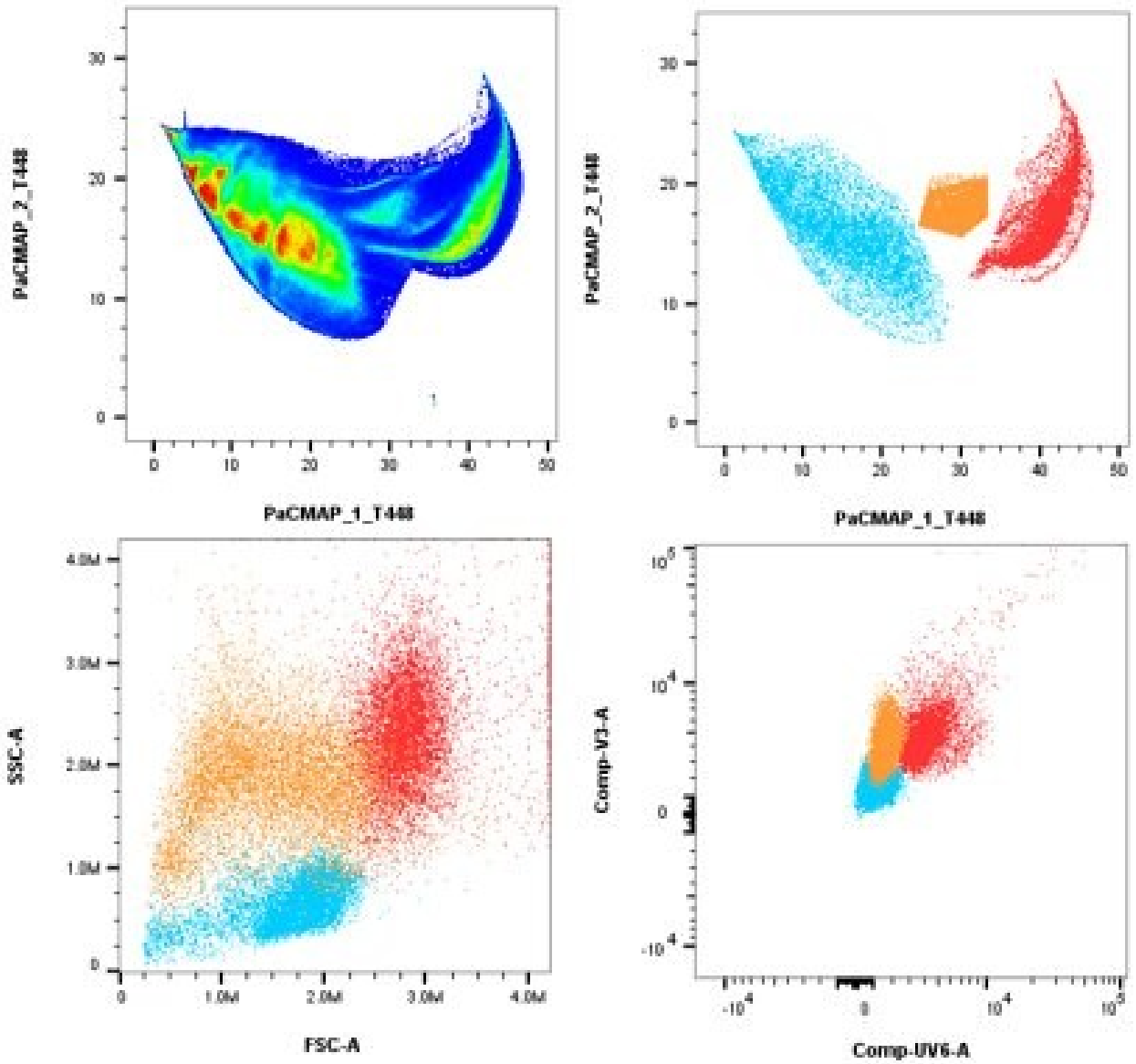
Original Article | Open Access

Unlocking autofluorescence in the era of full spectrum analysis: Implications for immunophenotype discovery projects

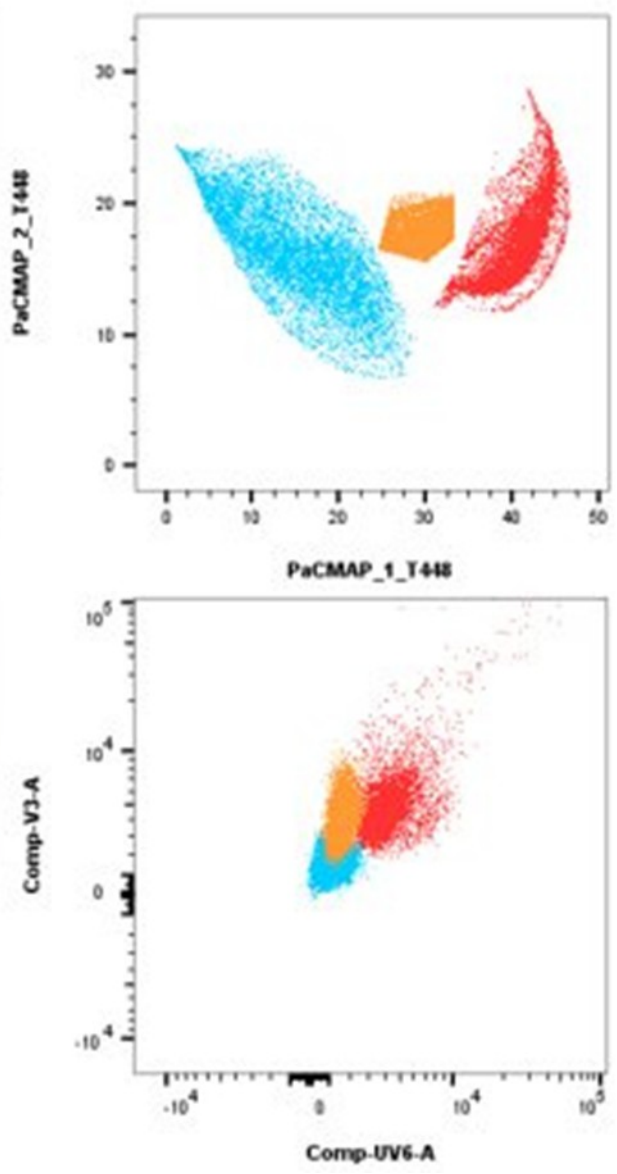
Vanta J. Jameson, Tina Luke, Yuting Yan, Angela Hind, Maximilien Evrard, Kevin Man, Laura K. Mackay, Axel Kallies, Jose A. Villadangos, Hamish E. G. McWilliam, Alexis Perez-Gonzalez

First published: 29 March 2022 | https://doi.org/10.1002/cyto.a.24555 | Citations: 5

2) The dimensionality reduction approach

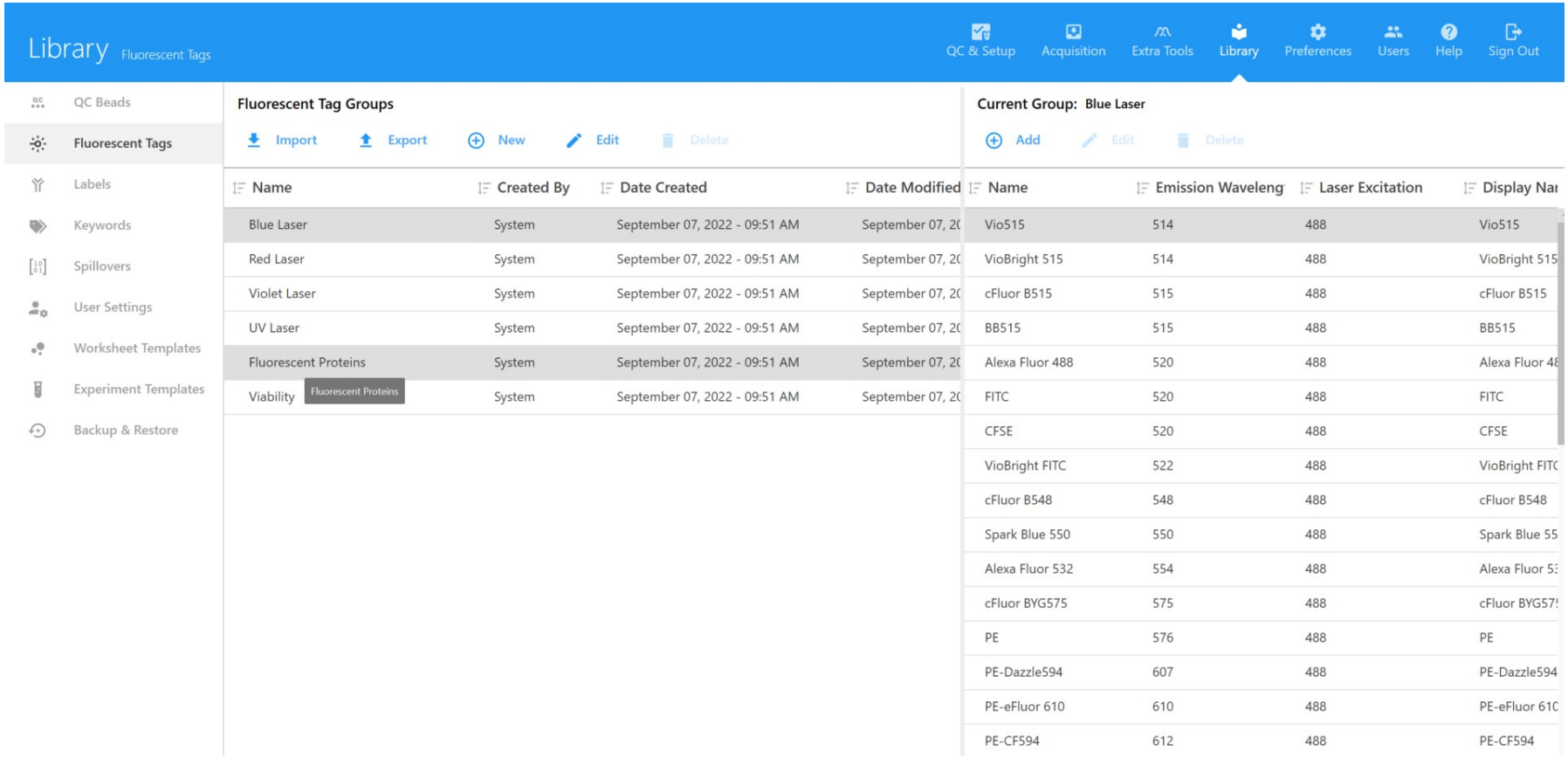


The Workflow:

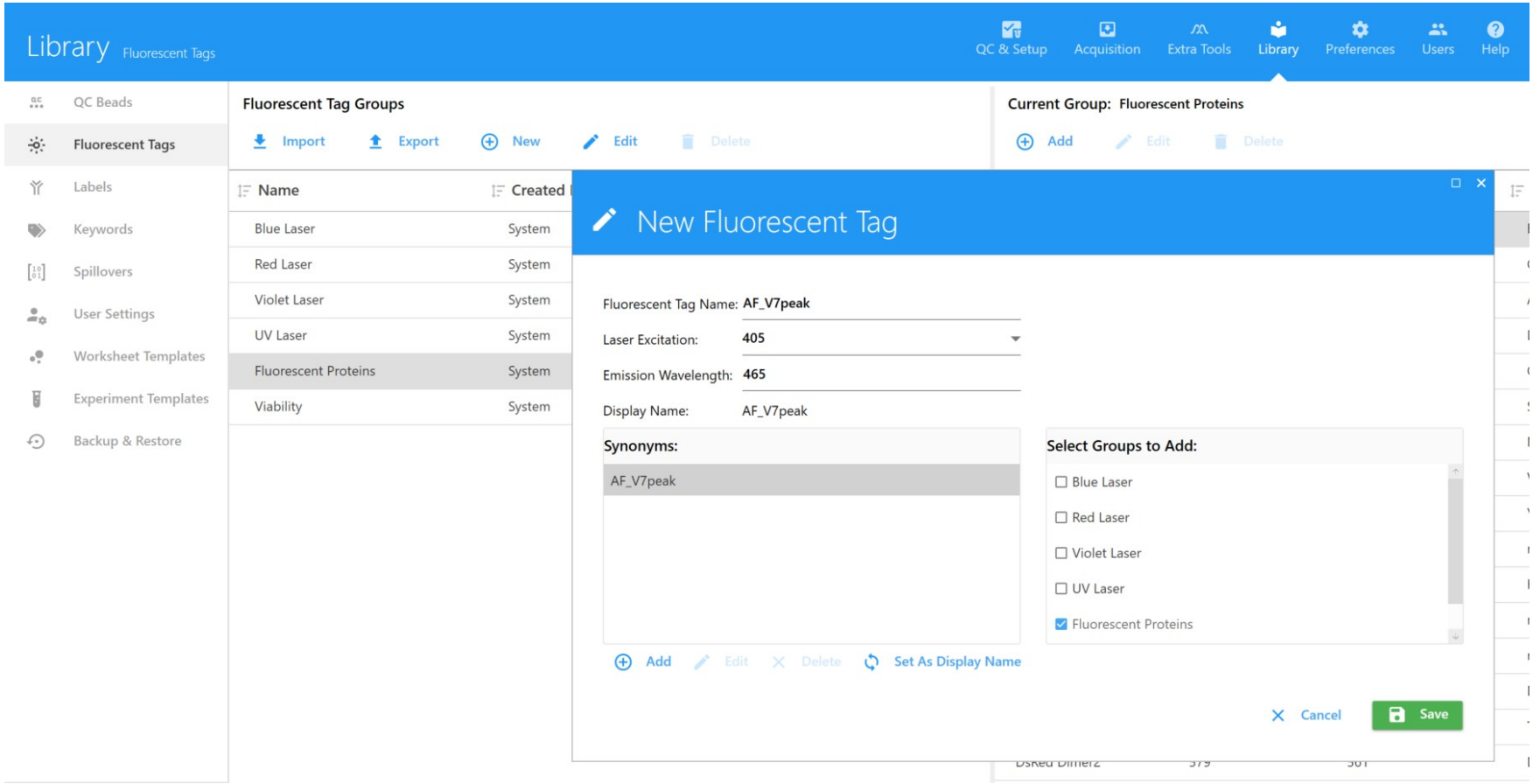


- Raw .FCS Unstained files
- tSNE, UMAP, PaCMAP.
- Select all detectors and run.
- Gate Islands
- Plot as an overlay
- Identify detectors where the autofluorescent islands differ
- Return to SpectroFlo, navigate to those detectors, gate similarly

Adding Autofluorescent Tags



Adding Autofluorescent Tags



SpectroFlo Autofluorescent Tag – Negative

- SpectroFlo still wants a negative to subtract out
- Your tag should be “pure signature” and not need subtraction

- We need to trick the software into thinking it subtracted.
- Gate and export as an AF .fcs file
- Gate on dimmest signal events and export as its own “Negative” .fcs file

- Reimport both to a single group
- Highlight both and right click. Select merge/concatenate and export combined .fcs file.

- Bring this in as it’s own fluorophore under reference group
- Set to use internal negative

- Gate all events fsc/ssc.
- Set positive for brightest AF
- Set negative for dim threshold cells
- Proceed with unmixing as normal

How bad is the unmixing?

Absolutely horrible? Likely partially panel design and need to move some fluorophore/marker combinations around to improve your panel.

Bad? Reference controls used are likely not accurately representing what is present on the full-stained cells, need to optimize your single color control preparation.

Pretty good except for this one marker? Minor compensation applied

Questions?