Panel Design for the ID7000 Spectral Cell Analyzer

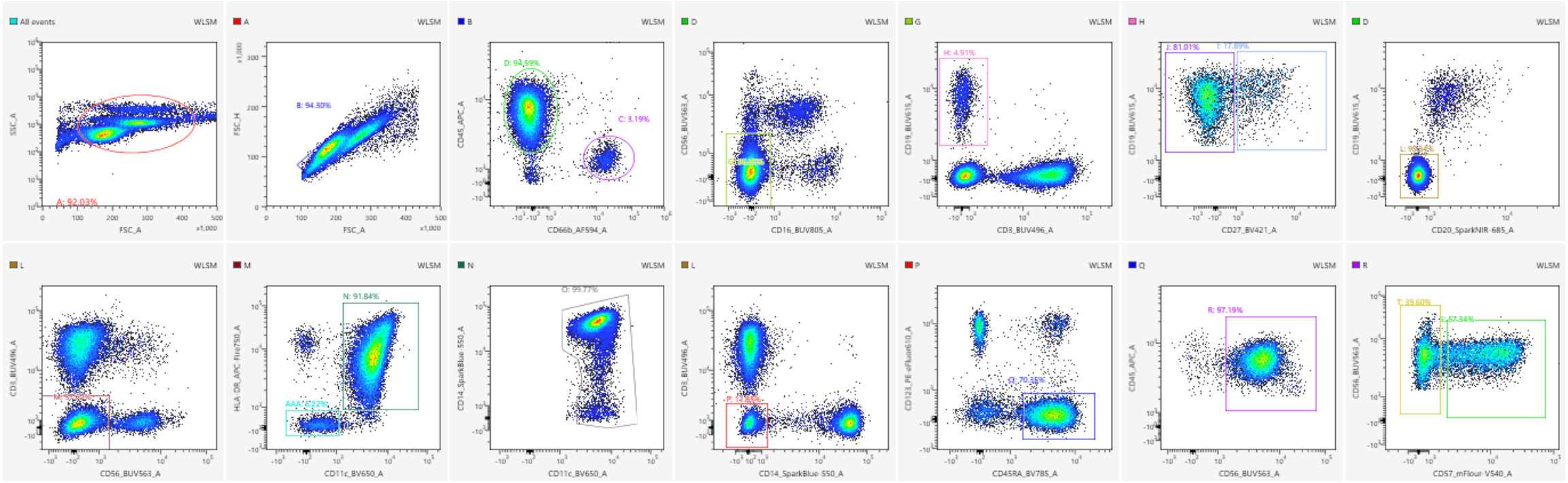
Insert Name

Agenda

- Core Concepts
- What do you need to build your panel?
- How do I make fluorochrome/marker assignments?
- Tips and Tricks

What is a Good Panel?

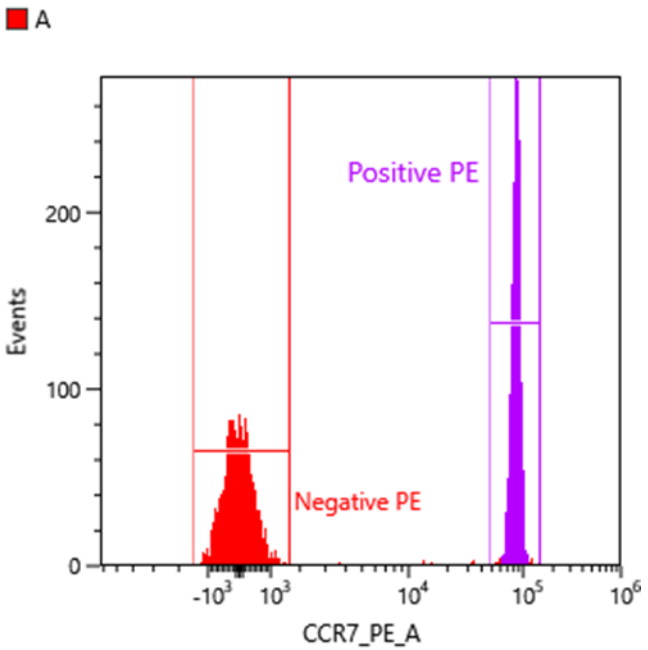
- A well-designed panel has clear resolution on all of the markers
- Resolution is the ability to distinguish positive populations from negative populations



Stain Index

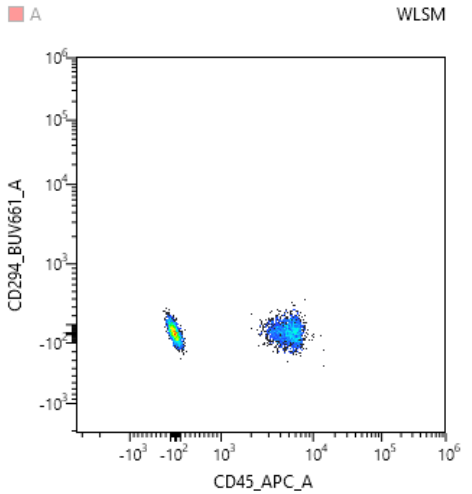
- This is the same calculation as from conventional flow
- A quantification of the separation of the positive and negative populations
- Used for titrations
- Important basic concept for panel design

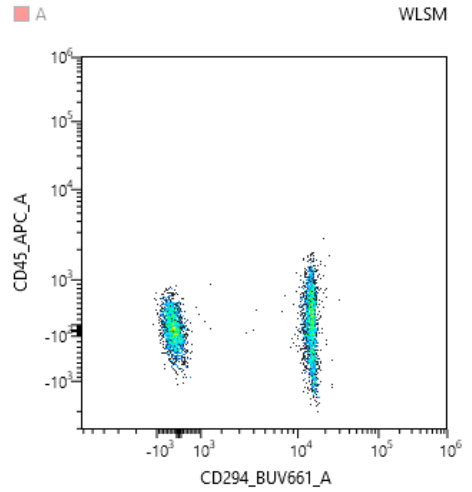
$$\text{Stain Index} = \frac{\text{MFI_Positive} - \text{MFI_Negative}}{2 * \text{SD_Negative}}$$

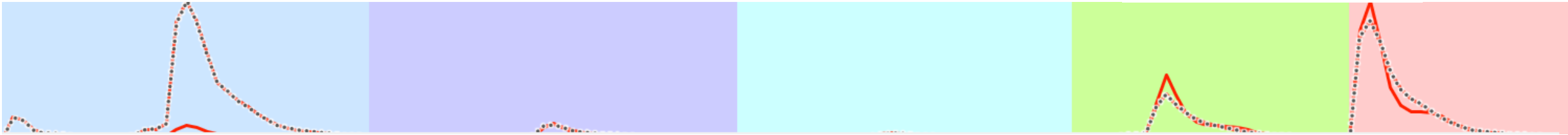


Similarity Matrix

- Measure of how similar two colors are
- Is NOT a good predictor of spread
- Try to not assign co-expressed markers to colors that are too similar
- Can be calculated using either Cosine Similarity (-1 to 1) or R^2 Similarity (0 to 1)
- Weaknesses
  - It does not show directional spread e.g. BUV661 and APC
    - BUV661 (highlighted) causes more spread in APC than APC causes in BUV661
  - Direct Overlap vs. Offset Overlap causes under-estimation/over-estimation of spread

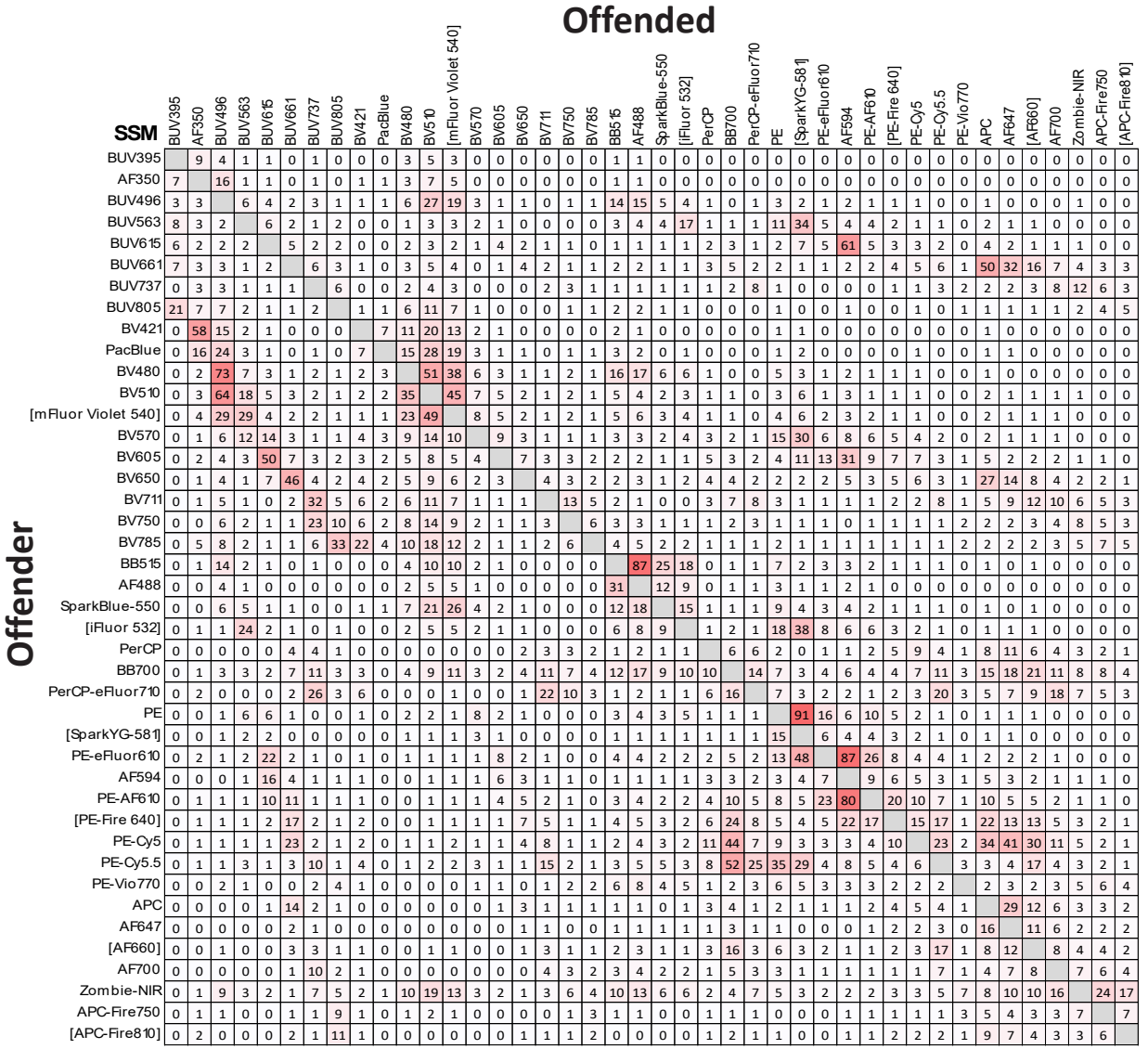






Spillover Spread Matrix

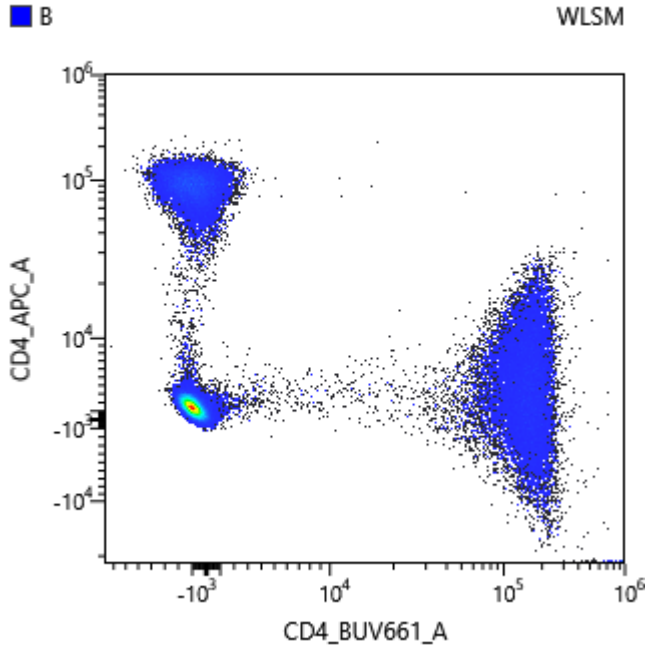
- Quantifies spillover spread
- Shows directional spread
- Single color controls must be run for every color in the panel and unmixed
- Does not account for intensity
- Overlap is also the source of unmixing errors
- Five Patterns of Spread

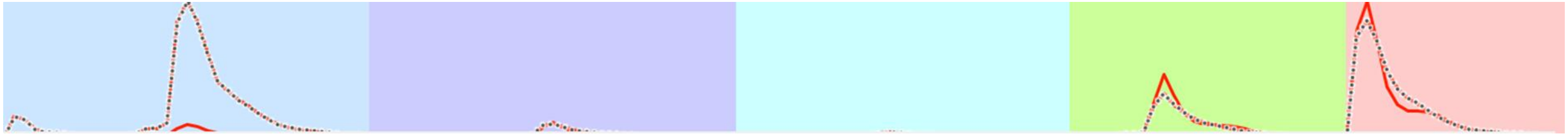


Nguyen, R., Perfetto, S.P., & Roederer, M. (2013). Quantifying spillover spreading for comparing instrument performance and designing multicolor panels. *Cytometry Part A*, 83(2), 179–181.

Patterns of Spread

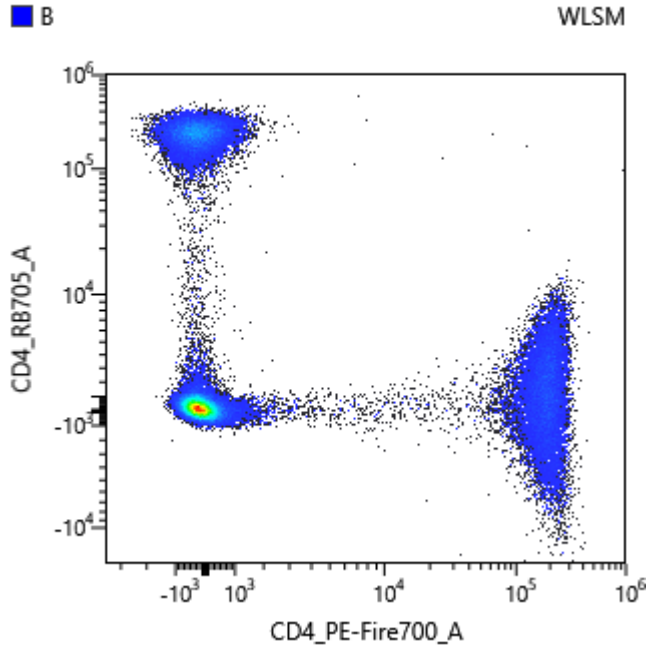
- **Direct Excitation of the Acceptor on a Tandem Dye**
- **BUV661 (Dotted Line) -> APC**





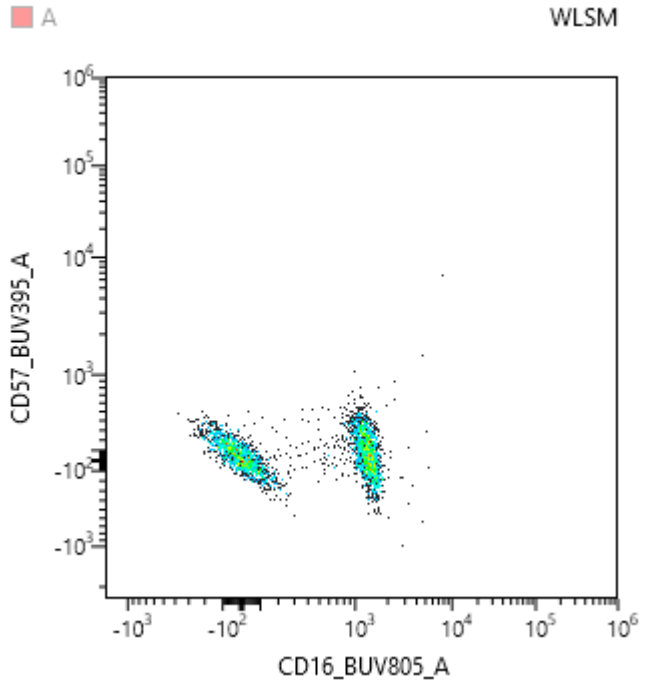
Patterns of Spread

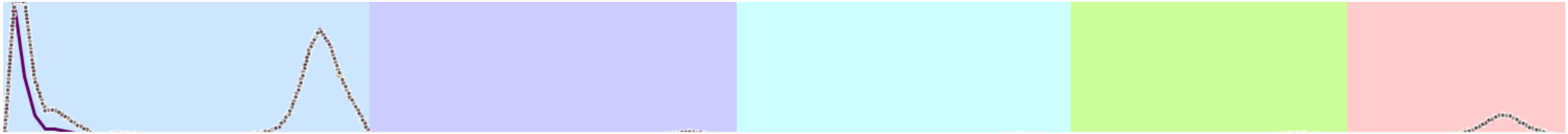
- **Excitation by a Lower Wavelength Laser**
- **PE-Fire700 (Dotted Line) -> RB705**



Patterns of Spread

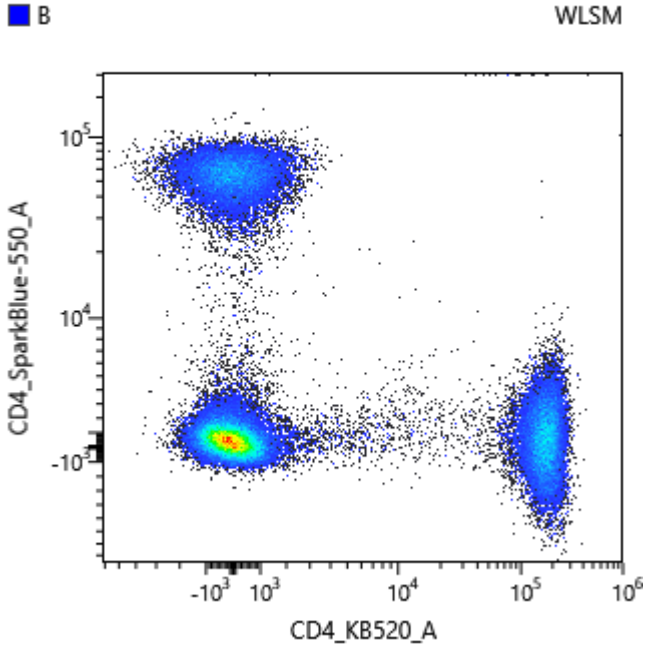
- **Tandem Dye Spilling back into its Donor**
- **BUV805 (Dotted Line) -> BUV395**

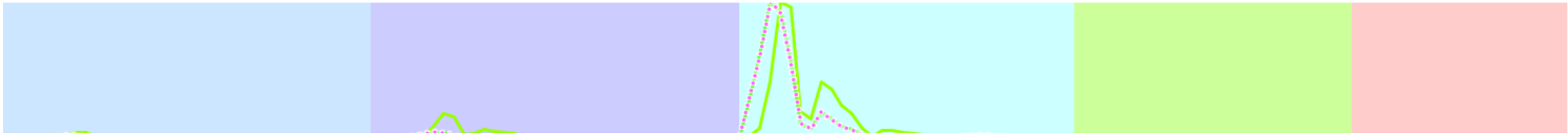




Patterns of Spread

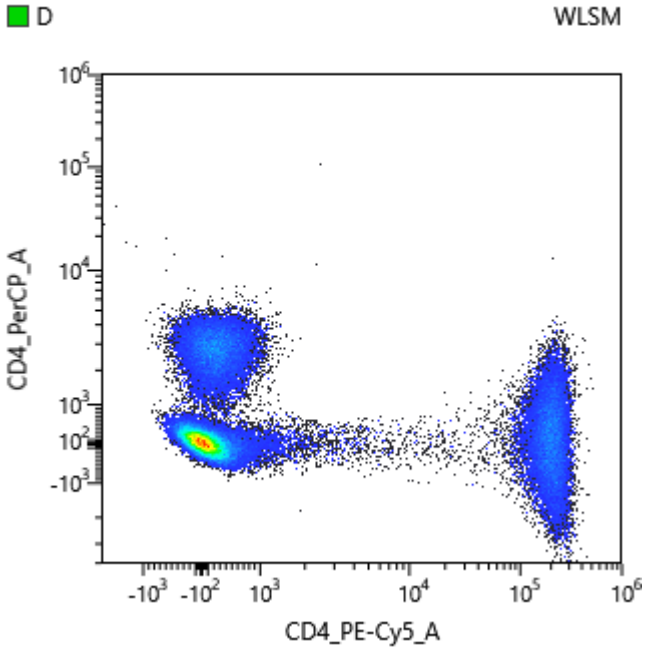
- **Next Door Neighbors**
- **KB520 (Dotted Line) -> SparkBlue550**

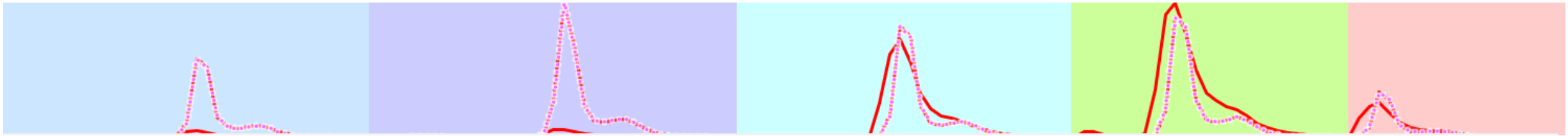




Patterns of Spread

- **Excitation by Many Lasers**
- **PerCP (Dotted Line) -> PE-Cy5**

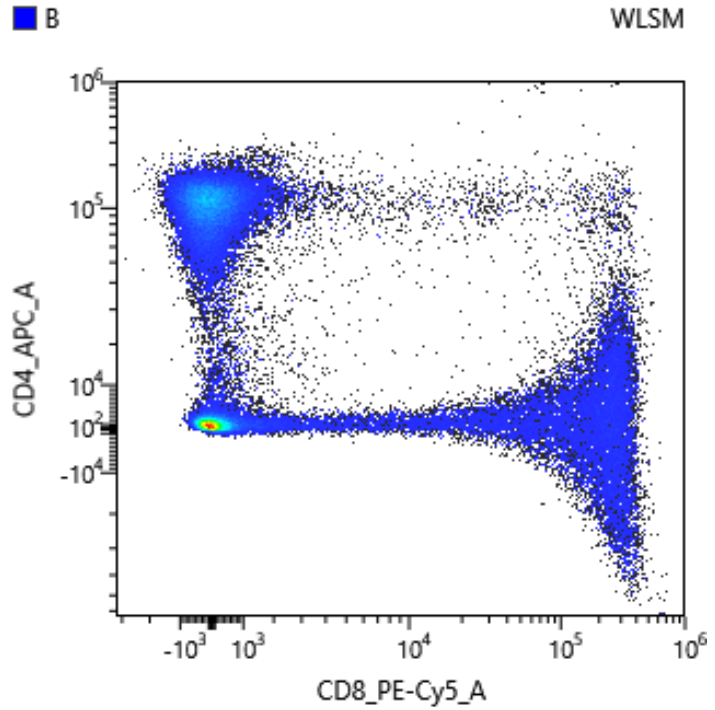




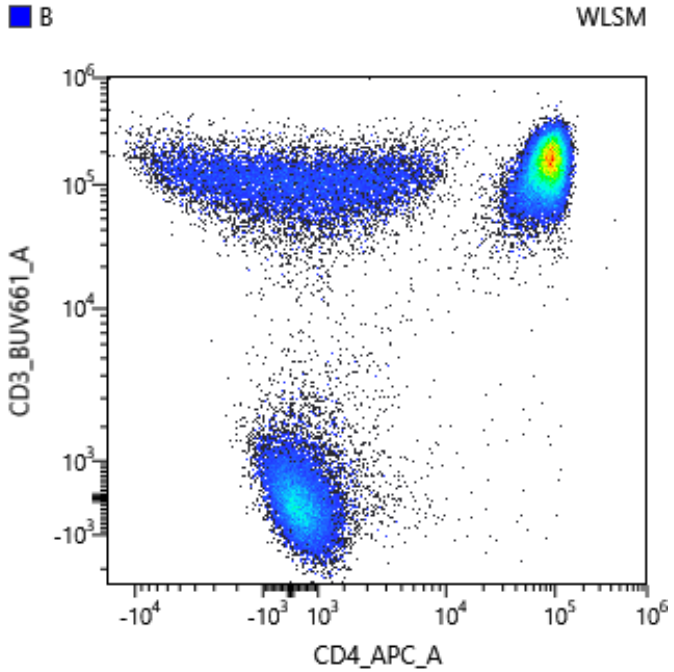
Pairwise Stain Index

- Concept that quantifies how spillover spread affects resolution
- Very similar calculation to SSM but SD of single positive population instead of SD of negative
- Spread of single positive population will show in the double negative population in other plots if not gated out

Well Designed Panel

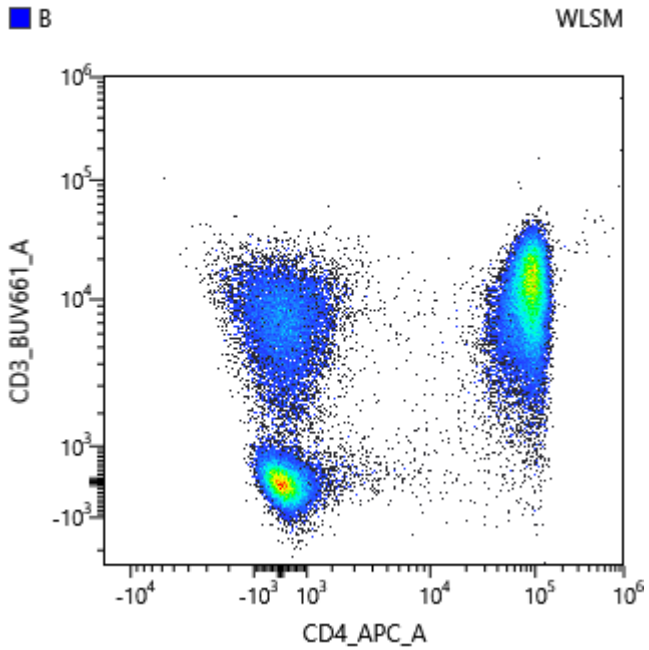


Badly Designed Panel

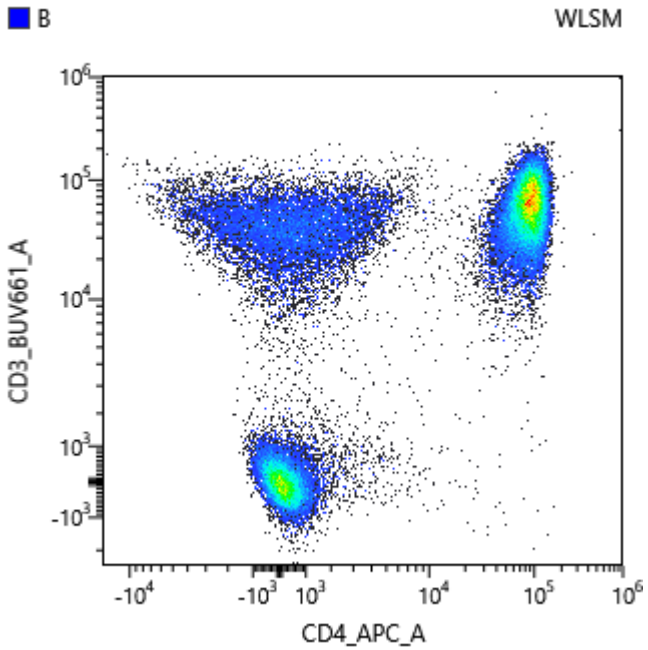


Brightness and the Trumpet Effect

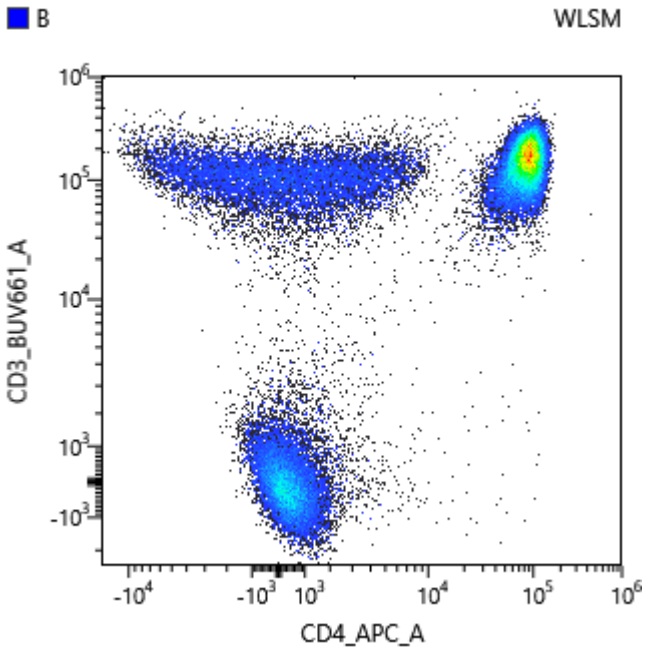
The brightness of a population is correlated to the amount of spread it will donate



Pairwise Stain Index	48.95
CD3+ CD4- Stain Index	9.22



Pairwise Stain Index	20.39
CD3+ CD4- Stain Index	37.54



Pairwise Stain Index	13.50
CD3+ CD4- Stain Index	68.48

What do you need to Build a Panel?

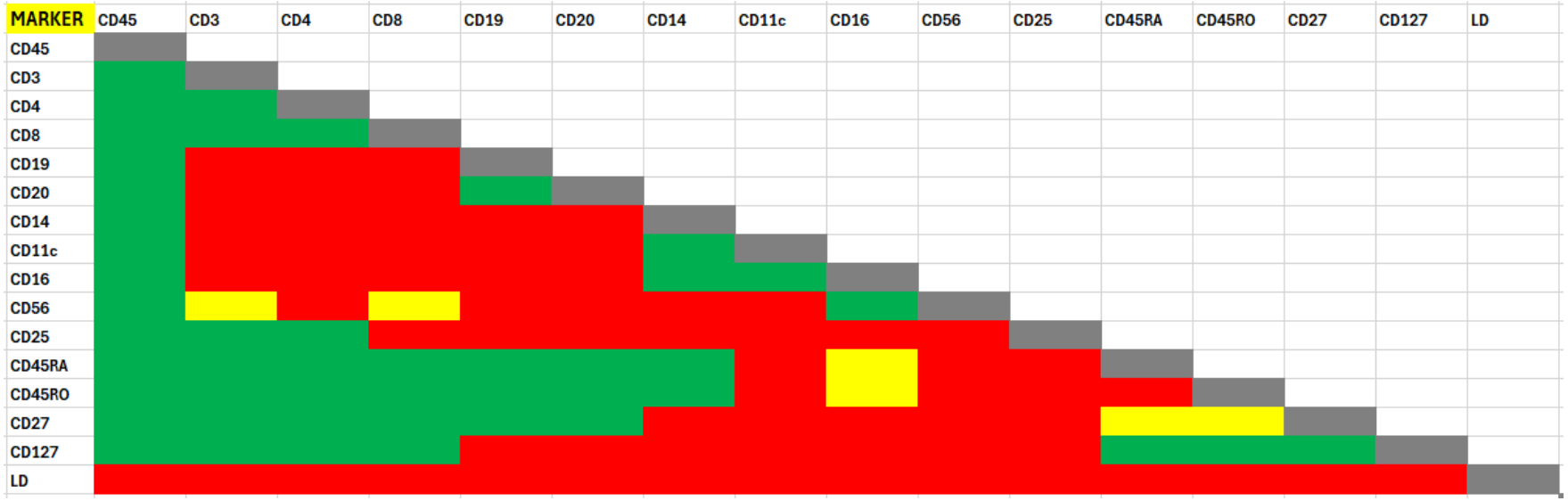
- What is the question that the experiment is trying to answer?
  - Type of sample (Fresh vs. Fixed vs. Frozen)
  - Viability
  - Condition (Unstimulated vs. Stimulated)
- What are the populations of interest?
  - What markers are necessary to identify my populations of interest?
    - Lineage Markers
    - Exclusion Markers
    - Activation Markers
    - Functional Markers
    - Fluorescent Proteins

Marker Information

Marker Information Needed	Reason
Marker Density	Bright vs. Dim Fluorochrome
Marker Location (Intranuclear, Cytoplasmic, or Surface)	Fluorochrome Size and Stability Permeabilization Method
Availability (How many and which fluorochromes are available conjugates)	Early vs. Late Marker Assignment
Specific Clone Needs	Some samples may already be fixed so there is a need for clones that target epitopes that are not fix sensitive Some epitopes may have steric hindrance concerns

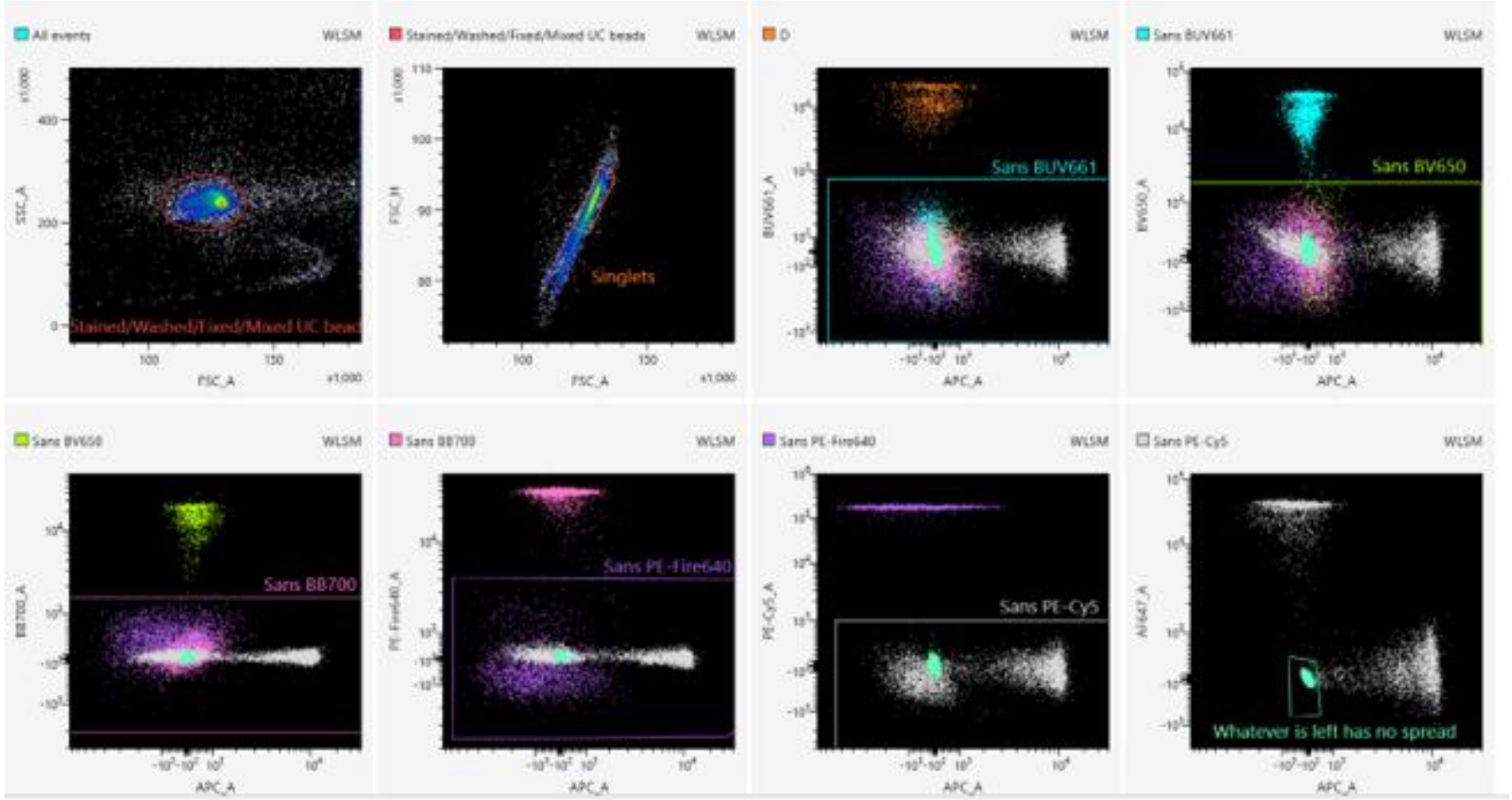
How do the Markers Relate to Each Other?

- Co-expression Matrix and Gating Strategy
  - Both will be a large help for guiding Marker-Fluorochrome assignments
  - Gating Strategy lets you know which markers will be gated out at which step of the analysis
    - If an offending fluorochrome is gated out before you look at a plot with an offended fluorochrome, this will improve the resolution on that plot



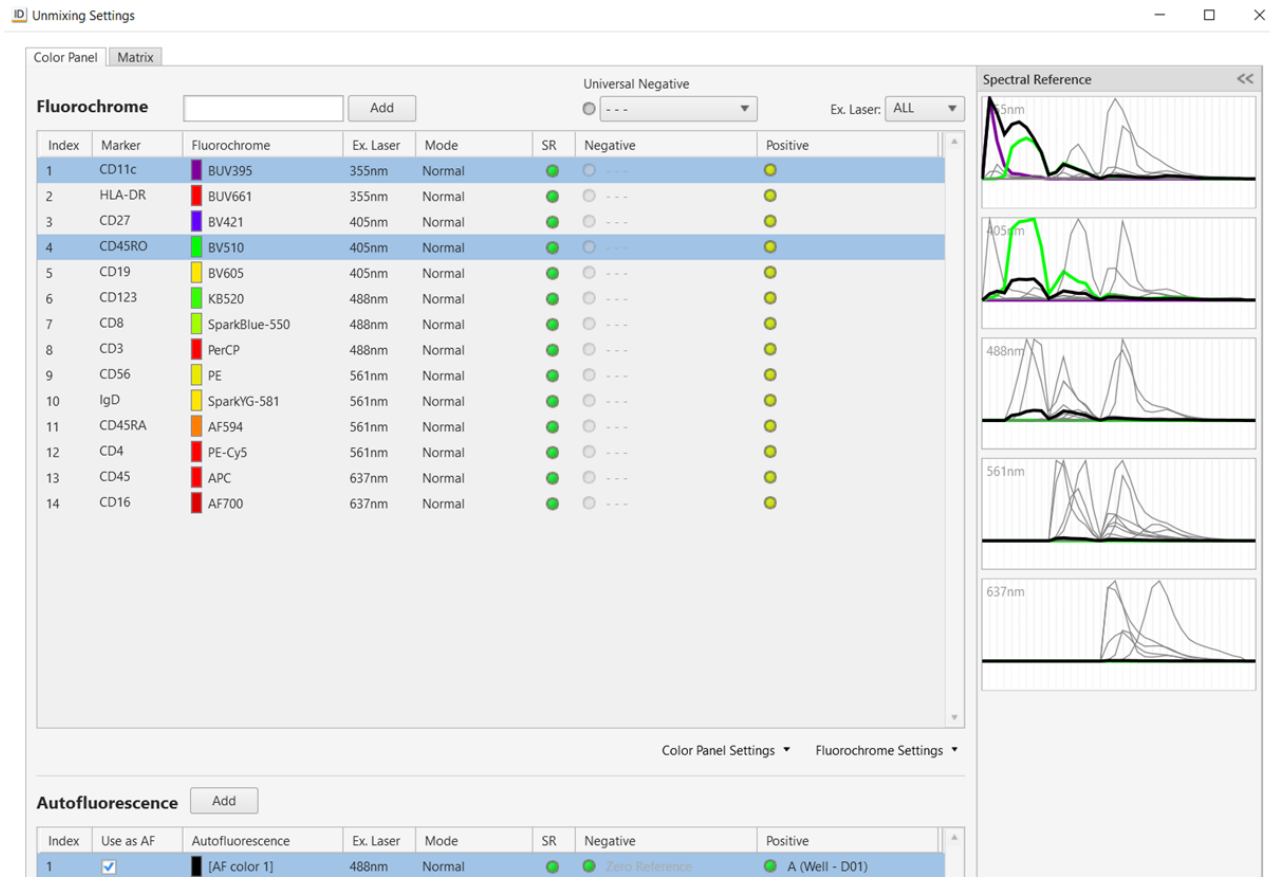
Miro's Most Beautiful Experiment

- Thought experiment done by staining single color controls beads and then fixing and mixing them
- Designed to show how the effects of spread can be mitigated through a proper gating strategy
- Each color that has a certain degree of spread into APC and is gated out individually in succession

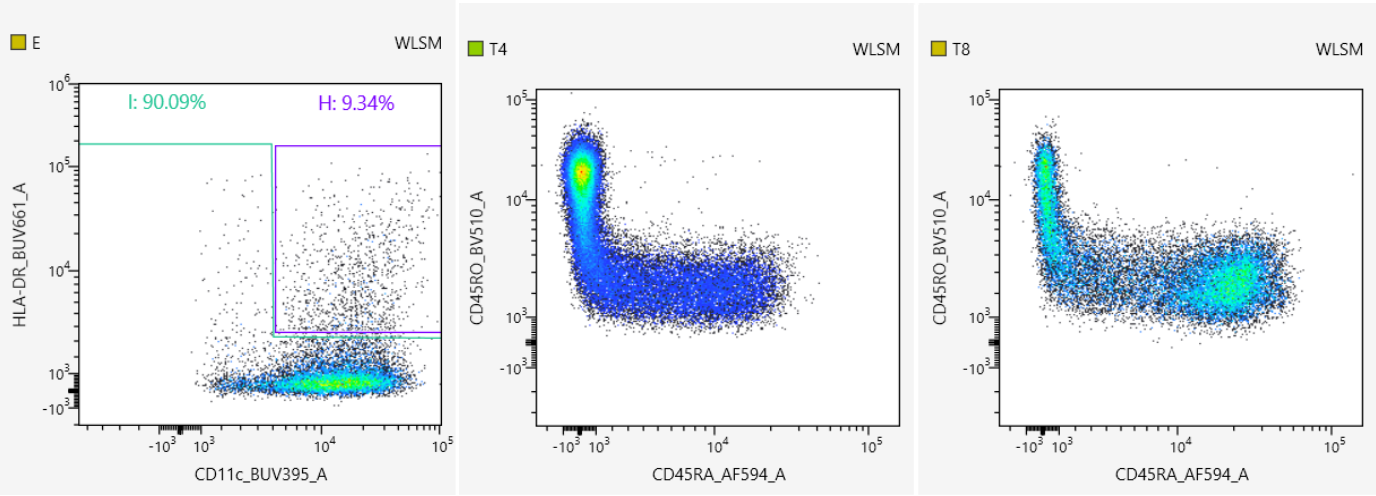


Autofluorescence Considerations

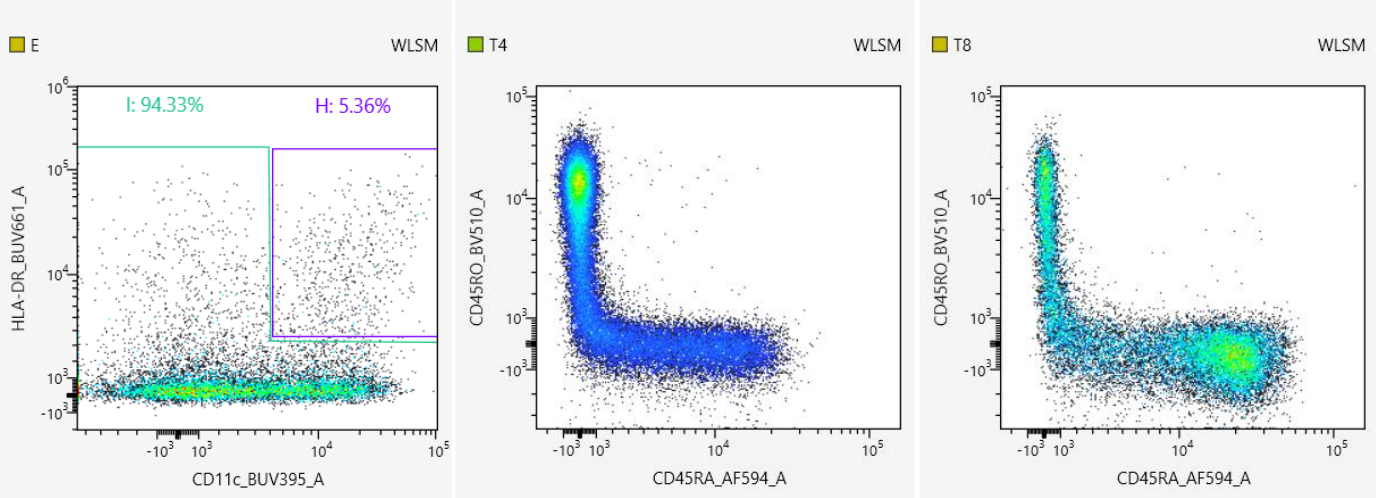
- The Autofluorescence of your sample can be a useful thing to know before building your panel
- Will spread the negative population in colors that it is similar to, reducing the resolution
- For PBMCs, AF is usually most similar to BUV496 and BV510
- For tissue digests (tumor, liver, brain, skin, lung, and gut) the multiple AF populations may look different and may be similar to unexpected fluorochromes



Autofluorescence Effects on Resolution



Autofluorescence NOT included in Unmixing



Autofluorescence IS included in Unmixing

How to do Marker-Fluorochrome Assignments

Availability

Assignment Early or Late

Co-Expression Difficulties

Location

Marker Density

Dye Brightness Needed

Surface vs. Cytoplasmic vs. Intranuclear

Dye Stability

Where to Start

Rare Markers

Many niche markers are only available on a few fluorochromes like AF488, PE, and APC

Dim/Intracellular Markers

Use bright dyes

Intracellular markers on smaller dyes but larger dyes can work (e.g. PE-Cy7 for TNFa)

Common Lineage Markers

Can be assigned to dimmer dyes

Wide Availability

Panel Hot Spots

- Once a panel reaches a certain size, it may be necessary to use fluorochromes that have a lot of spread
- Based on the patterns of spread, these “spready” fluorochromes can be grouped into “Hot Spots”
  - Mostly “Direct Excitation of Acceptor on a Tandem Dye” and “Excitation by Lower Wavelength Laser”

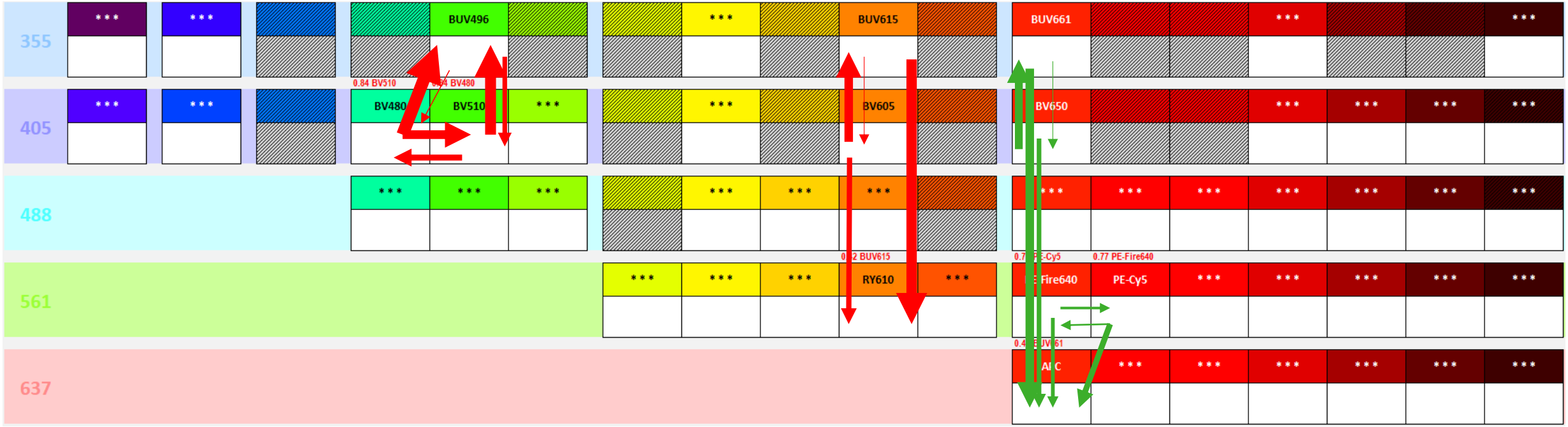
Offended

Offender

SSM	BUV395	AF350	BUV496	BUV563	BUV615	BUV661	BUV737	BUV805	BV421	PacBlue	BV480	BV510	[mFluor Violet 540]	BV570	BV605	BV650	BV711	BV750	BV785	BB515	AF488	SparkBlue-550	[iFluor 532]	PerCP	BB700	PerCP-eFluor710	PE	[SparkYG-581]	PE-eFluor610	AF594	PE-AF610	[PE-Fire 640]	PE-Cy5	PE-Cy5.5	PE-Vio770	APC	AF647	[AF660]	AF700	Zombie-NIR	APC-Fire750	[APC-Fire810]
BUV395		9	4	1	1	0	1	0	0	0	3	5	3	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AF350	7		16	1	1	0	1	0	1	1	3	7	5	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BUV496	3	3		6	4	2	3	1	1	1	6	27	19	3	1	1	0	1	1	14	15	5	4	1	0	1	3	2	1	2	1	1	1	0	0	1	0	0	0	1	0	0
BUV563	8	3	2		6	2	1	2	0	0	1	3	3	2	1	0	0	0	0	3	4	4	17	1	1	1	11	34	5	4	4	2	1	1	0	2	1	1	0	0	0	0
BUV615	6	2	2	2		5	2	2	0	0	2	3	2	1	4	2	1	1	0	1	1	1	1	2	3	1	2	7	5	61	5	3	3	2	0	4	2	1	1	1	0	0
BUV661	7	3	3	1	2		6	3	1	0	3	5	4	0	1	4	2	1	1	2	2	1	1	3	5	2	2	1	1	2	2	4	5	6	1	50	32	16	7	4	3	3
BUV737	0	3	3	1	1	1		6	0	0	2	4	3	0	0	0	2	3	1	1	1	1	1	1	2	8	1	0	0	0	0	1	1	3	2	2	2	3	8	12	6	3
BUV805	21	7	7	2	1	1	2		1	1	6	11	7	1	0	0	0	1	1	2	2	1	1	0	0	0	1	0	0	1	0	0	1	0	1	1	1	1	1	2	4	5
BV421	0	58	15	2	1	0	0	0		7	11	20	13	2	1	0	0	0	0	2	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
PacBlue	0	16	24	3	1	0	1	0	7		15	28	19	3	1	1	0	1	1	3	2	0	1	0	0	0	1	2	0	0	0	0	1	0	0	1	1	0	0	0	0	0
BV480	0	2	73	7	3	1	2	1	2	3		51	38	6	3	1	1	2	1	16	17	6	6	1	0	0	5	3	1	2	1	1	1	0	0	1	0	0	0	0	0	0
BV510	0	3	64	18	5	3	2	1	2	2	35		45	7	5	2	1	2	1	5	4	2	3	1	1	0	3	6	1	3	1	1	1	0	0	2	1	1	1	1	0	0
[mFluor Violet 540]	0	4	29	29	4	2	2	1	1	1	23	49		8	5	2	1	2	1	5	6	3	4	1	1	0	4	6	2	3	2	1	1	0	0	2	1	1	1	0	0	0
BV570	0	1	6	12	14	3	1	1	4	3	9	14	10		9	3	1	1	1	3	3	2	4	3	2	1	15	30	6	8	6	5	4	2	0	2	1	1	1	0	0	0
BV605	0	2	4	3	50	7	3	2	3	2	5	8	5	4		7	3	3	2	2	2	1	1	5	3	2	4	11	13	31	9	7	7	3	1	5	2	2	2	1	1	0
BV650	0	1	4	1	7	46	4	2	4	2	5	9	6	2	3		4	3	2	2	3	1	2	4	4	2	2	2	2	5	3	5	6	3	1	27	14	8	4	2	2	1
BV711	0	1	5	1	0	2	32	5	6	2	6	11	7	1	1	1		13	5	2	1	0	0	3	7	8	3	1	1	1	1	2	2	8	1	5	9	12	10	6	5	3
BV750	0	0	6	2	1	1	23	10	6	2	8	14	9	2	1	1	3		6	3	3	1	1	1	2	3	1	1	1	0	1	1	1	1	2	2	2	3	4	8	5	3
BV785	0	5	8	2	1	1	6	33	22	4	10	18	12	2	1	1	2	6		4	5	2	2	1	1	1	2	1	1	1	1	1	1	1	2	2	2	2	3	5	7	5
BB515	0	1	14	2	1	0	1	0	0	0	4	10	10	2	1	0	0	0	0		87	25	18	0	1	1	7	2	3	3	2	1	1	1	0	1	0	1	0	0	0	0
AF488	0	0	4	1	0	0	0	0	0	0	2	5	5	1	0	0	0	0	0	31		12	9	0	1	1	3	1	1	2	1	0	0	0	0	0	0	0	0	0	0	0
SparkBlue-550	0	0	6	5	1	1	0	0	1	1	7	21	26	4	2	1	0	0	0	12	18		15	1	1	1	9	4	3	4	2	1	1	1	0	1	0	1	0	0	0	0
[iFluor 532]	0	1	1	24	2	1	0	1	0	0	2	5	5	2	1	1	0	0	0	6	8	9		1	2	1	18	38	8	6	6	3	2	1	0	1	1	1	0	0	0	0
PerCP	0	0	0	0	0	4	4	1	0	0	0	0	0	0	0	0	2	3	3	2	1	2	1	1		6	6	2	0	1	1	2	5	9	4	1	8	11	6	4	3	2	1
BB700	0	1	3	3	2	7	11	3	3	0	4	9	11	3	2	4	11	7	4	12	17	9	10	10		14	7	3	4	6	4	4	7	11	3	15	18	21	11	8	8	4
PerCP-eFluor710	0	2	0	0	0	2	26	3	6	0	0	0	1	0	0	1	22	10	3	1	2	1	1	6	16		7	3	2	2	1	2	3	20	3	5	7	9	18	7	5	3
PE	0	0	1	6	6	1	0	0	1	0	2	2	1	8	2	1	0	0	0	3	4	3	5	1	1	1		91	16	6	10	5	2	1	0	1	1	1	0	0	0	0
[SparkYG-581]	0	0	1	2	2	0	0	0	0	0	1	1	1	3	1	0	0	0	0	1	1	1	1	1	1	1	15		6	4	4	3	2	1	0	1	1	0	0	0	0	0
PE-eFluor610	0	2	1	2	22	2	1	0	1	0	1	1	1	1	8	2	1	0	0	4	4	2	2	2	5	2	13	48		87	26	8	4	4	1	2	2	2	1	0	0	0
AF594	0	0	0	1	16	4	1	1	1	0	0	1	1	1	6	3	1	1	0	1	1	1	1	3	3	2	3	4	7		9	6	5	3	1	5	3	2	1	1	1	0
PE-AF610	0	1	1	1	10	11	1	1	1	0	0	1	1	1	4	5	2	1	0	3	4	2	2	4	10	5	8	5	23	80		20	10	7	1	10	5	5	2	1	1	0
[PE-Fire 640]	0	1	1	1	2	17	2	1	2	0	0	1	1	1	1	7	5	1	1	4	5	3	2	6	24	8	5	4	5	22	17		15	17	1	22	13	13	5	3	2	1
PE-Cy5	0	1	1	1	1	23	2	1	2	0	1	1	2	1	1	4	8	1	1	2	4	3	2	11	44	7	9	3	3	3	4	10		23	2	34	41	30	11	5	2	1
PE-Cy5.5	0	1	1	3	1	3	10	1	4	0	1	2	2	3	1	1	15	2	1	3	5	5	3	8	52	25	35	29	4	8	5	4	6		3	3	4	17	4	3	2	1
PE-Vio770	0	0	2	1	0	0	2	4	1	0	0	0	0	1	1	0	1	2	2	6	8	4	5	1	2	3	6	5	3	3	3	2	2	2		2	3	2	3	5	6	4
APC	0	0	0	0	1	14	2	1	0	0	0	0	0	0	1	3	1	1	1	1	1	0	1	3	4	1	2	1	1	1	2	4	5	4	1		29	12	6	3	3	2
AF647	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	3	1	1	0	0	0	1	2	2	3	0	16		11	6	2	2	2
[AF660]	0	0	1	0	0	3	3	1	1	0	0	1	1	0	0	1	3	1	1	2	3	1	1	3	16	3	6	3	2	1	1	2	3	17	1	8	12		8	4	4	2
AF700	0	0	0	0	0	1	10	2	1	0	0	0	0	0	0	0	4	3	2	3	4	2	2	1	5	3	3	1	1	1	1	1	1	7	1	4	7	8		7	6	4
Zombie-NIR	0	1	9	3	2	1	7	5	2	1	10	19	13	3	2	1	3	6	4	10	13	6	6	2	4	7	5	3	2	2	2	3	3	5	7	8	10	10	16		24	17
APC-Fire750	0	1	1	0	0	1	1	9	1	0	1	2	1	0	0	0	0	1	3	1	1	0	0	1	1	1	0	0	0	1	1	1	1	1	3	5	4	3	3	7		7
[APC-Fire810]	0	2	0	0	0	2	1	11	1	0	0	0	0	0	0	1	1	1	2	0	0	0	0	1	2	1	1	0	0	1	1	2	2	2	1	9	7	4	3	3	6	

Panel Hot Spots

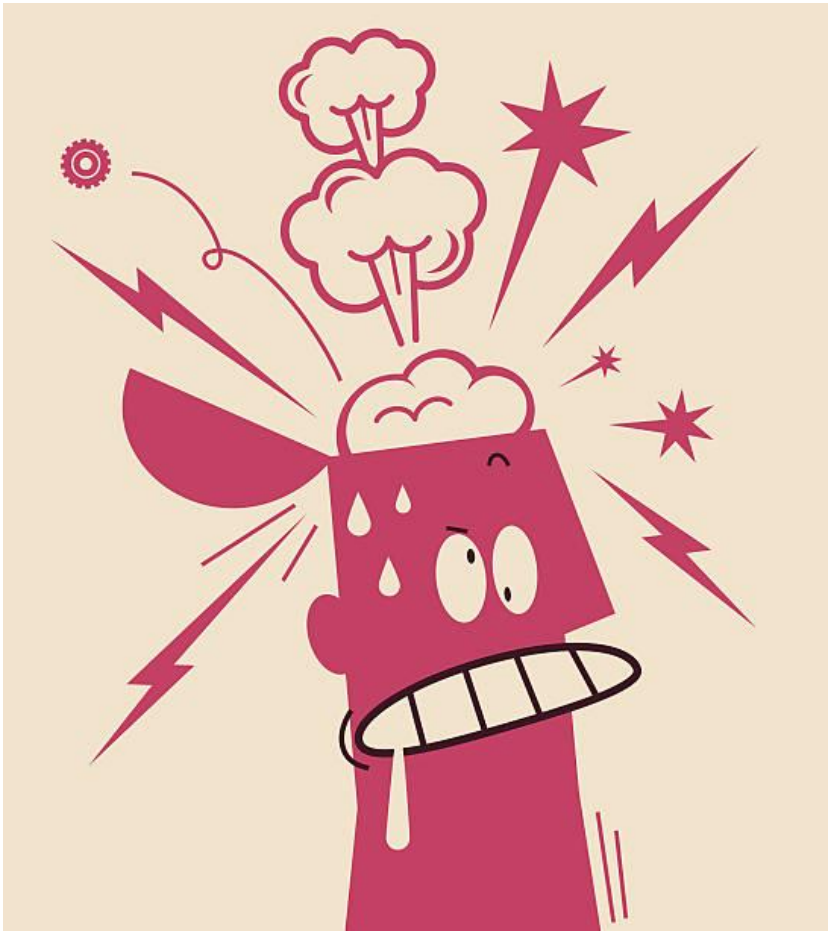
Attack these “Hot Spots” early to avoid shuffling markers later in the process



Three Major “Hot Spots” with arrows showing spread relationships. Arrow widths are correlated to SSM values from 42 color Application Note.

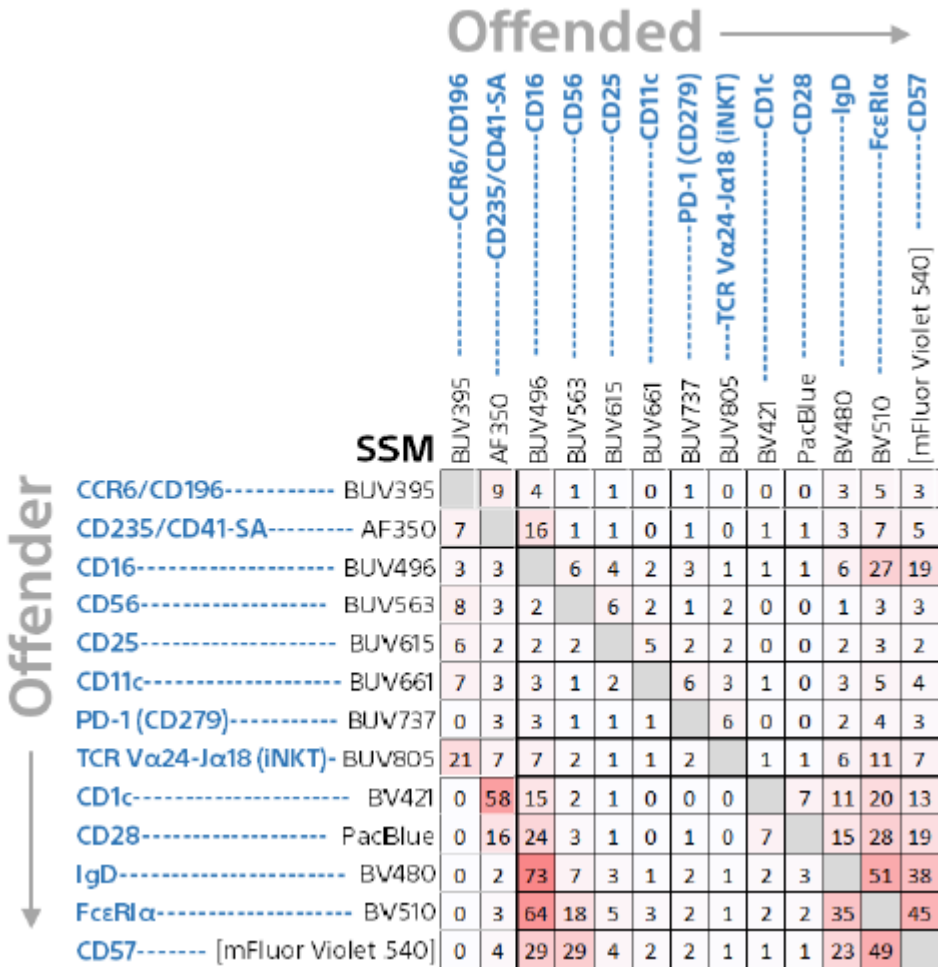
How to Avoid Getting Overwhelmed

- Take it one marker at a time
- Focus on what you need to know for that marker
  - Brightness of dye needed
  - Co-expression difficulty
  - Open spaces in the panel
- Take small breaks if you do get overwhelmed
  - Doing something else for 10-20 minutes can really help you to reset



Dump Channels in Spectral

- Try to use non-tandem dyes as dump channels
  - Tandem dyes can and will have different levels of breakdown and that will throw off the unmixing in a dump channel
  - Some can be under-unmixed and some over-unmixed
  - Add data with tandem dyes with different levels of breakdown
- BV510 or PacBlue are good choices for dump channels
  - Both are dim dyes that are also affected by autofluorescence
  - BV510 causes a lot of spread in BV480 and BUV496 so gating out the positive events in a dump channel can be a nice strategy in large parameter panels



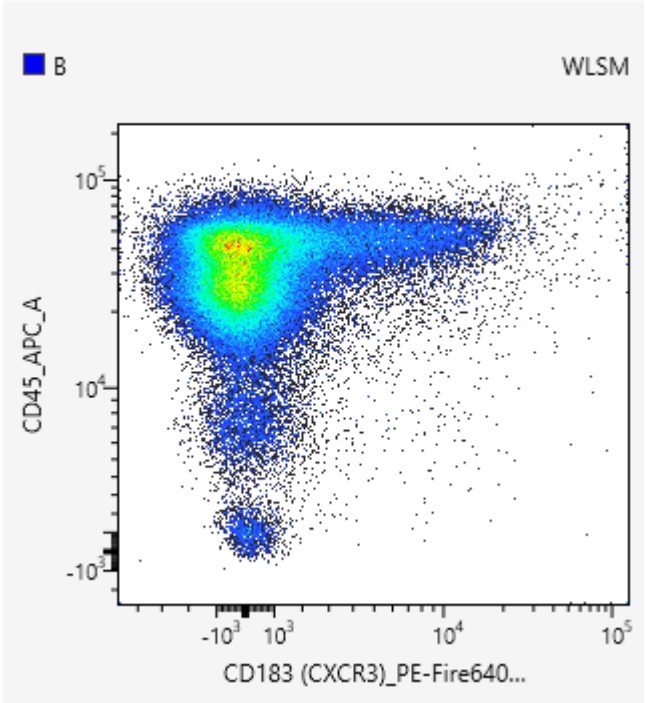
SSM	BUV395	AF350	BUV496	BUV563	BUV615	BUV661	BUV737	BUV805	BV421	PacBlue	BV480	BV510	[mFluor Violet 540]
CCR6/CD196 BUV395		9	4	1	1	0	1	0	0	0	3	5	3
CD235/CD41-SA AF350	7		16	1	1	0	1	0	1	1	3	7	5
CD16 BUV496	3	3		6	4	2	3	1	1	1	6	27	19
CD56 BUV563	8	3	2		6	2	1	2	0	0	1	3	3
CD25 BUV615	6	2	2	2		5	2	2	0	0	2	3	2
CD11c BUV661	7	3	3	1	2		6	3	1	0	3	5	4
PD-1 (CD279) BUV737	0	3	3	1	1	1		6	0	0	2	4	3
TCR Vα24-Jα18 (iNKT) BUV805	21	7	7	2	1	1	2		1	1	6	11	7
CD1c BV421	0	58	15	2	1	0	0	0		7	11	20	13
CD28 PacBlue	0	16	24	3	1	0	1	0	7		15	28	19
IgD BV480	0	2	73	7	3	1	2	1	2	3		51	38
FcεRIα BV510	0	3	64	18	5	3	2	1	2	2	35		45
CD57 [mFluor Violet 540]	0	4	29	29	4	2	2	1	1	1	23	49	

Drop-in Slots

- Drop-in slots are a great way to increase panel flexibility
  - 2-4 drop-in slots are typical in panels that want this flexibility
- Focus on using common, bright fluorochromes for drop-ins
  - BUV395, BV421, KB520/AF488/FITC, PE, APC
- Be careful with assignments on fluorochromes that cause spill in these drop-in slots
  - e.g. if PE is used as a drop-in slot, BUV563 and BV570
  - If a drop-in is designated for a single lineage, assign another lineage to offending fluorochromes

Tips and Tricks

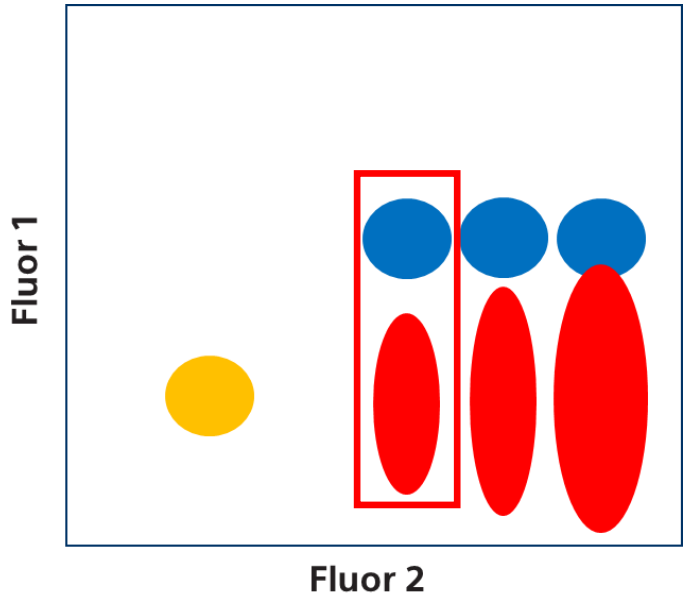
1. **Broadly expressed markers on heavily offended fluorochromes**
   - **e.g. CD45 on APC**
   - **Very useful strategy on high-parameter panels that have a large proportion of co-expressed markers**



Tips and Tricks

2. Offending fluorochromes – dim markers

- **A signal that is less bright will cause less spread into offended fluorochromes**
- **e.g. TCRgd vs CD8 on BV605**
  - **TCRgd will cause less spread into BUV615 and PE-Dazzle594/RY610 than CD8, maintaining the resolution of these colors better**
- **Very useful strategy on high-parameter panels that have a large proportion of co-expressed markers**



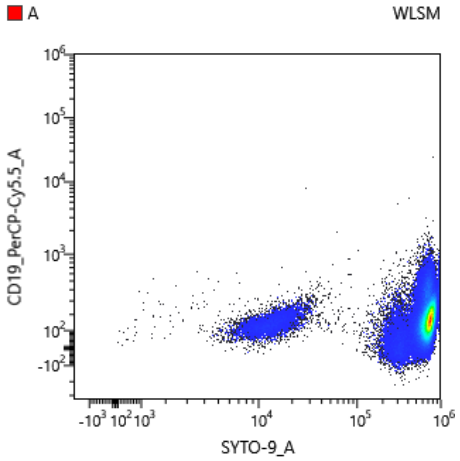
Tips and Tricks

3. Dealing with Over-Expressed Fluorescent Proteins

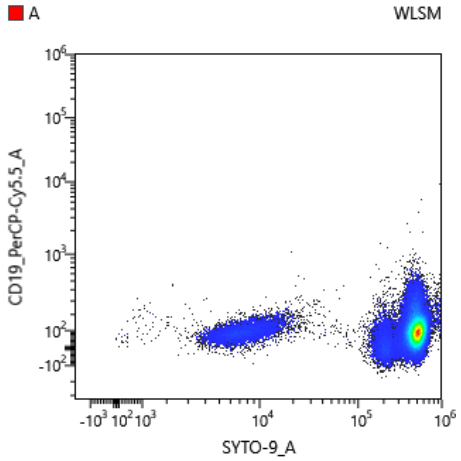
- **e.g. an extremely bright endogenous GFP**
  - **Be careful with assignments of fluorochromes off of the blue laser**
  - **If the detection deck off the blue laser has to be turned down, the resolution of signal of any other fluorochromes primarily excited by the blue will be compromised**

Very Bright Endogenous Fluorescent Protein

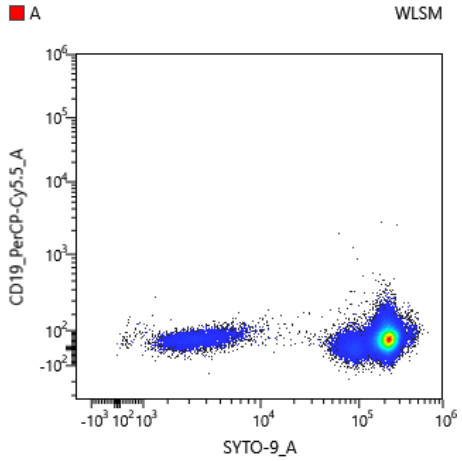
STD Voltage 4.43



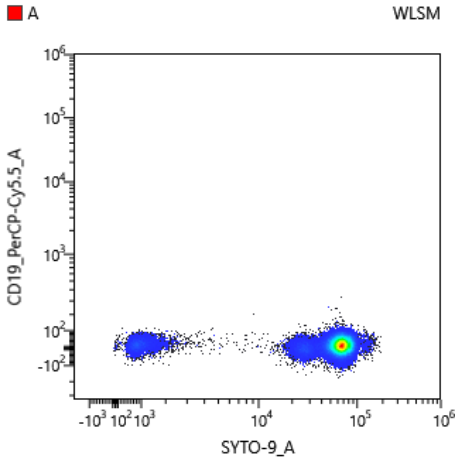
STD Voltage 4.23



STD Voltage 3.90



STD Voltage 3.38

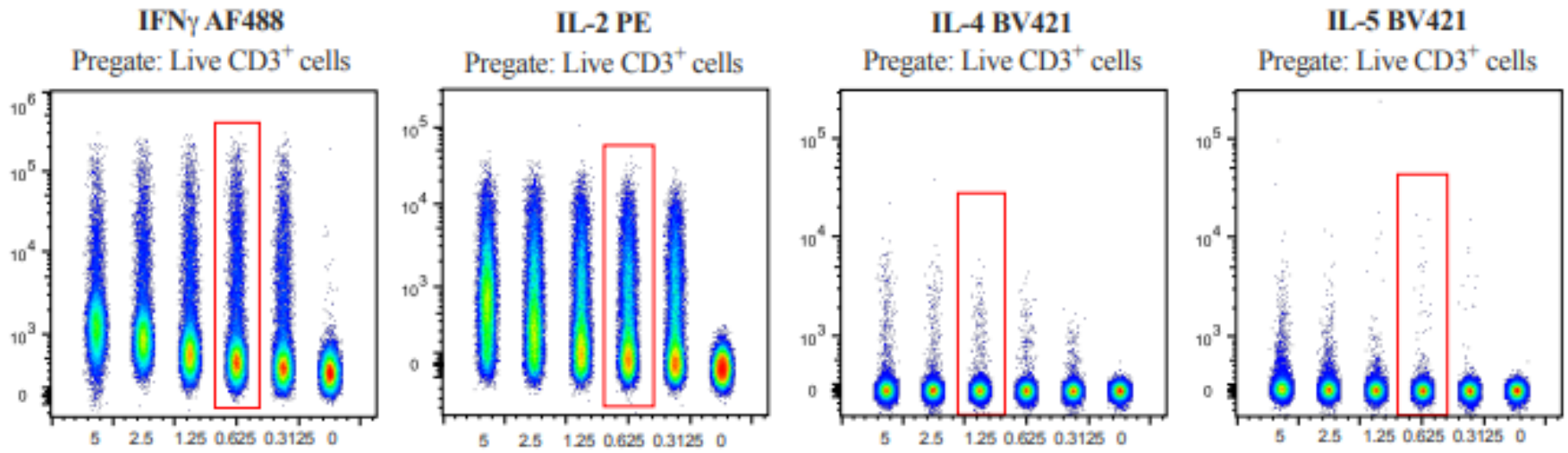


Voltage is decreased to get SYTO-9 signal out of saturation

CD19 positive population is lost

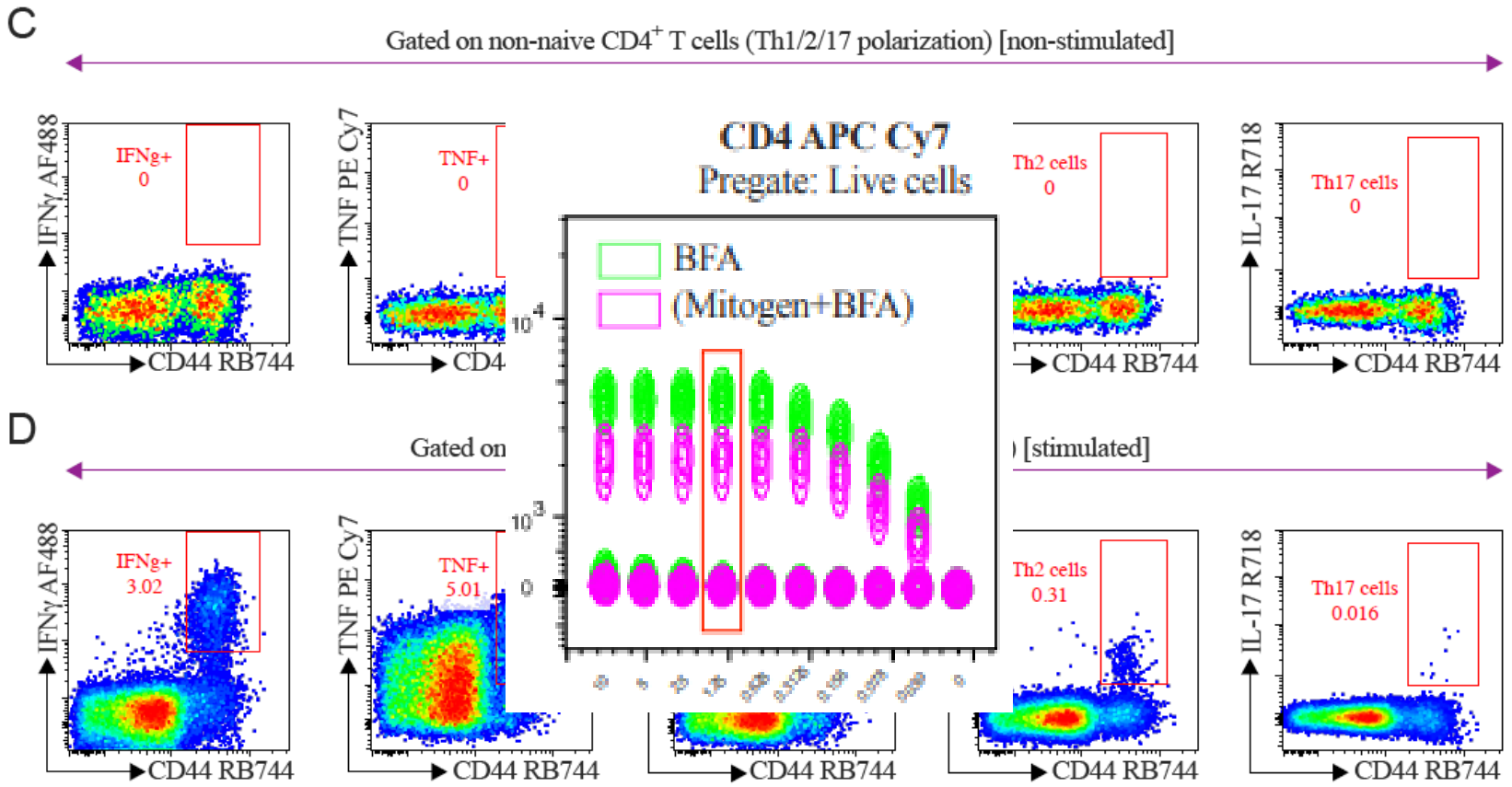
Titration

- **Always titrate new antibodies**
  - **Titrations are sample and protocol specific**
  - **Most of the time, titrations are transferable between instruments**
  - **Combo titrations are possible but do not use overlapping fluorochromes**



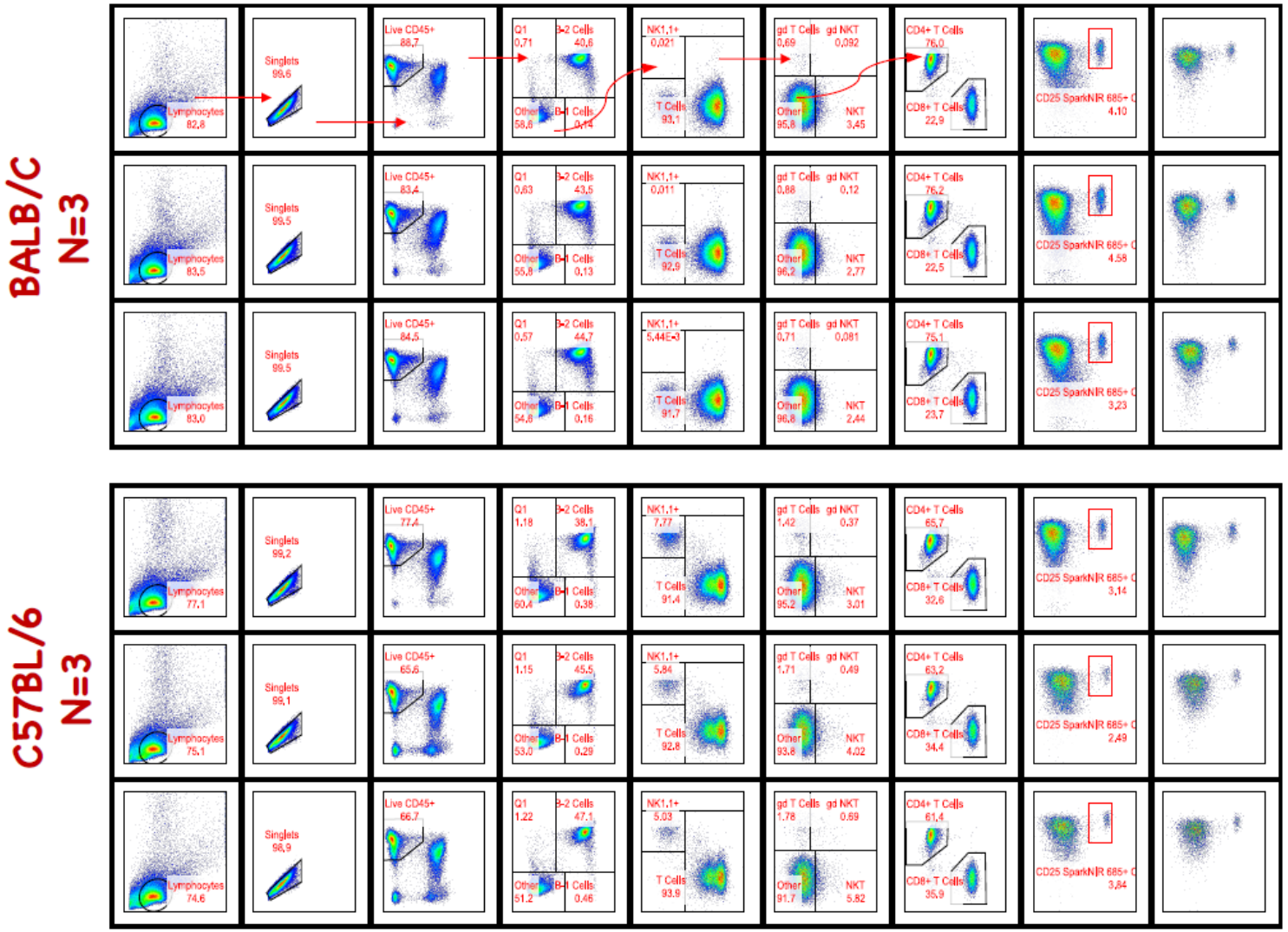
Titration and Validation Considerations

- **Driving expression for certain markers (e.g. activation markers)**
  - **CD3/CD28 beads, CytoStim, PMA-Ionomycin, Mitogen, LPS (old-school), co-culture with APCs, etc.**
  - **Brefeldin and Monensin are used to block the export of cytokines**
  - **Used for titration and validation/positive controls**
  - **Downregulation of surface markers – esp. CD3 and CD4**



Validation and Biological Controls

- Validation is key to building confidence in the data
  - Test on a variety of the samples that will be used during the study
  - Patient samples can have a lot of variability in expression levels
- Biological Controls build confidence in your conclusions



Buffer and Blocking Considerations

Brilliant Staining Buffer Plus

- Add to prevent polymer dye aggregation
- Only necessary when using multiple polymer dyes – not necessary to use in titrations

Monocyte Blocker

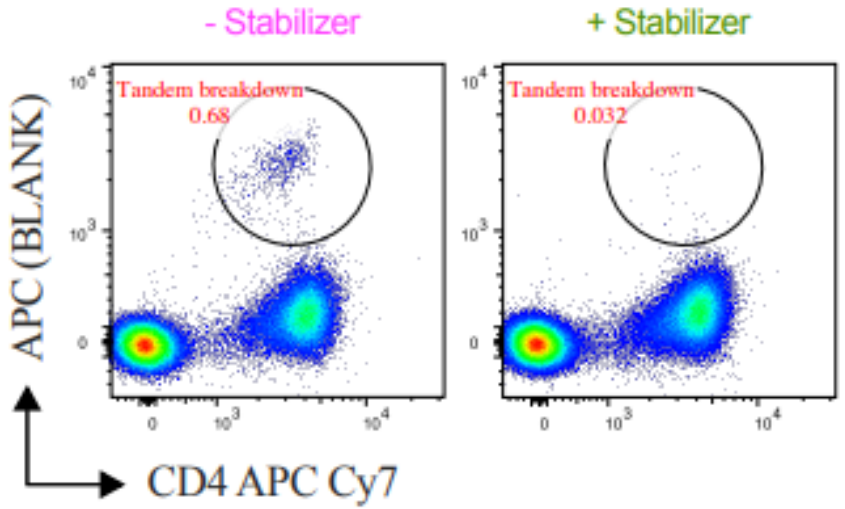
- Monocytes are prone to non-specific binding with Cyanines (Cy5, Cy5.5, Cy7)
- Protein and organic dyes to a lesser extent

Fc Block

- Prevents non-specific binding

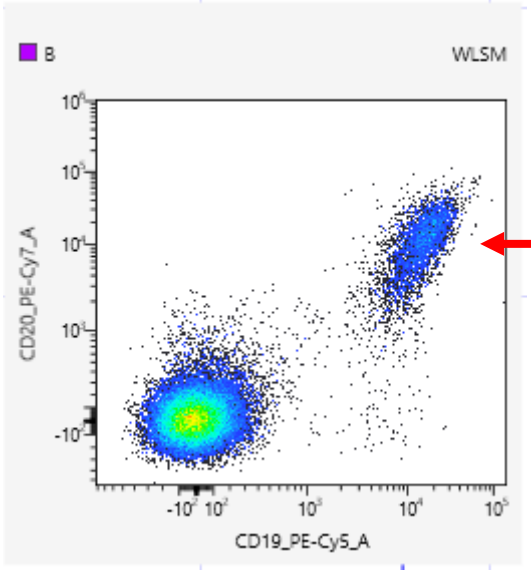
Fixative Considerations

- **Fix/Perm**
  - **Needed for intracellular staining**
  - **It is possible to stain for both cytoplasmic and nuclear targets in the same panel!**
  - **Tandem Stabilization Buffer**
    - **Fix/Perm can break down tandem dyes (esp. PE, APC, and PerCP tandems) on the surface of your cells**



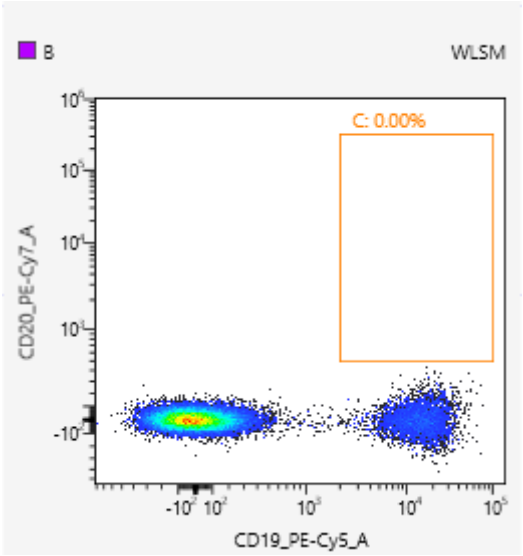
FMO Controls

- Full/Fluorescence Minus One
  - Used as a gating and unmixing control
  - Controls for effects of data spread
- FM2/FM3 controls
  - Streamlined approach for larger panels
  - Make sure that fluorochromes excluded do not overlap



Under-Unmixing or Coexpression?

Where to Place the Gate?



CD20 FMO Control shows accurate unmixing –> Coexpression

Location of single positive population on CD20 FMO gives most accurate gate placement for double positive population

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