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Aurora Panel Design

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Laura K Johnston, PhD

Scientific Associate Director

Cytometry and Antibody Technology Facility

University of Chicago

Steps for Successful Panel Design

1. Gather information beforehand about the markers and experiment
2. Pick fluorophores for the panel
3. Assign the fluorophores to the markers
 - Use the tools specific for your cytometer!
4. Test the panel on cells

For successful panel design, gather information beforehand

1. Decide on markers for the panel
2. Determine antigen classification (primary, secondary, tertiary), expression level, and co-expression
3. Make a rough gating strategy
4. Determine which fluorophores are available for each antibody in the panel

Decide on the Number of Markers

- How many markers are needed?
 - Are there any markers that would be nice to have, but not necessary?
- If many markers are needed, do they need to be in the same panel?
 - Two smaller panels will be easier than one large panel, but more cells and reagents will be needed for two panels

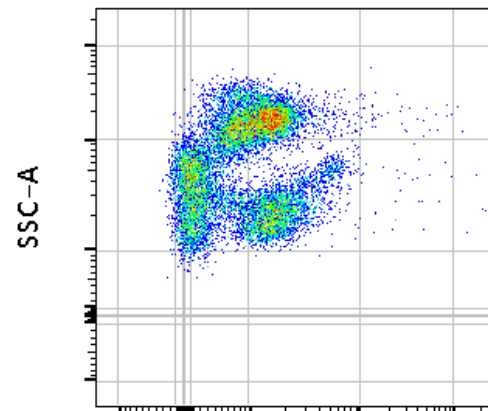
Panel Difficulty Levels

- Easier Panel
 - Markers are expressed on a variety of cell types (ex: immunophenotyping)
 - Lower number of co-expressed molecules (allows for easier placement of fluorophores)
- Harder panel
 - Many markers expressed on one or two cell types
 - High number of co-expressed molecules

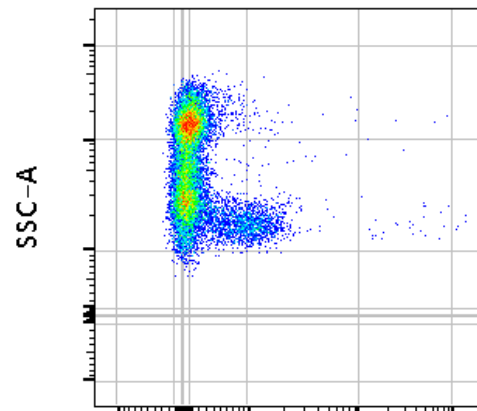
Decide on Markers – Antibody Clones

- Once markers have been decided determine if a specific clone is preferred

Two different clones for the same antibody can produce very different staining patterns:



CCR7-PE Clone: REA685



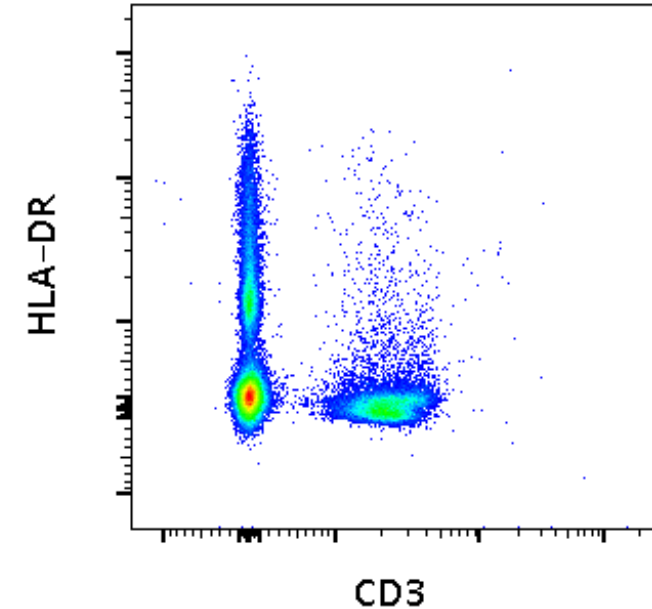
CCR7-PE Clone: REA546

Antigen co-expression

- What markers are you using to define/identify populations?
 - Helps identify markers that are mutually exclusive
- What markers characterize these populations once you have identified them (such as activation status)?
 - Characterization markers are usually co-expressed
- What markers are on a lot of cell types? (example: CD45, CD3 in whole blood)
- What markers are on a small number of cell types? (example: CD56, CD25 in whole blood)

Antigen Classification

- **Primary:** on or off (examples: CD3, CD4, CD45)
- **Secondary:** continuum (examples: CD45RA, HLA-DR)
- **Tertiary:** low or unknown expression (examples: FoxP3, ICOS)
 - If you are unsure about expression, you can check publications or the data provided on vendor websites for your antibody of interest (BioLegend, BD, eBioscience/Thermofisher, Miltenyi)



Antigen Density/Expression Level



Expression of Common Surface Molecules on Blood Cells

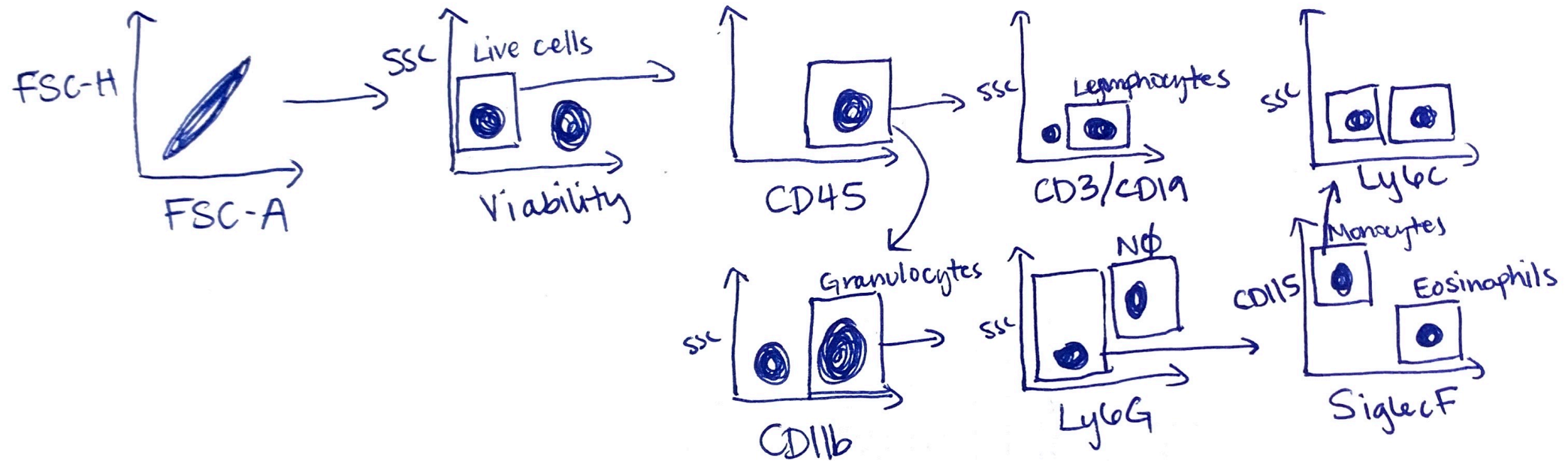
Cell	Antigen	Molecules per Cell	Reference
T cell	TCR	100,000	Cho, B. et al. 2000. <i>PNAS</i> . 98:1723.
	CD2	55,000	Ginaldi, L. et al. 1996. <i>J Clin Pathol</i> . 49:539.
	CD3	124,000	Ginaldi, L. et al. 1996. <i>Br J Haematol</i> . 93:921.
	CD5	90,000	Ginaldi, L. et al. 1996. <i>J Clin Pathol</i> . 49:539.
	CD7	20,000	Ginaldi, L. et al. 1996. <i>Br J Haematol</i> . 93:921.
	CD45	>200,000	Glattig, G. et al. 2006. <i>J Nucl Med</i> . 47:1335.
CD4+ T cell	CD4	100,000	Davis, K. et al. 1998. <i>Cytometry</i> . 33:197.
	CD28	20,000	Bryl, E. et al. 2005. <i>Arthritis Rheum</i> . 52:2996.
	CCR5	4,000-24,000	Reynes, J. et al. 2006. <i>J Infect Dis</i> . 181:927.
CD8+ T cell	CD8	90,000	Takada, S. et al. 1987. <i>J Immunol</i> . 139:3231.
	CD28	15,000	Bryl, E. et al. 2005. <i>Arthritis Rheum</i> . 52:2996.
B cell	CD19	18,000	Ginaldi, L. et al. 1998. <i>Pathobiology</i> . 66:17.
	CD20	109,000	Ginaldi, L. et al. 1998. <i>Pathobiology</i> . 66:17.
	CD21	210,000	Ginaldi, L. et al. 1998. <i>Pathobiology</i> . 66:17.
	CD22	14,000	Ginaldi, L. et al. 1998. <i>Pathobiology</i> . 66:17.
	HLA-DR	85,000	Ginaldi, L. et al. 1998. <i>Pathobiology</i> . 66:17.
	CD11a	10,000	Unternaehrer, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD40	2,000	Unternaehrer, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD86	16,000	Unternaehrer, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD80	2,000	Unternaehrer, J. et al. 2007. <i>PNAS</i> . 104:234.
Dendritic cell	CD11a	27,000	Unternaehrer, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD40	17,000	Unternaehrer, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD80	132,000	Unternaehrer, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD86	208,000	Unternaehrer, J. et al. 2007. <i>PNAS</i> . 104:234.
Monocyte	CD14	110,000	Antal-Szalmas, P. et al. 1997. <i>J Leukoc Biol</i> . 61:721.
	CD32	21,000	Antal-Szalmas, P. et al. 1997. <i>J Leukoc Biol</i> . 61:721.
	CD64	13,000	Antal-Szalmas, P. et al. 1997. <i>J Leukoc Biol</i> . 61:721.
Neutrophil	CD14	3,500	Antal-Szalmas, P. et al. 1997. <i>J Leukoc Biol</i> . 61:721.
	CD16	225,000	Antal-Szalmas, P. et al. 1997. <i>J Leukoc Biol</i> . 61:721.
NK cell	CD56	10,000	Ginaldi, L. et al. 1996. <i>J Clin Pathol</i> . 49:539.
Red Blood Cell	Glycophorin A	340,000	Antal-Szalmas, P. et al. 1997. <i>J Leukoc Biol</i> . 61:721.
Basophil	CD23	15,000	MacGlashan, D. et al. 2000. <i>J Leuk Biol</i> . 68:479.

Disclaimer: While these numbers are published data, actual numbers can vary significantly depending on factors such as antibody clone used, patient, method of molecule number calculation, flow cytometer and fluorochromes used. BioLegend recommends that these numbers only be used as relative indications of high, intermediate, or low expression of proteins on certain cell types. We also recommend viewing our product data sheets to view actual fluorescence data for fluorochrome-conjugated antibodies.

Decide on a rough gating strategy

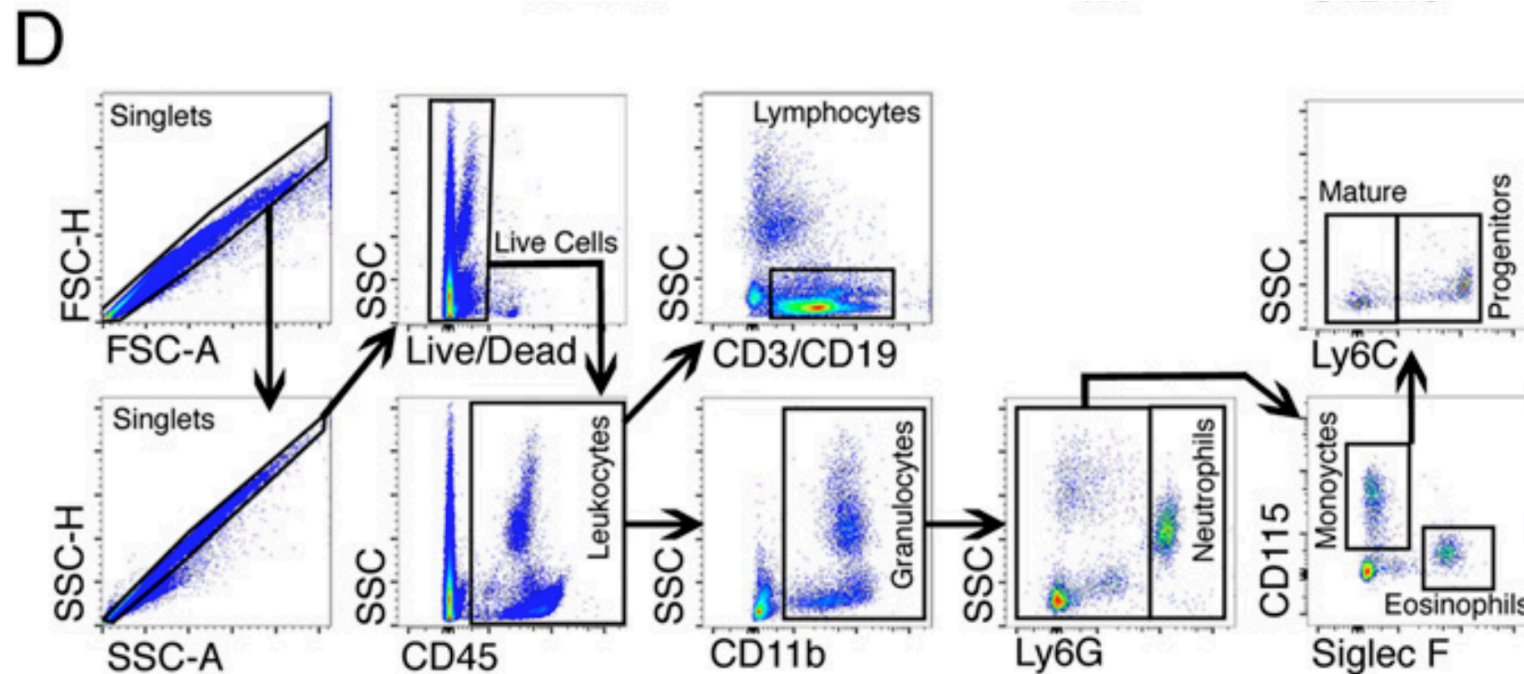
- This can still be helpful even if you are planning on using algorithms to analyze your data (tSNE, FlowSOM, etc.)
- Examples:
 - Schematic
 - Published data
- Tip: It can also be useful to identify a single plot that is critical and will be in the final figure. Plan to put those fluorophores on different lasers so that the plot looks great!
 - Example: looking at CD4/CD8 ratio – don't put CD4 and CD8 on the same laser (APC and AF647) or fluorophores that spread into each other (BUV661 and APC)

Gating Strategy Example: Schematic



Gating Strategy Example: Published Data

- Note how this gives information about antigen classification and coexpression!



Johnston LK, et al. [J Immunol.](#) 2016 Nov 1;197(9):3445-3453. Epub 2016 Sep 28.

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- 2. Pick fluorophores for the panel**
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Determine which fluorophores are available for each antibody in the panel

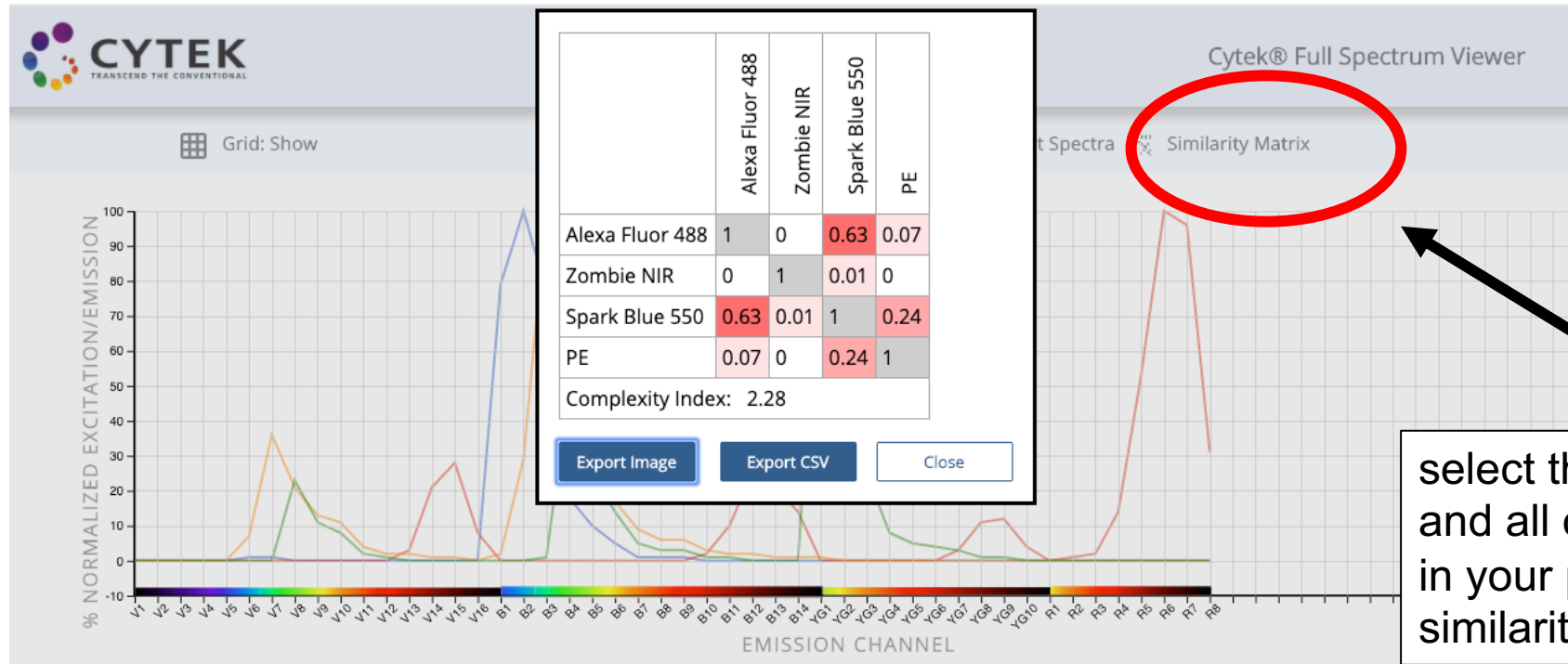
- What antibodies are commercially available for each antigen?
 - What fluorophores are available? – This is the most time consuming part of panel design
 - Are there multiple clones available? Is one clone better than the others?
- Fluorofinder antibody search:
<https://app.fluorofinder.com/searches/new>
- Some fluorophores have limited antibodies:
 - BV570, BV480, BUV563, BUV615, BUV661, AF532, BV750, BB515

Other considerations regarding fluorophores

- What fluorochromes can I use for this panel?
 - How many antigens do I want to detect?
 - What fluorophores are best for that number of antigens?
 - **Avoid using several fluorophores with excessive spill for smaller panels**, save those colors for when you don't have other options. (Example: don't use both BV421 and eFluor450 in an 8 color panel)

Cytek's Similarity Matrix and Complexity Index are Useful for Determining Which Fluorophores to Use

- Similarity matrix on Cytek's spectrum viewer:
<https://spectrum.cytekbio.com/>



select the 5L configuration and all of the fluorophores in your panel, then click the similarity matrix button

Understanding the Similarity Matrix and Complexity Index

- **Similarity matrix:** values from 0 to 1, tells you how similar two fluorophores are
 - Example: PE and PE-Dazzle594 or 67% similar, PE and APC are 4% similar

- **Complexity index**

- Combined index
- A smaller complexity index indicates less overall experimental spread
- Examples (5 laser)
 - 10 colors: 2.5
 - 20 colors: 7.3
 - 26 colors: 9.3
 - 30 colors: 29.75

	PE	PE-Dazzle594	Alexa Fluor 488	Alexa Fluor 532	APC	APC-Cy7	BUV737	BUV496
PE	1	0.67	0.07	0.4	0.04	0	0	0.03
PE-Dazzle594	0.67	1	0.05	0.3	0.16	0.02	0.02	0.02
Alexa Fluor 488	0.07	0.05	1	0.51	0	0	0	0.06
Alexa Fluor 532	0.4	0.3	0.51	1	0	0	0	0.02
APC	0.04	0.16	0	0	1	0.18	0.24	0
APC-Cy7	0	0.02	0	0	0.18	1	0.32	0
BUV737	0	0.02	0	0	0.24	0.32	1	0.01
BUV496	0.03	0.02	0.06	0.02	0	0	0.01	1
Complexity Index: 2.74								

- When to use? Example: 18-marker panel, should my last marker be BV711 or BV650? Try both panels in the spectra viewer and choose the one with the lower complexity index.

Don't Forget to Include a Live/Dead Dye

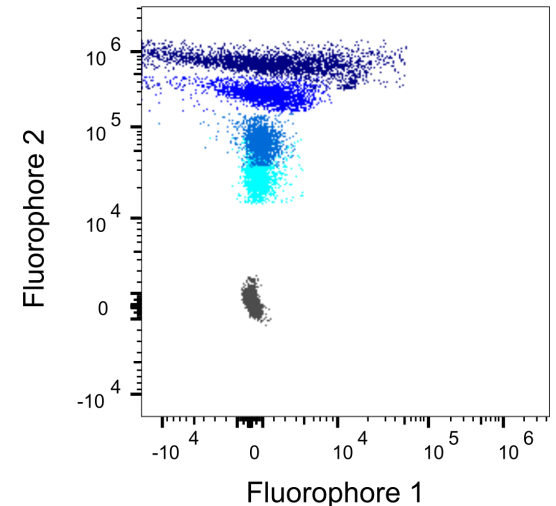
- Cyttek recommends BioLegend's Zombie NIR or ThermoFisher's Live/Dead Blue, but you can use any live/dead dye
 - See this [blog post](#) about the proper way to titrate fixable viability dyes
- DAPI and PI can be use for live/dead – just make sure you titrate!!!
 - Cell cycle analysis is more challenging – please contact me to discuss further

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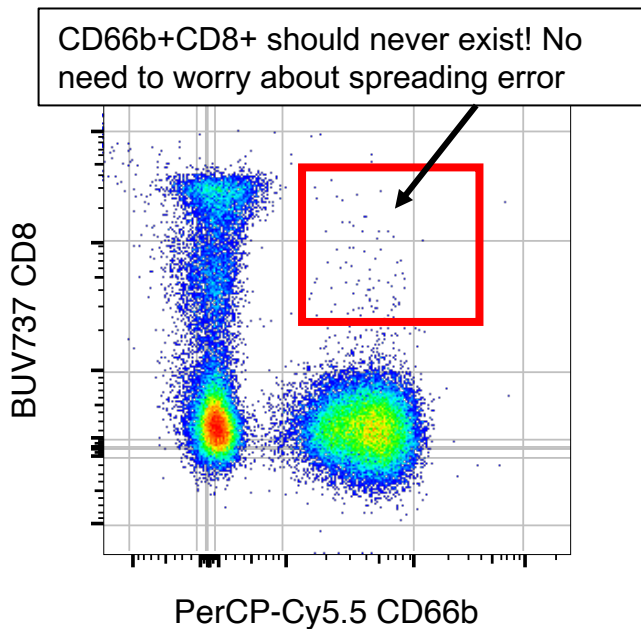
Remember Spillover-Spreading from Training!

- More spreading error occurs with more fluorophores in the detector
 - The more fluorophores in a panel, the more spreading error occurs
 - The complexity index is a rough estimate of this
- More spreading error occurs with brighter intensity
 - Assign bright fluorophores to low expressed markers, titrate reagents
- More spreading error occurs the more a fluorophore spills into another detector
 - Be cautious of fluorophores that are highly similar (can't be distinguished on a conventional flow cytometer)

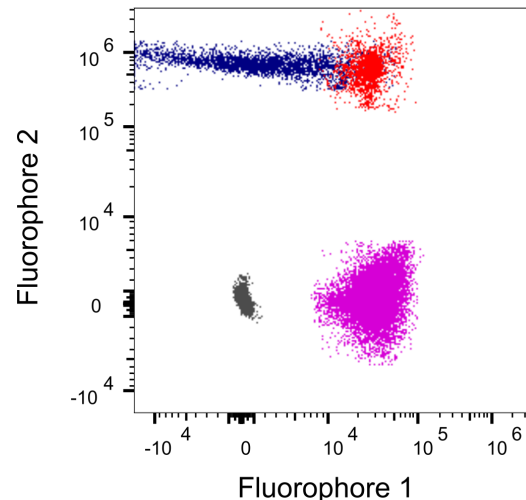


The goal of good panel design is to minimize spillover-spreading error

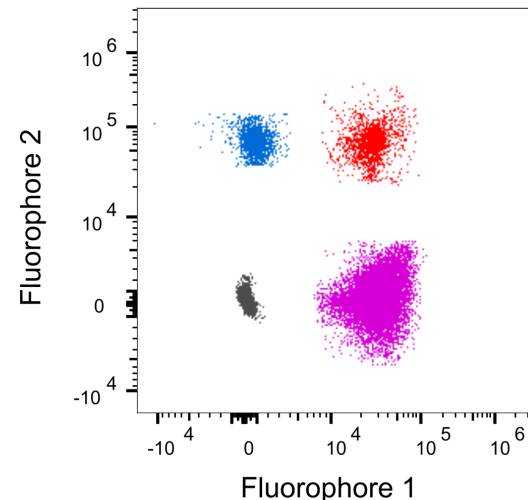
- Easiest solution: plan your panel so that fluorophores that spread into each other are on different cell types
- Be aware of spreading error with co-expressed markers when the double positive population is important



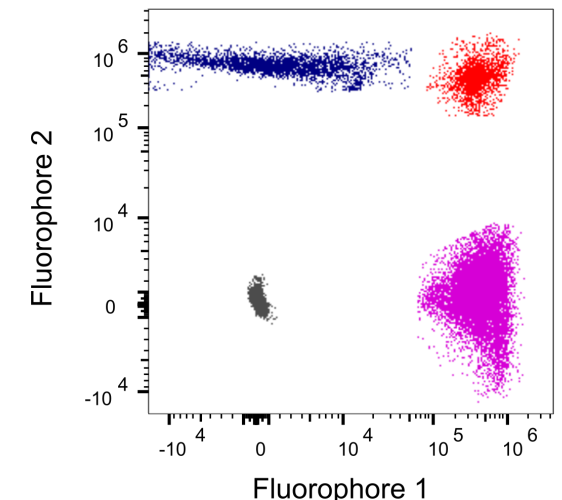
Problem: double positive (red) can not be resolved from blue population



Solution 1: dimmer fluorophore 2

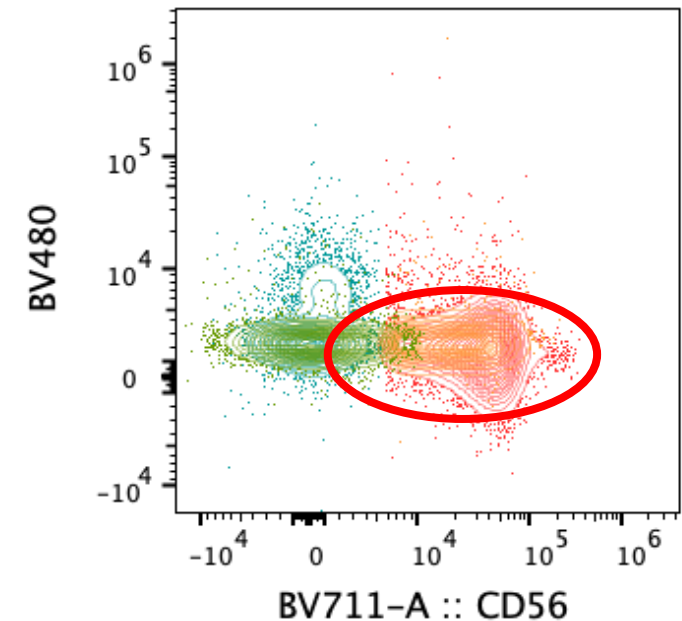
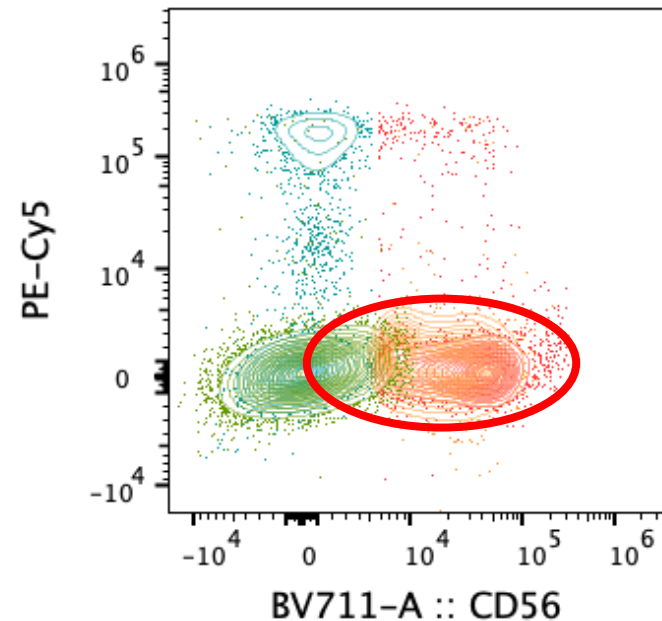
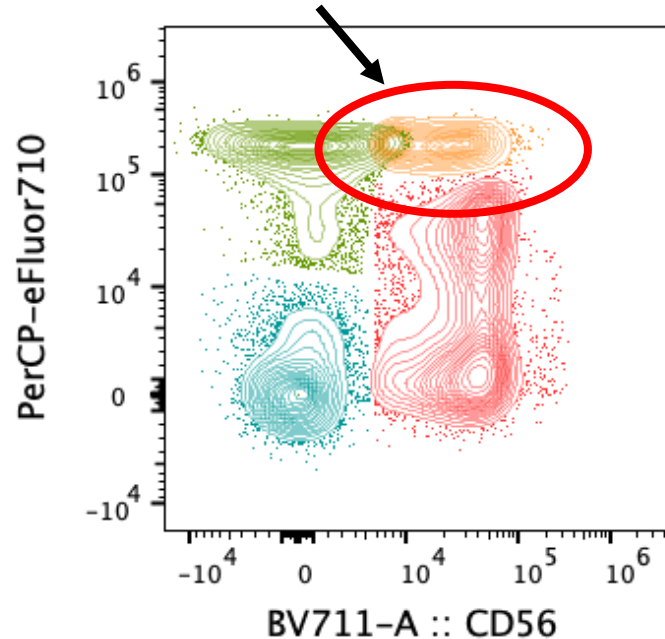


Solution 2: brighter fluorophore 1



Remember: Data has multiple dimensions, even when we only look at two dimensions

The orange double positive population is hard to separate from the green spreading error



Switching the Y-axis of the plot just moves the spreading error to the negative population. Unless there is another marker to separate the green and orange populations we will never be able to properly gate them without changing the panel

Understanding the spread matrix for Aurora panel design

- How to read this table: the fluor in the row impacts the one in the column. Red means the fluor in that row has significant spread into the dye in the column (for example PE into BV570). Areas in bright pink and red is where more attention to panel design is needed.
- This table was created with CD4 reference controls for all fluorophores
- This table assumes all markers are bright and all markers are in the tube

TABLE3.1	BUV395	BUV496	BUV563	BUV661	BUV737	BUV805	BV421	Super Bright 436	eFluor 450	BV480	BV510	BV570	BV605	BV650	BV711	BV750	BV785	BB515	Alexa Fluor 488	Alexa Fluor 532	PerCP-Cy5.5	PerCP-eFluor 710	PE	PE-Dazzle594	PE-Cy5	PE-Cy7	APC	Alexa Fluor 647	Alexa Fluor 700	APC-Fire 750
BUV395	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BUV496	16%	0%	2%	0%	0%	0%	0%	1%	0%	5%	14%	5%	0%	0%	0%	0%	0%	6%	17%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BUV563	11%	0%	0%	13%	6%	9%	0%	0%	0%	0%	7%	20%	4%	0%	0%	0%	33%	47%	64%	8%	0%	78%	12%	0%	0%	0%	0%	0%	0%	0%
BUV661	16%	0%	0%	0%	71%	65%	0%	0%	0%	0%	7%	0%	0%	42%	19%	18%	1%	0%	0%	0%	56%	30%	0%	0%	63%	0%	54%	59%	55%	10%
BUV737	13%	0%	0%	0%	0%	80%	0%	0%	0%	0%	0%	0%	0%	0%	14%	49%	28%	0%	0%	0%	7%	29%	0%	0%	0%	0%	6%	5%	68%	27%
BUV805	25%	0%	0%	0%	30%	0%	0%	0%	0%	0%	3%	0%	0%	0%	0%	0%	9%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	2%	27%
BV421	12%	0%	0%	0%	0%	0%	0%	72%	70%	36%	27%	8%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Super Bright 436	0%	0%	0%	0%	0%	0%	60%	0%	62%	32%	21%	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
eFluor 450	2%	0%	0%	0%	0%	0%	37%	48%	0%	11%	12%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BV480	5%	9%	0%	0%	0%	0%	19%	34%	39%	0%	46%	42%	29%	4%	0%	0%	0%	24%	31%	11%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BV510	5%	1%	4%	0%	0%	0%	0%	9%	10%	15%	0%	35%	22%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BV570	0%	0%	0%	0%	0%	0%	3%	15%	11%	0%	9%	0%	33%	10%	0%	0%	0%	0%	5%	21%	5%	0%	29%	0%	0%	0%	0%	0%	0%	0%
BV605	4%	0%	0%	31%	24%	26%	0%	11%	7%	0%	5%	21%	38%	26%	27%	15%	0%	14%	35%	37%	19%	0%	65%	6%	0%	10%	9%	0%	0%	
BV650	3%	0%	0%	31%	19%	23%	1%	14%	10%	0%	3%	0%	3%	0%	32%	30%	19%	0%	0%	26%	7%	0%	0%	16%	0%	15%	18%	11%	0%	
BV711	9%	0%	0%	0%	56%	61%	8%	19%	16%	0%	1%	0%	0%	0%	0%	68%	61%	0%	0%	17%	24%	0%	0%	0%	0%	11%	22%	63%	13%	
BV750	8%	0%	0%	0%	66%	66%	0%	7%	7%	0%	2%	0%	0%	0%	21%	0%	72%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	32%	0%	
BV785	7%	0%	0%	0%	44%	73%	6%	16%	11%	0%	3%	0%	0%	0%	0%	59%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	8%	15%	
BB515	2%	0%	0%	0%	0%	0%	0%	0%	0%	0%	8%	5%	0%	0%	0%	0%	0%	0%	84%	68%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Alexa Fluor 488	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	6%	0%	0%	0%	0%	0%	0%	65%	0%	48%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Alexa Fluor 532	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%
PerCP-Cy5.5	1%	0%	0%	0%	23%	23%	0%	0%	0%	0%	0%	0%	2%	20%	21%	6%	0%	0%	0%	0%	0%	53%	0%	0%	0%	12%	20%	20%	0%	
PerCP-eFluor 710	4%	0%	0%	1%	43%	47%	0%	0%	0%	0%	0%	0%	5%	55%	50%	41%	0%	0%	0%	63%	0%	0%	25%	20%	22%	29%	60%	7%	0%	
PE	1%	0%	22%	0%	0%	0%	0%	0%	0%	11%	62%	41%	16%	0%	0%	0%	64%	73%	84%	42%	31%	0%	54%	12%	0%	14%	8%	0%	0%	
PE-Dazzle594	2%	0%	0%	0%	0%	0%	0%	0%	0%	8%	25%	36%	20%	14%	0%	0%	47%	59%	73%	64%	53%	68%	0%	40%	0%	27%	25%	0%	0%	
PE-Cy5	0%	0%	0%	47%	35%	25%	0%	0%	0%	0%	0%	1%	52%	55%	27%	15%	12%	28%	45%	90%	84%	4%	0%	48%	64%	77%	56%	6%	0%	
PE-Cy7	0%	0%	0%	0%	59%	67%	0%	0%	0%	0%	0%	0%	0%	62%	27%	57%	0%	0%	3%	20%	72%	0%	0%	0%	0%	4%	47%	43%	0%	
APC	0%	0%	0%	20%	26%	18%	0%	0%	0%	0%	0%	0%	61%	15%	12%	0%	0%	0%	75%	47%	0%	0%	79%	0%	0%	67%	64%	25%	0%	
Alexa Fluor 647	0%	0%	0%	0%	6%	0%	0%	0%	0%	0%	0%	0%	14%	0%	0%	0%	0%	0%	45%	13%	0%	0%	56%	0%	58%	69%	36%	0%	0%	
Alexa Fluor 700	0%	0%	0%	0%	4%	3%	0%	0%	0%	0%	0%	0%	0%	0%	1%	3%	0%	0%	3%	4%	0%	0%	0%	1%	8%	0%	11%	0%	0%	
APC-Fire 750	0%	0%	0%	0%	3%	49%	0%	0%	0%	0%	0%	0%	0%	0%	0%	24%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Perpetrators and Victims: Another Way to View the Spread Matrix

- Download here: <https://voices.uchicago.edu/ucflow/spectral-flow-cytometry/>

	Perpetrator (>0%)	Perpetrator (>30%)	Perpetrator (>50%)		Victim (>0%)	Victim (>30%)	Victim (>50%)
Alexa Fluor 488	3	2	1	Alexa Fluor 488	9	5	3
Alexa Fluor 532	2	0	0	Alexa Fluor 532	10	7	4
Alexa Fluor 647	8	5	3	Alexa Fluor 647	13	3	3
Alexa Fluor 700	9	0	0	Alexa Fluor 700	13	9	7
APC	12	6	5	APC	12	3	3
APC-Fire 750	4	1	0	APC-Fire 750	11	2	0
BB515	6	2	2	BB515	7	4	2
BUV395	1	0	0	BUV395	19	0	0
BUV496	8	0	0	BUV496	2	0	0
BUV563	13	4	2	BUV563	3	0	0
BUV661	15	8	7	BUV661	6	3	0
BUV737	11	3	2	BUV737	17	7	4
BUV805	6	0	0	BUV805	15	8	6
BV421	6	3	2	BV421	7	2	1
BV480	12	5	0	BV480	5	2	0
BV510	9	1	0	BV510	20	1	0
BV570	10	1	0	BV570	10	3	1
BV605	20	5	1	BV605	8	3	0
BV650	18	3	0	BV650	12	4	2
BV711	15	5	5	BV711	12	4	3
BV750	9	4	3	BV750	12	5	2
BV785	10	3	2	BV785	12	4	3
eFluor 450	5	2	0	eFluor 450	10	3	2
PE	15	8	5	PE	4	2	2
PE-Cy5	19	11	7	PE-Cy5	9	4	3
PE-Cy7	11	7	5	PE-Cy7	3	1	0
PE-Dazzle594	16	8	5	PE-Dazzle594	3	2	2
PerCP-Cy5.5	12	1	1	PerCP-Cy5.5	17	8	5
PerCP-eFluor 710	15	7	3	PerCP-eFluor 710	13	6	4
Super Bright 436	5	3	2	Super Bright 436	11	3	1

Numbers represent the number of red/pink boxes in the panel design spread matrix. The values were counted from the actual values (see second excel sheet).

Perpetrators cause spreading in other channels.

Do not put highly and widely expressed markers on perpetrators with high values. Instead, assign low and/or rarely expressed markers.

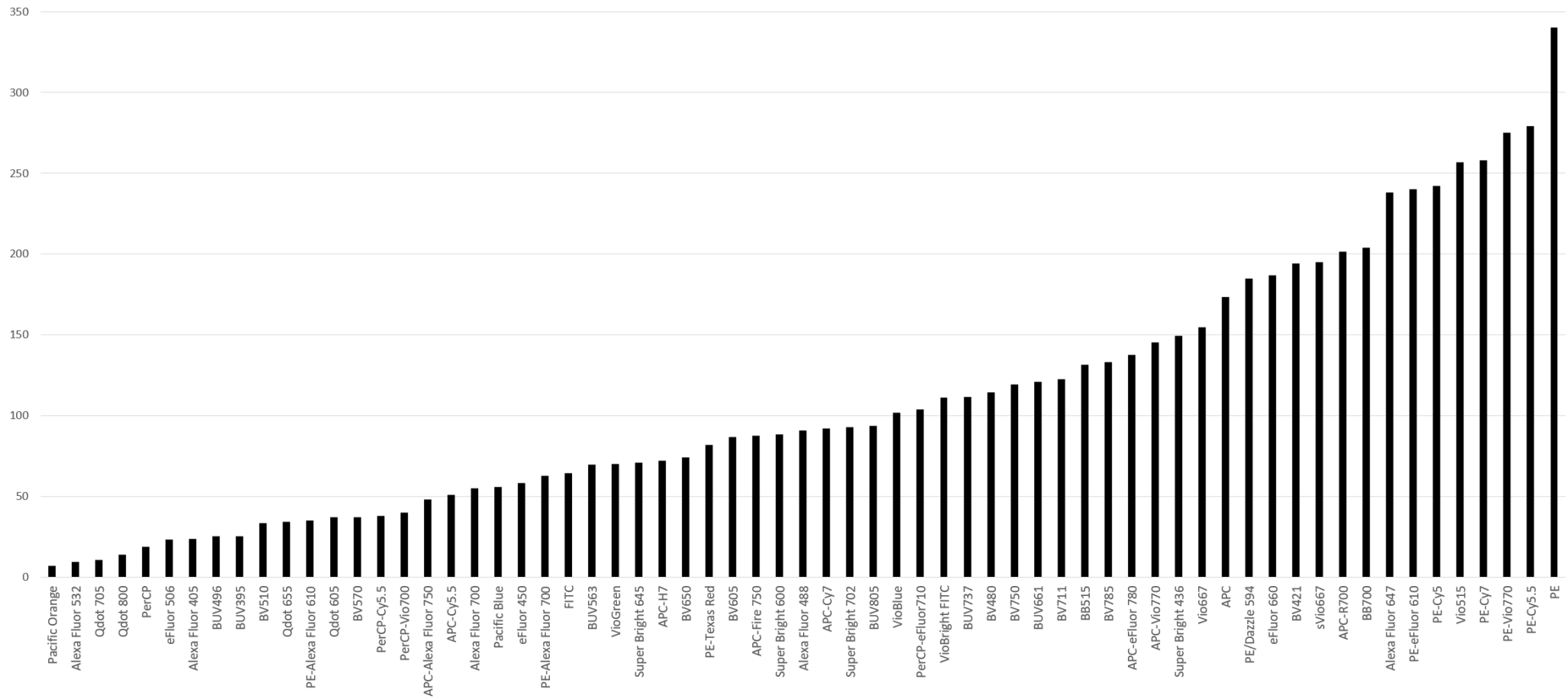
Victims are susceptible to spreading from other fluorophores.

Do not put low expressed markers on victims with high values

Basic rules for pairing markers and fluorophores

1. Always pair low expressed or unknown (tertiary) markers on the brightest fluorophores
2. High expressed markers can be paired with any fluorophore, but priority goes to rule 1. Therefore, high expressed markers usually end up on dim fluorophores.

Stain Index Ranking



How to assign fluorophores: Option 1

- If possible, you can start with Cytex's panel (or another aurora panel) and swap out markers that you aren't using

UV Excited Fluors	Violet Excited Fluors	Blue Excited Fluors	Yellow Green Excited Fluors	Red Excited Fluors
CD45RA BUV395	PD-1 BV421	CD86 BB515	ICOS PE	CD27 APC
CD16 BUV496	CD123 SB436	CD57 FITC	CD4 CF568	CD33 Alexa647
CD14 BUV563	CD38 eFluor450	CD19 CF514	CD24 PE/Dazzle594	CD127 APC-R700
CD11c BUV661	IgD BV480	CD11b PerCP-Cy5.5	CD25 PE-Cy7	CD161 APC/Fire750
CD56 BUV737	CD3 BV510	TCR $\gamma\delta$ PerCP-eF710		
CD20 BUV805	HLADR BV570			
	CD28 BV605			
	CXCR3 BV650			
	CCR6 BV711			
	CXCR5 BV750			
	CCR7 BV785			
	CD8 Qdot 800			

*Plus viability dye (Live/Dead Blue) for a 32 color panel

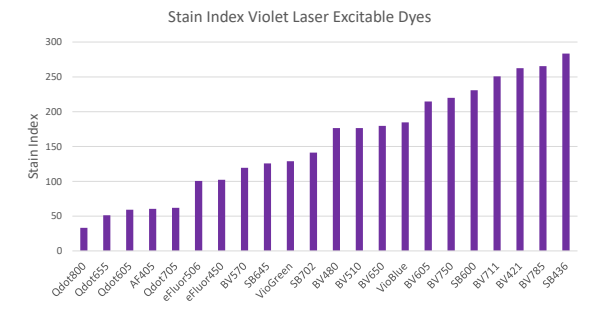
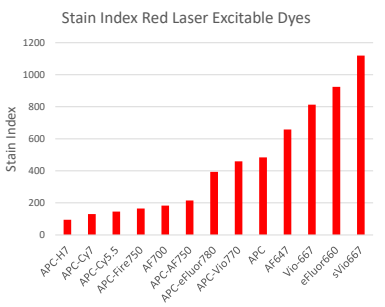
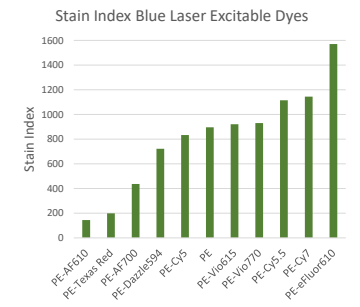
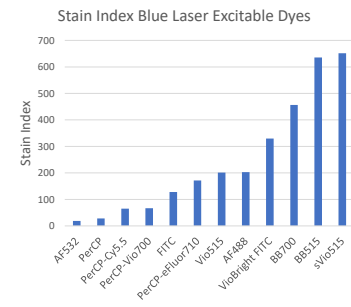
*** CF514 and CD568 are from Biotium, limited antibodies available, unique antibody clones

Panel from Cytex

How to assign fluorophores: Option 2

- Start from scratch and use the tools specifically made for the aurora!!!

	BUV395	BUV496	BUV563	BUV661	BUV737	BUV805	BV421	Super Bright 436	eFluor 450	BV480	BV510	BV570	BV605	BV650	BV711	BV750	BV785	BB515	Alexa Fluor 488	Alexa Fluor 532	PerCP-Cy5.5	PerCP-eFluor 710	PE	PE-Dazzle594	PE-Cy5	PE-Cy7	APC	Alexa Fluor 647	Alexa Fluor 700	APC-Fire 750	
BUV395	Black																														
BUV496		Black																													
BUV563			Black																												
BUV661				Black																											
BUV737					Black																										
BUV805						Black																									
BV421							Black																								
Super Bright 436								Black																							
eFluor 450									Black																						
BV480										Black																					
BV510											Black																				
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BV650														Black																	
BV711															Black																
BV750																Black															
BV785																	Black														
BB515																		Black													
Alexa Fluor 488																			Black												
Alexa Fluor 532																				Black											
PerCP-Cy5.5																					Black										
PerCP-eFluor 710																						Black									
PE																							Black								
PE-Dazzle594																								Black							
PE-Cy5																									Black						
PE-Cy7																										Black					
APC																											Black				
Alexa Fluor 647																												Black			
Alexa Fluor 700																													Black		
APC-Fire 750																													Black		



Fluorochrome assignment

1. Start with limiting reagents
 1. Make note of any fluorescent proteins (GFP, mCherry, etc.)
 2. Assign very rare markers to the only fluorophore options available (e.g. marker X is only available in PE)
 3. Assign the fluorophores that are only available in some markers (e.g. BV570, BV480, BUV563, BUV615, BUV661, AF532, BV750, BB515)

Adapted from L Ferrer-Font et al. <https://doi.org/10.1002/cpcy.70>

Fluorochrome assignment

2. Assign the remaining markers – be aware of co-expression
 - Assign bright fluorophores to low expressed markers and dim fluorophores to highly expressed markers
 - For co-expressed markers, assign fluorophores with minimal spillover into each other
 - Use the perpetrators and victims information:
 - Assign highly expressed markers to low perpetrators (e.g. BUV395, BUV496, AF532, AF488, APC-Cy7, BUV805, AF700, BV510)
 - Assign low expressed markers to low victim (e.g. BUV496, PE-Dazzle594, PE-Cy7, BUV563, PE, BV480, BUV661, BV421)

Adapted from L Ferrer-Font et al. <https://doi.org/10.1002/cpcy.70>

Fluorochrome assignment

- Be cautious of using and assigning fluorophores with lots of spreading (described on the next slide)
- If there is an extremely important biaxial plot (aka a plot that will be put in a final figure), consider assigning fluorophores that have no spread into each other. This will produce the best-looking and therefore most convincing data.

How to deal with fluorophores with high spreading error (many red boxes in the row of the spread matrix)

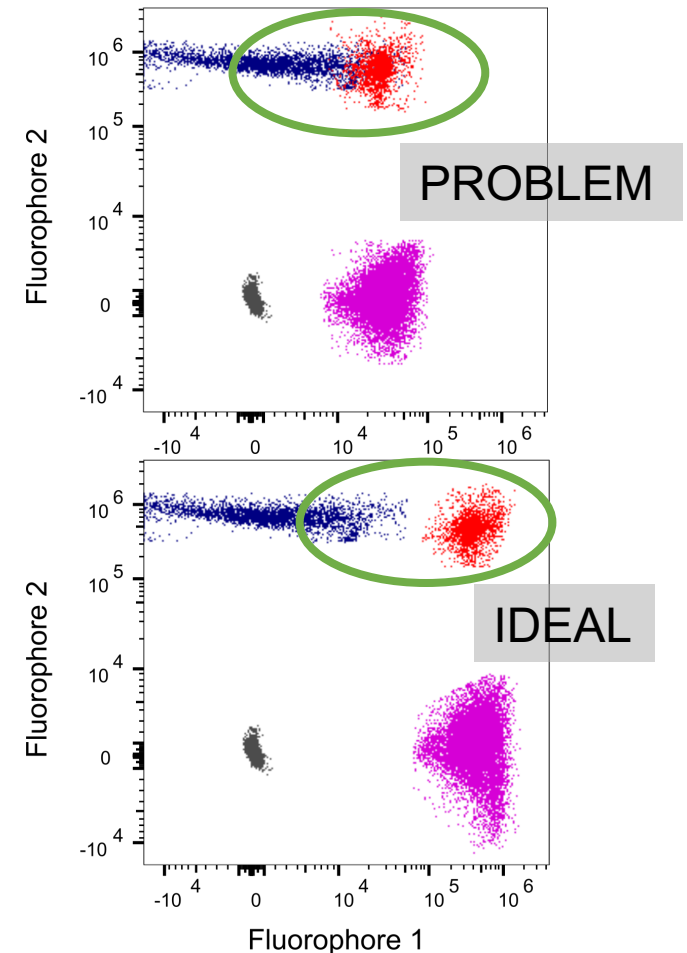
- Avoid assigning these fluorophores to highly and widely expressed markers. This depends on your experimental question: assigning CD8 to PE-Dazzle594 would be bad if CD8 T cells are of interest, but may be fine if the experimental goal is to examine CD4 T cells but NOT CD8 T cells.
- Assign them to a dump channel (one or more antibodies that remove cells that will not be analyzed). In this case, no cells of interest will have the offending fluorophore(s) and spreading can be ignored.
- Assign them to a low expressed or rarely expressed marker. In this case, the spreading will only be an issue for a small number of cells.
- Assign them to a marker that is on a cell subset that does not co-express any other markers in the panel. For example, assign it to a neutrophil marker when all other markers in the panel are expressed on lymphocytes and monocytes.

How to deal with highly similar fluorophores

- If you have a harder panel, I do not recommend including highly similar fluorophores (fluors that cannot be distinguished on a conventional cytometer like APC and AF647)
- For best results, place this type of fluorophore combination on markers that are not co-expressed
- Again, do not use these highly similar fluorophores until a panel is large enough that they must be used (fewer than 26 markers on the 5-laser instrument)

How to deal with fluorophores that receive spreading error from many fluorophores (many red boxes in the column of the spread matrix)

- Determine the staining pattern of the marker. Is it on/off with clearly defined and separated positive and negative populations like CD4? Is it a continuum like HLA-DR? Is it important to identify low expression of the marker?
- For fluorophores that receive a lot of spreading error, it is best to place a marker with on/off expression. In this case, it is more likely to end up with data where a double positive population can be resolved despite large spreading error.



Fluorochrome Assignment – Difficult Panels

- For the markers that define your cell type, place those on low perpetrator fluorophores
- For all markers that are co-expressed on that cell type, there isn't really a way to fully predict what will work. Just do your best and make sure you perform a panel quality check on your cells

After Completing Panel Design

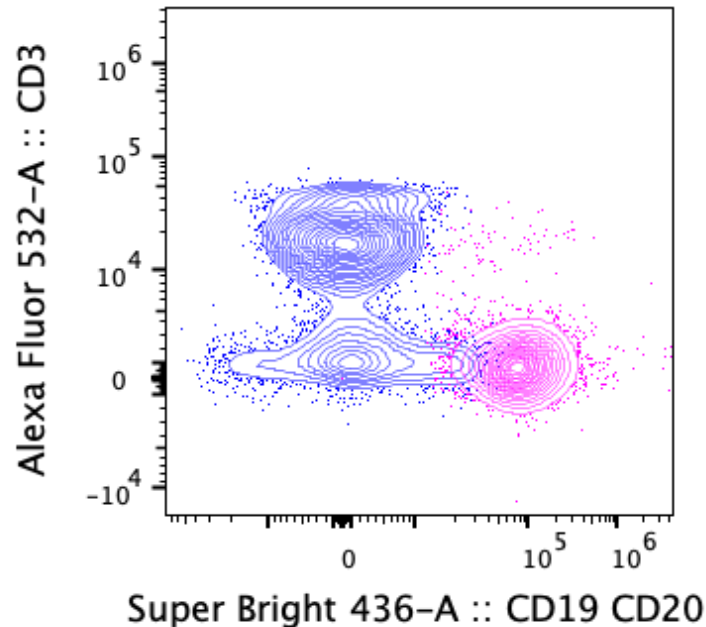
- Check your panel against the spread matrix
 - Example: I put CD3 on BV510. What boxes are pink/red in the BV510 row in the spread matrix? Are any of those markers co-expressed with CD3? If yes, am I concerned about spreading into that marker?
 - Primary markers (on/off) can handle spread better than secondary markers (continuum)
 - BV510 spreads into BV570 35%. If BV570 is on:
 - CD19, no problem. Not co-expressed with CD3.
 - CD4, it might be OK. CD4 is a primary marker.
 - CD45RA, I would be concerned about losing the resolution of the dim CD45RA+ cells from the CD3 spreading into CD45RA.
 - You may decide to keep the panel as is and remember to assess in a panel check

Steps for Successful Panel Design

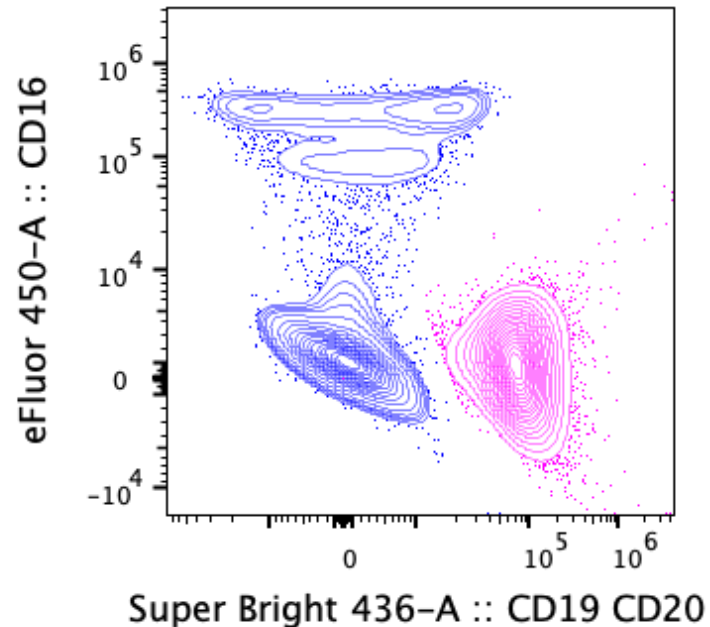
1. Gather information beforehand about the markers and experiment
2. Pick fluorophores for the panel
3. Assign the fluorophores to the markers
 - Use the tools specific for your cytometer!
4. Test the panel on cells

Know your panel!

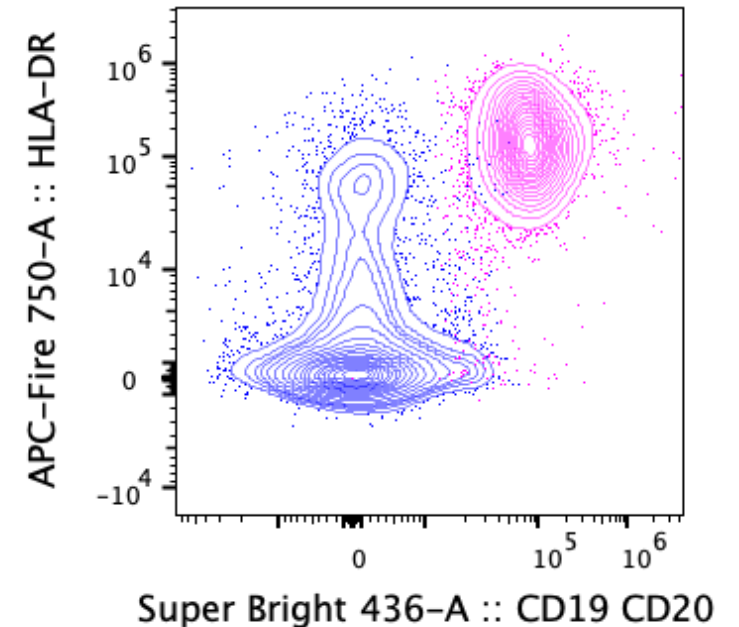
- Spreading error may effect how you need to draw manual gates



This is a popular plot choice, but my panel design does not allow me to gate B cells cleanly



Based on my panel design, this is the best way to draw a clean B cell gate



This plot is also an option for a B cell gate

A final note about panel design

- Keep in mind panel design is theoretical. There is no way to know if panel design is successful until it is tested on actual cells.
- The harder the panel, the harder it is to design a panel that will work as predicted.
- I strongly recommend performing a panel check on every new panel. Download the “How to perform a panel check” slides: <https://voices.uchicago.edu/ucflow/spectral-flow-cytometry/>