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BIOLOGICAL
SCIENCES

Flow Basics 2.3: Panel Design

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The Flow Basics 2.0 Series



Components of Successful Panel Design

1. Gather information beforehand about the markers and experiment
2. Know cytometer configuration
3. Assign the fluorophores to the markers
4. Test the panel on cells

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For successful panel design, gather information beforehand

1. Decide on markers for the panel, determine antibody availability
2. Identify antigen co-expression
3. Identify expression pattern/Classify antigens (primary, secondary, tertiary)
4. Decide on a rough gating strategy

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

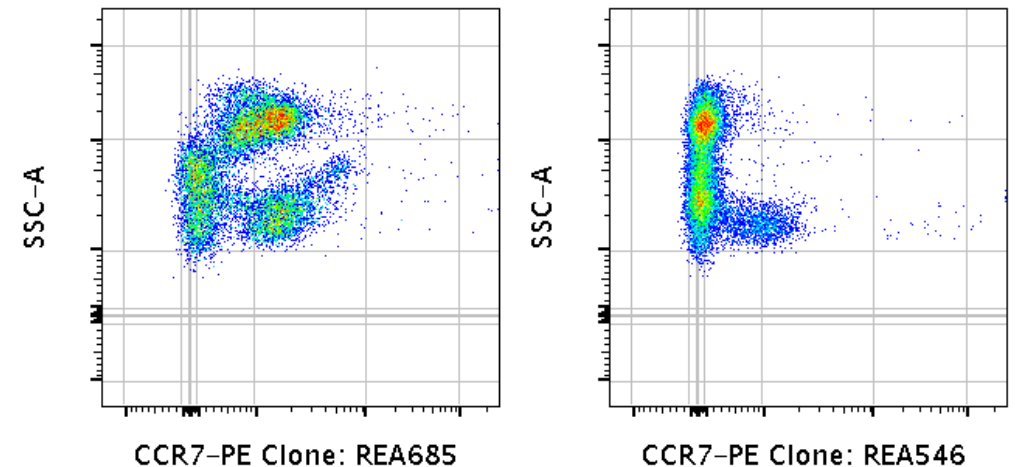
Panel Difficulty

- The larger the panel, the harder it is to design
- For a “larger” panel (about 10+ markers), the difficulty level also depends on antigen co-expression
 - Panels looking at a variety of cell types are easier to design (less co-expression)
 - Panels looking at many markers on a single cell type are harder to design (more co-expression)

Decide on Markers – Antibody Clones and Fluorophore Availability

- Once markers have been decided:
 - Determine if a specific clone is preferred
 - Create a list of fluorophores available for each marker (BioLegend, BD, Miltenyi, and ThermoFisher/eBioscience are the vendors we use the most) – I like FluoroFinder's [antibody search tool](#)

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

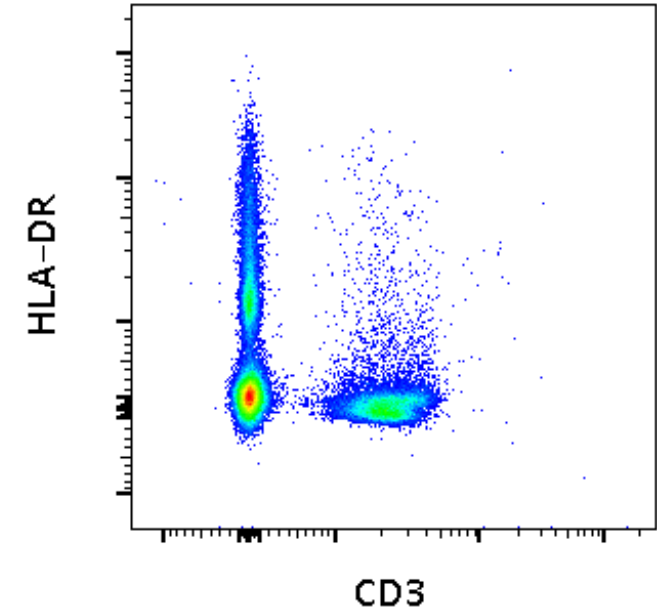


Identify Antigen co-expression

- 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)

Classify Antigens

- **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 the data provided on vendor websites for your antibody of interest (BioLegend, BD, eBioscience/Thermofisher, Miltenyi)



Determine Antigen Density/Expression Level



Expression of Common Surface Molecules on Blood Cells

- As part of classifying the antigens in your panel, you should also be aware of the expression level.
- Usually a general idea of high, low, or unknown is sufficient
- If a marker is secondary (continuum) and you are interested in high and low expressing cells, you can treat it as low expression

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	Glatting, 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	Unterhaeher, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD40	2,000	Unterhaeher, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD86	16,000	Unterhaeher, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD80	2,000	Unterhaeher, J. et al. 2007. <i>PNAS</i> . 104:234.
Dendritic cell	CD11a	27,000	Unterhaeher, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD40	17,000	Unterhaeher, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD80	132,000	Unterhaeher, J. et al. 2007. <i>PNAS</i> . 104:234.
	CD86	208,000	Unterhaeher, 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, patients, 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.

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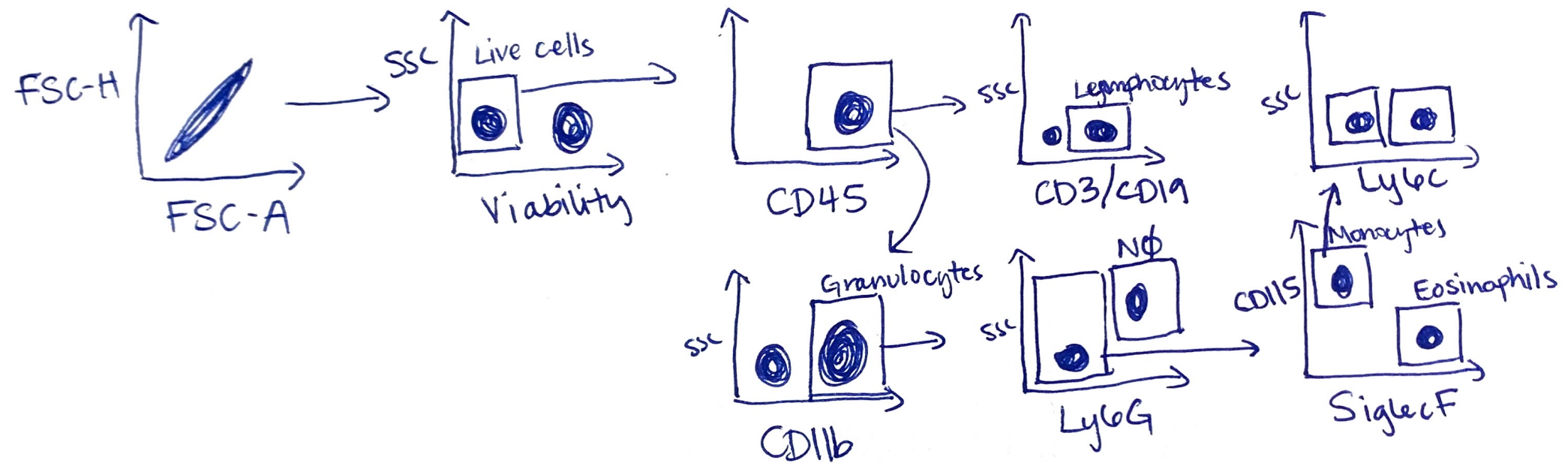
www.biolegend.com

Rev: 070808

Decide on a rough gating strategy

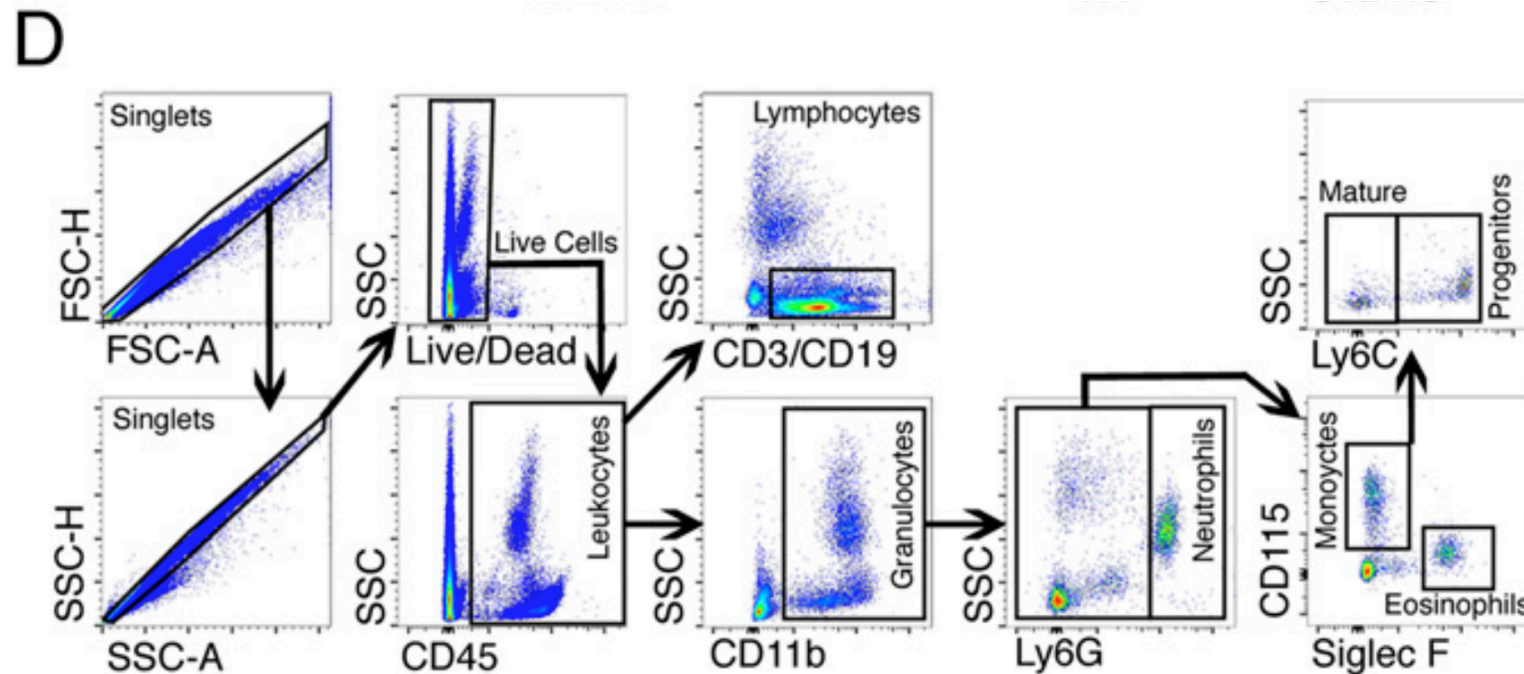
- This can still be helpful even if you are planning on using algorithms to analyze your data (tSNE, FlowSOM, etc.)
- Examples:
 - Schematic
 - Published data
- Tip: It can also be useful to identify a single plot that is critical and will be in the final figure. Plan to put those fluorophores on different lasers so that the plot looks great!
 - Example: looking at CD4/CD8 ratio – don't put CD4 and CD8 on the same laser (PE and PE-Cy7)

Gating Strategy Example: Schematic



Gating Strategy Example: Published Data

- Note how this gives information about antigen classification and co-expression!



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

Steps for Successful Panel Design

1. Gather information beforehand about the markers and experiment
2. Know cytometer configuration
3. Assign the fluorophores to the markers
4. Test the panel on cells

Know Cytometer Configuration

- Use the tools specifically made for the instrument you plan to use (if possible)
 - Tools for our instruments can be found here:
<https://voices.uchicago.edu/ucflow/panel-design/>
 - If you don't have an instrument-specific tool, look for a general brightness index. Spillover can be determined in spectra analyzers or Fluorofinder.com has general instrument configurations

Choose a Flow Cytometer

- How many antigens do I want to detect?
- What instrument am I planning on using?
 - At our facility, we label our instruments as as “number of lasers-number of maximum parameters”. So the X20 5-18 has 5 lasers and 18 detectors.
 - Instrument configurations for our facility can be found [here](#).
 - Note: for the X20, the instrument is configured to use either BV711 or BV650. Please talk to the staff if you would like to learn how to check or swap the filters, or if you need a special configuration.

Choose a Flow Cytometer

- Our “Benchtop analyzer available fluorophores” tool is a quick guide to our instrument configurations

		Fortessa X20	Fortessa 4-15	Fortessa 4-15 HTS	Attune NxT	LSR 4-12
UV	BUV396	X				
UV	DAPI	X				
UV	BUV496	X				
UV	BUV737	X				
UV	BUV805	X				
Violet	BV421	X	X	X	X	X
Violet	BV510	X	X	X	X	X
Violet	BV605	X	X	X	X	X
Violet	BV650	X (SWAP FILTER WITH BV711)	X	X	X	
Violet	BV711	X	X	X	X	
Violet	BV786	X			X	
Blue	FITC	X	X	X	X	X
Blue	PerCP-Cy5.5	X	X	X	X	X
Yellow/Green	PE	X	X	X	X	X
Yellow/Green	PE-Dazzle595	X	X	X	X	X
Yellow/Green	PE-Cy5		X	X		X
Yellow/Green	PE-Cy5.5		X	X		
Yellow/Green	PE-Cy7	X	X	X	X	X
Red	APC	X	X	X	X	X
Red	APCR700	X	X	X	X	X
Red	APC-Cy7	X	X	X	X	X

Choose fluorophores based on cytometer configuration and panel size

- The best way to start building a panel is to assign one fluorophore per laser.
 - Example: a 4-marker panel on a standard 4-laser instrument would be BV421, FITC, PE, and APC
- As you increase the number of markers, continue to spread the fluorophores out on the lasers
 - Example: an 8-marker panel would have 2 fluorophores on each laser.
 - Bad example: for a 5-marker panel, do not put all 5 markers on the violet laser
- Leave the fluorophores that spill into many detectors for last – don't use them if you don't have to

Why are fluorophores with high spillover annoying to use?

- More spillover means more compensation.
- More compensation means more time on the instrument, more time calculating/checking compensation, more chance of error (for beginners)
- Depending on the panel more spillover could mean more spreading error: the data has more variation instead of being a small, tight population
- Flow cytometry is hard enough, always take the easy route!

Fluorochrome Assignment Tools

- Our “Benchtop analyzer fluorophore equivalence table” tool is a quick guide to fluorophores that are detected by the same filter
 - How to use: Pick one fluorophore/dye per row when designing a panel

Fortessa X20

Laser	Laser	Filter	Fluorophore						Dye/Protein			
UV	355	379/28	BUV396									
UV	355	450/50	DAPI						DAPI	Hoechst	BFP	Zombie Violet
UV	355	515/30	BUV496						Zombie Aqua			
UV	355	740/35	BUV737									
UV	355	820/60	BUV805									
Violet	405	450/50	BV421	Pacific Blue	VioBlue	V450	SuperBright 436	eFluor 450	Sytox Blue	Zombie Violet	BFP	DAPI
Violet	405	525/50	BV510	Pacific Orange	V500	VioGreen	eFluor 506		AmCyan	Zombie Aqua	CFP	
Violet	405	610/20	BV605	eVolve 605	SuperBright 600							
Violet	405	660/20	BV650	eVolve 655	SuperBright 645							
Violet	405	710/50	BV711	SuperBright 702								
Violet	405	780/60	BV786									
Blue	488	530/30	FITC	AF488	BB515	VioBright FITC			GFP	Sytox Green		
Blue	488	710/50	PerCP-Cy5.5	BB700	PerCP-eFluor 710	PerCP-Vio700			Propidium Iodide			
Yellow/Green	561	586/15	PE	AF555					Propidium Iodide			
Yellow/Green	561	610/20	PE-Dazzle595	PE-CF594	AF594	PE-eFluor 610	PE-Texas Red	PE-Vio615	mCherry	tdTomato	7-AAD	Zombie Red
Yellow/Green	561	780/60	PE-Cy7	PE-Vio770								
Red	640	670/30	APC	AF647	eFluor 660				Cy5	Sytox Red		
Red	640	710/50	APCR700	AF700								
Red	640	780/60	APC-Cy7	APC-Fire 750	APC-H7	APC-Vio770	APC-eFluor 780					

Fluorochrome Assignment Tools

- A brightness index specifically generated for each of our Fortessas can be found on our website

Fluorophore tested	Separation Index	Brightness Estimation
APC	223	Very Bright
PE	222	Very Bright
PE-Dazzle595	214	Very Bright
BV421	198	Very Bright
PE-Cy7	181	Bright
BV786	178	Bright
BV711	172	Bright
BV605	135	Moderate
PerCP-Cy5.5	113	Moderate
BUV737	104	Moderate
AF700	86	Dim
BV510	85	Dim
AF488	85	Dim
BUV396	71	Dim
APC-Cy7	71	Dim
Pacific Blue	61	Dim
BUV496	33	Very Dim
BUV805	30	Very Dim
DAPI	not tested	

Steps for Successful Panel Design

1. Gather information beforehand about the markers and experiment
2. Use the tools available for panel design
3. Assign the fluorophores to the markers
4. Test the panel on cells

Fluorochrome assignment

1. Start with limiting reagents
 - Make note of any fluorescent proteins (GFP, mCherry, etc.)
 - Assign very rare markers to the only fluorophore options available (e.g. marker X is only available in PE)
2. Assign the remaining markers – be aware of co-expression
 - Utilize all of the information gathered previously
3. Assign viability dye

Fluorochrome Assignment: Fluor Brightness vs. Antigen Density

- Assign bright fluorophores to low expressed markers and dim fluorophores to highly expressed markers
- Low expressed markers should not be put on dim fluors –highly likely to not be able to resolve the positive population
- High expressed markers can be put on bright fluors if the bright fluors don't need to be saved for low expressed markers

Fluorochrome assignment: Co-Expression

- For co-expressed markers, assign fluorophores with minimal spillover into each other
- Be cautious of using and assigning fluorophores with lots of spillover
- If there is an extremely important biaxial plot (aka a plot that will be put in a final figure), consider assigning two fluorophores that have no spillover. This will produce the best-looking and therefore most convincing data.

What fluorophores have high spillover and should be avoided if possible?

- **PerCP-Cy5.5** and **PE/Dazzle 594** spill into many channels and we recommend using them only when needed (such as a 10 color panel, not a 4 color panel)
 - Using both together can be very challenging – choose one!
- **PE-Cy5** and **PE-Cy5.5** are not commonly used and not great fluorophores (lots of spillover), so we don't recommend using them
- **PerCP** is not a stable fluorophore, we recommend using PerCP-Cy5.5 instead

How to deal with fluorophores with high spillover

- 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 spillover can be ignored.
- Assign them to a low expressed or rarely expressed marker. In this case, the spillover 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 define lymphocytes and monocytes.

Other Notes About Fluorophores

- Some fluorophores have brighter or better alternatives (use brightness charts)
 - Example: AF488 is more stable than FITC, BV421 is much brighter than pacific blue, APCR700 is brighter than AF700
- The UV laser is dim and reagents are expensive, so we recommend that you only use it if you have to

How to utilize primary/secondary/tertiary information

- Tertiary markers must be placed on bright fluorophores
 - Placing a tertiary marker on a dim fluorophore will likely produce suboptimal results (unable to separate positive cells from negative cells)
- Secondary markers will benefit from moderate-bright fluorophores in order to resolve the dimmest population
 - Placing a secondary marker on a dim fluorophore will certainly resolve the high-expressing population, but it may be challenging to distinguish the low-expressing cells from the negative cells
- Primary markers can be placed on dim fluorophores
 - They can also be placed on bright markers, but those are often used up by secondary and tertiary markers

Fluorochrome assignment: Method 1

- Write out each marker you want in your panel (don't forget live/dead!) in a vertical list
- Next to each marker, list available fluorophores
 - You may try to utilize antibodies that your lab already has to minimize purchasing of new antibodies
 - Search BioLegend, eBioscience, Miltenyi, and BD for available antibodies
- Use your puzzle-solving skills to assign fluorophores to your markers. Remember there is no perfect panel!
 - Assign markers based on antigen classification, antigen co-expression and gating strategy

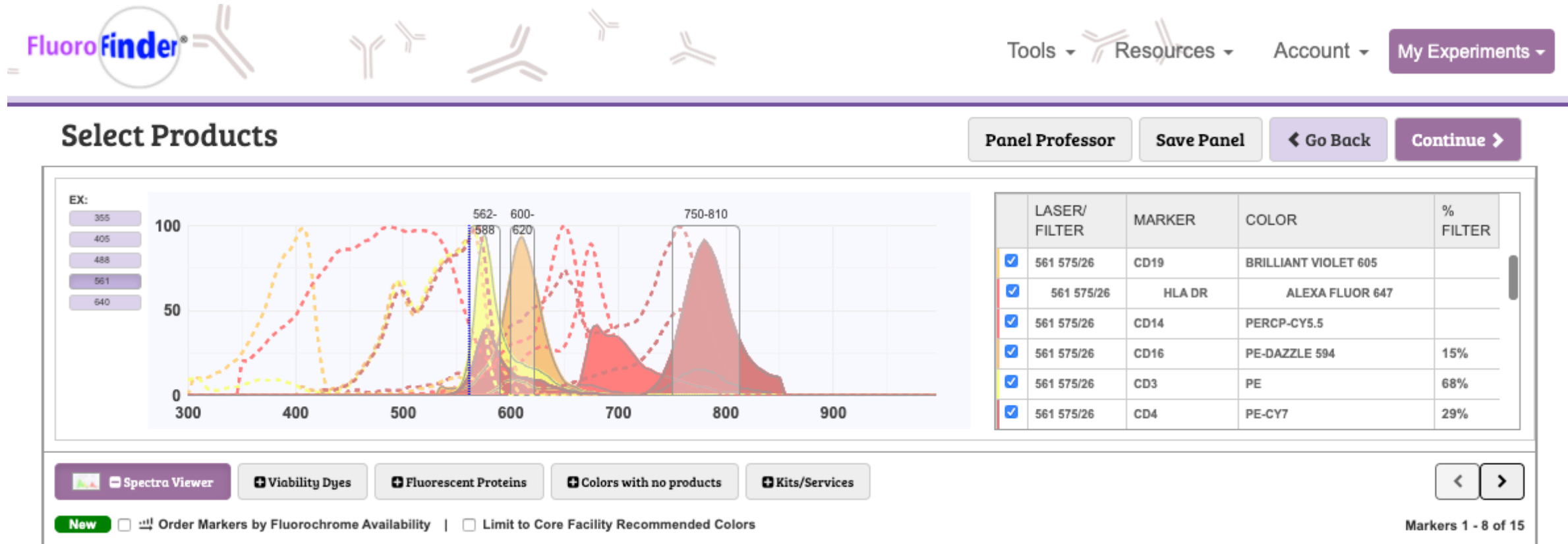
Fluorochrome assignment: Method 1

Marker				
CD3	AF488	PE	APC-Cy7	Pacific Blue
CD4	PE-Cy7	APC-Cy7	Pacific Blue	
CD8	PerCP-Cy5.5	PE-Cy7	APC	
CD11b	PE-Cy7	FITC	PE	
Ly6G	APC			
CD19	AF488	AF647	APC-Cy7	
Viability	PI	Live/Dead Aqua		

Fluorochrome assignment: Method 2

- All of our instruments can be found here: <https://app.fluorofinder.com/uchicago>
- Under “select markers” enter your markers of interest and target species (the website is a bit slow)
 - Viability stains and fluorescent proteins can be added on the next page
 - If you are using a fluorescent protein, add a marker name on this page
 - If you would like to use a dump channel, just enter one of the antibodies
 - You can go back to check the cytometer configuration at the top of the screen if you want
- Select continue, then select products
- Click a fluorophore to select a specific product
 - Note: We recommend choosing antibodies from BioLegend, eBioscience, BD, or Miltenyi
- As you select products, you will not be able to select interfering products
 - For dump channels you can purchase multiple antibodies in one color
 - At the top of the page, select viability dyes or fluorescent proteins if needed
- Click “show spectra viewer” at the top to assess spillover (see next slide)
- Hit continue or print or export as an excel sheet
- Feel free to have someone at the CAT facility check over your panel before you purchase antibodies or run your experiment!!

FluoroFinder's Panel Spectra Viewer



FluoroFinder's Panel Spectra Viewer

- After assigning fluorophores in a panel, FluoroFinder has a tool to determine predicted spillover
- For each laser and filter set, assess if the spillover is acceptable
- In this example the 561 575/26 filter is assigned to CD3
 - CD4 is 29% and coexpressed with CD3 – move this marker
 - CD16 is 15% but not coexpressed, no need to move

	LASER/ FILTER	MARKER	COLOR	% FILTER
☒	561 575/26	CD19	BRILLIANT VIOLET 605	
☒	561 575/26	HLA DR	ALEXA FLUOR 647	
☒	561 575/26	CD14	PERCP-CY5.5	
☒	561 575/26	CD16	PE-DAZZLE 594	15%
☒	561 575/26	CD3	PE	68%
☒	561 575/26	CD4	PE-CY7	29%

Final Notes

- Check the panel on test samples before running a full experiment
 - Can you resolve your populations of interest?
- Don't forget to titrate antibodies before testing the panel!

Stay Tuned for the Rest of the Flow Basics 2.0 Series

