# Panel Design

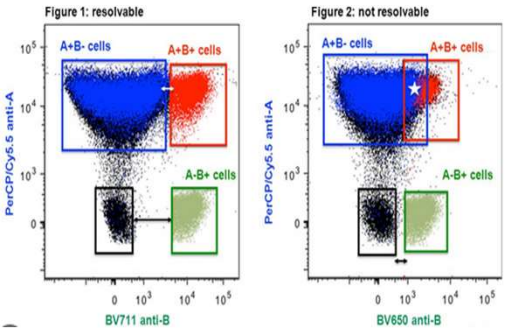
1

Today, we will be presenting and discussing steps needed to design a panel for flow cytometry.

## Why is panel design so important?

- In short, fluorochrome spillover produces background and spread which impacts the resolution of populations.

- PerCP/Cy5.5 (on Marker A) spreads into BV711 and BV650 s leading to a loss of resolution in both populations.




Ashurst TM, Smith AL, Cytometry tutorial: The impact of adjusting PMT voltages on spillover and compensation. Massachusetts Institute of Technology

Why is panel design so important?
-Goal—resolve populations of interest
Fluorochrome spillover and spread can impact population resolution
By designing a good panel, we can minimize the negative effects of things like fluor spillover and spread on population resolution to get best and most accurate results possible

Figure is an example demonstrating what fluor spread is:
- PerCP-Cy5.5 (on Marker A on y-axis) spreads into BV711 and BV650 filters (on Marker B on x-axes) leading to a loss of resolution in the PerCP-Cy5.5 single positive population in blue and double positive population in orange.
- Figure 1 (on left): BV711 is bright enough and marker B is expressed highly enough that A+B+ double positive population can be resolved from the single positive PerCP-Cy5.5 population.
- Figure 2 (on right): BV650 is not bright enough to resolve A+B+ double positive population from single positive PerCP-Cy5.5 population.

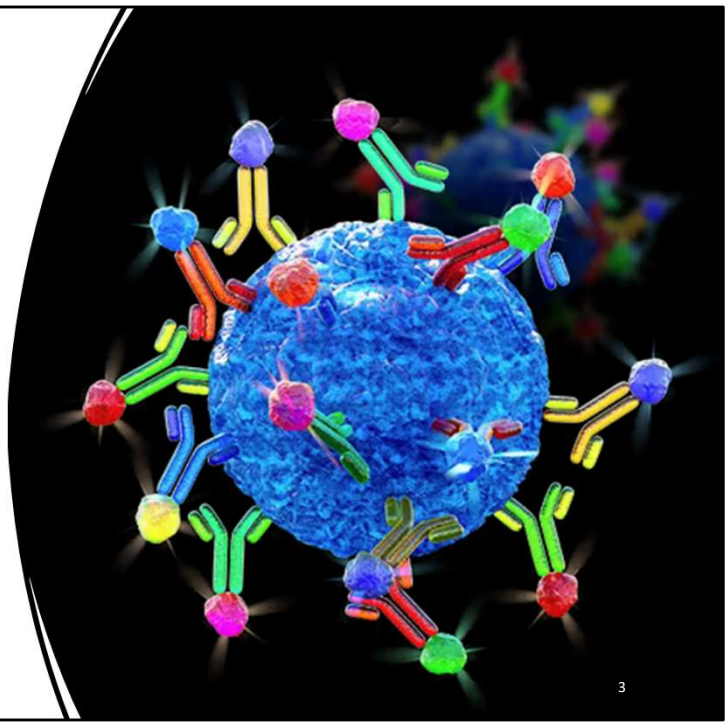
And that is why panel design is so important-to make sure populations can be resolved.

---

https://slideplayer.com/slide/12327816/
http://web.mit.edu/flowcytometry/www/Cytometry%20tutorial%20The%20impact%20of%20adjusting%20PMT%20voltages%20on%20spillover%20and%20compensation.pdf

# Topics Reviewed

1. The Biology: Cells / Markers
2. The Cytometer
3. Assigning Fluorochromes
4. Optimization / Panel Analysis




These are the steps we are going to discuss when designing a panel.

# 1. The Biology: Cells / Markers

4

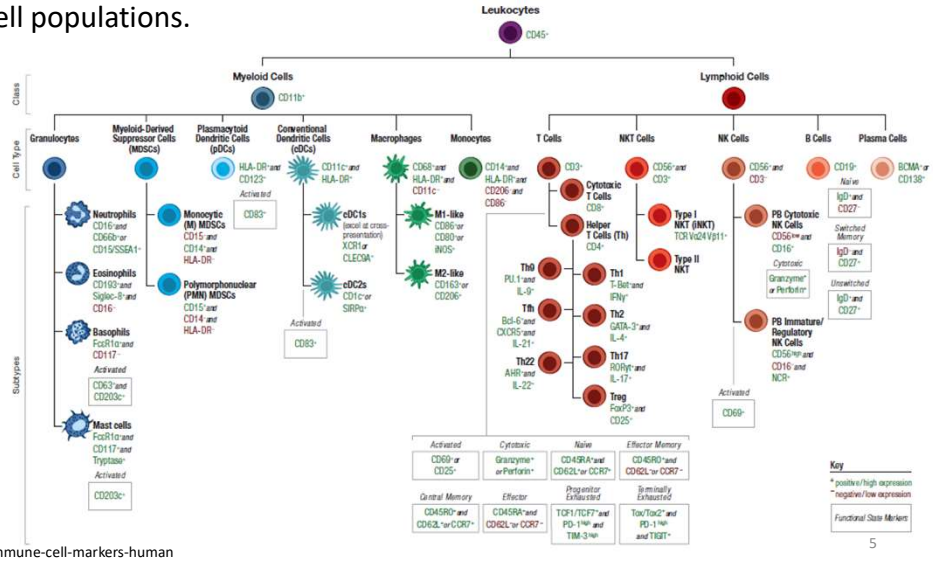
First, we need to gather information about the cells and their markers.

I will use the words Marker and Antigen interchangeably but mean the same thing regardless of which word I use.

# 1. Cells / Markers

**A.** Decide what cells are being identified and the markers needed to characterize different cell populations.

***Human Immune Cell Marker Guide for Flow Cytometry***




For pathway key and background, please visit: cst-science.cm/pathway

Cell Signaling TECHNOLOGY®

https://www.cellsignal.com/pathways/immune-cell-markers-human

We need to know:
- the cell populations of interest that need to be identified
- the markers that characterize the cell populations of interest.

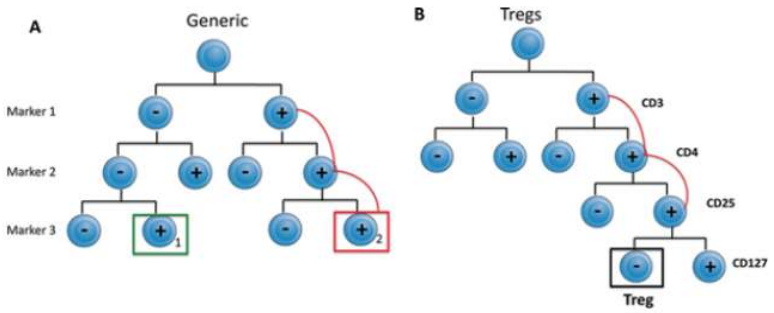
The figure shown here is an example of a flow chart or a cell hierarchy chart that shows markers for different human cell populations. Charts like these can be very helpful in figuring out which markers are needed to identify your cell populations of interest.

# 1. Cells / Markers

**B.** Identify markers (antigens) that are on several different cell types.

**C.** Identify co-expressed markers.

***Determine marker co-expression on target cells.***





- Maciorowski Z, Chattopadhyay PK, Jain P. Basic Multicolor Flow Cytometry. Curr Protoc Immunol. 2017 Apr 3;117:5.4.1-5.4.38. doi: 10.1002/cpim.26. PMID: 28369683.
- Bushnell T. The Fluorochrome Less Excited: How to Build A Flow Cytometry Antibody Panel. https://expert.cheekyscientist.com/the-fluorochrome-less-excited-how-to-build-a-flow-cytometry-antibody-panel/


6

Next, we need to find out if:

-the markers are on several cell types

-the markers are co-expressed with another marker on the same cell type.

This information is important for panel design because 2 or more markers on the same cell can lead to a loss of resolution with certain fluorochrome pairs due to the spreading of one fluorochrome into the filter for a different fluorochrome.

Shown here are 2 different cell tree examples that can help with determining marker expression patterns.

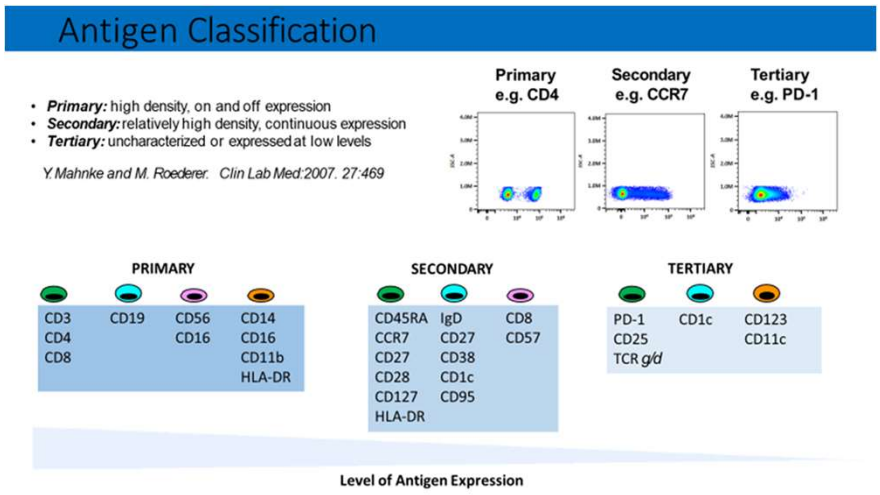
-Tree A is a generic cell tree showing which markers are co-expressed. The markers on the cell type in the red square are co-expressed with the markers on the cell types above it in the tree linked with the red lines but not with markers on the cell type in the green square.

-Tree B is an example that shows Treg cells co-express CD3, CD4, and CD25 but not CD127.

When different cell types have the same marker and when one cell type has co-expressed markers, it is extra important to select fluorochromes for those markers that do not spillover into each other's filters and cause spread.

# 1. Cells / Markers

**D.** Identify antigen classification




Then, we need to determine antigen, or marker classification.
Knowing antigen classification helps decide what fluorochrome to choose.
Antigens are classified as either primary, secondary, or tertiary.

**Primary Antigens:**
- Have expression that is either on or off (positive or negative)
- Often expressed in high levels and often define cell linages.
- Examples: CD3, CD4, CD19.

**Secondary Antigens:**
- Have a range of expression (or are a continuum).
- Some cells may express a lot of the antigen and other cells may express only a little which leads to a range of brightness for the antigen.
- Often well characterized.
- Examples: CD27 , CD28, CD45RA, CD45RO

**Tertiary Antigens:**
- Antigens that are expressed at low levels or have an unknown expression.
- Expression may vary upon activation—really important to keep in mind
- Often the critical markers in determining the cell population of interest

- Examples: CD25, STAT5, FoxP3

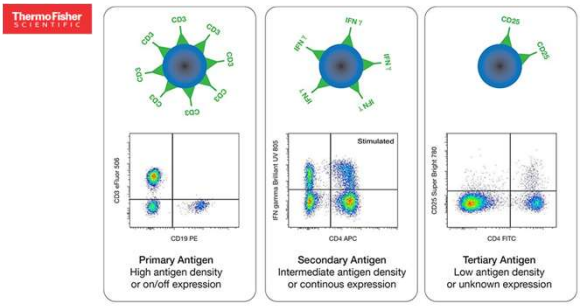
---

Antigen classification is type of expression
Antigen density is amount of expression

# 1. Cells / Markers

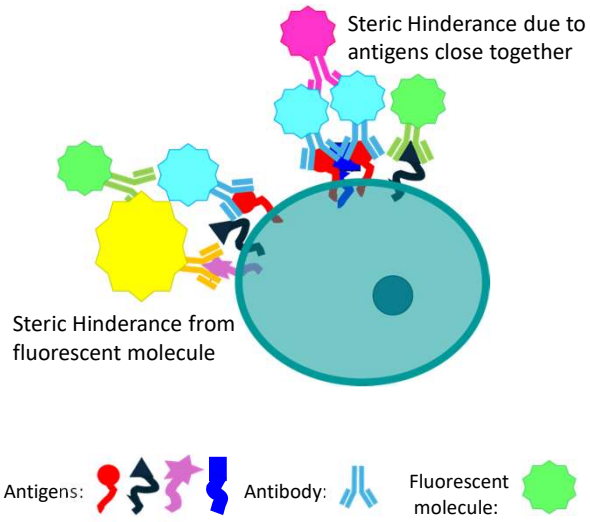
**E.** Be aware of antigen expression:

- Density (molecules/cell)
- Low or high expression
- Activated expression
- Are antigens expressed next to other antigens (steric hindrance).




https://www.thermofisher.com/us/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-learning-center/flow-cytometry-resource-library/flow-cytometry-methods/spectral-flow-cytometry-panel-design.html

**Steric Hindrance**




We also need to know antigen density or level of expression. This diagram illustrates what is meant by that.

- In the image farthest to the left, you can see that there are many CD3 molecules on the cell surface compared to CD25 on the right of this image and about middle of the slide.
- CD25 would be considered to have a low antigen density or low level of expression compared to CD3 which would be high.

When there is high density of markers, the possibility of steric hindrance needs to be kept in mind, especially if you have problems with staining or resolving a population.

Steric hindrance, as illustrated on the right, is when:
-one antibody prevents the binding of another to its respective target antigen when the reagents are used together.
-Basically, one antibody gets in the way of another one.
-This can be because the fluorescent molecule attached to the antibody hinders binding or because the antigens are too close together for so many antibodies to bind

Additionally, it is important to know if the cells need to be activated to express the marker or if activation changes the expression of the marker, and what activation state

your cells will be in for your flow experiment because the expression of the markers during the flow experiment is the expression level that needs to be used when designing a panel and selecting fluors.

Where can we find this information about our cells and markers?

---

-the reduction or absence of detectable fluorescence signals occurs because one monoclonal antibody hinders the binding of another one to its respective target antigen when the reagents are used conjointly (Matos, 2021)

# 1. Cells / Markers

Expression information can be found in:

- Publications
- OMIPs
- Preliminary experiments
- Company resources
- Online resources

Table: from BioLegend showing expression of some antigens on blood cells in terms of the number of molecules/cell

BioLegend®

**Expression of Common Surface Molecules on Healthy Blood Cells**

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

9

Expression information can be found in publications, OMIPs (which I'll discuss more in a few slides), preliminary experiments done by your lab, company resources, and online resources.

Shown here is an example of a table from Biolegend that shows the expression of some antigens on blood cells in terms of the number of molecules/cell. IMPT to NOTE: This info COULD BE DIFFERENT FOR ACTIVATED CELLS AND DIFFERENT CELL TYPES so always important to know the information for your exact cells as best as possible.

# 1. Cells / Markers

Interactive Cell Marker sites can help with cell expression information

- BioTechne: Interactive Cell Marker Tool: CD Markers and Beyond
  https://www.rndsystems.com/blog/interactive-cell-marker-tool-cd-markers-and-beyond

  An interactive cell marker tool to help find appropriate cell or organelle markers. Simply choose the appropriate category, such as immune cells, neural cells, stem cells or organelles, by clicking on an image of the cell of interest and browse a selection of highly sensitive and specific antibodies and immunoassays that can be used for detecting your marker of interest.

-  CellMarker 2.0

  http://bio-bigdata.hrbmu.edu.cn/CellMarker/
  Hu, Congxue et al. "CellMarker 2.0: an updated database of manually curated cell markers in human/mouse and web tools based on scRNA-seq data." *Nucleic acids research* vol. 51,D1 (2023): D870-D876. doi:10.1093/nar/gkac947

  CellMarker 2.0 is an updated database that provides a manually curated collection of experimentally supported markers of various cell types in different tissues of human and mouse. In addition, web tools for analyzing single cell sequencing data are described.

10

There are also interactive cell marker web sites that can also help with determining antigen expression information.

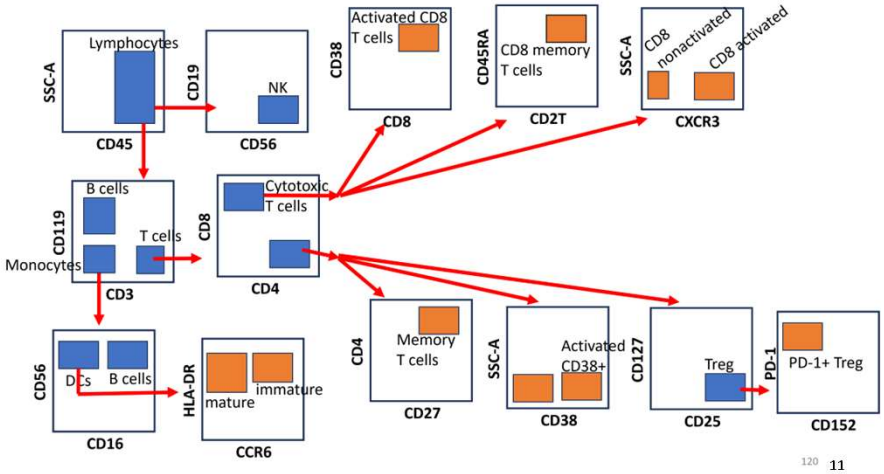
BioTechne: Interactive Cell Marker Tool: CD Markers and Beyond
Works by choosing the appropriate category, such as immune cells, neural cells, stem cells or organelles, by clicking on an image of the cell of interest and then you can browse a selection of highly sensitive and specific antibodies and immunoassays that can be used for detecting your marker of interest.

Cell Marker 2.0
CellMarker 2.0 is an updated database that provides a manually curated collection of markers in a variety of human and mouse tissues. It also has web tools for analyzing single cell sequencing data.

# 1. Cells / Markers

**F**. Think about the analysis strategy to determine the cells that need to be identified to get your cell population of interest.

Sketch of potential analysis strategy




120 11

Now we need to think about the analysis strategy for the population of interest.

This helps reveal some basic information like:
- the cell populations that need to be identified in order to get to the cells of interest
- the markers that characterize the different cell populations
- the gating strategy or path taken to identify the cells of interest

This is an example of an analysis strategy to get to PD-1+ Treg cells.
---

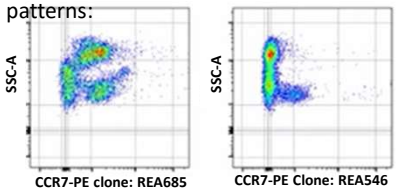
# 1. Cells / Markers

**G.** Determine what antibodies are available for each marker/antigen. What Ab clones to use.

**H.** Determine what fluorochromes are available for each marker/antigen.

Take note of the AB clones used.

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




**Sources for Antibodies and Fluors**
- Panel building programs
- Publications
- Company websites
- OMIPS
- Bench Sci
- Biocompare

UChicago Flow. (2020, June 12). *Flow Basics 2.3: Panel Design* [Video]. YouTube. https://www.youtube.com/watch?v=WHtqy3X_ch8

12

We also need to determine:
- what Abs are available for the markers you want to use
- the different fluors that are attached to the available Abs

In the figures here, you can see that two different clones **for the same marker** have very different staining patterns. Be sure to keep that in mind and make a note of which clones you prefer to use.

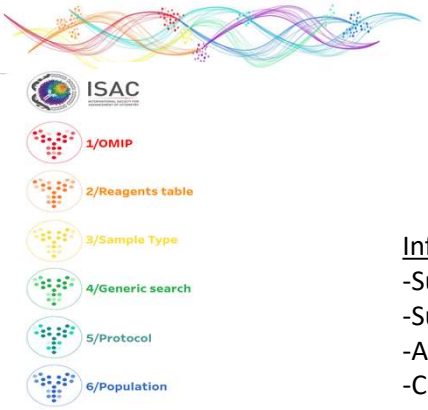
There are programs that can help you find available antibody clones and fluors for your markers of interest.
- Many companies that sell the fluorescent Abs will have helpful information, but they will show only the products they sell.
- However, there are other online resources to help find this information from companies that don't directly make or sell antibodies like FluoroFinder that provide information about available Abs and fluors from all companies.

This information can also be found in resources like publications (Current Protocols in Cytometry, or a journal that is specific for your field or cells), and OMIPs (Optimized Multicolored Immunofluorescence Panels), Cytek Cloud or other panel building programs.

# 1. Cells / Markers

OMIPs (Optimized Multicolored Immunofluorescence Panels).

- Peer reviewed publication that reports optimized multicolored panels for Flow Cytometry




**OMIP_100** 2024 A flow cytometry panel to investigate human neutrophil subsets

| Antigen | Fluorochrome | Clone | Catalog Num | Vendor | Antibody reactivity | Dilution | Volume |
|---|---|---|---|---|---|---|---|
| CD10 | PE | HI10a | 555375 | BD Bioscience | Null | 1:20 | 160 µL |
| CD11b | BUV737 | D12 | 748588 | BD Bioscience | Null | 1:80 | 160 µL |
| CD15 | BV786 | W6D3 | 323043 | BioLegend | Null | 1:160 | 160 µL |

Information Provided:

-Suggested antibodies
-Suggested fluors
-Antigen classification
-Co-expression
-Gating strategy
-Staining protocol
-Strategy behind selected panel

https://public.tableau.com/app/profile/fanny2212/viz/OMIP_ISAC/Menu

13

Now, we'll discuss OMIPs in greater detail. I've mentioned them at least a couple of times now as valuable resources, so what are they?

OMIP stands for Optimized Multicolored Immunofluorescence Panel

OMIPs are a great source of information—**emphasize that they are very helpful**
-peer-reviewed publications in *Cytometry A* journal that reports optimized panels for flow cytometry
-have been published for several species including human and mouse cells
-are over 100 of them published as of now
- Can save a lot of time in panel design because they give you a great starting point.
- Provide information such as a reagent/Ab information, staining figures, comparison to other OMIPS.

- Provide technical details such as the strategy of how the panel was developed, gating strategy, co-expression, antigen classification, maybe why they selected certain antibodies or fluors, titration information, staining protocols, instrument configuration.
- There is a database (link at the bottom of the slide) to search based on a bunch of different criteria like species, sample type, cell type, etc
- Impt to note that the **panels in the OMIPs could have been designed for a different instrument with a different configuration** than what we have in the RFCF or possibly

the same instrument as we have in the RFCF but with different configuration. That's why it's a good starting point—because your cells or the available cytometers and their configurations may vary some from the cells or instrument that the panel in the OMIP was designed for, like a different cell activation state, or different instrument configuration.

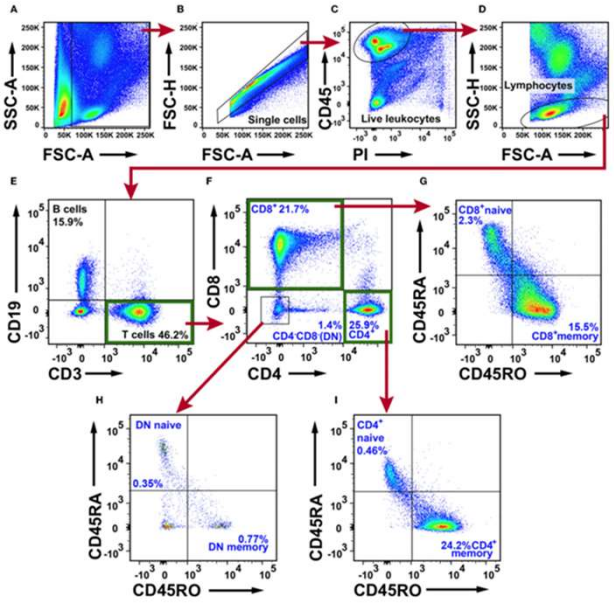
But these are a great resource, and if I had to design or redesign a panel, this is where I would start-I would first see if an OMIP existed for my cell type.

---

OMIP species: human, non-human primate, humanized mouse, mouse, rat, cattle, dog

# 1. Cells / Markers

- (Plot E): A Plot with 2 markers that have no double positive (DP) population shows that the markers are mutually exclusive, not co-expressed.
- Note the intensity of the populations in the plots which can show the expression of the marker.




Stankovic, Branislava et al. "Immune Cell Composition in Human Non-small Cell Lung Cancer." *Frontiers in immunology* vol. 9 3101. 1 Feb. 2019, doi:10.3389/fimmu.2018.03101 [14]

I've also mentioned a few times that publications are great resources to learn important cell information for panel design that is specific to your cells or area of interest.

This is just one figure from a publication that shows:

A. an **analysis strategy**, **markers, antigen classification and level of expression**, and **co-expression of markers** just in this one figure.
B. The publication text may have information about the antibodies and fluors used.

Discuss E and H for sure
Plot E:
- CD3 and CD19 are mutually exclusive markers; not co-expressed because we don't see a double positive population.
- CD3 is either on or off and therefore considered to be a primary antigen
- CD19 has more of a continuous expression pattern and is considered a secondary antigen

Plot F: Plot of CD19 cells
- CD19 cells may have either CD4 or CD8 but not both because CD4 and CD8 are not co-expressed here (No double positive population).

Plot H:

CD45RA and CD45RO have low levels of expression and are considered tertiary antigens.

# 1. Cells / Markers

**Panel Design Form**

- List markers and information such as preferred Ab clones and Ab/fluor combinations

**Panel Design Form**

| Marker | Cell type Expressing the Marker | Location (surface, intracellular) | Antigen Classification (1, 2, 3) | Expression Density (Low, Mid, High) | Co-expression with? (other marker in panel) | Ab clone (proposed) | Fluorochrome (proposed) |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |

15

Suggest that, in order to keep all this information that you are gathering organized that you complete a Panel Design Form like the one shown here in order to pull all the cell and marker information together and have it in one place for when you start selecting and assigning clones and fluors.

It includes markers to be used, expression density, antigen classification, co-expression, and if you have fluorochromes or Abs you know you want to use because they've worked well for you in the the past or you already have them.

If you request assistance from the RFCF in designing a panel, we will ask you to fill out as much information as possible on a form like this.

# 2. Cytometer

16

Next, we need to gather information about the cytometer or cytometers we plan to use. It is important to design panels for the cytometers you plan to use because different cytometers will have different characteristics.

# 2. Cytometer

Know the cytometer's lasers & filter configuration:

**FACSAria Fusion: Laser and filter configuration**

| Laser | Detector | Long Pass | Band Pass | Optimal fluors |
|---|---|---|---|---|
| 355 nm 60 mW | B | 410 | 450/50 | DAPI, Hoechst blue, Zombie UV, Live/Dead blue |
| | A | 635 | 670/14 | Hoechst red |
| 405 nm 100 mW | F | NA | 450/50 | BV 421, Pacific blue, eFluor 450, Alexa 405 |
| | E | 505 | 525/50 | BV 510, V500, eFluor 506, AmCyan, Live/Dead aqua |
| | D | 600 | 610/20 | BV 605, SB 600 |
| | C | 635 | 670/30 | BV 650, SB 645 |
| | B | 690 | 710/50 | BV 711, SB 702 |
| | A | 750 | 780/60 | BV 786, SB 780 |
| 488 nm 100 mW | B | 505 | 530/30 | FITC, Alexa 488, BB 515, GFP, YFP, Venus |
| | A | 690 | 710/50 | PerCP, PerCP-Cy5.5**, PerCP-e710, BB 700 |
| 561 nm 150 mW | E | 580 | 586/15 | PE, tdTomato, DsRed |
| | D | 600 | 610/20 | PE-CF594, PE-Dazzle594, PE-e610, mCherry, PI* |
| | C | 635 | 670/30 | PE-Cy5, 7-AAD* |
| | B | 690 | 710/50 | PE-Cy5.5**, NovaYellow 700 |
| | A | 750 | 780/60 | PE-Cy7 |
| 637 nm 100 mW | C | 665 | 670/30 | APC, Alexa 647, DRAQ7, Live/Dead far-red |
| | B | 690 | 710/50 | Alexa 700, DRAQ5 |
| | A | 755 | 780/60 | APC-Cy7, Zombie NIR, Live/Dead near-IR |

Symphony S6 — 30 colors — Cincinnati Children's | Research Flow Cytometry Core

| Laser | Detector | PMT | Long Pass | Band Pass | Optimal Fluors |
|---|---|---|---|---|---|
| 355 nm 60 mW | H | | NA | 379/28 BP | **BUV 395** |
| | G | | 410 LP | 450/50 BP | **DAPI**, Hoechst blue, Zombie UV, Live/Dead Blue |
| | F | | 450 LP | 515/30 BP | **BUV 496**, Live/Dead aqua, Zombie aqua |
| | E | | 550 LP | 586/15 BP | **BUV 563** |
| | D | | 595 LP | 610/20 BP | **BUV 615** |
| | C | | 635 LP | 670/30 BP | **BUV 661** |
| | B | | 690 LP | 740/35 BP | **BUV 737** |
| | A | | 770 LP | 820/60 BP | **BUV 805** |
| 405 nm 200 mW | H | | 410 LP | 431/28 BP | **BV 421**, SB 436, eFluor 450, Alexa 405, EBFP |
| | G | | 450 LP | 470/14 BP | **BV 480**, pacific blue, TagBFP, mTurquoise, sytox blue, CFP |
| | F | | 550 LP | 586/15 BP | **BV 570**, Live/Dead yellow |
| | E | | 600 LP | 610/20 BP | **BV 605**, SB 600 |
| | D | | 635 LP | 670/30 BP | **BV 650**, SB 645 |
| | C | | 690 LP | 710/50 BP | **BV 711**, SB 702 |
| | B | | 735 LP | 740/35 BP | **BV 750** |
| | A | | 750 LP | 780/60 BP | **BV 786**, SB 780 |
| 488 nm 150 mW | G | | NA | 488/10 BP | SSC |
| | F | | 505 LP | 515/20 BP | **BB 515**, FITC, Alexa 488, NovaBlue 510, GFP |
| | E | | 600 LP | 610/20 BP | **BB 630**, NovaBlue 610 |
| | D | | 635 LP | 670/30 BP | **BB 660**, PerCP, NovaBlue 660 |
| | C | | 690 LP | 710/50 BP | **BB 700**, PerCP-e710, PerCP-Cy5.5 |
| | B | | 730 LP | 750/30 BP | **BB 750** |
| | A | | 770 LP | 810/40 BP | **BB 790** |
| 561 nm 150 mW | E | | 570 LP | 586/15 BP | **PE**, tdTomato, DsRed |
| | D | | 600 LP | 610/20 BP | **PE-CF594**, PE-Dazzle594, PE-e610, mCherry, PI |
| | C | | 650 LP | 670/30 BP | **PE-Cy5**, 7-AAD |
| | B | | 690 LP | 710/50 BP | **PE-Cy5.5**, NovaYellow 700 |
| | A | | 750 LP | 780/60 BP | **PE-Cy7** |
| 637 nm 140 mW | C | | 650 LP | 670/30 BP | **APC**, af647, DRAQ7, TO-PRO-3, Live/Dead far-red |
| | B | | 690 LP | 710/50 BP | **Alexa 700**, DRAQ5 |
| | A | | 750 LP | 780/60 BP | **APC-Cy7**, Zombie NIR, Live/Dead near-IR |

17

First, we need to know the cytometer's lasers, number of parameters, and filter configuration.

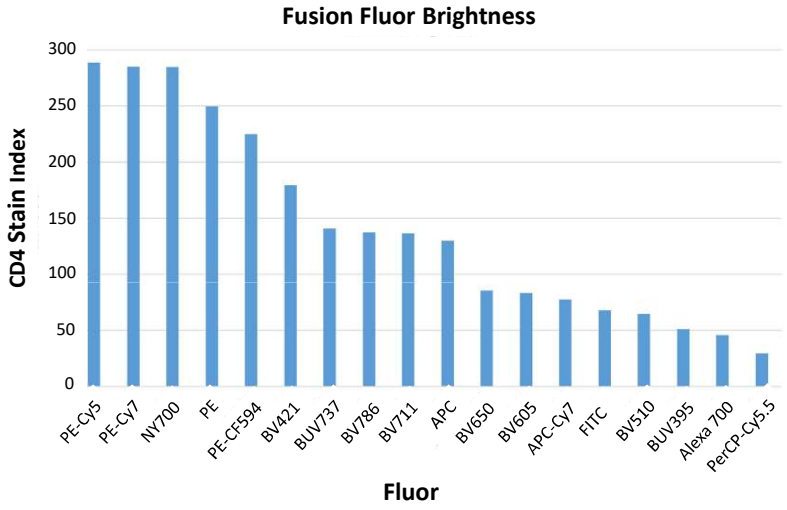
- It's important to know which lasers an instrument has to ensure you select fluors that can be excited by the lasers on the cytometer you plan to use. For example, if an instrument doesn't have a UV laser, you can't use Alexa Fluor 350 which is only excited by the UV laser.
- Be sure to select a cytometer that has enough parameters for your experiment. Shown here are laser and filter configurations for two of the RFCF's instruments: both have 5 lasers, but one has 18 parameters while the other has 31.
- different instruments have different default filter configurations, and the default filter may not be the best for a fluor you would like to use, so when designing a panel, it's important to know in advance if you can or need to swap filters to a more optimal configuration for a fluor you select.

It's also helpful to know if a panel can be used on more than one instrument. A panel designed for the instrument shown here with 18 parameters can probably be used on the instrument with 31 parameters, but a panel designed for the instrument with 31 parameters might not be able to be used on the instrument with 18 parameters.

Next, I'll go through cytometer resources that are very helpful in designing a panel.

# 2. Cytometer

The cytometer's Fluorochrome Stain Index (brightness) table




18

This is an example of something called a Stain Index or Brightness Index. This is:

-Basically, a ranking of the brightness of different fluors
- The larger the stain index, the brighter the fluor.

This table is on the RFCF website for most sorting instruments, the A5 SE analyzer, and all the Cytek analyzers but not the Fortessas or Cantos

Not every fluor that is available on the market is on the tables because there are so many, it isn't possible to do that. However, if you have a CD4 antibody with a fluorochrome you would like to know the brightness for, we can look at that for you on the instruments you're interested in.

These indices can be specific to an instrument or generic. If we don't have one specific for the instrument you are interested in using, you can find generic fluor brightness online...

---

EXTRA: technically, **the brightness is the ratio of the separation between the positive population and the negative population, divided by two times the standard deviation of the negative population**.

# 2. Cytometer

If the Brightness or Stain Index table is not available for the specific instrument, companies may have one.

**BioLegend Flow Cytometry Fluorophore Guide**

| Fluorophore | Emission Max | Similar Fluorophores | Brightness Index |
|---|---|---|---|
| Spark UV™ 387 | 387 nm | BUV395™ | ●●○○○ |
| Brilliant Violet 421™ | 421 nm | Pacific Blue™, V450, eFluor® 450, AF405 | ●●●●● |
| Spark Violet™ 423 | 415 nm, 423 nm | Super Bright 436 | ●●●○○ |
| Pacific Blue™ | 455 nm | BV421™, V450, eFluor® 450, AF405 | ●○○○○ |
| Brilliant Violet 510™ | 510 nm | V500, AmCyan, Pacific Orange™ | ●●○○○ |
| Spark Violet™ 538 | 538 nm | Pacific Orange™, Krome Orange™ | ●○○○○ |
| Brilliant Violet 570™ | 570 nm | - | ●●○○○ |
| Brilliant Violet 605™ | 603 nm | Qdot 605 | ●●●●○ |
| Brilliant Violet 650™ | 645 nm | Qdot 655 | ●●●●○ |
| Brilliant Violet 711™ | 711 nm | Qdot 705 | ●●●●● |
| Brilliant Violet 750™ | 750 nm | - | ●●●○○ |
| Brilliant Violet 785™ | 785 nm | Qdot 800 | ●●●●○ |
| Alexa Fluor® 488 | 519 nm | FITC, BD Horizon Brilliant™ Blue 515 | ●●●○○ |
| KIRAVIA Blue 520™ | 520 nm | FITC, AF488, BD Horizon Brilliant™ Blue 515 | ●●●●○ |
| FITC | 525 nm | AF488, BD Horizon Brilliant™ Blue 515 | ●●●○○ |
| Spark Blue™ 550 | 540 nm | AF532 | ●○○○○ |
| Spark Blue™ 574 | 574 nm | - | ●○○○○ |
| PerCP | 675 nm | - | ●○○○○ |
| PerCP/Cyanine5.5 | 690 nm | PerCP-eFluor™ 710, BD Horizon Brilliant™ Blue 700 | ●●○○○ |
| PE | 575 nm | - | ●●●●● |
| PE/Dazzle™ 594 | 610 nm | BD Horizon™ PE-CF594, ECD, PE-Texas Red®, AF594 | ●●●●● |
| PE/Fire™ 640 | 639 nm | - | ●●●●○ |
| PE/Cyanine5 | 670 nm | - | ●●●●● |
| PE/Fire™ 700 | 695 nm | PE/Cyanine5.5 | ●●●●● |
| PE/Cyanine7 | 774 nm | - | ●●●●○ |
| PE/Fire™ 810 | 806 nm | - | ●●●●○ |
| Spark YG™ 581 | 581 nm | cFluor® YG584 | ●●○○○ |
| Spark YG™ 593 | 593 nm | BD Horizon RealYellow™ 586 | ●●●○○ |
| APC | 660 nm | AF647, eFluor™ 660 | ●●●●○ |
| Alexa Fluor® 647 | 668 nm | APC, eFluor™ 660 | ●●●●○ |
| Spark NIR™ 685 | 685 nm | AF660 | ●●●○○ |
| Spark Red™ 718 | 711 nm | AF700, BD Horizon™ Red 718, APC-R700 | ●●●●○ |
| Alexa Fluor® 700 | 719 nm | Spark Red™ 718, BD Horizon™ Red 718, APC-R700 | ●●○○○ |
| APC/Cyanine7 | 774 nm | APC-H7, APC-eFluor™ 780 | ●●○○○ |

Like this one from BioLegend. However, since these are generic values, the actual brightness on the instrument you use may be different than what the information in the generic tables because of instrument specific laser power, laser wavelength, filters, etc.

For example, on this generic chart, BV421 is stated to be really bright but if we look back (go back to previous slide) at Fusion Brightness index at previous slide, BV421 is bright on this instrument but not as bright as other options.

---

**VERY IMPORTANT NOTE:** please note that the **values may vary for your particular instrument** because effected by laser wavelength/power and  used.

The stain index is **the ratio of the separation between the positive population and the negative population, divided by two times the standard deviation of the negative population**.

# 2. Cytometer

The cytometer's fluorochrome Spillover Spread matrix (or Spreading Error matrix)

**Spread Receiver (Filter)**

| Spread **Donor** (Fluor) | BUV396 | BUV737 | BV421 | BV510 | BV605 | BV650 | BV711 | BV786 | FITC | PCP-Cy5.5 | PE | PE-CF594 | PE-Cy5 | PE-Cy5.5 | PE-Cy7 | APC | Alexa 700 | APC-Cy7 | sum |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BUV 395 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BUV 737 | 0 | 0 | 0 | 0 | 0 | 19 | 56 | 68 | 0 | 192 | 0 | 0 | 15 | 50 | 52 | 28 | 260 | 78 | 818 |
| BV 421 | 0 | 0 | 0 | 10 | 14 | 0 | 7 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 37 |
| BV 510 | 0 | 15 | 0 | 0 | 54 | 41 | 34 | 32 | 7 | 14 | 18 | 20 | 0 | 8 | 0 | 21 | 49 | 0 | 312 |
| BV 605 | 0 | 27 | 0 | 0 | 0 | 83 | 83 | 59 | 0 | 75 | 69 | 90 | 96 | 53 | 32 | 52 | 114 | 0 | 835 |
| BV 650 | 0 | 36 | 10 | 0 | 53 | 0 | 109 | 79 | 0 | 62 | 19 | 32 | 92 | 50 | 28 | 122 | 196 | 25 | 913 |
| BV 711 | 0 | 71 | 17 | 0 | 12 | 35 | 0 | 157 | 0 | 160 | 0 | 0 | 41 | 71 | 53 | 70 | 461 | 68 | 1216 |
| BV 786 | 0 | 30 | 21 | 0 | 9 | 9 | 31 | 0 | 0 | 20 | 0 | 0 | 0 | 0 | 35 | 0 | 44 | 44 | 243 |
| FITC | 0 | 0 | 0 | 0 | 9 | 0 | 0 | 7 | 0 | 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 |
| PerCP-Cy5.5 | 0 | 23 | 0 | 0 | 6 | 28 | 49 | 46 | 0 | 0 | 0 | 0 | 43 | 50 | 35 | 55 | 118 | 24 | 479 |
| PE | 0 | 9 | 0 | 0 | 37 | 28 | 25 | 13 | 10 | 119 | 0 | 120 | 104 | 62 | 28 | 28 | 29 | 0 | 612 |
| PE-CF594 | 0 | 18 | 0 | 0 | 52 | 42 | 51 | 22 | 0 | 237 | 90 | 0 | 206 | 117 | 66 | 44 | 65 | 0 | 1011 |
| PE-Cy5 | 0 | 44 | 10 | 0 | 25 | 86 | 139 | 57 | 0 | 938 | 35 | 27 | 0 | 274 | 155 | 323 | 363 | 62 | 2539 |
| NovaYellow 700 | 0 | 18 | 0 | 0 | 9 | 35 | 71 | 17 | 0 | 339 | 62 | 44 | 236 | 0 | 104 | 264 | 1092 | 69 | 2362 |
| PE-Cy7 | 0 | 24 | 0 | 0 | 9 | 0 | 10 | 97 | 0 | 60 | 31 | 18 | 18 | 32 | 0 | 0 | 0 | 42 | 342 |
| APC | 0 | 27 | 0 | 0 | 9 | 31 | 34 | 24 | 0 | 93 | 0 | 11 | 170 | 66 | 41 | 0 | 276 | 41 | 824 |
| Alexa 700 | 0 | 20 | 0 | 0 | 0 | 0 | 25 | 21 | 0 | 34 | 0 | 0 | 24 | 38 | 35 | 25 | 0 | 45 | 268 |
| APC-Cy7 | 0 | 17 | 0 | 0 | 0 | 0 | 7 | 46 | 0 | 10 | 0 | 0 | 38 | 19 | 98 | 62 | 71 | 0 | 368 |
| | | | | | | | | | | | | | | | | | | | 8437 |

20

This is a:
- Spillover Spread Matrix (or Spreading Error Matrix). This shows which fluors will be detected by filters that are not it's main, primary filter which will then lead to population resolution problems.

How is this read? How do we use this?

- The fluors in the rows, labeled DONOR, spread into the filters for the fluors in the columns, labeled RECEIVER. It is a fluor spreading into a filter. Even though the columns are labeled as fluors, they are representative of filters.

- The combinations in red are combinations you want to avoid particularly for co-expressed markers. For example, PE-Cy5 spreads into the filter for PerCP-Cy5.5 a lot, that combination is red in about the middle of the table, so you don't want to have PE-Cy5 and PerCP-Cy5.5, or a different fluor that uses the PerCP-Cy5.5 filter as its primary filter, in the same panel if possible, but especially don't put them on co-expressed markers
- However, it's interesting to note that for this instrument PerCP-Cy5.5 doesn't spread into the filter for PE-Cy5 so spread doesn't always go both ways.

- There are two types of these tables: SSM (Spillover Spread Matrix) table and TSM (Total

Spread Matrix) tables. Either can be used; the TSM is usually more accurate, so if it's available, use a TSM.

- These can be instrument specific or generic, as well. Like with the Brightness or Stain Index, the RFCF has this resource for many instruments but not the Fortessa and Canto analyzers, so you can use a generic one keeping in mind the actual spread may be different on the instrument you use.

---

- The SSM represent the percentage of a signal that spills into another channel.
- The TSM tables also represent the percentage of spill into another channel but also takes into consideration of the fluorescent intensity of the fluor. Some feel more accurate

Note to self:
Don't get into difference between SSM and TSM unless someone asks—TSM takes fluor brightness into account which makes it more accurate than SSM
- Spread impacted by brightness of fluor and antigen density.
- Just because a fluor has spread, do not necessarily have to not use but use for mutually exclusive marker.
- If must use fluor that has spread, use a dim fluor to minimize spread impact.
- Far reds tend to have more spreading and are generally more expensive.

**What is a Total Spread Matrix (TSM)?**
While designing multicolor flow cytometry panels, it is important to assess fluorescence spillover to prevent or minimize loss of resolution due to spreading. The Spillover Spread Matrix (SSM) was developed as a tool to monitor and compare instrument performance over time, especially when experiments are standardized or calibrated across different instruments.(1)
The SSM is independent of fluorochrome brightness. While this feature is important for the comparison of instruments, it may lead to inaccurate spread prediction and sub-optimal panel design. The Total Spread Matrix (TSM) is the Spillover Spreading Matrix without normalization to the fluorescent intensity of the probe. This makes it possible to evaluate the true spreading in a given panel, based on the signal intensity of the different marker/fluorochrome combinations in each channel.
Please note for all tables shown below, the spread displayed is only found on the subpopulations that are positive for a given marker.
To view the instrument configuration on which these TSMs were collected, please scroll down.
Watch the webinar to learn more about TSM

https://lp.bd.com/202309-BDB23-EU_EN-Horizon_Tour-TSM_ebook-LP_LP-EN-01-MainLP.html?utm_source=ebook&utm_medium=text&utm_campaign=202309-BDB23-EU_EN-Horizon_Tour-TSM_ebook-LP

# 2. Cytometer

**CCHMC Analytic Cytometers**

| Instrument Name on Equipment Booking System* | Instrument Make/ Model** | Location*** | Number of Fluorescence PMTs: UV (355nm) | Violet (405nm) | Blue (488nm) | Yellow-Green (561nm) | Red (640nm) |
|---|---|---|---|---|---|---|---|
| Canto 1 | BD/FACSCanto | R5087A | | | 2 | 4 | 3 |
| Canto 2 | BD/FACSCanto II | R5087A | | 2 | 4 | | 2 |
| Canto 3 | BD/FACSCanto | R5087A | 3 | 5 | | 3 | |
| Fortessa 1 | BD/LSRFortessa | R5524 | 2 | 6 | 2 | 5 | 3 |
| Fortessa 2 | BD/LSRFortessa | R5524 | 2 | 6 | 2 | 5 | 3 |
| Fortessa 3 | BD/LSRFortessa | R5524 | 2 | 6 | 2 | 5 | 3 |
| A5 SE | BD/FACSymphony | R5524 | 10 | 14 | 9 | 9 | 6 |
| Aurora | Cytek/Aurora | R5509 | 16 | 16 | 14 | 10 | 8 |
| Maleficent | Cytek/Aurora | R5509 | 16 | 16 | 14 | 10 | 8 |
| Diablo | Cytek/Northern Lights | R5509 | | 16 | 14 | | 8 |

*Click on the instrument name for the configuration.

21

This is a table of the RFCF's Analytic Cytometers from website. For each analyzer, it shows:
- the available lasers and the number of filters on each laser.

**DEMO:** If you click on the instrument name in the table, you will get a PDF of the instrument configuration, which has the lasers and default filter information. For the bottom 4 instruments on this table, the brightness and spread matrices will be in the same PDF.

If you are interested in sorting, the RFCF has another table for the sorters with the same information plus the number of populations that can be collected by each sorter. Again, if you click on the instrument name you find the configuration of the instrument for all instruments, and, if we have it, the stain index and spread matrix. We do have the stain index and spread matrices for most of the sorters.

---

Extra

- Maybe the gate limitation of the cytometer. Aria's can you have a gating strategy using up to 8 consecutive gates. If you need more than 8 gates for your analysis strategy, you may need to choose a different cytometer which may have a different configuration for the lasers and s. The A5 SE can have as many as 16 consecutive gates.

If sorting, it is important to know the number of populations to collect and how many populations the cytometer can collect simultaneously. Aria's can collect up to 4 population at time when using FACs or microfuge tube. The S6 can sort up to 6 populations at a time if using FACS tubes or microfuge tubes for collection.

# 3. Assigning Fluorophores

22

Now that we have our information gathered, we are going to talk about assigning fluors.

# 3. Assigning fluorophores

**A**. Make note of fluorescent proteins (GFP, mCherry, RFP) so these filters are not used for other markers. Use a Panel Matrix to track your progression

**Panel Matrix**

| | UV 355 | | V 405 | | B 488 | | YG 561 | | R 640 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Emission | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor |
| 379/28 | | | | | | | | | | |
| 431/28 | | | | | | | | | | |
| 450/50 | L/D | DAPI | | | | | | | | |
| 470/15 | | | | | | | | | | |
| 488/10 | | | | | | | | | | |
| 515/30 | | | | | | GFP | | | | |
| 586/15 | | | | | | | CD95 | PE | | |
| 610/20 | | | | | | | | | | |
| 670/30 | | | | | | | | | Lin | APC |
| 710/50 | | | | | | | | | | |
| 740/35 | | | | | | | | | | |
| 750/30 | | | | | | | | | | |
| 780/60 | | | | | | | CD45RA | PE-Cy7 | | |
| 820/60 | | | | | | | | | | |

Dark grey indicates no filter for that laser.

**FACSAria Fusion: Laser and filter configuration**

| Laser | Detector | Long Pass | Band Pass | Optimal fluors |
|---|---|---|---|---|
| 355 nm 60 mW | B | 410 | 450/50 | DAPI, Hoechst blue, Zombie UV, Live/Dead blue |
| | A | 635 | 670/14 | Hoechst red |
| 405 nm 100 mW | F | NA | 450/50 | BV 421, Pacific blue, eFluor 450, Alexa 405 |
| | E | 505 | 525/50 | BV 510, V500, eFluor 506, AmCyan, Live/Dead aqua |
| | D | 600 | 610/20 | BV 605, SB 600 |
| | C | 635 | 670/30 | BV 650, SB 645 |
| | B | 690 | 710/50 | BV 711, SB 702 |
| | A | 750 | 780/60 | BV 786, SB 780 |
| 488 nm 100 mW | B | 505 | 530/30 | FITC, Alexa 488, BB 515, GFP, YFP, Venus |
| | A | 690 | 710/50 | PerCP, PerCP-Cy5.5**, PerCP-e710, BB 700 |
| 561 nm 150 mW | E | 580 | 586/15 | PE, tdTomato, DsRed |
| | D | 600 | 610/20 | PE-CF594, PE-Dazzle594, PE-e610, mCherry, PI* |
| | C | 635 | 670/30 | PE-Cy5, 7-AAD* |
| | B | 690 | 710/50 | PE-Cy5.5**, NovaYellow 700 |
| | A | 750 | 780/60 | PE-Cy7 |
| 637 nm 100 mW | C | 665 | 670/30 | APC, Alexa 647, DRAQ7, Live/Dead far-red |
| | B | 690 | 710/50 | Alexa 700, DRAQ5 |
| | A | 755 | 780/60 | APC-Cy7, Zombie NIR, Live/Dead near-IR |

23

One of the 1st things to do when assigning fluor is to make note of your fluorescent proteins that are being used.
And begin to fill out a Panel Matrix.

A Panel Matrix can be helpful when assigning fluors.

- On left are the filters used in the instrument and the lasers are across the top.
- When you see the same filter for different lasers, note that there is a filter for each laser. ie. 670/30 filter: There are 5 - 670/30 filters - one for each laser.
- Dark grey blocks indicate that that filter is not available for that laser.
- Place the Marker and the fluors in the matrix as you are assigning the fluors and it allows you to see if there might be problems due to overlap, spread.
    - Fluors that are dectected by the same wavelength filter (in the same row) or excited by the same laser (in the same column) may cause spread or spillover problems.
- This matrix helps keep track of the filters, lasers, and flours you are using.

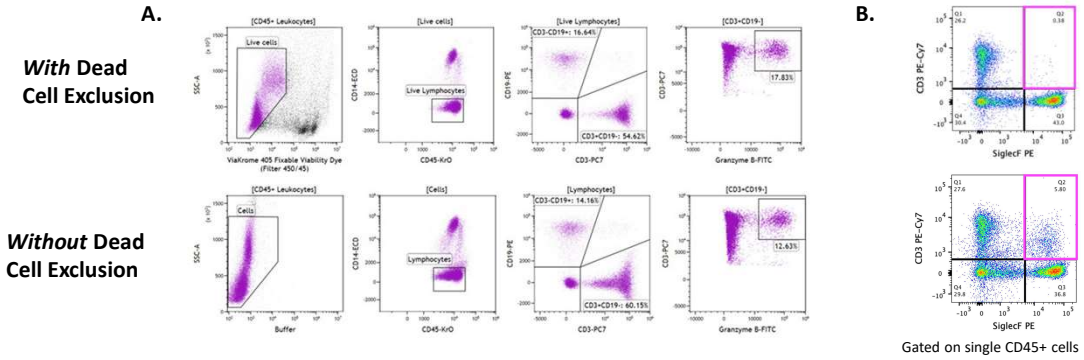
---

Note: - You can make a matrix using the instrument configuration or if you use a panel builder online site some of them will make one for you. You can also line up the spectra for each laser.

# 3. Assigning fluorophores

**B**. Assign viability dye:

Some viability dyes stain the DNA in dead cells and only work on unfixed cells (PI, DAPI, 7AAD, DRAQ-7). Zombie dyes can be used on fixed and unfixed cells. These dyes stain dead cells while other dyes stain live cells.




Beckman Coulter: https://www.beckman.com/reagents/coulter-flow-cytometry/cell-health-research-assays/viakrome-fixable-viability-dyes/why
University of Chicago: https://voices.uchicago.edu/ucflow/2020/11/19/how-to-identify-problems-with-panel-design-bad-data-part-2/

24

Next, assign a viability dye,
Viability dyes are good because they get rid of
- dead cells that **bind nonspecifically** with antibodies.
  - This causes false positives
- Reduces **autofluorescence** from dead cells that can also interfere with analysis.
-Also, gets rid of **artifacts** from dead cells that interfere with resolution of rare populations.
- **Not using a viability dye can change the statistics and lead to you making the wrong conclusions from your experiments because your population may include dead cells.**

There are different kinds of viability dyes.
- Some bind DNA in dead cells and the dead cells are positive.
- Some dyes stain live cells.
- Some dyes can with stand fixing process and others will not. Check this before use.

**Figure A.**
**Top Panel:** PBMCs analyzed with a viability dye
**Bottom Panel:** PBMCs analyzed without a viability dye.
- Notice the populations in all the plots in the top row are tighter, there is less spreading when a viability dye is used.

- The live lymphocyte population in upper panel is smaller (54%) than the lymphocyte population in the lower panel (60%). **However, they are all live in the upper panel.**
    - **Not using a viability dye will change the statistics and can lead to you making the wrong conclusions from the experiment.**

**Figure B.**
**-** In the lower image without a viability dye, a pink box highlights a population that should not be biologically possible without a viability dye. These would be considered false positives and affects the population percentages. Without the viability dye, this population is 5% of the cells and this affects all the populations percentages.
- See that the population does not exist when a viability dye is used.

---

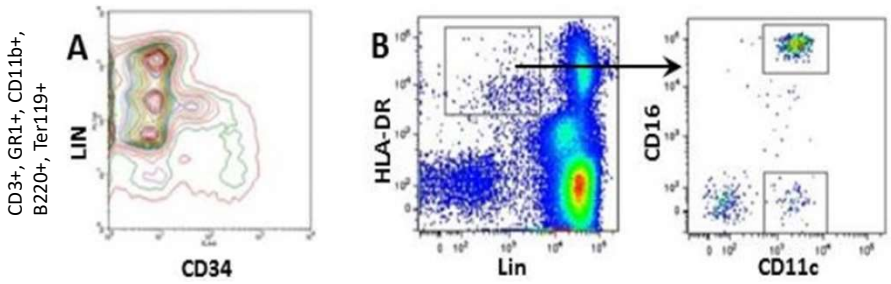
- However, overstaining with the viability dye can reck havoc on analysis

**Comparison of Sample Analysis Without and With Dead Cell Exclusion.** Thawed PBMCs were stressed by heat (55 °C for 10 minutes) prior to immunostaining staining without (A) or with (B) the addition of ViaKrome 405 Fixable Viability Dye. Cells were then processed with Perfix-nc Cellular Staining Preparation Kit (Part Number B10825) and stained with Granzyme B-FITC, CD19-PE, CD14-ECD, CD79a-PC5.5, CD3-PC7 and CD45-Krome Orange. Data was acquired using the CytoFLEX LX N-V-B-Y-R-I series flow cytometer and analyzed with Kaluza Analysis software. Gate statistics show the percent of the parent population and differ in the two conditions showing the effect of eliminating dead cells prior to sequential gating.

# 3. Assigning fluorophores

**C.** Assign dump or lineage channel to remove unwanted cells by labeling them all with one fluor (use any available channel/filter). Especially helpful when focusing on a rare population.

**Using lineage markers to help define rare populations**




Biolegend: https://www.biolegend.com/fr-lu/blog/key-sample-considerations-for-flow-cytometry-staining

25

It is very helpful if an enrichment of the cells of interest or a depletion of unwanted cells can be done.

- Especially helpful when focusing on a rare population.
- Some people use magnetic beads during the cell preparation to enrich or deplete for their cells of interest.
    - This is helpful, because you have fewer cells that the cytometer has to analyyze which will decrease the volume that needs to be analyzed this in turn means the samples will not take so long to run and the sample can be done sooner
    - This also usually increases the efficiency that the instrument operates at allowing for better data.

Using linage markers is like doing another enrichment or depletion and is very helpful to define a rare population.

A linage or dump channel can be made in a number of ways but basically all unwanted cells are labeled with a single fluor.

- Any available channel can be used when assigning a dump or lineage channel
- Generally, want to use a cheap fluor like FITC that has little overlap with other fluors.
- Do not use tandem dyes for linage markers because they are problematic with

degradation artifacts.
- Often used to analyze HSC (Hemopoietic stem cells) in Bone marrow. The HSCs are lin-

- Some make a dump channel by using biotin/streptavidin/fluor (FITC) to remove the unwanted cells .
- Some use linage markers characteristic to certain cell lineages for a dump channel.
- Some people make their own combination of markers for the dump channel while others use cocktails made by companies.

In the plots here See
A) Staining of hematopoietic progenitors using BioLegend mouse lineage cocktail which has antibodies that recognize CD3, GR1, CD11b, B220, and Ter119 in mouse bone marrow and all of the antibodies have the same fluor.
See that the lineage markers help separate out unwanted cells from the CD34 + cells.
B) Gating of human Dendritic Cells using BioLegend human lineage cocktail which has antibodies that recognize Human CD3, CD14, CD19, CD20, CD56 and human HLA-DR. Gating out the lineage cells helps to identify the CD11c cells and the Double positive CD16+CD11c+ population in the second plot.

---

Extra Note for self:
Some lymphoid tissues contain rare immune cell subsets (i.e. dendritic cells, innate lymphoid cells) and hematopoietic progenitors. To focus on rare populations such as bone marrow progenitors, gate out other populations by including lineage markers in your multicolor panel. Lineage markers are expressed only on terminally differentiated cells and include antigens such as Ter119 (erythrocytes), CD3 (T cells), CD19 (B cells), B220 (B cells), CD11b (myeloid cells), CD11c (Dendritic cells), etc. Cells positive for any of these markers can be grouped together on one "dump channel", minimizing the use of multiple fluorophores (Fig.1a and b). For your convenience, BioLegend offers wide variety of mouse and human Lineage Cocktails.

Examples of myeloid lineage markers include **pan-myeloid marker CD11b, CD206 for M2-type macrophages, CD68, and CD15 for neutrophils**.

# 3. Assigning fluorophores

**D**. Make note of any restrictions:

1. Preferred Ab-Fluor combinations in stock.
2. A very rare marker that is available with only one fluorophore option.
   (example: marker x is available with only PE). This would have to be assigned 1st.

Panel Matrix

| | UV 355 | | V 405 | | B 488 | | YG 561 | | R 640 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Emission | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor |
| 379/28 | | | | | | | | | | |
| 431/28 | | | | | | | | | | |
| 450/50 | | | | | | | | | | |
| 470/15 | | | | | | | | | | |
| 488/10 | | | | | | | | | | |
| 515/30 | | | | | | GFP | | | | |
| 586/15 | | | | | | | Lin | PE | | |
| 610/20 | | | | | | | | | | |
| 670/30 | | | | | | | | | Live/Dead | Far Red |
| 710/50 | | | | | | | | | | |
| 740/35 | | | | | | | | | | |
| 750/30 | | | | | | | | | | |
| 780/60 | | | | | | | | | | |
| 820/60 | | | | | | | | | | |

Panel Matrix

| | UV 355 | | V 405 | | B 488 | | YG 561 | | R 640 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Emission | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor |
| 379/28 | | | | | | | | | | |
| 431/28 | | | | | | | | | | |
| 450/50 | L/D | DAPI | | | | | | | | |
| 470/15 | | | | | | | | | | |
| 488/10 | | | | | | | | | | |
| 515/30 | | | | | | GFP | | | | |
| 586/15 | | | | | | | CD95 | PE | | |
| 610/20 | | | | | | | | | | |
| 670/30 | | | | | | | | | Lin | APC |
| 710/50 | | | | | | | | | | |
| 740/35 | | | | | | | | | | |
| 750/30 | | | | | | | | | | |
| 780/60 | | | | | | | | | | |
| 820/60 | | | | | | | | | | |

Next want to make note of any restrictions:

1. Preferred Ab-Fluor combinations that are in stock or that work well for you.
2. A very rare marker that is available with only one fluorophore option.
   An example is marker x is available with only PE. This would have to be assigned 1st.

In 1st panel, the Green Fluorescent Protein is added in the blue laser, the linage fluor is added in the Yellow-Green laser, and the Live/Dead marker is added in the red laser.
- However, maybe see that we have an anti-CD95 Ab with PE and want to use it. So, change the lineage marker fluorescent tag to APC.
- If use APC for linage tag, that means a different Live/Dead Dye needs to be chosen and choose DAPI in the UV laser

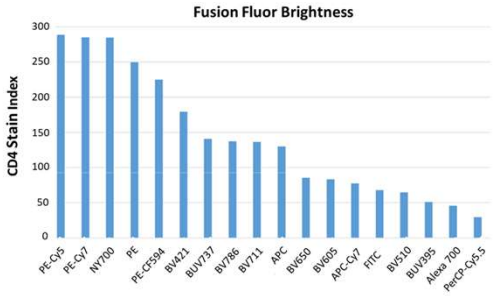
Assigned flours may have to be juggled and moved around in the matrix as things are added.
Again, the matrix allows you to keep track of those changes.

# 3. Assigning fluorophores

**E**. Assign fluorophores to:

**Tertiary** (3º) Antigens **first**
**Secondary** (2º) Antigens **next**
**Primary** (1º) Antigens **last**

- Assign bright fluors to low expressed (tertiary) antigens and dim fluors to high expressed (primary) antigens.
- Refer to instrument brightness index, available filters, and spread matrix.
- Save some of the brightest fluors in case the panel needs to be expanded later.




Panel Matrix

| | UV 355 | | V 405 | | B 488 | | YG 561 | | R 640 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Emission | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor |
| 379/28 | | | | | | | | | | |
| 431/28 | | | | | | | | | | |
| 450/50 | L/D | DAPI | | | | | | | | |
| 470/15 | | | | | | | | | | |
| 488/10 | | | | | | | | | | |
| 515/30 | | | | | | GFP | | | | |
| 586/15 | | | | | | | CD95 | PE | | |
| 610/20 | | | | | | | | | | |
| 670/30 | | | | | | | | | Lin | APC |
| 710/50 | | | | | | | | | | |
| 740/35 | | | | | | | | | | |
| 750/30 | | | | | | | | | | |
| 780/60 | | | | | | | | | | |
| 820/60 | | | | | | | | | | |

27

Once the restrictions are noted, want to start assigning fluorophores according to antigen classification.

Refer to the Panel Design Form you filled out to track the Markers and Fluors.

Assign fluors to the tertiary, then secondary and primary markers last.
- Assign bright fluors to low expressed (tertiary) antigens and dim fluors to high expressed (primary) antigens.
- Consult **brightness index**, spread matrix, and the list of the **cytometer filters** to assign fluors.
- Try to assess impacts of fluors on other markers as progress from tertiary, secondary and then primary markers. (will get better at this with time)
- Fill out the panel matrix as you progress.

---

# 3. Assigning fluorophores

**F**. Evaluate spread on co-expressed markers.

- Assign fluorophores with minimal spillover into each other using a spillover spread matrix or a spectral viewer.
- If using fluors on the same laser for co-expressed markers, spread the fluors out over the filters.

**Spread Receiver (filter)**

**Spread Donor (Fluor)**

**Panel Matrix**

| | UV 355 | | V 405 | | B 488 | | YG 561 | | R 640 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Emission | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor |
| 379/28 | | | | | | | | | | |
| 431/28 | | | | | | | | | | |
| 450/50 | L/D | DAPI | | | | | | | | |
| 470/15 | | | | | | | | | | |
| 488/10 | | | | | | | | | | |
| 515/30 | | | | | | GFP | | | | |
| 586/15 | | | | | | | CD95 | PE | | |
| 610/20 | | | | | | | | | | |
| 670/30 | | | | | | | | | Lin | APC |
| 710/50 | | | | | | | | | | |
| 740/35 | | | | | | | | | | |
| 750/30 | | | | | | | | | | |
| 780/60 | | | | | | | | | | |
| 820/60 | | | | | | | | | | |

28

As assign fluors, evaluate co-expressed markers fluor assignment.

a. Check to see that Assigned fluorophores have minimal spillover into each other (use spillover spread matrix or spectral viewer which we will discuss shortly)
b. Check that fluors on the same laser are spread out over the filters.
   - ie. Avoid filters that are right next to each other.

c. Avoid using flours that use a filter of the same wavelength even if on different lasers (ie. In same row) in case there is cross laser excitation.
d. If only fluors available have spread, use a dim fluor to minimize spread.

---.

# 3. Assigning fluorophores

**G**. As number of markers increases, continue to spread out the fluors on the lasers and filters noting expression levels and brightness of the fluor.

- Consult the previously suggested resources: panel design form, panel matrix, brightness index, spread matrix

**Panel Design Form**

| Marker | Cell type Expressing the Marker | Location | Antigen Classification (1, 2, 3) | Expression Density | Co-Expression | Fluor (proposed) |
|---|---|---|---|---|---|---|
| CD11b/lin | Myeloid cells | Extracellular | 1 | high | | |
| CD56/lin | NK cells | Extracellular | 1 | high | | |
| CD19/lin | B cells | Extracellular | 1 | high | | |
| CD3+ | T cells | Extracellular | 1 | high | CD95+ | |
| CD95 | Naïve T-cells | Extracellular | 3 | med | CD3+ | PE |

Panel Matrix

| | UV 355 | | V 405 | | B 488 | | YG 561 | | R 640 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Emission | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor |
| 379/28 | | | | | | | | | | |
| 431/28 | | | | | | | | | | |
| 450/50 | L/D | DAPI | | | | | | | | |
| 470/15 | | | | | | | | | | |
| 488/10 | | | | | | | | | | |
| 515/30 | | | | | | GFP | | | | |
| 586/15 | | | | | | | CD95 | PE | | |
| 610/20 | | | | | | | | | | |
| 670/30 | | | | | | | | | Lin | APC |
| 710/50 | | | | | | | | | | |
| 740/35 | | | | | | | | | | |
| 750/30 | | | | | | | | | | |
| 780/60 | | | | | | | | | | |
| 820/60 | | | | | | | | | | |

29

As increase number of markers, continue to spread out the fluors on the lasers and filters
- Noting expression, fluor brightness, and tables and matrices discussed previously.
- Remember to keep some bright fluors unused so they are available if need to expand panel later.

---

Noted earlier:
- Consult **instrument** brightness index and/or fluorochrome brightness chart.
  - Assign bright fluors to low expressed (tertiary) antigens and dim fluors to high expressed (primary) antigens.
  - Do not use all the brightest fluors up. Reserve some so the panel can be expanded

Note to self:
- UV laser is dim and reagents are moderate to dim. Although there are new fluorochromes that allow better use of the UV laser.
- The brightness is different for different instruments so you can find a “Relative Brightness Index” from some supplier companies but remember it may vary for your specific instrument due to laser power and laser wavelength.

# 3. Assigning fluorophores

**H.** Assess spillover of all flours:

a. Check spread values on table or use a spectral viewer.

b. Note that Far Red fluors (emission > 700) tend to have more spreading.

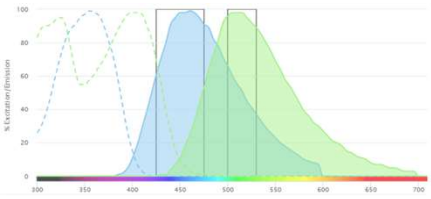
**Spread matrix**

**Spread Receiver (filter)**

**Spread Donor (Fluor)**

| Standardized settings | FITC 530/30 | PerCP-Cy5-5 710/50 | BV421 450/50 | BV510 525/50 | BV605 610/20 | BV650 670/30 | BV711 710/50 | BV786 780/60 | PE 586/15 | PE-CF594 610/20 | PE-Cy5 670/30 | PE-Cy5-5 710/50 | PE-Cy7 780/60 | APC 670/30 | APC-Alexa 700 710/50 | APC-Cy7 780/60 | BUV395 379/28 | BUV737 740/35 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mtC | | 34.9 | 56.3 | 17.4 | 34.7 | 54.5 | 56.8 | 57.9 | 51.9 | 51.9 | 26.4 | 25 | 53.6 | 58.5 | 35.9 | 53.6 | 21.2 | 51.4 |
| PerCP-Cy5-5 | 34.1 | | 66.8 | 30.3 | 34.7 | 27.7 | 45.6 | 41.3 | 31.4 | 31.9 | 47.8 | 48.9 | 31.5 | 53.8 | 113 | 25 | 21.7 | 23.4 |
| BV421 | 35.2 | 29.9 | | 20.7 | 36.8 | 34.7 | 36.8 | 37.9 | 31.9 | 33 | 27.2 | 25 | 34.1 | 39 | 36.9 | 34.1 | 22.3 | 31.4 |
| BV510 | 35.8 | 34.2 | 57.9 | | 46.2 | 39.7 | 35.3 | 32 | 20.1 | 22.8 | 27.2 | 25 | 33.6 | 28.2 | 58.2 | 34.1 | 21.2 | 17.4 |
| BV605 | 34.7 | 72.3 | 38.5 | 17.4 | | 77.2 | 71.2 | 56.5 | 66.3 | 76.7 | 88.5 | 52.2 | 31.5 | 52.7 | 106 | 34.7 | 21.2 | 28.2 |
| BV650 | 35.2 | 68 | 39.6 | 17.4 | 48.4 | | 94.7 | 70.7 | 22.3 | 23.9 | 84.8 | 50 | 28.8 | 108 | 180 | 24.6 | 22.3 | 33.7 |
| BV711 | 35.2 | 144 | 23.7 | 16.3 | 33.7 | 33.7 | | 137 | 11.9 | 11.9 | 41.1 | 61.4 | 49.4 | 62.5 | 365 | 64.7 | 22.3 | 63 |
| BV786 | 34.7 | 35.9 | 25.5 | 17.4 | 35.2 | 36.8 | 30.4 | | 11.9 | 11.9 | 27.7 | 25.5 | 33.1 | 22.8 | 92 | 38 | 21.7 | 29.2 |
| PE | 36.8 | 107 | 36.3 | 36.3 | 36.4 | 28.8 | 25.3 | 21.2 | | 109 | 99.6 | 54.3 | 28.8 | 32 | 46.9 | 34.1 | 21.7 | 34.7 |
| PE-CF594 | 34.7 | 231 | 36.8 | 36.3 | 45.9 | 39.7 | 45.1 | 27.2 | 79.4 | | 177 | 99.6 | 59.8 | 47.8 | 69.9 | 15.2 | 21.7 | 18.5 |
| PE-Cy5 | 35.2 | 694 | 20.1 | 36.9 | 23.4 | 76.7 | 133 | 51.1 | 33.1 | 26.6 | | 237 | 137 | 267 | 321 | 52.7 | 22.8 | 42.4 |
| BV650 | 35.2 | 286 | 36.8 | 36.9 | 35.7 | 33.3 | 60.3 | 23.9 | 50.5 | 37.5 | 252 | | 89.2 | 252 | 562 | 86 | 21.7 | 22.8 |
| PE-Cy7 | 35.2 | 58.7 | 17.9 | 35.8 | 34.1 | 34.7 | 20.3 | 82.7 | 28.8 | 20.6 | 33.7 | 35.8 | | 20.6 | 44 | 38.6 | 21.7 | 22.8 |
| APC | 35.2 | 84.3 | 33.8 | 36.3 | 33.2 | 90.4 | 33.7 | 27.2 | 11.9 | 11.9 | 146 | 62 | 40.7 | | 244 | 41.8 | 21.7 | 25 |
| Alexa 700 | 35.2 | 42.9 | 36.8 | 36.3 | 33.6 | 33.6 | 27.2 | 28.2 | 11.4 | 11.9 | 32.6 | 41.3 | 33.7 | 33.5 | | 41.8 | 21.2 | 22.3 |
| APC-Cy7 | 34.7 | 75.9 | 36.8 | 36.3 | 33.6 | 34.7 | 38.5 | 45.6 | 11.4 | 11.9 | 42.9 | 29.3 | 90.3 | 53.2 | 77.2 | | 21.2 | 58.5 |
| BUV395 | 35.2 | 29.9 | 36.3 | 36.9 | 33.6 | 33 | 36.8 | 17.4 | 11.4 | 11.9 | 26.6 | 23.9 | 34.1 | 17.9 | 36.4 | 33.6 | | 11.4 |
| BUV737 | 35.2 | 166 | 17.9 | 36.9 | 34.1 | 22.8 | 49.4 | 60.9 | 11.9 | 11.9 | 31.5 | 52.2 | 50 | 33.1 | 218 | 68.5 | 22.8 | |

**Spectral Viewer**




FluoroFinder: Introduction to Spectral Overlap and Compensation in Flow Cytometry
https://fluorofinder.com/newsletter-introduction-to-spectral-overlap-and-compensation-in-flow-cytometry/

30

Next:

Review and reassess the spillover of all fluors to see if there may be any conflicts.

- This can be done on the spread matrix or on a spectral viewer.

- This diagram is from a spectral viewer and shows the excitation and emission spectra of two flours and how the emission spectra may overlap.
    - Spectral Viewers are recommended because
        - they can be more accurate than a generic spread matrix
        - And you can add the specific fluor you are using which may not be on the spread matrix

---

Mentioned earlier by Krystal if you have the fluor you are interested in on an anti-CD4 antibody, we can check the spread matrix and the brightness index
for that particular fluor on a particular instrument.

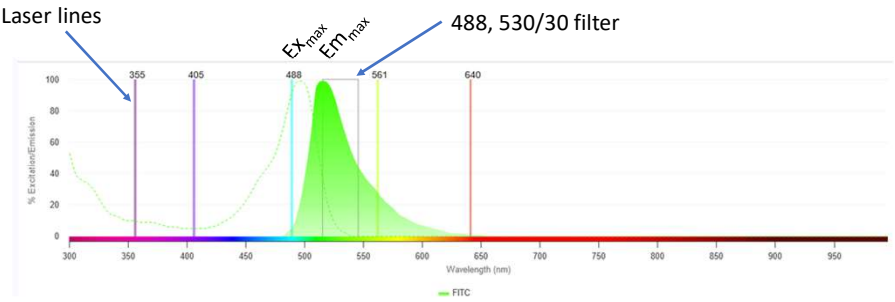
# 3. Assigning fluorophores

**Spectral Viewer:**

Dotted line = excitation profile
$EX_{max}$ = Maximum Excitation ƛ

Solid spectra = emission profile
$EM_{max}$ = Maximum emission ƛ

**FluoroFinder Spectral Viewer:**




**FITC $EX_{max}$ = 495 $EM_{max}$ = 520**

31

On many spectral viewers,

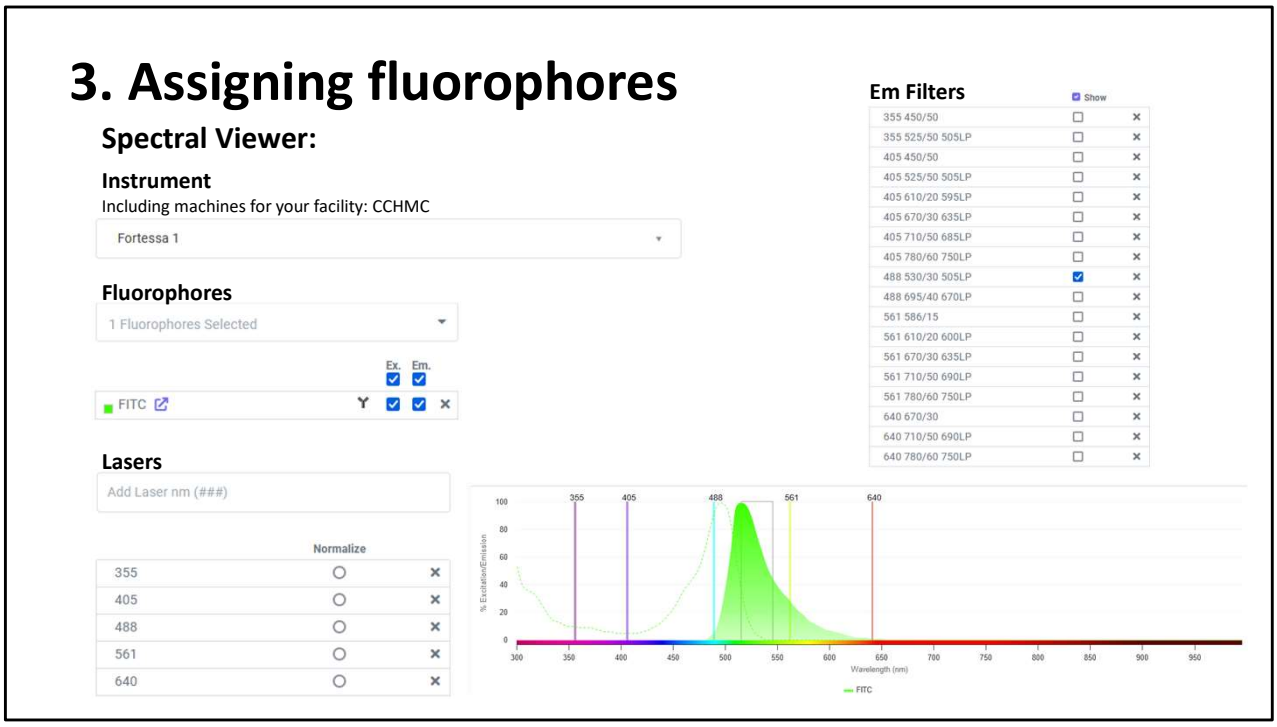
- The dotted line is the excitation profile.
    - FITC can absorb energy at all the wavelengths in the excitation spectra but absorbs best at the excitation maximum 495 nm which is closest to the Blue 488 nm laser. The FITC fluor will also be excited a little by the UV 355 nm laser and the Violet 405nm laser.

- The solid profile is the emission spectra of the FITC. FITC will fluoresce at all these wavelengths but its highest emission is at 520 nm.

•- Look at the excitation and emission spectra to determine which laser should be used to excite the fluorochrome and which filter to use to collect the signal.

- The box is the filter
    - You can see that the FITC emission will be captured by the blue 530/30 filter

# 3. Assigning fluorophores



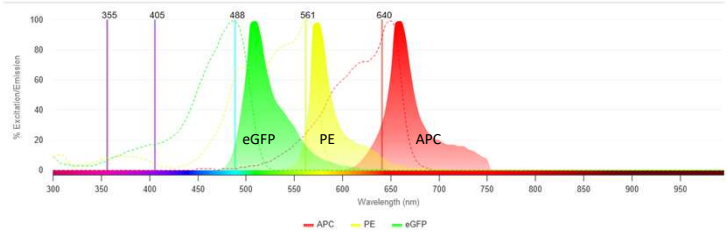

What to look at on a Spectral Viewer:
- I am using FluoroFinder program because I can choose my specific instrument at CCHMC. It shows the instruments lasers as lines, and I can choose the filters I want to use which is shown as the rectangle.
The laser and filter – explain more

-You can sign up for a free subscription to FluoroFinder and when you tell them your institution, you have access to the Children's instruments. Additionally, The RFCF has just purchased a site license for FluoroFinder which gives you access to an AI feature to help make panels, the program will make different matrices for you to help evaluate a panel such as a simplicity matrix and it will calculate a complexity score. The additional features are especially helpful with panels for conventional or spectral flow cytometry. Please email one of us or Celine Silva-Lages if you are interested in the advanced program on FluoroFinder.

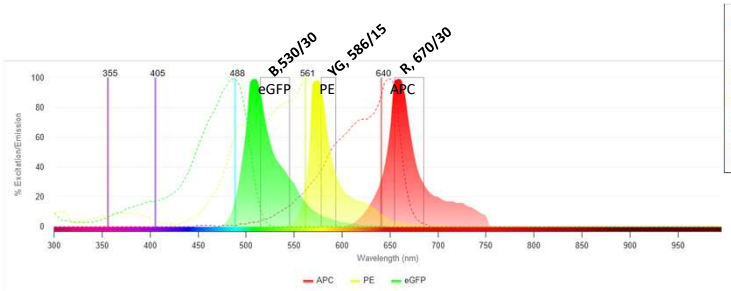
# 3. Assigning fluorophores

**Spectral Viewer: View APC (red), PE (yellow), eGFP (green)**

Ex, Em, Lasers




Ex, Em, Lasers, Filter




**eGFP**
$EX_{max}$ = 488 $EM_{max}$ = 507
**PE**
$EX_{max}$ = 565 $EM_{max}$ = 578
**APC**
$EX_{max}$ = 651 $EM_{max}$ = 660

33

Let's look at how the spectral viewers can be helpful when looking at multiple fluors in a panel. This is an example of 3 fluors, APC, PE and eGFP
The combination of eGFP, APC and PE is considered a pretty good combination of fluors because:
- The main excitation wavelength for each fluor is off of a different laser.
- Each of the fluors main emission wavelengths use different wavelength filters.

Let's see if there is spreading and spillover when using APC, PE and eGFP together.

If I look at only the top spectra, we see:
- eGFP is excited by the UV, Violet, and the Blue laser with the Blue laser being the primary wavelength of excitation. Its emission is 480 to 600
- PE is excited by the UV, Violet, Blue, and Yellow-Green is the primary excitation wavelength. It emission ranges from 550 to 670.
- APC is excited by the Blue, Yellow-Green laser and the Red laser is the primary laser of excitation. Its emission ranges from 625 to 750.
- This excitation by multiple lasers of different wavelengths is referred to as cross laser excitation and causes spillover of one fluors emission into the other fluors emission. If there is a lot of spillover, this indicates that a lot of compensation may be needed or that it will be difficult to see a rare population with two fluors that have a lot of spillover.

In the bottom panel, see:
- The primary filters for the fluors have been added.
- Let's look at eGFP;
    - although the tail of the emission peak extends into the PE 586 filter for the yellow green laser, the eGFP is not excited by the Yellow-Green laser (561). So, the spreading of the eGFP emission into the Yellow-Green 586 filter will have little to no affect on the detection of PE emission.
    - EGFP has very little to no fluorescence in the APC 760/30 filter and it is not excited by the 640 laser so it will not affect the detection of APC.

    Let's look at PE:
    - PE can be excited by the Blue and Yellow-green laser. And we see that PE emission spectra overlaps the eGFP and APC emission spectra. Even though PE is excited by the Blue lasers (488) , the emission spectra of the PE does not extend into the Blue laser 530 filter. Therefore, the PE will have little to no affect on eGFP detection.
    - The PE is not excited by the red laser so PE will not be detected by the red laser 670 filter therefore PE will have no affect on the detection of APC.

Lets look at APC:
- APC is excited by the Yellow –Green and Red lasers, and the emission spectra overlaps with the PE emission spectra. However, the emission spectra of the APC does not extend into the Yellow-Green 586 filter so the APC will have little to no affect on the PE detection.

- APC not activated by blue laser so affect on eGFP detection.

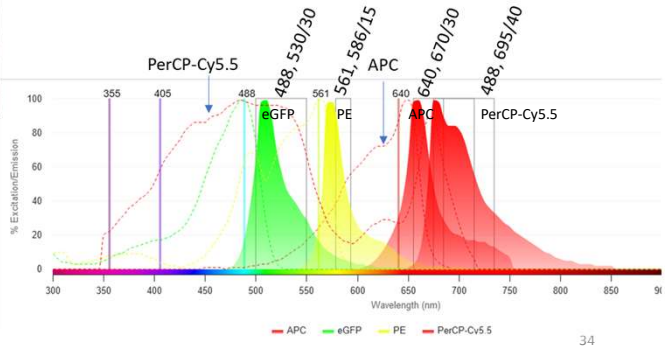
# 3. Assigning fluorophores

### Spectral Viewer: View

The Panel Matrix looks like the panel will be OK.
- Fluors are spread out across lasers
- Filters used are not next to one another or in same row

| Fortessa 1 | UV 355 | | V 405 | | B 488 | | YG 561 | | R 640 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Emission | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor |
| 450/50 | | | | | | | | | | |
| 488/10 | | | | | | | | | | |
| 525/50 | | | | | | | | | | |
| 530/30 | | | | | | eGFP | | | | |
| 586/15 | | | | | | | | PE | | |
| 610/20 | | | | | | | | | | |
| 670/30 | | | | | | | | | | APC |
| 695/40 | | | | | | PerCP-Cy5.5 | | | | |
| 710/50 | | | | | | | | | | |
| 780/60 | | | | | | | | | | |

The Spectral viewer shows a lot of spill over into the filters for the lasers that cross excite the PerCP-Cy 5.5.




eGFP: $EX_{max}$ = 488 $EM_{max}$ = 507
PE: $EX_{max}$ = 565 $EM_{max}$ = 578
APC: $EX_{max}$ = 651 $EM_{max}$ = 660
PerCP-Cy5.5: $EX_{max}$ = 482 $EM_{max}$ = 695

34

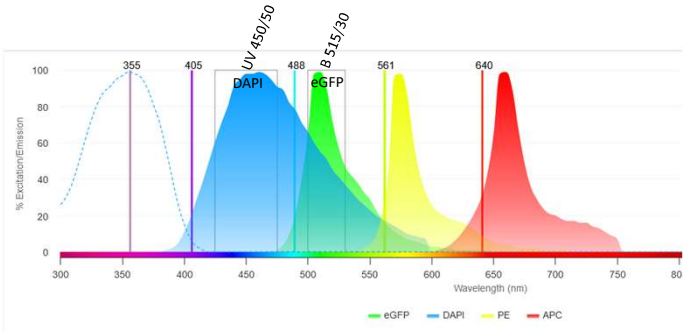
This example shows a bad combination of fluors for a panel.
- We previously showed that eGFP, PE and APC work pretty well together.
- Here we add PerCP-Cy5.5
- The Panel matrix looks like it might be a good choice because even though it is in the same laser as eGFP, the filter is far away.
- However, when we look at the spectra, PerCP-Cy5.5 spills into other filters a lot
- The PerCP-Cy5.5 excitation spectra shows that it can be excited by all of the laser, the UV laser, the Violet laser, the blue laser, the Yellow-green laser and the red laser.
- It emission spectra extends from 650 to 850 nm. This means that PerCP-Cy5.5 could spill into any filter on any laser that captures a wavelength in it emission spectra.
    - Ie. 670/30 filter on the Violet, the 670/30 on the yellow green and the 670/30 filter on the red laser.
    - The 710,/50 filters on those same lasers.
    - We do not have any fluors on the Violet or yellow green laser that are emitting into the 670/30 filter, but we do have APC off of the red laser.
    - We see PerCp-Cy5.5 emission spectra spilling a lot into the filter that APC will use.
    - PerCP-Cy5.5 excited by the red laser spills into the red 640/30 filter which also captures APC fluorescence. This cross laser excitation of PerCP-Cy5.5 will affect the detection of APC labeled population.

# 3. Assigning fluorophores

Panel with DAPI, APC, GFP, and PE fluors.

Panel Matrix

| A5 | UV 355 | | V 405 | | B 488 | | YG 561 | | R 640 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Emission | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor |
| 379/28 | | | | | | | | | | |
| 431/28 | | | | | | | | | | |
| 450/50 | L/D | DAPI | | | | | | | | |
| 470/15 | | | | | | | | | | |
| 488/10 | | | | | | | | | | |
| 515/30 | | | | | | GFP | | | | |
| 586/15 | | | | | | | CD95 | PE | | |
| 610/20 | | | | | | | | | | |
| 670/30 | | | | | | | | | Lin | APC |
| 710/50 | | | | | | | | | | |
| 740/35 | | | | | | | | | | |
| 750/30 | | | | | | | | | | |
| 780/60 | | | | | | | | | | |
| 820/60 | | | | | | | | | | |




DAPI: $Ex_{max}$ = 354 $Em_{max}$ = 456
eGFP: $Ex_{max}$ = 488 $Em_{max}$ = 507
PE: $Ex_{max}$ = 565 $Em_{max}$ = 578
APC: : $Ex_{max}$ = 651 $Em_{max}$ = 660

Lets go back to our previous panel we started with including eGFP, PE and APC.
Here is a histogram for our panel with DAPI (blue) added, and APC (red), GFP (green), and PE (yellow) spectra.

- We can see that there is some overlap with the DAPI and eGFP emission spectra.
    - DAPI is excited primarily by the UV laser and does not have the cross laser excitation by the blue laser and will not fall into the 515/30 filter that detects the eGFP or the filters for any other laser. SO we should have very little affect on the eGFP detection.
    - DA{I not excited by pther lasers so should not affect other fluors.
    - Additionally, DAPI stains the dead cells which will be removed right away so it will not affect resolution of down stream populations.
    - However, many people use the DAPI at the same level they use it for microscopy which is way too much for flow cytometry. Too much of any fluor will can cause so much scattering of light that it will mess up the whole panel.

- See that there is a nice separation and not a lot of overlap between the emission spectra for APC, GFP or PE flours.

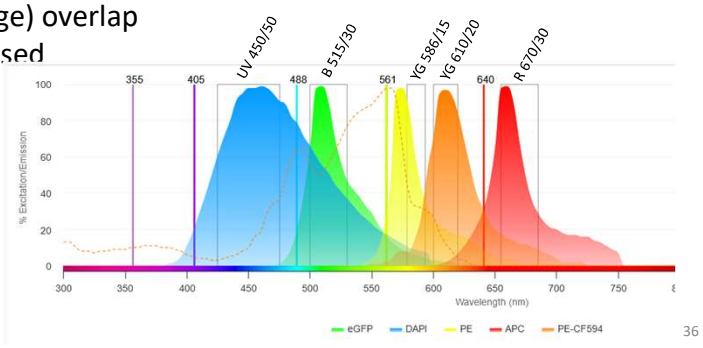
# 3. Assigning fluorophores

Another marker/fluor is added:

- CD45RA: PE-CF594
- See that PE and PE-CF594 are excited from YG-laser, the spectra of PE (in yellow) and PE-CF594 (in orange) overlap and the markers are co-expressed

Panel Matrix

| A5 | UV 355 | | V 405 | | B 488 | | YG 561 | | R 640 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Emission | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor |
| 379/28 | | | | | | | | | | |
| 431/28 | | | | | | | | | | |
| 450/50 | L/D | DAPI | | | | | | | | |
| 470/15 | | | | | | | | | | |
| 488/10 | | | | | | | | | | |
| 515/30 | | | | | | GFP | | | | |
| 586/15 | | | | | | | CD95 | PE | | |
| 610/20 | | | | | | | CD45RA | PE-CF594 | | |
| 670/30 | | | | | | | | | Lin | APC |
| 710/50 | | | | | | | | | | |
| 740/35 | | | | | | | | | | |
| 750/30 | | | | | | | | | | |
| 780/60 | | | | | | | | | | |
| 820/60 | | | | | | | | | | |




DAPI: $Ex_{max}$ = 354 $Em_{max}$ = 456
eGFP: $Ex_{max}$ = 488 $Em_{max}$ = 507
PE: $Ex_{max}$ = 565 $Em_{max}$ = 578
APC: : $Ex_{max}$ = 651 $Em_{max}$ = 660
PE-CF594: $Ex_{max}$ = 496 $Em_{max}$ = 612

36

Let's add another marker

– Let's add an anti-CD45RA antibody that is conjugated to PE-CF594.

- See that the filter PE-CF594 will use is next to the filter that will capture the CD95-PE emission spectra.
- Also, the PE-CF594 is excited by All but the red laser. The orange emission peak overlaps a lot with the Yellow PE emission and the red APC emission spectra. However, The PE-CF594 is not excited by the red laser so it will not affect APC much but, it is excited by the Yellow-green laser and will spill into the PE filter a lot.
- If CD95 and CD45RA are not co-expressed, the populations may resolve OK.
- If either of The markers is classified as a primary marker and have very good separation between + and – populations and only intermediate spillover again, these fluors may be OK.
- However, these two markers (CD95 and CD45RA) are co-expressed.
    - So, I am going to opt to change the fluor, **(move to next slide)**

---

- If there is an extremely important plot that might be used in a figure, consider 2 fluors that have no spillover.

# 3. Assigning fluorophores

Decide to choose another fluor:

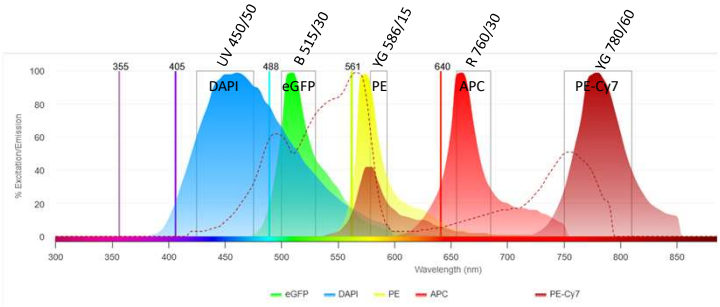
- with a filter in the same laser but with a filter further away: CD45RA: PE-Cy7.

OR

- With a filter in another laser but not in the same row.

Panel Matrix

| A5 | UV 355 | | V 405 | | B 488 | | YG 561 | | R 640 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Emission | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor | Marker | Fluor |
| 379/28 | | | | | | | | | | |
| 431/28 | | | | | | | | | | |
| 450/50 | L/D | DAPI | | | | | | | | |
| 470/15 | | | | | | | | | | |
| 488/10 | | | | | | | | | | |
| 515/30 | | | | | | GFP | | | | |
| 586/15 | | | | | | | CD95 | PE | | |
| 610/20 | | | | | | | | | | |
| 670/30 | | | | | | | | | Lin | APC |
| 710/50 | | | | | | | | | | |
| 740/35 | | | | | | | | | | |
| 750/30 | | | | | | | | | | |
| 780/60 | | | | | | | CD45RA | PE-Cy7 | | |
| 820/60 | | | | | | | | | | |




37

In picking another fluor, you can:

- Move to another laser and check on the overlap with the fluors chosen in that laser,
- You can move within the same laser on the Panel Matrix and choose filters further away from each other.
    - Here our CD95-PE is an antibody we have to use.
    - We can move within the same laser and choose an antiCD45RA antibody conjugated to PE-Cy7
    - The spectra of PE and PE-Cy7 which are both excited by the Yellow-green laser overlap much less than PE and PE-CF594 (in the previous slide)

    _____

    NOTE: Be prepared to discuss PE-Cy7 tandem overlap if asked.
    Will see this with all tandems but there's still good separation between primary peaks (another reason to check spread/spillover matrices)
    - While tandem dyes can serve as powerful research tools, they can also introduce a new set of problems to consider.
    - Tandem dyes refer to two covalently bonded fluorochromes, a donor and an acceptor. As the donor molecule becomes excited, it transfers photons to the acceptor molecule
    - Because the photon transfer efficiency in tandem pairs can differ each time the conjugation is performed, the resulting spectral overlap values can vary from lot-

to-lot.

- the covalent bonds that link tandem dyes may degrade over time and under certain conditions. For example, light exposure, temperature changes, lysing, and fixation can all affect tandem dye bond quality. Tandem dye breakdown can cause false-positive signals in the donor channel, giving the appearance of undercompensation. However, increasing compensation between channels will not resolve the problem, and may result in false negatives due to overcompensation.
- It should be noted that the efficiency of the transfer of energy between donor and the acceptor is never 100% and in flow cytometric detection, there is always some level of spillover or bleeding effect in the donor channel. As a rule of thumb, a higher FRET efficiency is evidenced by a stronger emission signal in the acceptor channel with a weaker emission signal in the donor channel.

# 3. Assigning fluorophores

An additional tool is a Similarity Index

- Compares how similar the spectra of two fluorophores are on a scale of 0–1 (1 = similar, 0 = unique)
- Very helpful with spectral flow cytometry

| | FITC | PE | PE-Cy5 | PE-Cy5.5 | PE-Cy7 | PE-Texas Red | PerCP-Cy5.5 | APC-C750 | APC | APC-R700 | BV421 | BV570 | BV711 | BV785 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FITC | 1 | | | | | | | | | | | | | |
| PE | 0.17 | 1 | | | | | | | | | | | | |
| PE-Cy5 | 0.02 | 0.13 | 1 | | | | | | | | | | | |
| PE-Cy5.5 | 0.03 | 0.18 | 0.74 | 1 | | | | | | | | | | |
| PE-Cy7 | 0 | 0.01 | 0.13 | 0.3 | 1 | | | | | | | | | |
| PE-Texas Red | 0.09 | 0.52 | 0.46 | 0.38 | 0.06 | 1 | | | | | | | | |
| PerCP-Cy5.5 | 0 | 0.06 | 0.81 | 0.91 | 0.27 | 0.34 | 1 | | | | | | | |
| APC-C750 | 0 | 0 | 0.07 | 0.07 | 0.19 | 0 | 0.12 | 1 | | | | | | |
| APC | 0 | 0.01 | 0.4 | 0.16 | 0.03 | 0.03 | 0.3 | 0.16 | 1 | | | | | |
| APC-R700 | 0 | 0 | 0.26 | 0.2 | 0.06 | 0.01 | 0.34 | 0.3 | 0.59 | 1 | | | | |
| BV421 | 0.01 | 0.01 | 0 | 0 | 0 | 0 | 0.01 | 0 | 0 | 0 | 1 | | | |
| BV570 | 0.04 | 0.48 | 0.05 | 0.08 | 0.01 | 0.3 | 0.05 | 0 | 0.03 | 0.01 | 0.16 | 1 | | |
| BV711 | 0 | 0.01 | 0.19 | 0.29 | 0.16 | 0.06 | 0.53 | 0.18 | 0.23 | 0.39 | 0.09 | 0.07 | 1 | |
| BV785 | 0 | 0 | 0.05 | 0.11 | 0.3 | 0.02 | 0.22 | 0.22 | 0.04 | 0.07 | 0.07 | 0.03 | 0.49 | 1 |

https://expert.cheekyscientist.com/panel-design-for-different-detection-modalities/

38

Another useful tool when designing a panel is the similarity matrix.
- This data lets researchers know when they may be using two incompatible fluorochromes because their spectra are alike.

- High scores (i.e. 0.98) indicate that the two spectra have a high degree of similarity, thus are more difficult for the instrument to discern between the fluors.
- Score of 1 means the spectra are identical.
- Lower values such as 0.3 are good and indicate that the spectra of the fluors can be distinguished separately.

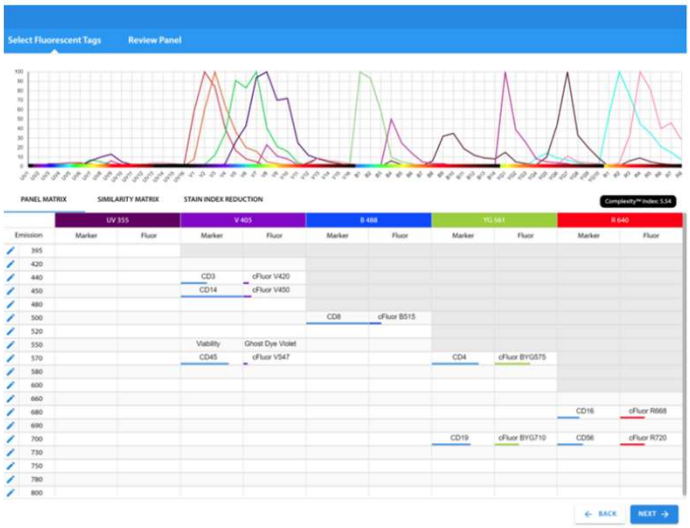
This is especially helpful when doing spectral analysis

---

A second value, the complexity index sums these up. The lower this number, the easier it is to work with the dyes, as the spread will be lower.

# 3. Assigning fluorophores

Online Panel Building Programs:
- FluoroFinder
- Cytek Cloud
- BD Research Cloud




39

There are online panel builders that help to build panels: the 3 that I think are the most popular are

- FluoroFinder (the Research Flow Cytometry Facility is getting a site license which allow CCHMC users to have access to some additional functions and evaluation tools)
- Cytek Cloud
- BD Research Cloud
- Antibody companies will often have panel builder programs but they will suggest their antibodies only; not other companies.
- Even if using a panel design program and AI sources, it is still helpful to understand the principles we just discussed. There is more than one good panel for each experiment and by the same token, there are many bad panels. It helps to understand why so you can fix a bad panel.

Fluorofinder:

- Can create free accounts
- We have our instruments registered on the Fluorofinder site and you can choose which of our specific instruments you are using
- Input the antigens to be used and their expression density.
- See antibodies from many companies for a particular antigen.
- Note if the antigens are co-expressed.

- The program makes the tables we have been discussing and a view of the spectra is made.
- Additionally, get a similarity matrix, complexity score

- You can sign up for a free subscription to FluoroFinder and when you tell them your institution, you have access to the Children's instruments. Additionally, The RFCF has just purchased a site license for FluoroFinder which gives everyone at Children's access to an AI feature to help make panels, the program will make different matrices for you to help evaluate a panel such as a simplicity matrix and it will calculate a complexity score. The additional features are especially helpful with panels for conventional or spectral flow cytometry. Please email one of us or Celine Silva-Lages if you are interested in the advanced program on FluoroFinder.

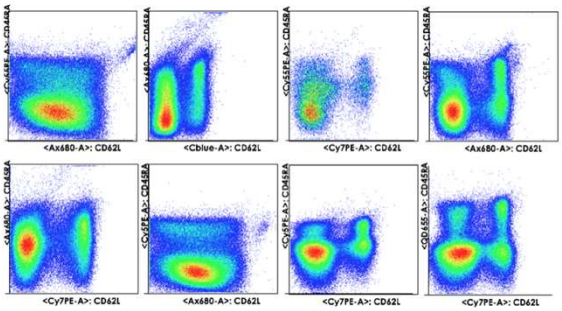
# 4. Optimization and Analysis of Panel

# 4. Optimization and Analysis of Panel:

Assess if the combination of markers and fluors resolves all markers by comparing single stained cells to a multi-colored sample.

Things that affect marker resolution include:

- Spread from panel design
- High Autofluorescence
- Antibody titer
- Unmixing or compensation errors
- Staining procedure steric hindrance




https://expert.cheekyscientist.com/flow-cytometry-data-analysis-tips-for-multi-color-experiments/

41

When checking the panel and optimizing it, we want to see if all of the markers are resolved by comparing single-stained controls to a multi-stained sample.

We will be checking things that affect marker resolution like:

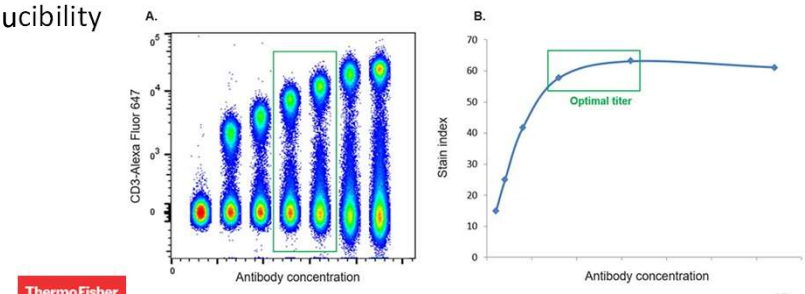
- Spread from the fluors
- High Autofluorescence
- Antibody titer
- Unmixing or compensation errors
- Staining procedure and possible Steric hindrance

In this example you can see that depending on what fluorochrome is used for these 2 markers (CD62L and CD45RA) you can completely lose resolution of the 4 populations.
- In the 1st plot (upper left-hand corner) the populations are not well defined. However, the combination of fluors in the last plot (lower right-hand corner) resolves 4 different populations.

# 4. Optimization and Analysis of Panel:

**A.** Titrate antibodies**:**

- Shows Ab concentration that gives best separation between positive and negative populations
- Titrating each lot reduces staining variability between lots
- Reduces cross-reactivity
- Reduces non-specific binding
- Increases reproducibility




ThermoFisher SCIENTIFIC

42

Start out by titrating the antibodies.

**Titrating antibodies is beneficial because it**

- Determines the amount of Ab that gives best separation of positive and negative populations.
- Titrating each lot reduces staining variability between lots and increases reproducbility
- determines the amount of Ab that gives best measure of expression levels.
- Reduces cross-reactivity
- Reduces non-specific binding
- Increases reproducibility between lots of antibody because using the correct amount of Ab

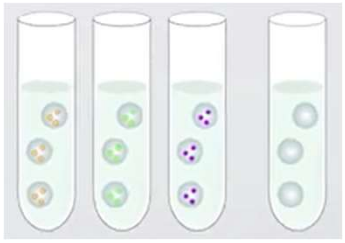
IF using too much of any antibody can mask or alter fluorescent et detection of all fluors.

Diagram:
The dilution which gives the brightest staining with minimum background and good separation between the positive and negative populations is the dilution to use.

Can look up titration method, company's have a protocol or ask us.

# 4. Optimization and Analysis of Panel:

**B.** Make reference controls:
- Consist of an unstained control and single-color controls
- Must be the same:
  - cell type
  - Source
  - Treatment
  - staining protocol
  - Instrument
  - Antibodies
  - Fluors



Single Color Unstained

https://cytekbio.com/blogs/spectrolearn

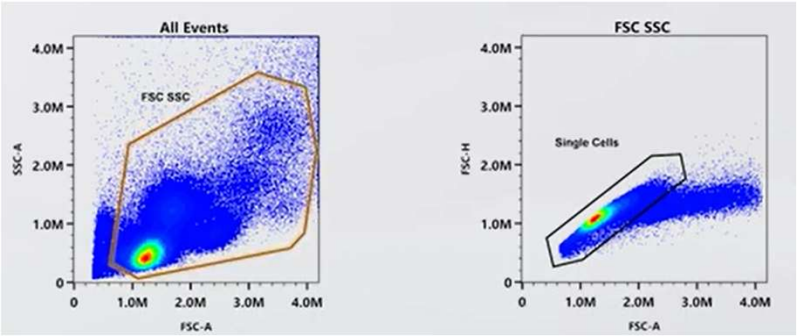
43

Next, make controls
- consisting of an unstained control and single-color controls.
  - True negative on eGFP expression cells do not have eGFP
- Do not want variability in controls and samples so make controls exactly the same as the sample.
  - The controls must be
    - The same cell type
    - From the same source
    - Have the same treatment
    - Use the staining protocol
    - Use the same instrument settings as the sample
    - Use same antibodies, fluors and cells

# 4. Optimization and Analysis of Panel:

**C**. Compare data acquired from single color and multi-color samples.

1. Perform compensation or unmixing.
2. Remove unwanted events with forward and side scatter gating and gating on single cells.




https://cytekbio.com/blogs/spectrolearn

44

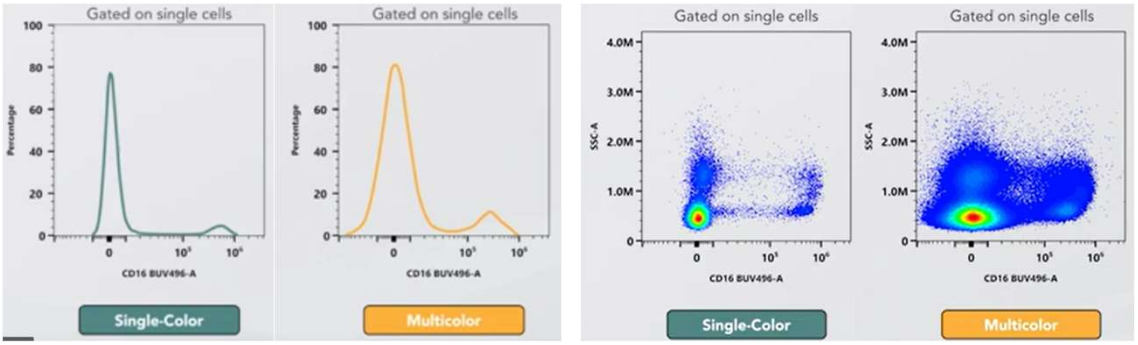
Next, Acquire data

To acquire data on the single-color controls and the multicolor sample:

- Start by performing compensation or unmixing
- Removing the unwanted events and look at the single cell populations. (double discrimination plots)

# 4. Optimization and Analysis of Panel:

**C.** 3. Create histograms or dot plots to compare each single-color control to the multi-color sample. Observe the marker resolution.




45

Off of the single cell gate make histograms and/ or dot plots and Compare the resolution of the single-color controls to the multicolor sample

Histogram plots:

- Can be viewed separately or overlaid.
- Here you can see the staining pattern is similar for both the single-color control and the sample.
- The negative population has a little more spread for the multicolor sample but does not seem to effect overall resolution (difference between the positive and negative populations).
    - This is Normal to see more spread on the multicolored sample. You can compare the % of parent population for the fully stained vs the single stained and if similar, the spread is OK.
- The intensity of the positive population is very low.

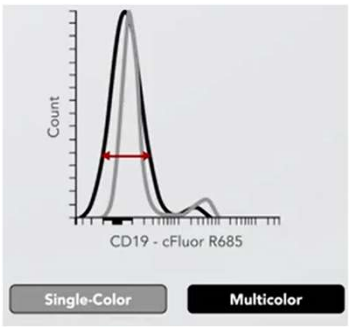
The Dot Plot, however, shows the same information. But I feel it gives more detail on the separation of the different populations and is a better choice when looking at rare populations because your positive peak on a histogram which is very small (low in height) we can see on the dot plot will be resolved OK. Dot plots better when looking at the rare populations.

If the low intensity or rare populations are not resolved in the dot plot, may want to test staining conditions such as Ab concentration, staining conditions, perform sequential staining which can help with steric hinderance.

# 4. Optimization and Analysis of Panel:

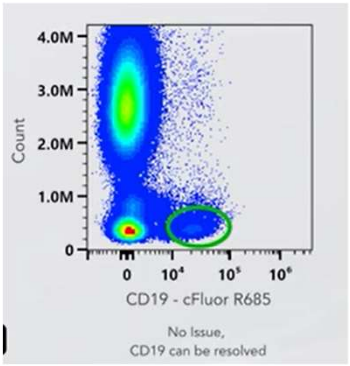
**C.** 3. In this histogram:

- Staining pattern similar
- Width of peaks shows more spreading in the neg population of multicolor sample
- Peak height shows intensity of the signal similar but rare and not resolved.




In this dot plot:

- See that CD19 population is better resolved even though there is spreading from the negative population.




46

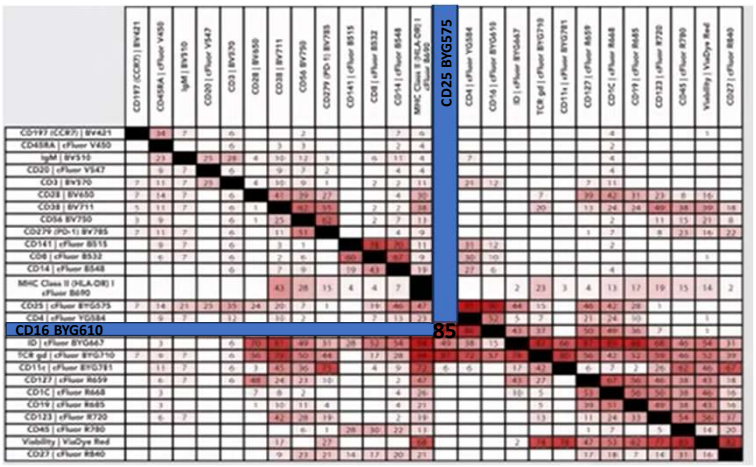
In this example:
In the Histogram the plots are overlaid:

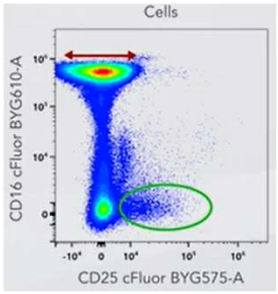
- Can see that the staining pattern is similar.
- The negative population has a little more spread for the multicolor, which again is normal, but does not seem to effect overall resolution (difference between the positive and negative populations).
- There are not a lot of cells for the positive population.

On the Dot plot you can evaluate if spread is affecting overall resolution of a population. You can see in the dot plot that you can resolve the CD19 population. So, OK

# 4. Optimization and Analysis of Panel:

**C.** 4. Consult spread matrix and check to see if fluors that contribute spread are affecting the resolution of populations




In optimizing your panel, Consult the spread matrix and check to see if which fluors that contribute spread and if they are affecting the resolution of populations

Here, according to the spread matrix, BYG610 CD16 affects the BYG575 on CD25 by a value of 85 .
When observing the dot plot, we see that the CD25 is resolved very well even though there is spread.

D16 and CD25 are not co-expressed so may be reason see minimal affect.

# 4. Optimization and Analysis of Panel:

## Is the panel optimal?

- Can the critical populations in the panel be resolved?
- Can gates be drawn easily for each population?

If **NO** to either question – the panel needs to be redesigned.

If **YES** to both of the questions – the panel is optimal.


48

Is the panel optimal?

- Can the critical populations in the panel be resolved?
- Can gates be drawn easily for each population?

If the answer is YES to both of the questions – the panel is optimal.

If the answer is NO to either question, then another version of the panel should be designed.