

### Flow Panel Design

Understanding the principals and mechanics of flow cytometry is critical for proper panel design and experiment setup. Flow cytometry is an incredibly powerful and useful technique, but as it is based on detecting the fluorescent emission of excited fluorochromes using light filters, so there is a lot of room for error. Flow cytometry is about knowing where in the spectrum of light your conjugated antibodies (Abs) fluoresce, whether these emissions overlap at all, and correcting for these overlaps. This protocol will follow detail what steps should be taken when designing a new flow panel and what can be done to minimize any potential errors or false positives. This protocol will **NOT** explain the mechanics of flow cytometry in great detail, for an in depth look at flow cytometry and at how the cytometers work, you can read “Practical Flow Cytometry” by Howard Shapiro (found on our lab server) and visit BD’s page and watch the video tutorials: [BD Flow Cytometry Web Training](#)

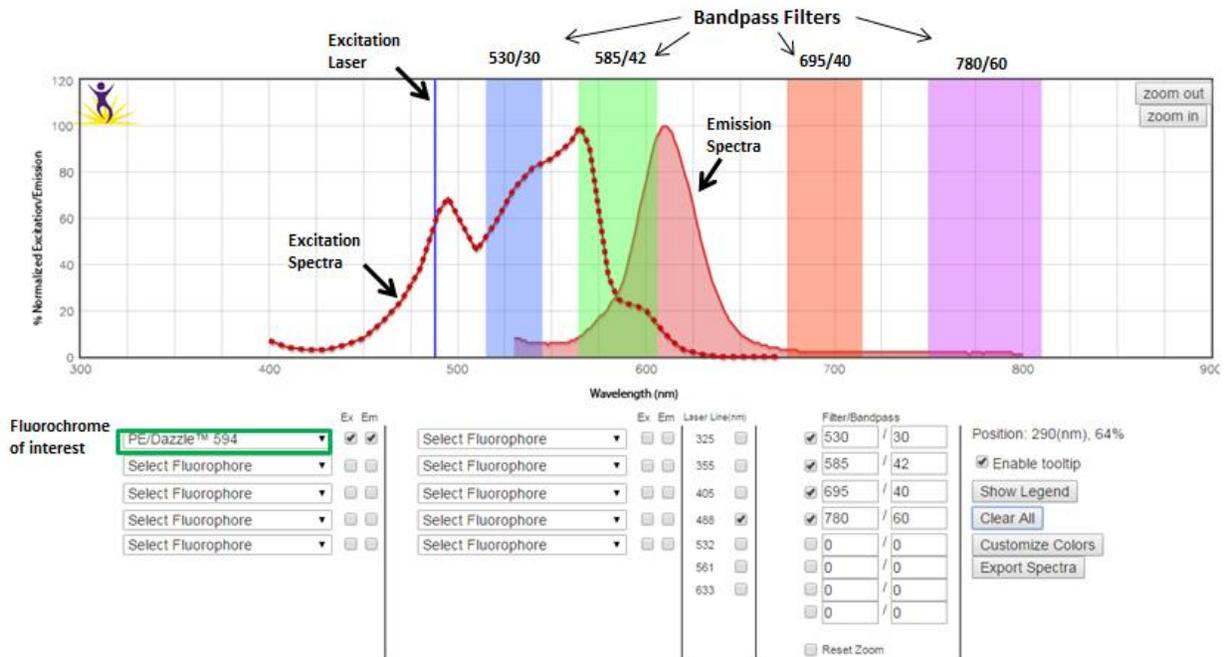
#### Know your cytometer

The first step to panel design is knowing which fluorochromes you can use with the available cytometer. Every cytometer is different and it is important to obtain all the necessary information beforehand. This includes:

- Which lasers does the cytometer have (Red, Blue, Violet, etc)
- At what wavelengths are these lasers fired at
- The bandpass filters the cytometer uses to capture emissions

Each fluorochrome is excited by a specific spectrum of light (some can be excited by multiple lasers) and will not fluoresce (or fluoresce poorly) if light of different wavelengths hits it (typically). Quite often, the cytometer’s caretakers already have a list which fluorochromes can be used with each bandpass filter on the cytometer. Our list is located on the lab server under “Flow Resources” for both the Canto and LSR II.

- Whenever you wish to use a fluorochrome that is not on the list, then check whether the fluorochrome can be excited by the cytometer’s lasers and whether its emission spectra is detected by a bandpass filter. For example, I want to use Biolegend’s new **PE-Dazzle594** fluorochrome. I check the Technical Data Sheet (TDS) on the manufacturer’s website and see it is excited by both the **Blue laser (488 nm)** and the **Green laser (532 nm)**, while the **emission** spectra peak is at **610 nm**. I check the Canto’s parameters and see that it can be excited by the Blue laser and my bandpass filter (585/42) should detect it, but it is not optimal (see next point).
- Another great resource is the [Biolegend Fluorescence Spectra Analyzer](#). Here you are able to check whether your cytometer can detect a fluorochrome by entering which fluorochrome you are interested in, clicking the excitation (Ex) and emission (Em) boxes, entering your bandpass filter values, and clicking which lasers/bandpass filters you wish to visualize. Below you can see I have entered all the bandpass filters that can be detected with the blue laser on the Canto as well as the laser excitation:



Notice how the emission peak is not inside the bandpass filter range. While half of the spectra is within range, the ideal situation is that the peak is in the middle of the bandpass filter range. In this example, this fluorochrome can be used, but a better alternative should be found instead.

### Know your fluorochromes

Once you know which fluorochromes your cytometer can detect, it is time to decide which combinations of fluorochromes to use. In an ideal world, each fluorochrome would fluoresce in a very narrow range of light, but that is rarely what happens. Some fluorochromes tend to have a very wide spectrum (PerCP-eFluor710), while others have a much better, more restricted range (Qdot605). The bandpass filters detect any emission of light within their range, regardless of how weak it is, so while the peak of a fluorochrome's emission spectrum may not be in the range of a particular bandpass filter, part of the emission may still spillover. For example:

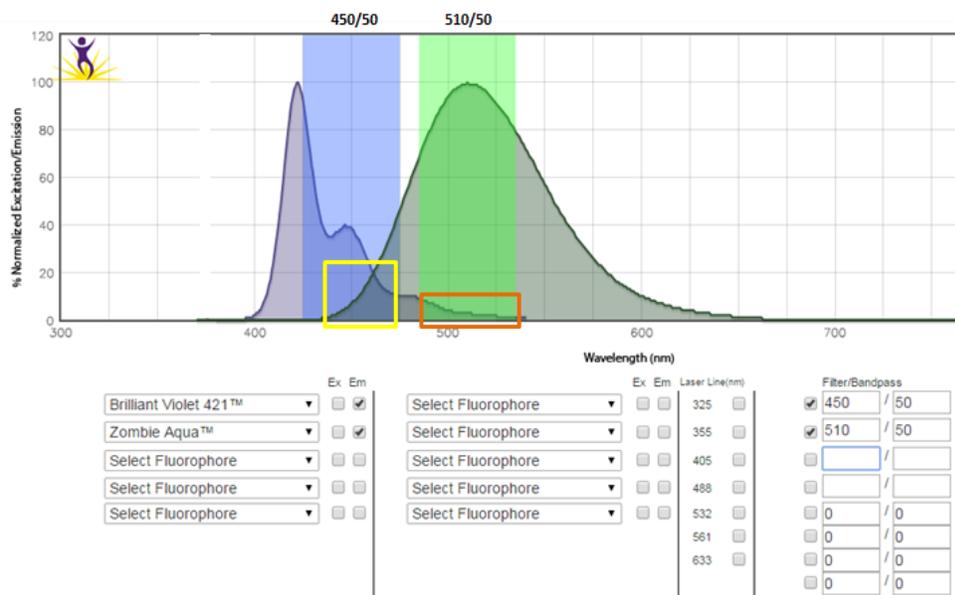


Figure 2

Here in Figure 2, the emission peaks of BV421 and Zombie Aqua are in primarily the 450/50 and 510/50 bandpass filters respectively, however both spectra spillover into the other bandpass filter as well. In the yellow box, the Zombie Aqua emission is at about 20% in the 450/50 bandpass filter. That means the cytometer will detect this light in the 450/50 filter when it should not, thus yielding either false positives and/or unreadable flow data. The same goes for the BV421 fluorochrome in the 510/50 bandpass filter to a lesser extent (orange box). Now these spillovers are fairly low and, with proper voltage adjustment on the cytometer and **compensation**, these can be minimized to the point where it will have little to no effect on your data. Figure 3 is an example where the spillover is too great and compensation would not be able to fix it:

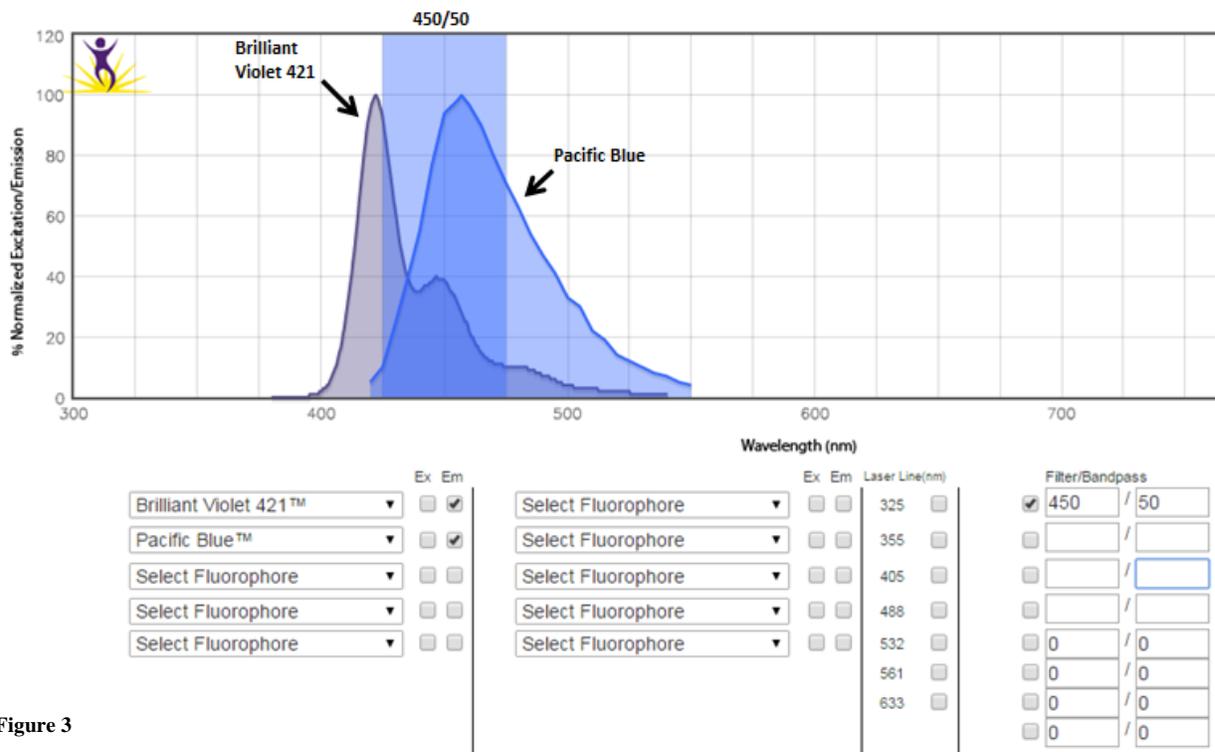


Figure 3

Both BV421 and Pac Blue are detected at greater than 80% in this bandpass filter. The overlap is too large and if both of these fluorochromes are used together, then there will be no way to tell the difference between the two.

In summary:

- Due to wide emission spectra, some fluorochromes may be weakly detected in several bandpass filters known as **spillover**
- Emission spectra and bandpass filters should be compared to determine whether some fluorochromes can be used together on a particular cytometer
- Minor spillovers are corrected by adjusting the cytometer's voltages for these bandpass filters and by performing **compensation**

### **Know your antibodies**

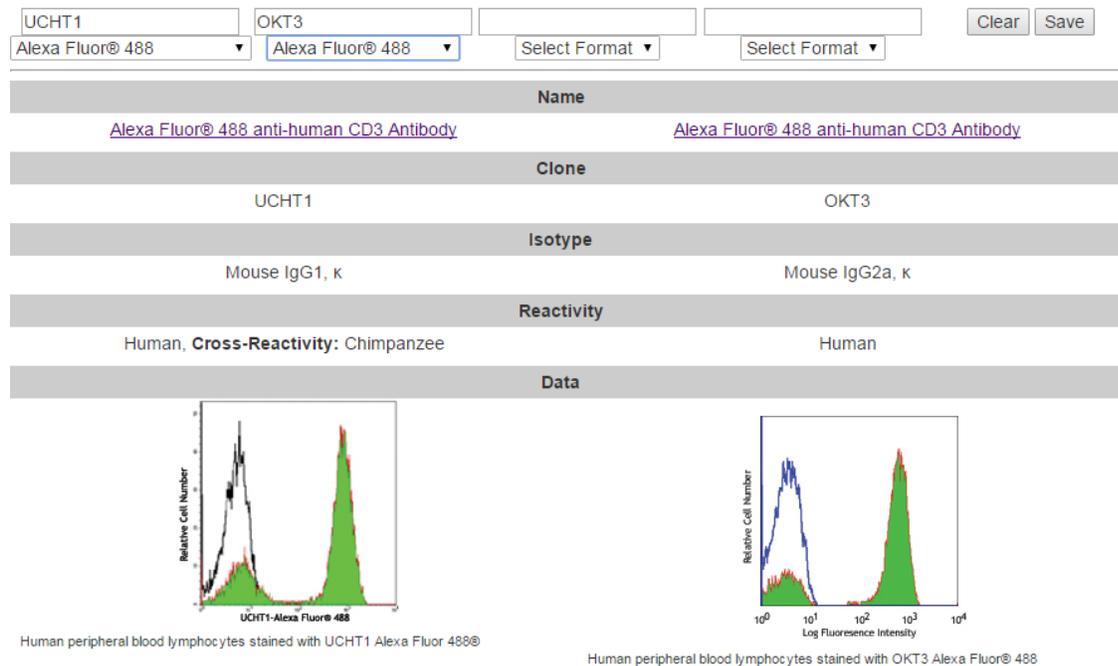
After determining what fluorochromes your cytometer can detect and which fluorochromes can be used together, it is time to decide which fluorochromes should go with each antibody. The purpose of flow cytometry is to determine whether your cell is expressing a particular protein by **staining** these proteins with fluorochrome conjugated antibodies that are specific for those proteins. Several companies sell directly conjugated antibodies such as BD, Biologend, and eBioscience, and many antibodies have a large variety of fluorochrome to choose from. There are several factors that affect the choice of which Ab (Ab from this point on will mean an antibody with a conjugated fluorochrome) to choose including:

- Brightness
- Availability
- Clone

**Brightness** – Every fluorochrome varies in terms of how bright they fluoresce. While the wavelength of their emission will not change, the intensity (how much light it produces) does vary. Every Ab manufacturer has a list of their available fluorochromes and their relative brightness. When deciding which antibody should go with which fluorochrome, it is good to know the relative abundance of the protein(s) being analyzed. For example, when someone wants to look at interferon gamma (IFN $\gamma$ ) in CD8 T cells, they will need Abs to detect both the CD8 and IFN $\gamma$  protein. CD8 is a surface protein with relatively high expression, while IFN $\gamma$  is a cytokine that varies in expression (depending on the stimulus) and it must also be detected by using intracellular staining (ICS). Since CD8 has a relatively higher expression and is found on the surface of the cell, one should use a relatively dim fluorochrome to detect it (e.g. Alexa Fluor 700) while IFN $\gamma$  requires a brighter fluorochrome for adequate detection (e.g. PE-Cy7). It is also important to note that the spillover and compensation can be affected by this. A fluorochrome with < 20% spillover into a particular bandpass filter can normally be compensated, but if it is a bright fluorochrome used on a highly expressed surface protein, then the compensation may prove to be more difficult due solely to the amount of light that must be corrected. So high expression = dim fluorochromes, low protein expression = brighter fluorochromes.

**Availability** – After choosing the best combinations of antibodies/fluorochromes, then the hunt for these Abs begins. Remember to search Abs with the correct reactivity (mouse/human) and search several sites to compare prices/quantity. If you are unable to find a particular Ab, the panel must be adjusted.

**Clone** – These conjugated antibodies are specific not only for particular proteins, but also to the part of the protein it binds to. Monoclonal antibodies are produced from a single, expanded B cell from an immunized mouse, thus creating a pool of identical antibodies. If an antibody is titrated/tested and it is found to perform poorly, a different clone can be tested before remaking the entire panel. As you would expect, different clones also affect the emission of certain fluorochromes. A great tool from Biolegend is their [Clone Comparison](#) chart in which you can compare the intensity of a fluorochrome on different clones (you can also use this to compare different fluorochromes simultaneously).



**Figure 4**

In Figure 4, we are comparing the fluorescence of Alexa Fluor 488 from two different clones of CD3 (UCHT1 and OKT3). Since UCHT1 (left) has a slightly higher intensity, this one should be tested first. Factors that can affect this decision include: intensity, separation, width of positive/negative populations, and any shift in the negative population.

In summary:

- There is a large variety of fluorochrome-conjugated antibodies (Abs) and several factors affect the choice of whether or not to use them.
- Fluorochromes have different relative levels of brightness. Dimmer fluorochromes should be used with proteins that have higher expression (surface markers/receptors) while brighter ones saved for weakly expressed proteins or those found within the cell (cytokines/transcription factors)
- Abs are specific for particular epitopes and this can affect the fluorescence, intensity, and binding affinity of the Ab.

### The final check

After choosing the optimal Abs and verifying that the cytometer can detect them all, it is time to do one last check with the [Spectra Analyzer](#). Below is a proposed panel that will be used to compare Th1 and Th2 CD4 T helper cells and it will be analyzed using the Canto cytometer. The color on the bandpass filter shows which laser is used for excitation (Blue 488nm, Violet 405nm, or Red 633 nm)

Antibody	Fluorochrome	Clone	
CD3	AF488	UCHT1	530/30
CD4	V450	RPA-T4	585/42
CD8	APC-Cy7	RPA-T8	695/60
Viability	Aqua		450/50
IFNg	PE-Cy7	B27	510/50
IL-4	PE	8D4-8	660/20
IL-13	APC	JES10-5A2	780/60

Every laser and its bandfilters should be checked separately. So for the Blue laser:

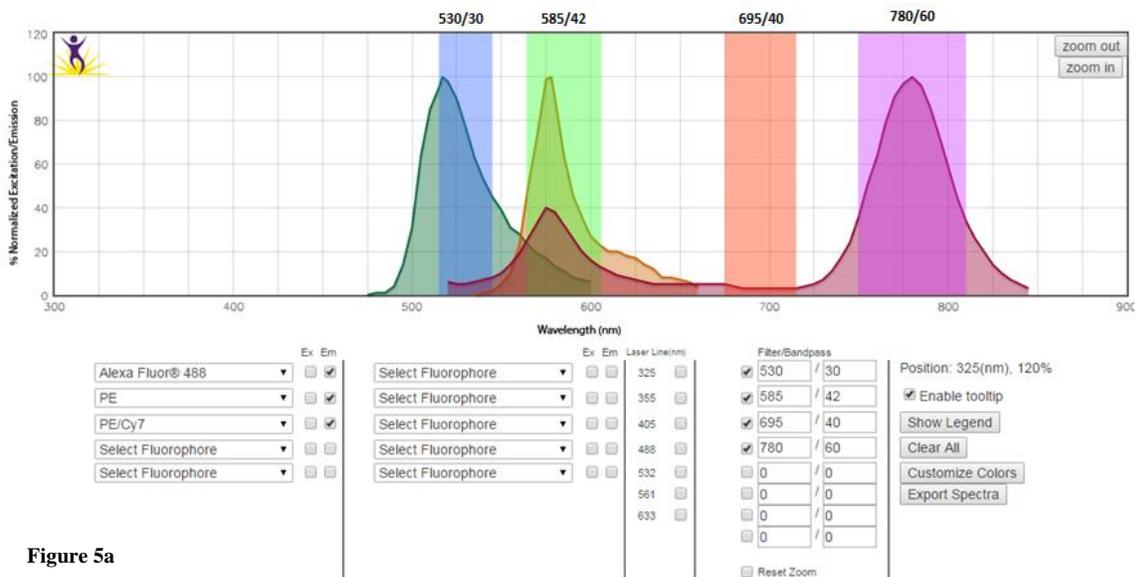


Figure 5a

In Figure 5a, each of our fluorochromes has their respective peak within one of the bandpass filter's range. However, PE-Cy7 has two peaks, the smaller of which is being detected by the wrong filter (the 585/42 filter). Since PE-Cy7 is

a conjugate of PE (basically it is PE with some extra stuff attached) it is not surprising that this is happening. This spillover can be compensated. There is nothing in the 695/40 filter because a fluorochrome that emits in this range was not included. Now we check the Violet laser:

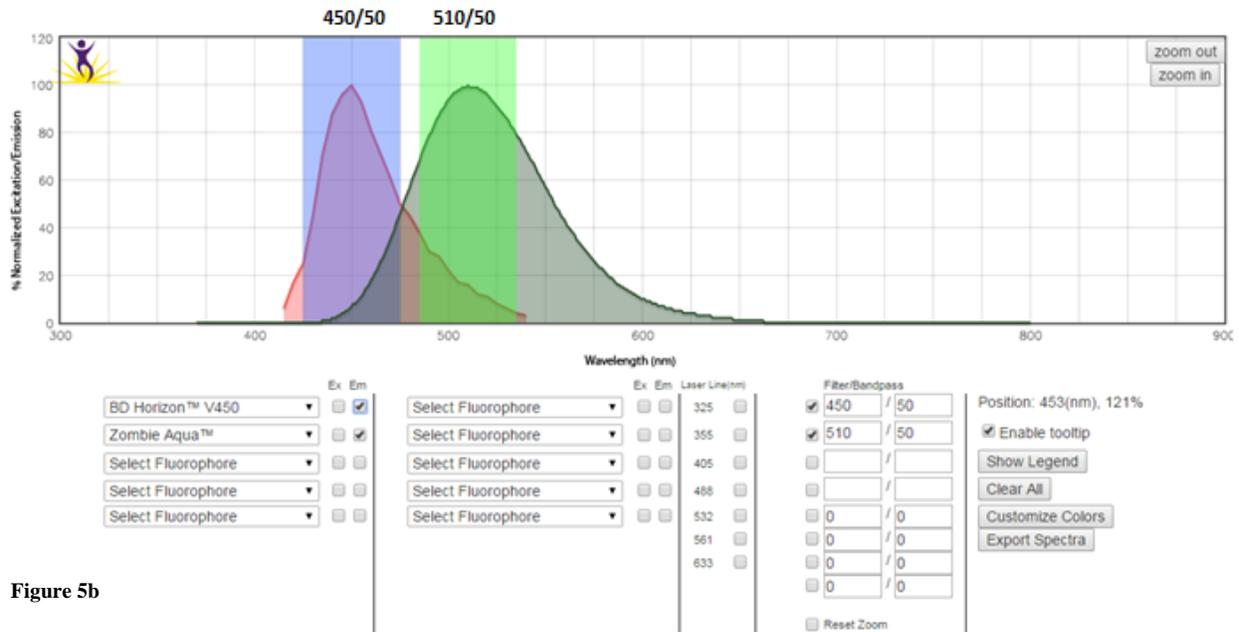


Figure 5b

And the Red laser:

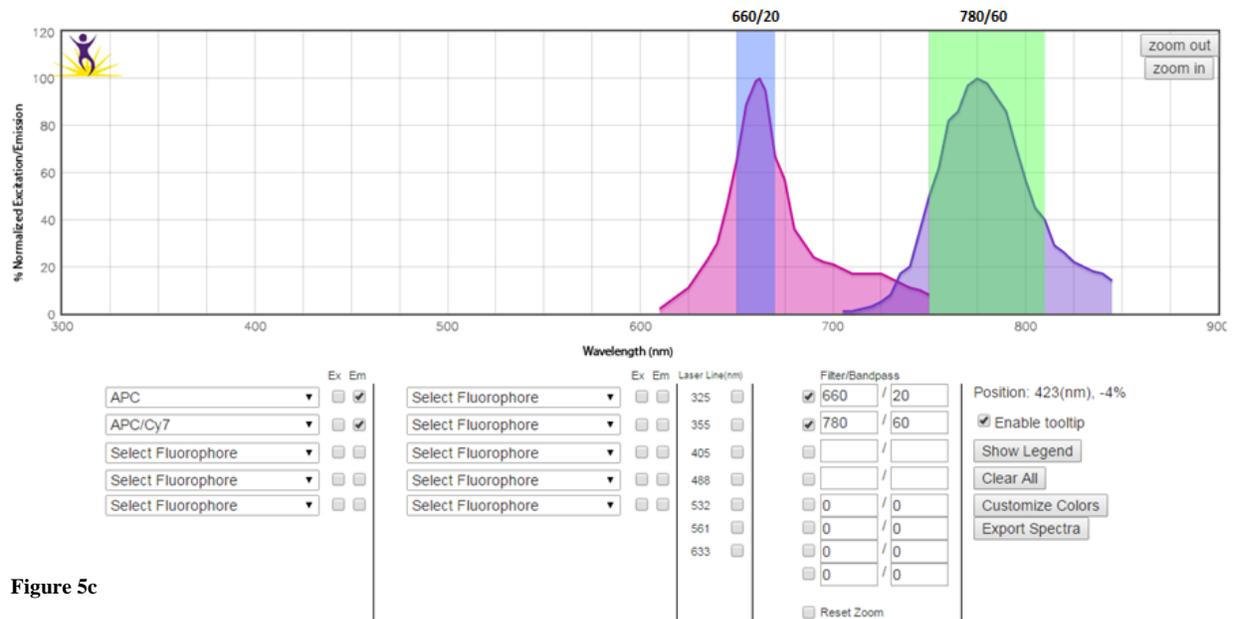


Figure 5c

All of the fluorochromes emit within a bandpass filter on this cytometer, each spillover can be theoretically compensated, and the cytokines have brighter fluorochromes while the surface markers' Abs are using dimmer ones. The next step is to titrate these antibodies, analyze full minus one samples (FMOs), run a compensation, and then finally try it on cells. These techniques are discussed in other protocols.