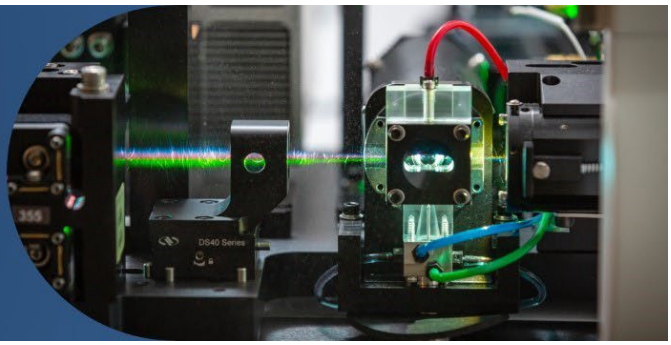
Fred Hutch Cancer Center

Flow Cytometry

Research Administration
Seattle, WA • 501(c)(3) Nonprofit



Fred Hutch's Shared Resources are catalysts for lifesaving discoveries. This uniquely centralized program of 15 specialized core facilities and scientific services drives advances by integrating dedicated experts and cutting-edge technologies across the entire research pipeline, from basic science to clinical trial.

LEARN MORE:

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Panel Design

For conventional and spectral flow cytometry and live-cell sorting

Know Your Instrument

- First, is it a sorter or analyzer? Analyzers only collect data, while sorters separate and collect populations for further assays.
- Is it a *conventional* or *spectral* system? This affects fluorophore selection. Laser wavelengths excite dyes (Ex_{max}), and detector arrays collect emitted wavelengths. Conventional systems collect peak intensity light (Em_{max}), allowing one similar Ex_{max} dye per channel. Em_{max} knowledge is vital for panel design and fluorophore separation. Spectral systems unmix the entire spectral signal, not just Em_{max} light, offering more flexibility and larger panels. However, knowing the system's lasers and similar spectrum dyes remains important. Instrument configurations are available on our website.

Designing Your Panel

- Separate your fluorophores as best you can. If your panel is small, this can be as simple as picking one color that's excited by each laser. You'll want to look at both the Ex_{max} and $Em_{max.}$ values for each fluorophore and attempt to separate the emission across detectors.
- Online tools for viewing spectra such as those found on the FluoroFinder, Cytek, BD, or antibody manufacturer websites can help you visualize potential spillover that may cause compensation challenges or data spread. A visual representation of the overlap fig.1, can be generated to assist with determining compatible fluors.
- Another consideration for panel design is *antigen density*. The online tools can help you match lower expressing markers with brighter dyes. For unusual markers you may also be limited by antibody availability.
- Pay attention to *tandem dye breakdown* for dyes that can separate into their components. A notorious example is BV785 which breaks down into BV421 when exposed to warmth or light.
- You can also use a *dump channel* and place several markers you're uninterested in on the same fluorophore, then plan to gate out any signal from this fluorophore.

Spectral Flow Cytometry

- Also known as "full-spectrum" and in contrast to conventional flow cytometry, spectral flow means the instrument uses detectors across the light spectrum from deep-UV to far-red, allowing the use of many more parameters together in a single experiment. The data must be *unmixed* mathematically in a process comparable to *compensation* on a conventional instrument to distinguish the signals from each fluorophore.
- The **Cytek Aurora** and **Sony ID7000** are our two spectral analyzers. Our BD Symphony 3 and 5 are spectral-equipped, which means they have filter sets capable of capturing the full spectrum. For *live-cell sorting*, we offer the native spectral **FACSDiscover S8** from BD and two BD Symphony S6 sorters (the S6-2 and S6-3) that have been spectrally equipped.
- Below in Fig.2 is a ribbon plot from the Sony ID7000 showing what a sample looks like while running on a spectral instrument. The signal across all detectors for each laser line is shown.
- Spectral cytometers can separate dyes that are typically impossible to distinguish on conventional cytometers, such as APC and AF647.

If you are interested in learning more about spectral flow and would like to be trained on the ID7000 or Aurora, please get in touch!

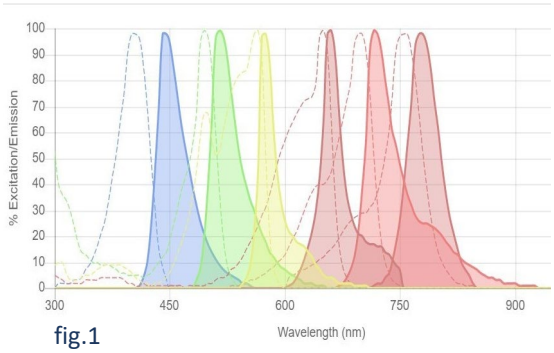

fig.1

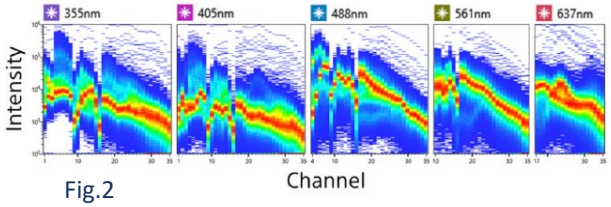

Fig.2

Controls for Compensation or Unmixing

- Controls are critical for any scientific experiment and successful analysis of flow cytometry samples is no different. Good controls are even more critical for spectral flow cytometry.
- The *unstained control* is needed for setting voltages so that the cells are on scale, and for determining the background or autofluorescence (AF). It can be helpful to prepare extra of this control when setting up a new cell type on a new instrument for the first time so that you don't run out of sample.
- You'll also want to prepare *single-color controls* for each color found in your samples. The positive population should be as bright or brighter than the sample and have at a minimum of 1000 events. If you use cells make sure that a good positive population is present, keep in mind this may require stimulation of cells for some markers. In some cases, it is recommended to use bead controls, particularly on the ID7000. On both spectral and conventional cytometers, you can use a combination of bead and cellular controls. Important note: the AF of the positive population needs to match the AF of the negative population i.e., if using beads, the negative population needs to be beads, if using cells, the negative should be the same cell type and treatment as the positive.
- For spectral flow it is critical that the fluorophore matches *identically* to what is in your samples. You cannot use a FITC control for GFP or an AF647 control for APC.

$$SI = \frac{(MFI_{Pos} - MFI_{Neg})}{2 \times rSD_{Neg}}$$

MFI= Median Fluorescence Intensity

Titrate Your Antibodies!

- It is critical to titrate each new antibody you are using so that you get the best *resolution*. You will get the best signal above background noise.
- It is best practice to titrate antibodies in the same conditions as your experimental cells. (Fixed or live? Permeabilized or not, etc.)
- You'll want to include a viability dye in your titration efforts because non-specific antibody binding increases on dead cells.
- Using an Fc blocking reagent is also recommended. Otherwise, the Fc region of fluorescent antibodies and Fc regions on cells can interact.
- Fig. 3 below illustrates the data from an antibody titration. The Stain Index (SI), using the equation, can guide us in determining the optimal concentration. As the concentration increases, the SI initially rises but then starts to decline due to increased background noise.
- It's important to note that using the concentration that yields the highest SI value is not mandatory. If there is a clear distinction from the unstained sample, opting for a lower concentration can be advantageous for two reasons: 1. It conserves reagents, thereby saving costs. 2. A less intense stain results in reduced spillover into other channels.

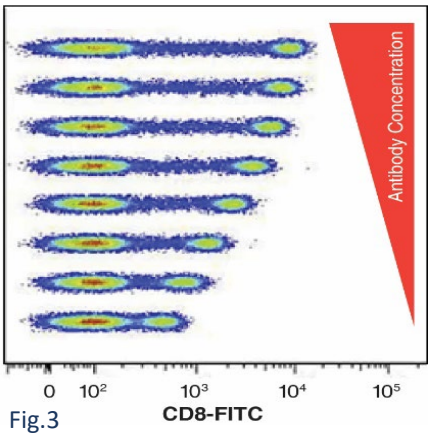

Fig.3

Some notes on Autofluorescence

- Autofluorescence (AF), literally self-fluorescence, can be a problem in flow cytometry. Some cell types are known to autofluorescence more than others. Flavins and NADPH are some of the culprits to this inherent property of cells.
- This unwanted fluorescence is important to account for, particularly in spectral flow cytometry. For both the Aurora and ID7000, it is critical to prepare an *unstained control for each cell type represented in your data.* The software can therefore account for the autofluorescence background.
- AF tends to be an issue in the lower end of the light spectrum, in *355, 405, and 488nm laser excitation and ~400-600nm emission*. Make sure if your marker is on a fluor in this range that the antigen density is high enough to separate from the AF, if not choose a fluorophore further out towards red can alleviate AF issues.
- Fixatives can affect both AF and some dye chemistries, and special attention needs to be paid to reduce the impacts of AF caused by fixation.