KNOW YOUR FLOW

Five Simple Rules for Optimal Panel Design

Brought to you by: FlowMetric™

Contents

Flow cytometry is a powerful technology that lets scientists observe and measure numerous aspects of thousands of cells in a single experiment. Flow cytometry has transformed biomedical research, especially in the fields of immunology, hematology, oncology, and genetics. Cells are at the heart of each flow cytometry experiment and are stained with a panel of fluorochrome-labeled antibodies to reveal characteristics about individual cells and the entire cell population.

Panel design is central to the success of a flow cytometry experiment in order to assure that cells are labeled with antibodies that recognize molecules of interest and that the fluorochromes linked to these antibodies are compatible with each other and with the flow cytometer.

In this white paper, we describe five basic rules that can help scientists get a better handle on panel design.

1 Rule 1: *Know Your Equipment*

Many researchers today have access to different types of flow cytometers either in their labs or through core facilities. Understanding what an instrument can measure and how it works is essential to panel design.

Lasers and Detectors

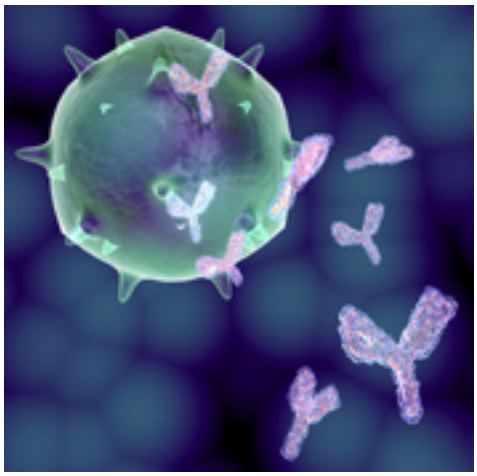
It's critical to understand what lasers are housed in the cytometer you are planning to use. Most cytometers contain multiple lasers, and each laser is tuned to a specific wavelength so that different types of fluorochromes can be detected discretely. You will need to know which lasers your cytometer has and at what wavelength each laser is tuned. You will also need to know which filters are contained in the detector downstream of each laser. Together this information will tell you which fluorochromes can be detected on the instrument and how many parameters can be measured.

Samples

You will want to consider which type of cytometer would be appropriate for the type of data you plan to collect. Are you measuring the frequency of different cell populations in your sample by immunophenotyping? Or, are you trying to sort and collect a particular cell subset that you go on to use in culture? These considerations will dictate the type of cytometer, such as a cell sorter, you will need for your experiment. Cytometers can also be equipped with multi-well samplers, which enable automated runs of cells in multi-well plates and can be essential for tackling large-scale experiments.

Events

The design of each cytometer controls the rate at which cells can be acquired during a run (event rate). Cytometers also have limits on how many total events (cells) can be acquired in a single file. Familiarizing yourself with these aspects of your cytometer, as well as considering if you are looking for rare or low frequency cell populations will help you figure out how many cells you will need each sample in order to yield usable data.

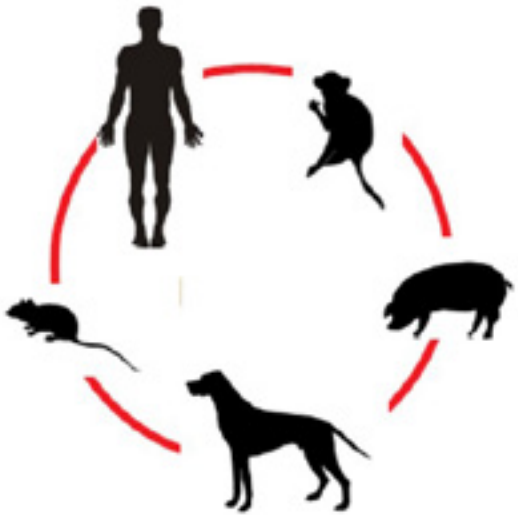


2 Rule 2: *Know Your Species*

We are all too busy planning experiments, writing proposals, or submitting abstracts for an upcoming meeting in an exotic location. We all know that taking the time to properly plan experiments can be hugely beneficial. But who has the time? Flow cytometry experiments usually involve precious samples and costly equipment and reagents, so we have come up with five simple rules to help you get the most out of your next flow experiment and truly accelerate your research.

Now you are ready to browse the multitude of paper and online catalogs to select the perfect flow cytometry panel of antibodies for staining your valuable samples. **Where you do you start?**

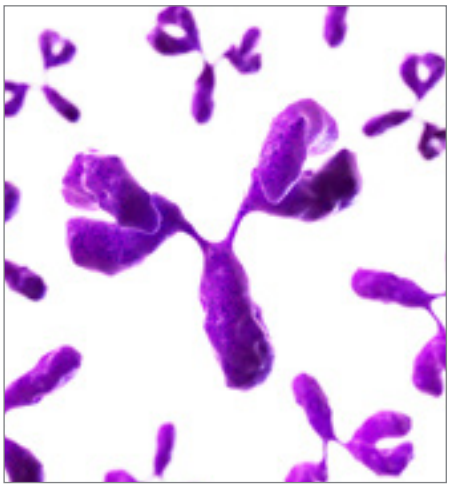
Species are Specific



Say you want to look at a population of mouse CD4+ cells. This sort of experiment has been done thousands of times by flow cytometry, and like it or not, there are dozens of ways to stain cells for this surface marker. So, how do you assure you are going to use an antibody that detects the right molecule in your sample?

Take a moment and consider the entire experiment as you build your flow cytometry panel: How many different molecules am I going to stain cells for? How many fluorochrome "colors" can I use at once (based on the cytometer)? And most importantly, what species are my samples from? This information alone will focus your search for the appropriate antibodies. The species of your sample (i.e., mouse, human) needs to match the species that the antibody was raised against. If you want to detect CD4 on the surface of mouse cells, you will have to browse "anti-mouse" antibodies and find "anti-mouse CD4 antibodies." Many companies now have web tools and apps to help you in searching their large antibody collections, and these tools will use the species information to refine your search.

57 Antibodies found… Now What?



Your search may give you more results than you can deal with. So how do you narrow your options? Multiple monoclonal antibody clones may exist for common surface markers, like CD4, and you may want to test a few different clones to find the one that works best for your samples. The antibody that binds to your target of interest is the "primary antibody." You can buy primary antibodies already conjugated with fluorochromes. If you use an unconjugated primary antibody, you'll need to select a fluorochrome-conjugated secondary antibody that matches the species of your primary antibody. So, if your primary anti-mouse CD4 antibody is from a rat, you'll need an anti-rat secondary antibody.

Pilot Experiment

This is a good point to consider doing a pilot experiment to test out different antibody clones. Some clones cross-react with off-target molecules, and some do not work well for a particular cell type. Not all antibodies are created equal.

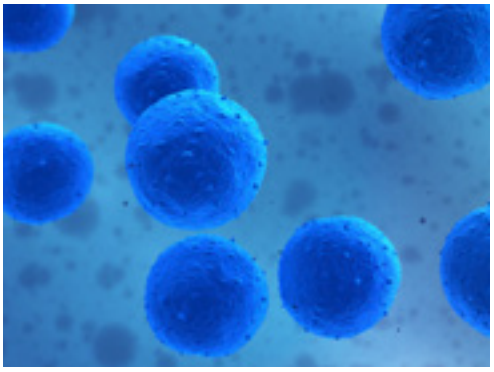
Now that your mind is spinning with species anti-species antibody choices, let's prepare as you create your optimal panel design.

3 Rule 3: *Know your Fluorochromes*

Precious resources, including your time, experimental samples, and reagents, are preserved as you take the time think about an optimal panel design for your first or thousandth flow cytometry experiment. We've walked you through the first two steps to consider as you design a flow cytometry staining panel.

In rule 3 you will learn about the importance of choosing the correct fluorochromes for your experiments.

How do Fluorochromes Work?



Fluorochromes (also known as fluorophores) are the fluorescent compounds at the heart of flow cytometry. These compounds can be used alone to label parts of cells but are more often linked to antibodies that bind to specific targets on cells, like CD4 molecules.

Flow cytometers house lasers that tap into the unique properties of fluorochromes to quantify and characterize multiple cell types simultaneously. Lasers produce a beam of light at a discrete wavelength. Fluorochromes absorb light photons from the laser and become "excited" as electrons shift to a higher energy state. Almost instantly, the fluorochrome's energy state falls and photons are released or "emitted." This emission wavelength is always higher than the excitation wavelength. Many fluorochromes on the market for use in flow cytometry work at discrete excitation and emission wavelengths. That's why most cytometers have multiple lasers and detectors to maximize the number of fluorochromes that can be used.

As you start to peruse your fluorochrome options, you'll typically notice important specs for each:

- Excitation (absorption) wavelength (the wavelength of the laser needed to excite the fluorochrome)
- Emission wavelength (the wavelength of emitted light can be measured using the corresponding detector)
- Excitation/Emission spectra graph

This information helps you start to work toward your optimal panel design as you try to follow one of the golden rule of flow cytometry: Avoiding spectral overlap!

Harnessing the Rainbow: AKA Avoiding the Specter of Spectral Overlap

Your optimal panel needs to include antibodies linked to fluorochromes that do not have overlapping emission spectra. This helps assure that each molecule you are staining for can be detected as a unique color. If you use fluorochromes that do have similar emission spectra, you may encounter "spectral overlap" and the flow cytometer may not be able to discriminate between these colors. That's why most researchers aim to use fluorochromes that span the light spectrum ("the rainbow"). There are some great tools on the web from flow cytometry supply companies that can help you figure out which fluorochromes will work together in an optimal panel design.

4 Rule 4: *Antibody Titration*

The Key to Customizing your Panel Design

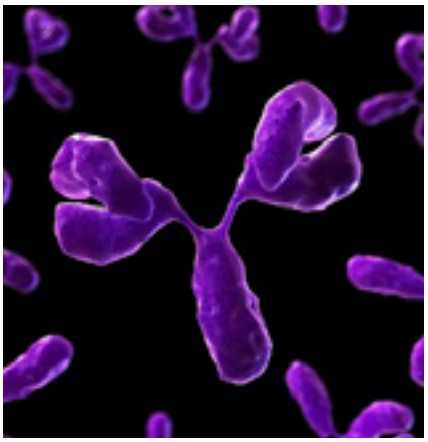
We've talked about the three key elements you need to design an optimal panel for your next flow cytometry experiment.

Now you might be ready to stain some cells, run them through the cytometer, and acquire some perfectly usable data. Not so fast! Your newly arrived fluorochrome-conjugated antibodies probably came with some general instructions, which included some beautiful histogram plots showing how well the antibody stained cells carrying that particular target. This doesn't mean your particular cell population will look anything like this when you blindly use the staining protocol recommended by the manufacturer.

Now is the time to do that "pre-experiment" type of experiment and titrate your antibody using cells and Antibodyconditions that most closely mimic your experimental conditions.

Thinking about Titration

Experienced flow cytometry users consider antibody titration a critical step for optimal panel design. Tony Chadderton, Senior Project Manager at FlowMetric, Inc. said, "Titrations are important to make sure that the fluorescence intensity of antibody stained cells is linearly proportional to the protein being measured and that the signal to noise ratio of the fluorescence for the antibody is maximum."



So now that you know you need to run a titration, think about these elements of your experiment:

1. Are you staining a target that is expected to be highly expressed or weakly expressed?

You'll want to titrate your antibody using the same or similar cell type as your plan to use in your experiments.

You may also consider using a cell type with maximal expression of your target so you can pinpoint the antibody concentration that can stain an abundant amount of target without causing too much non-specific background staining.

2. Is your target expressed on the surface or is it intracellular?

You'll want to use the same staining process for your titration as you'll use for your experiment, and this differs for surface or intracellular targets.

3. Do I need to treat my cells with something to block non-specific binding of my staining antibody?

Different antibody isotypes can bind non-specifically to Fc receptors on the cell surface. Check out the protocols provided with your antibody or in the methods section of scientific manuscripts that use this antibody to avoid this potential pitfall.

Doing the Dilutions

Now it's time to set up your titration. You'll want to use the same conditions as much as possible in your titration as you plan to use in your experiments. This means using the same number of cells in the same volume of liquid, the same buffers, the same number of wash steps—all those details are important. You'll probably want to start with a high concentration of antibody (like 1 mg antibody/100 ml sample) and make a series of 8 to 10 3-fold dilutions to get a broad range of antibody concentrations. As you set up your dilutions, you'll want to remember to reserve a sample with no antibody for use as unstained control cells. After you dilute your antibody, you can add your cells, incubate and wash them according to your experimental protocol, and read them on your cytometer.

So What Am I Looking For?

Now that you have run your titrated samples, you'll need to calculate the stain index to determine what that optimal antibody concentration is for your experiment. You'll notice that there will be both stained (positive) and unstained (negative) cells at each antibody concentration. Once you've acquired this data, you'll need to analyze each stained sample to determine the standard deviation (SDnegative) of the negative population (SDnegative) and the median fluorescence intensity (MFI) of the positive and negative populations.

The stain index can be calculated for each antibody concentration using this formula:

$$SI = (MFI_{positive} - MFI_{negative})/(2 \times SD_{negative})$$

Next, you can create a scatter plot with the log antibody concentration on the x-axis and the stain index on the y-axis. The peak of the curve generated by the scatter plot is the optimal antibody concentration.

5 Rule 5: *Conquer Compensation*

Unlocking your Optimal Panel Design

We've walked you through four essential elements to design an optimal panel for your next flow cytometry experiment.

Now you are ready to run a multicolor flow cytometry experiment and use a combination of your fluorochrome-linked antibodies to see your precious cells. But first we have to bring up a term that strikes fear and dread into most new flow cytometer users—Compensation!

Spillover Situations

In the “Know your fluorochrome” post, we discussed how to achieve an optimal panel design by using fluorochromes with different emission spectra in order to avoid spectral overlap or “spillover.” The reality of multicolor flow cytometry is that some level of spillover is likely to occur between detectors even with an optimal panel design when you have considered all things, for example, pairing high antigen expression with dimmer fluorochromes. This means that even with the best design a fluorochrome may be detected clearly in the appropriate detector, but another detector may also pick up some of a fluorochrome’s emission spectrum. If you were to run an experiment under these conditions, you might for example, detect a portion of a FITC-labeled sample in the PE detector. The use of more fluorochromes only adds potential for greater spillover effects into different channels.

The resolution sensitivity of the affected detector is irreversibly altered and the accuracy of the experiment is compromised if spillover effects are not addressed correctly. Fortunately, flow cytometry users can address this problem by using a compensation setup strategy.

Compensation Clarity

Compensation is run during the setup of your experiment on a flow cytometer to correct for spillover effects. Most modern cytometers walk you through the compensation process during setup. However, you need to think about your staining and compensation control samples when you are staining your cells because it will be too late to have an accurate batch of controls if you wait until you are warming up your cytometer.

Your experimental setup will need to include unlabeled cells and single color control cells stained with the individual fluorochrome-conjugated antibodies at the appropriate titer for a defined number of cells. Compensation beads like BD's CompBeads or Affymetrix's OneComp eBeads are widely used alternatives to single color cell controls. These beads contain a mixture of positive control beads that react with fluorochrome-conjugated antibodies and negative control beads that do not bind antibody.

Your compensation setup will also need fluorescence minus one (FMO) controls, which are cells stained with all antibodies except for the one being measured in the respective channel. FMO controls assure that spillover from fluorochromes into other channels is correctly identified.

Your cytometer will direct you to run your compensation samples based on the detectors you are using. After your samples are run, compensation analysis is typically automated, and the cytometer's settings will be adjusted accordingly to compensate for spillover in your experimental samples. Compensation needs to be run at the start of each cytometry experiment, and you can't assume compensation settings from an old experiment will be accurate and sufficient for a new experiment.

And with that, you can run your samples and complete your flow cytometry acquisition.

Data analysis? That's a whole other ballgame.

Quantifying Biological Response
Through Cytometry