The MMRI Beginners Guide to Flow Cytometry

Samantha Le Sommer

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Fluidics

The fluidics is what brings your sample up out of the tube and into the machine and then through the lasers. It seems simple but poorly working fluidics will ruin your data and experiments.

Sample is taking up into the machine, where it meets with the sheath fluid. The sample and sheath fluid do not mix. The sheath fluid uses hydrodynamic focusing to pull out the sample stream into a thin stream to march single cells through the lasers. This is essentially like focusing a microscope, if cells fall outside of the sample stream, then you can lose resolution and those cells become invisible the laser.

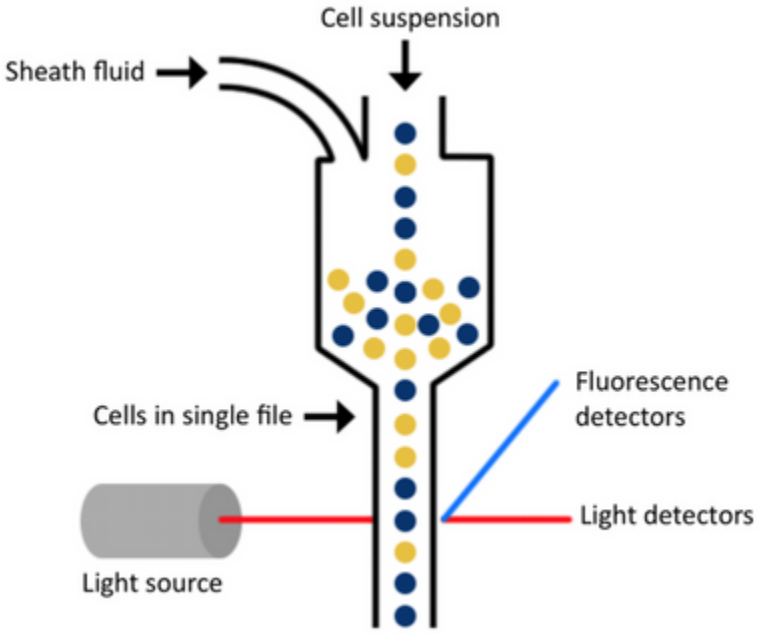

Figure 1: Fluidics of a standard flow cytometer. Asa Yokham, The Lab Managers Handbook, 2016.

Using laminar flow, the faster flowing sheath fluid drags the sample out into a thin stream encased within the sheath fluid. Every machine will have the ability to change the flow rate, increasing this rate increases the pressure with which the sample is injected into the laminar flow sheath fluid, making the sample core within the sheath fluid larger than diameter.

This has serious consequences, you will increase ‘coincidence’ ie have more than one cell passing through the laser at a time which can disrupt your data. You also increase the chance that cells pass through ‘off beam’ ie the stream of sample is wider than the laser diameter so you lose cells entirely and they can bypass the laser, losing resolution.

- Start with enough sheath buffer. Fluids require consistent pressure. Starting with enough buffer will ensure constant pressure.
- Keep the machine clean! Keep the lines clean! Meticulously clean each run otherwise the debris will ruin the machine by clogging it, making it harder to keep constant pressure.

- Run on low! If your samples are taking forever to run, then you can turn up the speed but run all samples at same speed, so every sample has the same degree of loss of resolution.
- Keep an eye on your fluorescence over time, this will give you an idea of how your fluidics is functioning inside the machine. Normal fluidics over time will give you a straight line. If you have a clumpy sample, or any other problem during the run, the fluorescence v time graph will not be straight. This indicates you had poor fluidics in your run.

Remember – *garbage in, garbage out*, the data you get out is only as good as the samples you put in. If your samples disrupt the fluidics it can ruin your data.

Optics

The optical systems are what allow us to sort the light coming off antibodies and dyes. The fluidics system transports the sample from the tube into the machine, in the flow cell the laser and fluidics system intersect, and then filters allow us to filter and detect the light.

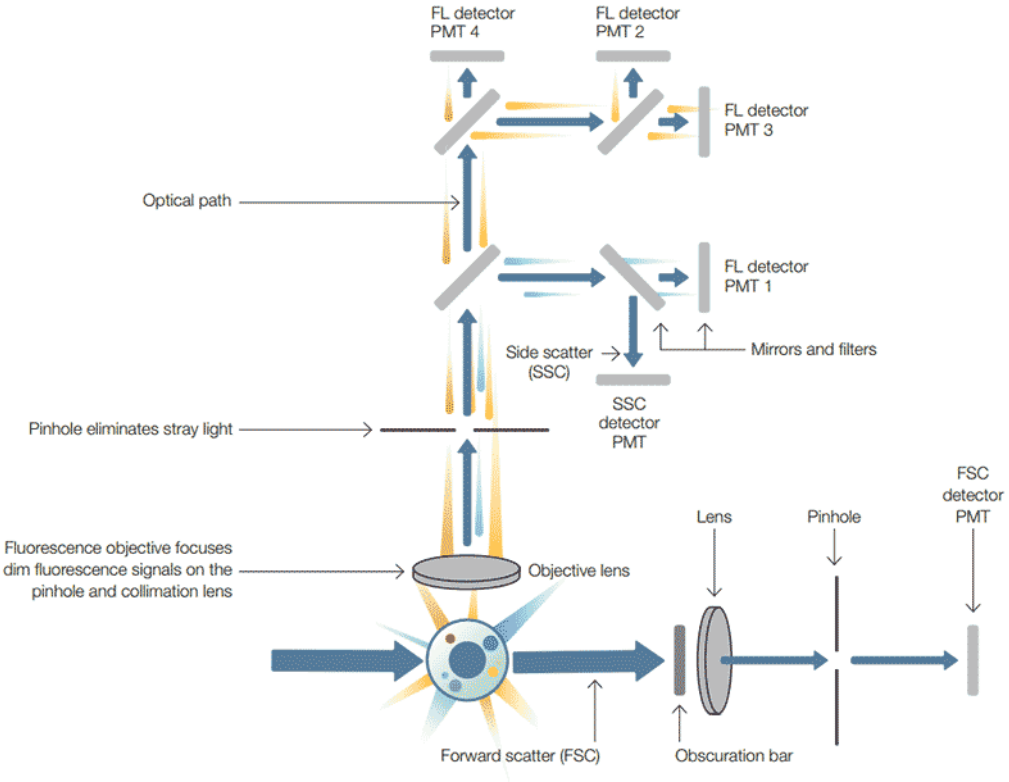

Figure 2: Optical systems of a flow cytometer. Sample is interrogated by the laser and photons are emitted from dyes and fluorochrome conjugated antibodies. The photons are emitted and travel through a system of mirrors and filters to detectors where they are converted into electrical signals.

Forward and side scatter

These are the two major properties of cells, the forward scatter is often called ‘size’ of a cell, but this isn’t really the case. It’s really a measure of the diffracted light, it’s called the forward scatter because the detector is at a forward angle compared to the laser. The side scatter detector is at a 90-degree angle to the sample and thus is the light that has hit stuff inside the cells and bounced to the side so is a rough measurement of cell complexity. Immunologists use this to discriminate between different immune cells such as lymphocytes and granulocytes because these cells have different properties. Though even non-immunologists can use forward and side scatter to gate out cellular debris.

What fluorochromes can you use?

The first step is to look at what lasers your machine has. You want to pick fluorochromes whose peak excitation matches the laser lines in your machine.

Downstream of the laser you have filters which break up the light and sends it to detectors. There are 3 kinds of lasers

- Long pass – lets light longer than a desired wavelength through
- Short pass – lets light shorter than a desired wavelength through
- Band pass – lets light between two wavelengths through

In the detector shown above, the light given off from the sample comes through and first hits a 735 long pass filter. All light longer than 735nm in wavelength will pass through this filter. They then hit a 710/50 band pass filter, this allows light between 685nm to 735nm through to the detector (or 50nm band centered on 710, so 25nm either side.) It hits detector A. The rest of the light hits a mirror and bounces off the mirror and travels until it hits the 630 long pass filter, allowing light longer than 630nm through. Again, it hits a 585/42 band pass filter and light between 564nm and 606nm through the mirror and detector. The light will bounce around the detectors until it finds a detector it can travel to.

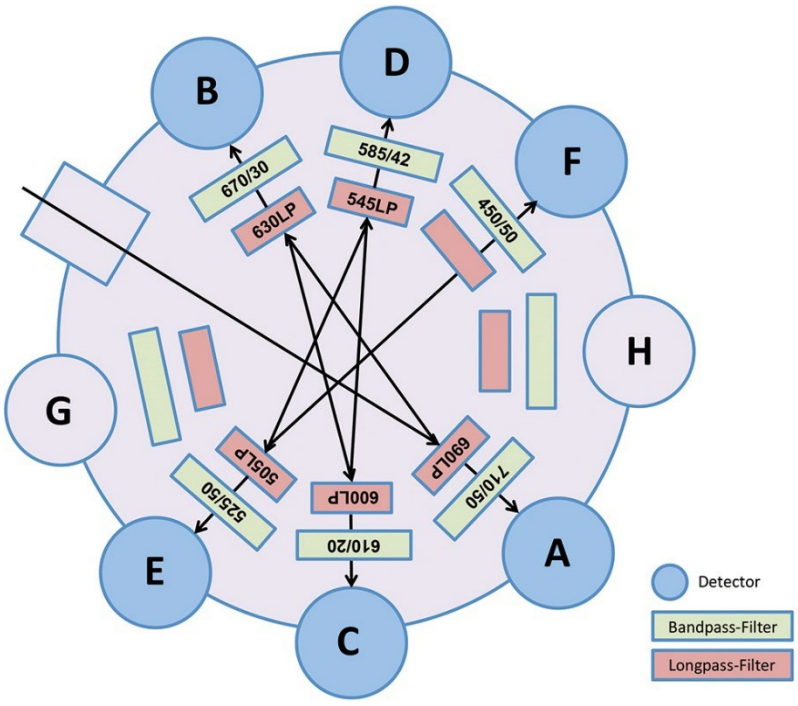

Figure 3: Example detection set up for the Violet 488 laser. Photons emitted from the sample travels through a series of filters and mirrors that allow the detection of specific wave lengths. Bio-Rad Guide to Flow Cytometry, 2009.

So, in order to use a fluorochrome its excitation wavelength muse correspond to a laser line, and its emission wavelength must correspond to a filter and detector set.

The voltage pulse

This is how light signals are converted into electricity. This is done through photo multiplier tubes (PMTs). The PMT takes your photons and converts them to an electrical signal in the form of a voltage pulse. As a cell begins to travel through the laser photons are given out, this will then peak, and then decrease again, as the cell passes through.

This is a voltage pulse. Every photon pulse has a height, an area, and a width of the signal. The brighter the cells, the higher the height in the voltage pulse. The voltage pulse for single cells are a nice bell curve which means we can also use it to discriminate single cell events from doublets.

In a correctly set up flow cytometer, the height and area of the voltage pulse should be directly proportional to each other. If you have doublets, the height of the voltage pulse doesn't change, however the area will increase, meaning the ratio is no longer proportional. You can use this to gate out any non-single cell events.

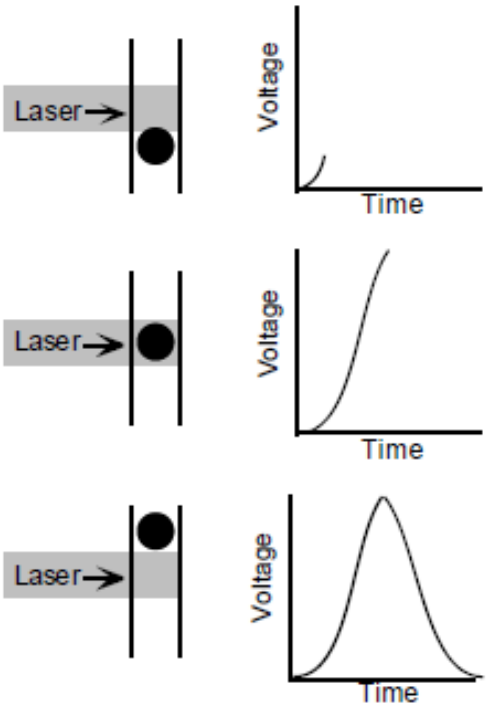

Figure 4: Generation of a voltage pulse. As a cell moves through the interrogation point, laser light hits the cell, and photons are emitted. These photons are detected and converted into an electrical signal. As the cell passes through the laser the emitted fluorescence increases, and then as the cell exits it decreases. This results in a bell shaped curve of voltage over time.

Antibodies

Antibodies are a Y shaped structure that can be split up into two regions, the Fab region is what targets the antigen and gives the antibody its specificity. It varies between antibodies. The back of the antibody is the Fc region which is constant. It is part of the complement cascade and is responsible for activating some immune cells such as macrophages.

When we design experiments, we want the antibody to bind to the target and light up. However, Fc receptors on some cells can bind antibodies via the Fc. This is often called nonspecific binding however its misnamed. This is not a nonspecific reaction. It is very specific between the Fc portion and the Fc receptor. It's just wanted. You can 'block' the Fc receptors by binding antibodies with no conjugate to the receptors.

If you are having major problems with Fc binding some companies sell antibodies that are just the Fab portion and lack the Fc region. This eliminates the problem altogether. True nonspecific binding occurs when antibodies bind by random regions to cells. This is rare in flow cytometry due to the BSA and FBS in the buffer, if you are unlucky enough to find nonspecific binding, you can alter the buffers to eliminate this. Isotype controls can be used to test nonspecific binding or to double check for it.

Choosing antibodies

Start with the vendor you will be purchasing the antibodies from. You can search on their website for the antigen specificity, the species, and the fluorochrome ie a FITC conjugated antibody that recognizes mouse CD3.

You will then get a list of clones that recognize CD3, with the FITC fluorochrome. The company will show you an example of the staining profile of the antibody, what they could do in their lab. If you see a reasonable separation between the positive and negative population, a list of the publications that have cited the antibody. A good indicator of a good antibody are the number of publications.

You can also use the website https://www.benchsci.com/ to check the antibodies in papers, it uses AI to search through papers, showing the appropriate figures, and you can see real life examples of the antibodies.

Designing a Flow Cytometry Panel

Panel design often feels like it's just a pile of markers with fluorochromes that are confusing. While there is science to panel design. There is also art. Below are some points to consider as you design a panel. No matter how many colors you are using the idea is the same, and panel design rarely works the first time round!

Always use a viability dye

No matter what you are staining for you must always have a viability dye.
Dead cells will destroy your data! You must always have a live/dead dye. No exceptions. It's recommended to put your live/dead dye onto channels that are underutilized such as the far-red channels where resolution is dimmest, or in channels that have dim fluorochromes such as AmCyan channel. The nice thing about viability dyes now is that they are so ubiquitously used is that they can be put in every channel.

Brightest fluorochromes give highest resolution.

When designing a panel for < 6 colors the best thing to do is to select a fluorochrome that is brightest from each laser line. By "bright" in flow cytometry we mean the fluorochrome with the brightest stain index, the gap between the negative and positive population. The brighter the fluorochrome, the wider the gap, the better the resolution. There are more than six very bright fluorochromes on the market, so when designing these smaller < 6 color panels the easier it is to put every color on bright fluorochromes to maximize the resolution. Below is an example of the stain index and "brightness" of fluorochromes.

		Fluorochrome		
Laser	Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon™ BUV563 BD Horizon™ BUV661 BD Horizon™ BUV737	BD Horizon™ BUV395 BD Horizon™ BUV496	BD Horizon™ BUV805
Violet (405 nm)	BD Horizon™ BV421 BD Horizon™ BV650 BD Horizon™ BV711	BD Horizon™ BV480 BD Horizon™ BV605 BD Horizon™ BV786	BD Horizon™ BV510	BD Horizon™ V450 BD Horizon™ V500
Blue (488 nm)	BD Horizon™ BB515 BD Horizon™ BB700 BD Horizon™ PE-CF594 PE-Cy™5	PE PE-Cy™7	FITC Alexa Fluor® 488 PerCP-Cy™5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon PE-CF594 PE-Cy5 PE-Cy7			
Red (640 nm)		APC Alexa Fluor® 647 BD Horizon™ APC-R700		Alexa Fluor® 700 APC-H7 APC-Cy7

Table 1: Relative brightness of the most common commercial antibodies. BD Biosciences 2019.

Reduce spill over.

This means spreading your fluorochromes out as much as possible. Start by selecting the first fluorochrome off each laser line this is generally the easiest way to design a four-color panel. We can add additional fluorochromes while reducing spill over by spreading them out across various filters. For example, coming off the 561 laser we know PE is a bright fluorochrome so firstly we select PE. PE-Cy5 and PE-Cy7 are both bright too, if we wanted to spread our fluorochromes out we would pick PE-Cy7 as it is further away from PE than PE-Cy5.

Test your panel.

Testing your panel can save you a lot of time and effort! It is highly recommended. Not just to test the antibodies but to make sure that the biology makes sense. You should have single stained cells, and then a sample with all of your antibodies in it. If you know one of your markers should be expressed on 20% of cells, but when you stain your sample we discover it is 1% or 80%, or biologically impossible populations appear that we know does not make sense. Then, you will have to troubleshoot or redesign your panel. You are also looking for resolution, if you find that you have much smaller resolution of a marker than you expected you may want to go back and look at different antibodies for that marker. Another thing to look for are diagonal populations. Very few markers are this closely co-expressed that you will have an entirely diagonal population. This is very rare and should be investigated, or an antibody replaced.

When designing panels > 6 colors, categorize your markers into high expression and low express, or high importance and low importance.

Making more complex panels can be more difficult, to make it easier to detect your most important markers to your assay such as cytokines which are harder to stain, rare markers, or markers on very low % populations, you want to put these antigens on the brightest fluorochromes. This gives you the best possibility of resolving these populations. You can then assign less bright fluorochromes to other markers such as CD3, CD4 or CD8 which have binary off – on expression or are less important to the actual assay and are used as cellular identifiers.

Avoid tandem fluorochromes in intracellular stains

No matter what it is you are staining for intracellular or the number of fluorochromes you have in your panel. Be it a transcription factor like FoxP3, or a cytokine like TNFα. Not only do you want to give these antigens the brightest fluorochromes available, but never give these antigens tandem fluorochromes! The fluorochromes that are attached to flow cytometry antibodies are big, it's like attaching a beach ball to a small antibody, so if you are using a tandem dye its like sticking two beach balls on! So not only does it make it far harder to get that antibody with two beach balls attached onto it into a cell, but also to get it back out if its unbound!

When making panels > 6 colors not only do we want to reduce spill over, but also spill over spread.

Compensation and spill over error is talked about in more detail in the compensation

section, but spill over spread is the background of the fluorescence. Spreading error can decrease the resolution, if our PE signal spreads, we may have difficulty detecting a PE-Cy5 population. It's fairly easy to figure out if you are doing to have a large spill over. To make a spill over spread matrix you just need compensation controls, if you have not made your own, you can use a panel design website using standard fluorochromes to give you an indication as to where you may encounter problems. When in doubt, test.

Flow Cytometry Buffers

We have banged on about the importance of sample prep. Your experiment will only be as good as the preparation.

Garbage in. Garbage out.

Buffers are an important part of your experiment. Dead cells will wreck your data, they clump, they have high background fluorescence, and are autofluorescence. Keeping your cells alive is a good start to getting good samples.

Flow Cytometry buffers all start with the common buffer base such as PBS, or HBSS. These two are the most common. Other bases can be used but they must follow the rules.

No phenol red. Ever. It will wreck your ability to detect fluorescence.

Avoid magnesium and calcium, these ions can bind to the cells and cause them to clump.

Protein additives

Additionally, all flow cytometry buffers will include a protein wither BSA or serum. For BSA a 0.1% to 1% additive or a 1-2% additive of serum Too much protein will affect your ability to stain the cells. The addition of a small amount of protein will firstly help block the cells and prevent things from binding and sticking to each other, and secondly, they can help keep cells happier and increase viability. You should always check if the % of FBS or serum works for your experiment.

EDTA

EDTA prevents clumping of cells in a cation dependent manner, it is usually added in a 0.5mM to 5mM concentrations. If you are working with sticky cells like macrophages, it will be essential to keeping a single cell suspension. Word of warning – too much EDTA can kill your cells. Double check your cells don't get too mad with you for using it.

HEPES

HEPES increases the Co2 buffering capacity of the buffer, 10-25mM concentrations are usually used in order to allow cells to respire normally without the pH getting too high. HEPES is helpful in situations where you have long incubations or when you are cell sorting, and the cells will be in the buffer for considerable time.

An added benefit of HEPES is that it seems to reinforce the cell membrane, we aren't sure why, but it can increase recovery in cell sorting as the cells are more sturdy when going through the machine.

DNase

When you are prepping samples from tissues that you have had to mash up, or digest, there will be a ton of DNA floating around in suspension in your sample. This often occurs in protocols where you have a large amount of cell death. DNA is sticky as all hell. It can make cells clump together. Get angry with each other. DNase additives can help break the cells back up and give you a single cell solution back together.

Warning – when you add DNase you will need to add magnesium chloride to facilitate the enzyme activity. If you can avoid cell death instead of using DNase it's better to make sure your sample prep avoids cell death rather than try and get DNase working. Sometimes, death is unavoidable.

Sodium Azide

Azide can be added to buffers to prevent bacterial and fungal growth. It is usually added in concentration between 0.05% and 0.1%.

However, azide will inhibit some cellular functions and metabolic activities. If you are sorting and using cells downstream azide is best avoided. It's simply better to make your buffer fresh every time.

It can be used as a control if you are looking at antigen internalization, shedding of internal antigens, antigen capping etc, you can use azide as a control as it will inhibit these functions.

Fixatives

Fixatives are fantastic for making experiments convenient, preventing contamination of samples by microbes, but they can affect fluorescence detection. There are two main kinds of fixatives – alcohol and aldehydes.

Alcohol fixatives

These fixatives are used mainly for cell cycle analysis. It is good for fixing DNA and nucleic acid but they can precipitate proteins and thus are poor protein fixers. They can result in antigens being hidden and destroying the structure of protein based fluorochromes. So, you may damage your antibody.

Once cells are fixed in methanol, they can be stored at -20 indefinitely. Alcohol fixatives will also permeabilize your cells as they dissolve the membrane but not all antibodies can withstand the methanol. It is critical to test your antibodies to make sure they can withstand the methanol.

Aldehyde fixatives.

These are more commonly used as they preserve proteins better. Aldehyde fixatives work by cross linking glycine residues, so they lock proteins together. This method results in less structure disturbance meaning you can fix and then stain your samples. Aldehyde fixatives however are bad for DNA.

The longer the cells are in the fixative the larger the autofluorescence. 10 to 30 minutes is more than enough time to fix cells. Aldehyde fixatives should be washed out after, and cells stored in PBS. Fixing in aldehyde is an exothermic reaction so aldehyde fixing should always be done cold. Aldehyde fixatives will also fix in any state it finds the cells – so if the cells are clumped up, its going to fix them into clumps! Having a single cell suspension will help fix them properly.

Permeabilization

If you are doing intracellular signaling you may see permeabilization steps which makes holes in the membrane so you can get antibodies through the membrane into the inside of the cell. Permeabilization must be done after fixing because if you do it before, you are going to explode the cell and there will be no cells left to stain. There are a number of different permeabilization methods.

Alcohols

As mentioned above alcohols will permeabilize your cells as you fix them. You'll probably only use alcohol as a method if you are only looking at nucleic acid content.

Detergents

Some detergents are stronger than others, you can play around with this depending on what you want to look at. Triton X is good for nuclear staining as it will permeabilize the plasma membrane and the nuclear membrane, whereas a weaker detergent like saponin is good for cytosolic staining, but not nuclear staining.

Some detergents cause permanent permeabilization, like Triton X, Tween-20, NP40, you only need to add once because the pores are permanent. These cells need to be

handled very gently. Be careful when vortexing or pipetting as the cells become fragile. These detergents also have a phenol ring that can absorb UV light, so be careful when using the with UV laser.

These detergents and their permanent permeabilization mean that you only need to add the detergent once, you can then switch back to your standard buffer. A word of caution, incubating too long in these detergents can lyse your cells.

Saponin however, creates small pores and selectively interacts with cholesterols in the membrane. It is also transient. As soon as you start working with saponin you must keep it in your buffer, or you will lose the permeabilization of the cell. If you do not, you will be able to get antibodies into the cell…and then not back out. It also is a poor permeabilizer of the nuclear membrane, so harsher detergents are normally used.

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Controls

Controls are both loved and hated, however, flow cytometry is impossible without them. Here we will look at the different types of controls and the consideration of them. There are five main types of controls. As you can see, these controls can stack up over time, and more often than not, you will end up more controls than samples.

Instrument controls

Every flow cytometer must be quality controlled to assure performance over time, on our BD machine these are done with CST beads and tracking beads. These beads monitor the fluidics, the detector sensitivity and laser delay settings. These calibrations are performed weekly by the core staff.

Compensation controls

Any time you have two or more colors you will need to run compensation (see the compensation settings for more details on how compensation works. It removes fluorescence detected from your antibody in channels other than the one designated for it.

You will need to follow the compensation rules which can be found in more detail in the compensation section but briefly:

- The fluorochrome in the compensation and experimental samples must match exactly and be from the same vial of the antibody.
- Compensation controls must be brighter than any of your experimental samples.
- Your universal negative must be the same across all compensation to match autofluorescence.
- Enough negative and positive events must be collected.

Isotype controls

Isotype controls are protocol controls. They are designed to measure the non-specific binding of the antibody, they are not gating controls. The isotype controls will tell you if your staining protocol is working. It will tell you if your blocking were complete enough, or if your washes were enough to wash away all the unbound antibody. The correct control for setting gates is the FMO (fluorescence minus one) control. I

FMO Controls

FMO controls contain all the dyes except for one. For example, in a four color panel you will have four FMO controls, where systematically one dye is removed from the sample. For the following panel: CD3 FITC, CD4 APC, CD8 PE, CD25 BV405 you could have the following controls

- CD3 FMO containing CD4 APC, CD8 PE, CD25 BV405
- CD4 FMO containing CD3 FITC, CD8 PE, CD25 BV405
- CD8 FMO containing CD3 FITC, CD4 APC, CD25 BV405
- CD25 FMO containing CD3 FITC, CD4 APC, CD8 PE.

FMOs must always be included in a panel. While some operators say that you can skip FMOs when you have discrete populations that are clearly positive or negative. However, this is bad practice. There should be an FMO for every single stain in your panel.

Biological Controls

You should always have biological controls in the experiment, for example, a cell sample that has not been treated with your drug that will tell you the background expression of a marker. Or if you are staining for viability and investigating if a drug kills cancer cells, you would want a sample of entirely dead heat shocked cells as a positive control to show that your viability dye works as intended.

Reference Controls

If you are doing longitudinal studies, for example assessing patients over time. A reference control is something you use every run to demonstrate your staining pattern isn't changing over time. It can be pooled blood, or a cell line, anything that shows your staining is standardized over time.

Compensation and Spreading Errors

These two concepts are important in every single flow cytometry experiment but are misunderstood.

Compensation

The emission spectra for a fluorochrome is almost always larger than the detector it is being detected in. Compensation tells the machine that a certain fluorochrome belongs in X filter, and that the signal from this fluorochrome in all the other filters and detectors, should be disregarded and subtracted from the final data. Compensation should be run every single time you are using more than one fluorochrome. No exceptions. Compensation is easy, all you require are single stained controls, almost always single stain Anti-IgG beads or amine reactive beads. The software will do the rest for you.

Problematic controls can ruin your experiment, for example, if you are working with a GFP+ cell line and you use this to create your compensation controls…and you stain this cell line with a single antibody then every single sample has two colors. The same thing with viability dyes, or DNA dyes. Compensation controls contain a single color. Nothing else. Ever. Anything else will not work.

There is still discourse between if cells or beads should be used for compensation, however it is generally accepted now that beads provide better compensation, as they are better for compensating low expressed markers, do not consume samples and provide the best fluorescent signal.

The best piece of advice can be to use beads, make them fresh, every single time. You should have no exceptions to this rule. You can destroy your experiment with dodgy compensation.

There are four basic rules for compensation controls.

The single-color sample you stain your compensation control with must exactly match the experiment.

Use the exact same antibody. They must match completely. The calculation of compensation relies on the emission profile of the fluorochrome and how it is detected in filters other than it's designated filter. If you do not use the exact same antibody with the exact same fluorochrome on it then the emission spectra of the control will not match that of the experiment and you compensation will not work. For example, GFP and FITC, they will be detected in the same filter, but they have different emission spectra so if you use FITC to compensate for a GFP+ cell then it will not properly compensate.

Tandem dyes also fall foul of this, because of the way tandem dyes are made they can vary lot to lot and vial to vial, so it's even more important that the same vial of antibody

is used. While it is fairly obvious if some dyes are tandems such as PE-Cy5 because they sound like they have two names, newer tandem dyes are not so easy to work out. There are some stealth tandems like BV650 – it is a tandem due even though it doesn't sound very different from its parent dye BV451.

Therefore, it is always best to use the exact same antibody from the exact same vial and get into good compensation practice from the very start.

Your compensation dyes must always be brighter (or at least as bright) than your experimental samples.

Compensation can not be extrapolated. The machine will accurately compensate the fluorescence up to the brightest point of the compensation controls. So, it is important, and it is good practice, to ensure that your compensation controls are always the brightest part of your experiment! Another important reason to use beads. By doing this you will ensure that all samples and all levels of fluorescence in your experiment are properly compensated for.

The autofluorescence of your sample must exactly match the compensation.

The easiest way to get around this is to draw a gate around your negative and positive population on the cytometer as you are acquiring samples. Thus, avoiding the use of a universal negative. If you want to use a universal negative, then the compensation should be done on the same materials. For example. Use unstained beads if you are using beads for compensation.

Collect enough events.

Accurate compensation depends upon the accurate calculation of the median fluorescence intensity for the positive and negative populations of every control. If you don't have enough events this will suffer. That simple. Collect at least the number of events the machine wants.

Avoid compensating by eye.

The machine will do all the math for you. You can do it if you want but the machine can do it better and faster. Avoid changing the compensation matrix by eye to make populations look "right." If you do this, you will not account for the changes this makes to other channels.

On that note, inspecting your compensation on n by n plots which plot every fluorochrome against every other fluorochrome to double check and see if there are any signs of over and under compensation. Double checking is good practice. If overcompensation is present, fluorescence will "frown" and signal will lean to wards the axis indicating values have been over subtracted. If fluorescence begins to smile, the converse has occurred and there has been an under subtraction.

If you see these avoid the urge to manually adjust, instead go back to your compensation controls and see what happened to cause this. It will always, always, come back to something having gone wrong with your compensation.

It is not worth losing your data to avoid the process of making a compensation control. The best piece of advice can be to use beads, make them fresh, every single time. Recycling a compensation matrix from even the day before can completely obliterate your data. It can not be stressed enough how important fresh, well made compensation controls are.

Compensating and voltages

It is important to remember that compensation is only valid at the same voltage as your experimental samples. Adjusting voltage will change your compensation values. Lowering your voltage will decrease your compensation, the lower the voltage the lower the compensation.

This may seem beneficial. It is not. It might not be what is best for your experiment.

If you have optimized your voltages, leave them. Remake your compensation beads. Lowering it to get the compensation "lower" will not help. If you have not optimized your voltage – start there.

Compensation is compensation. It is a mathematical compensation. There is no good. No bad. No high. No low. If you have done your controls properly the compensation you get is the compensation you get.

Spreading Error.

Often spreading error and spill over error are conflated with each other. Spill over error is due to the off-filter detection of fluorescence and can be corrected by compensation. Spreading error is very different and causes issues in detecting true positive populations, you have a spread of a population that means you may struggle to resolve that population. *Spreading error cannot be corrected for, nor does compensating create spreading error, it merely reveals it.*

Spreading error is caused by a mixture of the instrument and the fluorochrome, it is not predictable like compensation where we can look at the fluorochrome emission and know which other filters we will signal in. With spreading error you will not know if you have it until you have run the samples. This is another reason that testing your panel is incredibly important, you will not know you have it until you have tested for it.

The easiest way to look for spreading error is to create a spill over spread matrix. It sounds complex but FlowJo will calculate it for you, and you can simply look at the spill over spread matrix and check to see if you have this error in your data.

Theoretically we can reduce spreading error, but practically…not really. The main things we can do to decrease spreading error is to decrease the voltage, and decrease the amount of antibody, however this reduces the resolution of the population – its often better to just try a different fluorochrome if you have significant spreading error.