CRITICAL ASPECTS OF STAINING FOR FLOW CYTOMETRY

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Cell Concentration/Cell number:

For *each* sample, you will need between 10^5 and 10^6 cells. If you are new to flow cytometry, use the higher number of cells -- to give yourself a margin for error (you always lose more cells than you expect during the staining and washing procedures). Trying to stretch too few cells into too many sample tubes in order to maximize results from a single experiment almost always leads to no results at all (it's happened to all of us).

After staining and washing, resuspend the cell pellet in 0.3-1.0 ml, according to the configuration of the sample uptake on the cytometer. Use the smallest volume you can; the suspension can always be diluted with buffer later if cells are flowing too fast. When at the cytometer, adjust this final concentration of cells with more buffer if necessary, so that cells flow through the cytometer at about 100-1000 cells per second. Slower than 100 cells per second gets very boring. Faster than 1000 cells per second may lead to problems because the computer may not keep up or because two cells close together in the laser beam may be seen by the cytometer as a single "event." Constraints on adjusting cell concentration come from the total number of cells that are available and the minimum volume that your cytometer can handle. In general, you want to have cells at a high concentration so that you can use the lowest pressure possible to push them through the cytometer (but still achieve a flow rate close to 1000 cells per second). Pushing the cells too hard to achieve this high flow rate has deleterious effects on intensity resolution (by widening the sample core in the flow stream).

If you are setting up a new procedure or are needing to adjust instrument settings, it is especially helpful to have a few extra control samples with more cells in a larger volume. Running these cells initially, before you start collecting data , will give you time to adjust FSC and SSC parameters so that all cells are on scale, to make sure that background fluorescence is at an appropriately low position on the fluorescence scale, and to check out compensation if necessary for multicolor analysis.

Tubes vs Plates:

Cells can be stained in any container for which you have an appropriate centrifuge. You can stain in the test tubes that will eventually fit on your cytometer, although the large bottom diameter of these tubes will make it difficult to get consistent results with a small volume of staining solution. Eppendorf tubes are classical favorites because of their conical shape. Staining can take place in a small volume in an Eppendorf tube and then the suspension can be diluted to a full 1 ml for each washing step.

If you are staining more than a few samples and have a centrifuge with plate adaptors, consider using 96-well, round-bottomed microtiter plates. While the dilution factor in the washing step is not as effective in these plates (because the maximum volume is 200 ml in each well), the benefits that derive from not having to organize, label, and manipulate dozens of test tubes makes plate-staining highly attractive. Photocopy a template to use for identifying wells; plan the layout of samples in the plate with some thought in order to make addition of reagents easy. It usually works to add the antibody or antibodies to wells in advance while cells are being prepared, keep the plates covered in the dark at 4°C until the cells are ready, and then use a multi-channel or repeating pipette to add aliquots of the cell suspension to the rows of wells. Care needs to be taken not to drag antibody from one well to the next in this procedure. Although it takes a bit of practice to become confident about staining and centrifugation in plates, most people become converts very quickly.

Centrifugation and Washing:

The centrifugation step pellets cells from the staining suspension, thus permitting removal of the supernatant fluid, and the washing of unbound antibody from the cells. The unbound antibody is removed by dilution in the sequential washing steps. Exact advice about centrifugation speeds is not possible, as different centrifuges take more or less time to reach full speed. In general, you want to spin cells down hard enough that the supernatant fluid can be removed with little loss of cells, but not so hard that the cells are difficult to resuspend. If staining in Eppendorf tubes, a high speed microfuge with an angled rotor can be used; 1-2 seconds is long enough to pellet most cells well. If the Eppendorf lids are lined up in the rotor so that the hinges all point outward, then the cell pellets (even if not visible) will all be at the hinge side of the tube after spinning. The supernatant fluid can then be removed with confidence by inserting a glass disposable ("Pasteur") pipette into the other (non-hinge) edge of the tube. The disposable pipette should be hooked up to suction such that the aspirated fluid will go into a trap containing bleach. After addition of 1 ml of S/W buffer, the cells can be resuspended on a vortex mixer before spinning again.

If staining in microtiter plate wells, use a centrifuge with plate adaptors. At about 1,500 x g, cells will pellet well in about 2-3 seconds. Removing supernatant fluid after centrifugation can be done by flicking the plates and then blotting once on clean paper towels. More securely, the supernatant can be removed from each well with a glass disposable ("Pasteur") pipette hooked up to suction and a trap. Wash buffer can be added with a multi-channel pipette -- a squirt from this pipette is enough to resuspend a cell pellet if it has not been centrifuged too hard.

Specificity and Non-Specificity:

In order to draw conclusions about particular proteins on cells, we have to be sure that the stains that we use are specific in their binding to the proteins in which we are interested. Lack of specificity may occur as a result of several factors: Firstly, an antibody may bind to a common epitope on several different proteins. Secondly, different sorts of cells contain the same proteins, so a staining reaction may not be as specific as one would like in classifying different cell types. These first two types of problems require knowledge of the appropriate reagents for answering particular questions. Two other types of specificity problems are more difficult to control. Antibodies bind to many cell types by their non-specific (Fc) ends. Monocytes, in particular, professionally bind many antibodies through their Fc-receptors. The use of **Fab or F(ab')2 fragments** (antibodies without their Fc ends) is recommended whenever possible and helps to alleviate this problem. Blocking the Fc-receptors (see below) also helps. Finally, dead cells, with compromised membrane integrity, tend to be sticky and to bind all sorts of reagents with careless abandon. The dead cell problem can be helped by careful cell preparation or by "gating out" dead cells from analysis -- either through propidium iodide staining to positively identify dead cells by their membrane permeability or, less securely, through avoiding cells with low FSC intensity (cells that are dead usually show low FSC signals probably because the refractive index of their cytoplasm is similar to that of the surrounding medium). In the final analysis, however, in order to permit conclusions from fluorescence staining about the classification or chemical constituents of a cell, flow experiments must make use of careful controls.

Controls:

In an experiment with indirect staining, it is very necessary to stain cells with the secondary antibody alone to control for non-specific binding of this polyclonal antibody to dead or sticky cells. This is the so-called "secondary control" that marks the level above which fluorescence intensity can be considered specific. Comparing the intensity of cells stained with the secondary antibody alone to the background autofluorescence of unstained cells is often diagnostic of problems with the secondary reagent. Ideally, the secondary control should be no brighter than the unstained cells. It is also absolutely necessary to have singly-stained cells to set the spectral

cross-talk compensation for multicolor experiments. There is considerable debate, however, about the question of appropriate negative controls when staining cells with a direct protocol. If positive cells are much brighter than unstained (autofluorescent) cells and if the experiment contains some samples where no cells stain, then, arguably, the unstained cells within the stained samples are, in themselves, controls for non-specific staining. Most people, however, feel happier about ruling out problems with dead cells or Fc-receptor binding if their experiment contains samples stained with so-called isotype control antibodies. Isotype control antibodies are antibodies with no specificity for the cells in question but with all the non-specific characteristics of the antibodies used in the experiment. Since antibodies come in different classes ("isotypes") with different binding characteristics, an experiment should probably have an isotype control antibody for each class of antibody used for staining. Such antibodies are available from manufacturers of flow cytometric reagents. Sceptics will wonder about our ability to know the protein concentration of every antibody that we are using and to know the number of fluorochrome molecules conjugated to each of these antibodies. Compromises may be necessary, since each stain in a panel of many stains may be of a different isotype, at a different concentration, and with a different F/P ratio -- and will therefore require its own control. There are no perfect answers here; the requirement for controls is often determined by the nature of the experiment and needs to be considered carefully in relation to the stringency of the questions being asked of the resulting data.

Blocking:
One important way to minimize non-specific staining is by the use of a so-called blocking reagent. A blocking reagent contains a high concentration of immunoglobulin that will bind to the Fc-receptors on cells like monocytes, thereby blocking the non-specific binding of the staining antibody reagents to these receptors. The blocking reagent should be immunoglobulin from the species whose cells you are staining. For human cells, use the g-globulin fraction from human serum at a final concentration of 4 mg/ml. Alternatively, one can purchase specific antibodies directed against the Fc receptors of the cells in question; care needs to be taken here as there are different classes of Fc receptors and, in addition, not all antibodies against Fc receptors block the receptor binding site.

Titration and Proportionality:
Flow cytometry has the potential for providing quantitative information about the relative expression of membrane proteins on different cells. For example, we may want to determine if cells, when stimulated, express more of a given protein than they do under resting conditions. In order to be able to draw quantitative conclusions from flow cytometric data, we have to be sure that the fluorescence intensity of a stained cell is directly and linearly proportional to the protein we are measuring. This involves using concentrations of reagents that are not limiting, so that the staining intensity of a cell will be related to the amount of the protein on a cell and not to the number of cells present nor to pipetting inaccuracies in the exact amount of reagent used. Determining the amount of stain to use involves titrating each antibody reagent to find a concentration that approaches saturation within the time of the reaction. With low affinity/avidity antibodies, strict saturation may not be possible in short staining times at reasonable antibody concentrations; therefore use of such antibodies makes conclusions about relative intensities difficult.

To determine a saturating concentration, run a **titration curve** by using halving dilutions of antibody, covering a range above and below that suggested by the manufacturer (if any). Make this 1:2 dilution series (using 10 halving dilutions) of both your antibody of interest and also of an irrelevant antibody matched by concentration and isotype. Then stain cells with the standard staining protocol, using both the irrelevant antibody and the antibody of interest from each of the 10 dilutions. As your final working concentration, pick a dilution of stain well on the intensity plateau for the stained cells, where small changes in stain concentration have little effect on cell

fluorescence intensity. Very **high antibody concentration** is not, however, a panacea: cells often stain less well at very high antibody concentrations than they do at lower concentrations. Additionally, as can be seen from the cells stained with the control antibody, even negative controls often begin to stain non-specifically at high antibody levels. The important thing here is to maximize the signal-to-noise ratio: use a concentration on the plateau that gives maximal staining with the positive antibody but little staining with the control antibody.

With indirect staining, titration with halving dilutions is necessary for both the primary and the secondary antibody. Although it may seem as if there are too many unknowns in this equation, it is possible to guess at a reasonable concentration of one primary antibody, titrate halving dilutions of the secondary antibody with that concentration of the primary antibody, and then, using the concentration of secondary antibody determined to be optimal, titrate all primary antibodies in turn. Because non-specific staining will increase at high antibody concentrations, when titrating the secondary antibody it is particularly important to compare the staining of cells with and without primary antibody present. Cells stained with the secondary antibody, but without any primary antibody, are known as a "secondary control."As with titrating directly-conjugated antibodies, you want to use a level of secondary antibody that maximizes the signal-to-noise ratio, that is, gives a saturating signal when the primary antibody is present but little signal when the primary antibody is absent.

At staining concentrations that reach saturation, the amount of the stain present is hardly ever limiting: that is, there is usually enough antibody around to stain all the relevant cell proteins on all the cells without significantly lowering the concentration of free antibody (see Kantor and Roederer for examples of this calculation). The *number* of cells is not, therefore, critical to the staining intensity nor is the *volume* of the suspension. We simply need a high enough *concentration* of antibody so that effective equilibrium between bound and unbound antibody will be achieved in a reasonable staining time.

Staining Volume (and Expense!):
Many staining reagents, particularly monoclonal antibodies, are expensive. Any protocol for staining cells should have expense as one of the criteria for experimental design. Although the number of cells for an experiment is constrained by both experimental and flow cytometer design and the concentration of stain used is constrained by a desire to achieve near saturation (see above), the staining volume is under user control. The smaller the staining volume, the less stain will be required to achieve saturating concentration. Although many companies selling staining reagents suggest staining in 100 ml volume, it usually works to reduce this to 50 ml or even less. This reduces the requirement for stain by 2- or more-fold. The only considerations here are that you don't go to such a small volume that pipetting becomes inaccurate, nor that the amount of stain becomes limiting (that is, that there isn't enough stain to go around to all the receptors on all the cells present). With 50 ml of stain at a saturating concentration, 10^6 cells with antigens within the usual range of density will not use up a significant fraction of most antibodies. Check this out by using 2-fold more and 2-fold fewer cells than you are ever likely to have and convince yourself that the fluorescence intensity of the cells in all cases is the same.

Time and Temperature:
Antibody concentration, temperature, and time interact in the staining reaction: higher antibody concentrations reach effective staining saturation more quickly than lower concentrations and any concentration reaches saturation faster at a higher temperture. Even though azide is a metabolic poison (and some labs find that it allows them to stain cells at room temperature, rapidly and with impunity), most labs stain with all reagents/solutions at 4°C even in the presence of azide to be sure of minimizing capping / internalization/ miscellaneous loss of surface-bound antibodies. At 4°C, 30 minutes is a reasonable staining time. Antibody titrations can all be done at 4°C and for 30 minutes. After titration to determine a saturating antibody

concentration, check out the effect of time and use a time that is long enough that a bit more time or a bit less time makes no difference to the results.

Stability:

Once you have stained cells, it is necessary to know how long the cells with the fluorochrome on them will remain as you want them. Cells die with time, antibodies are internalized gradually at warm temperatures, fluorochromes become bleached in the light, and microbes will grow everywhere eventually. The answer to all of these problems is to keep cells (before and after staining) scrupulously at 4°C and in the dark, use a metabolic poison like azide in all buffers if you are not concerned with recovering cell function, and, finally, fix the cells with formaldehyde after staining if you are not going to analyze them within several hours on the flow cytometer. Although 1% formaldehyde fixation does change the FSC and SSC of some cells and may increase their background fluorescence and decrease their staining fluorescence just a bit, it provides great cell stability after staining and also a large margin of safety when dealing with potentially pathogenic samples. A good, general procedure for antibody-stained cells is to fix them in an equal volume of 2% formaldehyde after staining, let them sit at least 24 hours, (covered, in the cold and dark) to reach final FSC, SSC, and fluorescence levels, and then read them on the flow cytometer within 2 weeks (although most cells will last much longer). Check out your own cells with your own stains before deciding on the length of time that you are willing to risk this. But fixation after staining is definitely the way to go if possible --- giving great flexibility in planning time on that cytometer. As a reminder, you cannot fix cells before flow if you want to use a membrane-impermeant stain like propidium iodide to check cells for viability or to gate out dead cells in flow analysis. All cells become dead and take up the dye after formaldehyde fixation.

Safety:

Fixation of cells after staining and before running them through a flow cytometer is highly recommended if there are any questions about the pathogenicity of the cells involved. Since there are real concerns about the potential pathogenicity of all living cells, fixation after staining is always recommended unless the cells will be sorted and cultured afterward or unless you need to look at membrane integrity of the cells as a measure of viability.