New client guide for assisted data acquisition & analysis

Pre-Appointment Information

Controls

Flow cytometry is a reference based science. This means your flow cytometric data is only as good as your controls. These should ideally be the made from the same cells as your samples, but other cells of the same type or even beads can be used effectively when sample is limited.

Negative control (REQUIRED): *Cells with no staining*. Used to define the background fluorescence inherent in your cells. You should have a separate negative control for each cell type, because auto-fluorescence differences can be substantial. It is especially important that you bring a negative control to every appointment, because this is what is used to set detector voltages for light scatter properties (see below). If you don't bring a negative control, your fully stained sample will be used for this (effectively wasting some sample), which could be an issue for your data if your sample is limited.

Single Color controls (REQUIRED): *Cells stained with only one color* (fluorophore).

On conventional cytometers, these controls are used to determine how much the fluorophore spills over into other detectors, so compensation can be performed for the spectral overlap.

On spectral cytometers, these controls are used to identify the spectral signature, or where and how much each fluorophore or fluorescent protein emits in each detector, so unmixing can be performed.

The usefulness of these is dependent on having both negative and positive populations for every color. Therefore, consideration should be given to whether an experimental reagent is appropriate for single color controls in situations where target cells are rare; substitution of common markers works well for this (e.g. in immunology use CD3 for single color controls instead of a rare marker which wouldn't show enough positives to be useful for compensation.) Even when using beads, utility is dependent on having distinct negative and positive populations for every color.

The core sells UltraComp eBeads Compensation Beads that can be stained to create single color controls. Contact ccsc@bcm.edu to request a bottle of 25 tests (catalog # 01-2222-41) or 100 tests (catalog # 01-2222-42).

Fluorescence minus one (FMO) controls: *Cells stained with all but one color, using same reagents as sample*. *(Only for assays with 3 or more colors)* Used as the "gold standard" gating control to determine the extent the colors interact together with natural

auto-fluorescence to spill over into the detector for the missing color. Many clients forego these controls in simple analyses with consistently distinct populations, but FMO controls will always improve the reliability of your data by eliminating any false positives. In multicolor staining protocols, FMO controls are required, especially where populations are not consistently distinct and/or signal is dim. We recommend using these controls until convinced they are no longer necessary for your experiment.

Experimental/Biological controls: These are fully stained controls specific to your experiment, e.g. stimulated vs unstimulated, treated vs untreated, wildtype vs KO, etc. For mouse immunophenotyping of any tissue, it is almost always useful to prep and stain some spleen cells as an internal control. Spleen is also a good source of cells for single color and FMO controls in cases where sample is limited.

Isotype controls: *Cells stained with isotypic reagents* (same fluor and isotype as experimental antibody, but irrelevant specificity) were widely used in the past before being replaced by FMOs as the "gold standard" for gating controls. Currently accepted best practice is to avoid isotype controls unless there is a suspected problem with target cells binding the constant region (e.g. poor blocking of Fc receptors on myeloid cells) or reason to believe reviewers unfamiliar with current standards may request them. In the rare case they are used, they are usually only run once.

Analysis Strategy

Knowing which populations you are interested in and how they are defined through various markers is extremely important. You know your research far better than we do, and having a clear idea of what you are looking for will save you both time and money. We typically begin all cellular analyses with discrimination based on physical properties, before moving on to fluorescence information. We also highly recommend a viability stain.

Debris Removal: In a cellular analysis, we typically remove signal that is too small and uniform to be cells using a forward scatter (FSC) vs side scatter (SSC) plot.

Singlet Discriminations: Based on the area, height, and width of the FSC and SSC signals, we can remove signal likely to result from two or more cells stuck together or passing through the detector simultaneously.

Live/Dead Discrimination: Typically, researchers are interested in living cells. There are several live/dead (viability) stains available as well as ones that allow for viability staining and then fixation without losing the viability information (e.g. for intracellular staining protocols). The core will provide DAPI stain at cost to users in a convenient dropper format on request.

Populations: Basic experiments (up to 4 colors immunophenotyping, annexin/PI, typical cell cycle, GFP+/-, etc) will be routine for our techs to set up. However, when running multicolor experiments and/or novel assays for the first time, it's helpful to bring a reference with gating strategy, if available. Otherwise, please be ready to instruct us on which populations are of interest and how you want the analysis done. Consultation is always free, so please set up a time to come speak to us well before the experiment if you are unsure of your strategy.

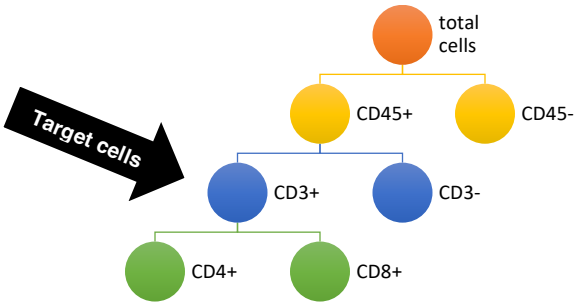
Sample Preparation and Staining

Cell cycle analysis: Please see our guide specifically for this technique linked from the web resources section of our website. Information below is primarily for other assays.

Cells harvested from cultures: Use of enzymes to loosen cells from plastic growth surfaces can result in loss of target epitopes from cell surface molecules, a classic example being a loss of mouse CD86 signal after trypsinization. Please be aware that this is a possibility and that alternative reagents/methods can be deployed to avoid it. More information on this is available on the web resources section of our website.

Fresh Preps: These often contain more debris than cells from culture and, depending on the tissue, may require additional steps to remove for best results (e.g. myelin removal in neural tissues). Experiments targeting rare populations often benefit from enrichment of the samples prior to analysis, depending on specific goals.

Sample concentration, cell number, and volume: The maximum volume for a 5mL tube to load on our instruments is 3.5mL and the minimum would typically be 200-300μL. For immunophenotyping, concentration should ideally be at least 10^5 cells/mL, not to exceed 10^7 cells/mL. The core will provide PBS to dilute samples if necessary. More information on sample preparation is available on the web resources section of our website. In some situations, cell numbers for flow are limited by the small yield of the prep. In other cases, there is an abundance of cells available and only an aliquot is needed for flow; to estimate how many total cells you'll need per sample, consider the relative rarity of your target population(s). For basic immunophenotyping aimed at generating percentages of parent populations, we generally recommend acquiring data on 10,000 to 20,000 target cells and doubling the expected total cell number when aliquoting samples. In the example on the left, if the goal is to determine %CD4 vs %CD8 cells, then the CD3+ population is the target cells. The table below shows calculations of total



cell numbers based on relative rarity of target cells. Keep in mind cell loss can be up to 25% at each wash step during staining.

Number of target cells needed	Target cells as percent total cells	Number of total cells in data file	Minimum number of total cells
10,000	100%	10,000	20,000
10,000	50%	20,000	40,000
10,000	25%	40,000	80,000
10,000	10%	100,000	200,000
10,000	5%	200,000	400,000

Titration of antibodies is the process of identifying the optimal concentration of antibody to use that will produce the best discrimination between the positive and negative cells. At the same time, nonspecific antibody binding is minimized. The most important measurement is the fluorescence intensity of staining (signal) vs. nonspecific binding (noise), the signal-to-noise ratio.

Clogs and partial clogs can greatly slow down data collection, and worse, have a huge impact on your data. There are several ways to prevent them.

Filter your cells: Passing your cells through a 40μm mesh filter will typically prevent clogs. We highly recommend you filter all your samples immediately prior to analysis. The core will provide filter-capped 5mL flow cytometry tubes at cost upon request.

Media: Keep protein concentration at 2% or lower. Calcium-free formulations and HEPES buffer can also help reduce aggregates and avoid pH rise (as is possible in culture media) in problematic samples.

EDTA treatment: Some cells are notorious for clumping together. Many of these cell-cell interactions require divalent cations which can be removed using EDTA. The usual suggested concentration is 1-5 mM of EDTA.

DNAse treatment: Sometimes clumping may result from the presence of DNA (usually released from dying cells). DNA issues frequently result in viscous tenacious samples. Cleaving the DNA by adding DNAse can resolve this issue. The usual suggested concentration is 200 μg/mL of DNAse.

Panel design is of utmost importance when setting out to analyze cells by flow cytometry. The CCSC recommends using the Fluorofinder panel designing tool (https://app.fluorofinder.com/bcm/panels/new) or EasyPanel (https://flow-cytometry.net/) to optimally match receptors, fluorophores, and cytometers at the core to best identify

your cells of interest. And don't forget, consultation with the core staff is always recommended and FREE!

Sample Tubes

Many of our instruments pressurize the flow tube in order push sample into the instrument, thus using the right size tube is critical. Our analyzers use the following 5 ml FACS tubes, and the packs can be purchased from the core upon request.

- BD 5mL 35um Filter Cap Tubes (352235): $25.00/pack of 25
- BD 5mL Flow Cytometry Tubes with caps (352054): $15.00/pack of 125
- BD 5mL Flow Cytometry Tubes without caps (352052): $12.50/pack of 125

Imaging Considerations (A8 only)

Cells can be imaged off the blue (488nm) laser *only*. There are 3 detectors (listed by emission wavelength):

- 534/46
- 600/60
- 788/225

For questions or tips on panel design please feel free to reach out to us at ccsc@bcm.edu. Imaging is optional when using the A8.

We also have an Image Scanning Cytometer, the Cytek ImagestreamX MarkII. Schedule a FREE consult with the core in iLab to discuss how to get started.