Flow Cytometry – A Guide to Experimental Design

Flow Cytometry is a widely employed technique in protein expression research and analysis. It also provides the researcher with a variety of additional data surrounding the various physical parameters such as cell count or number, cell size, shape. Moreover, specialized instruments can be used to identify and "sort" cells with desired characteristics.

An important aspect to successful flow cytometry data collection and analysis is good experimental preparation and design. This not only includes knowledge of the cellular target(s) of interest and its expected location and expression, but also that of the host cells themselves and their metabolism as well as the antibodies, cellular tools and stains used. While detailed protocols for various flow cytometry applications are covered in Technical Note #4, this article is intended to serve as a beginner's guide to Flow Cytometry experimental design. It intends to provide a few important, practical, yet critical tips and tricks that researchers should keep in mind while setting up and preparing cellular samples for flow cytometry expression discovery and analysis.

Before starting with your experiment, perform a quick background check on the target, and the availability of suitable primary and secondary antibodies, as well as the host cell line growth and expression characteristics expected for the target. It will do no good to perform flow on a cell line not expected to express the target, nor will it do any good to look for a target in the incorrect location or with subpar antibodies or reagents. Always use flow validated antibodies whenever possible.

✓ **But where to find such information, and why would be it be so important before the experiment starts?**

If the goal is to study cell expression of a protein of interest, then finding a positive control cell line that is known to express the target of interest is paramount to testing the experimental cell line alone. Information on the expression and the suitability of a control cell line can be obtained from literature. The Human Protein Atlas is a good site to refer to for protein expression in various human cell lines. In addition, the major search engines such as Google Scholar, PubMed or Scopus are helpful in obtaining and quickly scanning the published literature on the target, and assist a great deal in gathering important target and even application specific background information. Good preparation is key to experimental design.

✓ **How does knowing your target matter?**

Flow cytometry when used on intact cells can be used to detect proteins both extracellular and intracellular. Since they are intact cells in a fluid environment, in most cases cells can be stained without fixation, particularly for extracellular or secreted proteins. However, clearly for an internal protein once the cell membrane is compromised the cellular contents would be lost without fixation. Thus in many ways cellular fixation and permeabilization are crucially linked together. Based on the target's location and characteristics, cells may have to be treated for:

- No permeabilization (extracellular membrane protein, eg. p75 extracellular domain) - Cells can be unfixed or fixed depending upon specific target or experimental needs, typically they are used unfixed for most extracellular epitopes.

- Mild permeabilization and fixation (integral membrane, or semi-membrane bound intracellular antigen, eg. spectrin, gamma secretase) - Fixation here is intended to lightly secure targets to their current locations, but not crosslink proteins together into a web. Acetone or other volatile solvents are often good for this mild form of permeabilization and fixation. These solvents both fix and dissolve the membrane lipid, thereby permeabilizing the cells in one step.

- Permeabilization (intracellular antigen: i.e ER or structural proteins, nuclear transcription factors). Cells are typically fixed. Most commonly with 1-2% paraformaldehyde (PFA) is used but PLP, Zamboni's or even formalin fixations can be found in the literature. Permeabilization of the cellular membranes, both the plasma membrane as well as internal membranes is typically performed by some sort of detergent (i.e. Triton X-100, Tween 20) or organic solvent (acetone, methanol).

- Protein transport arrest (proteins internally localized and secreted, for instance neurotrophins). Cells are typically fixed for these applications, usually mildly but 1-2% PFA is quite common, too. The cells need to be permeabilized, but most typically some mild treatment is used to expose more of the target present inside the cell for better signals.

Permeabilization or the lack of it is critical for allowing detection molecules such as antibodies to enter the cell to find their target, and it can also serve to allow access to epitopes on target proteins that may not be fully accessible or in the case of no permeabilization, allow the adherence of the target to the cell of interest which would otherwise be lost to the extracellular environment. Fixation is obvious for internal proteins, for without it intact cells would simply dissolve in the fluid flow environment. Discovering which combination of fixation and permeabilization holds the target in place but allows sufficient penetration of the detection reagents is where much of the "art" of flow cytometry takes place.

Good sources for such target location information are Uniprot.org or again, the published literature. Flow cytometry fixation methods abound on the Internet and should be reviewed to find the ones that best suits the researcher's level of skill and that fits best with the experimental design.

✓ **Knowing your reagents, and why you really need to understand your antibodies:**

- Know your primary antibody – clonality (monoclonal/polyclonal), what is the host species (important if using secondary antibodies for increased signals), what is the antibody's target specificity, purity, cross-reactivity with closely related proteins, and epitope recognition site - all useful in knowing how to approach using the reagent as well as analysing the results from its use. Epitope recognition site is especially important for membrane spanning antigens, as antibodies may be raised against the intracellular C-terminal or extracellular N-terminal end of the protein of interest will determine how particular cells are treated for use in flow cytometry (i.e. an N-terminal ECD epitope antibody might be used on intact, unfixed cells, whereas an antibody directed to the same target but whose epitope was C-terminal and internal will need to have its host cells fixed and permeabilized).

- Antibodies successfully tested on applications such as Western Blotting or Immunohistochemistry may not be suitable for Flow cytometry analysis! Beware of vendors claiming application to Flow

cytometry without specific validation. Not all antibodies work in flow, and those that have not been validated will need to be tested on known positive controls *before* being used in an experimental situation. To do otherwise is per folly; good science is not gambling, at Biosensis, all our Flow cytometry antibodies are validated with detailed protocols and data available on the website.

- Know your secondary antibody – This applies to indirect flow cytometry analysis where a fluorophore labelled secondary antibody is being used for detection. The secondary antibody should be specific for primary antibody's host species and preferably cross-absorbed for the species from which the cell line was obtained for analysis to help eliminate non-specific background. If using monoclonal, subtype specific secondary antibodies can often be used and these typically provide very clean, albeit slightly less bright signals compared to whole molecule secondary antibodies and should be considered if backgrounds are high or signal-to-noise ratios low.

✓ **Proper controls, never assume, always know. That's what controls bring to an experiment!**

Use appropriate controls for your experiment. The idea is to demonstrate specificity of antigen-antibody interaction. In most cases, the four types of following controls can be used to demonstrate specificity:

- Unstained cells – Fluorescence emission arising from endogenous fluorophores or autofluorescence; may increase the population of positive cells. Prepare an unstained control to address false positive cells due to autofluorescence.

- Negative cells – If available, cell populations not expressing the protein of interest should be used as negative control. This serves as a control for target specificity of primary antibody.

- Isotype control – Isotype control is an antibody of the same class as the primary antibody, generated against an antigen not present in the cell population or an antibody with no known specificity (for eg. Non-specific Control IgG, Clone X63). A perfectly matched isotype control helps to assess undesirable background staining due to binding to Fc receptors.

- Secondary antibody control – This applies to indirect staining where a secondary antibody is conjugated to a fluorochrome and used for detection of bound primary/specific antibody. Prepare cells treated with only labelled secondary antibody to address non-specific binding of secondary antibody.

✓ **Additional Considerations, the "devils" are in the details!**

Use an appropriate blocker to mask non-specific binding sites and lower backgrounds to improve the all-important "signal-to-noise" ratio; the higher "the signal" and the lower the "noise" the better.

- Blocking cells with 10% normal serum from the same host species as labelled secondary antibody helps to reduce background. It is important to ensure however that the normal serum is NOT from the same host species as the primary antibody as this can lead to serious non-specific signals.

- Block with non-serum containing compounds to prevent primary antibody absorption. Many target proteins are highly conserved, for instance mature BDNF is virtually the same primary sequence in most mammals and present to some extent in their blood. Thus a serum may not be the best blocker in this case because of antigen conservation, thus a purified casein or albumin blocker would be preferred.

- Block Fc receptors on host cells. Fc receptors are present in one variety or another on most cells, particularly those used in research of rat, mouse or human. Antibodies can typically bind to these Fc receptors naturally. Thus many times, particularly when examining immune cell derived targets, or using immortalized immune variant cell lines the presence of Fc receptors on these cells can lead to highly specific binding of the primary host cell and lead to a false positive result or worse a poor detection of the true target of interest. Many commercial Fc blocking solutions are available and if your target will allow it diluted simple serum from an alternate host will often sufficiently block the target's cells Fc receptors to allow a clean and clear signal by the target specific binding of the primary and/or secondary antibodies.

- Perform a cell count and viability check before starting with your sample preparation. Dead cells give a high background scatter and may show false positive staining. Ensure that the cell viability is >90%.

- Use the appropriate cell number for your flow device. Cell concentration in the range of 10^5 to 10^6 is recommended to avoid clogging of the flow cell and to obtain a good resolution. However, if the entire sample preparation and staining protocol involves multiple washing steps, sometimes considerable loss in cell numbers can occur leading to low signals or incorrect population counts. Thus review the protocol carefully, and if there are a large number of steps, then starting with more, such as 10^7 cells/tube, will generally help to maintain the desired cell count.

- If you prefer using a same lot of cells over a period of time, it is a good idea to freeze down a healthy cell preparation. Cells frozen down in PBS can be stored at -20°C for at least one week before analysis.

- All steps of the flow protocol should be performed on ice. This prevents internalisation of membrane antigens. In addition, use PBS with 0.1% sodium azide to prevent internalisation.

- Perform at least 2 washes with PBS after harvesting of cells. Trace amounts of proteases in media may degrade antibodies added in subsequent steps.

- Do not centrifuge at >1000 g as this may damage cell integrity. After centrifugation, immediately decant solution using a narrow bore tip to avoid cell loss.

- Obtaining single cell suspension is important in flow analysis. Pipetting up and down helps breaking clumps of sticky cells. Do not vortex at any stage. During longer incubation periods in primary/secondary antibody solutions, give an intermediate gentle tap to keep cells in suspension.