

Immunostaining for Flow Cytometry

Rev.0

Background

The combination of single cell analysis using flow cytometry and the specificity of antibody-based immunostaining makes a powerful tool for the cell biologist, pharmacologist, drug developer and clinician. For example, with a homogenous cell population (e.g. a cell line), immunostaining and flow cytometry can be used to determine the percentage of cells responding to a given treatment at a given timepoint. With a heterogeneous cell population (e.g. blood), differential responses between distinct cell types can be discriminated. Depending on the laser and detector configurations of the flow cytometer, multiple analytes can be analyzed simultaneously.

MitoSciences has validated more than 50 of our monoclonal antibodies for use with flow cytometry. The result is an expanded toolkit for the assay of key metabolic, mitochondrial and apoptotic proteins in cultured cell lines, primary cells and complex cell mixtures.

The following protocol is a general guide to antibody staining of tissue culture cell lines for analysis by flow cytometry. All of MitoSciences flow cytometry validated antibodies were evaluated using the methods that follow.

Materials and equipment required.

Materials:

- Standard tissue culture materials and reagents
- 1X PBS
- Paraformaldehyde
- Methanol
- Triton X-100
- Bovine serum albumin (BSA)
- MitoSciences' flow cytometry validated primary antibody(ies)
- Appropriate detection antibodies (fluorochrome(s) suitable for available flow cytometer)

Equipment:

- Centrifuge
- Flow cytometer

Protocol.

I. Cell culture:

Cell culture and treatment conditions are dictated by the experiment at hand. As a general guideline, it is advisable to analyze at least 10,000 events (cells) on the flow cytometer per sample/datapoint, so at least four times that many cells should be collected to ensure sufficient material at the end of staining (some cell loss during processing is expected). It is possible to harvest a large number of cells and subsequently aliquot smaller numbers for individual experiments.

II. Harvest and fixation of cells:

It is critical that cells are fixed as a single cell solution; that is that all cells are dissociated from each other into individual cells and are not clumped together. This is generally a trivial matter with suspension cells but it is imperative to fully dissociate adherent cells by trypsinization.

For suspension cells: Gently pipette up and down to achieve a single cell suspension.

For adherent cells: Remove culture media, rinse with PBS and trypsinize cells as if passaging. After cells have detached from the plate and are dissociated into single cells, quench the trypsin with media and gently pipette up and down as necessary. It is important to save the culture media and PBS rinse so that any detached or loosely adherent cells (S-phase, apoptosing, necrosing) are also recovered (this is critical for experiments investigating cell death or cell cycle).

After generating a single cell suspension:

1. Pellet the cells (5min, 500xg) and aspirate the supernatant.
2. Resuspend in a suitable volume of PBS (e.g. 1mL per 2-5 million cells) and gently pipette up and down until fully resuspended.
3. Overlay with paraformaldehyde such that the final concentration is 4% (e.g. add 1mL 16% PFA to 3mL PBS). Mix well and incubate at room temperature for 15min.
4. Pellet the cells (5min, 500xg) and decant the paraformaldehyde-containing supernatant (*paraformaldehyde is toxic: handle with care dispose of according to local requirements*).
5. Resuspend the cells in small volume of cold PBS (e.g. 0.1mL per 1-2 million cells). Slowly add 9x volumes of ice-cold methanol to the cells while agitating; mix well and store at -20°C (final concentration is 90% methanol). Methanol acts as a secondary fixative as well as a permeabilization step. Samples should be stored at -20°C for 30 min before staining or can be stored at -20°C for at least 1 month.

Alternative to methanol permeabilization. Some antibodies have been empirically determined to provide better staining when permeabilized with Triton X-100 instead of methanol. After removing fixative (Step 4 above), resuspend cells in PBS + 0.1% Triton X-100 for 15min. Next, pellet the cells and proceed with staining (step 3 below). (MitoSciences antibodies that have superior staining with Triton permeabilization are indicated as such on their respective Technical Data Sheets (TDS)).

III. Antibody staining:

The block and incubation solution is 1% BSA in PBS and should be freshly prepared. Staining can be performed in eppendorf tubes, 96-well deep well blocks or polystyrene test tubes. The following volumes are suggested for the staining of 50,000 - 100,000 cells per tube.

1. Aliquot cells into assay tubes and wash two times with 20 volumes of block solution.
2. Resuspend cells in 50 μ L block solution for 20min at room temperature.
3. Add the primary antibody to the appropriate final concentration and incubate 1 hour at room temperature.
4. Wash two times with 20 volumes of block solution.
5. Add detection antibody in block solution to appropriate concentration.
6. Wash two times with 20 volumes of block solution.
7. Resuspend stained cells in 100 μ L PBS.

If using a directly labeled primary antibody: resuspend the stained cells in PBS following step 4 and proceed to analysis.

IV. Analyze on flow cytometer.

Specific methods depend on the available flow cytometer. It is important to appropriately establish forward and side scatter gates to exclude debris and cellular aggregates from analysis. If cells are stained with multiple fluorescent labels, attention must be paid to potential spillover between fluorochromes and detectors.

Considerations in designing flow cytometry experiments

Gating and Appropriate Controls.

Suitable controls to assess experimental antibody staining relative to background fluorescence are (1) a sample stained with secondary antibody only (no primary antibody) and (2) an isotype control antibody (that is, a non-reactive antibody that is the same isotype as the experimental antibody).

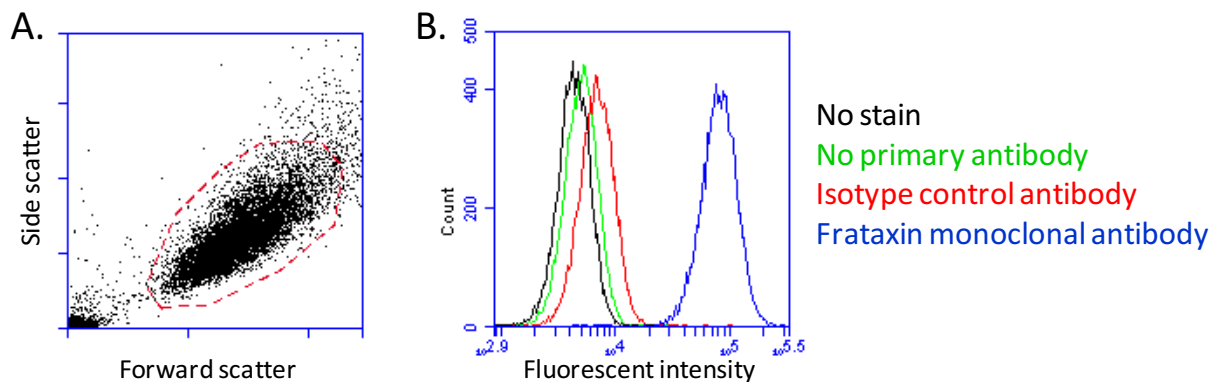


Figure 1. Cellular gate and fluorescence histogram from a typical HeLa flow cytometry experiment. A. The dotted red line indicates the gate for individual HeLa cells in a forward vs. side scatter dot plot. Establishing this gate excludes debris (lower left) and aggregated cells (upper right) from analysis. B. Fluorescent intensity histogram of control and experimental antibody stains. Unstained cells (black line) have some intrinsic fluorescence. Exposure to the secondary antibody only (no primary control) only marginally increases the fluorescent intensity (green) indicating that the detection antibody has minimal non-specific interaction with the cells. The isotype control antibody (red) has a slightly increased fluorescent intensity indicating some non-specific interaction. The experimental Frataxin antibody MSF01 (blue) has a significant shift in fluorescent intensity relative to the isotype control.

Selection of experimental antibodies.

All MitoSciences antibodies have been rigorously validated for target specificity by a combination of immunoprecipitation, western blot, immunocytochemistry and ELISA assays. MitoSciences flow-validated antibodies are all immunocytochemistry positive and have been assessed for staining in flow cytometry in both HeLa and HL-60 cells. All have at minimum a 5-fold greater staining intensity than the appropriate isotype control.

It is important to note that a strong fluorescent signal in a flow cytometry experiment does not provide any validation of antibody specificity. Specificity must be determined by complimentary experiments, for example:

- (1) Properly controlled immunoprecipitation and western blot experiments can be used to demonstrate antibody specificity. It is further required that the antibody give a positive immunocytochemistry result under the same conditions used in staining for flow cytometry (immunocytochemistry can also provide supporting specificity data based on sub-cellular localization).
- (2) Specificity demonstrated within flow cytometry by target modulation. A convincing demonstration of an antibody's specificity for a given target is to modulate the target levels by genetic manipulation (e.g. knock-down or knock-out) or culture conditions (e.g. drug treatment or growth conditions). For example, the specificity of the Frataxin antibody MSF01 is demonstrated in Figure 2 using primary patient cell lines with defined relative levels of the protein Frataxin.

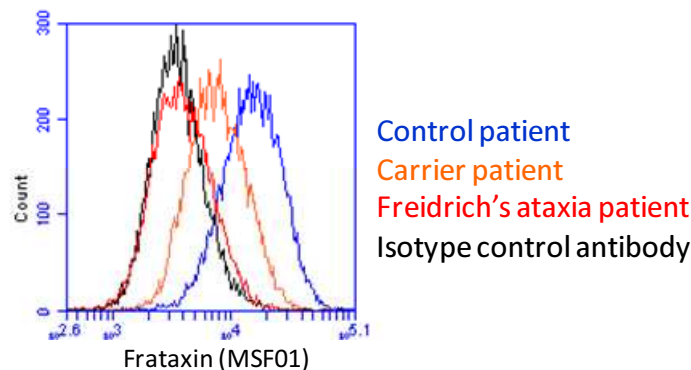


Figure 2. Frataxin protein levels assayed by flow cytometry. Human lymphocytes from control (blue), carrier (orange) and Freidrich's ataxia (FA) (red) patients are assessed for Frataxin protein levels by flow cytometry using the anti-Frataxin antibody MSF01. Isotype control is shown in black. The FA patient has marginal amounts of Frataxin and the carrier patient has Frataxin levels intermediate to the FA and control patients.

Selection of fluorochromes.

Selection of fluorochromes is dependent on laser and detector configuration of the available flow cytometer. Typical flow cytometers have at minimum a green laser and a detector for FITC (and comparable fluorochromes) whereas more advanced instruments have additional lasers and detectors allowing simultaneous multi-fluorochrome detection. If a single antibody is being used to stain a sample there is freedom to select any bright and available fluorochrome. If co-staining with multiple fluorochromes it is important to determine if there is any cross-talk between detectors and to correct as necessary.

EXPERIMENTAL FLOW CHART

For quick reference only. Be completely familiar with previous details of this document before performing the assay.

