

Flow cytometry is a technique for analyzing individual cells in suspension. It uses a stream of fluid to direct the cells in single file past an interrogation point, where light scatter and the expression of specific cellular markers are measured to enable characterization of distinct sub-populations. Fluorescently-labeled antibodies are essential tools for flow cytometry, where they are often combined in a panel and used to detect multiple markers simultaneously. To maximize the value of a flow cytometry experiment, several best practices are recommended.



1. Take care when collecting and storing samples

Materials analyzed by flow cytometry includes blood, tissue, and cultured cells, and must always be handled carefully to preserve sample integrity. Blood and tissue samples can benefit from being preserved in specialized storage reagents at -80°C upon collection, especially where they are acquired from large cohort studies over an extended period of time. Cultured cells should be maintained in log-phase growth, and the use of harsh detachment reagents should be optimized and high-speed centrifugations avoided, to prevent cell death that can lead to clumping.

2. Always check cell concentrations

Irrespective of the sample type, cells should always be counted before running a flow cytometry experiment to safeguard experimental consistency. It is recommended that a density of $10^5 - 10^7$ cells/mL is used for optimal results; loading too many cells can mean events are lost from the analysis, while

loading too few cells can extend workflows by increasing the time taken to obtain statistically relevant numbers of events. Automated cell counting is preferred over manual counting as it removes user bias for more reproducible results.

3. Consider enriching rare cells

Where a flow cytometry experiment is designed to analyze rare cell types such as hematopoietic stem cells, circulating tumor cells, or antigen-specific T lymphocytes, an enrichment step may need to be performed. A popular approach is to use magnetic beads coated with antibodies against specific cellular markers to extract a particular subset (known to contain the target cell type) from a heterogeneous population. This increases the likelihood of obtaining the target in sufficient numbers for reliable analysis.

4. Take steps to remove aggregates

Cellular aggregates, tissue fragments, and clumps of unwanted debris can block the flow cytometer. They can also complicate data analysis (e.g., doublets may be incorrectly identified as dividing cells during cell cycle analysis) and should always be removed before samples are injected into the instrument. Cell strainers with different mesh sizes are available to fit most commonly used tubes and provide a quick and easy way of producing a uniform single-cell suspension. Adding DNase to buffers eliminates DNA (released from damaged cells) that can cause aggregation. Gating can also be performed to identify and exclude aggregates from single-cell analysis.

5. Include a viability stain

Cellular viability stains provide an assessment of cell health and can alert researchers to any harmful effects of sample handling. They also provide a more accurate means of eliminating dead cells and cellular debris from flow cytometry analysis than gating on light scatter properties alone. Common viability dyes include Acridine Orange / Propidium Iodide (AO/PI) that stains live cells green and dead cells red; DAPI that enters only cells with a compromised plasma membrane; and Annexin V that binds the apoptotic marker phosphatidylserine.

6. Consider staining for extracellular markers before fixing cells

While many flow cytometry experiments are designed to measure both extracellular and intracellular markers, some fixatives can alter the antibody binding sites of markers expressed at the cell surface. To prevent this, it is recommended that extracellular markers are stained prior to fixation; cells can then be permeabilized to enable staining of intracellular targets such as cytoplasmic proteins or nucleic acids.

7. Block samples appropriately

Blocking is critical to prevent non-specific antibody binding that can produce unwanted background signal and is typically achieved by incubating samples with reagents such as [bovine serum albumin \(BSA\)](#) or [normal serum](#). Where samples contain immune cells such as monocytes, macrophages, or B lymphocytes, an additional Fc blocking step is advised to prevent antibodies from binding to Fc receptors expressed on the surface of these cell types. Normal serum can be used to provide a source of IgG to block the Fc receptors.

8. Include suitable controls

Positive and negative controls are fundamental to any immunoassay, where they are used to monitor assay performance and validate results. However, flow cytometry requires that several other types of control are included, largely to ensure fluorescent signals are interpreted correctly. Unstained controls omit antibody reagents and are used to determine background fluorescence; compensation controls are samples stained with a single fluorophore that allow spectral overlap to be assessed; fluorescence minus one (FMO) controls comprise samples stained with all but one fluorophore and are used to evaluate fluorescence spread. Additionally, isotype controls (antibodies that share the same isotype as marker-specific antibodies but do not recognize the target) provide valuable insights into sources of background staining.

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9. Ensure antibodies are from a reliable source

Antibodies must always be sourced from a trusted manufacturer for reliable results. Primary antibody datasheets should show application-specific validation data, including a recommended protocol for use, and should include details of the antibody concentration, isotype, formulation, and storage conditions, as well as the fluorophore characteristics of any labeled antibody reagents. Because multicolor flow cytometry experiments usually combine antibodies from several host species, cross-adsorbed secondary antibody conjugates are recommended to minimize non-specific background signal.

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10. Be diligent with panel design

Factors to consider when building a multicolor flow cytometry panel include the relative abundance of any target antigens, and the properties of the fluorophores that will be used to detect them. Rare antigens should be paired with bright fluorophores, and vice versa, and it is important to check that fluorophore excitation and emission maxima are compatible with the flow cytometer's lasers and detectors, respectively. Online [Spectra Viewer](#) and Panel Builder tools can simplify reagent selection, while Optimized Multicolor Immunofluorescence Panels (OMIPs) can save considerable time when designing panels that will be used to evaluate similar cell types.

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