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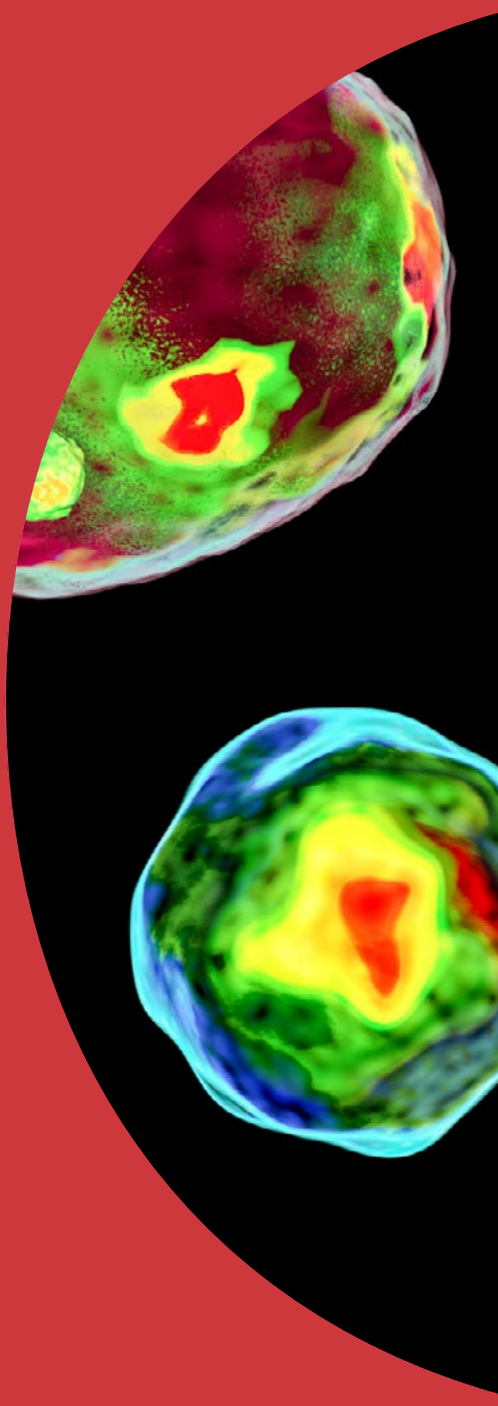

Flow Cytometry: Principles, Best Practices, and Considerations for Experimental Design

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INTRODUCTION

Flow cytometry is a technique for studying individual cells in suspension. It achieves this using a combination of fluidics, optics, and electronics to measure light scatter and fluorescence intensity, which can reveal unique insights into the relative abundance and functionality of different cell types within a sample. The multiparametric, high throughput nature of flow cytometry has led many researchers to regard it as the ultimate single-cell analysis technique and its use continues to grow year on year.

When fluorescence-based flow cytometry was first developed, researchers were limited to measuring just one or two parameters using antibodies they had painstakingly labeled in-house. Now, advances in instrumentation, reagents, and software have made multiparametric analysis routine. Yet, the same fundamental rules for experimental design and execution still apply and following established best practices is essential to ensure that flow cytometry data is both accurate and reproducible.

This guide provides an overview of the basic principles of flow cytometry, including an explanation of fluorescence and how key fluorochrome properties are relevant to multicolor panel design. It also describes each step of the flow cytometry workflow, from sample preparation through to immunostaining, and offers guidance for analyzing flow cytometry data. Links to relevant resources are shared throughout to help streamline reagent selection and provide researchers with practical support.

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1. Background

Unlike bulk population analysis methods such as enzyme-linked immunosorbent assay (ELISA) and Western blot, flow cytometry enables characterization of individual cells within a heterogenous sample. Moreover, it accomplishes this at speed, with modern instruments being capable of processing thousands of cells per minute. These properties of flow cytometry have led to its use for many different applications, including drug screening, cell cycle analysis, and an almost infinite number of immunophenotyping studies, as well as a broad range of diagnostic and prognostic uses. Importantly, by interrogating samples at the single-cell level, flow cytometry can reveal insights that might otherwise go unnoticed using other techniques.

Basic principles of flow cytometry

Flow cytometers comprise three main systems – fluidics, optics, and electronics – that are used in combination for characterizing multiple cellular properties. The fluidics system functions to transport the cells through the instrument and consists of a central core into which the sample is first introduced, surrounded by an outer sheath fluid. Following sample injection, narrowing of the sheath increases the fluid velocity to create a stream of cells in single file, a process known as hydrodynamic focusing. This is shown in **Figure 1**.

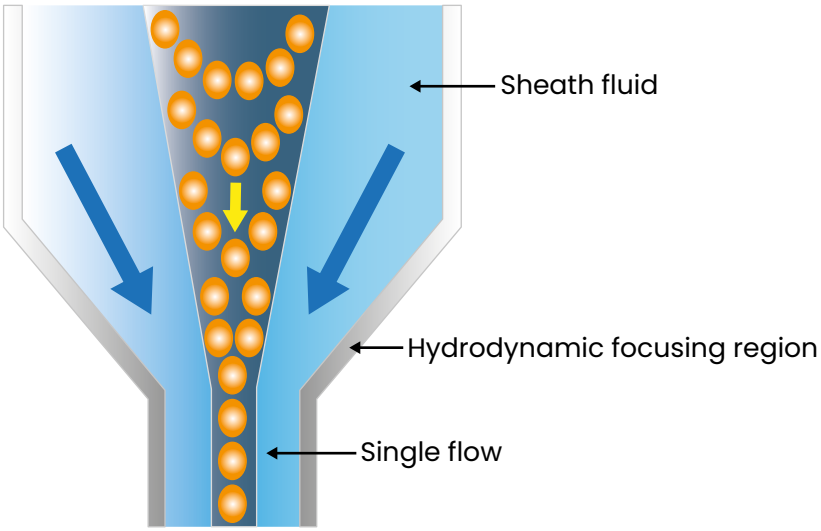


Figure 1. Hydrodynamic focusing creates a stream of cells in single file.

After hydrodynamic focusing, the cells pass through an interrogation point where one or more lasers intercept. Each laser produces a single wavelength of light at a specific frequency, ranging from ultraviolet to infrared, and has a variable power level that is defined as photon output/time, measured in milliwatts (mW). As the cells cross the interrogation point, they scatter the laser light, which is directed by a series of dichroic mirrors and filters toward photomultiplier tubes (PMTs) for detection. At the same time, any fluorochrome-labeled antibodies that are bound to the cells fluoresce, also producing a measurable signal. A typical flow cytometer setup is shown in **Figure 2.**

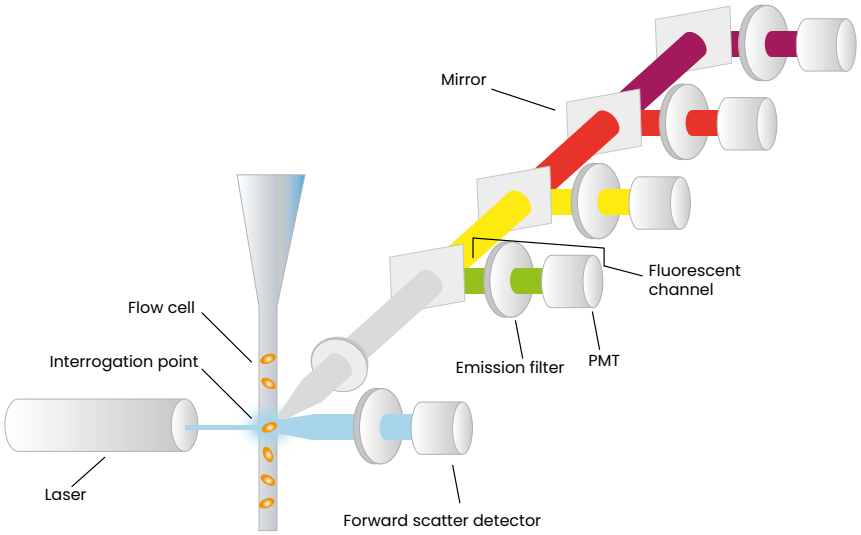


Figure 2. A typical flow cytometer setup showing mirrors, filters, and PMTs.

When the light reaches a PMT, it generates an electrical current that is reflective of the cell's passage through the interrogation point. This is known as an event and, essentially, comprises a voltage pulse that rises as the cell enters the interrogation point and falls as it leaves. While the height (H) and area (A) of each event correlate with the signal intensity, the width (W) corresponds to the time taken for the cell to pass through the interrogation point. This is shown in **Figure 3.**

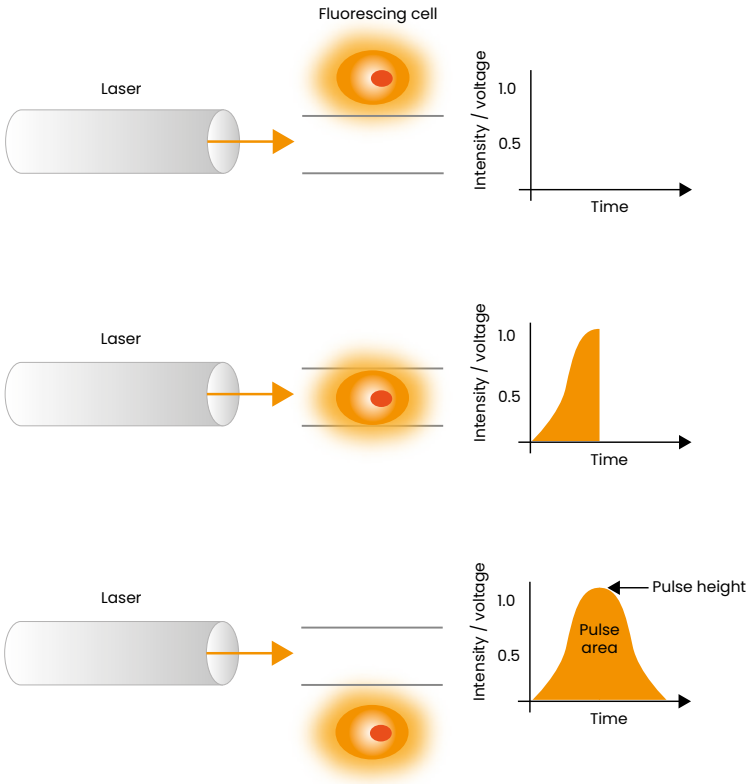


Figure 3. Each cell generates a voltage pulse known as an event as it passes through the interrogation point.

To avoid small particles such as dust and cellular debris being mistakenly identified as cells, a dedicated PMT is used to set a threshold, usually based on forward scatter. This ensures that only events generating above a certain signal intensity are registered by the flow cytometer's detectors, minimizing unwanted background signal that could lead to inaccurate results.

Scatter-based analysis

Light scatter measurements for flow cytometry fall into two categories – forward scatter (FSC) and side scatter (SSC). FSC is proportional to the cell surface area or size and is typically measured at a 20o offset from the incident laser beam. In general, larger cells refract more light than smaller cells; however, FSC is influenced by factors including the wavelength of the laser, the collection angle, and the refractive index of the sheath fluid. For this reason, FSC is usually combined with other readouts. SSC correlates with cell granularity and is measured at a 90o angle to the laser line; like FSC, it is unique to every cell and used in conjunction with other measurements. The light scattering properties of a cell are shown in **Figure 4.**

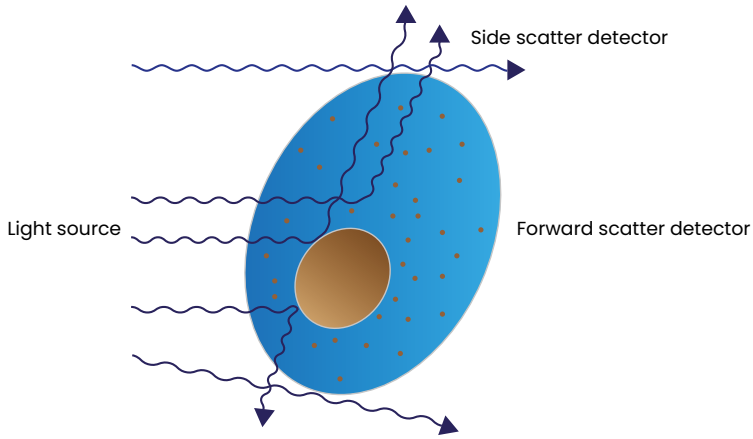


Figure 4. Cellular light scattering properties.

Using FSC and SSC, it is possible to identify major sub-populations within a complex sample such as blood. This type of analysis is shown in Figure 5. However, it is more common for light scatter measurements to be combined with fluorescence-based analysis for a more complete picture of the sample in question.

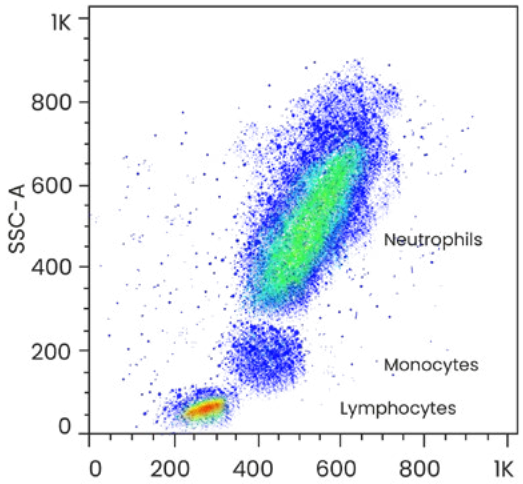


Figure 5. Identification of cellular sub-populations in lyzed whole blood using FSC and SSC.

Fluorescence-based analysis

Fluorescence-based flow cytometry uses antibodies labeled with fluorochromes for detecting specific cell surface markers or intracellular targets. Provided fluorochromes are carefully selected to avoid spectral overlap, as many as forty different analytes can be measured in the same flow cytometry experiment – although detecting 5-10 targets is more common. Critically, fluorochromes should be matched to the flow cytometer's lasers and detectors, with each detector being used for measuring a distinct target.

2. Fluorochromes

Fluorochromes are core reagents for flow cytometry, where their unique properties are exploited for simultaneous detection of multiple targets. They comprise both naturally occurring fluorescent proteins and an extensive array of synthetic dyes and are typically supplied conjugated to antibody reagents that recognize and bind analytes of interest.

Basic principles of fluorescence

Fluorescence occurs when a specialized protein or dye (the fluorochrome) absorbs a photon of light at a specific wavelength and emits it at another, longer wavelength. During this process, the fluorochrome's electrons transition from a resting state (S0) to an excited electronic singlet state (S2) before some of the energy is released as heat to enable their return to a more stable condition (S1). The remaining energy is then emitted as fluorescence. This is shown in **Figure 6**.

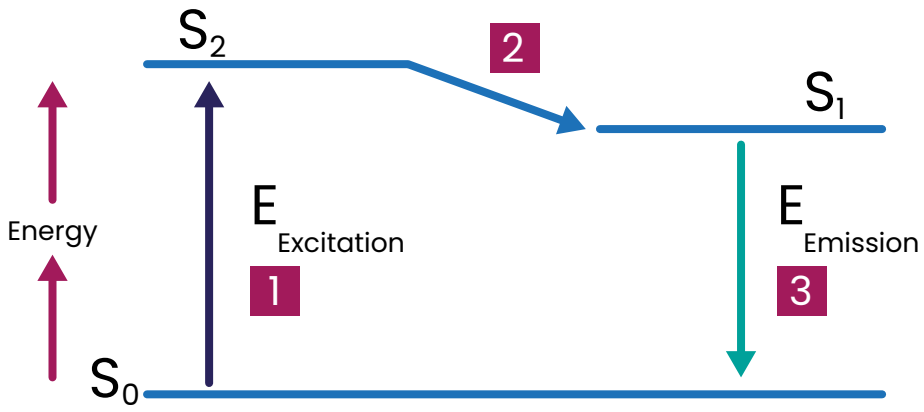


Figure 6. Basic principles of fluorescence. Upon excitation (1), the fluorochrome's electrons transition from a resting state (S0) to an excited electronic single state (S2). Energy is then released as heat (2) and fluorescence (3) as the electrons return via a more stable state (S1) to S0.

Because this cycle repeats thousands of times for each fluorochrome, and because multiple fluorochromes are bound to each antibody used for target detection, the fluorescent signal is amplified and can be measured using techniques such as flow cytometry.

Fluorochrome properties

Although each fluorochrome absorbs maximally at a specific wavelength (the excitation maximum), it will also absorb light at wavelengths either side of this value (the excitation spectrum). Likewise, the emitted light spans a range of wavelengths (the emission spectrum), peaking at the emission maximum. When designing a flow cytometry experiment, it is vital to match the excitation and emission maxima to the instrument's lasers and detectors, respectively, since this will increase the likelihood of target detection.

The Stokes shift is another important fluorochrome property to consider during experimental design. This represents the difference between the excitation and emission maxima and is shown in **Figure 7**. Where there is a need to combine fluorochromes that are excited by the same laser in a single experiment, selecting fluorochromes with different Stokes shifts is a popular strategy for ensuring the resultant signals are spectrally distinct.

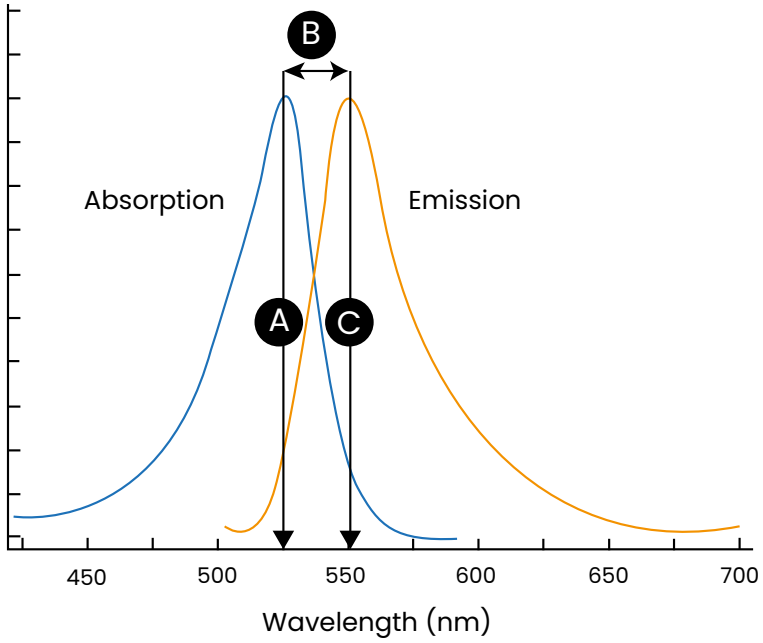


Figure 7. Excitation maximum (A), emission maximum (C) and Stokes shift (B) of a fluorochrome.

Other fundamental fluorochrome properties include the brightness, pH sensitivity, and susceptibility to photobleaching – the light-induced degradation of fluorochromes that can limit their performance. It is recommended that bright fluorochromes be paired with less abundant targets, and vice versa, and that staining conditions for multicolor flow cytometry (including the composition and pH of the antibody diluent) are carefully optimized to ensure they are suitable for all of the chosen fluorochromes. Where flow cytometry studies will use strong lasers or take place over an extended time period, fluorochromes that are resistant to photobleaching represent a sensible choice.

Introduction to panels

With most flow cytometry experiments being used for measuring multiple targets simultaneously, panel design is critical to avoid generating misleading results. Numerous tools have been developed to simplify this process, including:

- Spectra Viewers for comparing fluorochrome excitation and emission spectra and investigating Stokes shifts
- Brightness tables for matching fluorochromes to target abundance
- Panel Builders for identifying the right fluorochrome combinations for a particular flow cytometer
- Optimized Multicolor Immunofluorescence Panels (OMIPS) for characterizing a pre-defined cell state or response

Additionally, where antibody reagents are not available with the desired conjugate, antibody manufacturers can offer guidance on alternative fluorochromes or provide a custom labeling service. Figure 8 shows the excitation and emission maxima of some common fluorochromes and includes examples of corresponding dyes.

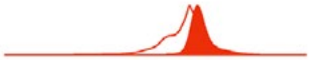
Fluorochrome	Excitation Max (nm)	Emission Max (nm)	Spectrum	Corresponding Dyes
FITC	495	519		Alexa Fluor® 488, ATTO® 488, CF®488A, Cy2®, DyLight® 488, fluorescein
PE	565	576		Alexa Fluor® 568, ATTO® 565, CF®568
Cy5®	649	666		Alexa Fluor® 647,ATTO® 647N, CF®640R, CF®647, DyLight® 650
Cy5.5®	672-673	690		Alexa Fluor® 680, CF®680, DyLight® 680, IRDye® 680

Figure 8. Characteristics of some common fluorochromes and their corresponding dyes.

Traditional versus modern fluorochromes

Compared to when fluorescence-based flow cytometry was first developed, modern-day researchers have access to an almost limitless choice of fluorochromes. Traditional fluorescent proteins such as R-phycoerythrin (RPE), allophycocyanin (APC) and green fluorescent protein (GFP) remain popular, as do established synthetic dyes like the DyLight®, Cy® dye, and Alexa Fluor® product ranges. More recently, these have been complemented by products promising features such as greater sensitivity and resolution, improved compatibility with antibody diluents, and the capacity to fill spectral gaps between existing fluorochromes.

Tandem dyes are also increasingly used for greater flexibility in panel design. These sophisticated reagents comprise a pair of conjoined fluorochromes, whereby excitation of one molecule results in a transfer of energy to the other, causing it to emit a fluorescent signal. Incorporating tandem dyes into a flow cytometry experiment can allow several readouts to be obtained from a single laser, meaning panel size can be extended when the capabilities of the instrument are a limiting factor. A schematic representation of a tandem dye is shown in **Figure 9**.

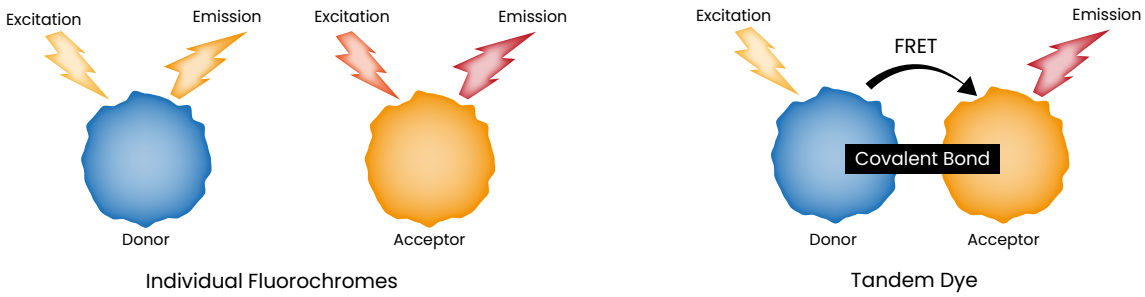


Figure 9. Comparison of individual fluorochromes and tandem dyes.

3. The flow cytometry workflow

A typical flow cytometry experiment starts with the collection of sample material. Common sample types include cultured cells, whole blood, and tissue biopsy material, as well as more novel sample materials like induced pluripotent stem cells (iPSCs), organoids, and spheroids. Following collection, each sample is processed into a single-cell suspension and is then handled according to whether cell surface or intracellular markers will be detected, and whether the cells will be used live or fixed.

Where cell surface markers are of interest, it is typical to stain for these prior to fixation since some fixatives can adversely affect antibody binding sites. However, because many flow cytometry experiments are designed to detect both cell surface and intracellular markers, a conventional approach is to stain for cell surface markers before fixing and permeabilizing the cells for detection of intracellular targets. Optimization is vital to both maximize the assay window and ensure that fluorochromes are not compromised by fixation and/or permeabilization methods.

Irrespective of whether cells are live or fixed, a blocking step is essential to prevent non-specific antibody binding. (Where fixed cells are used, the blocking step follows fixation and permeabilization). At this stage in the workflow, Fc receptor blocking may also be carried out to prevent unwanted antibody binding to Fc receptors on immune cells. The cells are then stained using either labeled primary antibodies for direct detection or unlabeled primary antibodies and labeled secondary antibodies for indirect detection. Finally, after washing to remove any unbound antibody reagents, the cells are introduced into the flow cytometer. The flow cytometry workflow is shown in Figure 10.

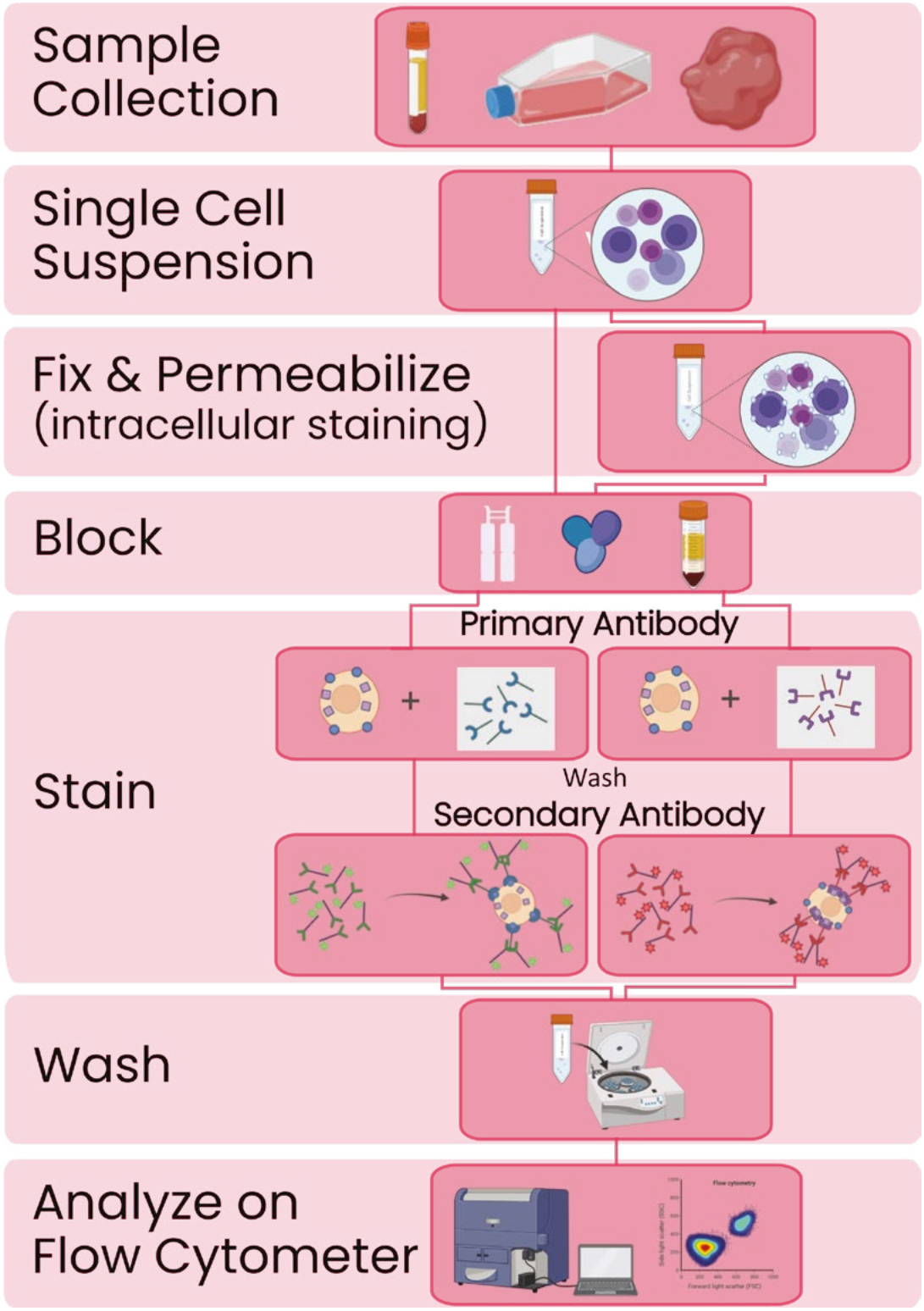


Figure 10. A typical flow cytometry workflow.

Find flow cytometry resources, protocols, and answers to frequently asked questions on our flow cytometry page.

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4. Sample preparation

Sample preparation is a key step in generating reliable flow cytometry data and should be carefully optimized to preserve cellular integrity. General good practice guidelines include being consistent with any treatments to prevent assay variability; checking cell concentrations to avoid missing events (due to using too many cells) or extending run times (due to using too few cells); and using resuspension buffers that contain DNase and EDTA and are free of Ca2+/Mg2+ ions to prevent clumping.

Other recommendations include using a viability stain for gating out dead cells and debris, which is widely recognized for being more accurate than using plots of FSC versus SSC; filtering samples through an appropriately-sized cell strainer to remove clumps; and performing pre-enrichment of rare cell types such as circulating tumor cells, hematopoietic stem cells, or antigen-specific T cells using antibodies conjugated to magnetic beads.

Common Viability Stains

Dead cell exclusion is important for generating accurate flow cytometry results. Not only can dead cells bind non-specifically to antibodies and cause clumping due to the release of DNA, but they also exhibit higher autofluorescence than living cells. Including a viability stain in flow cytometry experiments allows dead cells to be gated out during analysis. Several different options are available to researchers.

DNA binding dyes

When cells die, the plasma and nuclear membranes become permeable, allowing DNA binding dyes to enter the cells and attach to double-stranded DNA. Here, they produce a fluorescent signal upon excitation with an appropriate laser. An advantage of DNA binding dyes is that their use is relatively straightforward; however, because they rely on membrane integrity, they cannot distinguish between live and dead cells in fixed samples. Common DNA binding dyes include:

- 7-AAD (7-Aminoactinomycin D) - excited by the 488 nm laser and has an emission maximum of 617 nm
- DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) - excited by the 405 nm laser and has an emission maximum of 461 nm
- PI (propidium iodide) - excited by the 488 nm laser and has an emission maximum of 647 nm

Fixable viability dyes

Fixable viability dyes function by binding to primary amine groups which, in the case of living cells, are found at the cell surface. When the cell membrane is compromised, the dyes can enter the cell, where a higher number of primary amine groups is present. Consequently, dead cells exhibit higher fluorescence than live cells. Because fixable viability dyes attach covalently to proteins, they remain bound when the cells are subsequently fixed and permeabilized. Fixable viability dyes are available in a broad range of excitation and emission spectra. Examples include Zombie Dyes™, Ghost Dyes™, and Phantom Dyes.

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Suspension cells

Non-adherent cells represent the simplest sample type for flow cytometric analysis, since they are both quick and easy to prepare. After being collected by centrifugation and washed in ice cold phosphate buffered saline (PBS) containing a low concentration of blocking agent to remove any residual media components, the cells are resuspended in PBS at an appropriate concentration, ready for immunostaining.

Adherent cells

Adherent cells are slightly trickier to handle as they must first be detached from the culture vessel. Importantly, the detachment method should be optimized for the target of interest to avoid damaging antibody binding sites. Common detachment methods include gently scraping the cells into culture media, and washing the cells with PBS before incubating briefly with a detachment agent such as Accutase™, 0.25% trypsin, or an enzyme-free alternative.

Tissue homogenates

Tissue homogenates present unique challenges for flow cytometry since they require mechanical and/or enzymatic dissociation (e.g., using collagenase or liberase) to produce a single-cell suspension. Critically, overly aggressive dissociation methods should be avoided, especially when processing delicate sample types like spleen or lymph nodes, and optimization is essential to prevent target epitopes from being destroyed.

Cell counting

The number of cells used for a flow cytometry experiment requires careful optimization. Where cell counts are too high, rare events may be missed, while using too few cells can lead to long run times that could compromise cell health or lead to photobleaching. Accurate cell counting is vital to ensure experimental reproducibility and, when combined with viability staining, also helps safeguard sample quality. Using an automated cell counter rather than performing a manual count with a hemocytometer is a popular strategy for standardizing the flow cytometry workflow.

5. Fixation

Fixation is the process of preserving cells at a specific point in time and is used to prevent sample degradation. While some fixatives function by forming protein cross-links that can mask antibody binding sites, others disrupt the cell membrane to both fix and permeabilize the sample in a single step. Factors to consider for fixation include whether fixation is actually required, the choice of fixative agent, and the optimal incubation conditions.

Live versus fixed cells

Where the aim of a flow cytometry experiment is to detect only cell surface markers, staining and analysis can often be performed on live cells. Advantages of using live cells are that the experimental workflow is shortened through removal of both the fixation and permeabilization steps and the cells can subsequently be used for downstream applications like in vitro culture. However, a limitation of using live cells is that samples must be processed straight away, which precludes sample collection over time. Live cell staining is also poorly suited to situations where immediate access to the flow cytometer cannot be guaranteed. Where both cell surface and intracellular markers will be detected, a popular approach is to stain for cell surface markers first, before fixing and staining for intracellular targets.

Download flow cytometry protocols for extracellular and intracellular targets here.

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Types of fixatives and tips for use

The two major types of fixative used when preparing cells for flow cytometry are formaldehyde/glutaraldehyde (typically diluted to a concentration of 0.5 – 4% in PBS depending on the sample type) and alcohols such as ethanol and methanol (generally used as a 70% solution).

Formaldehyde is generally more popular than glutaraldehyde as it is less likely to cause autofluorescence. Yet, because formaldehyde gives rise to protein cross-links (especially if incubated with the sample for > 1 hour) and necessitates a separate permeabilization step, it may not always be the first choice.

Alcohols simultaneously fix and permeabilize the cells and are compatible with long term storage at 4oC or -20oC. However, like formaldehyde, they can mask epitopes if their use is not carefully optimized.

More recently, reagents have been developed that combine gentle fixation and permeabilization into a single step, circumventing some of the challenges of using traditional methods.

6. Permeabilization

Permeabilization is necessary whenever intracellular targets are of interest. These include cytokines, transcription factors, and DNA, as well as recombinantly expressed proteins. Permeabilizing agents function by disrupting the cell membrane and/or nuclear membrane, allowing antibody reagents to access their targets, and should be carefully matched to both the sample type and target location, as well as to the chosen fluorochrome.

Types of permeabilizing agents and tips for use

Mild detergents such as Tween® 20, Saponin, and Digitonin are commonly used as permeabilizing agents when detecting cytoplasmic proteins, soluble nuclear antigens, or targets that are expressed at the cytoplasmic face of the plasma membrane. These are usually diluted to a concentration of 0.1 – 0.5% in PBS and function to disrupt the cell membrane without altering the morphological scatter properties of the cell.

Harsher detergents such as Triton® X-100 or NP-40 are generally preferred for nuclear antigen staining. Used at a concentration of 0.1 – 1% in PBS, these reagents permeabilize both cellular and nuclear membranes and are associated with decreased light scattering due to the loss of cell membrane and cytoplasm.

Alcohol-based permeabilization reagents such as ethanol and methanol are likewise used for nuclear antigen staining. They are also popular for phosphoprotein detection.

7. Blocking

Blocking is essential for any flow cytometry experiment to prevent unwanted background signal and avoid false positive results. It should be performed immediately prior to immunostaining and must typically address both non-specific antibody binding interactions and antibody binding to Fc receptors on immune cells.

Blocking non-specific binding

Non-specific antibody binding is blocked by incubating the sample with a protein solution such as bovine serum albumin (BSA), a commercially available protein-free blocker, or a normal animal serum (a serum containing antibodies from the same species as that of the secondary antibody). Blocking agents are usually diluted to a concentration of 5% in PBS (which should also include the permeabilizing agent where necessary) and should be optimized during experimental design. An effective blocking agent will show minimal affinity for the target, exhibit high binding to non-target sites, and will also function to stabilize cellular morphology.

Fc receptor blocking

Antibody binding to Fc receptors (FcRs) present on the surface of immune cells such as macrophages, monocytes, B lymphocytes, and dendritic cells can be a major problem for flow cytometry, where it yields false positive results. Fc receptor blocking ensures only antigen-specific binding is observed and involves simply incubating the sample with a dedicated FcR blocking agent (**e.g., Purified Human IgG-Fc Fragment, normal serum**) prior to adding the target-specific antibody.

8. Washing

Wash steps are included in the flow cytometry workflow to eliminate debris and residual media components from the cells and to remove unbound antibody reagents that could yield misleading results. The washing protocol should be carefully optimized during experimental design to determine the correct number, duration, and volume of wash steps required.

Buffers

Flow cytometry wash buffers usually comprise the same solution used for antibody dilution, namely a low concentration of the blocking agent in PBS, which may also include the permeabilizing agent used for detecting intracellular targets, as well as EDTA to prevent cells from clumping.

9. Immunostaining

Immunostaining is one of the most challenging aspects of the flow cytometry workflow and increases in complexity as multiparameter panels grow in size. Identifying well-validated antibody reagents for the targets of interest is critical and should be followed by rigorous in-house testing and optimization for the experimental model in question. It is also important to decide whether to use direct or indirect detection, or a combination of both, and to incorporate any relevant controls.

Bethyl Antibody Validation Pillars

At Bethyl, we adhere to six antibody validation pillars to ensure our antibodies are of the highest possible quality:

Pillar 1: Independent Antibodies - This pillar requires that two or more antibodies directed against different epitopes of a protein generate similar results.

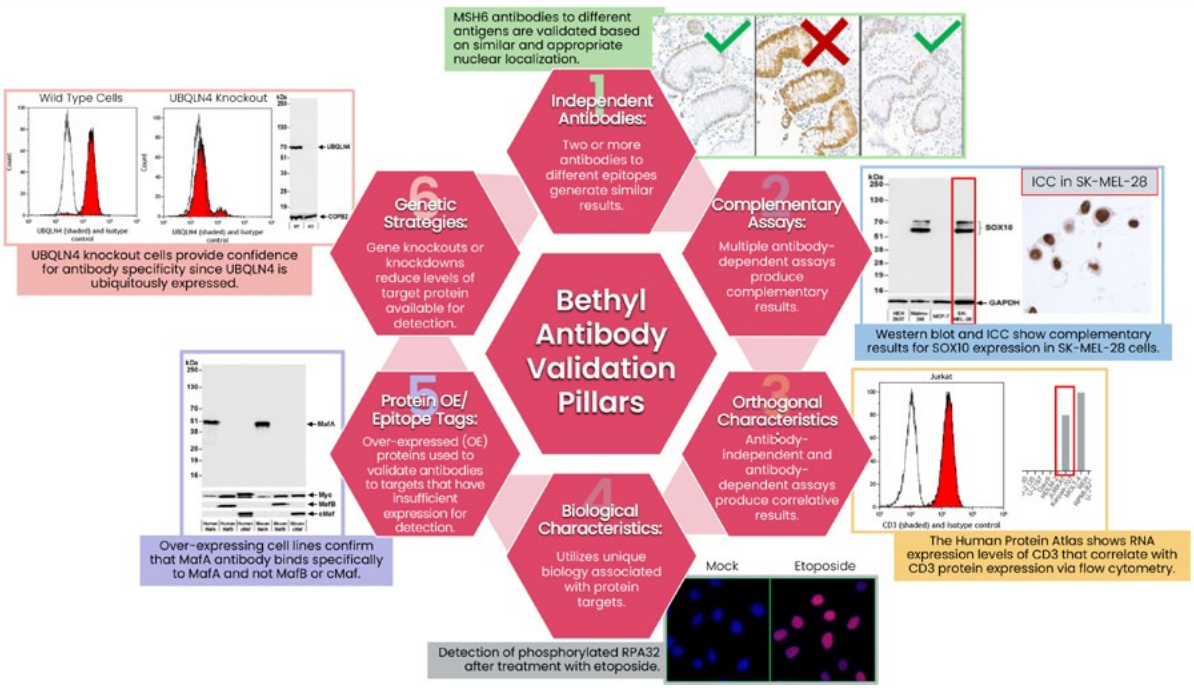
Pillar 2: Complementary Assays - This pillar requires that multiple, antibody-dependent assays produce complementary results.

Pillar 3: Orthogonal Characteristics - This pillar requires that antibody-independent and antibody-dependent assays produce results that are correlative.

Pillar 4: Biological Characteristics - This pillar takes advantage of the unique biology associated with some protein targets.

Pillar 5: Protein OE/Epitope Tags - This pillar uses over-expressed (OE) proteins to validate antibodies against targets where we cannot identify a natively expressing cell line, or the protein is expressed at levels insufficient for detection.

Pillar 6: Genetic Strategies - This pillar uses gene knockout or knockdown to reduce the levels of target protein available for detection.



Primary antibody selection

There are numerous factors to consider when selecting primary antibodies for flow cytometry, not least antibody specificity for the target of interest. For example, where the aim of the experiment is to detect a single member of a closely related protein family, it is critical to see validation data showing that cross-reactivity is not an issue. Antibody sensitivity is equally important; where the target of interest is scarce, comparing several different primary antibodies can help identify a more sensitive product to maximize the chances of successful detection.

Another key factor is the antibody host species, which is especially of relevance to panel design. Where secondary antibodies will be used for indirect detection, primary antibodies should be sourced from different (ideally unrelated) host species to avoid unwanted cross-reactivities. Similarly, primary antibodies should be derived from a different host species than the sample material to avoid secondary antibody binding to endogenous proteins.

An established solution to the innate challenges of using secondary antibodies for multiparameter flow cytometry is to source isotype- or subclass-specific secondaries for detection. Provided these have been cross-adsorbed to eliminate the risk of off-target binding, they provide a quick and easy means of increasing panel flexibility. An alternative approach is to circumvent secondary antibody use altogether by introducing labeled primary antibodies into the flow cytometry workflow.

Finally, irrespective of whether labeled primary antibodies or secondary antibody reagents will be used for detection, it is essential to validate antibody performance in your own experimental system.

We offer an extensive selection of antibodies that are validated for flow cytometry; simplify your search for a suitable product by filtering on criteria such as host species, clonality, reactivity, and available conjugates.

Direct versus indirect detection

During direct detection, labeled primary antibodies are used to recognize and bind the target of interest. Advantages of this approach are that it shortens the experimental workflow and increases flexibility for panel design by removing the need for primary antibodies to be sourced from different hosts. However, direct detection provides only limited sensitivity since it does not include any form of signal amplification.

Indirect detection uses unlabeled primary antibodies for target recognition, followed by detection with labeled secondary antibodies. Because multiple secondary antibodies can bind each primary antibody, indirect detection provides signal amplification and can increase the likelihood of identifying less abundant targets. However, the experimental workflow is extended by the additional incubation and wash steps and it is vital to ensure that secondary antibodies are not exhibiting unwanted cross-reactivities. This can be addressed by using secondary antibodies that have been cross-adsorbed against the sample species, as well as including relevant controls (e.g., secondary antibody only controls).

Both direct detection and indirect detection are shown in **Figure 11**.

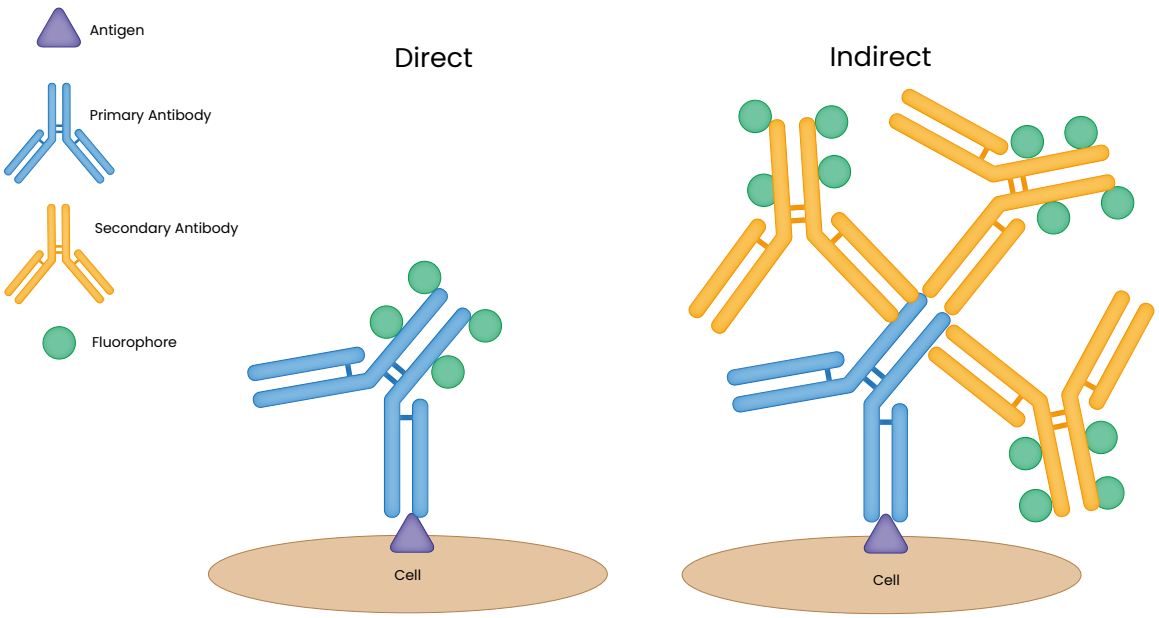


Figure 11. Direct versus indirect detection.

If you're struggling to find a particular antibody-fluorochrome conjugate, try our custom antibody conjugation service.

Sequential staining

Sequential staining is an approach used for detecting both cell surface markers and intracellular targets in the same flow cytometry experiment. Because some fixatives can adversely affect antibody binding sites, researchers often choose to stain for cell surface markers first before fixing and permeabilizing the cells for detecting intracellular targets. Although this extends the experimental workflow, it is a recognized strategy for multiparameter flow cytometry that is open to automation for reduced sample handling time. A typical sequential staining workflow is shown in **Figure 12.**

Figure 12. Sequential staining for flow cytometry.

10. Controls

Controls are used to monitor assay performance and validate results, making them a key consideration for any experimental workflow. Due to its unique nature, flow cytometry requires several types of controls that are not applicable to other immunoassay formats, several of which are used for resolving the inherent complexities of multicolor analysis.

Biological controls

Biological controls comprise known positive and known negative sample types. They include cells where target expression (or a lack of expression) is described in the literature; cells that have been transfected or engineered to yield positive staining; and cells in which target expression has been knocked down or knocked out using techniques such as RNAi or CRISPR, respectively. Additionally, for flow cytometry experiments that will measure some form of cellular response to a stimulus or a drug, controls may encompass both treated and untreated samples. **Figure 13** is an example of assessing phosphorylation after treatment with etoposide, where the untreated cells provide information about normal or background levels of phosphorylation.

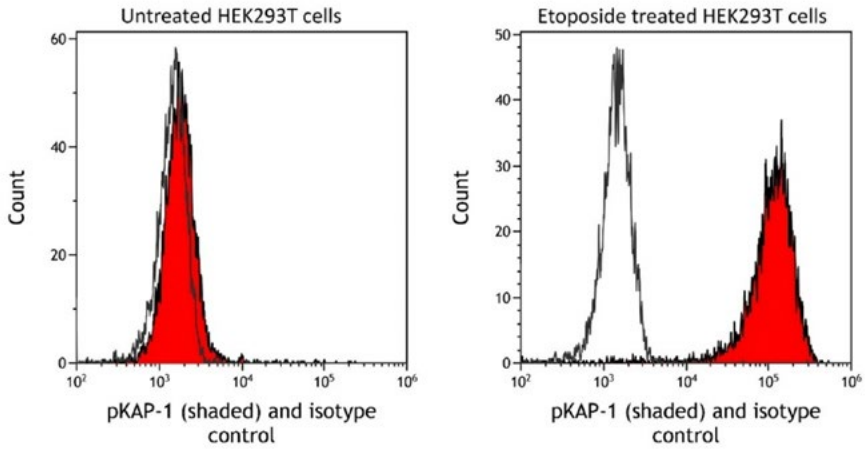


Figure 13. Example of biological controls. Etoposide treatment is used as a positive control (shaded in red) and compared to an untreated control (unshaded).

Unstained controls

Unstained samples are useful for identifying autofluorescence, which can vary based on cell type. They are produced by treating the designated control samples similarly to test samples, but with the omission of any primary and secondary antibody reagents.

Isotype controls

Isotype controls are antibodies that share the same isotype as target-specific antibodies but that were raised against an antigen known to be lacking from the cell type of interest. They function to ensure any observed staining is due to specific binding and provide information about whether antibody reagents are recognizing Fc receptors, fluorochromes, or other off-target experimental components. As well being matched to the host species and isotype of primary antibodies, isotype controls should also match the fluorochrome. For example, a suitable isotype control for an RPE-conjugated mouse IgG1 antibody would be a mouse IgG1 isotype control labelled with RPE. **Figure 14** shows a V5 tag antibody being used as an isotype control for a target monoclonal antibody.

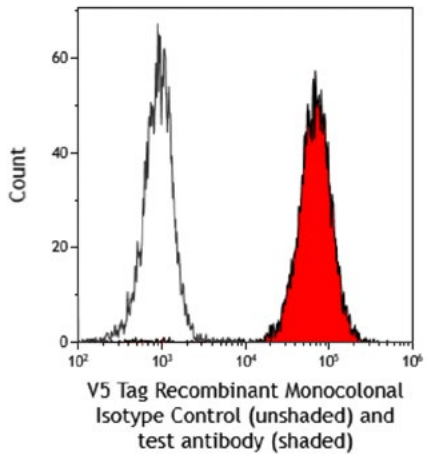


Figure 14. Example of an isotype control. V5 tag recombinant monoclonal antibody is used as an isotype control (unshaded) and compared to a test antibody (shaded in red).

Secondary antibody only controls

Secondary antibody only controls provide insights into non-specific secondary antibody binding. They comprise samples incubated only with the secondary antibody (no primary antibody incubation step) and are useful for flow cytometry experiments that employ indirect detection.

Fluorescence-minus-one (FMO) controls

Fluorescence minus one (FMO) controls are critical for multicolor flow cytometry, where they are used for setting gates. They consist of samples that have been stained with all but one of the fluorochromes used in the multicolor panel and provide an indication of how fluorescence spread affects the channel of interest.

Compensation controls

Because fluorochromes exhibit spectral overlap, the signal from any fluorochrome used in a multicolor flow cytometry panel will be measured across all of the detectors, a phenomenon known as fluorescence spillover. Compensation is an algorithm for removing fluorescence spillover that relies on compensation controls – samples where both a positive population and a negative population are each stained with just one fluorochrome. Compensation usually employs automated software for calculating and applying spillover values, although it can also be performed manually.

11. Data analysis

The defining property of flow cytometry is the ability to collect information about cell populations at the single cell level, yet this makes data analysis one of the technique's most challenging components. Depending on the experimental design, data analysis can range from a simple single-color analysis of a single cell population to a complex multi-color analysis of a heterogeneous sample. In either case, large multifaceted data sets are produced that require special analysis tools and software to decipher.

Process overview

Data analysis begins with selecting the cell population of interest based on light scatter and fluorescent properties. From here, cells are analyzed for targets of interest based on the expression of as many as forty different markers. Specifically, using gating strategies and fluorescence intensity, data is gathered to answer defined experimental questions, with statistical tools often being used to determine the significance of experimental results.

Gating

Gating describes the process of identifying and selecting specific cell populations for further investigation. While this process can be subjective, it is one of the most important parts of data analysis since the final conclusions of any flow cytometry experiment will be dictated by the initial gating strategies.

Proper experimental controls (see the Controls section) are vital for establishing gates. Used in combination, unstained samples, isotype controls, and secondary antibody only controls enable gates to be set such that targets/cells of interest are included but background fluorescence is not. Fluorescence minus one (FMO) controls ensure that gates are being set around positively fluorescing samples rather than background or spectral overlap. Positive controls likewise help to confirm that gates have been created around the correct cell populations, as well as being beneficial for back-gating (discussed later).

After control samples have been used to create gates, the gates should remain unchanged for the entirety of the data analysis for that experiment. If gates are shifted (and sometimes this might be necessary), they should be moved for all of the samples to ensure accurate comparisons and analysis.

Sequential gating

Often, initial gates are created using forward and side scatter data. Then, from this information, additional gates can be set around single cells (rather than debris or cell clumps) and around specific cell subsets, which can subsequently be investigated further until the final, desired target population is examined. This process of classifying a broad grouping of cells to a more specialized cell population is termed sequential gating (1). An example of sequential gating is shown in **Figure 15.**

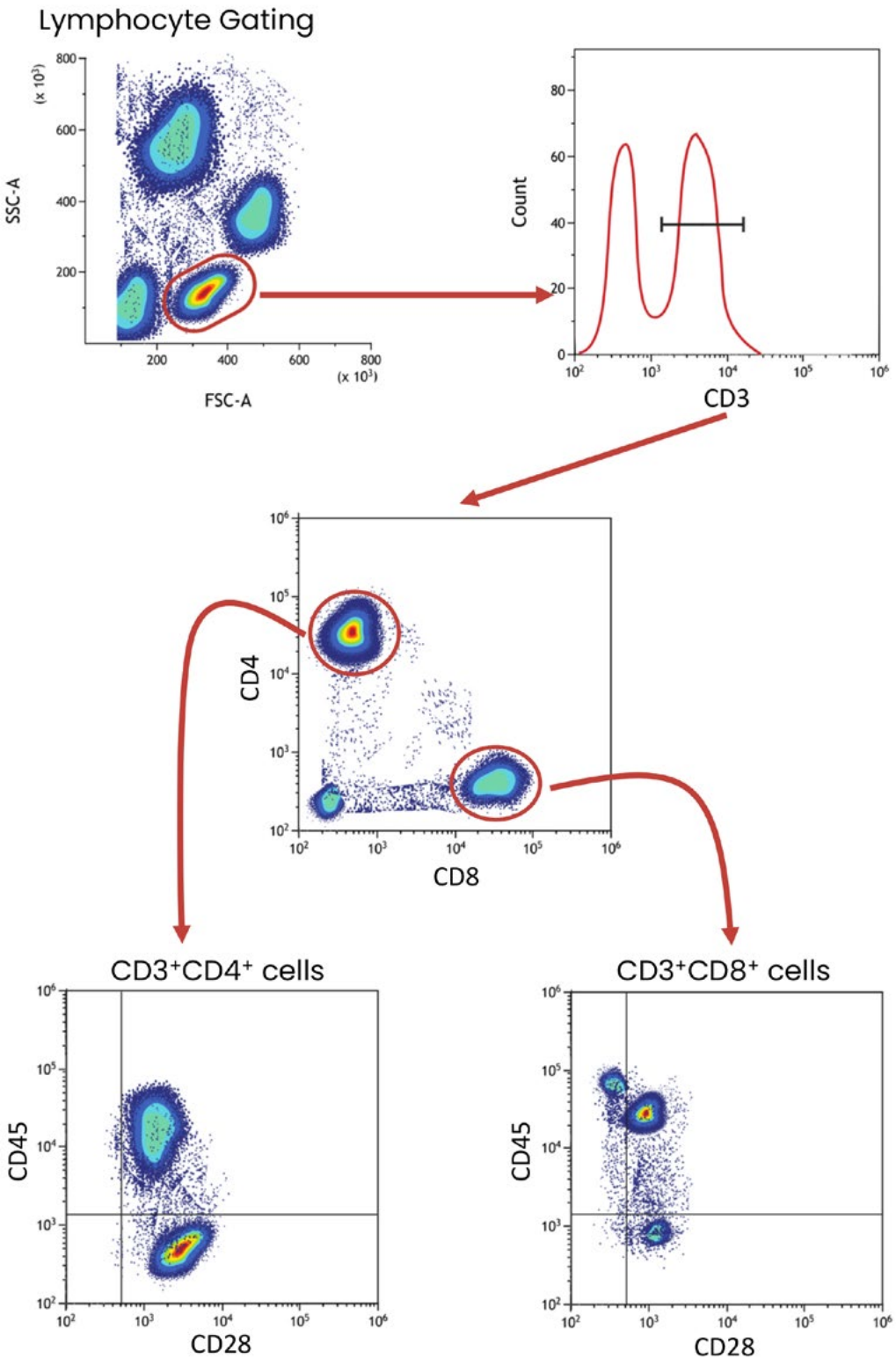


Figure 15. Example of sequential gating to investigate specific T cell populations. First, the lymphocyte population is identified, before being narrowed down to the T cells (CD3+). The T cells are then further classified as CD4+ or CD8+ cell populations. Subsequently, CD28 and CD45RA, the targets of interest, can be examined in both the CD4+ and CD8+ populations.

Back gating

Another gating process, termed back gating, can be useful in confirming that a target cell population has been included in the initial gating strategy. Here, understanding the markers used is critical, since a gate used for back gating cannot be used in the initial gating strategy.

Using Figure 15 as a reference, back gating could involve examining the CD3/PE fluorescent channel prior to creating the gate around the lymphocytes. Gating on the CD3+ cells and then identifying that population on the scatter plot would help ensure the appropriate region is selected to set the lymphocyte gate. Often, data analysis programs will create a color specific to the gate that can be seen on the scatter plot. If the marker used for back gating is present on more than one cell population, this information needs to be taken into consideration as the gating strategy is developed (2).

Because of the subjective nature of gating and its effect on subsequent data collection and analysis, new methods of automation are being developed to increase accuracy, consistency, and throughput (3).

Data types

Flow cytometry data differs considerably in terms of its complexity, but can broadly be considered as either single parameter or multiparameter, with fluorescence intensity being represented in one of several different ways.

Single parameter data

Single parameter data is the collection of information about one specific element – either fluorescence or light scatter – and is frequently represented by histograms. Usually, the element of interest is displayed on the x-axis and the count on the y-axis. **Figure 16** provides an example of single parameter data generated for the protein Smad4.

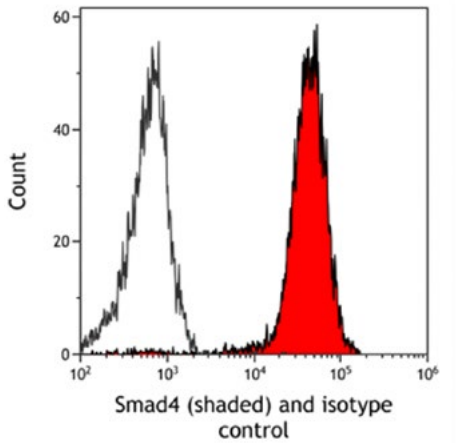


Figure 16. Example of single parameter data represented in a histogram. Fluorescent staining is representative of the amount of Smad4 present, where the red filled histogram shows an increased level of Smad4 expression compared to the isotype control (unshaded histogram).

Multiparameter data

Multiparameter data is the collection of information about several specific elements. It can be used to identify distinct cell populations in a heterogeneous cell sample or to characterize changes in cell populations due to treatments and/or experimental conditions. This type of data is typically represented as dot plots, which are named for the fact that each event is displayed as a dot on the graph. Multiparameter data can also be represented as density plots, which are multi-color dot plots where different colors equate to varying amounts of events. Often, multiparameter data is presented as Fluorescence 1 versus Fluorescence 2 or as Fluorescence versus Light Scatter.

Figure 17 provides an example of a dot plot showing the expression of two T cell markers, CD3 and CD8. The lower left quadrant shows cells that express neither, or only low levels, of CD3 and CD8. The upper left quadrant shows cells that express high levels of CD3 but not CD8. The lower right quadrant shows cells that express high levels of CD8 but not CD3. The upper right quadrant shows cells that express high levels of both CD3 and CD8. The numerical values represent the percent of cells present in each quadrant based on the initial gated lymphocyte population, although this data could also be depicted in absolute numbers. Using this data, double positive cells (expressing both CD8 and CD3) could be gated and further investigated for additional targets of interest to provide more detailed information about this particular T cell population.

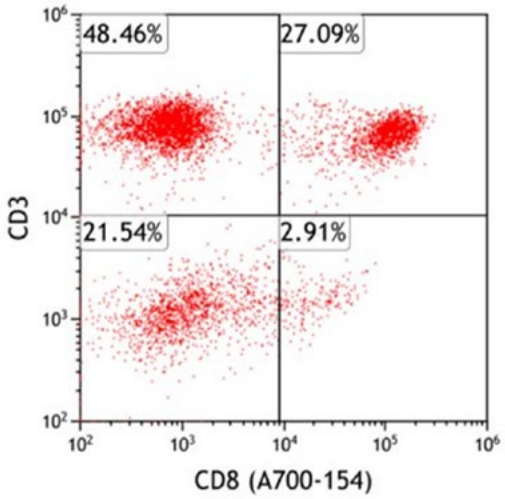


Figure 17. Example of multiparameter data represented in a dot plot. The data is divided into quadrants based on the amount of CD3 and CD8 expression.

Relative Fluorescence Intensity

Flow cytometry experiments are often designed to compare changes in target expression across different experimental conditions. Quantifying this information is not only useful in determining a more complete picture of the changes occurring throughout the experiment, but also for statistical analysis. Commonly, fluorescence intensity is used to determine levels of target expression, but there are multiple ways in which fluorescence intensity can be represented.

Mean fluorescence intensity can represent either the Arithmetic or Geometric Mean. The Arithmetic Mean is the number of events in each fluorescent channel divided by the number of channels. The Geometric Mean reflects the fact that fluorescent intensity increases logarithmically, and creates an average based on this compounding effect. The Geometric Mean is thought to be a better representation of fluorescence intensity for flow cytometry than the Arithmetic Mean. However, a more accurate measure still is the Median Fluorescence Intensity, which uses the midpoint of the fluorescent population in a flow cytometry sample (4).

Differences in background fluorescence have led to the use of Relative Fluorescence Intensity when comparing target expression between different cell populations or different experiments. Specifically, by comparing the average (mean or median) fluorescence intensity of one population with another, the relative fluorescence intensity allows fluorescence levels of targets of interest to be compared to isotype controls or amongst positive and negative cell populations. Creating these relative differences helps improve experimental consistency.

Statistical methods

Statistical analysis for flow cytometry is dependent on the experimental design and the questions being addressed. If pair-wise comparisons are examined, statistical methods like T-test or similar non-parametric tests (e.g., Kruskal-Wallis or Mann-Whitney U) can be used. If more complicated comparisons are being made, different statistical methods like ANOVA or regression analysis will be needed (2).

12. Conclusion

By allowing researchers to study individual cells in suspension, flow cytometry can reveal valuable insights that might otherwise be missed using conventional bulk analysis techniques. Critically, despite flow cytometry experiments becoming increasingly complex in recent years, adhering to best practices for experimental design and execution remains key to generating reliable results. The information contained here is intended as a broad overview of flow cytometry for researchers. Should you have further questions, including around reagent selection, our technical support and customer service teams are always happy to help. Contact us today to discuss how we can help advance your research.

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