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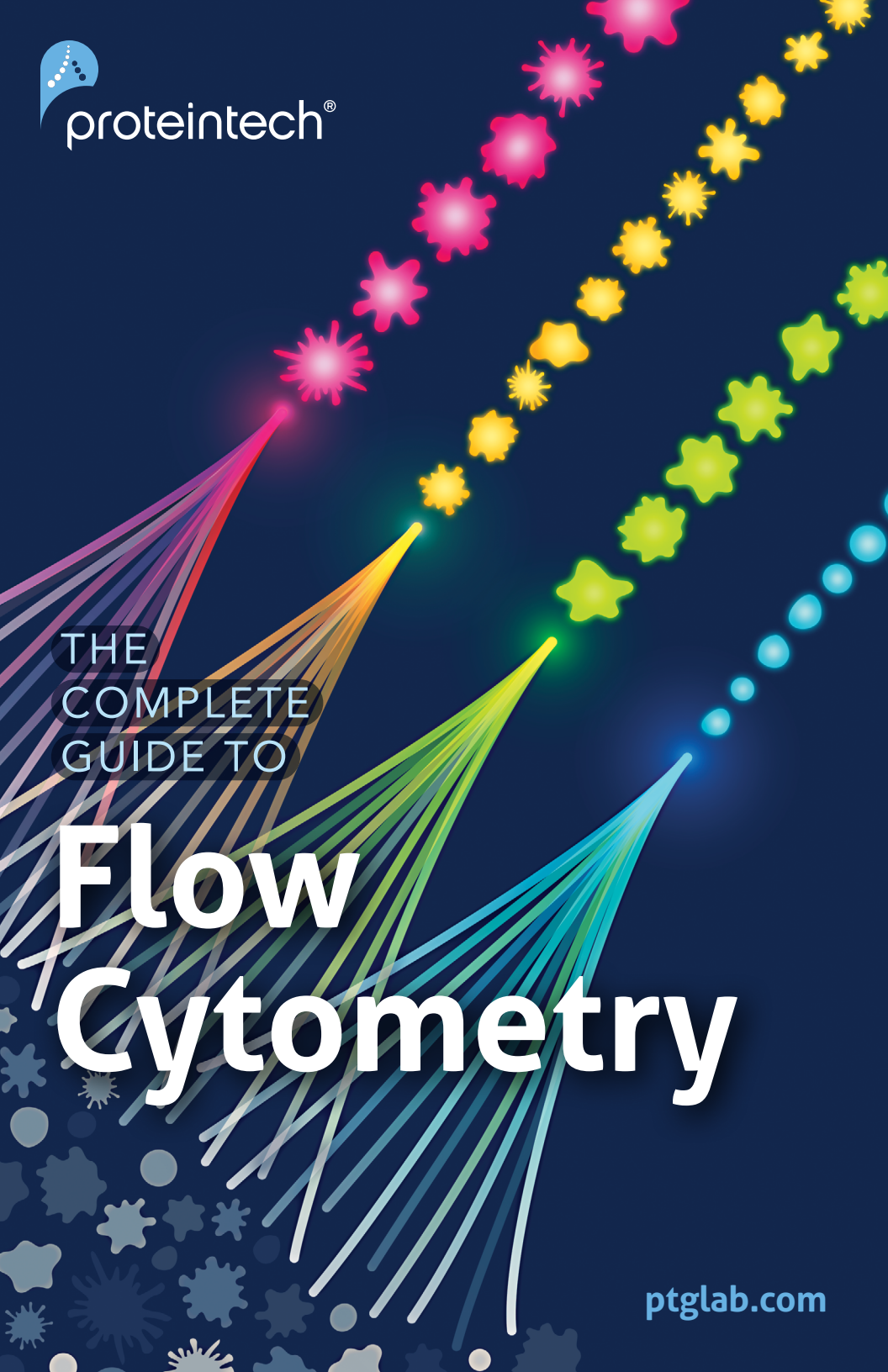

THE COMPLETE GUIDE TO

# Flow Cytometry

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# WELCOME

This booklet aims to give you a complete guide to flow cytometry. It will cover a comprehensive background to flow cytometry, step-by-step protocols with useful technical tips, and troubleshooting.

Flow cytometry is a technique that enables the measurement of properties of individual particles (cells) in a heterologous population. This allows researchers to phenotype each cell based on these properties and, in some cases, to sort cells into different subpopulations (fluorescence-activated cell sorting or FACS). In a flow cytometer, cells in suspension pass through a laser in a single file, which enables the measurement of particle size and granularity. Further phenotyping occurs by using fluorescently conjugated antibodies that bind to target proteins on the cell surface or intracellularly, enabling the measurement of fluorescence intensity as another parameter.

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# WHAT IS FLOW CYTOMETRY?

## How does Flow Cytometry Work?

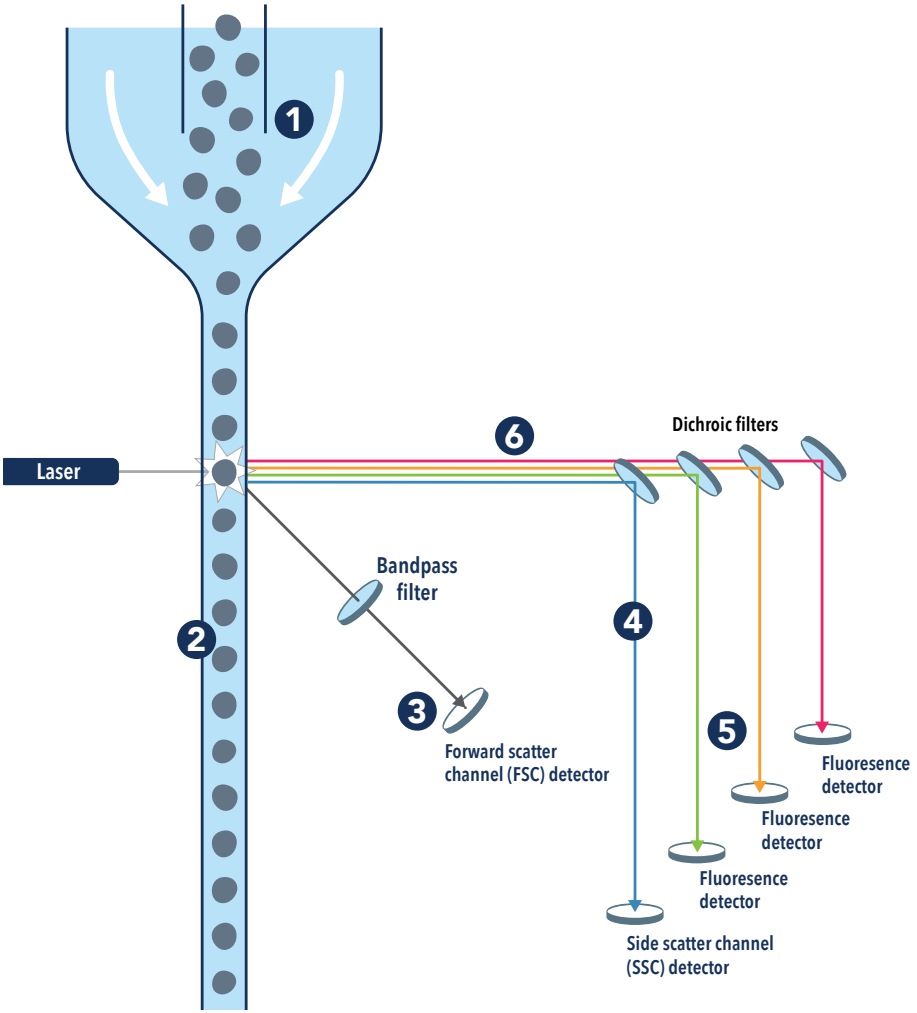



FIGURE 1: **Overview of a flow cytometer**

In general, a flow cytometer is composed of three systems: the fluidics, optics, and electronics systems. The fluidics system focuses a cell suspension into a single stream by forcing the cells through the sheath towards a narrow nozzle, **Figure 1 (1)**. This hydrodynamic focusing enables cells to pass one by one through one or more lasers **(2)**. Light scattered in the forward direction to that of the laser is then collected by a photomultiplier tube (PMT) and is referred to as the forward scatter channel (FSC) **(3)**. This measurement provides information about the size of the particles as, in general, larger particles refract more light than smaller ones. A PMT measuring light at roughly 90° to the light beam is called the side scatter channel (SSC) **(4)**. This provides information on the granularity of the cells. For example, neutrophils are highly granular cells compared to macrophages.

Fluorescent light detected at different wavelengths by PMTs **(5)** provides further information for cell identification based on the combination of fluorescently tagged cell markers. This is achieved using specialized filters that can transmit certain wavelengths of light while blocking other wavelengths. There are three main types of filters: short-pass filters that allow wavelengths of light below a specified threshold, long-pass filters that allow wavelengths of light above a specified threshold, and dichroic mirrors **(6)** that allow certain wavelengths to pass through but reflect others. Using a combination of these filters in a certain order enables the detection of multiple signals simultaneously.

When a particle passes through a laser, it creates a pulse of information, which is termed an 'event.' Each event can be analyzed for its size (area under the curve; the time it takes for the particle to pass through the beam) and signal intensity (height). Thresholds for these parameters are set to determine which events correspond to cells, rather than smaller particles, which will be ignored by the detectors. Fluorescence data is displayed in a logarithmic scale to facilitate easy visualization on a histogram due to the wide range in signal distribution by strong and weak signals. Each parameter is displayed according to its height, area, and width by specialist software. This is then interpreted to compare populations according to your experimental design.

## Flow Cytometry Applications

- **Analysis of plasma membrane receptor expression**
- **Analysis of cell viability, apoptosis, and necrosis**
- **Cell phenotyping**
- **Cell cycle analysis**
- **Cell sorting**
- **Analysis of intracellular protein expression**
- **Biomarker detection**
- **Cell counting**

## Fluorophores and Dyes

The majority of flow cytometry experiments involve the use of fluorophores. Fluorophores act by accepting the energy of a certain wavelength (for example from a laser) that causes the electrons in the fluorophore to move from a resting to an excited state. When the electron returns to its resting state, energy is released as fluorescence at a lower energy wavelength. The excitation-emission spectrum of a fluorophore defines its properties when used for designing panels in multiplex experiments. When conjugated to primary or secondary antibodies, fluorophores in flow cytometry are used to identify and quantify populations of cells based on targeted cell surface markers or intracellular targets. Cells manipulated to express fluorescent proteins such as GFP or Venus can use their fluorescent properties in a similar manner, with the added benefit that these can be used to tag intracellular markers in live cells without the need for membrane permeabilization.

Single-dye organic fluorophores such as FITC, APC, PE, and PerCP have been around for many years. More recently, synthetic dyes such as Alexa Fluor® and CoraLite® Plus have been designed with enhanced brightness and greater photostability. Tandem dyes are also available and commonly used in flow cytometry, whereby two fluorophores are covalently coupled together. When the first dye (donor) is excited, it transfers energy to the second dye (acceptor), which then becomes activated, resulting in fluorescence. Thus, tandem dyes enable more colors to be detected from a single laser wavelength.

TABLE 1: **Excitation-emission details of common fluorophores and their relative brightness**

| Fluorophore | Fluorescence Color | Max Excitation | Max Emission | Relative Brightness |
|---|---|---|---|---|
| CoraLite Plus 405 | | 399 nm | 422 nm | 1 |
| Pacific Blue™ | | 410 nm | 455 nm | 1 |
| CoraLite Plus 488 | | 493 nm | 522 nm | 3 |
| FITC/FITC Plus | | 490 nm | 525 nm | 3 |
| PE | | 490; 565 nm | 578 nm | 5 |
| CoraLite Plus 555 | | 554 nm | 570 nm | 2 |
| CoraLite 594 | | 590 nm | 617 nm | 4 |
| Texas Red® | | 596 nm | 615 nm | 3 |
| APC | | 650 nm | 661 nm | 4 |
| CoraLite Plus 647 | | 654 nm | 674 nm | 4 |
| PerCP | | 490 nm | 675 nm | 2 |
| CoraLite Plus 750 | | 755 nm | 780 nm | 3 |

**PE is the same as R-phycoerythrin Cy = cyanine. APC = allophycocyanin. FITC = fluorescein isothiocyanate. PE = phycoerythrin. PerCP = peridinin chlorophyll pr otein.*

# EXPERIMENTAL DESIGN

## Choosing a Fluorophore

There are many different types of fluorophores available, and their different properties can be used to best suit the desired targets and the experimental setup:

1. To minimize spectral overlap, avoid using fluorochromes with similar emissions spectra if they are excited by the same laser.
2. Use bright fluorophores with low expression markers to resolve their signal from background noise, whereas abundantly expressed markers can be paired with dyes that are less bright.
3. Some fluorochromes add significant molecular weight to the labeling reagents – this may reduce their ability to permeate the cell membrane to stain intracellular targets.
4. Be sure to properly handle tandem dyes – avoid freeze-thaw cycles and exposure to light to minimize decoupling.

TABLE 2: **Types of fluorophore reagents commonly used in flow cytometry**

| Reagent | Example | Live cells | Fixed cells | Permeabilization |
|---|---|---|---|---|
| Viability dyes | Propidium iodide | ✓ | ✗ | ✗ |
| Apoptotic markers | Fluorescently labeled annexin V | ✓ | ✗ | ✗ |
| Fluorescently labeled antibodies against extracellular proteins | GFP-LC3 | ✓ | ✓ | Not required |
| Fluorescent proteins expressed by cells | CD3 | ✓ | ✓ | Not required |
| Typically | TDP-43 | Technically challenging | ✓ | Required |

*Pacific Blue™ and Texas Red® are registered trademarks of Thermo Fisher Scientific.*
*Alexa Fluor® is a registered trademark of Life Technologies Corporation.*

## Flow Cytometry Multicolor Panel Design

Many flow cytometry experiments use more than one fluorophore to stain samples. Multicolor experiments are a powerful tool that not only enables cost savings by analyzing fewer samples at a time but also allows for the gathering of more information using single-cell analysis. They also make it possible to examine relationships between targets – e.g., examining whether cells positive for one target also express another. However, multicolor experiments require careful design.

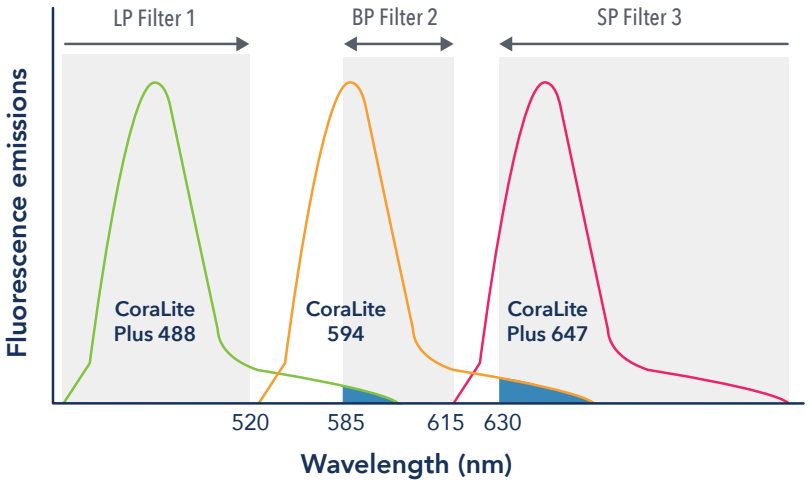
## Filters in Flow Cytometers

Every fluorophore has characteristic excitation and emission spectra. The wavelength of the laser light is chosen based on its excitation spectrum. Before detection, the emitted light is filtered through sets of optical filters. There are three types of filters:

- long-pass (LP) – filter light above a certain wavelength
- bandpass (BP) – filter light between certain wavelengths
- short-pass (SP) – filter light below a certain wavelength

It is easy to choose appropriate filters in single fluorophore experiments. However, in multicolor experiments with fluorophores excited by the same laser, sets of filters need to be chosen with caution. The example setup depicted in **Figure 2** shows the emission spectra of three fluorophores. LP filter 1 reflects light below 520 nm, which allows it to gather the spectrum of fluorophore 1. BP filter 2 (600/15 nm) collects light from fluorophore 2, while SP filter 3 (630 nm) collects most of the spectrum of fluorophore 3. Using appropriate filter sets, the user can efficiently collect signals originating from different fluorophores.

FIGURE 2: **Considerations for light filtering and compensation using LP, BP, and SP filters based on fluorophore emissions spectra**



## Controls in Flow Cytometry

**Unstained controls** are required to assess the level of cell autofluorescence in your sample and are used to determine your voltage settings and negative gates.

**Isotype controls** utilize antibodies raised against antigens that are not present in cells and are used as a form of negative control in place of the target antibody to assess the level of non-specific binding. These controls should be used with caution and must be the same host, class, and subclass, conjugated to the same fluorophore, and used at the same working concentration as the target antibody. If all other controls are performed, then an isotype control may not be necessary.

**Fc block controls** are performed using species-specific serum, such as mouse or human IgG or commercial reagents. This prevents Fc-mediated binding of conjugated target antibodies to Fc receptors on cell types such as B lymphocytes, NK cells, granulocytes, monocytes, macrophages, or platelets. This non-specific Fc binding can cause false positive results; the Fc block thus ensures that the observed signal is specific to antibody paratope/cell-epitope binding.

**Biological controls** should include negative and positive samples for your target antigen or cell lines where the target has been overexpressed or knocked down (e.g., siRNA constructs) or out (e.g., CRISPR constructs). These controls are necessary to ensure that your protocol is working correctly and validate any negative results. In experiments where stimulation is required to observe the target antigen, a non-stimulated sample should also be included.

TABLE 3: **Example experimental design for FMO controls**

| Antigen | CoraLite Plus 488 | PE | Cy5-PE | APC |
|---|---|---|---|---|
| Unstained | — | — | — | — |
| CD3 FMO | — | CD4 | CD8 | CD19 |
| CD4 FMO | CD3 | — | CD8 | CD19 |
| CD8 FMO | CD3 | CD4 | — | CD19 |
| CD19 FMO | CD3 | CD4 | CD8 | — |

*Table 3 demonstrates a typical experimental design for unstained and FMO controls in a multiplex panel. The '-' indicates which antibody(s) is removed from each control.*

## Compensation Controls

When the emission spectra of different fluorophores overlap (**Figure 2** – marked in blue), additional fluorescence compensation is needed. In the example given, filters 2 and 3 detect some of the light emitted from fluorophores 1 and 2 respectively. To determine whether cells in the analysis are truly positive or negative for a given fluorophore, compensation is required. There are two types of compensation controls: 1) single stained controls and 2) fluorescence minus one (FMO) controls.

Single-stained controls are cell samples or beads stained with only one fluorophore. Cells used for compensation should be a mix of both positive and negative cells for a certain fluorophore reagent. This way it is possible to distinguish between positive and negative events within a single channel. Commercially available compensation beads are composed of a mix of beads where a fraction has binding sites for antibodies. Beads can provide better distinction between negative and positive events and reduce the number of cell samples used per experiment but are not compatible with all antibody species. Single-stained samples should be performed for every fluorophore used.

FMO controls are vital in experiments using more than two fluorophores. FMO controls are samples stained with all used fluorophores in the experiment apart from one. They are important for assessing fluorescence spreading and for final gating.

TABLE 4: **Controls in flow cytometry experiments**

| Control | Reason to include |
|---|---|
| Single stained control | To account for spectral overlap |
| Fluorescence minus one (FMO) control | To adjust for spectral overlap in multicolor experiments |
| Unstained control | To account for cell autofluorescence |
| Isotype control | To account for non-specific binding with a species-matched antibody |
| Fc block control | To block Fc receptors in immune cells – essential in staining B cells, dendritic cells, monocytes, and macrophages |
| Biological control | To ensure that the staining procedure is successful |

## Optimizing for Different Target Cell Types

Cell frequency, or the percentage of cells in the sample that are the target cell population, is an important factor to consider when designing the experiment to ensure that the correct conclusions can be drawn from the data. **Tables 5-7** list the expected frequencies of commonly studied cell populations.

### Number of Events

If a cell population is rare, the number of "events" collected should be increased to ensure that the target population is large enough to be detected and statistical significance achieved, as a large percentage of events will be gated out and excluded from analysis.

### Fluorophore Selection

The choice of fluorophores is also influenced by the frequency of the marker. It is recommended to use a brighter fluorophore for rare markers to account for background fluorescence and dimmer ones for highly expressed markers. A bright fluorophore on a very abundant marker can lead to samples appearing off the chart limits. Adjusting the voltage on your cytometer before running your sample is also crucial for making sure the full range of fluorescent intensity can be captured.

## Expected Cell Frequencies in Common Samples

TABLE 5: **Cell frequencies for common human leukocytes**

**Lymphoid cells**

| CELL TYPE | PERCENTAGE |
|---|---|
| T cells | 7-25% |
| CD4+ T cells | 4-20% |
| CD8+ T cells | 2-11% |
| Regulatory T cells | 0.2-1.4% |
| γδ T cells | 1-5% |
| NKT cells | 0.01-0.5% |
| B cells | 1-10% |
| Naïve B cells | 0.5-5% |
| Memory B cells | 0.4-2% |
| NK cells | 1-6% |
| Innate lymphoid cells | 0.01-0.1% |

**Myeloid cells**

| CELL TYPE | PERCENTAGE |
|---|---|
| Granulocytes | 7-25% |
| Neutrophils | 4-20% |
| Eosinophils | 2-11% |
| Basophils | 0.2-1.4% |
| Dendritic cells | 1-5% |
| Monocytes | 0.01-0.1% |

TABLE 6: **Cell frequencies for common murine samples**

### Mouse Spleen

| CELL TYPE | PERCENTAGE |
|---|---|
| T cells | 20-35% |
| CD4+ T cells | 13-25% |
| CD8+ T cells | 4-15% |
| γδ T cells | 0.5-1% |
| NKT cells | 44-70% |
| NK cells | 1-5% |
| iNK cells | 1-2% |
| Granulocytes | 1-2% |
| Dendritic cells | 1-3% |
| Monocytes | 3-5% |

### Mouse Bone Marrow

| CELL TYPE | PERCENTAGE |
|---|---|
| T cells | 10-25% |
| B cells | 15-25% |
| Granulocytes | 45-70% |
| Monocytes | 5-20% |
| Erythoid cells | 20-45% |

### Mouse Thymus

| CELL TYPE | PERCENTAGE |
|---|---|
| CD4+ T cells | 4-6% |
| CD8+ T cells | 1-2% |
| CD4+/CD8+ T cells | 85-95% |

### Mouse Peripheral Blood

| CELL TYPE | PERCENTAGE |
|---|---|
| T cells | 17-20% |
| CD4+ T cells | 8-12% |
| CD8+ T cells | 7-10% |
| B cells | 35-58% |
| NK cells | 4-7% |
| iNK cells | 0.2-0.5% |
| Neutrophils | 4-6% |
| Eosinophils | 1-2% |
| Monocytes | 2-3% |

### Mouse Lymph Node

| CELL TYPE | PERCENTAGE |
|---|---|
| T cells | 65-78% |
| CD4+ T cells | 35-64% |
| CD8+ T cells | 19-30% |
| Regulatory T cells | 2-3% |
| B cells | 9-15% |
| Dendritic cells | 0.5-1% |
| Macrophages | 1-2% |
| Stromal cells | 0.3-0.5% |

TABLE 7: **Cell frequencies for common rat samples**

**Rat Spleen**

| CELL TYPE | PERCENTAGE |
|---|---|
| T cells | 31-34% |
| CD4+ T cells | 22-27% |
| CD8+ T cells | 14-18% |
| B cells | 51-59% |

**Rat Thymus**

| CELL TYPE | PERCENTAGE |
|---|---|
| CD4+ T cells | 5-10% |
| CD8+ T cells | 2-7% |
| CD4+/CD8+ T cells | 83-88% |
| CD4-/CD8- T cells | 1-2% |

**Rat Peripheral Blood**

| CELL TYPE | PERCENTAGE |
|---|---|
| T cells | 51-67% |
| CD4+ T cells | 32-40% |
| CD8+ T cells | 18-26% |
| γδ T cells | 1-2% |
| B cells | 22-41% |
| NK cells | 6-10% |
| Neutrophils | 14-20% |
| Eosinophils | 1-4% |
| Monocytes | 1-6% |

## Optimizing for Different Target Cell Types

As in any antibody-based technique, biological samples require preparation before staining and analysis. Cells require disassociation and are often fixed and permeabilized when analyzing cytoplasmic and nuclear proteins. Samples need to be washed after staining to remove any excess of unbound fluorescent reagents. Please refer to our experimental workflow **(Figure 3)** and sample preparation table **(Table 8)**.

FIGURE 3: **Experimental workflow for analyzing protein targets in flow cytometry**

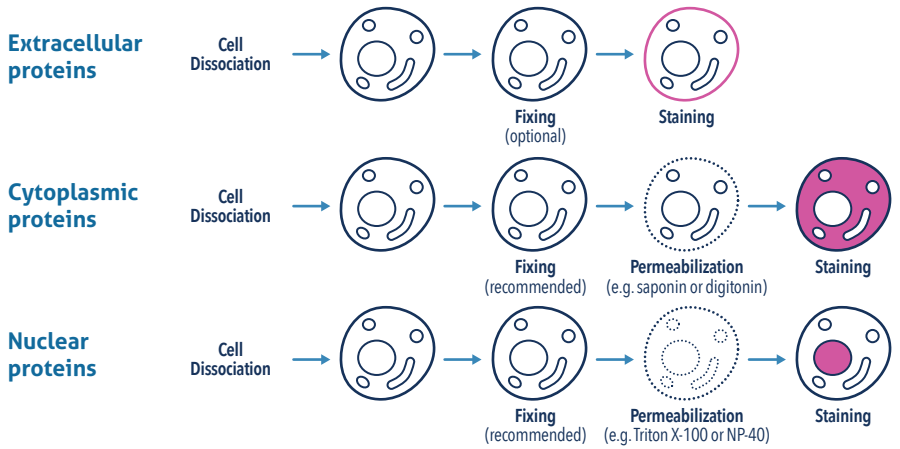


TABLE 8: **Sample preparation steps in flow cytometry experiments**

| Sample preparation step | Description and tips |
|---|---|
| Cell dissociation | • Adherent cells need to be detached before flow cytometry analysis using detaching agents, e.g., trypsin. Milder enzymes are frequently used for cell detachment to preserve plasma membrane receptors if they are subject of analysis<br>• Use appropriate buffers to prevent cell clumping – avoid calcium and magnesium salts. If they are present, a chelating agent, e.g., EDTA, can be added to samples.<br>• Tissue samples require more stringent dissociation methods – either mechanical or enzymatic (e.g., trypsin, collagenase). |
| Fixation | • Advantageous for sample preservation but can increase autofluorescence levels.<br>• Two types of fixatives are available: alcohols (methanol or ethanol) and aldehydes (formaldehyde or glutaraldehyde). |
| Permeabilization | • Necessary for staining of intracellular proteins.<br>• Mild detergents, such as saponin or digitonin, are used for analysis of cytoplasmic proteins.<br>• Harsher detergents, e.g., Triton X-100 or NP-40, need to be used for analysis of nuclear proteins to ensure permeability of both the plasma membrane and the nuclear envelope. |
| Incubation with fluorophores, dyes, or antibodies | • Optimization of used concentrations needed to achieve the best signal to noise ratio and to reduce non-specific binding.<br>• Choice of dyes influences other steps (e.g., compatibility with used fixatives).<br>• Careful design is needed for multicolor analysis (see page 8). |
| Biological control | • It is necessary to remove remnants of fixatives, permeabilization agents, and unbound fluorophores. |

The proper execution of sample preparation steps helps to reduce noise and ensure good-quality data are obtained. The presence of many dead cells, cell clumps, and high cell autofluorescence can usually be avoided. For example, the presence of dead cells can be spotted by analyzing FSC vs SSC plots – dead cells have low FSC and high SSC compared to live cells. It is good practice to include viability dyes in experiments as this provides a definite method of excluding dead cells from analysis. Dead cells are particularly sticky to antibodies and can therefore significantly influence and falsify results if they are not excluded.

Cell concentration for flow cytometry should range from $10^5$ to $10^7$ for optimal detection without blocking the fluidics of the cytometer. Event acquisition should not exceed 10,000 events to reduce doublets and for the best detection by the cytometer.

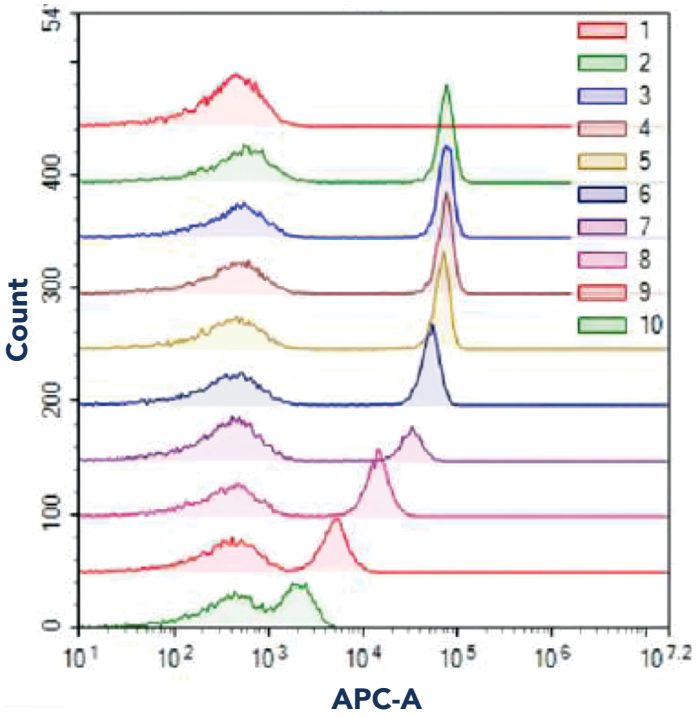
TABLE 9: **Common tissue types and preparation tips**

| Tissue Type | Description and tips |
|---|---|
| Tissue Culture Cells | • Adherent cells need to be detached before flow cytometry analysis using detaching agents, e.g., trypsin. Milder enzymes are frequently used for cell detachment to preserve plasma membrane receptors if they are a subject of analysis (e.g. Accutase).<br>• Use appropriate buffers to prevent cell clumping– avoid calcium and magnesium salts. If they are present, a chelating agent, e.g., EDTA, can be added to samples. |
| Solid Tissue | • Solid tissue (e.g. tumors, liver, lungs) must be digested into a single-cell suspension before staining.<br>• The tissue should be digested mechanically then enzymatically to break down the extracellular matrix and allow cell dissociation.<br>• Enzymatic digestion uses proteases or collagenases (or enzymatic cocktails) supplemented with DNase to reduce cell clumping. This must be performed in serum-free conditions to prevent enzyme saturation. At end of digestion, the enzymes must be neutralized with serum to stop digestion.<br>• Once the tissue is digested, it should be filtered through a cell strainer to create a single-cell suspension.<br>• For tissue containing many red blood cells (RBCs) such as the spleen, we recommend carrying out a red blood cell lysis step. This makes it easier to isolate the lymphocytes and leads to better antibody binding. |
| Bone Marrow | • The cells in the bone marrow are isolated by flushing the long bones of mice with PBS using a syringe and needle. Cell aggregates are separated by passing through a needle and then a cell strainer to create a single-cell suspension.<br>• Bone marrow has a high concentration of red blood cells and RBC lysis is recommended. |
| Whole Blood | • Depending on the size of the blood sample, the RBCs can be removed by lysis or by gradient separation. Removal of RBCs is recommended to concentrate the sample with cells of interest and increase specific antibody binding.<br>• Via RBC lysis<br>  ○ If the blood volume is < 1mL, the red blood cells can be removed by RBC lysis.<br>• Via gradient separation<br>  ○ For a larger blood volume, like for the isolation of human PBMCs, the blood is separated using gradient separation.<br>  ○ Whole blood is overlaid onto separation media and centrifuged at 300g for 30 mins at room temperature, without brakes.<br>  ○ The PBMCs are found at the interphase between plasma and separation media and can be taken up with a pipette. |

## Optimal Antibody Concentration

It is important to optimize the dilutions of antibodies and other probes used for staining. This not only allows the saving of reagents but also reduces the background from non-specific binding. The correct dilution depends on the antibody's affinity to the target and target abundance. Very high dilutions are recommended for abundant targets. As seen in **Figure 4**, it is possible to easily discriminate between CD4-positive and CD4- negative lymphocytes stained with even very high dilutions of the anti-CD4 antibody.

FIGURE 4: **Dilution of antibodies for sample staining in flow cytometry**




◀ $10^6$ human peripheral blood lymphocytes were surface-stained with (1) APC-Mouse IgG2b isotype control at 0.25 µg, or (2-10) APC-Anti-Human CD4 (APC-65134, clone OKT4) using 2-fold serial dilutions from 0.25 µg to $10^{-4}$ µg. Samples were not fixed.

## Adding Intracellular Targets

Surface markers usually identify cell type while intracellular targets indicate cell behavior. Viability dyes are also intracellular and often included in flow experiments for live/dead gating.

Some surface antigens are damaged by fixative and permeabilization agents such as methanol or paraformaldehyde. However, cells must undergo fixation and permeabilization to detect intracellular markers.

### Preparing your sample for dual surface and intracellular staining

| | |
|---|---|
| Surface marker is **not** sensitive to fixation | Fix cells and stain with both antibodies at once (ensuring no spectral overlap if directly conjugated, and different species if unconjugated). |
| Surface marker is damaged by fixation | First live-stain cells with surface markers antibodies, then fix and perm and stain with intracellular marker.<br>*Careful: dyes such as APC or PE are damaged by methanol permeabilization. Ensure the extracellular antibody is conjugated to a fluorophore that can withstand methanol or choose a different permeabilization method.* |

# PROTOCOLS

## Protocol for Studying Extracellular and Intracellular Proteins

### Materials and equipment:

- Cell dissociation agent (e.g., trypsin) – for adherent cell lines
- Cell culture medium containing FBS or trypsin inhibitors
- PBS or HBSS
- Benchtop centrifuge
- Fixative (e.g., 3% (w/v) Paraformaldehyde (PFA))
- Permeabilization solution (e.g., 0.1% (w/v) saponin or 0.1% (w/v) Triton X-100)
- Primary and secondary antibodies
- Flow cytometer

### Experimental procedure:

1. Adherent cells:
   a. Remove medium and wash cell monolayer with PBS.
   b. Add dissociating agent (e.g., trypsin) and incubate at 37°C for 5 min or until cells detach from the cell culture dish.
   c. Add serum-containing medium or trypsin inhibitors to inactivate dissociating agent.
   d. Transfer cell suspension into a microcentrifuge tube.
2. Pellet cells by centrifugation (300 G's, 5 min at room temperature). Remove medium and resuspend in PBS or HBSS.
3. Count cells and take 1 million ($10^6$) cells per condition:
   a. Unstained sample – for establishing autofluorescence levels
   b. Sample stained with antibody A
   c. Sample stained with antibody B
   d. Sample stained with both antibodies

   ***Note 1:*** *Consider using an isotype control to compensate for non-specific binding with a species-matched antibody.*

   ***Note 2:*** *Fluorophores conjugated to antibodies A and B must have different spectra to distinguish their staining. Please refer to page 8 for tips on multicolor panel design.*
4. *Optional:* fix cells with a fixative agent (e.g., incubated with 3% (w/v) paraformaldehyde (PFA) solution for 20 min at room temperature). Centrifuge as in step 2 and wash cells with PBS.

   ***Note:*** *This step is recommended for staining intracellular targets.*
5. For intracellular targets, permeabilize cells.

a. For cytosolic protein staining, use milder detergents – e.g., incubated with 0.1% (w/v) saponin solution for 5-10 min.

b. For nuclear protein staining and proteins inside cellular organelles, use harsher detergents – e.g., incubated with 0.1% (w/v) Triton X-100 solution for 5 min. Centrifuge as in step 2 and wash cells with PBS or HBSS.

6. Add antibodies A and/or B according to requirements in step 2 and incubate for 30 min at room temperature (include milder detergent if it was used for permeabilization).

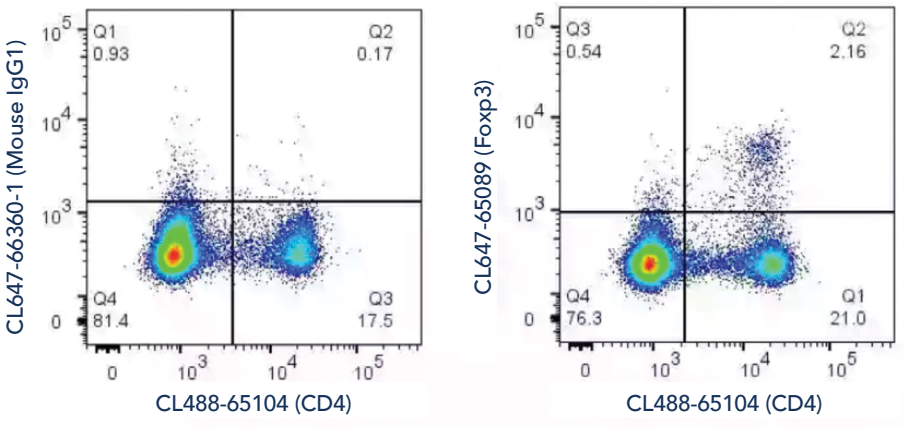
7. Pellet cells by centrifugation (300 G's, 5 min at room temperature). Wash cells twice with PBS or HBSS.

8. If using unlabeled primary antibodies, incubate with secondary antibodies for 20 min at room temperature. Wash cells as in noted in step 7.

   ***Note:*** *When using secondary antibodies, primary antibodies must be raised in different species to distinguish their staining.*

9. Analyze cells on a flow cytometer. If the cell population or marker of interest is rare, increase the number of events acquired (minimum 100,000 events) to ensure detecting the cell population after gating and achieving statistical significance.

FIGURE 5: **An example of a flow experiment where cells were stained for a surface marker (CD4), a nuclear target (Foxp3), and an isotype control.**




▲ 1X10$^6$ mouse splenocytes were surface stained with CoraLite® Plus 488-conjugated Anti-Mouse CD4 (GK1.5) (CL488-65104) and then fixed and permeabilized with Transcription Factor Staining Buffer Kit (PF00011). Cells were then stained with CoraLite® Plus 647-conjugated Mouse IgG1 Isotype Control (CL647-66360-1) or 5 µl CoraLite® Plus 647-conjugated Anti-Mouse Foxp3 (3G3) (CL647-65089).

## Protocol for Studying Cell Viability and Apoptosis

### Materials and equipment:

- Cell dissociation agent (e.g., trypsin) – for adherent cell lines
- Cell culture medium containing FBS or trypsin inhibitors
- PBS or HBSS supplemented with calcium chloride
- Benchtop centrifuge
- Fluorescent annexin V probe
- Viability dye (e.g., propidium iodide)
- Flow cytometer

### Experimental procedure:

Cell viability experiments are commonly performed using flow cytometry. They are used to study the effect of different agents, e.g., cytotoxic drugs, on the cell viability of various cell lines. They can be used to establish the minimum concentration of antibiotics required to cause cytotoxic effects, which is useful for stable cell generation using antibiotic markers. They are often used when studying apoptosis and other forms of cell death. Viability dyes are recommended in flow cytometry experiments and are performed on unfixed cells to exclude dead cells from analysis.

Viability dyes can penetrate dead cells and bind to their DNA, while live cells and early apoptotic cells are impermeable to viability dyes due to their intact cell membrane **(Figure 6)**. Annexin V binds to phosphatidylserine found on the outer layer of early apoptotic cells. Annexin V also binds to a pool of phosphatidylserine present on the outer and inner layers of dead cells due to their compromised cell membrane integrity.

1. Adherent cells:
   a. Remove medium and wash cell monolayer with PBS.
   b. Add dissociating agent (e.g., trypsin) and incubate at 37°C for 5 min or until cells detach from the cell culture dish.
   c. Add serum-containing medium or trypsin inhibitors to inactivate dissociating agent.
   d. Transfer cell suspension into a microcentrifuge tube.
2. Pellet cells by centrifugation (300 G's, 5 min at room temperature). Remove medium and resuspend in PBS or HBSS containing calcium ions.

   ***Note:*** *Annexin V requires calcium for interaction with phospholipids. Supplement buffers with calcium salts and avoid chelating agents such as EDTA or EGTA.*
3. Count cells and take 1 million ($10^6$) cells per condition:
   a. Unstained sample – for establishing autofluorescence levels.
   b. Sample stained with a viability dye.
   c. Sample stained with an annexin V probe.

**d.** Sample stained with a viability dye and an annexin V probe.

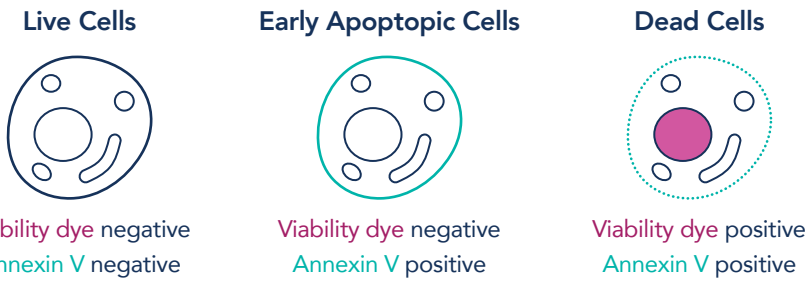
***Note 1:*** *Annexin V and the viability dye must have different excitation spectra to distinguish their staining. Please refer to page 8 for tips on multicolor panel design.*

***Note 2:*** *Consider including a positive control sample where cells are treated with an cell death-inducing agent to validate the performance of used probes.*

4. Add fluorescently labeled annexin V and incubate for 15 min at room temperature. Annexin V preferentially binds to phosphatidylserine, which is found on the inner layer of the plasma membrane in living cells. In early apoptotic cells, phosphatidylserine is translocated to the outer layer, making it accessible for annexin V binding.
5. Pellet cells by centrifugation (300 G's, 5 min at room temperature). Remove medium and resuspend in PBS or HBSS.
6. Add a viability dye (e.g., propidium iodide) and incubate for 5-20 min at room temperature. Propidium iodide (PI) is a DNA intercalating agent – it is used as a fluorescent dye that binds to DNA. Dead cells lose cell membrane integrity, which allows the dye to reach the nucleus and bind to nucleic acids.
7. Analyze cells on a flow cytometer.

   ***Note:*** *Do not wash cells before analysis to avoid washing out the viability dye accumulated in dead cells.*

FIGURE 6: **Analysis of cell viability and apoptosis by flow cytometry**



# DATA ANALYSIS AND INTERPRETATION

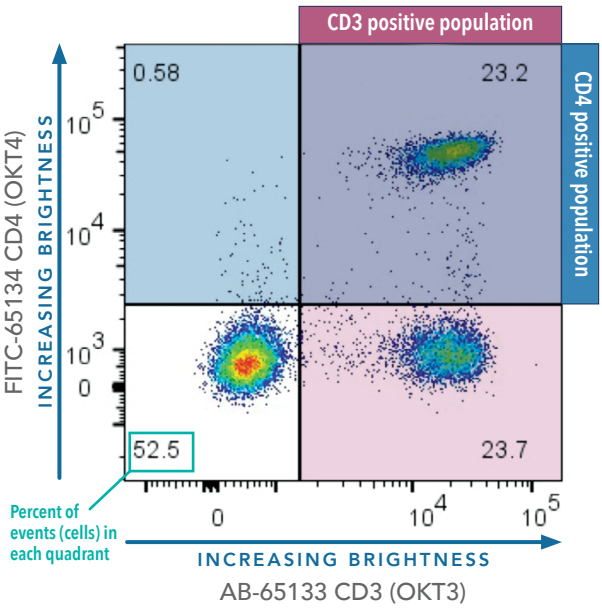
## Reading Flow Data

Flow cytometry data is usually presented in two main ways.

### Dot or Density Plots

Dot or density plots organize individual events according to two parameters (x and y axis) and is used for visualization and segregation of sub-populations. The axes may represent fluorophore intensity, as in this example, or light scatter, as in an FSC/SSC plot. Individual events may be shown and density represented by color, or the data can be presented as a contour plot. Quadrants may be drawn to separate populations that are positive and negative on each axis.

FIGURE 7: **Anatomy of a dot plot**

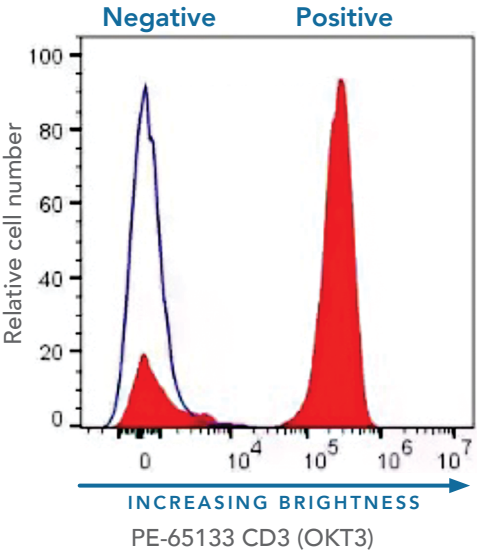



▲ 1x10⁶ human PBMCs were surface stained with 5 µl Atlantic Blue Anti-Human CD3 (AB-65133, Clone: OKT3) and 5µl FITC Plus Anti-Human CD4 (FITC-65134, Clone: OKT4). Cells were not fixed. Lymphocytes were gated.

## Histograms

Histograms visualize the distribution of events along a single parameter of one or more populations of cells where the area under the curve represents the number of cells. Histograms can be used for adjusting the voltage on your cytometer, single-stain gating, and easy comparison of target and control cell populations.

FIGURE 8: **Anatomy of a Histogram**




▶ Histogram showing cell populations negative and positive for CD3. 1X10$^6$ human PBMCs were surface stained with 5µl PE Anti-Human CD3 (PE-65133, Clone: OKT3, red filled) or Mouse IgG2a Isotype Control (blue line). Cells were not fixed. Lymphocytes were gated.

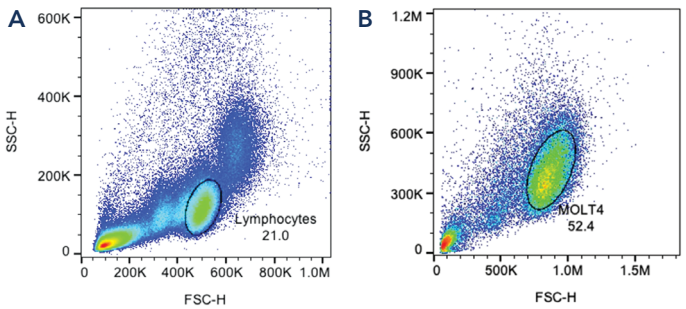
## Flow Cytometry Gating

After samples are run, gating is used to exclude unwanted cells from analysis and home in on the cell population of interest. When gating, cells inside the gate are included in further analysis, while cells outside the gate are excluded.

### Gating FSC/SSC

Gating begins with forward scatter (FSC) and side scatter (SSC). FSC increases with the size of cells, while SSC increases with the granularity, or complexity, of the cells. If cell lines are being used, the FSC/SSC should show one main population of cells; this population should make up the initial gate. This initial gate excludes non-cell events, such as cell debris or air bubbles. For primary cell analysis, lymphocytes, monocytes, granulocytes, or a combination of these is the initial gate on the FSC/SSC plot.

FIGURE 9: **FSC/SSC plots**




◀ **A.** FSC/SSC plot of PBMCs with gating for lymphocytes based on size and granularity. 21.0% of the events collected were included in the lymphocyte gate. **B.** FSC/SSC plot of a cancer cell line (MOLT4). 52.4% of events were included in this initial gate.

## Gating for single cells

Clumps of multiple cells can cause false positive results and gating them out is another crucial preliminary step. This is done by looking at FSC-A vs FSC-H (or FSC-W) or SSC-A vs SSC-H (or SSC-W); the latter is more sensitive for gating out doublets. When multiple cells are collected in a single event, the height (H) of the peak tends to be greater than the overall area (A) of the peak for that event. Width (W) can also be used for this analysis in place of height. Width and height are proportional to area for single cells, and single cells will fall on the diagonal on a plot of Area vs Height or Area vs Width, while multiples will fall outside of the diagonal line. This step can also be done before broad gating of cells of interest.

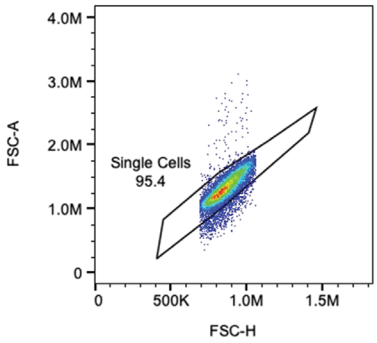



FIGURE 10: **Gating along the diagonal for single cells in an FSC-A vs FSC-H plot**

## Gating for live cells

Once single cells of interest have been isolated, dead cells can be gated out. Dead cells can be sticky and pick up dye non-specifically, causing inaccurate results and false conclusions. Typically, viability dyes stain dead or dying cells, so the live cells will be in the negatively stained population in the viability dye channel.

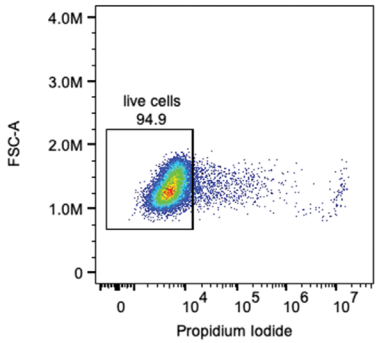



FIGURE 11: **Gating for live cells (negative for propidium iodide)**

## Finding your cells of interest

After these preliminary gating steps, only live single cells of interest should be left. Gating after this point is dependent on the target cell population and panel design. Begin with broad markers (i.e., CD45 for lymphocytes) and increasingly narrow your cells down to the desired population (i.e. CD3+/CD19-/CD8+ for CD8 cytotoxic T lymphocytes). This specific cell population can then be analyzed for its expression of various specialized markers, such as interferon-gamma (IFNγ) in this example.

The presentation of flow data can be changed in multiple ways. Axes can be adjusted to better separate positive and negative populations. Additionally, large or brightly stained cells may be beyond the axis maximum depending on the settings, and adjusting the minimum and maximum of the axis can bring these cells into view. You can also toggle between dot and contour-style plots through the "smoothing" tool, and these plots can be colored according to the density of events. These tools can help to delineate cell populations for more accurate gating.

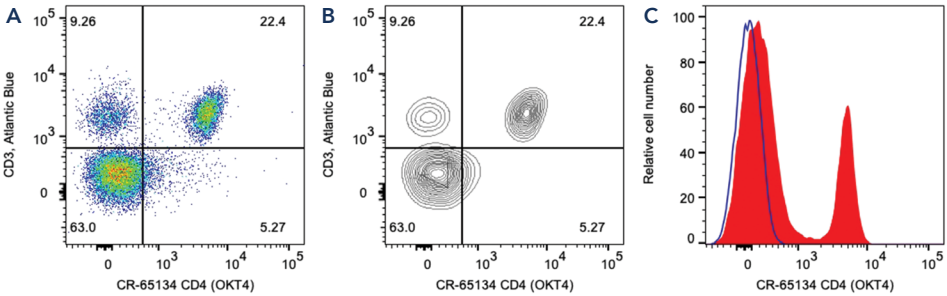



FIGURE 12: **Three ways to present flow data.** A. Lymphocytes stained with CD3 (y-axis) and CD4 (x-axis), viewed as a dot plot and colored to show density of events. B. The same data displayed as a contour style plot. C. A histogram showing the distribution of CD4 expression in the same lymphocytes.

# TROUBLESHOOTING

| Problem | Possible causes and solutions |
|---|---|
| No or weak signal | • **Incorrect antibody storage:** Ensure that all antibodies are stored according to the manufacturer's instructions (e.g., PE should not be frozen).<br>• **Wrong dilution:** Perform a titration to check the required antibody concentration.<br>• **Incorrect laser:** Confirm correct laser is in use and check laser alignment.<br>• **Intracellular target is inaccessible or the fluorochrome is too large:** Ensure adequate permeabilization and change the fluorochrome to a lower molecular weight fluorochrome.<br>• **Target protein has low or no expression:** Use a positive control to check staining procedure and use a brighter fluorophore for the target.<br>• **Soluble target protein has been excreted:** Implement a Golgi-block step. |
| Non-specific staining | • **Autofluorescence:** Include an unstained sample control.<br>• **Fc mediated binding:** Include an Fc block control for B cells, dendritic cells, monocytes, and macrophages.<br>• **Insufficient washing:** Add more wash steps.<br>• **Antibody concentration too low:** Test-titrate antibodies, non-specific binding favored at lower concentrations. |
| High fluorescence intensity | • **Antibody concentration too high:** Perform a titration to check the required antibody concentration.<br>• **Insufficient blocking:** Increase blocking reagent and include blocking reagent with antibody incubation buffer.<br>• **Antibody trapped for intracellular targets:** Increase number of wash steps and add low concentrations of a permeabilization reagent in wash buffers. |
| Non-specific staining | • **Lysed or broken cells:** Use fresh samples, avoid excessing vortexing and high centrifuge speeds.<br>• **Activation:** Some activation methods affect scatter profiles. |

## Further resources and support:

If you have any questions regarding these protocols or any of our products for flow cytometry, our expert technical support team are always happy to help. Contact proteintech@ptglab.com or visit our website www.ptglab.com for more contact details. Additional information is also available on our website where you can find expert interviews, recorded webinars, and educational blogs.

For further detailed information on flow cytometry principles and protocol, please see this highly comprehensive review by Cossarizza et al. 2019, Eur J Immunol (PMID: 31633216). Additionally, for more specialized protocols we recommend: Chow et al. 2003, Cytometry Part A (PMID: 16080188) for a whole blood fixation and permeabilization protocol, and Krutzik and Nolan, 2003, Cytometry Part A (PMID: 14505311) for post-translational modifications such as phosphorylation.

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