



Flow Cytometry Basics Guide



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1 Principles of the Flow Cytometer

Fluidics System

One of the fundamentals of flow cytometry is the ability to measure the properties of individual particles. When a sample enters a flow cytometer, the particles are randomly distributed in the 3-D space of the sample line, the diameter of which is quite a bit larger than the diameter of most cells. The sample must therefore be ordered into a stream of single particles that can be interrogated individually by the machine's detection system. This process is managed by the fluidics system.

The fluidics system consists of a central core through which the sample fluid is injected, enclosed by an outer sheath fluid. These are pushed through the system at slightly different pressures. The sheath fluid is under high pressure and, therefore, moves faster. As the sheath fluid moves, it creates a drag effect that causes the sample in the central core to narrow (Figures 1). This creates a single file of particles and is called hydrodynamic focusing. Under optimal conditions (laminar flow) the fluid in the central chamber will not mix with the sheath fluid.

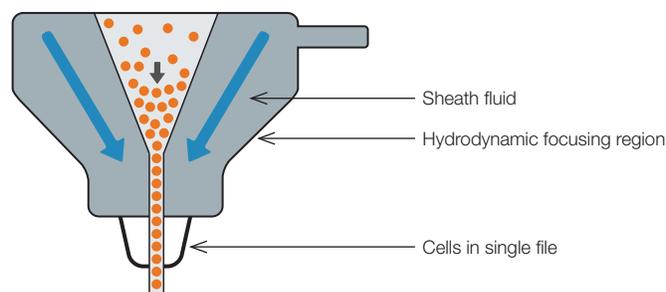


Fig. 1. Hydrodynamic focusing produces a single stream of particles.

Without hydrodynamic focusing, the nozzle of the instrument (typically 70 or 130 μm) would become blocked. The cells flow in single file through the illumination source, allowing them to be analyzed one cell at a time.

Optics and Detection

After hydrodynamic focusing, each particle passes through one or more beams of light. Light scattering or fluorescence (FL) emission (if the particle is labeled with a fluorophore) provides information about the particle's properties. Lasers are the most commonly used light sources in modern flow cytometry.

Lasers produce a single wavelength of light (a laser line) at one or more discrete frequencies (coherent light). They are available at different wavelengths ranging from ultraviolet to far red and have a variable range of power levels (photon output/time).

Light that is scattered in the forward direction, typically up to 20° offset from the laser beam's axis, is collected by a photomultiplier tube (PMT) or photodiode known as the forward scatter (FSC) channel. The FSC equates roughly to the particle's size. Typically, larger cells refract more light than smaller cells.

Light measured at an approximately 90° angle to the excitation line is called side scatter (SSC). The SSC channel provides information about the relative complexity (for example, granularity and internal structures) of a cell or particle. Both FSC and SSC are unique for every particle, and a combination of the two may be used to roughly differentiate cell types in a heterogeneous sample such as blood. However, this depends on the sample type and the quality of sample preparation, so fluorescent labeling is always preferred.

Fluorescence measurements taken at different wavelengths can provide quantitative and qualitative data about fluorophore-labeled cell surface receptors or intracellular molecules such as DNA and cytokines. Flow cytometers use separate channels and detectors to detect light emitted. The number of detectors will vary according to the instrument and its manufacturer. Detectors are either silicon photodiodes or photomultiplier tubes. Historically, silicon photodiodes were used to measure forward scatter for strong signals. More commonly now, PMTs are used even in the FSC channel. PMTs are more sensitive detectors and are ideal for scatter and fluorescence readings.

The specificity of detection is controlled by optical filters, which block certain wavelengths while transmitting (passing) others. There are three major filter types. Long pass filters allow light through above a cutoff wavelength, short pass filters permit light below a cutoff wavelength, and band pass filters transmit light within a specified narrow range of wavelengths (termed a band width). All these filters block light by absorption (Figure 2).

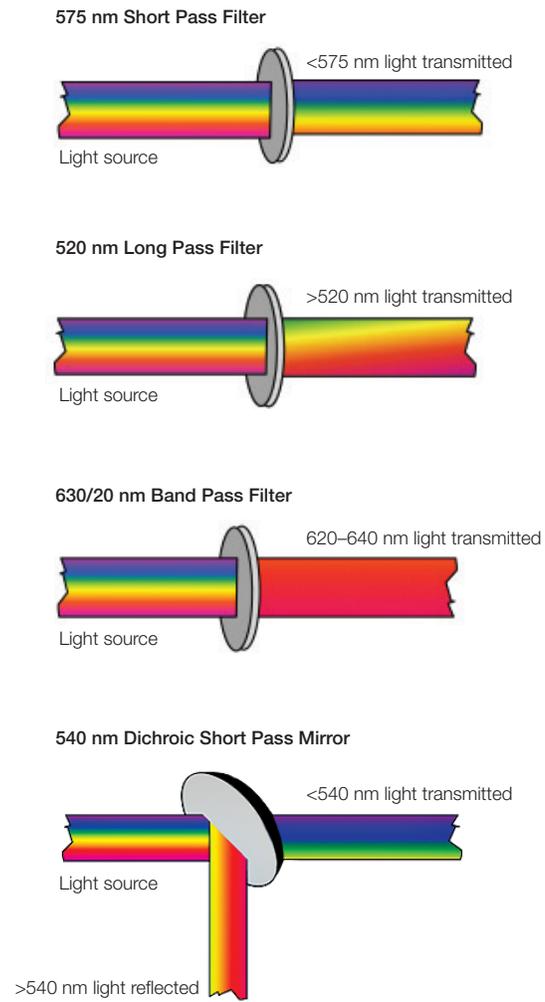


Fig. 2. Different types of optical filters.

A dichroic filter/mirror has a filter placed at an angle to the oncoming light. This type of filter performs two functions, first, to pass specified wavelengths in the forward direction and, second, to deflect blocked light at a 90° angle. To detect multiple signals simultaneously, the precise choice and order of optical filters is an important consideration (Figure 3).

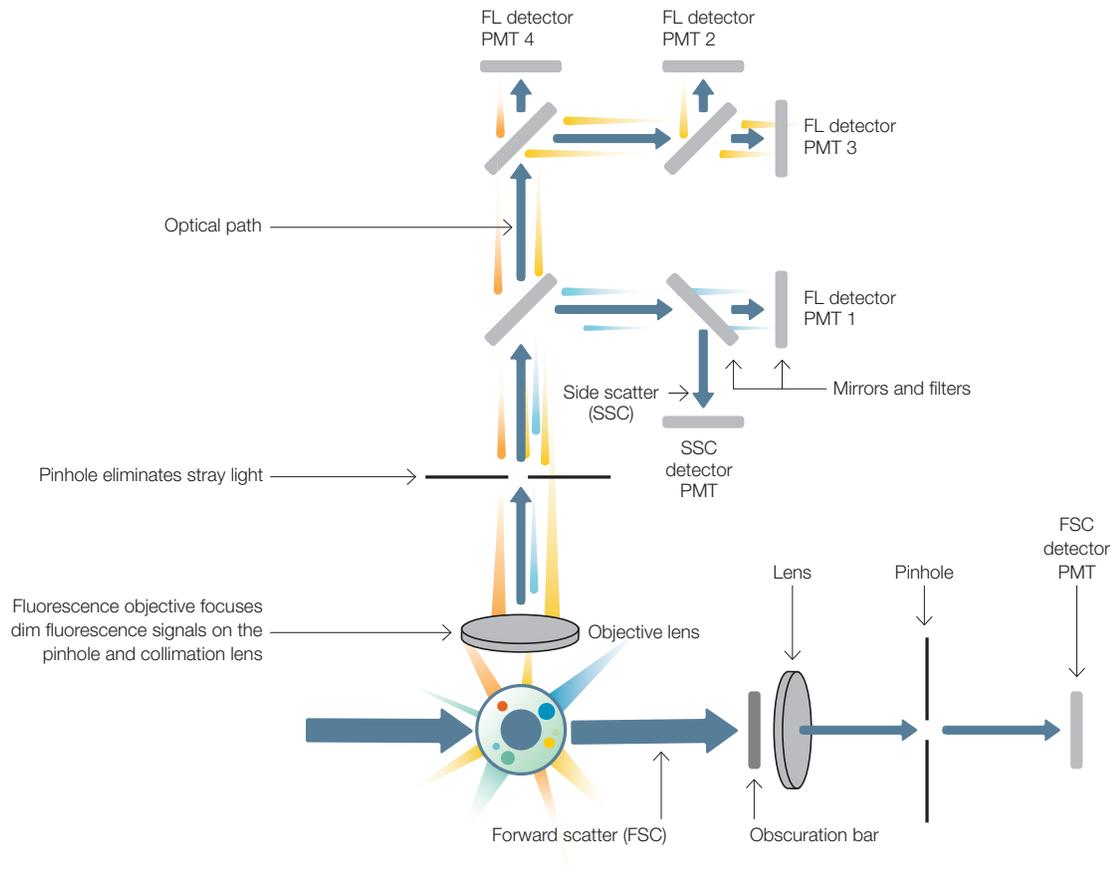


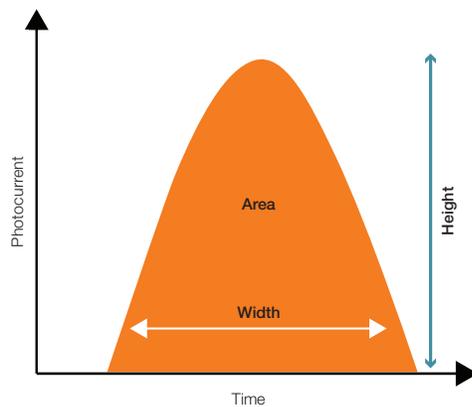
Fig. 3. Schematic overview of a typical flow cytometer setup. FL, fluorescence; PMT, photomultiplier tube; SSC, side scatter; FSC, forward scatter; blue arrow, light path.

Signal and Pulse Processing

Any time a relevant particle passes through the interrogation point and generates a signal a pulse is generated in every PMT detector. These pulses reflect the passage of the cell through the laser beam and the signal generated at each point in the cell's path. These pulses can be mapped by plotting signal as a function of time.

As the particle enters the laser beam spot, it will generate scattered light and fluorescence signals, which will ultimately manifest in a stream of electrons (current) from the anode of the PMT. The magnitude of the current is proportional to the number of photons that hit the photocathode and thus is also proportional to the intensity of the scatter or fluorescence signal generated by the particle. As the particle enters the laser beam spot, the output of the PMT will begin to rise, reaching peak output when the particle is located in the center of the laser beam (Figure 4).

At this point, the particle is fully illuminated (the laser beam's photons are at highest density in the center of the laser beam) and will produce a maximum amount of optical signal. As the particle flows out of the laser beam, the current output of the PMT will drop back to baseline. This generation of a pulse is termed an "event."



Height: The maximum amount of current output by the PMT.

Area: The integral of the pulse.

Width: The time interval during which the pulse occurs.

Signal intensity can be measured by either height or area.

The width parameter measures the **time that the cell spends in the laser.**

Fig. 4. Quantifying the pulse by measuring its height, area, and width.

However, not all pulses produced by particles will be considered events. This determination is made based on the trigger parameter and threshold level. PMTs are extremely sensitive and detect signal from a variety of sources that are irrelevant to experimental data — stray light, dust, very small particles, and debris. The number of these pulses in the system is usually orders of magnitude higher than the number of pulses that are generated by experimental particles, so including these in the dataset would substantially drown out relevant data points. Therefore, it is desirable and necessary to “threshold out” this nonessential data. This is done by designating a parameter as the trigger, usually forward scatter, and setting a level in that parameter as the threshold. Any pulse that fails to exceed the threshold level is ignored in all detectors (Figure 5A); any pulse that surpasses the threshold level is fully processed by the electronics (Figure 5B).

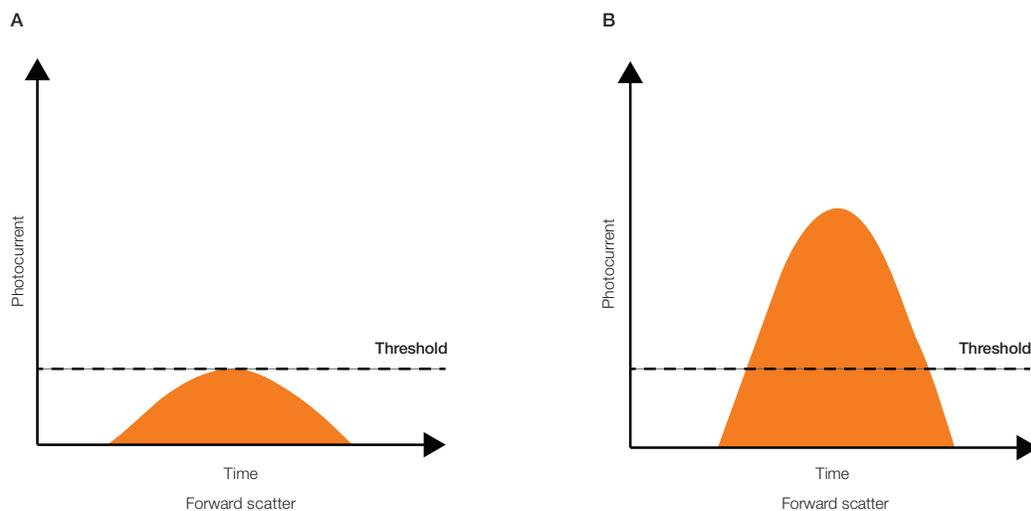


Fig. 5. Determining whether a pulse is ignored (A) or fully processed (B).

As the pulses are generated, their quantification is necessary for fluorescence signals to be displayed on plots, analyzed, and interpreted. This is the job of the signal processing electronics. The majority of flow cytometers and cell sorters are now digital systems. The analog current from the PMT is first digitized or broken down into very small slices by the analog to digital converter (ADC). This process is called “sampling.” A sample of a pulse captures the signal at an instant in time and stores it as a digital value. Together these samples represent the entire pulse and optical signal from the particle.

The electronics quantify the entire pulse by calculating its height, area, and width. The height and area, or maximum and integral, respectively, are used to measure signal intensity because their magnitudes are proportional to the number of photons that interacted with the PMT. The width, on the other hand, is proportional to the time that the particle spent in the laser and can be used to distinguish doublets (that is, two particles that pass through the laser so closely that the system assigned both of them to a single pulse and event) from singlets.

Log amplification is normally used for fluorescence studies because it expands weak signals and compresses strong signals, resulting in a distribution that is easy to display on a histogram. Linear scaling is required when very small differences in fluorescence signal must be assessed, for example in DNA analysis.

The measurement from each detector is referred to as a parameter. Each parameter can be displayed in height, area, and width values on the histograms and dot plots in flow cytometry software. These are used to measure fluorescence intensity, compare populations, and designate sorting decisions.

Electrostatic Cell Sorting

A cell sorter provides the ability to separate cells identified by flow cytometry. Cell sorters first analyze the particles but also have hardware that can generate droplets and a means of deflecting or directing wanted particles into a collection tube. Droplets can be formed by using high-frequency (cycles/second, Hz) vibration of the nozzle at an optimal amplitude (in volts) over a period of time. This is typically created by a piezoelectric crystal.

There are two types of electrostatic sorters, which differ by where the particles are interrogated by the laser. Sense-in-air sorters illuminate particles as they exit the nozzle and enter the stream. In cuvette sorters, particles are illuminated in a quartz cuvette before they enter the stream. After the particles are illuminated at what is called the interrogation point, they continue down the stream. Data collected from the particles as they pass through the lasers at the interrogation point is sent to a computer, where the decision is made whether a given cell meets the criteria the user has defined for a desired particle. As the particle continues to travel down the stream, the stream eventually breaks into droplets, and the particle of interest is captured in a drop. One of the most critical parameters of sorting is to measure the distance between the point of interrogation and the exact point where the droplet breaks off. This distance is called the drop delay. When the cell gets to the last connected drop, the entire stream is charged at the nozzle. As the cell of interest-containing drop breaks off, the drop becomes charged. The droplet then passes through an electrical field, and is deflected into a tube or plate. Uncharged particles pass into the waste (Figure 6).

To prevent the break-off point happening at random distances from the nozzle and to maintain consistent droplet sizes, the nozzle is vibrated at high frequency. The droplets eventually pass through a strong electrostatic field and are deflected left or right based on their charge (Figure 6). Uncharged droplets pass into the waste.

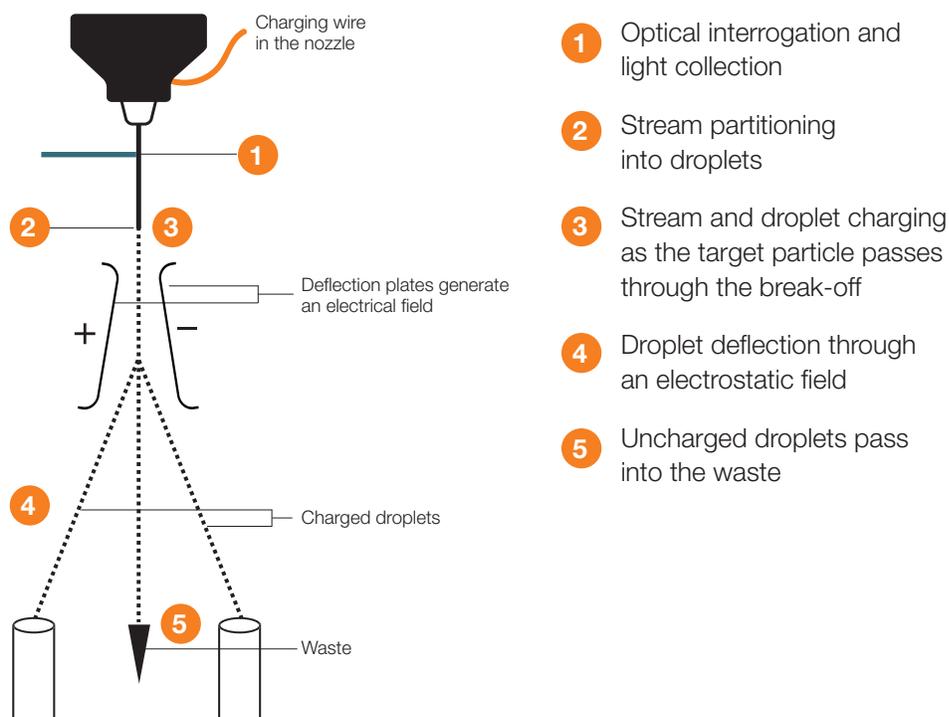


Fig. 6. Electrostatic flow sorting.

The speed of flow sorting depends on several factors, including particle size and the rate of droplet formation. A typical nozzle is 70–130 μm in diameter and can produce 10,000–100,000 droplets per second. The stability of the break-off dictates the accuracy of the sorting.

2 Principles of Fluorescence

Fluorophores and Light

A fluorophore is a fluorochrome conjugated to a given macromolecule. Fluorophores are fluorescent markers used to detect the expression of proteins and nucleic acids, which functionally accept light energy (for example, from a laser) at a given wavelength and re-emit it at a longer wavelength. These two processes are called excitation and emission. Emission follows excitation extremely rapidly, commonly in nanoseconds, and is known as fluorescence. Before considering the different types of fluorophores available for flow cytometry, it is necessary to understand the principles of light absorbance and emission.

Light is a form of electromagnetic energy that travels in waves. These waves have both frequency and length, the latter of which determines the color of the light. The light that can be visualized by the human eye represents a narrow wavelength band (380–700 nm) between ultraviolet (UV) and infrared (IR) radiation (Figure 7). Sunlight, for example, contains UV and IR light that, although invisible to the eye, can still be felt as warmth on the skin and measured scientifically using photodetectors. The visible spectrum can further be subdivided according to color, often remembered by the mnemonic ROY G BV, standing for red, orange, yellow, green, blue, and violet. Red light is at the longer wavelength end (lower energy) and violet light is at the shorter wavelength end (higher energy).

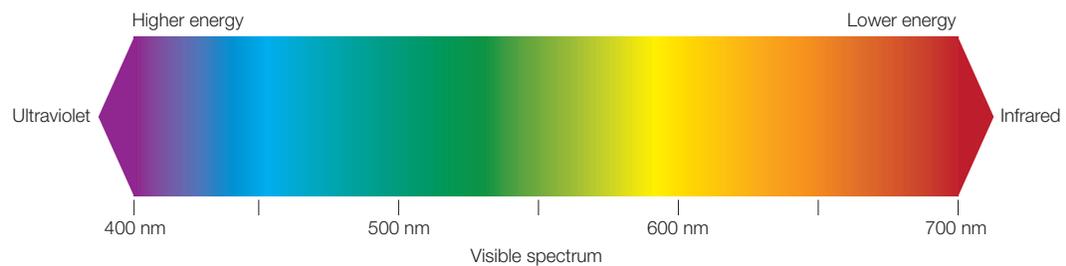


Fig. 7. The electromagnetic spectrum.

Fluorescence

When a fluorophore absorbs light, its electrons become excited and move from a resting state (S_0 , Figure 8A) to a maximal energy level called the excited electronic singlet state (S_2) (1). The amount of energy required for this transition will differ for each fluorophore. The duration of the excited state depends on the fluorophore and typically lasts for 1–10 nanoseconds. The fluorophore then undergoes conformational change, the electrons fall to a lower, more stable energy level called the electronic singlet state (S_1), and some of the absorbed energy is released as heat (2). The electrons subsequently fall to the resting state (S_0) releasing the remaining energy (E_{Emission}) as fluorescence (3). The difference between wavelengths of the emission and excitation maxima is called the Stokes shift (Figure 8B). This cycle can repeat several thousand times for a single fluorophore, which allows recycling of fluorophores and thus amplification of the signal.

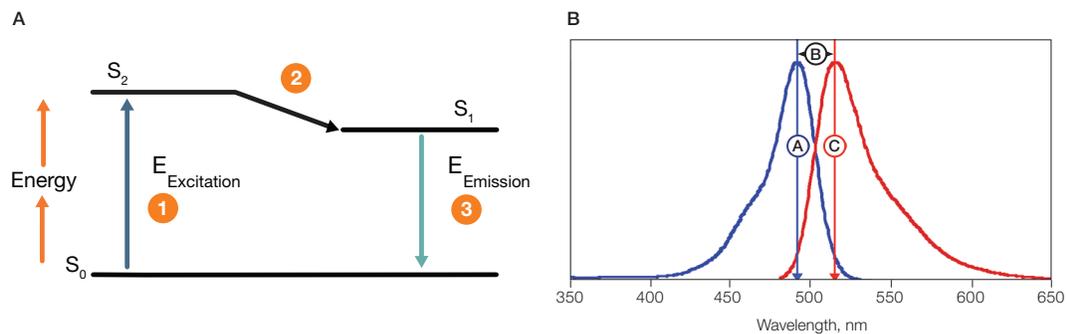


Fig. 8. Stokes shift. **A**, upon excitation, 1, electrons in a fluorophore move from a resting state, S_0 , to the excited electronic single state, S_2 . Some energy is released as heat, 2. The remaining energy is released as fluorescence, 3, as the electrons return to their ground state, S_0 . **B**, the difference between the excitation maxima, (A), and the emissions maxima, (C), of a fluorophore is called its Stokes shift, (B).

Emitted light typically contains less energy than was originally put into the fluorophore to excite it. Therefore, the emission wavelength of any fluorophore is longer (lower energy) than its excitation wavelength and thus a different color.

The wavelength of excitation is critical to the total photons of light that the fluorophore will absorb. Fluorescein isothiocyanate (FITC), for example, will absorb light from 400 to 530 nm but absorbs most efficiently at its peak or excitation maximum of 490 nm wavelength. It is desirable to excite fluorophores at their excitation maximum because the more photons are absorbed, the more intense the fluorescence emission will be. The wavelengths of greatest absorption and emission are termed maximal absorbance and maximal emission wavelengths.

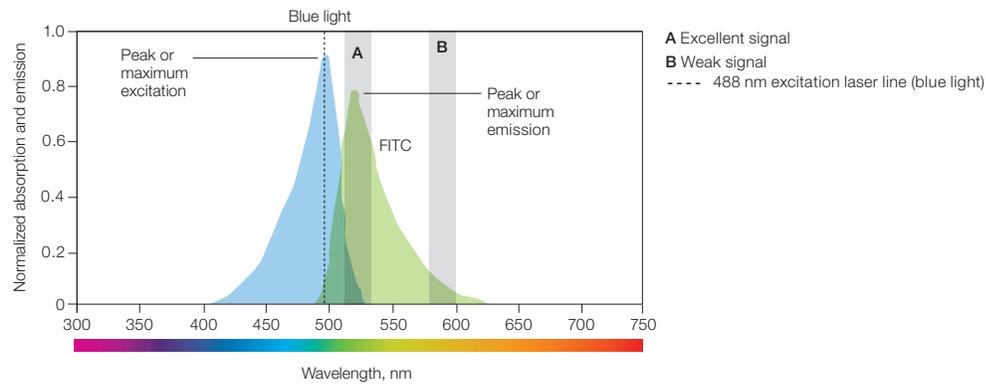


Fig. 9. Spectral profiles. Light absorbance and light emission of fluorescein isothiocyanate (FITC).

Maximal absorbance informs the laser spectral line that is used for excitation. In the case of FITC, its maximum absorption falls within the blue spectrum. Therefore, the blue 488 nm laser, which is close to FITC's absorbance peak of 490 nm, is commonly used to excite this fluorophore. FITC emits fluorescence from 475 to 650 nm, peaking at 525 nm, which falls in the green spectrum. How the flow cytometer is set up determines how the fluorophore is detected. If filters are used to screen out all light other than that measured at the maximum via channel A (Figure 9), FITC will appear green. Fluorescence color usually refers to the color of light a fluorophore emits at its highest stable excited state.

However, if FITC fluorescence is detected only via channel B (Figure 9), it will appear orange and be much weaker in intensity. How the flow cytometer is set up to measure fluorescence will thus ultimately determine the perceived color of a fluorophore. Because the color of the exciting and emitting light is different, they can be separated from one another by using optical filters.

Why Use a Fluorescent Marker?

The purpose of a fluorescent marker, such as a fluorophore-conjugated antibody, is to directly target an epitope of interest and to allow its biological and biochemical properties to be measured. Fluorescent markers are useful in a wide range of applications, including identifying and quantifying distinct populations of cells, cell surface receptors, or intracellular organelles; cell sorting; immunophenotyping; calcium influx experiments; determining nucleic acid content; measuring enzyme activity; and for apoptosis studies. Several fluorophores can be excited by a single laser. And by using filters to segregate the light to the proper detectors and using more than one fluorophore, it is possible to analyze several parameters of the sample at any one time. This forms the basis of multicolor fluorescence studies.

Which Fluorophores Are Useful for Flow Cytometry?

There are many of fluorescent molecules (fluorophores) with a potential application in flow cytometry. The list is ever growing, but it is not the scope of this applications guide to cover them all. Some of the most useful fluorophores for surface or intracellular epitope detection are described in Tables 1 and 2. There is enough variation in the two tables to cover most researchers' needs.

Single and Tandem Dyes

Single dyes such, as FITC, PE, APC, and PerCP have been the standard for many years but are now facing competition from alternatives like Alexa Fluor Dyes, which offer the user greater photostability and brighter fluorescence.

Tandem dyes comprise a small fluorophore covalently coupled to another, larger fluorophore. When the first dye is excited and reaches its maximal excited electronic singlet state, its energy is transferred to the second dye (an acceptor molecule). This activates the second fluorophore, which then produces the fluorescence emission. The process is called fluorescence resonance energy transfer (FRET). It is a clever way to achieve a higher Stokes shift and, therefore, increase the number of colors that can be analyzed from a single laser wavelength.

The majority of tandem dyes have been manufactured for the standard 488 nm laser, which is found in most flow cytometers. Tandem dyes are very useful for multicolor fluorescence studies, especially in combination with single dyes. For example, Alexa Fluor 488, phycoerythrin (PE), peridinin chlorophyll protein (PerCP)–Cy5.5, and PE-Texas Red can all be excited at 488 nm, but will produce green, yellow, red, and infrared emissions, respectively, which can be measured using separate detectors.

Fluorescent Proteins

Fluorescent proteins (FPs), such as green fluorescent protein (GFP), have become an integral tool for understanding protein expression in many scientific disciplines. Other fluorescent proteins, such as mCherry and eYFP, have also become widely used for flow cytometry analysis and cell sorting.

Often, FPs are co-expressed with or directly conjugated to the protein of interest. The use of FPs allows the quantitation of intracellular markers without requiring permeabilization of the cell membrane.

Table 1. Fluorophores for flow cytometry.

Fluorophores	Fluorescence color	Maximal absorbance, nm	Maximal emission, nm	Relative brightness
DyLight 405		400	420	3
Alexa Fluor 405		401	421	3
Pacific Blue		410	455	1
DyLight 488		493	518	4
Alexa Fluor 488		495	519	3
FITC		490	525	3
DyLight 550		562	576	4
PE*		496, 546	578	5
Texas Red		596	615	2
APC		650	661	4
Alexa Fluor 647		650	665	4
Cy5		649	670	3
DyLight 650		654	673	4
PerCP		490	675	2
DyLight 680		692	712	4
Alexa Fluor 700	Infrared	702	723	2
DyLight 755	Infrared	752	778	4
DyLight 800	Infrared	777	794	4

* PE is the same as R-phycoerythrin.

APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Table 2. Tandem dyes for flow cytometry.

Fluorophores	Fluorescence color	Maximal absorbance, nm	Maximal emission, nm	Relative brightness
PE–Alexa Fluor 647		496, 546	667	4
PE–Cy5		496, 546	667	5
PE–Cy5.5		496, 546	695	4
PE–Alexa Fluor 700	Infrared	496, 546	723	2
PE–Alexa Fluor 750	Infrared	496, 546	779	4
APC–Alexa Fluor 750	Infrared	650	779	4
PE–Cy7	Infrared	496, 546	785	2
APC–Cy7	Infrared	650	785	2

* PE is the same as R-phycoerythrin.

APC, allophycocyanin; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Fluorescence Compensation

One consideration when performing multicolor fluorescence studies is the possibility of spectral overlap. Because the fluorophores used in flow cytometry emit photons of multiple energies and wavelengths, a mathematical method called compensation was developed to address the measurement of the photons of one fluorophore in multiple detectors. Due to the nature of flow cytometry measurements, a particle's emission is measured not in a single detector, but in all the detectors being used in the experiment (Figure 10). For example, FITC emits photons that are green, yellow, and orange, all of which can be detected on a multidetector instrument with the corresponding detectors. In some experiments FITC may be combined with other dyes that emit yellow and orange photons. In those cases the relative contribution of each fluorophore to the signal in a given detector must be determined (Figure 11).

You can avoid the need for compensation by using dyes that don't have overlapping emission spectra, but this is practically impossible in multicolor flow cytometry.

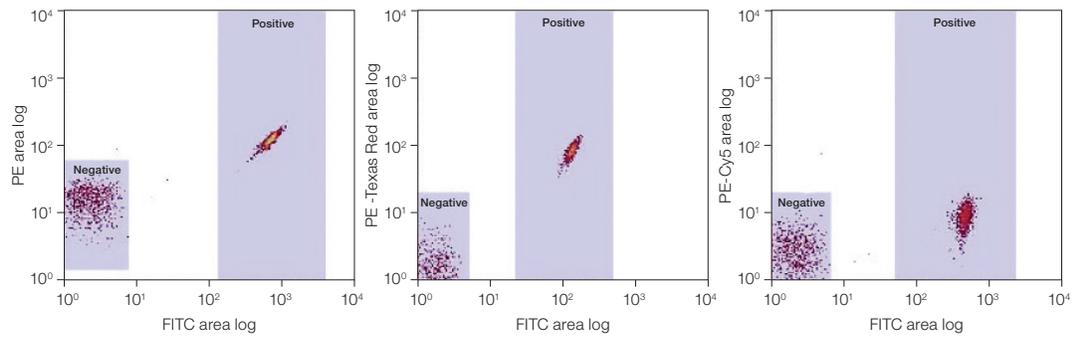


Fig. 10. FITC spillover into other channels. FITC single-stained beads show spillover into PE, PETR, and PE-Cy5 detectors. Gates are set to identify positive and negative populations.

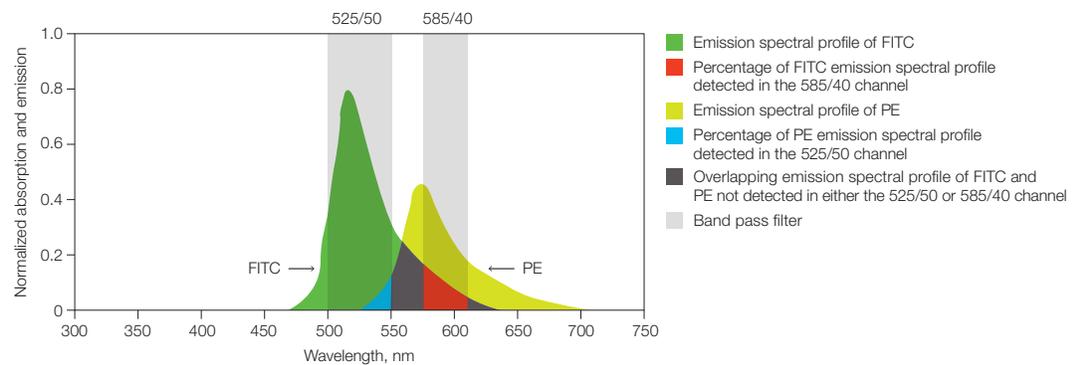


Fig. 11. Fluorescence compensation. Emission spectra of two fluorophores commonly used in flow cytometry, FITC and PE are shown. Also shown is a graphical representation of two commonly used filters, 525/50 and 585/40, to detect these fluorophores. Shown in red is the portion of the FITC spectrum that will be detected in the PE detector (585/40) and that must be subtracted from the PE signal using compensation. This process becomes even more complicated when photons from multiple dyes are detected in each PMT.

Compensation Controls

There are a few basic principles to remember when designing compensation controls for an experiment. Since compensation controls are critical to the determination of what we call positive or negative for a given marker, they are absolutely critical to the success of the instrument. The definition of a compensation control is simple: for each fluorophore used in the experiment, a single-stained cell or bead sample must also be prepared.

The important rules to remember are:

1. The staining of the compensation control must be as bright as or brighter than the sample. Antibody capture beads can be substituted for cells and one fluorophore conjugated antibody for another, as long as the fluorescence measured is brighter for the control. The exceptions to this are tandem dyes, which cannot be substituted.

Note: Although it would seem safe to assume that all tandem dyes created with the same donor and acceptor would have the same emission, this is not the case. Tandem dyes from different vendors or different batches must be treated like separate dyes, and a separate single-stained control should be used for each because the amount of spillover may be different for each of these dyes.

2. The compensation algorithm needs to be performed with a positive population and a negative population (Figure 10). Whether each individual compensation control contains beads, the cells used in the experiment, or even different cells, the control itself must contain particles with the same level of autofluorescence. The entire set of compensation controls may include individual samples of either beads or cells, but the individual samples must have the same carrier particles for the fluorochromes.
3. The compensation control must use the same fluorophore as the sample. For example, both GFP and FITC emit mostly green photons, but have vastly different emission spectra. You thus cannot use one of them for the sample and the other for the compensation control.
4. Enough events must be collected for the software to make a statistically significant determination of spillover. Ideally about 5,000 events for both the positive and negative population is ideal.

Fortunately, compensation is easily accomplished by software when the correct controls are used. The software will calculate spillover values and apply them to the data, and the data will be properly compensated (Figure 12).

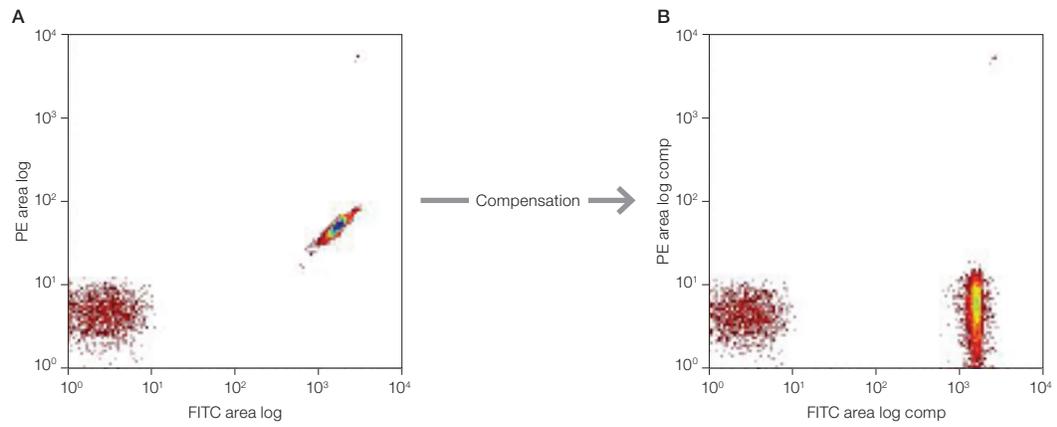


Fig. 12. Fluorescence compensation corrects for spectral overlap. FITC single-stained cells showing fluorophore being detected in PE channel before (A) but not after compensation (B).

3 Data Analysis

Gates and Regions

Flow cytometry data analysis is fundamentally based on the principle of gating. Gates, or regions, are drawn on fluorescence scatter plots and histograms to selectively focus on populations of interest. These gates can then be applied to view and analyze specific populations.

The first step in gating is typically to distinguish the cells based on their light scatter properties. For instance, subcellular debris can be distinguished from single cells by relative size, estimated by forward scatter. Also, dead cells tend to have lower forward scatter and higher side scatter than living cells. Lysed whole blood cell analysis is the most common application of gating, and Figure 13 depicts typical graphs for SSC vs. FSC when using large cell numbers. The different light scatter signals of granulocytes, monocytes, and lymphocytes allow them to be distinguished from each other and from cellular debris.

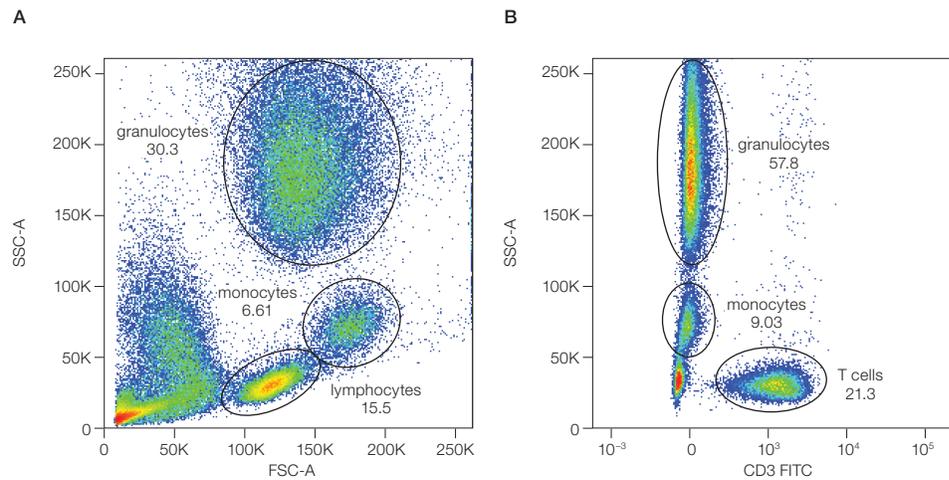


Fig. 13. Analysis of lysed whole blood. **A**, SSC vs. FSC density plot; **B**, SSC vs. CD3 FITC fluorescence density plot. FITC, fluorescein isothiocyanate; FSC, forward scatter; SSC, side scatter.

Data can be analyzed as histograms or in two-parameter dot or density plots. On a density plot, each dot or point represents an individual cell that has passed through the instrument. The plots in Figures 13A and 13B are color intensity plots and are the most common way to represent a density plot. Here, the red/yellow/green/blue hot spots indicate increasing numbers of events resulting from discrete populations of cells. The colors give the graph a three-dimensional feel. With a little experience, discerning the various subtypes of blood cells becomes relatively straightforward.

While not shown in this guide, another less common way to represent a density plot is by a contour diagram. The graph takes on the appearance of a geographical survey map, which in principle closely resembles the density plot. It is a matter of preference, but sometimes discrete populations of cells are easier to visualize on contour diagrams.

In Figure 13B, the same cells are now plotted as SSC on the y-axis vs. CD3 fluorescence on the x-axis. CD3 is a marker that is expressed on T lymphocytes but is absent on other white blood cells. This highlights the usefulness of gating strategies that combine a scatter parameter with a fluorescence parameter.

Single-Parameter or Univariate Histograms

These are histograms that display a single measurement parameter (relative fluorescence or light scatter intensity) on the x-axis and the number of events (cell count) on the y-axis.

The histogram in Figure 14 looks very basic but is useful for evaluating the total number of cells in a sample that possesses the physical properties selected for or which express the marker of interest. Cells with the desired characteristics are known as the positive dataset.

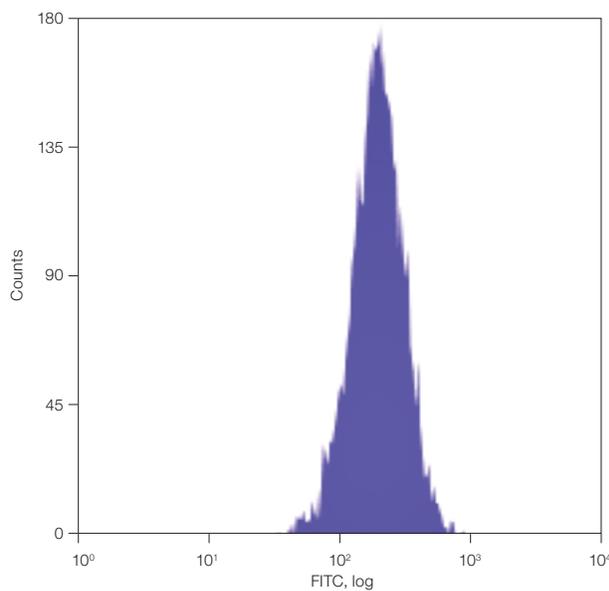


Fig. 14. A single-parameter histogram. FITC, fluorescein isothiocyanate.

Ideally, flow cytometry will produce a single distinct peak that can be interpreted as the positive dataset. However, in many situations, flow analysis is performed on a mixed population of cells, resulting in several peaks on the histogram. In order to identify the positive dataset, flow cytometry should be repeated in the presence of an appropriate control (Figure 15).

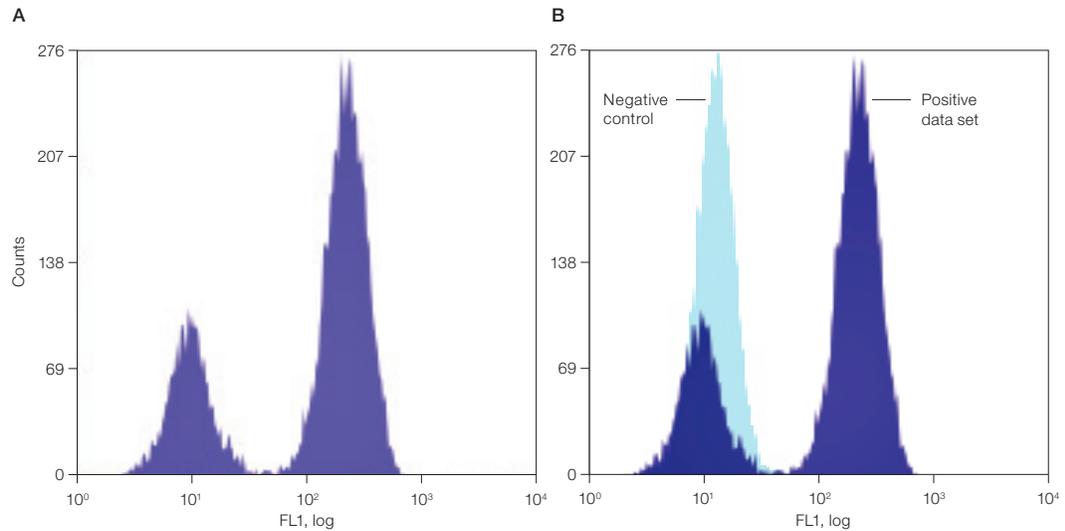
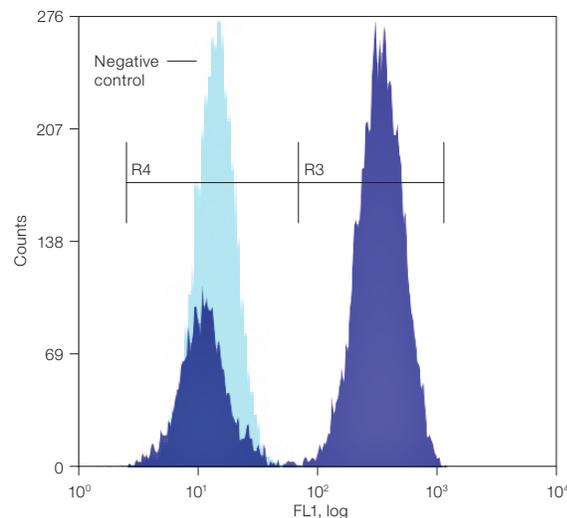


Fig. 15. Which is the positive dataset? **A**, using rat anti-mouse F4/80 conjugated to FITC to stain mouse peritoneal macrophages produces two peaks. **B**, by running an appropriate control (rat IgG2b negative control conjugated to FITC) and overlaying its image on the histogram (blue peak), the positive dataset is identified as the taller purple peak on the right. FL, fluorescence.

Analytical software packages that accompany flow cytometry instruments make measuring the percentage of positive-staining cells in histograms easy. For example, the F4/80 histogram is shown again in Figure 16 with statistics for R3 and R4 (known on this type of graph as bar regions).



Label	Total, %	Plot, %	Median	Standard Deviation
Total	58.87	100.00	202.00	146.59
R3	42.28	71.82	242.00	108.67
R4	16.38	27.82	10.00	5.65

Fig. 16. Statistical analysis. FL, fluorescence.

In Figure 16, 100.00% of the negative control (blue peak) is in R4, and 27.82% of cells stain negative for F4/80 (R4) compared to 71.82% in the positive dataset (R3). Additional statistics about the peaks (median and standard deviation) are also provided automatically here, but this will vary with the software. A similar type of analysis will be generated for two-parameter histograms.

Two-Parameter or Bivariate Histograms

These are graphs that display two measurement parameters, one on the x-axis and one on the y-axis, and the cell count as a density (dot) plot or contour map. The parameters could be SSC, FSC, or fluorescence.

An example is the dual-color fluorescence histogram in Figure 17. Lymphocytes were stained with anti-CD3 in the FITC channel (x-axis) and anti-HLA-DR in the PE channel (y-axis). CD3 and HLA-DR are markers for T and B cells, respectively. See Figures 15 and 16 for additional examples of two-parameter histograms.

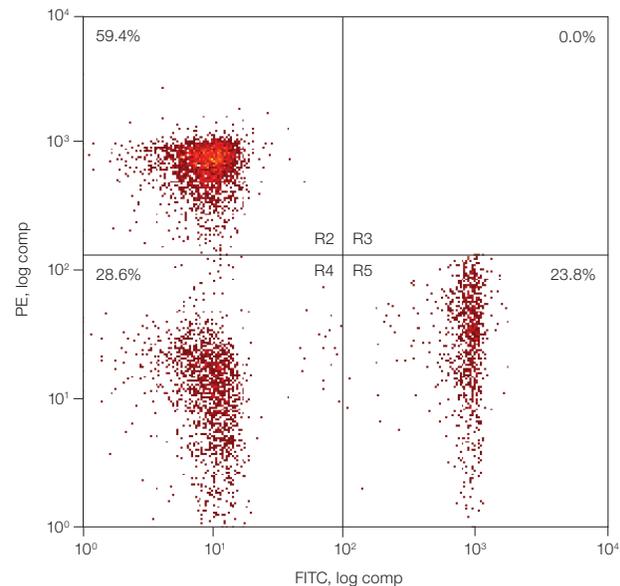


Fig. 17. Two-parameter (dual-color fluorescence) histogram. FITC, fluorescein isothiocyanate; log comp, logarithmic scale with compensation applied; PE, phycoerythrin.

In Figure 17, R2 encompasses the PE-labeled B cells — note their positive shift along the PE axis. R5 contains the FITC-labeled T cells (positively shifted along the FITC axis). The top right quadrant, R3, would show the cells that are stained for both antibody markers, in this case making them activated T cells. In this sample there are no activated T cells. R4 contains cells negative for both FITC and PE (no shift).

Currently, flow cytometry can be performed on samples labeled with ≥ 17 fluorescence markers simultaneously (Perfetto et al. 2004). Therefore, a single experiment can yield a large set of data for analysis using various two-parameter histograms.

Intracellular Antigens

Staining intracellular antigens like cytokines can be difficult because antibody-based probes cannot pass easily through the plasma membrane into the interior of the cell. In order to accomplish this, cells should first be fixed in suspension and then permeabilized before adding the fluorophore. This allows probes to access intracellular structures while leaving the morphological scatter characteristics of the cells intact. Triton is one example of a permeabilization reagent (Figure 18). There are also many commercial kits available today that provide the reagents to carry out these crucial steps, for example, Leucoperm™.

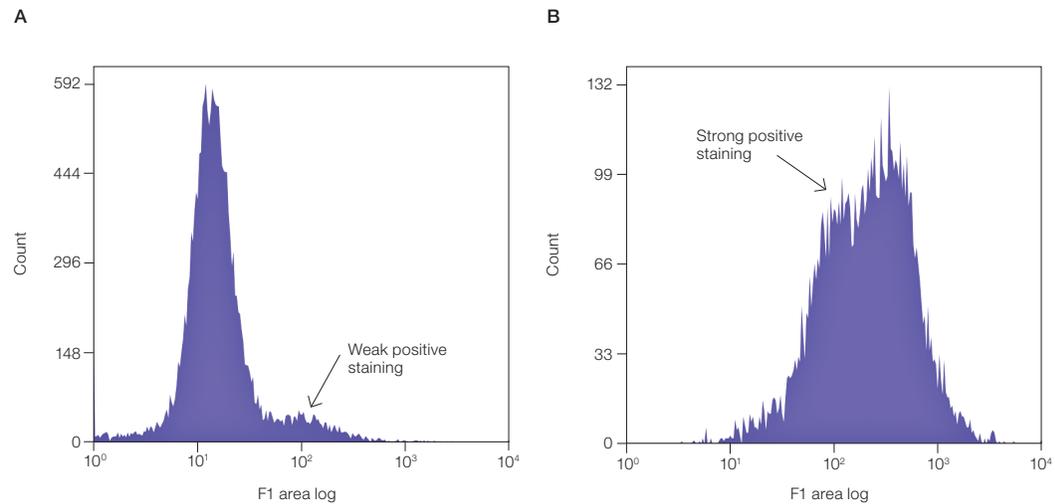


Fig. 18. 0.1% Triton used in conjunction with phalloidin, which recognizes and binds to filamentous actin. **A**, before treatment of cells; **B**, after fixation and permeabilization. Notice how distinctive the positive dataset becomes. FL, fluorescence.

4 Common Protocols

Sample Preparation

Single cells must be suspended at a density of 10^5 – 10^7 cells/ml to keep the narrow bores of the flow cytometer and its tubing from clogging up. The concentration also influences the rate of flow sorting, which typically progresses at 2,000–20,000 cells/second. Higher sort speeds can result in lower yield or recovery.

Phosphate buffered saline (PBS) is a common suspension buffer. The most straightforward samples for flow cytometry include nonadherent cells from culture, waterborne microorganisms, bacteria, and yeast. Even whole blood is easy to use — red cells are usually removed by a simple lysis step. It is then possible to quickly identify lymphocytes, granulocytes, and monocytes by their FSC and SSC characteristics (see Figure 15).

However, researchers may also wish to analyze cells from solid tissues, for example, liver or tumors. In order to produce single cells, the solid material must be disaggregated. This can be done either mechanically or enzymatically. Mechanical disaggregation is suitable for loosely bound structures such as adherent cells from culture, bone marrow, and lymphoid tissue. It involves passing a suspension of chopped tissue through a fine-gauge needle several times followed by grinding and sonication as necessary.

Enzymes are used to disrupt protein-protein interactions and the extracellular matrix that holds cells together. Their action depends on factors including pH, temperature, and cofactors, so care must be taken when choosing an enzyme. For example, pepsin works optimally between pH 1.5 and 2.5, but the acidic conditions would damage cells if left unneutralized for too long, and cell surface antigens of interest might be lost. Chelators like ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) can remove divalent cations responsible for maintaining cell function and integrity, but their presence may inhibit certain enzymes. For example, collagenase requires Ca^{2+} for activity. Optimizing the isolation of an epitope under investigation via disaggregation, either enzymatic or mechanical, is often a trial and error process.

To study intracellular components, for example, cytokines, by flow cytometry, the plasma membrane of the cell must be permeabilized to allow dyes or antibody molecules through while retaining the cell's overall integrity. Low concentrations (up to 0.1%) of nonionic detergents like saponin are suitable. In summary, the method for sample preparation will depend on the starting material and the nature of the epitope. Although it is not possible to describe every method here, some standard protocols are provided in this chapter.

Preparation of Cells for Flow Cytometry

Single cells must be suspended at a density of 10^5 – 10^7 cells/ml to keep the narrow bores of the flow cytometer and its tubing from clogging up. The concentration also influences the rate of flow sorting, which typically progresses at 2,000–20,000 cells/second. Phosphate buffered saline (PBS) is a common suspension buffer.

The most straightforward samples for flow cytometry are nonadherent cells from tissue cell culture. Here we describe methods for both tissue culture cell lines grown in suspension and adherent tissue culture cell lines. Analysis may be required of cells derived from other sources. Protocol FC4 provides instructions for the preparation of human peripheral blood mononuclear cells and protocol FC5 for the preparation of peritoneal macrophages, bone marrow, thymus, and spleen cells.

It is recommended that all containers that have come into contact with human blood or cells should be considered hazardous waste and discarded appropriately.

The following should be considered when designing your flow cytometry experiments:

1. Fc receptors need to be blocked with FcR blocking reagents such as [Mouse Seroblock FcR Reagent](#) (AbD Serotec product code BUF041) when working with cell types such as spleen cells.
2. Appropriate controls should always be carried out, including:
 - [Isotype controls](#) to determine specificity of staining
 - Unstained cells to monitor autofluorescence

For all multicolor flow cytometry experiments it is advisable to include compensation controls and fluorescence minus one (FMO) controls, which assist in identifying gating boundaries.

Note: These methods provide general procedures that should always be used in conjunction with the product- and batch-specific information provided by the supplier. A certain level of technical skill and immunology knowledge is required for the successful design and implementation of these techniques. These are guidelines only and may need to be adjusted for particular applications.

FC1. Preparation of tissue culture cells stored in liquid nitrogen

This method provides a general procedure for use with tissue culture cells stored in liquid nitrogen.

Reagents

- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)

Method

1. Prepare PBS/BSA.
2. Carefully remove cells from liquid nitrogen storage.
3. Thaw cells rapidly in a 37°C water bath.
4. Resuspend cells in cold PBS/BSA and transfer them to a 15 ml conical centrifuge tube.
5. Centrifuge at 300–400 g for 5 min at 4°C.
6. Discard supernatant and resuspend pellet in an appropriate amount of cold (4°C) PBS/BSA, such as 10^7 cells/ml. **Note:** higher viability can be obtained by allowing the cells to recover in culture media overnight.

FC2. Preparation of tissue culture cells in suspension

This method provides a general procedure for use with tissue culture cells in suspension.

Reagents

- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS

Method

1. Prepare PBS/BSA.
2. Decant cells from tissue culture flask into 15 ml conical centrifuge tube(s).
3. Centrifuge at 300–400 g for 5 min at room temperature.
4. Discard supernatant and resuspend pellet in 10 ml room temperature PBS/BSA.
5. Centrifuge at 300–400 g for 5 min at room temperature.
6. Discard supernatant and resuspend to a minimum concentration of 1×10^7 cells/ml in cold (4°C) PBS/BSA.

FC3. Preparation of adherent tissue culture cells

This method provides a general procedure for use with adherent tissue culture cells.

Reagents

- Phosphate buffered saline (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- 1x Accutase solution
- 0.25% trypsin

Method

1. Prepare PBS/BSA.
2. Harvest cells by enzymatic release using a solution containing 1x Accutase or 0.25% trypsin, followed by quenching with media containing serum. (**Note:** epitopes may be cleaved when using the enzymatic digestion method. Cells can also be harvested by gently scraping them into culture media.)
 - i. Remove the culture medium and eliminate residual serum by rinsing cell monolayers with sterile, room temperature PBS.
 - ii. Slowly add 1x Accutase Solution or 0.25% trypsin to cover the cell monolayer.
 - iii. Incubate at 37°C for up to 10 min.
 - iv. After incubation gently tap the flask and the cells will detach and slide off in one sheet to the bottom of the flask.
 - v. Add growth medium and resuspend the cells by gently pipetting.
3. Centrifuge at 300–400 g for 5 min at room temperature.
4. Discard supernatant and resuspend pellet in fresh, room temperature PBS/BSA to wash off any remaining cell debris and proteins.
5. Centrifuge at 300–400 g for 5 min at room temperature.
6. Discard supernatant and resuspend pellet in an appropriate amount of room temperature PBS/BSA.
7. Count cells using a hemocytometer or an automated cell counter such as the **TC20™ Automated Cell Counter** (Bio-Rad product code 1450102)
8. Once counted, dilute the cells with cold (4°C) PBS/BSA to a minimum concentration of 1×10^7 cells/ml.

FC4. Preparation of human peripheral blood mononuclear cells

This method provides a general procedure for use with peripheral blood mononuclear cells.

Reagents

- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- Histopaque or Ficoll

Method

1. Allow separation media such as Histopaque or Ficoll to equilibrate to room temperature.
2. Dilute blood in equal volumes of room temperature PBS/BSA (for example, add 3 ml of PBS/BSA to 3 ml of blood).
3. Carefully overlay whole blood onto an equal volume of separation media in a 15 ml conical centrifuge tube.
4. Centrifuge at 300–400 g for 30 min in a 20°C temperature controlled centrifuge with no brake. **Note:** Centrifugation at 4°C or with brake reduces efficiency of cell recovery.
5. Harvest cells from the serum/separation media interface using a pipet.
6. Place harvested cells in a 15 ml conical centrifuge tube.
7. Adjust the volume to 10 ml with room temperature PBS/BSA.
8. Centrifuge at 300–400 g for 5 min at room temperature.
9. Discard supernatant and resuspend pellet to a final concentration of at least 1×10^7 cells/ml with cold (4°C) PBS/BSA.

FC5. Preparation of peritoneal macrophages, bone marrow, thymus, and spleen cells

This method provides a general procedure for use with cell suspension cells acquired from the peritoneum, bone marrow, thymus, or spleen.

Reagents

- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- Ammonium chloride lysis buffer: 0.16 M ammonium chloride, 0.17 M Tris, pH 7.2
- Optional: PBS/BSA with 25 µg/ml DNase I or 5 mM EDTA to reduce cell aggregates

Method

1. Prepare a single cell suspension from relevant tissue. Keep cells on ice to minimize cell death, which can lead to cell aggregation. Addition of DNase I or EDTA can also reduce aggregation. Large aggregates can be removed by passing the cell suspension through a 40 μm cell strainer.
2. Centrifuge at 300–400 g for 5 min at 4°C.
3. Discard supernatant and resuspend pellet in 10 ml ammonium chloride lysis buffer.
4. Mix and incubate for 2 min at 4°C. **Do not exceed this time.**
5. Centrifuge at 300–400 g for 5 min at 4°C.
6. Discard supernatant and resuspend pellet in 10 ml cold (4°C) PBS/BSA.
7. Centrifuge at 300–400 g for 5 min at 4°C.
8. Discard supernatant and resuspend pellet to a final volume of 10 ml with cold (4°C) PBS/BSA.
9. Count cells using a hemocytometer or an automated cell counter such as the [TC20™ Automated Cell Counter](#) (Bio-Rad product code 1450102).
10. Adjust suspension if necessary to give a final count of $0.7\text{--}1.2 \times 10^7$ cells/ml.

Staining of Cells for Flow Cytometry

FC6. Direct immunofluorescence staining of surface epitopes of cells and blood

Applicable when the fluorophore is directly linked to the primary antibody, for example, RPE, FITC, and Alexa Fluor conjugates. RPE conjugates should always be handled in the dark.

Note: Specific methodology for blood appears in [] brackets.

Reagents

- [Phosphate buffered saline](#) (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- [Erythrolyse red blood cell lysing buffer](#) (AbD Serotec product code BUF04)
- Anticoagulant (**Note:** for basic staining any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. In some instances specific anticoagulants may be required.)
- Optional: 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)

Method

1. Prepare cells appropriately; refer to the Preparation of Cells for Flow Cytometry section for further information. Adjust the cell suspension to a concentration of 1×10^7 cells/ml with cold (4°C) PBS/BSA.

[Whole blood samples may be used undiluted unless the cell count is high, as in leukemia samples. Use appropriate anticoagulant.]

2. Aliquot 100 μ l of the cell suspension [or whole blood] into as many test tubes as required.
3. Add antibody at the vendor-recommended dilution. Mix well and incubate at 4°C for at least 30 min, avoiding direct light.
4. Wash cells with 2 ml cold (4°C) PBS/BSA, centrifuge at 300–400 g for 5 min at 4°C, and discard the resulting supernatant.

[To the blood suspension add 2 ml freshly prepared erythrolyse red blood cell lysing buffer and mix well. Incubate for 10 min at room temperature. Centrifuge at 300–400 g for 5 min at room temperature and discard the supernatant. Wash with 2 ml room temperature PBS/BSA, centrifuge at 300–400 g for 5 min at room temperature, and discard the supernatant. Proceed to step 5.]

5. Resuspend cells in 200 μ l cold (4°C) PBS or with 200 μ l 0.5% paraformaldehyde in PBS if required.
6. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

FC7. Indirect immunofluorescence staining of surface epitopes of cells and blood

This technique is applicable when using unconjugated or biotin-conjugated monoclonal and polyclonal antibodies recognizing cell surface antigens. A conjugated secondary reagent must be used to visualize the primary antibody, for example, streptavidin in the case of biotin.

Note: Specific methodology for blood appears in [] brackets.

Reagents

- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- **Erythrolyse red blood cell lysing buffer** (AbD Serotec product code BUF04)
- Anticoagulant (**Note:** for basic staining any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. In some instances specific anticoagulants may be required.)
- Optional: 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)

Method

1. Prepare cells appropriately; refer to the Preparation of Cells for Flow Cytometry section for further information. Adjust the cell suspension to a concentration of 1×10^7 cells/ml with cold (4°C) PBS/BSA.

[Whole blood samples may be used undiluted unless the cell count is high, as in leukemia samples. Use appropriate anticoagulant.]

2. Aliquot 100 μ l of the cell suspension [or whole blood] into as many test tubes as required.
3. Add primary antibody at the vendor-recommended dilution. Mix well and incubate at 4°C for at least 30 min.
4. Wash cells with 2 ml cold (4°C) PBS/BSA, centrifuge at 300–400 g and 4°C for 5 min, and discard the supernatant.

[To the blood suspension add 2 ml freshly prepared erythrolyse red blood cell lysing buffer and mix well. Incubate for 10 min at room temperature. Centrifuge at 300–400 g and room temperature for 5 min and discard the supernatant. Wash with 2 ml room temperature PBS/BSA, centrifuge at 300–400 g for 5 min at room temperature, and discard the supernatant. Continue to step 5.]

5. Add an appropriate secondary reagent at the vendor-recommended dilution. Mix well and incubate at 4°C for at least 30 min, avoiding direct light.
6. Centrifuge at 300–400 g for 5 min at room temperature and discard the supernatant.
7. Resuspend cells in 200 μ l cold (4°C) PBS or with 200 μ l 0.5% paraformaldehyde in PBS if required.
8. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

FC8. Direct immunofluorescence staining of immunoglobulin light chains on B lymphocytes in whole blood

For use with directly conjugated antibodies recognizing human kappa and lambda immunoglobulin light chains.

Note: The immunofluorescence staining of B lymphocyte immunoglobulin in whole blood requires removal of serum immunoglobulins, which otherwise interfere with staining with immunoglobulin-specific antibodies.

Reagents

- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- **Erythrolyse red blood cell lysing buffer** (AbD Serotec product code BUF04)
- Anticoagulant (**Note:** for basic staining any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. In some instances specific anticoagulants may be required.)
- Optional: 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)

Method

1. Collect blood into appropriate anticoagulant, such as EDTA, heparin or acid citrate dextrose.
2. Aliquot 2–3 ml of whole blood into a 50 ml centrifuge tube.
3. Add 20–25 ml PBS/BSA, prewarmed to 37°C and mix well.
4. Centrifuge at 300–400 g for 5 min at 37°C. Carefully aspirate the supernatant, taking care not to disturb the cell pellet, and resuspend the pellet in the residual supernatant.
5. Repeat steps 3 and 4 twice more for a total of three washes.
6. Resuspend in 2–3 ml cold (4°C) PBS/BSA. Aliquot 100 µl of the washed blood into the required number of test tubes. Add appropriate volume of antibody at the vendor-recommended dilution and incubate at 4°C for at least 30 min, avoiding direct light.
7. Add 2 ml freshly prepared erythrolyse red blood cell lysing buffer and mix well.
8. Incubate for 10 min at room temperature.
9. Centrifuge at 300–400 g for 5 min at room temperature and discard supernatant.
10. Wash with 2 ml room temperature PBS/BSA, centrifuge at 300–400 g for 5 min at room temperature, and discard the supernatant.
11. Resuspend cells in 200 µl cold (4°C) PBS or with 200 µl 0.5% paraformaldehyde in PBS if required.
12. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

The cell washing procedure described above has no effect upon other cell surface antigens, and has been shown not to affect the recovery of individual cell subsets.

Reference

Reynolds WM et al. (1992). A simple technique for the determination of kappa and lambda immunoglobulin light chain expression by B cells in whole blood. *J Immunol Methods* 151, 123–129.

FC9. Direct staining of intracellular antigens and cytokines: Leucoperm Accessory Reagent method

Method for cell permeabilization required prior to intracellular staining using Leucoperm™ Accessory Reagent.

The detection of intracellular antigens requires a cell permeabilization step prior to staining. For the detection of cell cycle antigens such as PCNA and BrdU, methanol modification is recommended (refer to protocol FC10).

Note: Specific methodology for blood appears in [] brackets.

Reagents

- **Leucoperm Accessory Reagent** (AbD Serotec product code BUF09). Includes Reagent A (cell fixation agent) and Reagent B (cell permeabilization agent)
- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- **Erythrolyse red blood cell lysing buffer** (AbD Serotec product code BUF04)
- Anticoagulant (**Note:** For basic staining any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. In some instances specific anticoagulants may be required.)
- Optional: 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)

Method

1. Harvest cells after appropriate treatment and determine the total number present. Adjust cell suspension to a concentration of 1×10^7 cells/ml with cold (4°C) PBS/BSA.

[Whole blood samples may be used undiluted unless the cell count is high, as in leukemia samples. Use appropriate anticoagulant.]
2. Add 100 µl of cell suspension [or whole blood] to the appropriate number of test tubes.
3. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
4. Wash cells once in 2 ml cold (4°C) PBS/BSA and discard the supernatant.
5. Resuspend cells in 100 µl cold (2–8°C) Leucoperm Reagent A (cell fixation agent) per 1×10^6 cells. Incubate for 10 min at 2–8°C.
6. Add 3 ml room temperature PBS/BSA and centrifuge for 5 min at 300–400 g at room temperature.

7. Remove supernatant and add 100 μ l Leucoperm Reagent B (cell permeabilization agent per 1×10^6 cells. Add the directly conjugated antibody at the vendor-recommended dilution and incubate at 4°C for at least 30 min, avoiding direct light.

[To the blood suspension add 2 ml freshly prepared erythrolyse red blood cell lysing buffer and mix well. Incubate for 10 min at room temperature. Centrifuge at 300–400 g and room temperature for 5 min and discard the supernatant. Wash with 2 ml room temperature PBS/BSA, centrifuge at 300–400 g for 5 min at room temperature, and discard the supernatant. Continue to step 8.]

8. Wash once in PBS and then resuspend in 200 μ l cold (4°C) PBS for immediate analysis or with 200 μ l 0.5% paraformaldehyde in PBS if required.
9. Acquire data by flow cytometry. Analyze fixed cells within 24 hours.

FC10. Direct immunofluorescence staining of intracellular antigens: methanol plus Leucoperm Accessory Reagent method

Alternative method for the cell permeabilization step required prior to intracellular staining.

The detection of intracellular antigens requires a cell permeabilization step prior to staining. This method provides an alternative procedure for use when protocol FC9. Direct staining of intracellular antigens and cytokines: Leucoperm™ Accessory Reagent method does not provide the desired results. This method is particularly suitable for the detection of nuclear antigens, such as PCNA and Ki67.

Note: Phycoerythrin conjugates are not suitable for the detection of cell surface antigens using this method due to damage of RPE at low temperatures.

Note: Specific methodology for blood appears in [] brackets.

Reagents

- **Leucoperm Accessory Reagent** (AbD Serotec product code BUF09). Includes Reagent A (cell fixation agent) and Reagent B (cell permeabilization agent)
- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- **Erythrolyse red blood cell lysing buffer** (AbD Serotec product code BUF04)
- Anticoagulant (**Note:** for basic staining any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. In some instances specific anticoagulants may be required.)
- Optional: 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)

Method

1. Harvest cells and determine the total number present. Adjust cell suspension to a concentration of 1×10^7 cells/ml with cold (4°C) PBS/BSA.

[Whole blood samples may be used undiluted unless the cell count is high, as in leukemia samples. Use appropriate anticoagulant.]
2. Add 100 µl of cell suspension [or whole blood] to the appropriate number of test tubes.
3. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
4. Wash cells once in 2 ml cold (4°C) PBS/BSA and discard the supernatant.
5. Resuspend cells in 100 µl cold (2–8°C) Leucoperm Reagent A (cell fixation agent) per 1×10^6 cells. Incubate for 10 min at 2–8°C.
6. Add 500 µl ice cold absolute methanol, vortex, and incubate for 10 min at 2–8°C.
7. Add 3 ml cold (4°C) PBS and centrifuge for 5 min at 300–400 g at 4°C.
8. Remove supernatant and add 100 µl Leucoperm Reagent B (cell permeabilization agent per 1×10^6 cells. Add the directly conjugated antibody at the vendor-recommended dilution and incubate at 4°C for at least 30 min, avoiding direct light.

[To the blood suspension add 2 ml freshly prepared erythrolyse red blood cell lysing buffer and mix well. Incubate for 10 min at room temperature. Centrifuge at 300–400 g and room temperature for 5 min and discard the supernatant. Wash with 2 ml room temperature PBS/BSA, centrifuge at 300–400 g for 5 min at room temperature, and discard the supernatant. Continue to step 9.]
9. Resuspend cell in 2 ml cold (4°C) PBS and centrifuge at 300–400 g at 4°C. Discard supernatant.
10. Resuspend cells in 200 µl cold (4°C) PBS for immediate analysis or with 200 µl 0.5% formaldehyde in PBS if required.
11. Acquire data by flow cytometry. Analyze fixed cells within 24 hours.

FC11. Direct immunofluorescence staining of intracellular antigens: digitonin method

Alternative method for cell permeabilization required prior to intracellular staining that does not require the use of [Leucoperm™ Accessory Reagent](#).

The detection of intracellular antigens requires a cell permeabilization step prior to staining. This method provides an alternative procedure for use when protocol FC9. Direct staining of intracellular antigens and cytokines: Leucoperm Accessory Reagent method does not provide the desired results. This method is particularly suitable for the detection of T cell antigen receptor zeta chain using [MCA 1297](#).

Note: This procedure causes a reduction in Forward Scatter (FSC) signal, so the flow cytometer setup may need to be adjusted to compensate.

Reagents

- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)
- 0.05% (v/v) Tween 20 in PBS
- 10 µg/ml digitonin in PBS.

Method

1. Prepare cells appropriately; refer to the Preparation of Cells for Flow Cytometry section for further information. Adjust the cell suspension to a concentration of 1×10^7 cells/ml with cold (4°C) PBS/BSA.
2. Aliquot 100 µl of the cell suspension into the required number of tubes.
3. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
4. Wash twice in cold (4°C) PBS/BSA by pelleting cells at 300–400 g and 4°C for 5 min. Discard supernatant.
5. Add 50 µl PBS/BSA to each tube, followed by 100 µl 0.5% paraformaldehyde.
6. Incubate for 20 min at room temperature.
7. Wash twice with 3 ml 0.05% Tween 20. Pellet cells at 300–400 g and 4°C for 5 min.
8. Decant supernatant. Add 100 µl of cold 10 µg/ml digitonin and the directly conjugated antibody at the vendor-recommended dilution and incubate for at least 30 min at 4°C, avoiding direct light.
9. Wash twice with 3 ml 0.05% Tween 20. After each wash step pellet cells at 300–400 g and 4°C for 5 min.
10. Resuspend in 200 µl PBS.
11. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

FC12. Direct immunofluorescence staining of intracellular cytokines in blood

For staining of intracellular antigens in whole blood using directly conjugated antibodies.

This is a rapid and simple approach for the analysis of intracellular cytokines in whole blood by flow cytometry. It permits the analysis of small samples and avoids generating artifacts due to the separation of peripheral blood cells by density gradient centrifugation. All blood samples must be collected into heparin anticoagulant. EDTA interferes with the cell stimulation process and therefore must be avoided.

The detection of intracellular antigens requires a cell permeabilization step prior to staining. The method described below has been found to provide excellent results in our hands; however other permeabilization techniques have been published and may also be used successfully in this application.

Note: Resting cells often require stimulation in vitro for the detection of intracellular cytokines.

Reagents

- **Leucoperm™ Accessory Reagent** (AbD Serotec product code BUF09). Includes Reagent A (cell fixation agent) and Reagent B (cell permeabilization agent)
- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- **Erythrolyse red blood cell lysing buffer** (AbD Serotec product code BUF04)
- Cell culture medium
- Monensin
- Ionomycin
- PMA
- Optional: 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)

Method

1. Aliquot 500 μ l of blood into as many tubes as required, including 2 extra control tubes, and then add 500 μ l of cell culture medium (without any additives) to each sample.
2. To one tube (the resting population), add monensin to a final concentration of 3 μ M.
3. To another tube (activated cells), add PMA to a final concentration of 10 ng/ml, ionomycin to 2 μ M, and monensin to 3 μ M.
4. To the rest of the tubes (experimental samples) add monensin to 3 μ M and treat as required by the experiment.
5. Incubate for 2–4 hr at 37°C in a 5% CO₂ atmosphere.

6. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
7. Wash cells once with PBS/BSA and discard supernatant.
8. Add 100 µl of Leucoperm Reagent A (cell fixation agent) and incubate for 10 min at 2–8°C.
9. Add 2 ml cold (4°C) PBS/BSA and centrifuge for 5 min at 300–400 g at room temperature.
10. Remove supernatant and add 100 µl Leucoperm Reagent B (cell permeabilization agent) per 1×10^6 cells. Add the directly conjugated antibody at the vendor-recommended dilution and incubate for at least 30 min at 4°C, avoiding direct light.
11. Add 2 ml freshly prepared erythrolyse red blood cell lysing buffer to the blood suspension and mix well.
12. Incubate for 10 min at room temperature.
13. Centrifuge at 300–400 g for 5 min and discard the supernatant.
14. Wash once in PBS/BSA, and then resuspend in 200 µl PBS for immediate analysis or with 200 µl 0.5% paraformaldehyde in PBS if required.
15. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

FC13. Direct immunofluorescence staining of Intracellular antigens: paraformaldehyde/saponin method

Alternative method for cell permeabilization required prior to intracellular staining that does not require the use of Leucoperm™ Accessory Reagent.

The detection of intracellular antigens requires a cell permeabilization step prior to staining. This method provides an alternative procedure for use when protocol FC9. Direct staining of intracellular antigens and cytokines: Leucoperm Accessory Reagent Method does not provide the desired results. This method is particularly suitable for use with directly conjugated FITC monoclonal antibodies.

Reagents

- **Phosphate buffered saline** (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- 4% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)
- 0.1% (w/v) saponin in PBS
- Optional: 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)

Method

1. Prepare cells appropriately; refer to the Preparation of Cells for Flow Cytometry section for further information. Adjust the cell suspension to a concentration of 1×10^7 cells/ml with cold (4°C) PBS/BSA.
2. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C , avoiding direct light.
3. Following staining, wash cells once in 3 ml PBS/BSA, pellet cells at 300–400g and 4°C for 5 min, and discard supernatant.
4. Resuspend cells in 100 μl 4% paraformaldehyde per 1×10^6 cells. Incubate for 20 min at room temperature. Wash once in 3 ml PBS/BSA.
5. Resuspend cells in 100 μl 0.1% saponin per 1×10^6 cells. Incubate for 15 min at room temperature.
6. Aliquot 100 μl of cell suspension into the required number of tubes containing directly conjugated antibody at the vendor-recommended dilution. Incubate for at least 30 min at 4°C , avoiding direct light.
7. Wash once in 3 ml 0.1% saponin and resuspend in 200 μl 0.5% paraformaldehyde.
8. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

Reference

Sewell WA et al. (1997) Determination of intracellular cytokines by flow cytometry following whole blood culture. *J Immunol Methods* 209, 67–74.

5 Troubleshooting

Troubleshooting Guide

If something doesn't work, check through the following guidelines to identify and resolve the problem. If there are still difficulties and you have purchased any Bio-Rad or AbD Serotec reagents, our Technical Services Team will be happy to offer further advice.

Problem	Course of Action
No staining	<ol style="list-style-type: none"> 1. Confirm that all antibodies have been stored correctly according to the manufacturer's instructions. 2. Confirm that commercial antibodies have not exceeded their date of expiration. 3. Make sure that appropriate primary or secondary antibodies have been added. 4. Make sure that antibody is conjugated to a fluorophore. If not, confirm that an appropriate fluorophore-conjugated secondary antibody is being used. 5. Confirm that secondary antibody is active. Has it been used successfully with other primary antibodies? 6. Make sure a secondary antibody that will recognize your primary antibody is being used. 7. If the fluorophore used is phycoerythrin or allophycocyanin based, make sure that the product has not been frozen. 8. Is the target antigen present on test tissue? Check literature for antigen expression and incorporate a positive control of known antigen expression alongside test material. 9. Does antibody recognize antigen in test species? Check that antibody cross-reacts with species being used. Not all antibodies will react across species. 10. Confirm that correct laser is being used to excite fluorophore, and that correct channel is being used to analyze emissions.
No staining with PE antibody but same FITC antibody gives good results.	<ol style="list-style-type: none"> 1. PE conjugate may have been frozen. If so, purchase another vial of antibody. 2. Paraformaldehyde (PFA) may be a problem. Breakdown of PFA may release methanol, which will affect staining. Make up fresh PFA. Cells can be analyzed immediately without fixing.

Problem	Course of Action
No staining	<ol style="list-style-type: none"> 1. Confirm that all antibodies have been stored correctly according to the manufacturer's instructions. 2. Confirm that commercial antibodies have not exceeded their date of expiration. 3. Make sure that appropriate primary or secondary antibodies have been added. 4. Make sure that antibody is conjugated to a fluorophore. If not, confirm that an appropriate fluorophore-conjugated secondary antibody is being used. 5. Confirm that secondary antibody is active. Has it been used successfully with other primary antibodies? 6. Make sure a secondary antibody that will recognize your primary antibody is being used. 7. If the fluorophore used is phycoerythrin or allophycocyanin based, make sure that the product has not been frozen. 8. Is the target antigen present on test tissue? Check literature for antigen expression and incorporate a positive control of known antigen expression alongside test material. 9. Does antibody recognize antigen in test species? Check that antibody cross-reacts with species being used. Not all antibodies will react across species. 10. Confirm that correct laser is being used to excite fluorophore, and that correct channel is being used to analyze emissions.
No staining with PE antibody but same FITC antibody gives good results.	<ol style="list-style-type: none"> 1. PE conjugate may have been frozen. If so, purchase another vial of antibody. 2. Paraformaldehyde (PFA) may be a problem. Breakdown of PFA may release methanol, which will affect staining. Make up fresh PFA. Cells can be analyzed immediately without fixing.
Nonspecific staining	<ol style="list-style-type: none"> 1. May be due to autofluorescence. Solution: check levels of autofluorescence by including only a tube of cells (that is, without any antibody) in your panel. 2. Certain cells express low-affinity Fc receptors CD16/CD32, which bind whole antibodies via Fc region. For mouse cells, dilute antibody in Mouse SeroBlock FcR (AbD Serotec product code BUF041A or B). 3. May be due to the secondary antibody. Select a secondary antibody that will not cross-react with target tissue. 4. Make sure that sufficient washing steps have been included. 5. Titrate test antibody carefully. Nonspecific staining may be reduced at lower antibody concentrations.

<p>Weak staining</p>	<ol style="list-style-type: none"> 1. May be due to overdilution of antibodies. Confirm that antibodies are used at the correct concentration by titrating antibodies before use. 2. Weak staining in indirect staining systems may be due to prozoning effect, where highly concentrated antibodies may give weak results. Titrate antibodies carefully. 3. May be due to an excessive number of cells. Adjust cell population to recommended density. 4. May be due to the antigen expression. Check literature for expected levels of expression. 5. If antigen expression is weak, select an antibody that is conjugated to a brighter fluorophore. 6. May be seen if using a cross-reacting antibody rather than one specific for the target species. 7. Optimize incubation time and temperature with either primary or secondary antibody.
<p>Unusual scatter profiles</p>	<ol style="list-style-type: none"> 1. Make sure that cells as fresh as possible are used. Profile may be showing dead cells and debris. 2. Activation methods may affect scatter characteristics of cells. 3. If you are using lysing solution, confirm that this is fresh and has been made up correctly.
<p>Unexpected staining</p>	<ol style="list-style-type: none"> 1. Some reagents may affect certain antigens and, therefore, may need reviewing. For example, EDTA will affect some platelet markers. 2. Lysing solutions may affect certain antigens. Select a method that does not interfere with antigen detection. 3. Some antigens are expressed intracellularly, therefore cell permeabilization methods may be required. Check manufacturer's datasheet for correct permeabilization reagent.

Recommended Reading

Givan AL (2001). *Flow Cytometry: First Principles*, 2nd Edition (New York: Wiley-Liss, Inc.).

Haugland RP (2005). *The Handbook: A Guide to Fluorescent Probes and Labeling Technologies*, 10th Edition (Invitrogen — Molecular Probes).

Macey MG (1994). *Flow Cytometry: Clinical Applications* (Oxford, United Kingdom: Blackwell Scientific Publications).

Ormerod MG, ed. (2000). *Flow Cytometry: A Practical Approach*, 3rd Edition (Oxford, United Kingdom: Oxford University Press).

Perfetto SP et al. (2004). Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol* 4, 648–655.

Shapiro HM (2003). *Practical Flow Cytometry*, 4th Edition (New York: Wiley-Liss, Inc.).

Stewart CC and Nicholson JKA, eds. (2000). *Immunophenotyping* (New York: Wiley-Liss, Inc.).

Watson JV (2004). *Introduction to Flow Cytometry* (Cambridge, United Kingdom: Cambridge University Press).

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