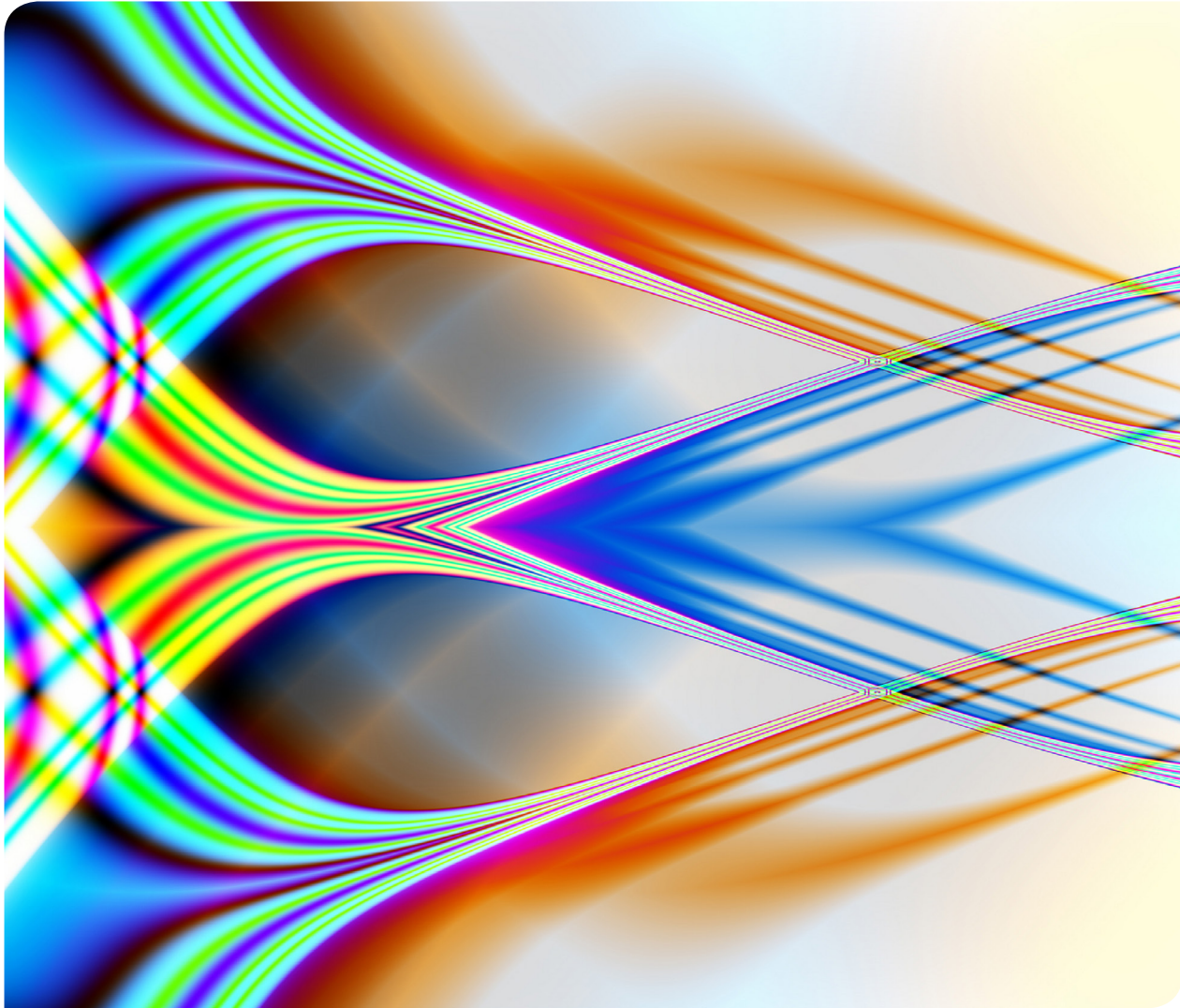




Flow Cytometry Basics Guide

BIO-RAD

Table of Contents

Chapter 1	Principles of the Flow Cytometer	
	Fluidics System	3
	Optics and Detection.	4
	Signal and Pulse Processing	6
	Electrostatic Cell Sorting	9
Chapter 2	Principles of Fluorescence	
	Fluorophores and Light	11
	Fluorescence.	12
	Why Use a Fluorescent Marker?	13
	Which Fluorophores are Useful for Flow Cytometry?.	13
	Single and Tandem Dyes.	14
	Fluorescent Proteins	14
	Fluorescence Compensation.	16
	Compensation Controls.	18
Chapter 3	Data Analysis	
	Gates and Regions	19
	Single-Parameter or Univariate Histograms.	21
	Two-Parameter or Bivariate Histograms	22
	Backgating to Confirm Gating Strategies.	24
Chapter 4	Controls in Flow cytometry	
	Unstained Controls	25
	Isotype Controls	26
	Single Staining and Compensation Controls	27
	Fc Blocking Controls	28
	Fluorescence Minus One Controls.	29
	Intracellular Staining Controls	30
	Biological Controls	30
Chapter 5	Optimizing your Experiments	
	Sample Preparation.	31
	Live/Dead Exclusion	32
	Autofluorescence.	34
	Doublet Discrimination.	34
	Collect a Statistically Relevant Number of Cells.	35
	Permeabilization and Fixation for Intracellular Antigens	36

Chapter 6	Multicolor Panel Building	
	Resolution of Signal	37
	Instrument Configuration	38
	Fluorophore Separation	38
	Antigen Density	38
	Fluorophore Properties	38
	Marker Expression Patterns	39
	Dump Channels	39
	Antibody Titration	39
	Panel Building Tools	40
 Chapter 7	 Common Applications and New Technology	
	Immunophenotyping	41
	Apoptosis	42
	Proliferation and Cell Cycle	43
	Signaling and Phosphoflow	44
	Small Particle Detection	44
	Gene Expression and Transfection	44
	Absolute Quantification	45
	Particle Internalization	45
	Fluorescence <i>in situ</i> Hybridization and RNA Detection	45
	Innovations in Flow Cytometry	45
	Recommended Reading	46
 Chapter 8	 Common Protocols	
	Sample Preparation	47
	Preparation of Cells for Flow Cytometry	48
	Preparation of Tissue Culture Cells Stored in Liquid Nitrogen	49
	Preparation of Tissue Culture Cells in Suspension	49
	Preparation of Adherent Tissue Culture Cell Lines	50
	Preparation of Human Peripheral Blood Mononuclear Cells	51
	Preparation of Peritoneal Macrophages, Bone Marrow, Thymus, and Spleen Cells	51
	Direct Immunofluorescence Staining of Surface Epitopes of Cells and Blood	52
	Indirect Immunofluorescence Staining of Surface Epitopes of Cells and Blood	53
	Direct Staining of Intracellular Antigens and Cytokines: Leucoperm Accessory Reagent Method	54
	Direct Immunofluorescence Staining of Intracellular Antigens: Methanol plus Leucoperm Method	56
	Direct Immunofluorescence Staining of Intracellular Cytokines in Blood	57
	Propidium Iodide Staining of Cells for Cell Cycle Analysis	60
	BrdU Staining of Cells for Cell Cycle Analysis and Apoptosis	61
 Chapter 9	 Troubleshooting	
	Troubleshooting Guide	63
	Glossary	65

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 significantly 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 instrument'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. Due to narrowing of the sheath (in a nozzle or cuvette) the fluid velocity is increased. The sample is introduced into the center and is focused by the Bernoulli effect (Figure 1). This allows the creation of a stream of particles in single file and is called hydrodynamic focusing. Under optimal conditions (laminar flow) there is no mixing of the central fluid stream and the sheath fluid.

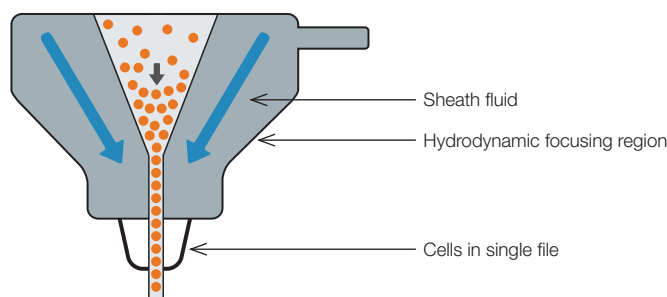


Fig. 1. Hydrodynamic focusing produces a single stream of particles.

Without hydrodynamic focusing, the cuvette (typically 250 x 250 μm or 180 x 480 μm) or nozzle of the instrument (typically 70-130 μm) would not create a focused stream of cells and analysis of single cells would not be possible. With hydrodynamic focusing the cells flow in single file through the illumination source, called the interrogation point, allowing single cell analysis.

Optics and Detection

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

Lasers produce a single wavelength of light (a laser line) at a specific frequency. They are available at different wavelengths ranging from ultraviolet to far red and have a variable range of power levels (photon output/time typically specified in mW).

Light that is scattered in the forward direction after interacting with a particle, typically up to 20° offset from the laser beam's axis, is collected by a photomultiplier tube (PMT) or photodiode and is known as the forward scatter (FSC) channel. This angle can however vary depending on your instrument, leading to variation of FSC signals between different machines. This FSC measurement can give an estimation of a particle's size with larger particles refracting more light than smaller particles, but this can depend on several factors such as the sample, the wavelength of the laser, the collection angle and the refractive index of the sample and sheath fluid. A good example of this is in the detection of small particles. When the particles are smaller than the wavelength of the illumination source, e.g. a 200 nm exosome passing through a 488 nm laser, does not necessarily scatter light in a forward direction.

Light measured at a 90° angle to the excitation line is called side scatter (SSC). The SSC can provide information about the relative complexity (for example, granularity and internal structures) of a cell or particle; however as with forward scatter this can depend on various factors. 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 population such as blood. However, this depends on the sample type and the quality of sample preparation, so fluorescent labeling is generally required to obtain more detailed information.

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. Most flow cytometers use separate channels and detectors to detect emitted light, the number of which vary according to the instrument and the manufacturer. Detectors are either photomultiplier tubes or avalanche photodiodes (APD). PMTs are the most commonly used detectors.

The specificity of detection is controlled by optical filters, which block certain wavelengths whilst 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 certain wavelength and band pass filters transmit light within a specified narrow range of wavelengths (termed a band width). These dichroic filters can block light by phased reflection allowing certain light to pass through and interfering with other wavelengths (Figure 2).

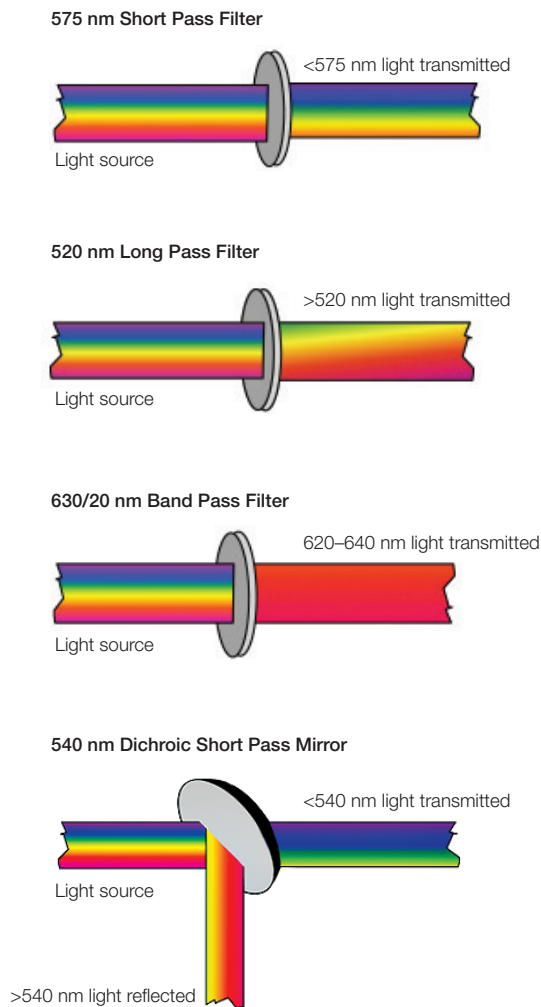


Fig. 2. Different types of optical filters.

A dichroic filter is also a mirror when placed at an angle to the oncoming light. This type of filter can now perform two functions. First, it allows specific wavelengths to pass in the forward direction, second it can reflect light at a 90° angle. This allows the light path to be passed through a series of filters. The precise choice and order of the filters can be arranged so that multiple signals can be detected simultaneously (Figure 3).

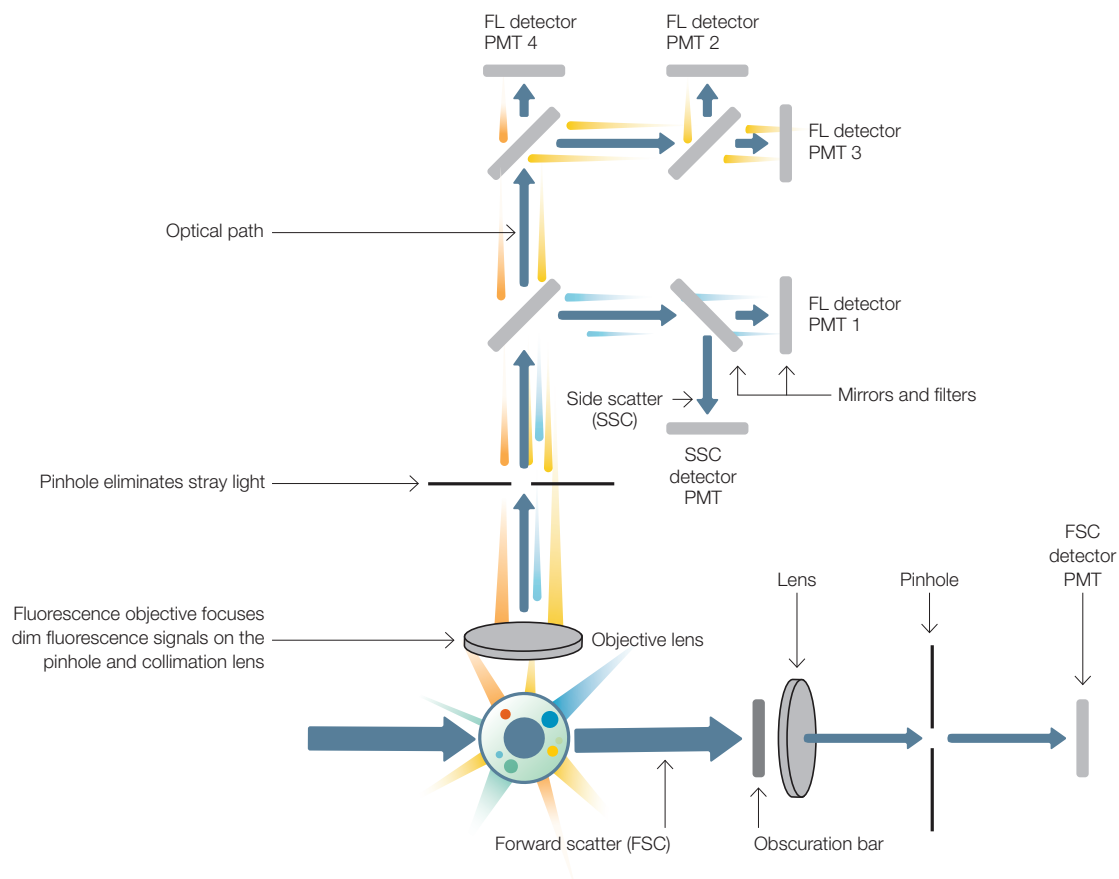


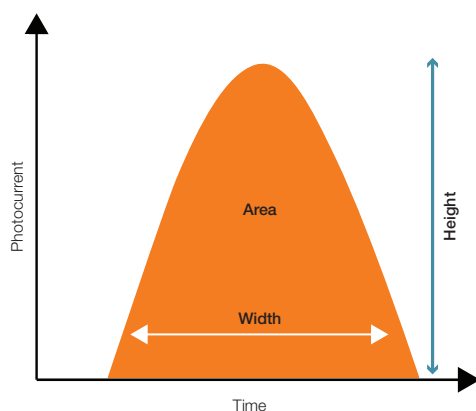
Fig. 3. Schematic overview of a typical flow cytometer setup. FL, fluorescence; PMT, photomultiplier tube; SSC, side scatter; FSC, forward scatter; blue arrow, light path.

Signal and Pulse Processing

Any time a particle passes through the interrogation point and generates a signal a pulse is generated in every detector. These pulses reflect the passage of the particle through the laser beam or beams 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 focus) 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 signals that are generated correspond to a particle of interest. To avoid the processing of unwanted signals a decision is made upon the signal intensity (threshold) of a dedicated detector, the trigger channel. This determination is made based upon the trigger parameter and threshold level. PMTs are extremely sensitive and detect signal from a variety of sources that are irrelevant to experimental data including stray light, dust, very small particles and debris. The number of these pulses in the system can be orders of magnitude higher than the number of pulses that are generated by experimental particles. Including these in the dataset would give high levels of background, substantially mask out relevant data points and overload the electronic's ability to process relevant signals. Therefore it is desirable and necessary to have a threshold below which non-essential data is not detected. This is done by designating a parameter as the trigger for recording events, 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).

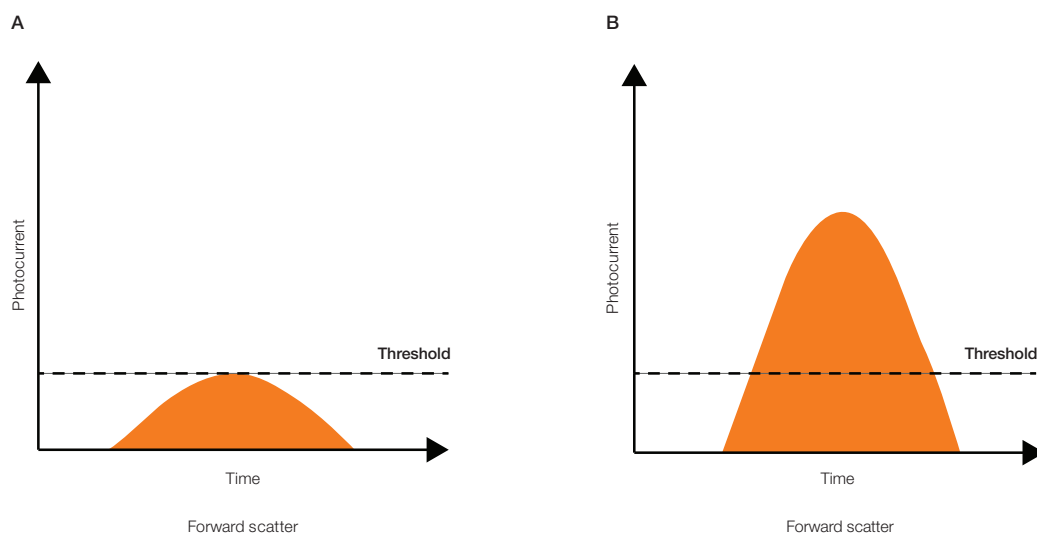


Fig. 5. Determining whether a pulse is ignored (A) or fully processed (B).

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 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 particles spent in the laser and can be used to distinguish between single particles or closely interacting particles and doublets (this will be discussed later).

Although data is collected in a linear scale, data display is usually log scale 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 display is required when very small differences in fluorescence signal must be assessed, for example in DNA analysis where there may only be a two-fold increase in fluorescence.

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. Droplet based 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 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 particle 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. 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.

One of the most critical parameters of sorting is to measure the time between the point of interrogation and the exact point where the droplet breaks off. The time and therefore the distance is called the drop delay. When the particle gets to the last connected drop, the entire stream is charged at the nozzle. As the particle 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).

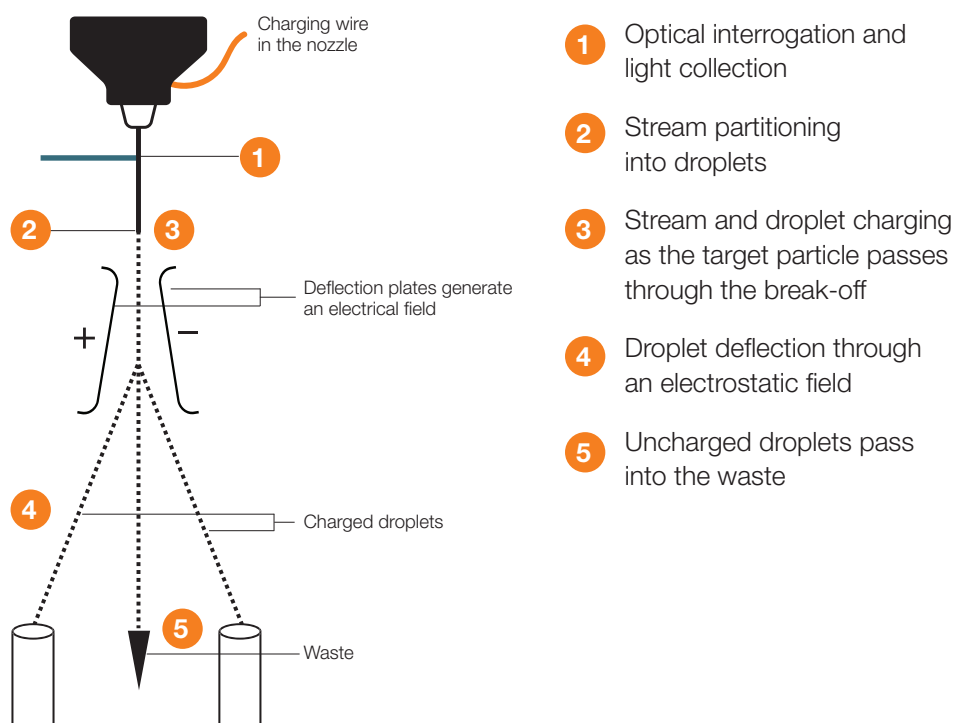


Fig. 6. Electrostatic flow sorting.

The speed of cell 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 10,000-90,000 droplets per second. The stability of the break-off dictates the accuracy of the sorting.

Common uses of cell sorting include analysis of identified cell populations and single cells in subsequent downstream applications where DNA, protein or cellular function is investigated. Purifying cells based on markers such as CD34 in hematopoietic stem cells or viability is often used, as is selecting cells, either in populations or single cell cloning, for further culture.

2 Principles of Fluorescence

Fluorophores and Light

Fluorophores are fluorescent markers used to detect the expression of cellular molecules such as proteins or nucleic acids. They 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 a 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 be felt as warmth on the skin and measured scientifically using photodetectors. The visible spectrum can be further subdivided according to color, red, orange, yellow, green, blue and violet. Red light has a longer wavelength and lower energy, whereas violet light has a shorter wavelength and higher energy.

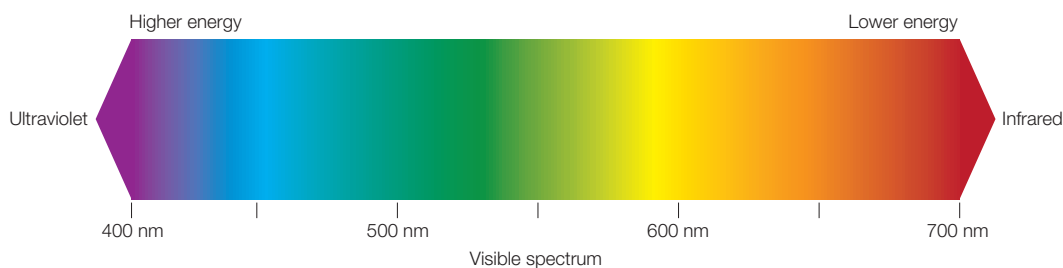


Fig. 7. The electromagnetic spectrum.

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 a 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 back to their 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.

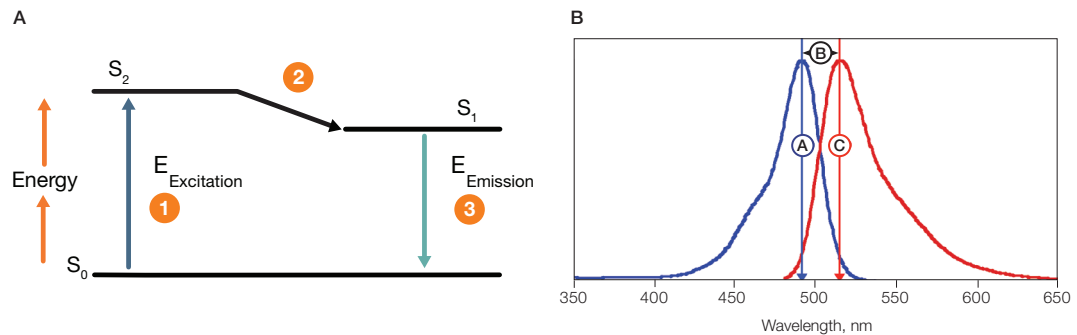


Fig. 8. Stokes shift. **A**, upon excitation, 1, electrons in a fluorophore move from a resting state, S_0 , to the excited electronic single state, S_2 . Some energy is released as heat, 2. The remaining energy is released as fluorescence, 3, as the electrons return to their ground state, S_0 . **B**, 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 400-530 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.

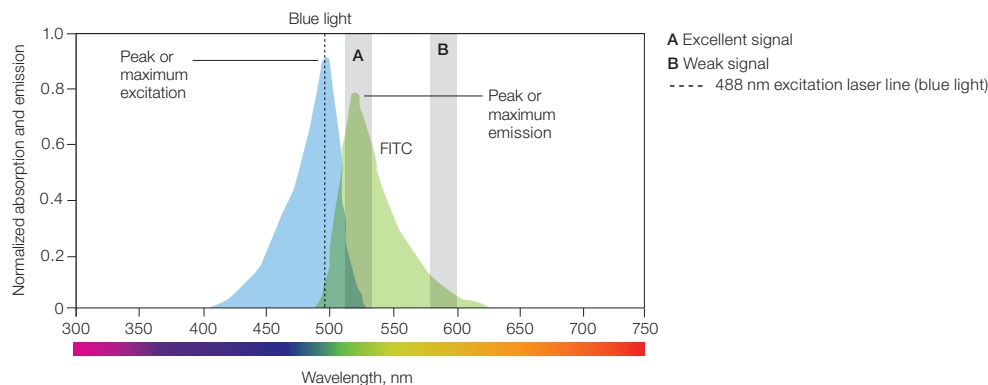


Fig. 9. Spectral profiles. Light absorbance and light emission of fluorescein isothiocyanate (FITC).

A fluorophore's maximal absorbance informs you which laser line is optimal to be used for excitation. In the case of FITC, its maximum absorbance 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 the filters are used to screen out all light other than that measured at the maximum absorbance 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 targets; cell sorting; immunophenotyping; calcium flux experiments; determining nucleic acid content; measuring enzyme activity; and apoptosis studies. Several fluorophores can be excited by a single laser. By using filters 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 fluorescent molecules (fluorophores) with a potential application in flow cytometry. The list is ever growing and we will not cover all of them here. The fluorophores directly conjugated to antibodies currently available from bio-rad-antibodies.com are described in Tables 1 and 2. With new fluorophores constantly being added, please visit our website to check out our latest offerings.

Single and Tandem Dyes

Single dyes such as FITC, PE, APC and PerCP have been available for many years, but there are now alternatives available from Alexa Fluor dyes, which offer users greater photostability and brighter fluorescence. In addition alternative laser lines are becoming more affordable so dyes excited by 355 nm and 405 nm lasers are increasing the options for multiplexing.

Tandem dyes comprise a small fluorophore covalently coupled to another 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 488 nm and 640 nm lasers which are found in most 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 then be measured using separate detectors.

Fluorescent Proteins

Fluorescent proteins, 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 yellow fluorescent protein (YFP), have also become widely used for flow cytometry analysis and cell sorting. The fluorescent proteins are often co-expressed or expressed as a fusion with the protein of interest. The benefit of these fluorescent proteins is the quantitation of positive cells and therefore intracellular markers in live cells without requiring permeabilization of the cell membrane. Common fluorescent proteins are listed in Table 3.

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
Alexa Fluor 488		495	519	3
FITC		490	525	3
DyLight 549		562	576	4
PE*		496, 546	578	5
APC		650	661	4
Alexa Fluor 647		650	665	4
DyLight 650		654	673	4
PerCP		490	675	2
Alexa Fluor 700	Infrared	702	723	2

* 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 750	Infrared	496, 546	779	4
PE–Cy7	Infrared	496, 546	785	2

* PE is the same as R-phycoerythrin.

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

Table 3. Fluorescent proteins in flow cytometry.

Fluorophores	Fluorescence Color	Maximal Absorbance, nm	Maximal Emission, nm	Relative Brightness
EBFP		383	445	2
CFP		439	476	2
EGFP		484	509	4
YFP		514	527	5
RFP		558	583	4
mCHERRY		587	610	3

Fluorescence Compensation

One consideration when performing multicolor fluorescence studies is the possibility of spectral overlap between fluorophores. 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. 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 (Figure 10).

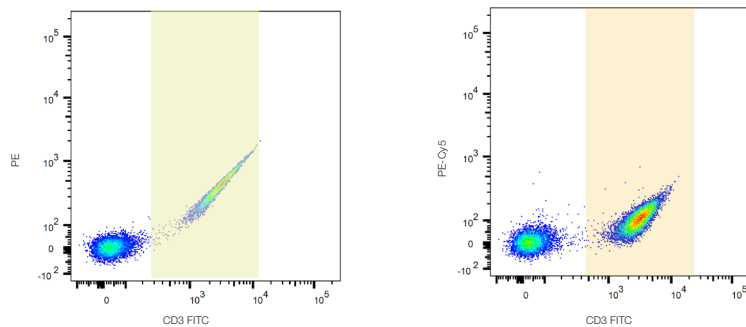


Fig. 10. FITC spillover into other channels. FITC single stained lymphocytes show spillover into PE and PE-Cy5 detectors. Gates are shown to identify the FITC positive cells and their spillover.

In some experiments FITC may be combined with other dyes, for example PE, 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).

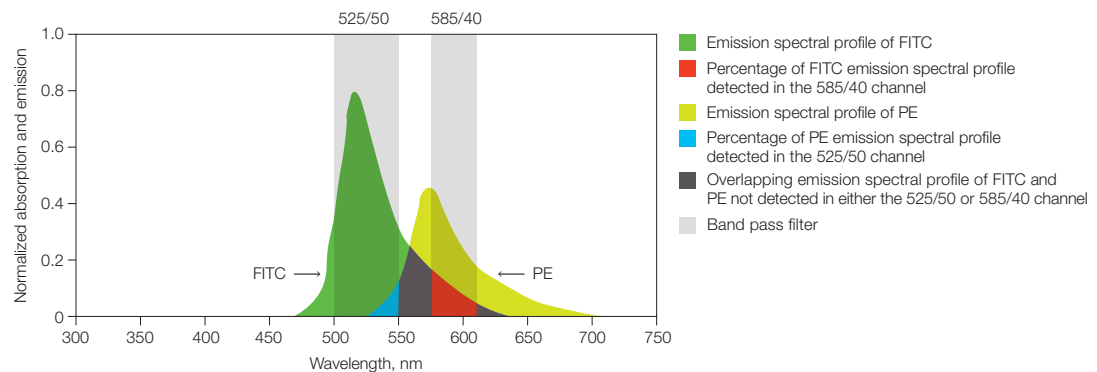


Fig. 11. Fluorescence compensation. Emission spectra of two fluorophores commonly used in flow cytometry, FITC and PE are shown. Also shown is a graphical representation of two commonly used filters, 525/50 and 585/40, to detect these fluorophores. Shown in red is the portion of the FITC spectrum that 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.

We can see in Figure 12 how compensation can be applied to a sample stained with antibodies conjugated to FITC and PE. Single stained samples reveal the amount of spectral overlap. When the sample is stained with both fluorophores, without compensation, a double positive population is observed. However when the correct level of compensation is applied using software the true level of staining is revealed. The software calculates spillover values and will apply this to the data to obtain correctly compensated data. After compensation we can see that in fact there are no double positive cells, which is to be expected from these mutually exclusive markers.

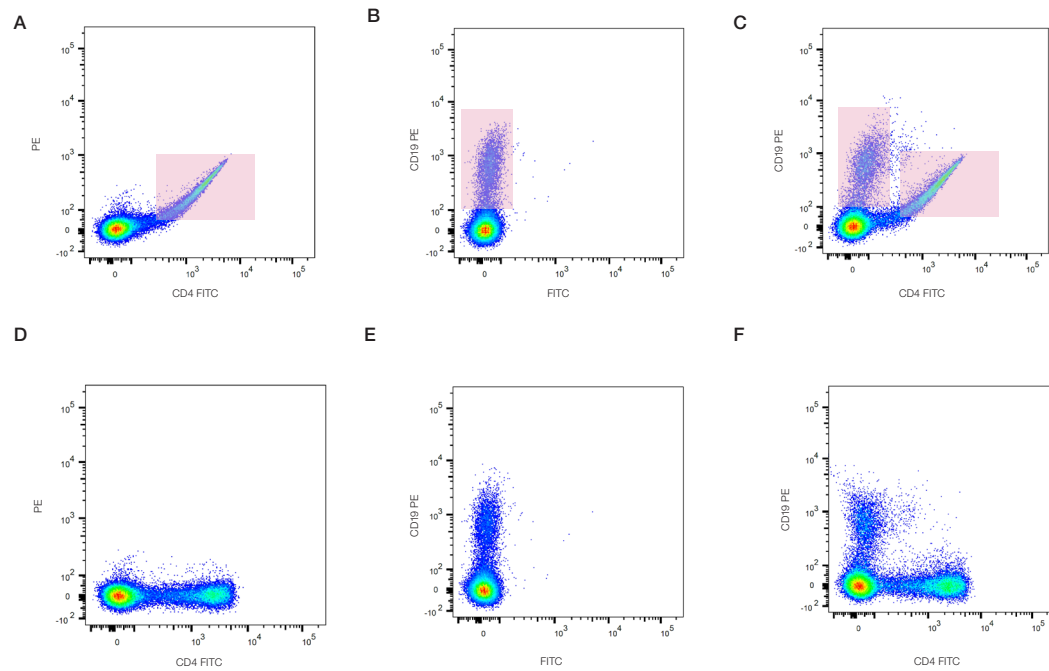


Fig. 12. Fluorescence compensation corrects for spectral overlap. Peripheral blood was singly stained with CD4 FITC, CD19 PE, or both CD4 FITC and CD19 PE. When compensation was not applied, fluorescence spillover can be seen (top panel), which is removed after compensation (bottom panel).

You can avoid the need for compensation by using fluorophores that do not have overlapping emission spectra. Alternatively you can combine fluorophores that can only be activated by specific individual laser lines (providing the lasers are spatially separated), but as you increase the number of fluorophores this becomes more difficult.

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 in an experiment, they are absolutely critical to the success of the experiment. 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. We will go into more detail regarding the important rules in Chapter 4, Controls in Flow Cytometry.

3 Data Analysis

Gates and Regions

Flow cytometry data analysis is fundamentally based upon the principle of gating. Gates and regions are placed around populations of cells with common characteristics, usually forward scatter, side scatter and marker expression, to investigate and quantify these populations of interest. Here we will show what the common flow cytometry graph outputs look like and how in a few simple steps you can identify different cell populations that have been stained with antibodies conjugated to fluorophores.

The first step in gating is often distinguishing populations of cells based on their forward and side scatter properties. Forward and side scatter give an estimation of the size and granularity of the cells respectively, although this can depend on several factors such as the sample, the wavelength of the laser, the collection angle and the refractive index of the sample and the sheath fluid. Distinguishing populations of cells can be relatively straight forward for cell lines where there is only one type of cell, but it can be more complex for samples where there are multiple cell types. As can be seen in the density plots in Figure 13, red cell lysed whole blood has several distinct populations. The red/yellow/green/blue hot spots indicate increasing numbers of events resulting from discrete populations of cells. The light scatter patterns of granulocytes, monocytes and lymphocytes allow them to be distinguished from cellular debris and dead cells. Debris and dead cells often have a lower level of forward scatter and are found at the bottom left corner of the density plot. The forward scatter threshold can be increased to avoid collecting these events, or they can be removed by gating on the populations of interest (Figure 13A). Data can also be plotted as a combination of fluorescence and forward or side scatter (Figure 13B).

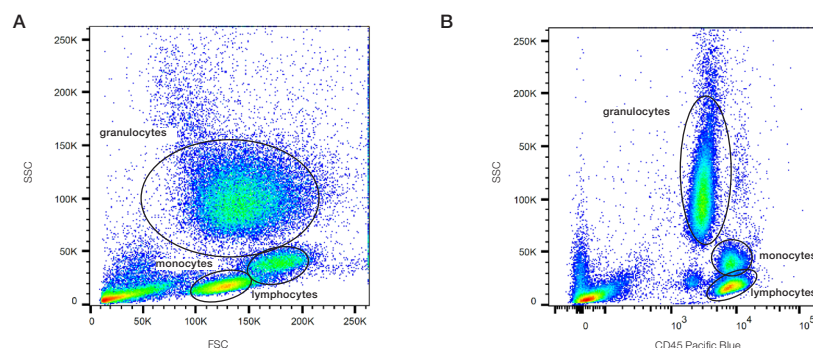


Fig. 13. Analysis of lysed whole blood. A. SSC vs. FSC density plot. **B.** SSC vs. CD45 PB fluorescence plot. PB, Pacific Blue; FSC, forward scatter; SSC, side scatter.

Events can also be displayed as a dot plot where no density information is shown or as a contour map to show the relative intensity of scatter patterns. Examples of contour maps are shown in Figure 14. It is down to the user preference as to which display is preferred, but sometimes discrete populations of cells are easier to visualize on contour diagrams.

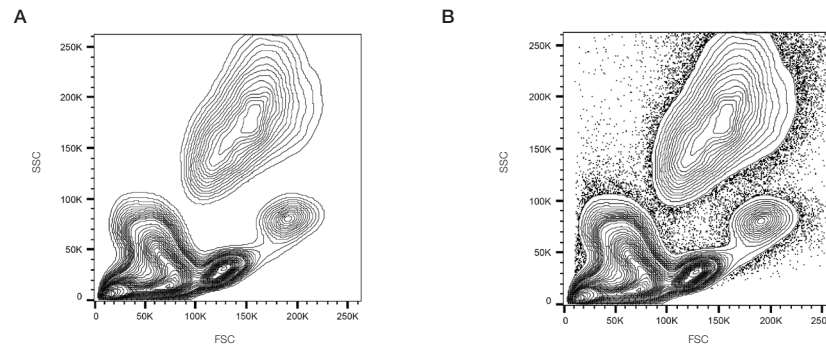


Fig. 14. Analysis of lysed whole blood. **A.** SSC vs. FSC contour plot. **B.** SSC vs. FSC contour plot plus outliers; FSC, forward scatter; SSC, side scatter.

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 data is expressed in a histogram which can be all the data collected or a selected (gated) population. While simple, it is useful for evaluating the total number of cells in a sample that possesses the selected physical properties or express the marker of interest. Cells with the desired characteristics are called the positive dataset. An example can be seen in Figure 15. Peripheral blood was stained for CD3 and then gated on the lymphocytes using forward and side scatter. There are two peaks which can be interpreted as the positive and negative dataset. In this example the CD3 positive T cells represent around 61% of the cells within the lymphocyte gate.

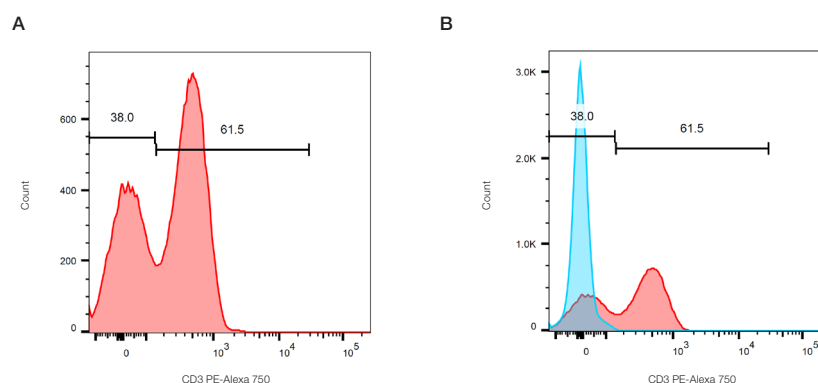


Fig. 15. Single parameter histograms. **A.** Cells within the lymphocyte gate defined in Figure 13A, are represented in a histogram to evaluate the relative expression of CD3. **B.** Overlay of a control population onto the stained population allows easy identification of the positive cells.

In order to accurately identify the positive dataset, flow cytometry should be repeated in the presence of appropriate controls, discussed in Chapter 4. This is particularly necessary if a single distinct peak is observed, however often in flow cytometry multiple peaks are observed due to mixed populations. Figure 15B shows a control histogram (in this case an isotype control), in blue, overlaid onto the stained positive dataset, in red, allowing the background staining levels to be accurately defined.

Using analytical software, measurements and statistics can be obtained for many parameters in addition to the number of cells and percentage of cells within the gate. This can include measurements such as median and mean fluorescence intensity (MFI) often used when there are small increases or decreases in fluorescence.

Two-Parameter or Bivariate Histograms

These graphs display two measurement parameters, one on the x-axis and one on the y-axis and the events displayed as a density (or dot) plot. The parameters can be fluorescence, FCS or SSC depending on what you want to show. In Figure 16, the lymphocytes determined by forward and side scatter (Figure 16A) are stained with CD3 and CD19 to identify the T and B cell populations. The relative proportion of B and T cells can then be quantified by placing gates or quadrants around the distinct populations (Figures 16B and C).

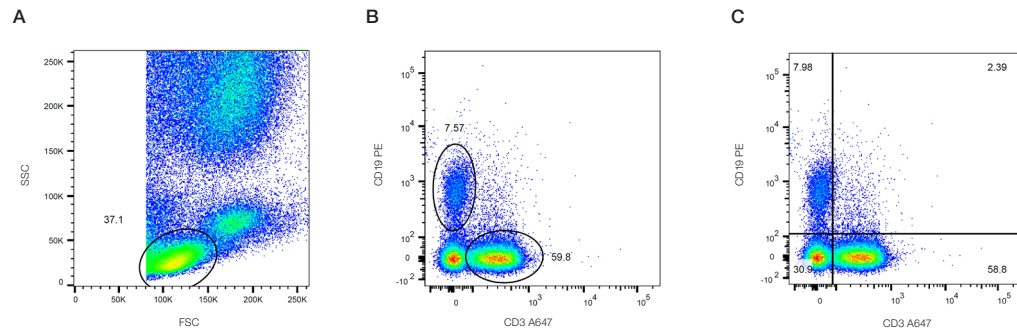


Fig. 16. Two-parameter (dual color fluorescence) density plot. Red cell lysed whole blood was stained with CD3 A647 and CD19 PE. The relative populations were determined using different gating methods.

In this case, there are 7.5% B cells and 59.8% T cells (Figure 16B). This data can also be visualized where the density plot is split into four quadrants allowing you to determine the cells single positive for each marker and both double negative and double positive (Figure 16C). When the expression levels do not show distinct populations or are not mutually exclusive the appropriate controls will help determine the positive and negative populations.

Gating is a powerful flow cytometry tool that can be used multiple times on a dataset to refine a population of interest. The simple principle of sequential gating can be applied again and again to further determine the expression patterns on particular cell types. This is particularly useful as the number of markers and fluorophores in a single experiment increases. Currently flow cytometry can be performed on samples labeled with over 17 fluorescence markers simultaneously, although cytometers are available which can detect up to 28 colors, e.g. the ZE5™ from Bio-Rad. A single experiment can therefore yield a large data set for analysis. For example, a 17 color panel would yield 136 two parameter tables.

An example of a multicolor experiment where simple sequential gating has been used to identify specific cell populations is shown in Figure 17. Briefly the lymphocytes were identified and gated by their forward and side scatter (Figure 17A). The CD3 positive T cells were then further identified (Figure 17B) and gated by the expression of CD4 and CD8 (Figure 17C). The relative expression of CD28 and CD45RA to identify CD45RA⁺CD28⁺ naïve cells, CD45RA⁺CD28⁺ memory cells and CD45RA⁺CD28⁻ effector cells were then determined on both the CD4 and CD8 populations (Figures 17D and E).

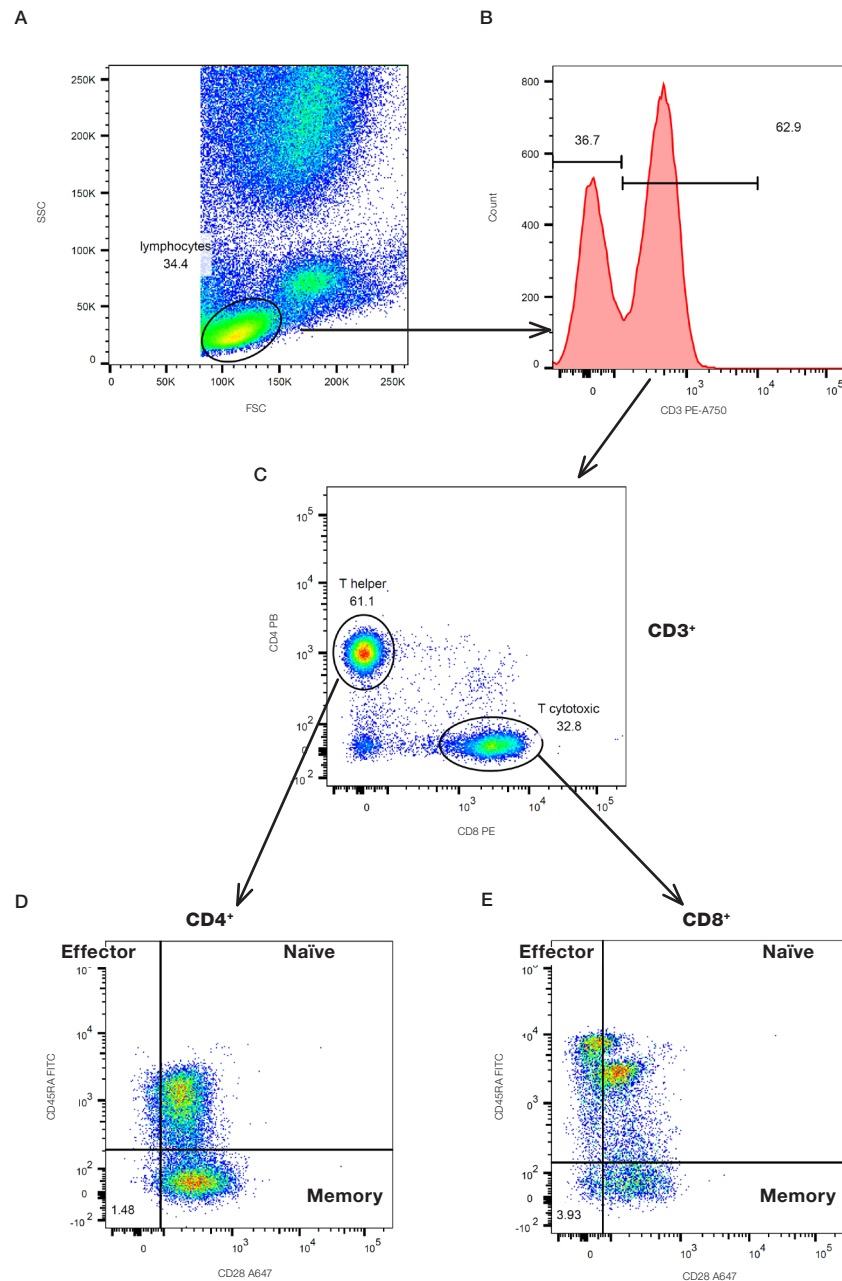


Fig. 17. Sequential gating to identify specific T subsets. Red cell lysed whole blood was stained with CD3, CD4, CD8, CD45RA and CD28 in the presence of propidium iodide to remove the dead cells. The gating strategy is shown by the arrows.

Backgating to Confirm Gating Strategies

Backgating is a useful method of identification of cells to confirm a staining pattern or gating method. It allows you to analyze cells identified in a gate on dot plots with different parameters. This can be useful if you are unsure of your gates, the expression levels, non-specific binding, whether you have identified your cells of interest or the presence of dead cells and need additional information to identify your cells (Figure 18).

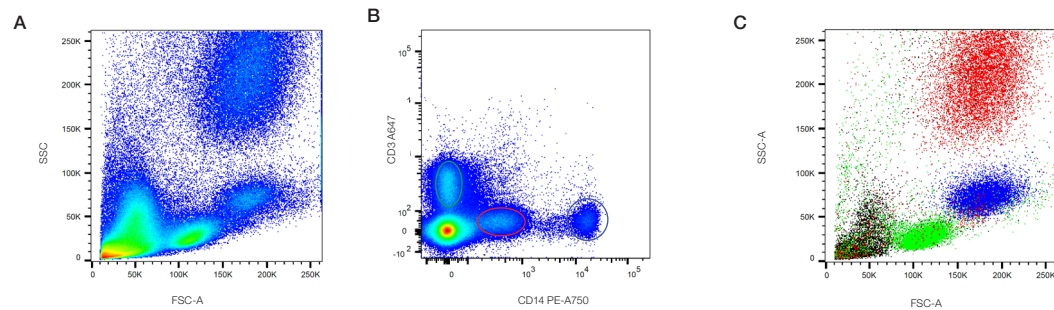


Fig. 18. Backgating to identify leukocyte subsets. A. Red cell lysed whole blood. B. Stained for CD3 and CD14. C. Cells in the green, blue and red gates were backgated onto FSC vs. SSC to confirm leukocyte populations.

Gating does not need to be a daunting process and by following just a few simple steps you can quickly begin to analyze specific cell populations. As you increase the number of stains and fluorophores you will be able to identify more specific cell populations. However, make sure you perform the right controls and have an adequate sample size because as you increase the fluorescence you increase the background and non-specific binding, making the data harder to analyze.

4 Controls in Flow Cytometry

Unstained Controls

One of the first things to identify in flow cytometry is your cell population. This can be done by its forward and side scatter characteristics or using side scatter plus a marker such as CD45 for cell population identification. After this you need to determine the location of the negative population. To do this you should always use an unstained sample. This will allow you to determine the level of background fluorescence or autofluorescence and set your voltages and negative gates appropriately.

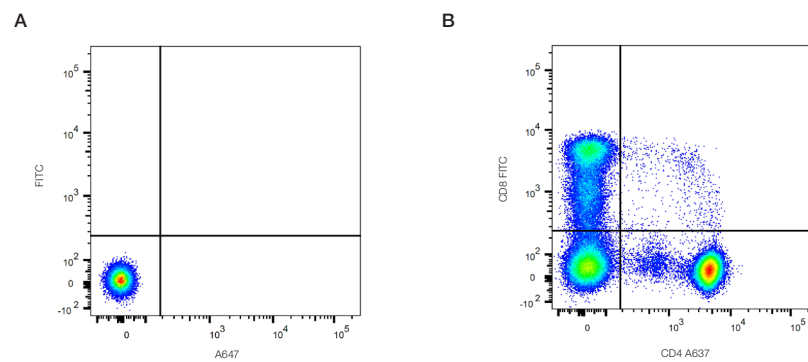


Fig. 19. Unstained controls. Unstained lymphocytes (A) are used to determine the background autofluorescence to set the negative population allowing stained cells to be visualized (B).

Isotype Controls

Isotype controls are antibodies raised against an antigen not found on the cell type or sample analyzed. They have been developed for surface staining and their role is to ensure the observed staining is due to specific antibody binding to the target rather than an artifact.

An isotype control will:

- Determine the non-specific binding of an antibody to Fc receptors found on monocytes, macrophages, dendritic and B cells
- Ensure the observed staining is due to specific binding rather than an artifact
- Reveal other non-specific binding of the antibody or fluorophores to cellular components (e.g. RPE and FITC, Takizawa et al, 1993, Hulspar et al. 2009)

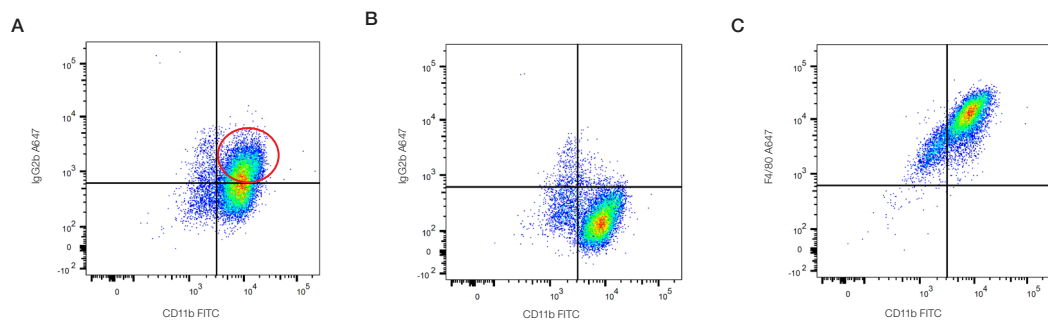


Fig 20. Isotype controls. J774 macrophages were stained in the absence (A), or in the presence (B and C) of Fc block for CD11b and F4/80, or its isotype, in the presence of 7-AAD to exclude dead cells. Without the Fc block there is background staining denoted by the red circle.

The most appropriate isotype control matches:

- The host species
- Ig subclass
- Fluorophore of the primary antibody

If you are using a mouse IgG1 monoclonal antibody that is conjugated to FITC, you should select a mouse IgG1 isotype control conjugated to FITC.

As the fluorophore conjugation to the antibody (known as the F/P ratio) can vary between suppliers, it is best to purchase the isotype from the same supplier as the primary. It is also advisable if possible to use them at the same concentration as the primary antibody.

Single Staining and Compensation Controls

As mentioned in Chapter 2, single staining will reveal the level of spectral overlap between different fluorophores and allow you to remove or compensate for this overlap (Chapter 2, Figure 12). This spectral overlap should be compensated for every fluorophore used.

The important rules to remember when using single stained samples for compensation 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. 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.
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. About 5,000 events for both the positive and negative population is ideal, but less can be used if necessary.

Fc Blocking Controls

Fc receptors are found on monocytes, macrophages, dendritic cells and B cells. As the name suggests they bind antibodies via their constant Fc domain rather than the antigen specific Fab domain.

This type of binding can lead to false positives and meaningless data. In order to prevent this type of binding, Fc blocking reagents (e.g. Human Fc Seroblock and Murine Fc Seroblock) have been developed which, when added to a staining protocol, can ensure that only antigen specific binding is observed.

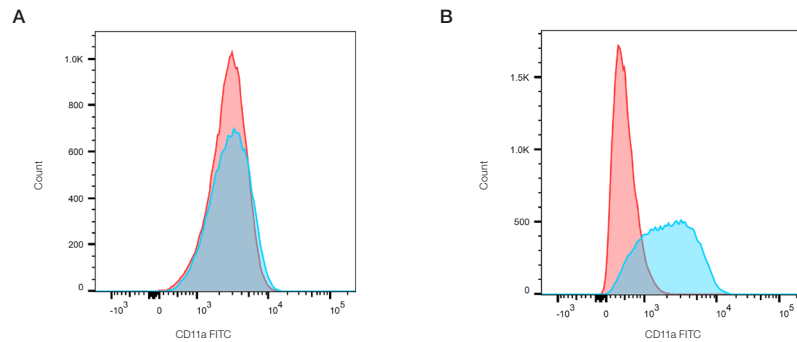


Fig 21. Fc blocking. THP-1 cells stained with mouse anti human CD11a (blue) or mouse IgG2a isotype control (red) in the absence (A) and presence (B) of human Seroblock (BUF070A).

Fluorescence Minus One Controls

Fluorescence minus one (FMO) controls are important when building multicolor flow cytometry panels as they will help you determine where your gates should be set. When acquiring data, there is fluorescence spread, especially with brighter fluorophores, which is particularly noticeable after compensation and cross-laser excitation. FMO controls are the experimental cells stained with all the fluorophores minus one fluorophore. An example of an FMO matrix is shown below in Table 4. Figure 22 shows how the fluorescence spread from other channels can affect the data ensuring you can position your gates accordingly, taking into account the fluorescence spread.

FMO controls should be done for all the fluorophores in your panel when starting a new multicolor experiment. This will then allow you to assess the spread of all the fluorophores into your missing channel and set your gates accordingly.

Table 4. FMO Matrix

Tube	FITC	PE	PE-Cy5	PE-A750
Unstained	-	-	-	-
FITC-FMO	-	+	+	+
PE-FMO	+	-	+	+
PE-Cy5-FMO	+	+	-	+
PE-A750-FMO	+	+	+	-

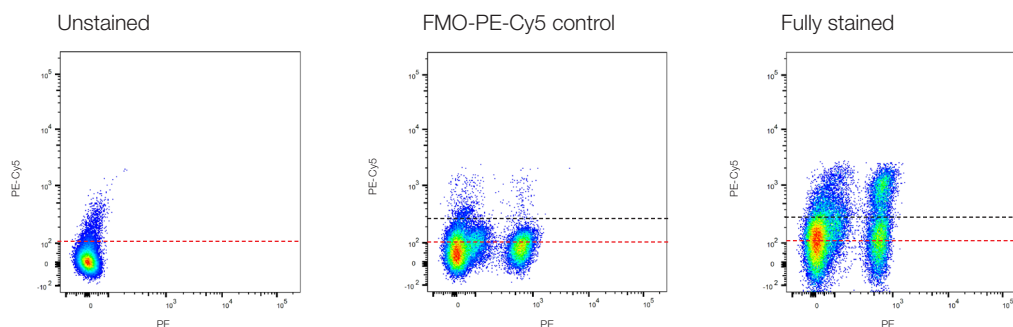


Fig. 22. FMO controls to determine fluorescence spread. Dot plots of multicolor flow cytometry showing the fluorescence spread into the PE-Cy5 channel shown by the FMO control compared to an unstained control. Black dotted line represents FMO gating boundary compared to unstained boundary in red.

Intracellular Staining Controls

Isotype controls have been optimized for cell surface staining to control for non-specific binding of antibody and fluorophore. They are therefore only one of several controls that may be performed to accurately detect your positive population. Because intracellular staining requires fixation and permeabilization which can affect antigen detection, autofluorescence, fluorophore brightness and cell morphology, other controls are necessary. To find out more about optimizing your intracellular staining go to our intracellular staining tips page ([bio-rad-antibodies.com/intracellularflow](https://www.bio-rad-antibodies.com/intracellularflow)).

Biological Controls

Biological controls are important for all staining, but are especially needed in intracellular staining which can have higher background fluorescence. Controls include known negative samples and known positive samples. Examples of these are: cells known from the literature to either express or lack expression of the antigen of interest, cells where the antigen has been knocked down or out, using RNAi or CRISPR technology to produce a negative cell, or cells which have been transfected and the antigen is being over expressed to ensure positive staining. For some experiments such as cytokine release measurement, an unstimulated and fully stimulated sample is important to determine both positive results and the dynamic range of fluorescence staining.

5 Optimizing your Experiments

Sample Preparation

There are many considerations for sample preparation to optimize your flow cytometry. The first one is to take into account your starting sample. Frozen cells will need to be treated differently to an adherent cell line for example; however there are some basic rules that can be followed for most samples that will help you optimize your experiments.

1. Defrost cells as quickly as possible and remove the DMSO by resuspending in a large volume of cold media or PBS containing BSA or FCS. Cells may need to go into culture after defrosting to restore epitope expression.
2. Adherent cells may need gentler treatments than those required for passaging. Trypsin is a harsh treatment that can often destroy cells, creating lots of cell debris, as well as destroying epitopes. More gentle methods of creating single cell suspensions may be needed.
3. Take care not to be over aggressive when using mechanical disaggregation of tissues such as spleen or lymph nodes. Filtering the sample can help to remove any unwanted clumps of cells.
4. The extraction of some cells from secondary lymphoid tissue, e.g. F4/80 positive macrophages and Follicular DCs, requires additional enzymes such as collagenase or liberase. However there may also be some unwanted removal of epitopes so optimization may be required.
5. Remove any unwanted contaminating material. For example when flushing bone marrow from bones, remove as much muscle as possible. Again filtering can remove any unwanted bone and muscle.
6. Use the appropriate anticoagulant for peripheral blood. EDTA should not be used when detecting intracellular cytokines or some surface markers that require Ca^{2+} ions such as integrins.

7. Removal of contaminating red blood cells from peripheral blood samples can be performed using hypotonic lysis using a red cell lysis buffer such as Erythrolyse (BUF04B). Care needs to be taken not to leave the samples for too long in the buffer. Alternatively a density gradient can be used. After centrifugation leukocytes are trapped at the interface of the higher density liquid whereas red cells pass through. Unfortunately granulocytes also pass through the interface so this method is not suitable if this is the cell population you are interested in.
8. Avoid vortexing and excessive centrifugation of your samples and take care to avoid leaving the cells as a dry pellet. Excessive bubbles when resuspending can increase cell death as can suspension at high concentrations. Keeping your cells on ice can improve the viability as this slows down necrosis and the cell metabolism.
9. All sample preparation should be as short as possible as the time taken to prepare your cells can have a large effect on the cell viability.

Live/Dead Exclusion

The presence of dead cells in your sample can greatly affect your staining and therefore the quality of your data. This is because dead cells have greater autofluorescence and increased non-specific antibody binding, which can lead to false positives and reduce the dynamic range. This may make identification of weakly positive samples and rare populations difficult. Whilst using gates based on the forward and side scatter can help to remove debris and dead cells it will not exclude them all. Because of this, dyes have been developed to distinguish live from dead cells.

One group of viability dyes are the nucleic acid binding dyes. Examples of these include propidium iodide (PI) and 7-AAD which are excitable by both the 488nm and 561nm lasers. When they bind to double stranded nucleic acid they fluoresce. They are excluded by live cells, as these dyes are not membrane permeable. They can be added directly to samples after being stained with antibodies and after a brief incubation acquired as normal. The dead cells can then be identified and removed from the final analysis by gating on the unstained population (live cells). As these dyes rely on membrane integrity it is not possible to fix the samples.

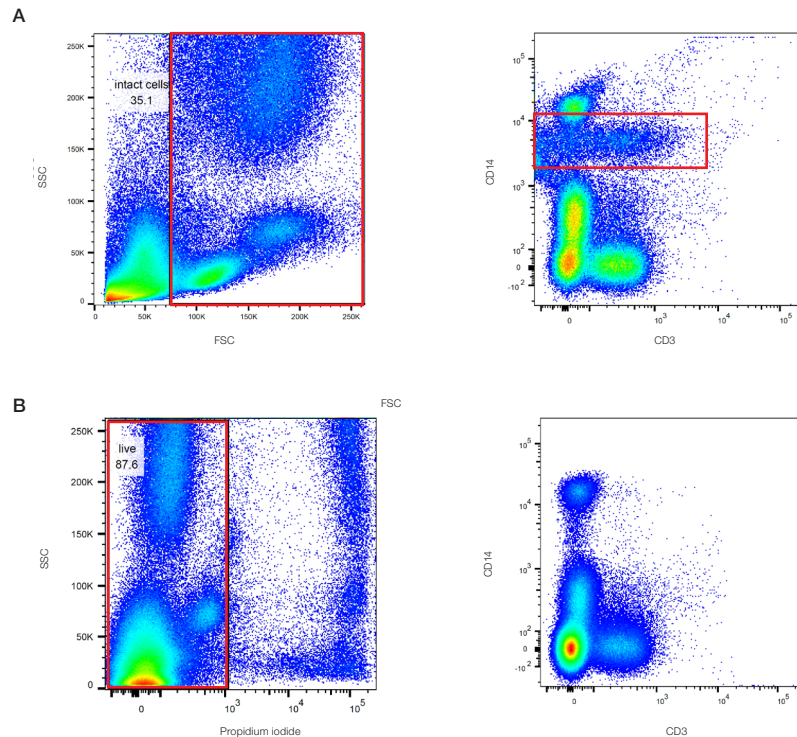


Fig. 23. Using a live/dead stain can improve your staining. **A.** Use of forward and side scatter gating (red rectangle) may not remove all dead cells and some non-specific binding may still be present. **B.** Exclusion of dead cells using propidium iodide staining (red rectangle) means less non-specific binding and easier identification of positively stained populations. Images shown here are human peripheral blood stained with CD14 and CD3.

There are a second group of viability dyes available to discriminate dead cells from your samples. These are protein binding dyes rather than DNA binding dyes. These dyes will bind to both live and dead cells. However when a cell has a compromised membrane as seen in dead and dying cells there is access to a greater amount of protein therefore they have higher fluorescence. Similar to the DNA binding dyes, the dead cells can be excluded by gating on the less stained population (live cells).

The benefit of these dyes is that once the cells are stained with the viability dyes they can be fixed (they can also be used unfixed) without any reduction in the resolution between live and dead cells. In addition, they are available in a broader range of excitation and emission spectra than DNA binding dyes for convenient addition to multi-color flow cytometry panels.

Autofluorescence

Cells have a natural level of fluorescence, called autofluorescence, which can be a problem in flow cytometry data analysis. Cellular autofluorescence can be due to the presence of collagen and elastin, cyclic ring compounds such as NADPH and riboflavin, aromatic amino acids and cellular organelles such as mitochondria and lysosomes. The level in cells can vary due to variances in the levels of these cellular compounds and organelles that give rise to the fluorescence. In general, larger cells and more granular cells have increased autofluorescence due to an increase in the number of fluorescent compounds.

Most autofluorescence is detected at shorter light wavelengths with most absorbing at 350-500 nm and emitting at 350-550 nm. Autofluorescence can therefore be a problem in these light ranges as the signal to noise ratio is increased resulting in decreased sensitivity and false positives. In addition autofluorescence spillover into another channel can mask low expressers.

The level of autofluorescence can be determined using unstained controls. As there is less autofluorescence at longer light wavelengths, fluorophores which emit above 600 nm will have less autofluorescence interference.

Doublet Discrimination

Doublet discrimination is to ensure you count single cells and exclude doublets from your analysis. This can be critical in cell sorting, cell cycle and DNA analysis. If a doublet containing a fluorescence positive and negative cell passes through the laser it will produce a positive pulse leading to false positives in both analysis and sorting experiments.

Doublet exclusion is performed by plotting the height or width against the area for forward scatter or side scatter (Figure 24). Doublets will have double the area and width values of single cells whilst the height is roughly the same. Therefore disproportions between height, width and area can be used to identify doublets.

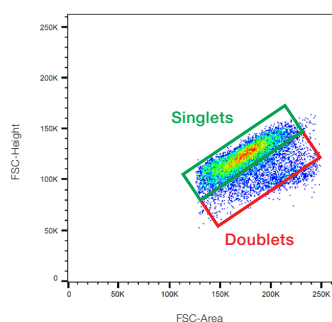


Fig. 24. Doublet discrimination. Doublets (red) can be distinguished from single cells (green) by plotting FSC height vs FCS area. Doublets have increased area whilst similar height to single cells.

Removal of doublets is also crucial in cell cycle analysis. It is important to distinguish between single cells that have double the amount of DNA and doublets as they will both show increased fluorescence when stained with a DNA dye such as propidium iodide. Fortunately using the height or width vs area allows you to separate the doublets from the single cells containing 4n amounts of DNA. Cells containing 4n amounts of DNA have double the area and height values whilst the width is roughly the same as cells containing 2n amounts of DNA.

Collect a Statistically Relevant Number of Cells

The number of cells you need to collect during analysis, to have statistically significant results, can vastly differ depending on the sample and frequency of your cells. If you have a sample with an abundant cell type such as T cells in human peripheral blood, which represent around 20% to total mononuclear cells, you will have to collect and stain less cells than if you are looking at NK cells which have a frequency around 5% or less. The table below shows an example of how the frequency of cells can affect the number of cells collected.

Table 5. Cell Frequency

Starting Population	Frequency	Number Collected
1,000,000	10%	100,000
1,000,000	1%	10,000
1,000,000	0.1%	1,000

In addition to the number of cells, the number of markers simultaneously detected to look at cell subsets can affect the number of cells that are needed to be acquired; generally an increase in markers requires more cells. Finally performing the right controls, to determine the variation and allow definition of a positive or negative, is also very important. More detailed information on collecting enough events can be found in an article by M Roederer in Cytometry Part A. (Roederer M (2008). How many events is enough? Are you positive? Cytometry, 73A:384-385).

Permeabilization and Fixation for 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 antibody. The choice of fixative is an important first step. Formaldehyde and glutaraldehyde create bonds between lysine residues resulting in cross-linked proteins, however glutaraldehyde increases autofluorescence. The fixative is usually used at concentrations of 0.5-4% in PBS depending on the sample. If you are going to store your samples for longer periods of time they should be removed from the fixative after 1-2 hours.

Formaldehyde will not permeabilize the samples so a separate permeabilization step is needed. This allows probes to access intracellular structures while leaving the morphological scatter characteristics of the cells intact. Triton, digitonin and saponin are examples of permeabilization reagents which act by disrupting the cellular membrane. The level of permeabilization is important as epitope access may require different levels of permeabilization (e.g. cytoplasmic vs nuclear epitopes) and unbound antibody has to be sufficiently washed out of the cells. There are also many commercial kits available today that provide the reagents to carry out both fixation and permeabilization, for example, Leucoperm™ (Figure 25).

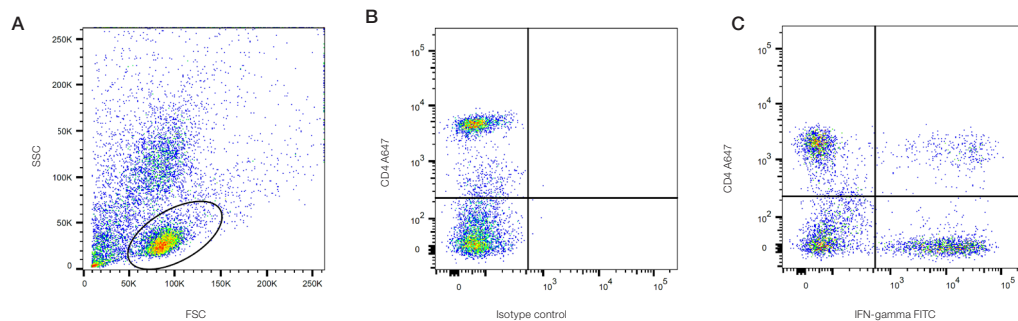


Fig. 25. Intracellular staining. Human whole blood (A) was surface stained for CD4 followed by intracellular staining for IFN-gamma, on resting lymphocytes (B) or lymphocytes stimulated with PMA/ionomycin and treated with monensin (C) prior to staining.

Alcohols are also used as fixatives, typically as a cold 70% solution, that fix by denaturing proteins. The benefits here are that they also permeabilize the cell membrane and are suitable for long term storage at 4°C or -20°C. Epitopes can however be masked by the denaturing process with alcohol fixation, so optimization may be required. Alcohols as a fixative are most commonly used for DNA analysis.

6 Multicolor Panel Building

Resolution of Signal

Multicolor flow cytometry is the terminology used when analyzing multiple fluorescent parameters in one sample, may it be surface markers, intracellular markers, DNA or all combined. In addition to ensuring the right controls (discussed in Chapter 4), optimizing your experimental procedure (discussed in Chapter 5) and careful sample preparation, there are a few other considerations which should be taken into account to ensure meaningful results can be obtained. This is because each additional fluorophore you add to your flow cytometry panel has the potential to influence another fluorophore. The result of this is unwanted fluorescence in additional channels which has to be compensated for (discussed in Chapter 2) and a loss in resolution. High levels of noise caused by non-specific staining, high background staining, cell autofluorescence and spillover can all contribute to reduction in sensitivity and resolution.

Resolution can be described as the potential of an instrument together with a combination of fluorophores to separate a positive population from a negative population. To ensure optimal resolution there are a few simple rules that can be followed that will help form the basis of all panel design.

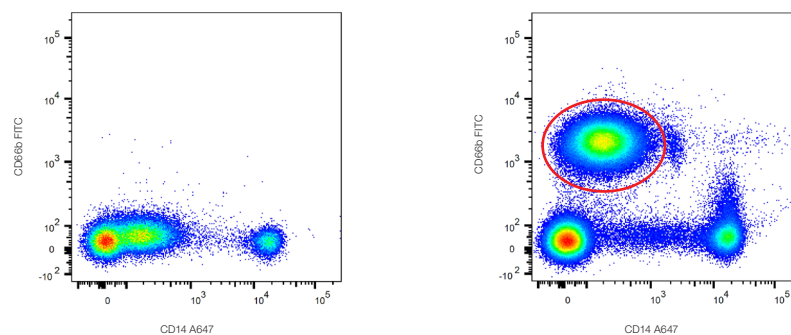


Fig. 26. Cell population resolution. Using an additional marker (CD66b FITC), the granulocytes (circled in red) that express low levels of CD14 A647 can be resolved from lymphocytes which are negative for CD14 and CD66b.

Instrument Configuration

Instrument configuration is important to understand before you start to build your panel. You simply cannot use a fluorophore your instrument is not configured for, regardless of which is the theoretical best fit. Instrument configuration is simply the set-up of the lasers, optics and filters that are contained within the cytometer. This can vary significantly between cytometers. For instance, the S3e™ Cell Sorter from Bio-Rad has 2 lasers and 4 fluorescence detectors, whereas the ZE5 Cell Analyzer has 5 lasers and the potential to simultaneously detect 28 fluorophores.

Fluorophore Separation

Ideally, when building multicolor panels, it is best to separate fluorophore excitation across lasers, and where possible, the emission across the detectors. This will minimize the amount of spillover and therefore compensation you will need to do. It will also reduce the effect that fluorescence spread will have on your data. However as you increase the number of fluorophores in your panel, this will not always be possible. Therefore other considerations need to be included in your design.

Antigen Density

The relative antigen density is of vital importance when choosing your fluorophores. As a general rule it is best to match bright fluorophores (e.g. PE) with low expressing markers and dimmer fluorophores (e.g. Pacific Blue) with highly expressed markers. Spread from a bright fluorophore will mask low level fluorescence in nearby channels. Careful choice of fluorophore will help with the resolution of your cell populations. Be careful however as fluorophore brightness can be a double edged sword. If you have a bright fluorophore and an abundant antigen this will create more spillover into adjacent channels, possibly masking any true signal into that channel from other markers.

Fluorophore Properties

You should be aware of cross laser excitation, for example APC can be excited by the 405 nm laser as well as a 640 nm laser. In addition to the relative brightness of fluorophores the amount they spread is important. In general, brighter fluorophores will have greater spread and this can be more pronounced at longer wavelengths. In addition, although tandem dyes will give greater flexibility across lasers, allowing increased multiplexing capability, they should receive special care and attention. Take into account the possible donor fluorophore emission, e.g. for PE tandems there may be some emission at 578 nm. The acceptor excitation and emission should also be taken into consideration. Careful handling of tandem dyes, as they are sensitive to light and fixation, to avoid breakdown is recommended. In addition there can be lot-to-lot variation due to variation in the quantity of acceptor dyes on the donor molecule.

Marker Expression Patterns

One common method of reducing the effect of spillover and spread is to carefully choose your fluorophores based upon the expression pattern of your antigens. This can be done by placing fluorophores with spillover onto mutually exclusive markers, e.g. CD3 and CD19. The effect of the spillover can be compensated fairly easily. In contrast for markers where there is co-expression, such as cell subsets or unknown expression levels, fluorophores with minimal spillover should be chosen. This should also be the case for antigens where the expression pattern is continuous such as those seen in activation markers. Another way of minimizing the effect of spillover is to use the parent descendant rule. This is where the spillover does not matter as the marker is expressed anyway and the relative amount may not be important. An example of this could be spillover of CD4 fluorescence into the CD3 channel in T cells. As all the T cells will express CD3 anyway it does not matter.

Dump Channels

A dump channel, as the name suggests, removes all the unwanted sample by placing it in a channel that will be ignored. This is particularly useful when looking at rare cells, such as hematopoietic stem cells, as any cells that are not required can be excluded by labeling all the cells with one fluorophore. Often the easiest way to do this is to use biotinylated primary antibodies and a streptavidin secondary of whatever color is being used for the dump channel. The viability stain can also be included in this channel for convenience as the negatives will be the live cells. If you remove the cells you are not interested in, any unwanted binding or fluorescence spillover and spread caused by those cells, will also be removed.

Antibody Titration

Another important consideration when building multicolor panels is titration of your antibodies. Excess antibody will bind at low affinity and create background that will reduce the resolution and therefore cloud your results. In addition too much antibody may result in a false negative prozone effect. It is therefore important to determine the right amount of antibody needed for your specific sample. To determine the best antibody concentration, the stain index, which is defined as the ratio of the separation between the positive and negative population divided by two times the standard deviation of the negative population, can be used as a guide (Figure 27).

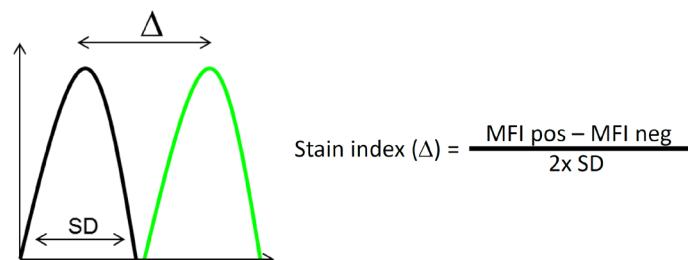


Fig. 27. Stain index. The stain index is the ratio of the separation between the positive population (green) and the negative population (black), divided by two times the standard deviation of the negative population.

Titration requires dilutions of antibody to be made and the same number of cells stained in the same volume. The dilution that represents the best stain index is the dilution to use. In the graph below, the points in the green box (Figure 28) represent the best concentrations that will generate specific staining with the least amount of background.

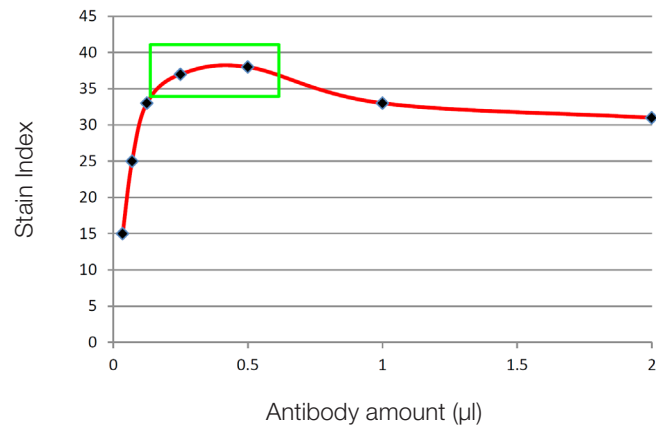


Fig. 28. Antibody titration. Plotting the stain index for each concentration of antibody will allow you to titrate the optimal amount of antibody for your experiment.

Panel Building Tools

There are useful tools that can help with panel design. Spectra viewers will help you determine the amount of spillover and excitation by each laser. Relative brightness tables will give you information on pairing targets with fluorophores and marker expression data will help to determine expression patterns. Panel building websites can help with panel design and there are published examples of optimized multicolor immunofluorescence panels (OMIPS) which will also help with your panel design.

7 Common Applications and New Technology

Immunophenotyping

The most common use of flow cytometry is in the identification of markers on cells, particularly the immune system or immunophenotyping. Immunophenotyping (Figure 29) can be simply identifying a cell by a single marker or more complex identification of cells, using homing profile, activation states and cytokine release all in one panel. As a consequence, experimental protocols are often a combination of surface and intracellular staining. In addition to basic research, immunophenotyping is now routinely used in clinical applications to diagnose disease or monitor and evaluate residual disease. Additional information in a more complex panel may include the subsets of T cells, e.g. T helper, T cytotoxic and T regulatory, using markers such as CD4, CD8, CD25, CD127 and FOXP3, whether they are naïve, memory or activated, using markers such as CD45RA, CD45RO, CD62L, CD69 and HLA-DR and their cytokine profile such as IFN- γ , IL-2, IL-17, IL-9. It is easy to see how an 18 color panel can be quickly built. Refer to Chapter 6 for more information on building multicolor panels.

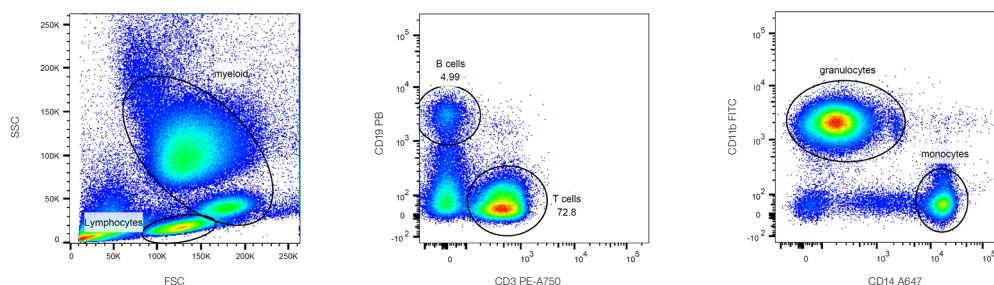


Fig. 29. Immunophenotyping of whole blood. Simple 4 color immunophenotyping panel using CD3, CD19, CD66b and CD14 to identify T, B, granulocyte and monocytes respectively.

Apoptosis

One of the most common features of apoptosis that can be measured by flow cytometry is externalization of phosphatidylserine (PS), a phospholipid found in the inner membrane of healthy cells. Annexin V binds to phosphatidyl serine and thus annexin V labeled with fluorophores allow apoptosis to be assessed, usually in combination with a viability dye such as propidium iodide (PI) to distinguish apoptotic from necrotic cells (Figure 30).

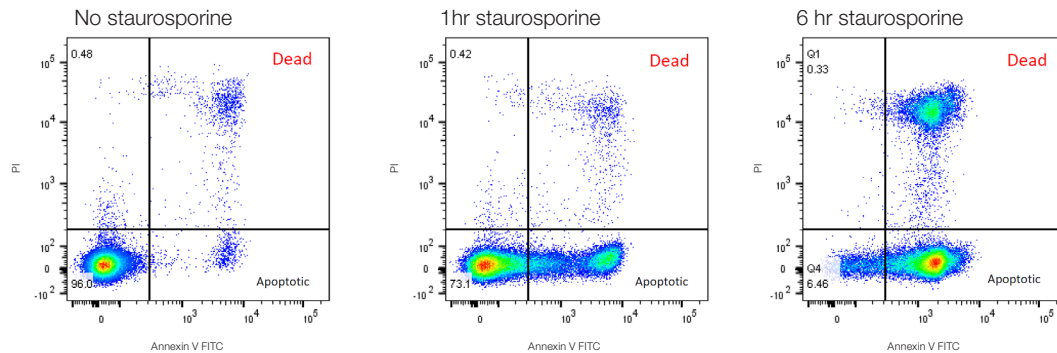


Fig. 30. Annexin V staining to measure apoptosis. Jurkat cells were treated with staurosporine at 1 mM for 0 hr, 1 hr and 6 hr to induce apoptosis. The cells were then stained with annexin V FITC (ANNEX300F) and ReadIDrop™ propidium iodide (1351101). Apoptotic cells positive for annexin V can be seen in the bottom right quadrant and dead cells positive for both annexin and PI in the top right quadrant. Healthy cells are negative for both stains.

DNA fragmentation, which occurs during the late stages of apoptosis, can also be measured by flow cytometry using the sub-G1 assay. The small, ~180 bp, DNA fragments generated during apoptosis leak out of cells, decreasing the total DNA content of apoptotic cells. By staining DNA with PI, hypodiploid apoptotic cells can be counted in the sub-G1 peak of the PI histogram. Staining with DNA markers will also allow measurement of cell shrinkage in combination with a reduction in the FSC signal.

Early apoptosis can also be measured by potentiometric dyes which assess the reduced mitochondrial potential of cells. Examples of these include; tetramethylrhodamine ethyl ester (TMRE), tetramethylrhodamine methyl ester (TMRM), and JC-1. These lipophilic dyes aggregate in mitochondria of non-apoptotic cells and brightly fluoresce. When the mitochondrial membrane potential collapses the dye disperses into the cytoplasm in its monomeric form leading to reduced fluorescence or a change in color. These dyes can be combined with other apoptosis markers such as fluorophore labeled inhibitor of caspase assays (FLICA) which fluoresce in the presence of caspase and with antibodies against specific caspases.

Proliferation and Cell Cycle

Cell proliferation can be measured by flow cytometry using several methods. One method is to stain with an antibody against a proliferation marker such as Ki67, MCM2 or PCNA. Alternatively you can incubate your cells with BrdU, which incorporates into DNA during S-phase of the cell cycle. Incorporated BrdU can be detected using fluorescently labeled anti-BrdU antibodies. When combined with a DNA stain such as PI or DAPI, the relative proportion of cells in S-phase can be determined (Figure 31).

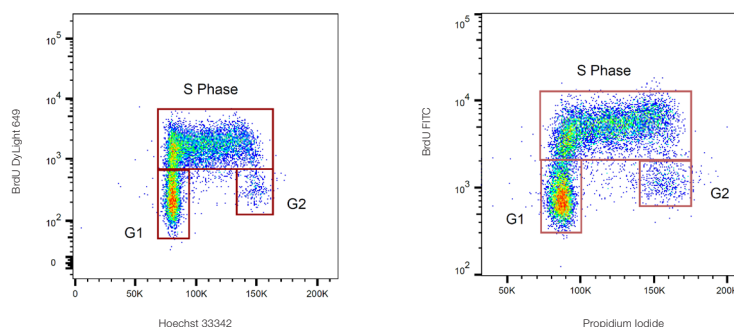


Fig. 31. BrdU staining for proliferation. Proliferating cells were stained for incorporated BrdU against total DNA content using A. Hoechst or B. Propidium Iodide. BrdU was detected by labeling a mouse primary anti-BrdU antibody (MCA2483) with the appropriate secondary antibody attached to a fluorophore.

In addition to using antibodies, cytoplasmic dyes, such as CFDA-SE, can be used to measure proliferation. Cells are incubated with the protein binding dyes and as the labeled cells divide, the concentration of the dye is halved and the proliferation measured based upon the reduced levels of fluorescence in subsequent generations. The advantage of these dyes is that, as they are non-toxic and available in a wide variety of colors, they can be combined with immunophenotyping and do not require the sample to be fixed.

The proportion of cells within each stage of the cell cycle can also be determined using DNA binding dyes such as PI, 7-AAD, Hoechst 33342 and DAPI that bind in a stoichiometric manner. This way cells in G2, which have twice as much DNA as cells in G1, will fluoresce twice as bright. To ensure good staining the cells should be fixed in cold 70% ethanol. This can interfere with other staining protocols, see our protocols section for advice on the different methods.

Signaling and Phosphoflow

Flow cytometry offers a quick and effective way to measure signaling at a specific time point in individual cells. The staining methods for detecting signaling molecules and phosphorylated proteins may differ from normal intracellular staining and specific experimental controls may be required. Typically cells need to be rapidly fixed to avoid dephosphorylation and stronger permeabilization methods may be required to ensure permeabilization of the nuclear membrane. As with cell cycle staining, fixation and permeabilization may alter your ability to measure cell surface molecules. Care should be taken with the buffers used as they can interfere with signaling e.g. EDTA. Signaling can also be measured using dyes which fluoresce in response to changes in calcium. Cells are loaded with dyes such as indo-1 to determine a baseline level of signaling and then stimulated. The change in fluorescence denotes a change in intracellular calcium levels.

Small Particle Detection

The type of small particles now being detected and evaluated by flow cytometry is increasing. Small particles can include platelets, which are typically 2-3 μm in diameter, bacteria, which can range from 0.3 μm -5 μm and cellular extravesicles, which can be further split into apoptotic bodies, microvesicles and exosomes, with the smallest being exosomes which are as little as 50 nm in diameter. Exosomes are thought to be important in cross-talk and regulation of cells by transferring proteins and RNA between cells. Detection of these particles can be problematic as light scatter depends on the particle diameter, the wavelength of the interrogation light (it is difficult to detect particles smaller than the wavelength), the particle refractive index, the angle of collection and the intensity of the laser. To account for this cytometers have been developed that have extra PMT detectors in the forward scatter (e.g. the ZE5 from Bio-Rad) and the trigger for data collection changed to a fluorescent signal or multiple fluorescent markers. Alternative methods for enhanced detection include using shorter light wavelengths, as this generally results in increased scatter, and using antibody coated beads to increase the size of the particle being detected. Care should be taken to reduce the noise by filtering the sheath fluid and carefully setting the threshold level. It should be noted however that as the particles decrease in size the available antigen will also reduce leading to decreased sensitivity or resolution.

Gene Expression and Transfection

Fluorescent proteins to determine gene expression and transfection efficiency in both live and fixed cells are widely used in flow cytometry, and are particularly useful when performing cell sorting experiments. They can be used as reporters of transcription factors, promoter activity and cellular expression patterns as well as screening for RNAi and CRISPR activity due to the high throughput capacity of flow cytometry. Initially only green fluorescent protein, there are now over a hundred fluorescent proteins which excite and emit at various wavelengths, making them perfect for multicolor flow cytometry.

Absolute Quantification

Although flow cytometry can quantify expression of markers both on and in cells they do not provide information on the cell concentration or necessarily absolute quantification. To overcome this, fluorescent beads can be added and counted. If a known amount of beads at a known concentration are added to your sample and acquired, the number of beads will be relative to the number of cells. Some cytometers can give accurate cell counts by measuring the volume of sample acquired and in this case the number of cells per μl can be measured.

Particle Internalization

Internalization of particles, cell surface markers and antigens can occur through various cellular processes, e.g. phagocytosis. Flow cytometry has proved to be an effective method of quantifying this through fluorescently labeling the particle that is to be internalized. Utilizing dyes which either alter their fluorescent characteristics when internalized, or by quenching surface bound fluorescence, the difference between surface and internalized particles can be measured.

Fluorescence *in situ* Hybridization and RNA Detection

Fluorescence *in situ* hybridization (FISH) was first performed in flow cytometry in the late 1990's to determine telomere length. Fluorescent nucleic acid probes were used to highlight specific repeat sequences and then fluorescence measured using specific software. Since then RNA expression protocols have been developed which allow quantification of the levels of mRNA. This is a powerful tool as it can be performed in combination with surface staining to identify specific cells and subsets, whereas quantitative RTPCR, whilst very sensitive, will only give information on a cell population.

Innovations in Flow Cytometry

Flow cytometry has become more accessible to researchers through a reduction in the complexity of instrument set up, true automation, increased sensitivity and more user friendly software. Although multicolor flow cytometry using fluorescent markers is still one of the most powerful tools in research, there are some new innovations. Imaging flow cytometry allows capture of images of the particles as they pass through the laser using a CCD camera. Multiple (spectrally different) images can be captured simultaneously allowing composites to be made and analysis of antigen location to be determined.

Another innovation is mass cytometry. The introduction of multiple laser containing flow cytometers capable of detecting 28 fluorescent parameters e.g. the ZE5 from Bio-Rad, has vastly increased the complexity of fluorescent flow cytometry experiments. Mass cytometry however has the capability to detect in 135 channels, allowing very large multiplex panels to be built and currently over 40 markers can be measured per cell. Mass cytometry relies on labeling the samples with antibodies bound to metal isotopes which can then be measured by analyzing the time each isotope takes to pass through an electric field towards the detector. The larger the isotope the longer it takes. Sample acquisition is slower with mass cytometry and as the cells are vaporized only analysis can be performed, however there are fewer problems with spillover and compensation. Analysis of the sample can however be time consuming and problematic as it requires specialized software due to the number of parameters that can be collected on one cell.

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).
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- Takizawa et al. (1993). J Imm. Methods Jun 18; 162(2):269-72.
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8 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 13).

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 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'-tetra acetic 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

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.

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

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.

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** (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.

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** (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.

Preparation of Adherent Tissue Culture Cell Lines

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

Reagents

- **Phosphate buffered saline** (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.

Preparation of Human Peripheral Blood Mononuclear Cells

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

Reagents

- **Phosphate buffered saline** (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.

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** (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.

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 are light sensitive and therefore exposure to light should be minimized.

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

Reagents

- **Phosphate buffered saline** (BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- **Erythrolyse red blood cell lysing buffer** (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 µl 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 µl cold (4°C) PBS or with 200 µl 0.5% paraformaldehyde in PBS if required.
6. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

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** (BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- **Erythrolyse red blood cell lysing buffer** (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 µl 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 µl cold (4°C) PBS or with 200 µl 0.5% paraformaldehyde in PBS if required.
8. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

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 methanol modification is recommended.

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

Reagents

- **Leucoperm Accessory Reagent** (BUF09). Includes Reagent A (cell fixation agent) and Reagent B (cell permeabilization agent)
- **Phosphate buffered saline** (BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- **Erythrolyse red blood cell lysing buffer** (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 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.

[Whole blood samples may be used undiluted unless the cell count is high, as in leukemia samples. Use appropriate anticoagulant]
8. Wash once in PBS and then resuspend in 200 µl cold (4°C) PBS for immediate analysis or with 200 µl 0.5% paraformaldehyde in PBS if required.
9. Acquire data by flow cytometry. Analyze fixed cells within 24 hours.

Direct Immunofluorescence Staining of Intracellular Antigens: Methanol plus Leucoperm Accessory Reagent Method

Alternative method for 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 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** (BUF09). Includes Reagent A (cell fixation agent) and Reagent B (cell permeabilization agent)
- **Phosphate buffered saline** (BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- **Erythrolyse red blood cell lysing buffer** (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.

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** (BUF09). Includes Reagent A (cell fixation agent) and Reagent B (cell permeabilization agent)
- **Phosphate buffered saline** (BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- **Erythrolyse red blood cell lysing buffer** (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 µl of blood into as many tubes as required, including 2 extra control tubes, and then add 500 µl 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 µM.
3. To another tube (activated cells), add PMA to a final concentration of 10 ng/ml, ionomycin to 2 µM, and monensin to 3 µM.
4. To the rest of the tubes (experimental samples) add monensin to 3 µM 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. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
8. Wash cells once with PBS/BSA and discard supernatant.
9. Add 100 µl of Leucoperm Reagent A (cell fixation agent) and incubate for 10 min at 2–8°C.
10. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
11. Wash cells once with PBS/BSA and discard supernatant.
12. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.

13. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
14. Wash cells once with PBS/BSA and discard supernatant.
15. Add 100 µl of Leucoperm Reagent A (cell fixation agent) and incubate for 10 min at 2–8°C.
16. Add 2 ml cold (4°C) PBS/BSA and centrifuge for 5 min at 300–400 g at room temperature.
17. 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.
18. Add 2 ml freshly prepared erythrolyse red blood cell lysing buffer to the blood suspension and mix well.
19. Incubate for 10 min at room temperature.
20. Centrifuge at 300–400 g for 5 min and discard the supernatant.
21. 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.
22. 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.

Propidium Iodide Staining of Cells for Cell Cycle Analysis

This method provides a general procedure for DNA staining for cell cycle analysis using propidium iodide (PI). Although other nucleic and binding dyes such as DAPI, Hoechst and 7AAD are available. These are guidelines only and the incubation times may need to be adjusted for different cell types.

Reagents

- Phosphate buffered saline (BUF036A)
- 70% Ethanol in DI water
- Nucleic acid staining solution (1x PBS, 100 µg/ml RNase A)

Method

1. Prepare cells appropriately; refer to the Preparation of cells for flow cytometry section for further information.
2. Fix in 2-5 ml cold (4°C) 70% ethanol. Add drop wise to cell pellet while vortexing. This should ensure fixation of all cells and minimise clumping.
3. Fix for at least 30 min on ice. Note: specimens can be left at this stage for several weeks.
4. Centrifuge at 500 g for 10 min, decant supernatant.
5. Wash twice with 3 ml PBS at 300-400 g and 4°C for 5 min and discard supernatant.
6. Resuspend cell pellet in 500 µl Nucleic acid staining solution. Mix well.
7. Add propidium iodide e.g. 1-2 drops of ReditDrop Propidium Iodide (Bio Rad product code. 1351101)
8. Analyze by flow cytometry. The propidium iodide should be read on the appropriate channel in the linear scale. Doublets should be gated out using the Area vs Height or Width depending on your instrument.

BrdU Staining of Cells for Cell Cycle Analysis and Apoptosis

BrdU is an analogue of thymidine readily incorporated into DNA during DNA synthesis and is an accurate method to monitor proliferation and apoptosis. The following methods were used and provide a useful guide for using anti-BrdU antibodies.

Note: The acid treatment to unwind the DNA may affect surface immunophenotyping. Staining of cells with BrdU using DNaseI may be applicable if this is required.

Reagents

- Phosphate buffered saline (BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- 2 M HCl containing 0.5% Triton X-100
- 0.05% (v/v) Tween -20 in PBS
- Propidium iodide
- 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$, pH 8.5

Method

1. Add BrdU to the cell suspension in culture medium to a final concentration of 10 μM and incubate for at least 30 min at 37°C in a CO_2 incubator.
2. Wash cells twice with PBS/BSA, at 500 g for 10 min at room temperature, decant supernatant.
3. Resuspend in 2-5 ml cold (4°C) 70% ethanol. Add drop wise to cell pellet while vortexing. Fix for at least 30 min on ice.
4. Centrifuge at 500 g for 10 minutes, decant supernatant.
5. Resuspend the pellet in 2 ml of 2 N HCl containing 0.5% Triton X-100. Incubate for 30 min at room temperature (preferably on a rocking platform).
6. Centrifuge at 500 g for 10 minutes, decant supernatant. Resuspend in 3 ml of 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$, pH 8.5 for 2 min at room temperature.
7. Centrifuge at 500 g for 10 min, decant supernatant, and resuspend in room temperature PBS/BSA + 0.05% Tween-20. Adjust cell concentration to 1×10^7 cells/ml.
8. Aliquot 100 μl of the cell suspension into required number of FACS tubes.
9. Incubate with antibody at the recommended vendor dilution overnight at 4°C avoiding direct light.
10. Resuspend in 2 ml of room temperature PBS/BSA. Centrifuge at 500 g for 10 min at room temperature.

11. If a secondary antibody is required, then decant the supernatant, add 100 μ l of PBS/BSA and incubate with the secondary antibody at the vendor recommended dilution for at least 30 min at 4°C.
12. Wash with 2 ml of PBS/BSA, centrifuge at 500 g for 10 min.
13. Re-suspend cells in 1 ml of PBS. Add propidium iodide e.g. 1-2 drops of RaddiDrop™ Propidium Iodide (Bio Rad product code. 1351101).
14. Analyze by flow cytometry. The propidium iodide should be read on the appropriate channel in the linear scale. Doublets should be gated out using the Area vs Height or Width depending on your instrument.

9 Troubleshooting

Problem	Course of Action
No staining	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.	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
Nonspecific staining	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 (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.
Weak staining	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.
Unusual scatter profiles	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.
Unexpected staining	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.

Glossary

Apoptosis – Programmed cell death through various tightly regulated biological pathways.

Antibody – A specialized protein of the immune system that can identify and neutralize specific targets called antigens.

Area – The integral of the pulse.

Autofluorescence – Natural levels of fluorescence found within cells due to the presence of fluorescent compounds.

Backgating – A useful gating control to ensure you have identified the right cell population using traditional gating.

Band pass filter - Allow light through within a specified narrow range.

Cell sorting – The ability to separate cells, identified by specified characteristics, within droplets using an electrical charge.

Compensation – Mathematical algorithm for removing fluorescence spillover of one fluorophore into multiple detectors.

Doubles – Where two particles pass through the laser at the interrogation point.

Drop delay – The time between the interrogation and the point where a droplet breaks off during cell sorting.

Event – Any particle which generates enough signal when it passes through the laser to be recorded as a signal or pulse.

Fc receptors – Antibody receptors on certain cells which bind antibodies via their constant region to elicit immune responses.

Fixation – Crosslinking of cellular proteins to preserve from decay and allow permeabilization without loss of cell contents and structure for intracellular staining.

Forward scatter – Light that is scattered in the forward direction (up to 20°) after interacting with a particle.

Flow cytometer – Instrument which allows the measurement of properties of individual particles as they pass through a laser.

Fluorescence minus one control – Specific control, where one fluorophore is removed from the staining panel, to account for fluorescence spread.

Fluorescent protein – A protein which can accept light energy and re-emit at longer wavelengths and can be expressed in cells for live marking.

Fluorophore – Fluorescent markers that accept light energy at a given wavelength and re-emit at a longer wavelength.

Gating – Placing of regions around populations of cells with common characteristics to quantify and further investigate.

Height – The maximum amount of current output by the PMT of the pulse.

Histograms – Plots which display a single measurement parameter.

Hydrodynamic focusing – A faster outer sheath fluid around the sample stream allows narrowing of the stream creating a stream of single cells.

Immunophenotyping – Identification of cells in a population through the staining and identification of specific markers.

Instrument configuration – The set-up of lasers, optics and filters contained within the cytometer.

Isotype controls – Antibodies raised against an antigen not found on the cell being analyzed to help determine specific antibody binding.

Laser – A device that emits optically amplified light at a single wavelength.

Long pass filter – Allow light through above a certain wavelength.

Maximal emission – The wavelength at which a fluorophore emits the most photons.

Maximal excitation – The wavelength at which a fluorophore is excited the most photons.

Parameter – The measurement from any given detector which can be displayed in height, area or width.

Photomultiplier tube – A photoemissive detection device in which the absorption of a photon results in the emission of an electron.

Permeabilization – Creation of holes in the cell membrane using detergents to allow large molecules (such as antibodies) to enter the cell for intracellular staining.

Resolution – The ability to separate a positive from a negative population.

Short pass filter – Allow light through below a certain wavelength.

Side scatter – Light that is scattered at 90° after interacting with a particle.

Spillover – The overlap of one fluorophore emission spectra with another.

Spread – The increase in a population's fluorescence into another channel after compensation.

Stain index – The point where there is maximal separation between the positive and negative population on stained samples.

Stokes shift – The difference between the maximal excitation and emission wavelengths of a fluorescent molecule.

Tandem dye – A fluorescent dye which is two molecules covalently coupled together.

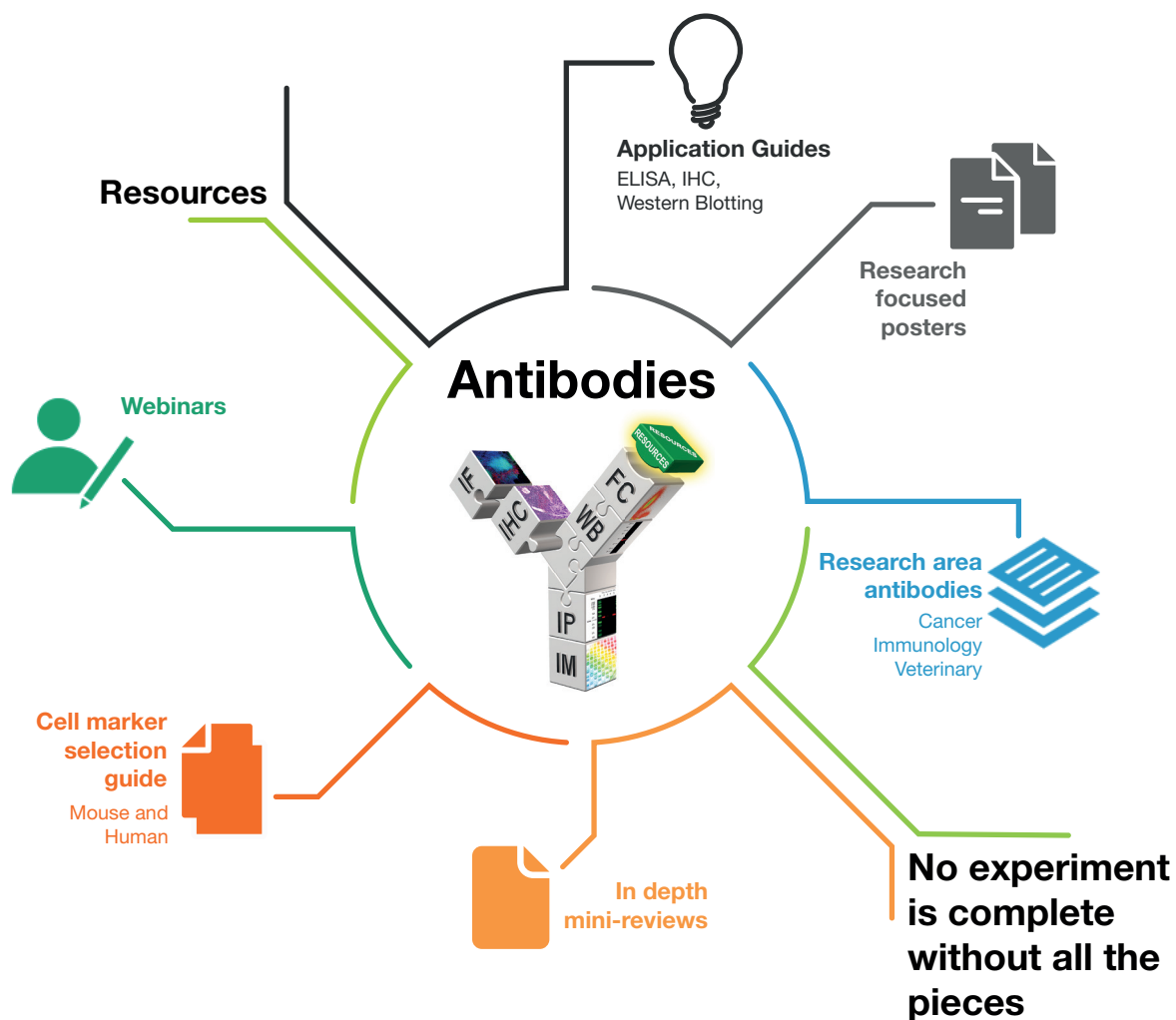
Threshold – Signal intensity below which the flow cytometer does not record an event.

Titration – Dilution of an antibody to the concentration at which there is an optimal stain index.

Viability dye – Dye which allows identification of dead cells through a reduction in cell membrane integrity.

Width – The time interval during which the pulse occurs.

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Notes

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