

Flow Cytometry Basics Guide

Practical Advice to Get You Started in Flow Cytometry



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1. Principles of the Flow Cytometer

A flow cytometer is composed of fluidic, optic, and electronic systems. This chapter explains the role of each system, and how they work together. You will also learn the basics of electrostatic cell sorting.

Fluidics System

One of the fundamentals of flow cytometry is the ability to measure the properties of individual particles as they pass through one or more laser beams. 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 a single file, called hydrodynamic focusing. Under optimal conditions (laminar flow), there is no mixing of the central fluid stream and the sheath fluid.

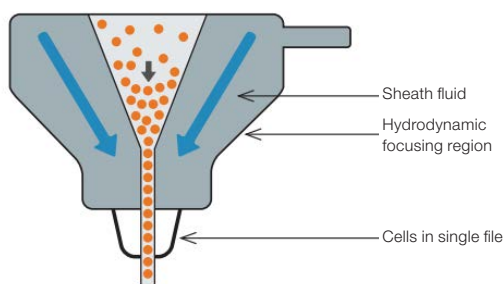


Fig. 1. Hydrodynamic focusing produces a single stream of particles.

Without hydrodynamic focusing, the cuvette (typically 250 x 250 μm or 180 x 480 μm) or instrument nozzle (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 a 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 focused laser beams. Light scattering or fluorescence emission from the particle 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 beam) at a specific frequency. They are available at different wavelengths ranging from deep ultraviolet (UV) to infrared (IR), 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 and 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 particle size because larger particles refract more light than smaller particles. However, this depends on several factors such as the sample, laser wavelength, collection angle, refractive index of the sample, and sheath fluid. The detection of small particles provides a good example. When the particles are smaller than the wavelength of the illumination source, such as a 200 nm exosome illuminated using a 488 nm laser, the light is not necessarily scattered in a forward direction. Therefore, forward scatter may not be a good estimate of size.

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 lymphocytes, monocytes, and granulocytes in peripheral blood. However, FSC and SSC characteristics vary based on the sample type and 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 varies according to the instrument and manufacturer. Detectors are either PMTs or photodiodes, specifically avalanche photodiodes (APD). PMTs are the most commonly used detectors, but APDs are becoming more popular due to the cost and having improved sensitivity when detecting longer wavelengths.

In a conventional flow cytometer, such as the Bio-Rad ZE5 Cell Analyzer, detection specificity is controlled by optical filters that block certain wavelengths while transmitting (passing) others. There are three major filter types. Long pass filters allow light through above a cutoff wavelength, short pass filters permit light below a certain wavelength, and band pass filters transmit light within a specified narrow range of wavelengths (termed a bandwidth). These dichroic filters can block light by phased reflection, allowing certain light to pass through and interfering with other wavelengths (Figure 2).

A dichroic filter is also a mirror when placed at an angle to the oncoming light. This type of filter can perform two functions. Firstly, it allows specific wavelengths to pass in the forward direction. Secondly, 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).

Visit [bio-rad.com/CellAnalysis](https://www.bio-rad.com/CellAnalysis) to learn how the flexible configuration of the ZE5 Cell Analyzer can help you design complex experiments with high speed and sensitivity.

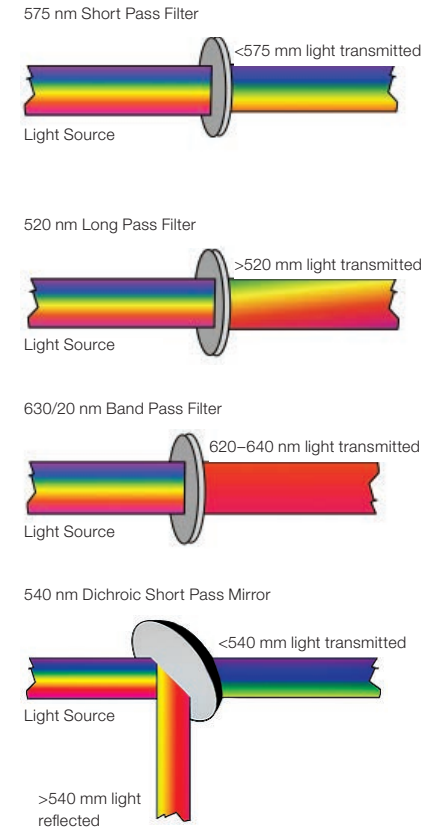
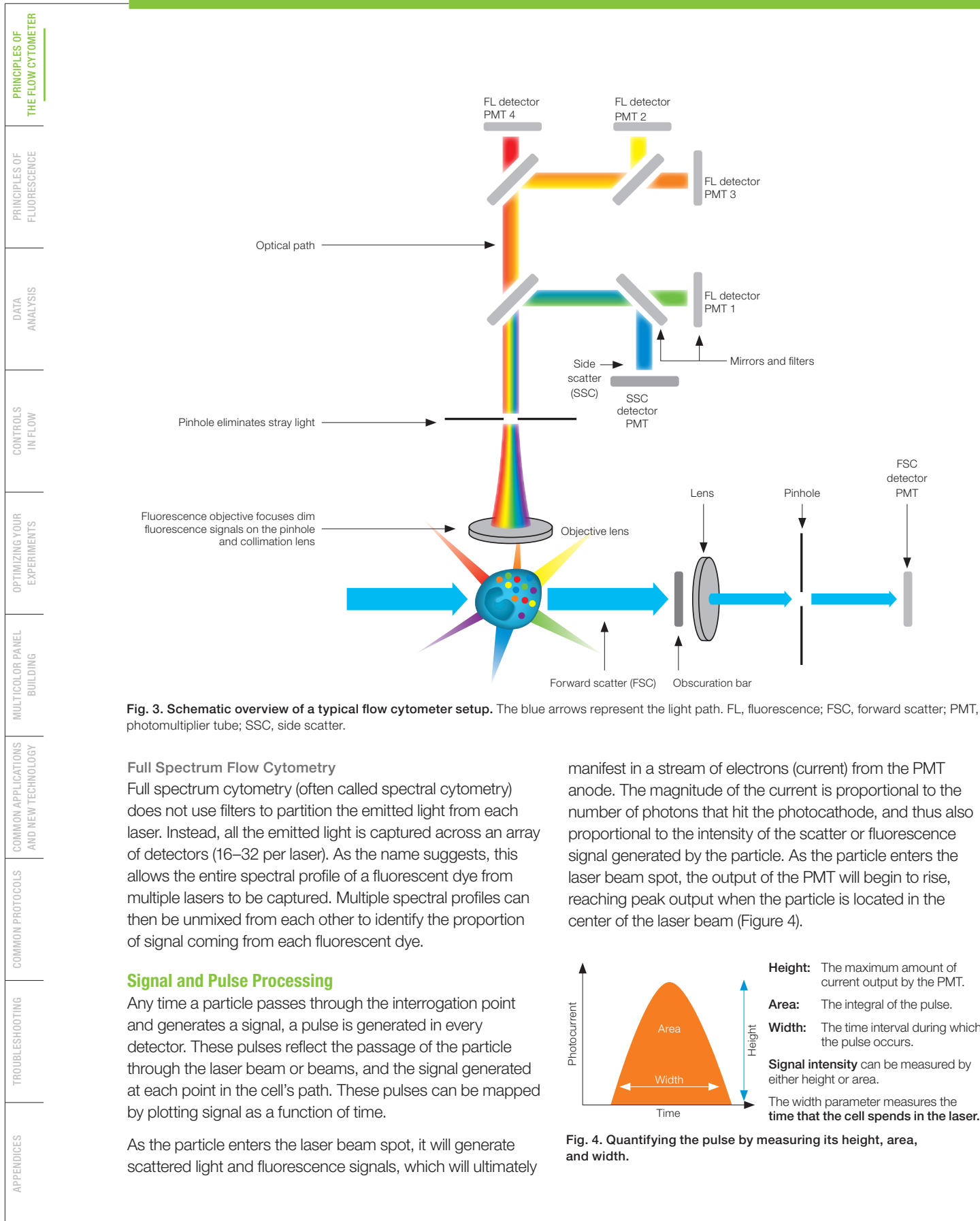


Fig. 2. Different types of optical filters.

PRINCIPLES OF THE FLOW CYTOMETER	PRINCIPLES OF FLUORESCENCE	DATA ANALYSIS	CONTROLS IN FLOW	OPTIMIZING YOUR EXPERIMENTS	MULTICOLOR PANEL BUILDING	COMMON APPLICATIONS AND NEW TECHNOLOGY	COMMON PROTOCOLS	TROUBLESHOOTING	APPENDICES
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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 maximal 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.

However, not all generated signals 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 called the trigger channel. This determination is made based upon the trigger parameter and threshold level. PMTs or APDs 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 those generated by experimental particles. Including these in the dataset would give high levels of background and substantially mask out relevant data points, and overload the electronics ability to process relevant signals. Therefore, it is desirable and necessary to have a threshold below which nonessential 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).

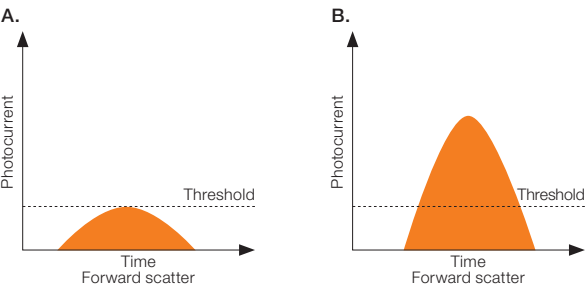


Fig. 5. Signal discrimination. A, an ignored signal; B, a fully processed signal.

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 or APD 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 beam, and can be used to distinguish between single particles or closely interacting particles and doublets.

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 (for example, the Bio-Rad S3e 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 (in cycles/sec, Hz) vibration of the nozzle at an optimal amplitude (in volts) over a period of time. This is typically created by a piezoelectric crystal. As with cell analyzers, sorting can now be performed using conventional and full-spectrum flow cytometers.

There are two types of electrostatic sorters, which differ in where the particles are interrogated by the laser beam. 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 the interrogation point, they continue down the stream. Data collected from the particles as they pass through the laser beam, at the interrogation point, are sent to a computer, where the decision is made whether a given

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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).

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 sec. The stability of the break-off dictates the accuracy of the sorting.

Cell sorting is commonly used to isolate cell populations and single cells for downstream experiments analyzing DNA, protein, or cellular function. 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. Visit [bio-rad.com/S3e](https://www.bio-rad.com/S3e) to learn more about the Bio-Rad S3e Cell Sorter, the first truly walk-away cell sorter that simplifies experiments while being accessible to both novice and expert users.

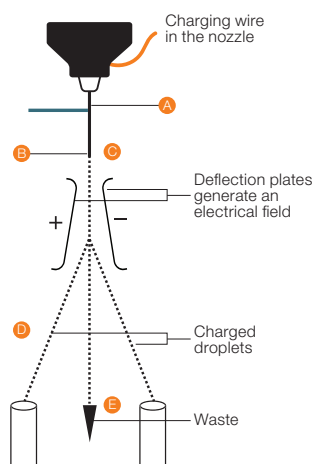


Fig. 6. Electrostatic flow sorting. A, optical interrogation and light collection; B, stream partitioning into droplets; C, stream and droplet charging as the target particle passes through the break-off; D, droplet deflection through an electrostatic field; E, uncharged droplets pass into the waste.



2. Principles of Fluorescence

Flow cytometry is underpinned by the principles of fluorescence, which are covered in this chapter. We also present useful fluorescent dyes for flow cytometry and explain fluorescence compensation for multicolor panels.

Fluorescent Dyes and Light

Fluorescent dyes are markers used to detect the expression of cellular molecules such as proteins or nucleic acids. They accept light energy (for example, from a laser) at a given wavelength and reemit 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, which determine the color of the light. The light that can be visualized by the human eye represents a narrow wavelength band (380–700 nm) between UV and 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.

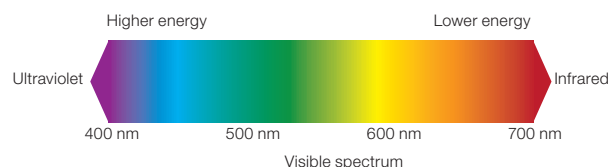


Fig. 7. The electromagnetic spectrum.

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) ❶. The amount of energy required for this transition will differ for each fluorophore. The duration of the excited state depends on the fluorescent dye and typically

lasts for 1–10 ns. The fluorescent dye 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 ❷. The electrons subsequently fall back to their resting state (S_0), releasing the remaining energy (E_{Emission}) as fluorescence ❸. 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 signal amplification.

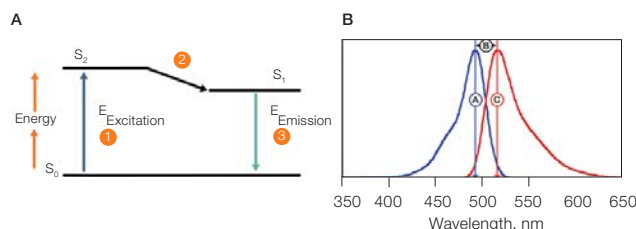


Fig. 8. Stokes shift. **A**, upon excitation, 1, electrons in a fluorophore move from a resting state, S_0 , to the excited electronic singlet state, S_2 . Some energy is released as heat, 2. The remaining energy is released as fluorescence, 3, as the electrons return to their ground state, S_0 . **B**,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

Emitted light from a fluorescent dye typically contains less energy than that used to excite it; therefore, the emission wavelength of any fluorescent dye is longer (lower energy) than its excitation wavelength, and thus corresponds to a different color on the electromagnetic spectrum.

The excitation wavelength is critical to the total number of photons of light that the fluorophore will absorb. Fluorescein isothiocyanate (FITC), for example, absorbs 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 greater the number of photons absorbed, the more intense the fluorescence emission will be. The wavelengths of greatest absorption and emission are termed maximal absorbance and maximal emission wavelengths.

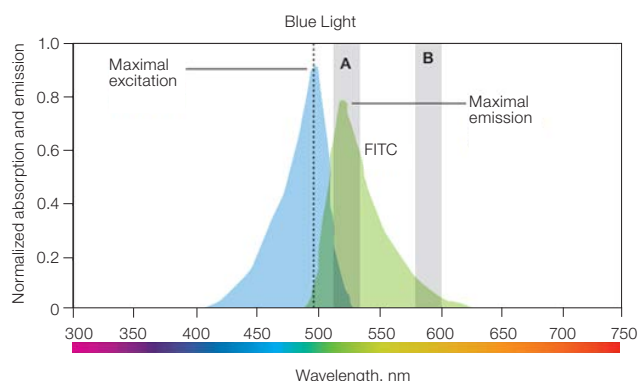


Fig. 9. Spectral profiles showing light absorbance and light emission of FITC. A, excellent signal; B, weak signal; ----, 488 nm excitation laser line (blue light). FITC, fluorescein isothiocyanate.

A fluorophore's maximal absorbance indicates the optimal laser line for excitation. In the case of FITC, its maximal 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 fluorescent dye is detected. If the filters are used to screen out all light other than that measured at the maximum via channel A (Figure 9), FITC will appear green. Fluorescence color usually refers to the color of light a fluorophore emits at its highest stable excited state.

However, if FITC fluorescence is detected in channel B only (Figure 9), it will appear orange and be much weaker in intensity. How the flow cytometer is set up to measure fluorescence will ultimately determine the perceived color of a fluorescent dye. Because the color of the exciting and emitting light is different, they can be separated from one another 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 allow its biological and biochemical properties to be interrogated. Fluorescent markers are useful in a wide range of applications, including identifying and quantifying distinct cell populations, cell surface receptors, or intracellular targets; cell sorting; immunophenotyping; analyzing calcium flux; determining nucleic acid content; measuring enzyme activity; and studying apoptosis. 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 Fluorescent Dyes Are Useful for Flow Cytometry?

A wide range of fluorescent dyes can be used for flow cytometry. The list is ever growing and we will not cover all of them here. The fluorescent dyes currently available from [bio-rad-antibodies.com](https://www.bio-rad-antibodies.com) are described in Tables 1 and 2. With new fluorophores constantly being added, check out our latest offerings at [bio-rad-antibodies.com/flow](https://www.bio-rad-antibodies.com/flow).

Single and Tandem Dyes

Single dyes like FITC, phycoerythrin (PE), allophycocyanin (APC), and peridinin chlorophyll protein (PerCP) have been available for many years, but there are now alternatives available, including Bio-Rad StarBright Dyes, which offer greater photostability and much brighter fluorescence. (Visit [bio-rad-antibodies.com/StarBright](https://www.bio-rad-antibodies.com/StarBright) to learn more.)

In addition, alternative laser lines are becoming more affordable, so dyes excited by 355 nm laser lines are increasing the options for multiplexing.

A tandem dye comprises one fluorescent dye covalently coupled to another fluorescent dye. When the first dye (the donor) is excited and reaches its maximal excited electronic singlet state, its energy is transferred to the second dye (the acceptor). This activates the second dye, which then produces the fluorescence emission. The process is called Förster resonance energy transfer (FRET). It is a clever way to achieve a greater Stokes shift and therefore increase the number of colors that can be analyzed from a single laser wavelength.

Tandem dyes are very useful for multicolor fluorescence studies, especially in combination with single dyes. For example, Alexa Fluor (A) 488, PE, PerCP-Cyanine (Cy) 5.5, 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 integral tools for understanding protein expression in many scientific disciplines. Other fluorescent proteins, like mCherry and yellow fluorescent protein (YFP), have also become widely used for flow cytometry and cell sorting. Fluorescent proteins are often co-expressed or expressed as a fusion with the protein of interest. The benefit of these fluorescent proteins is to allow the quantitation of intracellular markers in live cells, without requiring permeabilization of the cell membrane. Common fluorescent proteins and dyes are listed in Table 3.

Table 1. Single dyes.

Fluorescent Dyes	Laser Line, nm	Fluorescence Emission Color	Maximal Absorbance, nm	Maximal Emission, nm	Relative Brightness
StarBright UltraViolet 400 Dye	355		335	394	3
StarBright UltraViolet 445 Dye	355		347	440	3
StarBright UltraViolet 510 Dye	355		340	513	3
StarBright UltraViolet 575 Dye	355		340	569	4
StarBright UltraViolet 605 Dye	355		340	609	4
StarBright UltraViolet 665 Dye	355		340	669	4
StarBright UltraViolet 740 Dye	355	infrared	344	743	4
StarBright UltraViolet 795 Dye	355	infrared	340	792	2
DyLight 405	405		400	420	3
StarBright Violet 440 Dye	405		383	436	5
Pacific Blue	405		401	452	1
StarBright Violet 475 Dye	405		406	479	4
StarBright Violet 515 Dye	405		402	516	5
StarBright Violet 570 Dye	405		404	571	4
StarBright Violet 610 Dye	405		403	607	5
StarBright Violet 670 Dye	405		401	667	5
StarBright Violet 710 Dye	405	infrared	402	713	5
StarBright Violet 760 Dye	405	infrared	403	754	4
StarBright Violet 790 Dye	405	infrared	402	782	4
DyLight 488	488		493	518	4
A488	488		493	519	3
FITC	488		490	525	3
PerCP	488		490	675	2
StarBright Blue 700 Dye	488		473	703	5
DyLight 550	561		562	576	4
PE	488/561		496/562	578	5
APC	640		650	661	4
A647	640		650	665	4
DyLight 650	640		654	673	4
DyLight 680	640	infrared	692	712	3
A700	640	infrared	702	723	2

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

Table 2. Tandem dyes.

Fluorescent Dyes	Laser Line, nm	Fluorescence Emission Color	Maximal Absorbance, nm	Maximal Emission, nm	Relative Brightness
PerCP-Cy5.5	488		490	695	3
PE-A647	488/561		496/562	667	4
PE-Cy5	488/561		496/562	667	5
PE-Cy5.5	488/561		496/562	695	4
PE-A750	488/561	infrared	496/562	779	4
PE-Cy7	488/561	infrared	496/562	785	4
APC-Cy7	640	infrared	650	785	2

Axxx, Alexa Fluor; APC, allophycocyanin; Cy, cyanine; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Table 3. Fluorescent proteins.

Fluorescent Dyes	Laser Line, nm	Fluorescence Emission Color	Maximal Absorbance, nm	Maximal Emission, nm	Relative Brightness
EBFP	405		383	445	2
CFP	405		439	476	2
GFP/EGFP	488		484	509	4
YFP	488		514	527	5
RFP	561		558	583	4
mCherry	561		587	610	3

CFP, cyan fluorescent protein; EBFP, enhanced blue fluorescent protein; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; RFP, red fluorescent protein; YFP, yellow fluorescent protein.

Fluorescence Compensation

One consideration when performing multicolor fluorescence studies is the possibility of spectral overlap between fluorescent dyes. 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 used in the experiment. For example, two of the most common fluorescent dyes, FITC and PE, maximally emit photons that are green (525 nm) and yellow (578 nm), respectively. But as can be seen in Figure 10, they also emit photons of longer wavelength, all of which can be detected on a multidetector instrument with the corresponding detectors.

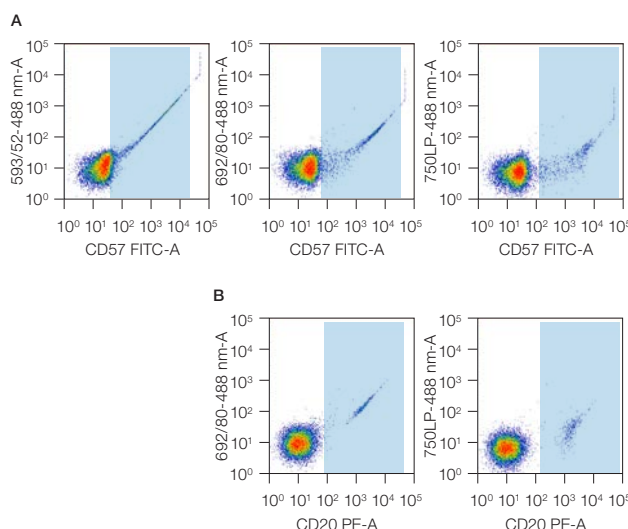


Fig. 10. FITC and PE spillover into other channels. Gates (gray) are shown to identify the FITC and PE positive cells and their spillover. **A**, FITC-stained lymphocytes; **B**, PE-stained lymphocytes. FITC, fluorescein isothiocyanate; LP, long pass; PE, phycoerythrin.

In some experiments, multiple fluorescent dyes are used; for example, FITC may be used in combination with PE. In these cases, the relative contribution of each fluorescent dye to the signal in a given detector must be determined. In Figure 11, you can see that there is some FITC signal in the neighboring PE channel (593/52 filter), the PerCP/PerCP-Cy5.5 channel (692/80 filter), and even the PE-Cy7 channel (750LP filter), all of which will need to be removed. As more fluorescent dyes are added, the signal from each additional dye must be taken into account. In Figure 11, you can see how PE, when excited by the 488 nm laser, also emits in the PerCP/PerCP-Cy5.5 channel (692/80 channel), and the PE-Cy7 channel (750LP filter), and again would need to be

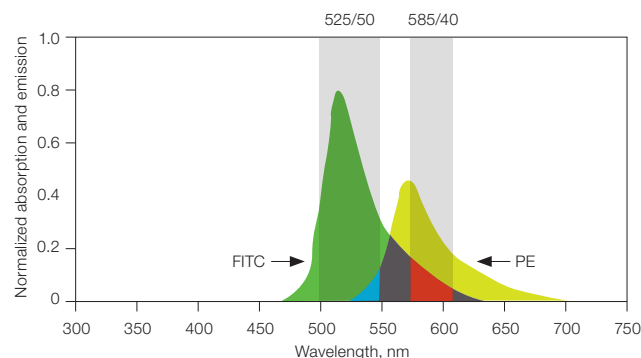


Fig. 11. Fluorescence compensation. ●, emission spectrum of FITC; ●, emission spectrum of PE; ●, band pass filters; ●, portion of FITC emission detected in the 585/40 channel; ●, portion of PE emission detected in the 525/50 channel; ●, overlapping emission spectral profile of FITC and PE detected by neither the 525/50 nor the 585/40 channel. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

removed. In a multiplexing experiment, if PerCP-Cy5.5 was added, which is another 488 nm excitable dye detected in the 692/80 channel, both FITC and PE signal would have to be subtracted from this channel.

In Figure 12, you can see how compensation can be applied to a sample stained with antibodies conjugated to the StarBright Violet 710 or StarBright Violet 790 Dyes. Single stained samples reveal the amount of spectral overlap. When the sample is stained with both fluorescent dyes, without compensation, a double positive population is observed. However, when the correct level of compensation is applied using specific software, the

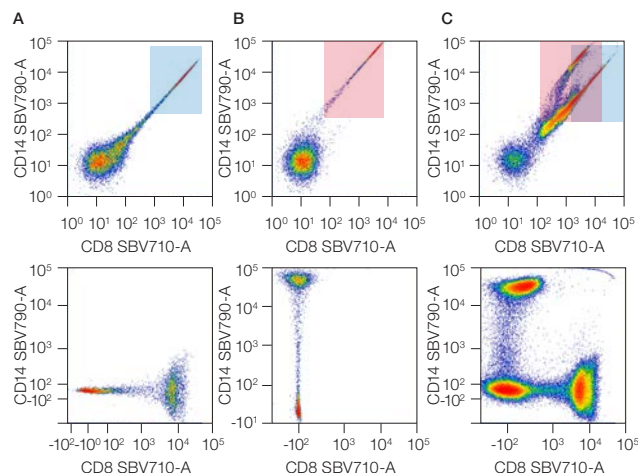


Fig. 12. Fluorescence compensation corrects for spectral overlap.

A, peripheral blood stained with Mouse Anti-Human CD8:StarBright Violet 710 (CD8SBV710; #MCA1226SBV710). **B**, peripheral blood stained with Mouse Anti-Human CD14:StarBright Violet 790 (CD14SBV790; #MCA1568SBV790). **C**, dual-stained peripheral blood. When compensation was not applied (top panels), spillover can be seen and removed after compensation (bottom panels). SBV, StarBright Violet.

true level of staining is revealed. The software calculates spillover values and applies this to the data to obtain correctly compensated data. After compensation, no double positive cells are observed, which is to be expected from these mutually exclusive markers.

You can avoid the need for compensation by using fluorophores that do not have overlapping emission spectra. Alternatively, you can combine fluorescent dyes that can be activated only by different laser lines (providing the lasers are spatially separated), but as you increase the number of fluorescent dyes this becomes practically impossible. We have created common human immunophenotyping panels, using four fluorescent dyes, which require no compensation. They are useful to identify common populations or as a start, to build larger and more complex panels. Visit the [No Compensation Panels](#) web page to find out more about our B, T, NK, and myeloid cell panels.

PRINCIPLES OF THE FLOW CYTOMETER	PRINCIPLES OF FLUORESCENCE	DATA ANALYSIS	CONTROLS IN FLOW	OPTIMIZING YOUR EXPERIMENTS	MULTICOLOR PANEL BUILDING	COMMON APPLICATIONS AND NEW TECHNOLOGY	COMMON PROTOCOLS	TROUBLESHOOTING	APPENDICES
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3. Data Analysis

Understanding how to analyze your data is critical for success with flow cytometry. In addition, since some analysis steps happen in real time with flow cytometry, it's important to have a plan before you start an experiment. In this chapter, you will learn how to place gates and regions for cell analysis, and how to backgate. We also examine the different plot types used to analyze and display flow cytometry data.

Gates and Regions

Flow cytometry data analysis is fundamentally based on the principle of gating. Gates and regions are placed around cell populations with common characteristics, usually forward scatter, side scatter, and marker expression, to investigate and quantify these populations. Here we cover how to interpret the common flow cytometry graphs. Visit bio-rad-antibodies.com/gates-regions to download a handy resource on this topic as a quick reference.

The first step in gating is often to distinguish cell populations based on their forward and side scatter properties.

Forward and side scatter give an estimation of cell size and granularity respectively, although this can depend on several factors such as the sample, laser wavelength, sample collection, angle refractive index of the sample, and the sheath fluid. Distinguishing cell populations can be relatively straightforward for samples containing one cell type but can be more complex for samples containing multiple cell types. As can be seen in Figure 13, red cell-lysed whole blood contains several distinct cell types. The blue/green/yellow/red hot spots indicate increasing numbers of events resulting from discrete cell populations (Figure 13A). The light scatter patterns of granulocytes, monocytes, and lymphocytes allow them to be distinguished from cellular debris and dead cells. The latter often have a lower level of forward scatter and are highlighted 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. Data can also be plotted as a combination of fluorescence and forward or side scatter, stained with CD8SBV710 (Figure 13B) and CD14SBV790 (Figure 13C). Forward and side scatter gating is often used to remove dead cells that have increased autofluorescence and nonspecific binding of antibodies. However, including a viability dye is a much more reliable method.

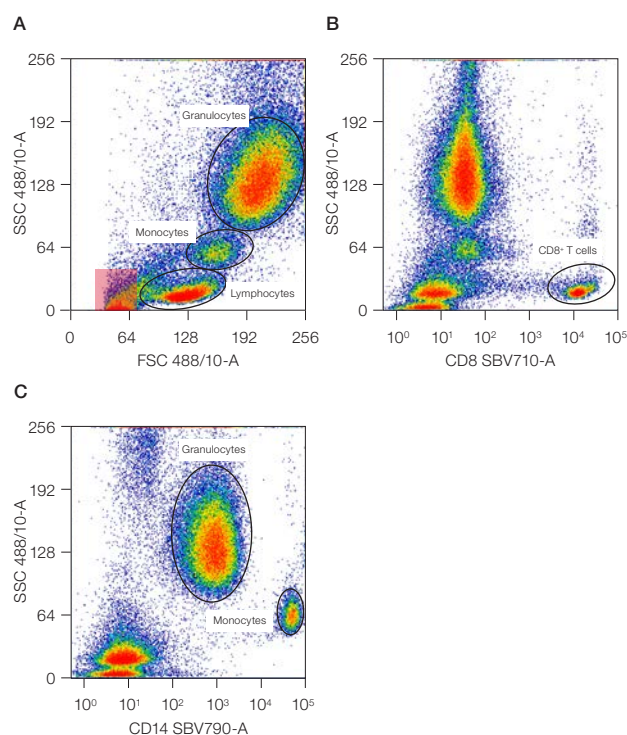


Fig. 13. Analysis of lysed whole blood. **A**, SSC vs. FSC density plot; **B**, SSC vs. CD8SBV710 fluorescence plot; **C**, SSC vs. CD14SBV790 (#MCA1568SBV790) fluorescence plot. FSC, forward scatter; SBV, StarBright Violet; SSC, side scatter.

Events can also be displayed as a dot plot where no density information is shown or as a contour map, with or without outliers, to show the relative intensity of scatter patterns. Examples of contour maps are shown in Figure 14. Although users are free to choose either display, sometimes discrete cell populations are easier to visualize on contour diagrams.

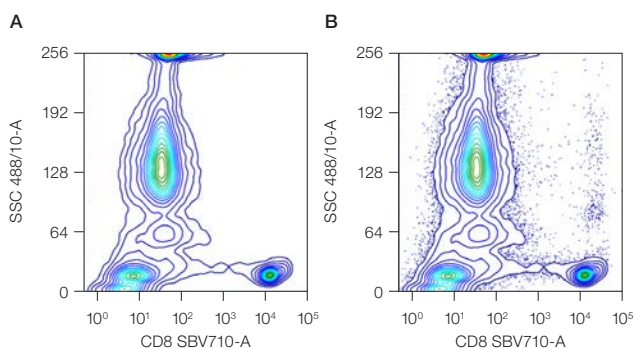


Fig. 14. Contour plots for analysis of lysed whole blood. **A**, SSC vs. Mouse Anti-Human CD8:StarBright Violet 710 (CD8SBV710; #MCA1226SBV710) without outliers; **B**, SSC vs. Mouse Anti-Human CD8:StarBright Violet 710 (CD8SBV710) with outliers. SSC, side scatter; SBV, StarBright Violet.

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 are expressed in a histogram that includes 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 possess specific properties or express a marker of interest. Cells with the desired characteristics are called the positive dataset. An example can be seen in Figure 15B. Peripheral blood was stained for CD4 and then gated on the lymphocytes using forward and side scatter. Two peaks can be interpreted as the positive and negative dataset. In this example, the CD4 positive T cells represent around 35% of the cells within the lymphocyte gate.

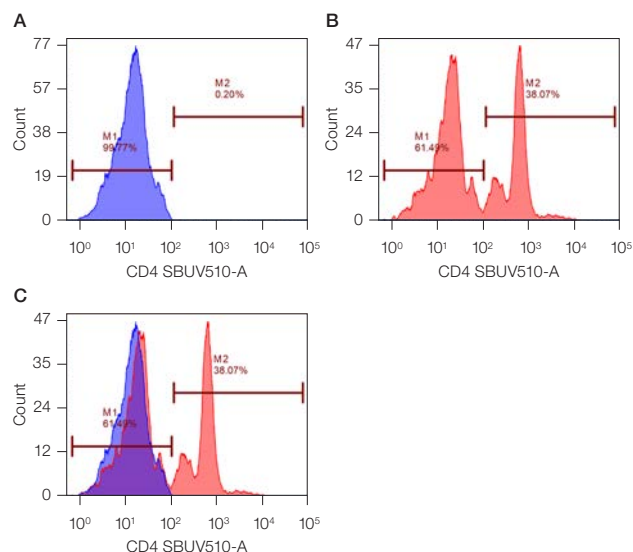


Fig. 15. Single parameter histograms. **A**, unstained cells within the lymphocyte gate (Figure 13A) show the background level of fluorescence. **B**, cells within the lymphocyte gate (Figure 13A) stained with Mouse Anti-Human CD4:StarBright UltraViolet 510 (CD4SBUV510) to define the CD4 expression. **C**, overlay of the control (unstained) population onto the stained population allows easy identification of the positive cells. M1, marker 1; M2, marker 2; SBUV, StarBright UltraViolet.

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 where there are no negative cells to compare against; however, often in flow cytometry multiple peaks are observed due to mixed populations. Figure 15A shows a control histogram. In this case, an unstained peripheral blood sample, in blue, is 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 and percentage of cells within the gate. This can include measurements such as mean fluorescence intensity (MFI) which allows you to assess how bright a population is, often used when there are small increases or decreases in fluorescence.

Two-Parameter Plots

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, FSC, SSC, or a combination thereof, depending on what you want to show. The lymphocytes determined by forward and side scatter (Figure 16A) are stained with CD3SBUV400 and CD19SBUV605 to identify the T and B cell populations, respectively. The relative proportion of B and T cells can then be quantified by placing gates or quadrants around the distinct populations (Figure 16 B and C).

In this case, there are around 5% B cells and 78% 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). As CD3 and CD19 are mutually exclusive to T and B cells, there are no double positive cells. 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.

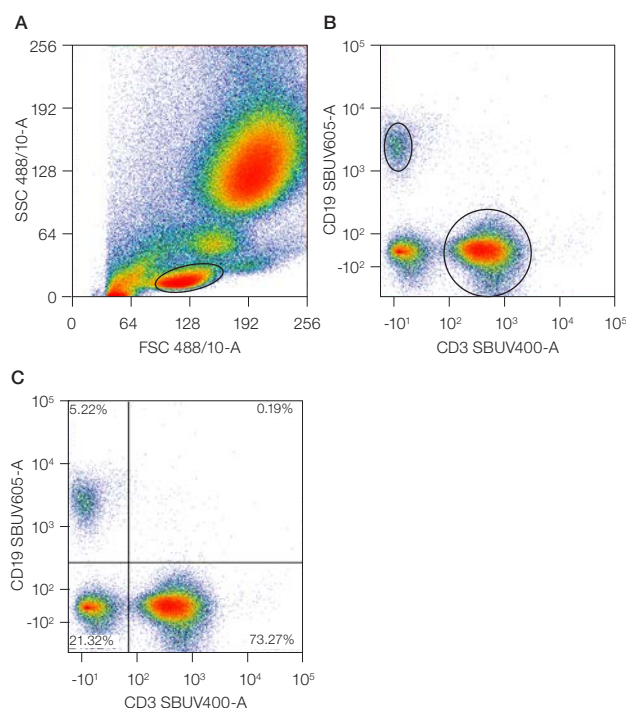


Fig. 16. Two-parameter (dual color fluorescence) density plots. Red cell lysed whole blood was stained with CD3SBUV400 and Mouse Anti-Human CD19:StarBright UltraViolet 605 (CD19SBUV605). The relative populations were determined using different gating methods. **A**, SSC vs. FSC; **B**, CD19SBUV605 vs. CD3SBUV400; **C**, quantification of relative B and T cell populations. FSC, forward scatter; SBUV, StarBright UltraViolet; SSC, side scatter.

Gating 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 specific cell types. This is particularly useful as the number of markers and fluorophores in a single experiment increases.

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 identified (Figure 17B) and gated by the expression of CD4 and CD8

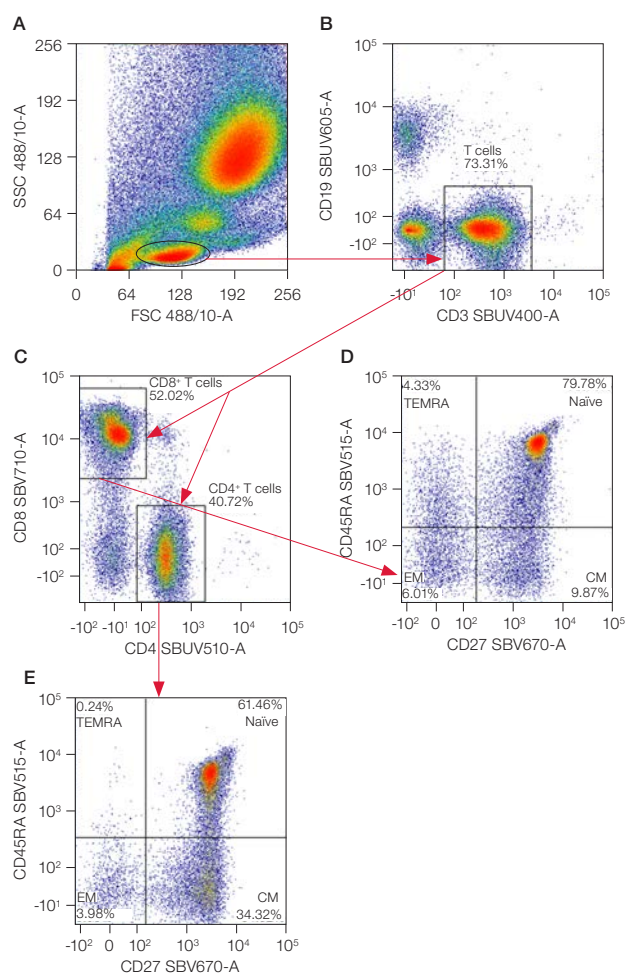


Fig. 17. Sequential gating to identify specific T subsets. Red cell lysed whole blood was stained with CD19SBUV605, CD3SBUV400, CD4SBUV510, CD8SBV710, Mouse Anti-Human CD45RA:StarBright Violet 515 (CD45RASBV515), and Mouse Anti-Human CD27:StarBright Violet 670 (CD27SBV670) in PBS containing 1% (w/v) BSA. The gating strategy to identify different T cell subsets is shown by the arrows. **A**, SSC vs. FSC; **B**, CD19SBUV605 vs. CD3SBUV400; **C**, CD8SBV710 vs. CD4SBUV510; **D**, CD45RASBV515 vs. CD27SBV670 (CD8 T cells); **E**, CD45RASBV515 vs. CD27SBV670 (CD4 T cells). BSA, bovine serum albumin; CM, central memory; EM, effector memory; FSC, forward scatter; PBS, phosphate buffered saline; SBUV, StarBright UltraViolet; SBV, StarBright Violet; SSC, side scatter; TEMRA, CD45RA⁺ effector memory T cells.

(Figure 17C). The relative expression of CD45RA and CD27, which were used to identify naïve cells, central memory (CM) cells, effector memory cells (EM), and terminal effector memory RA⁺ cells (TEMRA), was then determined on both the CD4⁺ (Figure 17D) and CD8⁺ populations (Figure 17E). This principle can be continued with additional markers, but it is worth noting that as the cell populations become more defined, there are fewer events within each gate, showing the importance of collecting enough cells to answer the experimental questions.

Sequential gating is the most common way of identifying cells of interest. However, as the number of fluorescent parameters within an experiment has increased, alternative methods of data analysis have emerged. These large datasets are often generated in spectral flow cytometry, where panels of over 40 colors have been developed. These alternative analysis methods will be discussed in Chapter 6.

Backgating to Confirm Gating Strategies

Backgating is a useful method of validating 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, marker expression levels, nonspecific binding; whether you have identified your cells of interest or the presence of dead cells; or need additional information to identify your cells. A simple backgating strategy can be seen in Figure 18, where the CD3⁺ cells in gate 1 show FSC and SSC characteristics of lymphocytes, the CD3-CD14^{hi} cells in gate 3 show FSC and SSC characteristics of monocytes, and CD3-CD14^{lo} cells in gate 2 show FSC and SSC characteristics of granulocytes.

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, you need to ensure you perform the right controls and have an adequate sample size because as the fluorescence increases, so will the background and nonspecific binding, making the data harder to analyze.

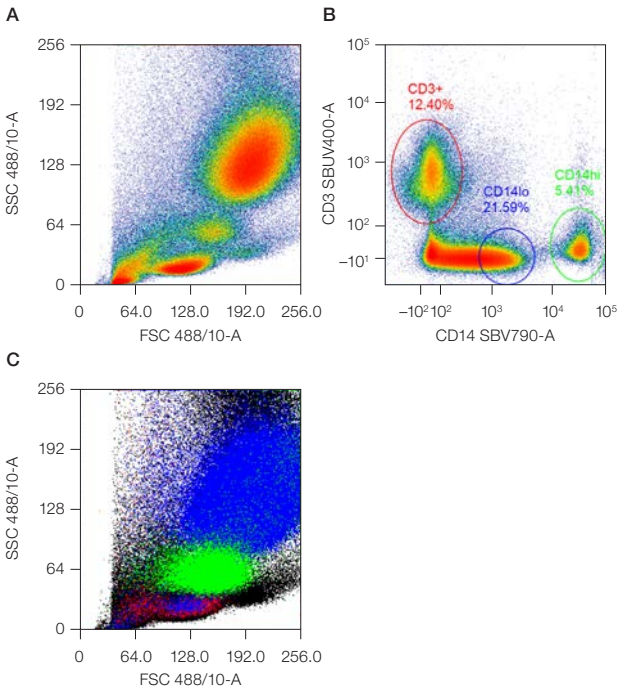


Fig. 18. Backgating to identify leukocyte subsets. **A**, red cell lysed whole blood, **B**, stained with CD3SBUV400 and CD14SBV790. **C**, FSC and SSC characteristics of cells in the green, blue, and red gates. FSC, forward scatter; SBUV, StarBright UltraViolet; SBV, StarBright Violet; SSC, side scatter.

PRINCIPLES OF THE FLOW CYTOMETER	PRINCIPLES OF FLUORESCENCE	DATA ANALYSIS	CONTROLS IN FLOW	OPTIMIZING YOUR EXPERIMENTS	MULTICOLOR PANEL BUILDING	COMMON APPLICATIONS AND NEW TECHNOLOGY	COMMON PROTOCOLS	TROUBLESHOOTING	APPENDICES
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4. Controls in Flow

Controls are vital in any experiment to reliably distinguish your results from background variation and nonspecific effects. Some controls are also specific to flow cytometry and should be included in every experiment. These include unstained controls, viability controls, and compensation controls. Here, we discuss some essential controls for flow cytometry that will help you obtain publication-quality data.

Unstained Controls

One of the first things to identify in flow cytometry is your cell population. Use unstained cells to set up your instrument so that all your cells can be easily visualized on an FSC and SSC plot. Unstained cells can also be used to set your PMT voltage so that you can distinguish dim signals from autofluorescence and electronic noise while keeping the brightest cells within the scale. Historically, this was done by altering the PMT voltage so that the unstained cells lie within the first log decade for each fluorescent parameter used in your experiment (Figure 19). Using the data from unstained cells (Figure 19B), signals from stained cells can be distinguished from autofluorescence.

More recently, an alternative method for setting the PMT voltages, called volttration, has gained popularity. This method uses beads with multiple fluorescence intensities to set the PMT voltage at their optimum sensitivity. The voltage is increased incrementally, and the optimal voltage is where the largest difference between the two peaks with the lowest fluorescence intensities is observed. Using this method, the negative population is not necessarily located within the first log decade and very bright signals could appear off scale. Therefore, some adjustments may be required for each experiment, but they should not be made between samples within an experiment.

Isotype Controls

In flow cytometry, background levels of staining can be a problem, especially with rare populations, cells with low expression levels, and when building multicolor panels. Isotype controls are antibodies raised against an antigen not found on the cell type or sample analyzed. Developed for surface staining, their role is to confirm the observed staining is due to a specific antibody binding to the target rather than an artifact. They should not be used to determine positive versus negative cells or to set gates, and may not be suitable for intracellular staining.

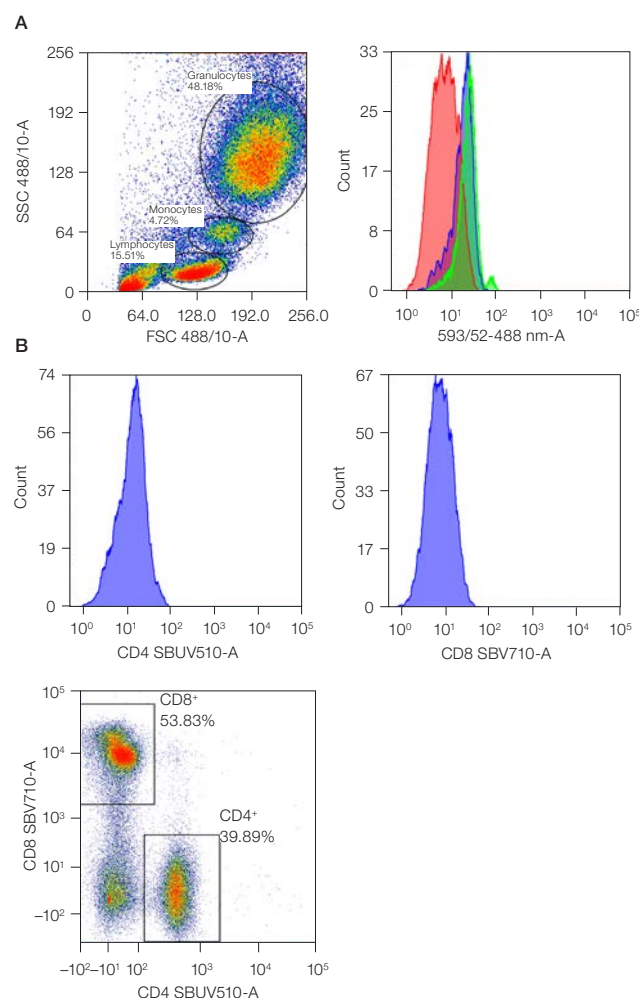


Fig. 19. Unstained controls. **A**, unstained peripheral blood was used to set the FSC and SSC to visualize the cells of interest and show the autofluorescence in the three marked populations (histogram key: red, lymphocytes; blue, monocytes; green, granulocytes). **B**, unstained cells (histograms) were used to set the PMT voltages for fluorescence channels so that fully stained cells (in the dot plot) can be evaluated. FSC, forward scatter; PMT, photomultiplier tubes; SBUV, StarBright UltraViolet; SBV, StarBright Violet; SSC, side scatter.

An isotype control will:

- Determine the nonspecific binding of an antibody to Fc receptors found on monocytes, macrophages, and dendritic and B cells (Fc receptors can be blocked to reduce nonspecific binding, explained later in this chapter)
- Confirm the observed staining is due to specific binding rather than an artifact
- Reveal other nonspecific binding of the antibody or fluorophores, such as R-phycoerythrin (RPE or PE) and FITC (Takizawa et al. 1993, Hulspas et al. 2009), to cellular components

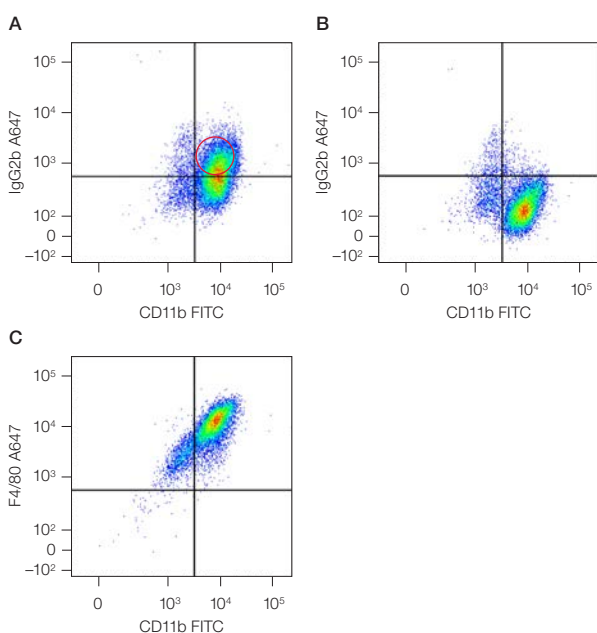


Fig. 20. Isotype controls. J774 macrophages were stained for antibodies against CD11b and F4/80 (or isotype control IgG2b) in the presence of 7-AAD (to exclude dead cells). **A**, CD11b and IgG2b without Fc block; **B**, CD11b and IgG2b with Fc block; **C**, CD11b and F4/80 with Fc block. Without the Fc block, there is background staining denoted by the red circle. 7-AAD, 7-aminoactinomycin D; Axxx, Alexa Fluor; Fc, fragment crystallizable; FITC, fluorescein isothiocyanate; IgG2b, immunoglobulin G2b.

The most appropriate isotype control matches the:

- Host species
- Immunoglobulin (Ig) subclass
- Fluorescent dye on 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 number of fluorophore molecules conjugated to each antibody (called the F/P ratio) differs between suppliers, it is best to purchase the isotype control from the same supplier as the primary antibody. It is also advisable, if possible, to use isotype controls at the same concentration as the primary antibody.

Nonspecific antibody binding can be reduced by:

- Blocking Fc receptors
- Adding excess protein such as bovine serum albumin (BSA) to your buffer
- Titrating your antibody
- Gating out dead cells using a live/dead cell marker
- Performing extra washing steps after staining

Isoclonic Controls

One alternative to an isotype control is the isoclonic control. This is where cells are stained in the presence of an excess of identical unlabeled antibody. The latter takes up all the binding sites, preventing the labeled antibody from binding specifically. Thus, any signal that is detected must come from nonspecific binding (Figure 20).

Viability Controls

Dead cells have a high level of autofluorescence and nonspecific antibody binding, which can lead to false positives, a reduction in the detectable dynamic range, and a loss of resolution. This will make it difficult to detect weakly positive samples and rare populations. Using a forward and side scatter gate will allow you to exclude debris and some dead cells, but it will not remove them all. Therefore, a viability dye should be included in your flow cytometry panel. Figure 21 illustrates an example in murine bone marrow, where combining a viability dye with a forward and side scatter gate significantly improves the data quality. By using a forward and side scatter gate to exclude dead cells, you can identify myeloid cells positive for both GR-1 and CD11b (upper right quadrant) in murine bone marrow. When a viability dye is introduced, in this case DAPI, you can see that the same forward and side scatter contains both live and dead cells.

When the viability dye and forward and side scatter gate are combined, the staining for CD11b and GR-1 is clearer and some staining, presumably from dead cells, disappears.

There are two types of viability dyes. Nucleic acid binding dyes, such as propidium iodide, 7-AAD, and DAPI, fluoresce upon binding to nucleic acid, but cannot pass through an intact cell membrane. Therefore, only dead cells, with a permeable membrane, will fluoresce.

The second type of viability dye, protein binding dyes, covalently bind to free primary amines on proteins, which are present on the surface of cells. When the membrane is compromised, the dyes permeate the cells and react with intracellular primary amines. Greater fluorescence is observed in dead cells due to their increased content of accessible free amines, allowing them to be easily distinguished from live cells. VivaFix Cell Viability Assays are fixable viability dyes, available in a wider range of

excitation and emission spectra than nucleic acid binding dyes, for convenient analysis and addition to multicolor flow cytometry panels.

As mentioned in Chapter 2, single staining will reveal the level of spectral overlap between different fluorescent dyes and allow you to remove or compensate for this overlap (Figure 12). As the addition of each new fluorophore can have an effect on the existing fluorophores in a panel, this spectral overlap should be compensated for every fluorophore used.

Here are some important rules to remember when using single-stained samples for compensation:

1. The staining of the compensation control must be as bright as or brighter than the sample to replicate any spillover in your experiment. Antibody capture beads can be substituted for cells, and one fluorophore

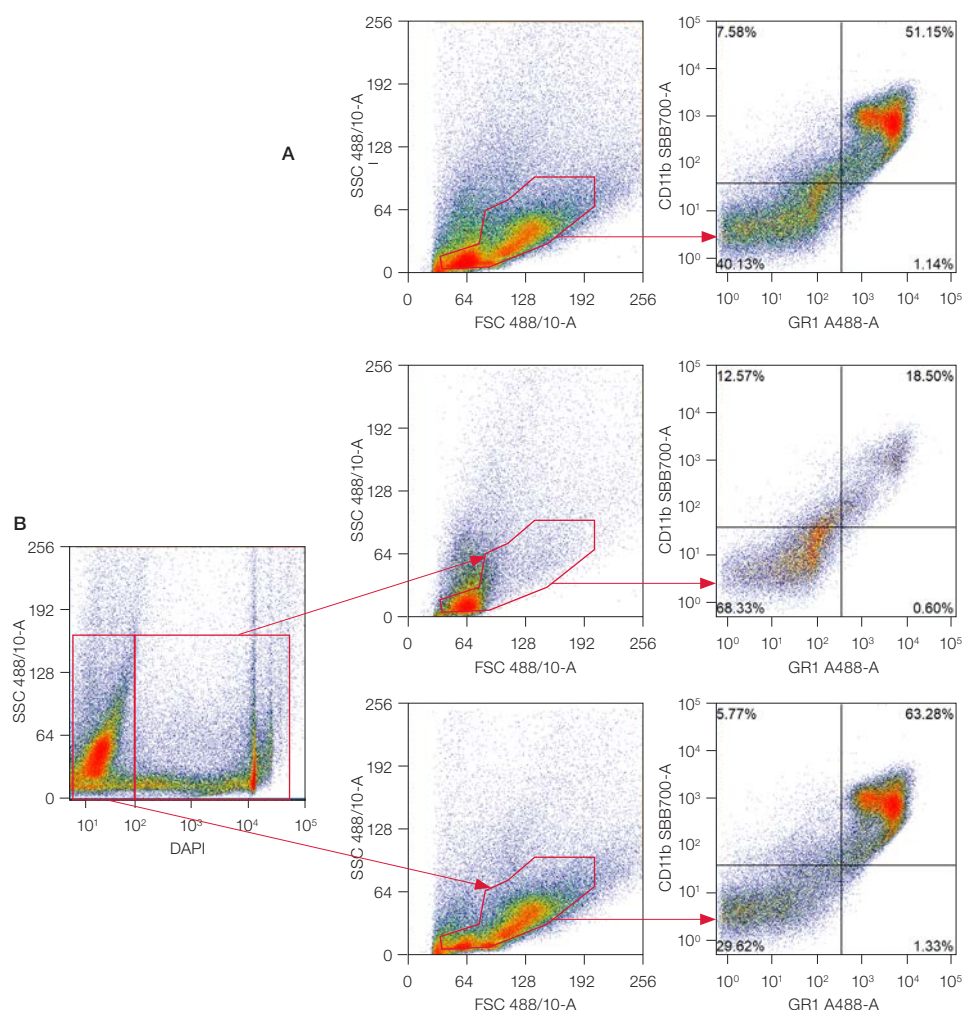


Fig. 21. Viability controls. **A**, a forward and side scatter gate was used to select GR-1 and CD11b positive cells in murine bone marrow (upper right quadrant). **B**, the viability dye ReadDrop Propidium iodide (#1351101) shows that using forward and side scatter gates is not the most effective strategy for eliminating dead cells from analysis. Using a combination of the viability dye propidium iodide and a forward and side scatter gate, CD11b and GR-1 positive cells can be identified in murine bone marrow. Axxx, Alexa Fluor; FSC, forward scatter; SBB, StarBright Blue; SSC, side scatter.

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 due to varying levels of FRET.

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 all of these dyes.

- The compensation algorithm needs to be performed with a positive population and a negative population (Figure 22). If you have a sample that is 100% positive (for example, CD45 in peripheral blood), unstained cells can be spiked into the sample to provide a negative cell 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. The entire set of compensation controls may include individual samples of either beads or cells, but the individual samples must have the same carrier particles (cells or beads) as the fluorophores.
- 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. Thus, you cannot use one of them for the sample and the other for the compensation control.
- 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 populations is ideal, but fewer can be used if necessary.
- Compensation can be applied during or after acquisition. Once you have set the PMT voltages and have applied compensation, do not change the voltages, as this will invalidate the compensation. Whenever you apply compensation, it is best to avoid doing so manually. Modern software, such as FCS Express, has automated compensation, which is much more accurate than manual methods.

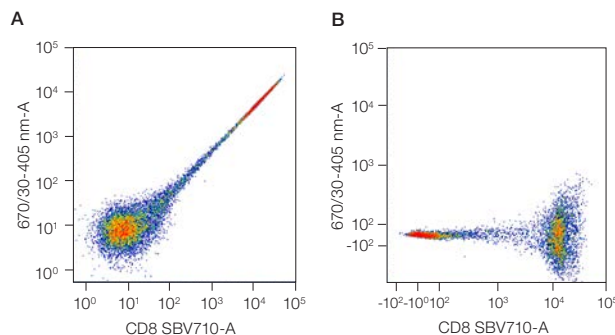


Fig. 22. Compensation controls correct spectral overlap. Human peripheral blood was stained with Mouse Anti-Human CD8:StarBright Violet 710 (CD8SBV710). **A**, staining without compensation. **B**, staining after compensation has been applied. SBV, StarBright Violet.

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 fragment antigen-binding (Fab) domain.

This nonspecific binding can lead to false positives and meaningless data. To prevent this, Fc blocking reagents (for example, Human Fc Seroblock and Murine Fc Seroblock) block Fc receptors and ensure that only antigen-specific binding is observed (Figure 23). An alternative to specialized blocking reagents is diluted serum from the same sample type (for example, mouse serum, if staining mouse cells). If used in excess, immunoglobulins in the serum will compete for the Fc receptors, preventing binding of the conjugated antibodies.

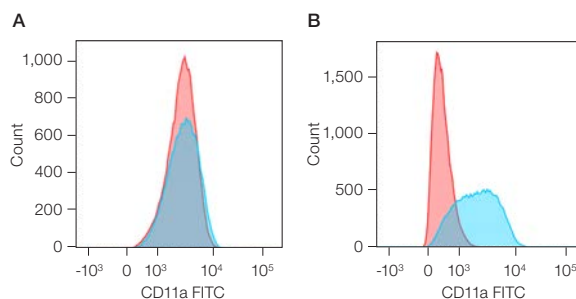


Fig. 23. Fc blocking. THP-1 cells stained with Mouse Anti-Human CD11a Antibody (#MCA6115F) (blue) or Mouse IgG2a Negative Control Antibody (#MCA929F) (red). **A**, with or **B**, without Human Seroblock (#BUF070A). FITC, fluorescein isothiocyanate.

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. Fluorophore spread occurs when you have multiple fluorophores compensated in a panel. Examples of the levels of spread of the positive population can be seen in Figure 24.

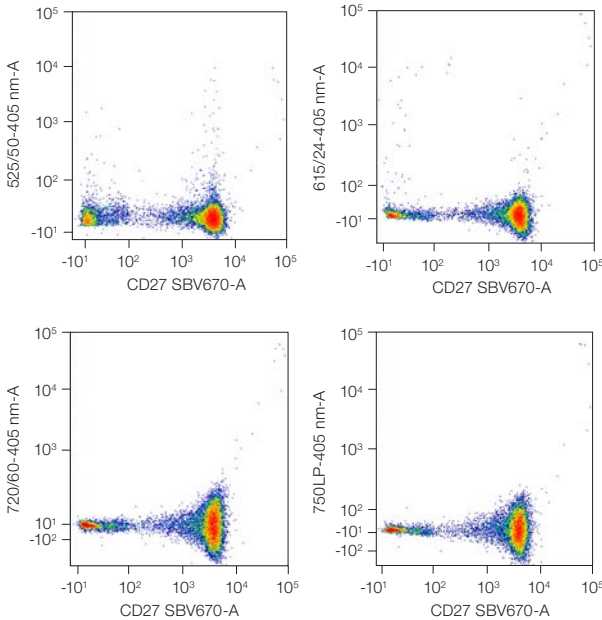


Fig. 24. Fluorescence spread. Density plots of Mouse Anti-Human CD27:StarBright Violet 670 (CD27SBV670; #MCA755SBV670) on human peripheral blood-compensated data showing the spread into neighboring channels. LP, long pass; SBV, StarBright Violet.

The spread increases with the number and brightness of the fluorophores used. Although careful experimental design, avoiding channels with a large amount of spreading, and antibody titration will help reduce this influence, FMO controls are still important. 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 25 shows how the fluorescence spread from other channels can affect the data, ensuring you can position your gates accordingly.

FMO controls should be included 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	+	+	+	–

Axxx, Alexa Fluor; FITC, fluorescein isothiocyanate; FMO, fluorescence minus one; PE, phycoerythrin.

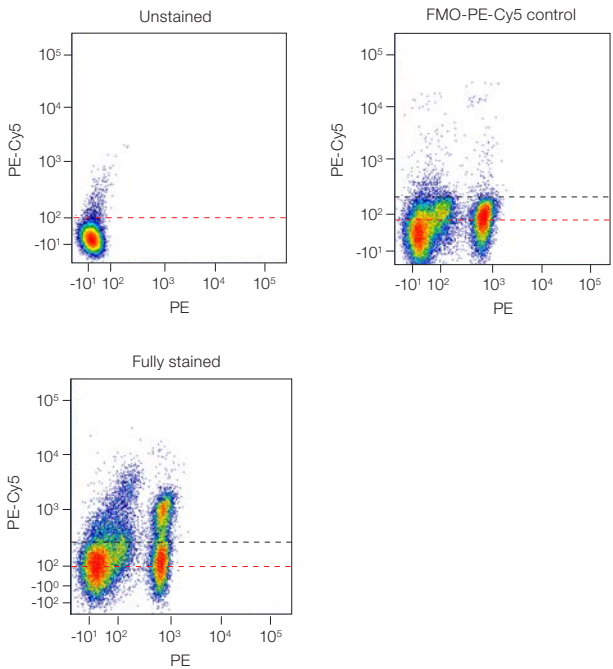


Fig. 25. Use of FMO controls to determine fluorescence spread and set accurate gates. Dot plots of multicolor flow cytometry showing the fluorescence spread into the PE-Cy5 channel shown by the FMO control compared to an unstained control. Black dotted line represents FMO gating boundary compared to unstained boundary in red. FMO, fluorescence minus one; PE, phycoerythrin; PE-Cy5, phycoerythrin-cyanine 5.

Intracellular Staining Controls

Intracellular staining can be more problematic than surface staining, often due to higher levels of background within cells caused by protein-protein interactions. Because it requires fixation and permeabilization, which can affect antigen detection, autofluorescence, fluorophore brightness, and cell morphology, other controls are necessary. Controls such as a negative cell line, an antibody known to be negative on your cells, or a secondary antibody alone (if using primary and secondary antibodies) can be useful to determine specific binding in intracellular staining. An example of using these controls in intracellular staining can be seen in Figure 26.

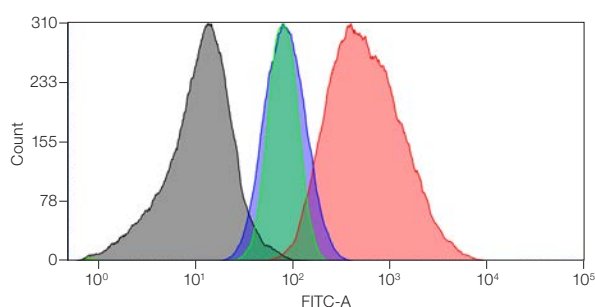


Fig. 26. Intracellular controls. Cas9-positive (red) or Cas9-negative (green) HEK-293T cells were stained with an Anti-Cas9 Antibody and a secondary antibody conjugated to FITC (#STAR121F). An unstained control (gray) and a secondary-only control (blue) were included. FITC, fluorescein isothiocyanate.

Using an internal control within your sample is a good example of control for intracellular staining. When staining for a T cell-specific marker in peripheral blood, check that the B cells and monocytes are negative within the same sample. Additionally, because intracellular staining requires fixation and permeabilization, staining without permeabilizing the cells will give you some information on the levels of background surface staining. As well as controlling for the antibodies, appropriate controls for the other reagents must also be included. The fixative and permeabilization buffers used for intracellular staining may affect your cells' morphology, so you may have to alter the position of the gates normally used in analysis. The fixation and permeabilization conditions may need optimizing and some reagents, such as the fluorophore APC-Cy7, may not be suitable.

Biological Controls

In addition to staining and isotype controls, you should also consider biological controls that will enable you to determine staining specificity and experimental limitations. They are important for all staining but especially intracellular staining, which can have higher background

fluorescence. Controls include known negative samples and known positive samples. These include cells known to either express or lack expression of the antigen of interest, where the antigen has been knocked down or out using RNA interference (RNAi) or CRISPR technology to produce a negative cell, or that have been transfected and the antigen is being overexpressed to verify positive staining.

For some experiments, such as cytokine release measurement, both an unstimulated and a fully stimulated sample is important to determine both positive results and the dynamic range of fluorescence staining, and to confirm the antibody is performing as expected.

An example of this can be seen in Figure 27, in which human peripheral blood lymphocytes were stained for CD154 and CD3 on stimulated and unstimulated cells. The pattern of antibody staining matches what would be predicted from these cell types, indicating that the antibodies are working as intended. Using these antibodies, positive samples can be distinguished from negative, demonstrating that the antibody has an appropriate dynamic range.

This type of control may also help you choose the most appropriate fluorophore, as small changes may have better resolution with brighter fluorophores. Understanding your experiment and sample is important when choosing the right biological control.

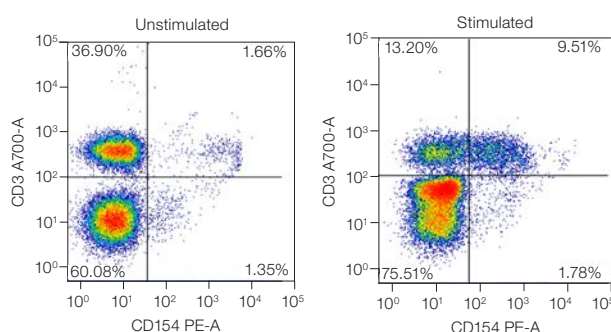


Fig. 27. Use of stimulated and unstimulated controls. Human peripheral blood was unstimulated or stimulated with PMA and ionomycin for 5 hr and then stained with Mouse Anti-Human CD3:Alexa Fluor 700 (#MCA463A700) and Rat Anti-Human CD154:RPE (#MCA1938PE). Axxx, Alexa Fluor; PMA, phorbol 12-myristate 13-acetate; PE, phycoerythrin.



5. Optimizing Your Experiments

One of the fundamentals of flow cytometry is the ability to measure single particles as they pass through a laser beam. However, the cytometer can measure only what is put in, and a poor sample will generally lead to poor data. It is essential to start with the best possible sample and treat your samples as gently as possible so that you have a viable cell suspension and obtain good data.

Sample Preparation

There are many considerations for sample preparation to achieve optimum results. The first one is the sample itself. For example, frozen cells will need to be treated differently than an adherent cell line. Following some basic rules, like the ones highlighted below, will help you optimize your experiments.

1. Defrost cells as quickly as possible and remove the dimethyl sulfoxide (DMSO) by resuspending the cells in a large volume of cold media or phosphate buffered saline (PBS) containing BSA or fetal calf serum (FCS) prior to centrifugation. Cells may need to go into culture after defrosting to restore epitope expression.
2. When preparing cells for flow cytometry, you 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 the epitopes that you want to detect using flow cytometry.
3. Be gentle when using mechanical disaggregation of tissues such as spleen or lymph nodes. Filtering the sample can help remove any unwanted clumps of cells.
4. The extraction of some cells from secondary lymphoid tissue (for example, F4/80-positive macrophages and follicular dendritic cells [DCs]) requires additional enzymes like collagenase or liberase. However, these enzymes may inadvertently remove epitopes if applied for too long, 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 by hypotonic lysis using Erythrolyse Red Blood Cell Lysing Buffer (#BUF04B) or a comparable lysis buffer. Care needs to be taken not to leave the samples in the buffer for too long. 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 density gradients are not recommended for this cell type.
8. Avoid vortexing and excessive centrifugation, or leaving the cells as a dry pellet. Creating too many bubbles while resuspending cells or resuspending cells at high concentrations can increase cell death. Keeping your cells on ice can improve the viability, as this slows down necrosis and cell metabolism.
9. Make sure you are using the right tubes. Many cell types (for instance, monocytes) show stronger adherence to polystyrene but adhere less to polypropylene. If cell numbers are low, avoid polystyrene tubes to reduce the loss of these cell types.
10. All sample preparation should be as short as possible, as the time taken to prepare your cells can have a significant effect on the cell viability.

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 like mitochondria and lysosomes. The autofluorescence 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 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. It can therefore be a problem in these wavelength ranges, as the signal-to-noise ratio is decreased (there is more noise), resulting in decreased sensitivity and false positives. In addition, autofluorescence spillover into other channels can mask low expressers.

The level of autofluorescence can be determined using unstained controls (Figure 28). As autofluorescence weakens at longer light wavelengths, fluorophores emitting above 600 nm have less autofluorescence interference. The use of a very bright fluorophore will also reduce the impact of autofluorescence.

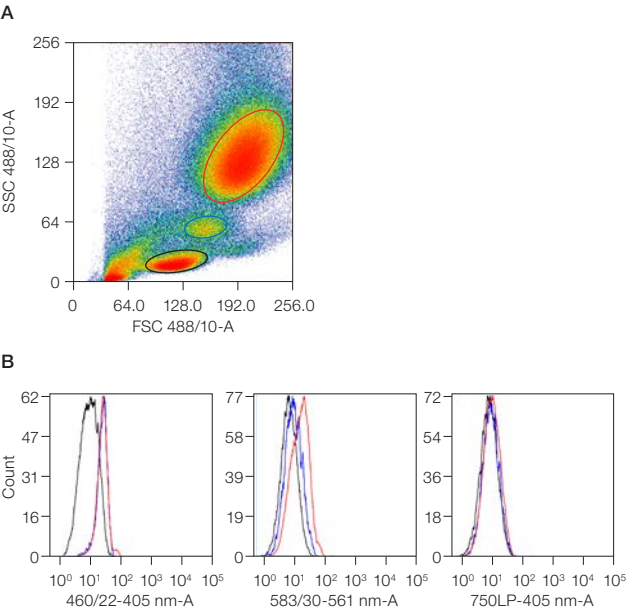


Fig. 28. Autofluorescence levels. Fig. 28. Autofluorescence levels. The levels of autofluorescence in unstained peripheral blood can differ depending upon the wavelength of the laser beam, emission wavelength, and lineage. **A**, FSC vs. SSC plot showing gates for granulocytes (red), monocytes (blue), and lymphocytes (black). **B**, histograms showing increased levels of autofluorescence in granulocytes (red) and monocytes (blue) versus lymphocytes (black) at short (left and middle histograms) but not long wavelengths (right histogram). FSC, forward scatter; LP, long pass; SBV, StarBright Violet; SSC, side scatter.

Live/Dead Exclusion

The presence of dead cells in your sample can greatly affect your staining and therefore the quality of your data. As discussed in Chapter 4, dead cells have greater autofluorescence and increased nonspecific antibody binding compared with live cells, leading to false positives and reducing the dynamic range. This may make identification of weakly positive samples and rare populations difficult. Including a viability control, not relying on FSC and SSC, will improve your data.

DNA binding dyes, such as propidium iodide (PI), 7-AAD, and DAPI, are not membrane permeable and are therefore excluded from live cells. They can be added after staining just before acquisition but cannot be used on fixed cells, as the fixation process renders the membrane permeable.

Protein binding dyes are a second group of viability dyes that can be used to discriminate live and dead cells in your samples. These bind to primary amines and both live and dead cells. However, when a cell has a compromised membrane, as seen in dead and dying cells, a greater amount of protein is accessible, therefore the cells have higher fluorescence. Protein binding dyes have two main benefits. Firstly, they can be fixed (they can also be used unfixed) without any reduction in the resolution between live and dead cells. Secondly, they are available in a broader range of excitation and emission spectra than DNA binding dyes, making their addition to multicolor flow cytometry panels convenient (Table 5).

As the dead cells are excluded from the analysis, unwanted spillover from these dyes is also excluded from the analysis, but you should always include a single stain to enable compensation.

Table 5. Viability dyes for flow cytometry.

Viability Dye	Laser Line	Maximal	
		Excitation, nm	Emission, nm
VivaFix 353/442	355	353	442
DAPI	355 405	359	461
Propidium iodide	355 488 561	490	617
VivaFix 410/450	405	410	450
VivaFix 408/512	405	408	512
VivaFix 398/550	405	398	550
VivaFix 498/521	488	498	521
7-AAD	488 561	546	647
VivaFix 547/573	561	547	573
VivaFix 583/603	561	583	603
VivaFix 649/660	640	649	660

7-AAD, 7-aminoactinomycin D; DAPI, 4',6-diamidino-2-phenylindole.

Doublet Discrimination

Doublet discrimination, as the name suggests, allows you to count single cells and exclude doublets from your analysis. As the sample passes the interrogation point, it creates a signal pulse, giving you the height, time (width), and area of the signal for each parameter. If more than one cell passes through, the cytometer will register them all as one cell. This leads to false statistics, over- or underrepresentation of subsets, and false staining patterns.

Doublet exclusion is performed by plotting the height or width against the area for forward scatter or side scatter. Doublets will have double the area and width values of single cells while the height is roughly the same. Therefore, disproportions between height, width, and area can be used to identify doublets (Figure 29).

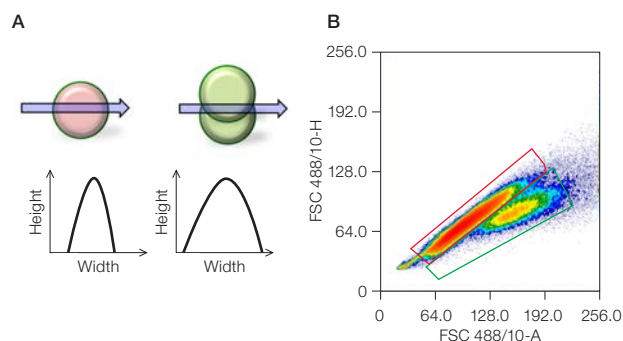


Fig. 29. Doublet discrimination. **A**, doublets (green) can be distinguished from single cells (red) by plotting FSC height vs. FSC area. **B**, doublets (green gate) have an increased width (and therefore area) and a similar height to single cells (red gate). Blue arrows represent the laser beam. FSC, forward scatter.

While doublet discrimination is important in all acquisitions, it is especially important in cell sorting, cell cycle, and DNA analysis. In general, when acquiring a multicolor panel of peripheral blood, a doublet of a B and T cell would be positive for both the T cell marker CD3 and the B cell marker CD19. In cell sorting, if a fluorescence-positive cell and a negative cell pass through the laser at the same time, it will produce a positive pulse, leading to false positives and poor sorting results (Figure 30).

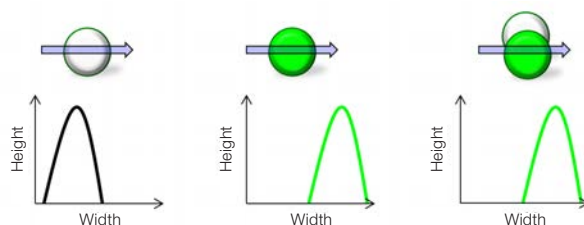


Fig. 30. Doublet discrimination in cell sorting. A doublet of a positive and a negative cell will register as one positive cell, leading to poor purity in sorting experiments.

In cell cycle analysis, it is important to distinguish between doublets and single cells that have twice the amount of DNA, as both show increased fluorescence when stained with a DNA dye. Fortunately, doublets can be distinguished from single cells. Doublets contain two cells and therefore four complete copies of DNA, whereas single cells late in their cell cycle contain only two copies of DNA. These values can be denoted as $4n$ and $2n$, respectively, where n equals the number of DNA copies.

Collect a Statistically Relevant Number of Cells

The number of cells you need to collect during analysis to have enough statistical power can vastly differ depending on the sample and frequency of your cells. Theoretically, with perfect controls, just one event can be seen as a positive result, but in practice this is unlikely to be publication-quality. If you have a sample with an abundant cell type, such as T cells in human peripheral blood, which represent around 20% of total mononuclear cells, you will have to collect and stain fewer cells than if you are looking at natural killer (NK) cells, which have a frequency around 5%, to collect the same number of events. Table 6 shows an example of how the frequency of cells can affect the number of cells collected.

Table 6. Cell frequency.

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

In addition, the number of markers simultaneously required to look at cell subsets can affect the number of cells that need to be acquired. Generally, using more markers requires more cells, as more sequential gates are required to identify the subsets of interest. An example of this can be seen in Figure 31. Despite acquiring 600,000 cells after several rounds of gating to identify resting regulatory T cells (Tregs), the final number of positive cells was ~0.25% or roughly 1,500 events.

Finally, performing the right controls to determine the variation in your experiment and allow definition of a positive or negative population is also very important and will allow you to make accurate judgements on low numbers of events. More detailed information on how to collect enough events can be found in an article by Roederer (2008).

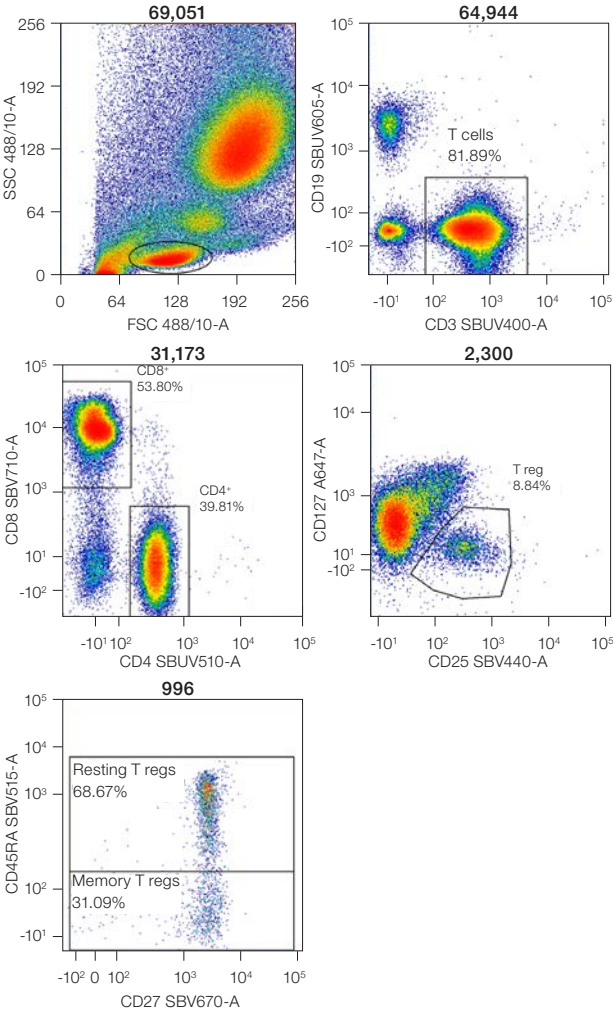


Fig. 31. Effect of gating on cell number. A total of 600,000 cells was stained with CD3SBUV400, CD19SBUV605, CD4SBUV510, CD8SBV710, Mouse Anti-Human CD25:StarBright Violet 440 (CD25SBV440), Human Anti-Human CD127:Alexa Fluor 647 (CD127A647), and CD45RASBV515 to identify resting Treg cells. The gating strategy shows that even though 600,000 cells were stained initially, after several gates have been applied, the final population of resting Tregs contains only 996 cells. SBUV, StarBright UltraViolet; SBV, StarBright Violet; Treg, regulatory T.

Permeabilization and Fixation for Intracellular Antigens

Staining intracellular antigens (cytokines, for example) can be difficult because antibody-based probes cannot pass easily through the plasma membrane into the cell. 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 crosslinked proteins, and glutaraldehyde increases autofluorescence. Fixatives are 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 and stored in PBS at 4°C.

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 that act by disrupting the cellular membrane. The level of permeabilization is important, as the epitope access may require different levels of permeabilization (for example, cytoplasmic vs. nuclear epitopes) and the 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 Reagent (Figure 32).

Alcohols are also used as fixatives, typically as a cold 70% (v/v) 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 be masked by the denaturing process with alcohol fixation, resulting in no detectable antibody staining. Optimization may be required in this case. Alcohols are most commonly used as fixatives for DNA analysis.

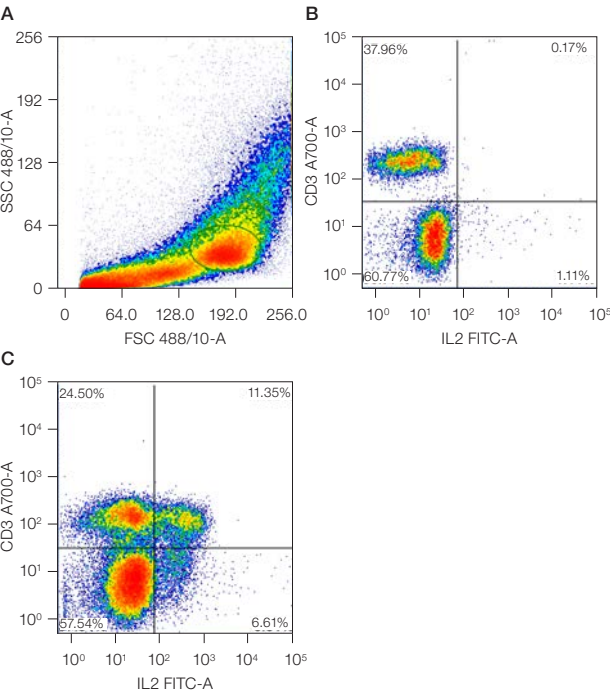


Fig. 32. Intracellular staining. Human whole blood surface stained with Mouse Anti-Human CD3:Alexa Fluor 700 (#MCA463A700) followed by permeabilization using Leucoperm Reagent (#BUF09) and intracellular staining with Rat Anti-Human Interleukin-2:FITC (# MCA1553F). **A**, FSC vs. SSC plot; **B**, resting lymphocytes; **C**, lymphocytes stimulated using Cell Stimulation Reagent with Brefeldin A (#BUF077A) for 5 hr prior to staining. Axxx, Alexa Fluor; FITC, fluorescein isothiocyanate; FSC, forward scatter; SSC, side scatter.



6. Multicolor Panel Building

Multicolor flow cytometry involves analyzing multiple fluorescent parameters in one sample. These parameters may be surface markers, intracellular markers, DNA, or a combination. In addition to using the right controls (Chapter 4), optimizing your experimental procedure (Chapter 5), and carefully preparing your samples, there are still other factors to consider to achieve meaningful results with multicolor flow cytometry. When multiple dyes are used, signal from each dye can spill over into neighboring channels used to detect other dyes. This spillover can cause a loss in resolution and needs to be compensated for (Chapter 2). Other factors including high levels of noise caused by nonspecific staining, high background staining, and cell autofluorescence, can also contribute to reductions in sensitivity and resolution.

Signal Resolution

Resolution can be described as the ability to distinguish positive and negative populations. In practice, resolution depends on the instrument, fluorescent dye compatibility, and gating. This could involve detecting B and T cells in a lymphocyte gate and then determining which type of T cells are present within the T cell population (Figure 33). To achieve optimal resolution, there are a few simple rules, discussed later, that will help form the basics of all panel design. These best practices may not always result in a perfect panel at first, but they will ensure you have a solid starting point.

Rules for Optimized Panel Building

When building a multicolor panel, you should consider a wide variety of factors, as discussed below.

Experimental Design

Before you build your panels, you should have a clear idea of how you will design your experiment. Considerations include:

- Are you looking at one cell type or a subset?
- Will there be an increase or decrease in the number of cells in a population of interest?
- Will you need to change your FSC or SSC settings?
- Are you looking at an activation marker or a change in cell frequency?
- Are the markers co-expressed?
- Is the marker expression pattern known?
- What are the appropriate controls?

Remember, when designing any experiment, controls are important to confirm the data you are seeing is significant. Some specific flow cytometry controls are discussed in Chapter 4.

Instrument Configuration

It is important to know the configuration of your instrument before you start to plan your panel. You cannot use a fluorescent dye that is not configured for your instrument, regardless of which is the theoretical best fit. Instrument configuration includes the setup of the lasers, optics, and filters in the cytometer. This can vary significantly between cytometers. The Bio-Rad S3e Cell Sorter has two lasers

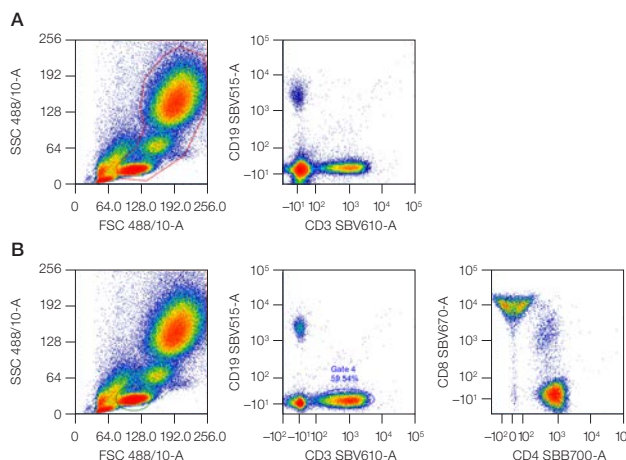


Fig. 33. Cell population resolution. A, B and T lymphocytes can be resolved from all the cells in human peripheral blood, using a simple forward and side scatter gate and staining for CD19 and CD3. B, using a lymphocyte gate and additional markers for CD4 and CD8, not only can B and T lymphocytes be determined but also the ratio of T helper and T cytotoxic cells within the T cell population. FSC, forward scatter; SBB, StarBright Blue; SBV, StarBright Violet; SSC, side scatter.

and four fluorescence detectors with co-linear lasers. The Bio-Rad ZE5 Cell Analyzer has five spatially separated lasers and the potential to simultaneously detect 27 fluorescent dyes. A fluorescent dye's excitation and emission peaks will determine whether it is compatible with your instrument. FITC is excited at 490 nm and emits at 520 nm; therefore, a 488 nm laser and a 525/35 filter (or similar) is required. If you have a fluorescent dye excited at 355 nm and do not have an ultraviolet laser, it won't be detected in your cytometer. Similarly, if you do not have a filter with the right band pass (for example, a 670/30 is required for StarBright Violet 670 Dye), you will either see no signal or a weak one, as only a proportion of the signal will be detected.

Fluorescent Dye Separation

Ideally, when building multicolor panels, it is best to separate fluorescent dye excitation across lasers, and where possible, the emission across the detectors. This will minimize the amount of spillover and therefore the need for compensation. It will also reduce the effect that fluorescence spread will have on your data. However, as you increase the number of

fluorescent dyes in your panel, it will not always be possible to separate emission across detectors. Therefore, other considerations need to be included in your design. The example below shows how using the Bio-Rad Spectraviewer can help you pick fluorescent dyes that have more distinct excitation and emission profiles, which will therefore have less influence on the other fluorescent dyes in the panel. As shown in Figure 35, a five-color panel of SBV440, FITC, SBB700, PE, and A647 dyes is relatively easy to build without spectral overlap. Three more fluorescent dyes can be added, for example SBV515, SBV610, and APC-Cy7, to make an eight-color panel and still minimal compensation is required. When SBV670, SBV790, PE-A647, PE-A750, and A700 are added to make 13-color panels, more compensation is needed and there are fewer channels where extra fluorescent dyes can be added without significant influence on the existing fluorescent dyes. The addition of an ultraviolet 355 nm laser to your cytometer is a great enhancement to the instrument, as you will be able to add fluorescent dyes to make larger panels or change dyes to avoid spectral overlap.

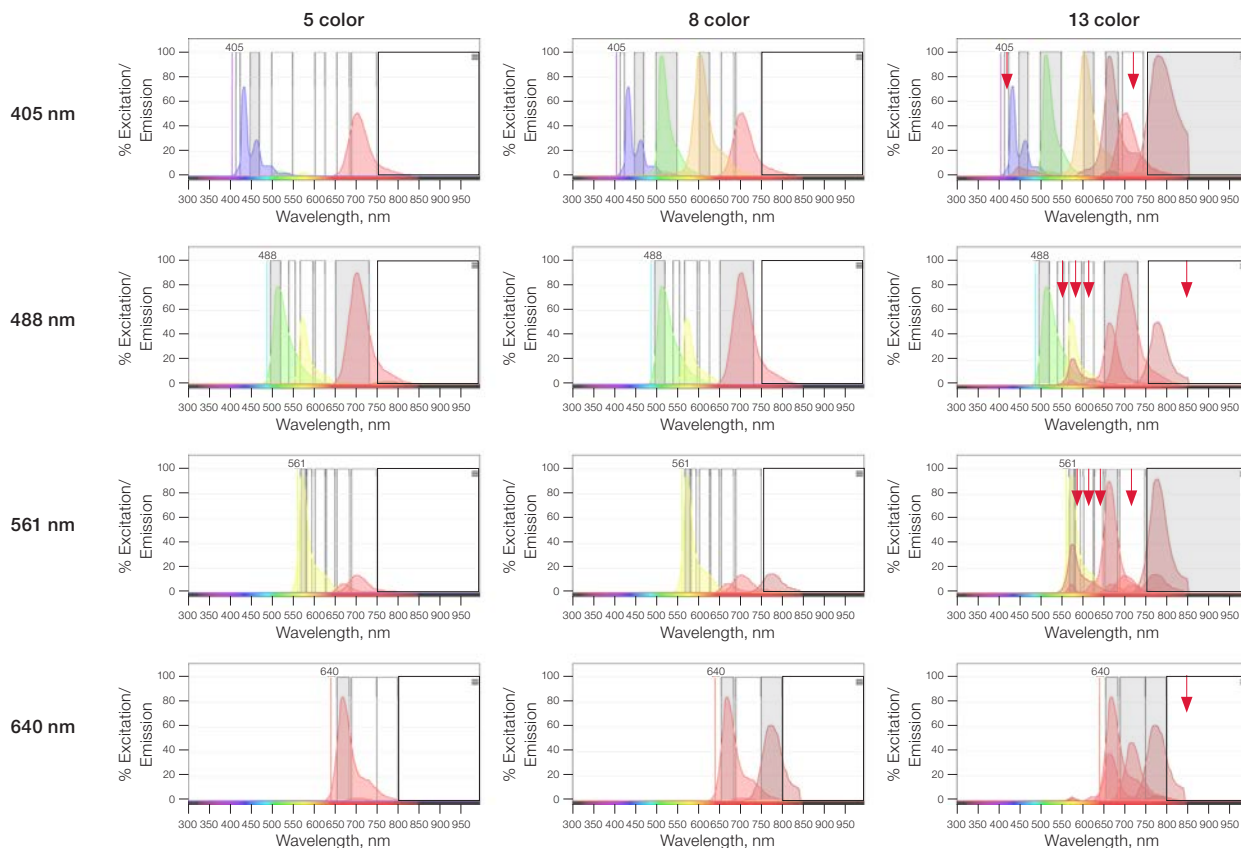


Fig. 34. Using the Bio-Rad Spectraviewer to avoid spectral overlap. When building larger panels (for example, moving from a five-color panel to a 13-color panel), Bio-Rad Spectraviewer can help pick lasers and filters that are unused and have little influence with other fluorescent dyes, minimizing compensation and spread. Gray boxes represent the channels used in the panel, and red arrows show the remaining empty channels within a 13-color panel. Images generated using the Bio-Rad Spectraviewer with a preloaded, four-laser (405, 488, 561, and 640 nm) ZE5 Cell Analyzer configuration.

Titration requires samples with the same number of cells, in the same volume, but with serial dilutions of antibody between samples. If 5 μ l of an antibody was recommended, then doubling dilutions of 2.5 μ l, 1.25 μ l, 0.6125 μ l, and so on, would be suitable. You should use the dilution with the highest stain index. The points in the green box of Figure 36 represent the concentrations that will generate specific staining with a minimal amount of background.

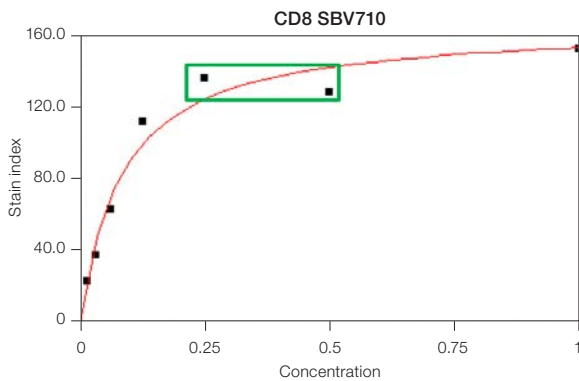


Fig. 36. Antibody titration. Plotting the stain index for each concentration of antibody will allow you to titrate the optimal amount of antibody for your experiment. SBV, StarBright Violet.

Panel Building Tools

There are useful tools that can support panel design. Spectraviewers (Figure 34) help you determine the excitation and emission and amount of spillover by each laser. Relative brightness tables give you information on pairing targets with fluorescent dyes, and marker expression data help determine cellular expression patterns.

Online panel building apps can also assist with panel design. They allow you to choose your instrument, determine the antigen density, pick the correct fluorescently labeled antibody, and build a panel that takes these things into consideration. Visit bio-rad-antibodies.com/flow to access these resources.

There are also published examples of optimized multicolor immunofluorescence panels (OMIPS), which can help with your panel design and are often a good starting point when designing new panels, as both the panel and gating strategy are shown.

Finally, remember compromises will have to be made due to the dye, antibody, and cytometer availability, as well as the cell type you are working with. Several iterations of a panel may have to be designed and improved upon, but following these rules should reduce the number of iterations and therefore time and effort required.

Example of Conventional Flow Cytometry Multicolor Panel

This section outlines an example of an 18-color panel acquired on the ZE5 Cell Analyzer, including the experimental design and optimization steps.

- SAMPLE PREPARATION** — peripheral blood was collected in heparin and red cells lysed prior to staining.
- INSTRUMENT** — this panel was acquired on a five-laser ZE5 Cell Analyzer, capable of detecting 27 different fluorescent parameters.
- FLUORESCENT DYE SEPARATION** — the fluorescent dyes used in the panel are shown in Figure 37. As there were 18 dyes in total (Table 7), it was impossible to avoid spectral overlap, but all five lasers were used to minimize this.
- ANTIGEN DENSITY AND MARKER EXPRESSION PATTERNS** — the dye choice for each marker was determined by the brightness, spillover, antigen density, and expression patterns. Phycoerythrin-Alexa Fluor 647 (PE-A657) was placed on CD45, as it had significant spillover into many channels. Because CD45 is expressed on all leukocytes and expected to be present on every cell, it is less of a problem. StarBright UltraViolet 575 (SBUV575) Dye has spillover into StarBright UltraViolet 605 (SBUV605) Dye, so they were both placed onto mutually exclusive markers. StarBright Violet 440 (SBV440) Dye was placed on the low-density marker CD25 because of its minimal interference from other dyes.

Table 7. Antibodies used in the panel. Only mouse anti-human antibodies were used.

Target	Fluorescent Dye	Target	Fluorescent Dye
CD3	SBUV400	CD14	SBV790
CD4	SBUV510	CD57	FITC
CD28	SBUV575	CD10	SBB700
CD19	SBUV605	CD20	PE
CD25	SBV440	CD45	PE-A647
CD45RA	SBV515	CD38	PE-A750
CD45RO	SBV610	CD127	A647
CD27	SBV670	CD16	A700
CD8	SBV710	Live/Dead	VivaFix 583/603

Axxx, Alexa Fluor; FITC, fluorescein isothiocyanate; PE, phycoerythrin; SBB, StarBright Blue; SBV, StarBright Violet; SBUV, StarBright UltraViolet.

- DUMP CHANNEL AND ANTIBODY TITRATION** — no dump channel was included in this panel, as we were interested in all the cell populations. However, all the antibodies were titrated prior to building the 18-color panel to optimize the staining.

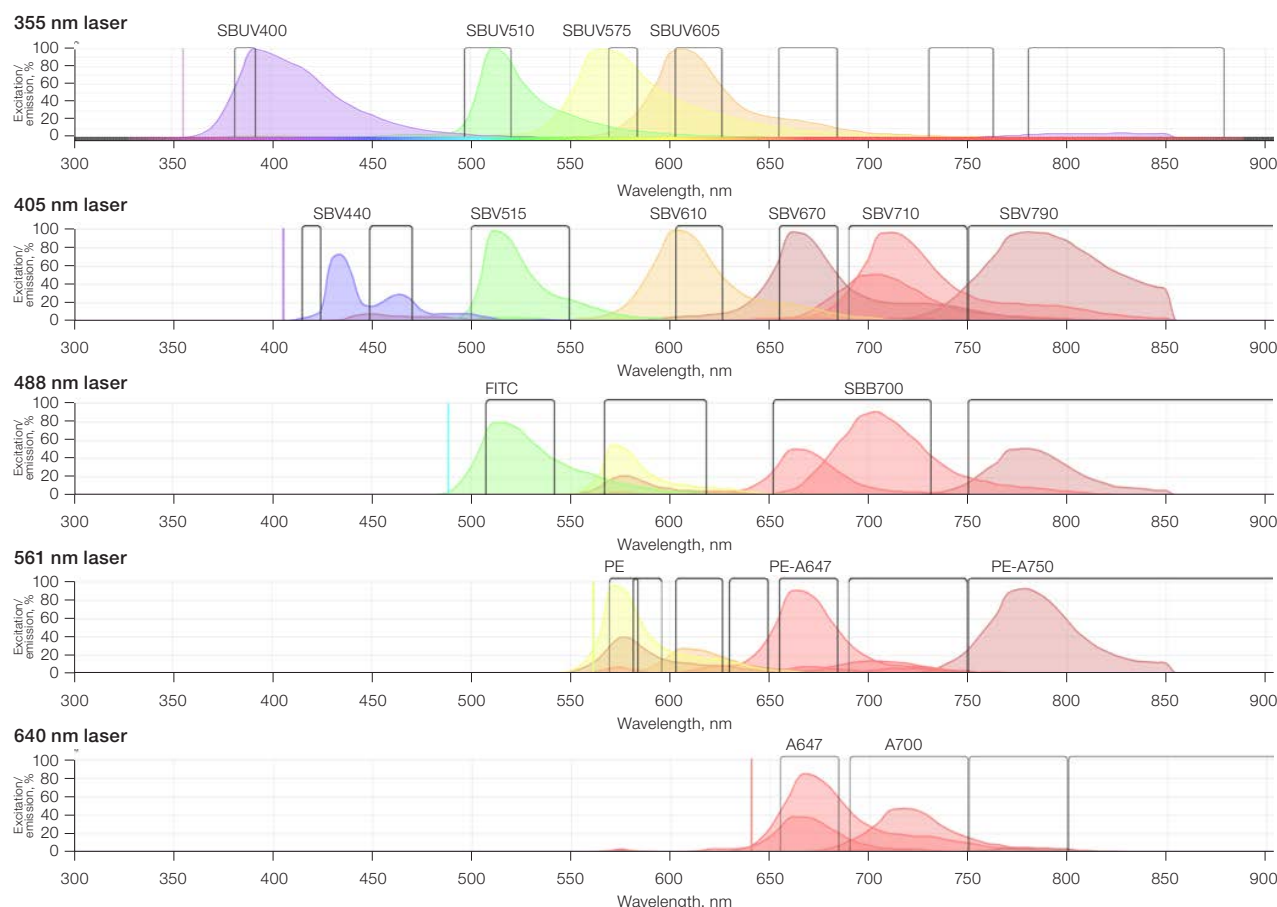


Fig. 37. Spectral traces of all fluorescent dyes used in the panel. Traces were acquired using the preloaded five-laser ZE5 Cell Analyzer configuration. Axxx, Alexa Fluor; FITC, fluorescein isothiocyanate; PE, phycoerythrin; SBB, StarBright Blue; SBV, StarBright Violet; SBUV, StarBright Ultraviolet.

6. **STAINING** — cells were washed after red cell lysis and blocked in 10% human serum prior to staining. The staining was done in 1% BSA in PBS for 1 hr at room temperature. After staining, the cells were washed three times before acquisition. No intracellular staining was performed.
7. **CONTROLS** — unstained controls were used for instrument setup and single stained controls were used for compensation. Beads were used for some markers due to low level staining or insufficient positive and negative signal. Cells heated to 65°C for 10 min were used as a live/dead cell compensation control. A viability dye was included in the panel to exclude dead cells from the analysis. FMO controls were also performed to confirm gating strategies (data not shown). As we were not comparing against any other sample, no experimental controls were required.
8. **SAMPLE ACQUISITION** — the sample was acquired immediately without fixation. To ensure identification of small populations, 600,000 cells were collected.
9. **DATA ANALYSIS** — see Figures 38–41. The dead cells and doublets were first excluded from the analysis. Lymphocytes, monocytes, and granulocytes were identified by FSC and SSC characteristics. Sequential gating was performed to identify specific populations based on marker expression. T and B lymphocyte, monocyte, and granulocyte lineages with subsets and activation states can be seen. In addition, dimensional reduction was performed to create a t-distributed stochastic neighbor embedding (tSNE) plot. This clusters cells with similar staining together allowing identification of “islands” with similar properties. Examples of tSNE plots gated by markers are shown in Figure 42.

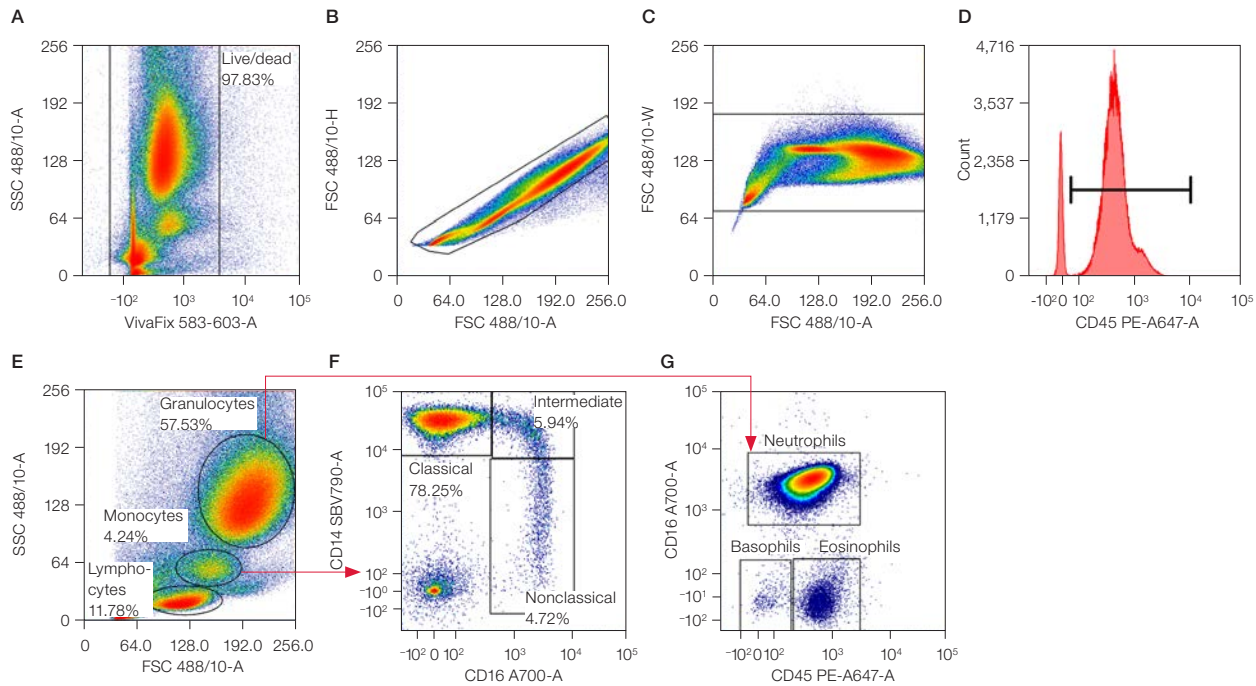


Fig. 38. 18-color conventional flow cytometry panel. After removing the dead cells and the doublets, cells were identified as lymphocytes, monocytes, or granulocytes based on their CD45 expression and forward and side scatter. Monocyte and granulocyte subsets were determined by CD14 and CD16 expression. See Figures 39–41 for lymphocyte subsets. Axxx, Alexa Fluor; FSC, forward scatter; PE, phycoerythrin; SBV, StarBright Violet; SSC, side scatter.

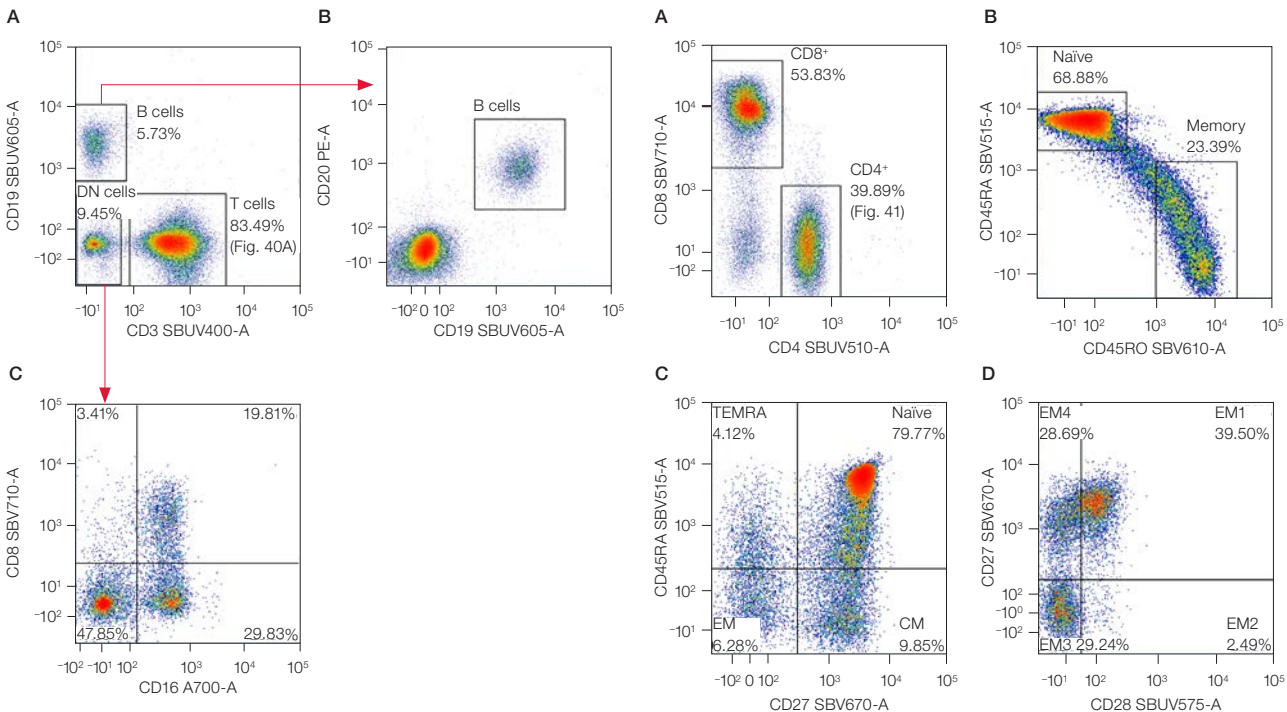


Fig. 39. 18-color conventional flow cytometry panel. T, B, and NK cell subsets (A) were identified within the lymphocyte gate (Figure 38E) based on CD3⁺ (Figure 40A), CD19⁺CD20⁺ (B), and CD3⁺CD19⁺ (C), respectively. CD8 and CD16 expression within the NK cell population confirmed NK cells were present. Axxx, Alexa Fluor; DN, double negative; PE, phycoerythrin; SBV, StarBright UltraViolet; SBV, StarBright Violet.

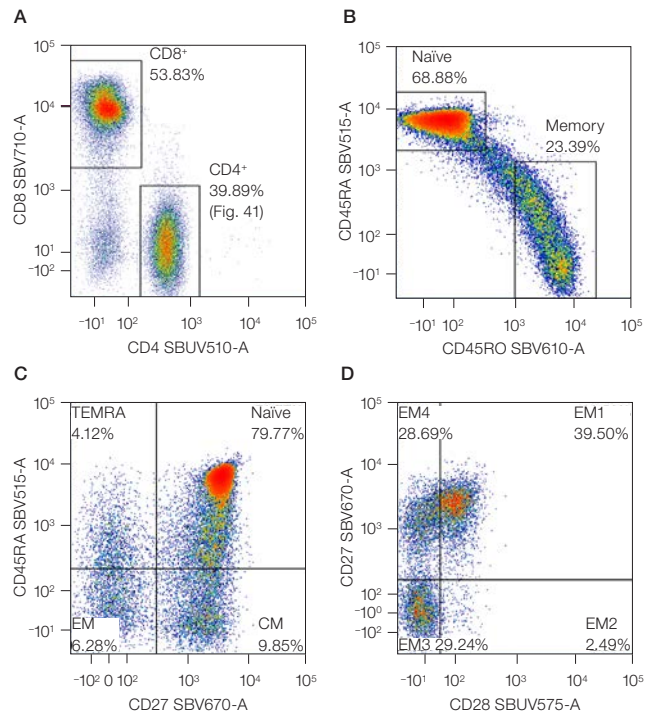


Fig. 40. 18-color conventional flow cytometry panel. Within the CD3⁺ population (Figure 39B), the CD8⁺ T cytotoxic cells (A) could be further split into various subsets (B–D) based on CD45RA, CD45RO, and CD27 expression. Furthermore, the EM population (C) could be split into EM1–4 populations (D) based on CD27 and CD28 expression. CM, central memory; EM, effector memory; SBV, StarBright UltraViolet; SBV, StarBright Violet; TEMRA, CD45RA⁺ effector memory T cells.

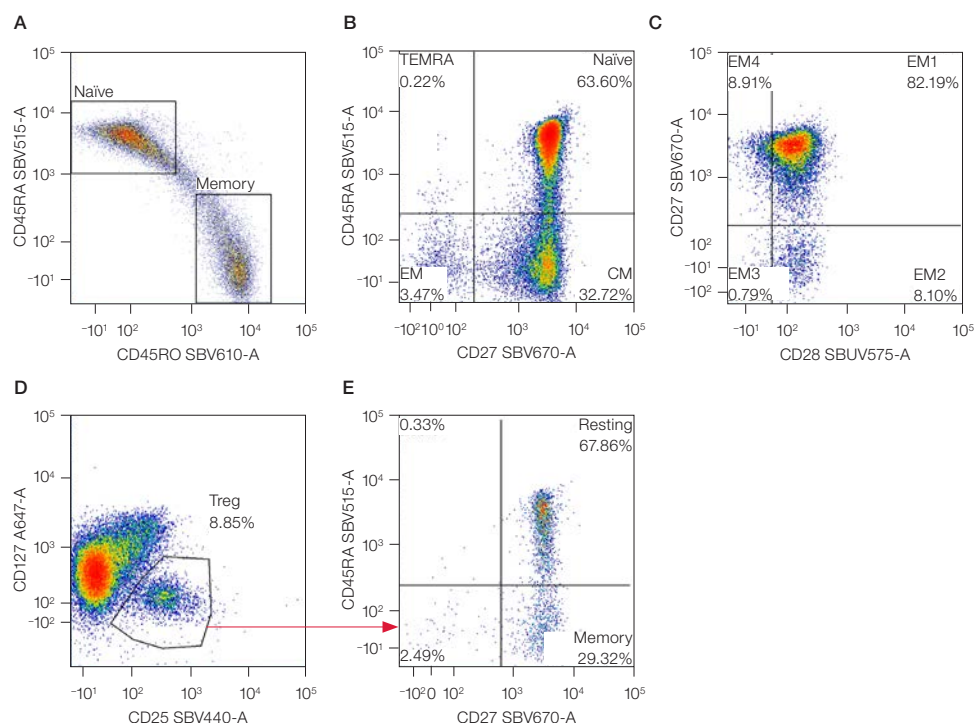


Fig. 41. 18-color conventional flow cytometry panel. Within the CD3⁺ population (Figure 39B), the CD4⁺ T helper cells (Figure 40A) could be further split into various subsets based on CD45RA, CD45RO, and CD27 expression (B). Furthermore, the EM population could be split into EM1-4 populations based on CD27 and CD28 expression (C). T regulatory cells could be identified by CD127⁺CD25⁺ expression (D) and further split into resting and memory based on CD45RA and CD27 expression (E). Axxx, Alexa Fluor; CM, central memory; EM, effector memory; SBV, StarBright Violet; TEMRA, CD45RA⁺ effector memory T cells; Treg, T regulatory.

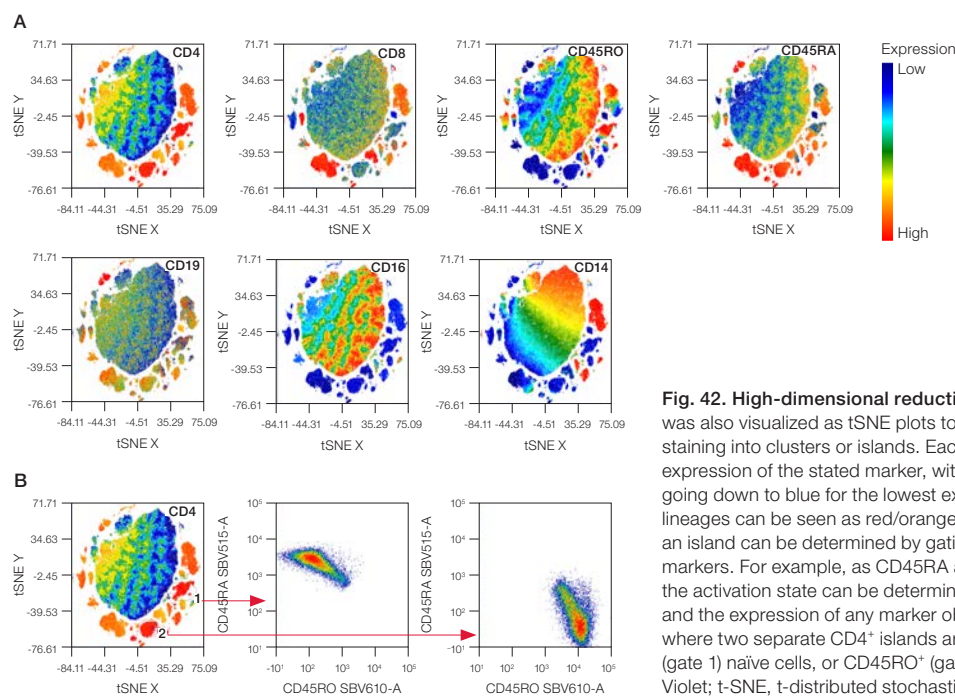


Fig. 42. High-dimensional reduction analysis. A, The 18-color panel was also visualized as tSNE plots to group together cells with similar staining into clusters or islands. Each tSNE density plot shows the relative expression of the stated marker, with red having the highest expression going down to blue for the lowest expression. T cell, B cell, and myeloid lineages can be seen as red/orange islands. B, further characterization of an island can be determined by gating on an island and looking at other markers. For example, as CD45RA and CD45RO is included in the panel, the activation state can be determined by placing gates on red clusters and the expression of any marker observed. This can be seen for CD4 where two separate CD4⁺ islands are revealed to be either CD45RA⁺ (gate 1) naive cells, or CD45RO⁺ (gate 2) memory cells. SBV, StarBright Violet; t-SNE, t-distributed stochastic neighbor embedding.

Full Spectrum Flow Cytometry Panel Building

The constraints of separating fluorescent signals using combinations of band pass and dichroic mirrors are not present in full spectrum flow cytometry. Spectrally similar fluorescent dyes can be effectively used in panels; for example, APC and Alexa Fluor 647 can be separated. This has led to the development of larger panels of over 40 fluorescent dyes. However, each fluorescent dye must have a unique spectral signature and a similarity index of less than 0.98 (where 1 would represent identical dyes).

The rules of panel design still apply to full spectrum flow cytometry and there are some additional considerations:

- Tandem dyes should not be used for dump channels, as there can be some variation in the spectral signature
- Single stained spectral reference controls should have the same spectral signature as the experimental sample
- Using preloaded spectra from previous experiments is not recommended
- The use of some buffers can affect the profile of some beads

More information can be found in Ferrer-Font et al. (2020).

Example Full Spectrum Multicolor Panel

1. **SAMPLE PREPARATION** — peripheral blood was collected in heparin and red cell lysed prior to staining.
2. **INSTRUMENT** — this panel was acquired on a four-laser Aurora Spectral Analyzer (Cytek Biosciences) with 58 detectors.
3. **FLUORESCENT DYE SEPARATION** — as there were 27 dyes in total (Table 8), it was impossible to avoid spectral overlap, but combinations were chosen to ensure unique spectral profiles with similarity scores below 0.98 for all fluorescent dyes chosen.

4. **ANTIGEN DENSITY AND MARKER EXPRESSION PATTERNS** — the dye choice for each marker was determined by the dye brightness, spillover, antigen density, and expression patterns to minimize spread. Of note, CD3 and CD19 were placed on StarBright UltraViolet 400 (SBUV400) Dye and StarBright UltraViolet 445 (SBUV445) Dye due to spillover, and IgD and CD2 on Brilliant Violet 510 (BV510) and StarBright Violet 515 (SBV515) Dye due to similarity between these dyes. CD14 and CD16 were separated to avoid spillover by using StarBright Violet 790 (SBV790) Dye and PE. The dim marker CD25 was placed on StarBright Violet 610 (SBV610) Dye, a bright fluorescent dye. As with the conventional panel, populations of interest such as CD45RA/CD27 and CD27/CD28 positive T cells, which could be both positive and negative for each marker, were placed on dyes that had minimal spillover. Spectral flow cytometry allows you to combine existing dyes in configurations not possible in conventional flow. StarBright Dyes enable novel combinations.

Novel combinations include:

- StarBright Blue 700 (SBB700) Dye/PerCP-Cy5.5
- Pacific Blue/Brilliant Violet 421 (BV421)/StarBright Violet 440 (SBV440) Dye
- StarBright Violet 475 (SBV475) Dye/BV510/SBV515 Dye
- Brilliant Violet 605 (BV605)/SBV610 Dye
- StarBright Violet 710 (SBV710) Dye/Brilliant Violet 711 (BV711)

Table 8. Antibodies used in the panel.

Target	Fluorescent Dye	Target	Fluorescent Dye	Target	Fluorescent Dye
CD3	SBUV400	CD19	SBUV445	CD4	SBUV510
CD28	SBUV575	HLA DPDQDR	BUV615	CD56	BV421
CD11b	Pacific Blue	CD45	SBV440	CD8	SBV475
IgD	BV510	CD2	SBV515	CD33	SBV570
CD11c	BV605	CD25	SBV610	CD27	SBV670
CD10	SBV710	TCR	BV711	CD14	SBV790
CD57	FITC	CD45RO	SBB700	CD20	PerCP-Cy5.5
CD16	PE	CD38	PE-A750	CD45RA	A700
CD127	A647	CD40	APC-Cy7	Viability	Live/Dead Fixable Blue

Axxx, Alexa Fluor APC, allophycocyanin; BUV, Brilliant Ultraviolet; BV, Brilliant Violet; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SBB, StarBright Blue; SBUV, StarBright UltraViolet; SBV, StarBright Violet

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PRINCIPLES OF THE FLOW CYTOMETER	5. DUMP CHANNEL AND ANTIBODY TITRATION — no dump channel was included in this panel, as we were interested in all cell populations. However, all the antibodies were titrated prior to building the 27-color panel to optimize the staining. 6. STAINING — cells were washed after red cell lysis and blocked in 10% human serum prior to staining. The staining was done in BD Brilliant Stain Buffer for 1 hr at room temperature. After staining, the cells were washed three times before acquisition. No intracellular staining was performed. 7. CONTROLS — unstained controls were used for instrument setup and single stained controls were used for compensation. Beads were added for some markers due to low-level staining or insufficiently positive and negative signals. Cells heated to 65°C for 10 min were used as a live/dead compensation control. A viability dye was included in the panel to exclude dead cells from the analysis. FMO controls were also performed to confirm gating strategies (data not shown). As we were not comparing against any other sample, no experimental controls were required. 8. SAMPLE ACQUISITION — the sample was acquired immediately without fixation. To ensure we would be able to identify small populations, we collected 400,000 cells. 9. DATA ANALYSIS — see Figure 43. The dead cells and doublets were first excluded from the analysis. Lymphocytes, monocytes, and granulocytes were identified by FSC and SSC characteristics. Sequential gating was performed to identify specific populations based on marker expression. T and B lymphocyte, monocyte, and granulocyte lineages with subsets and activation states can be seen. In addition, dimensional reduction was performed to create a tSNE plot (Figure 44). This clusters cells with similar staining together, allowing identification of “islands” with similar properties. This way, differences between multiple samples can be easily visualized and rare populations can be identified more easily than by using sequential gating.

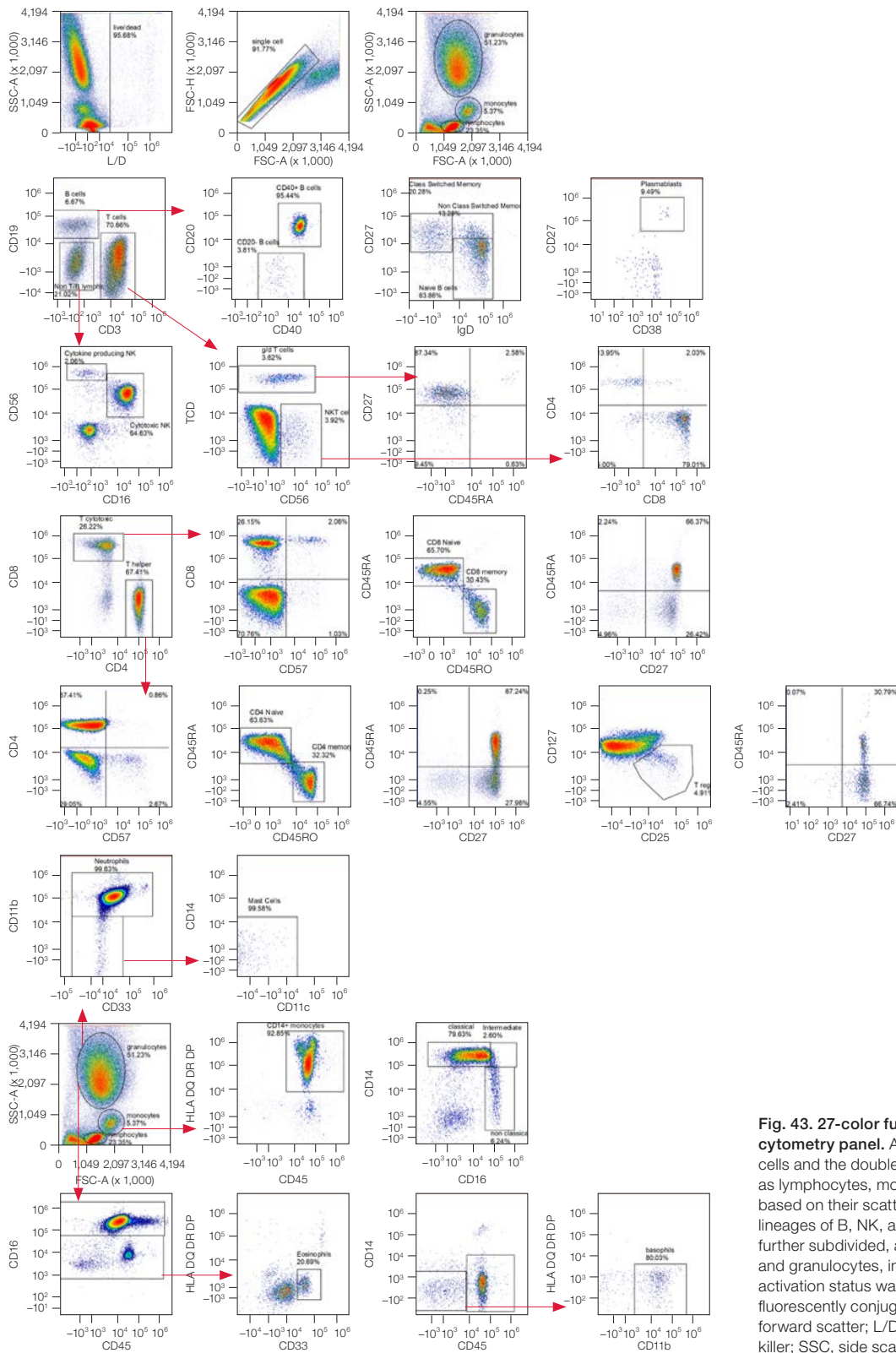


Fig. 43. 27-color full spectrum flow cytometry panel. After removing the dead cells and the doublets, cells were identified as lymphocytes, monocytes, or granulocytes based on their scatter properties. The major lineages of B, NK, and T lymphocytes were further subdivided, as were monocytes and granulocytes, into subsets, and the activation status was also determined using fluorescently conjugated antibodies. FSC, forward scatter; L/D, live/dead; NK, natural killer; SSC, side scatter.

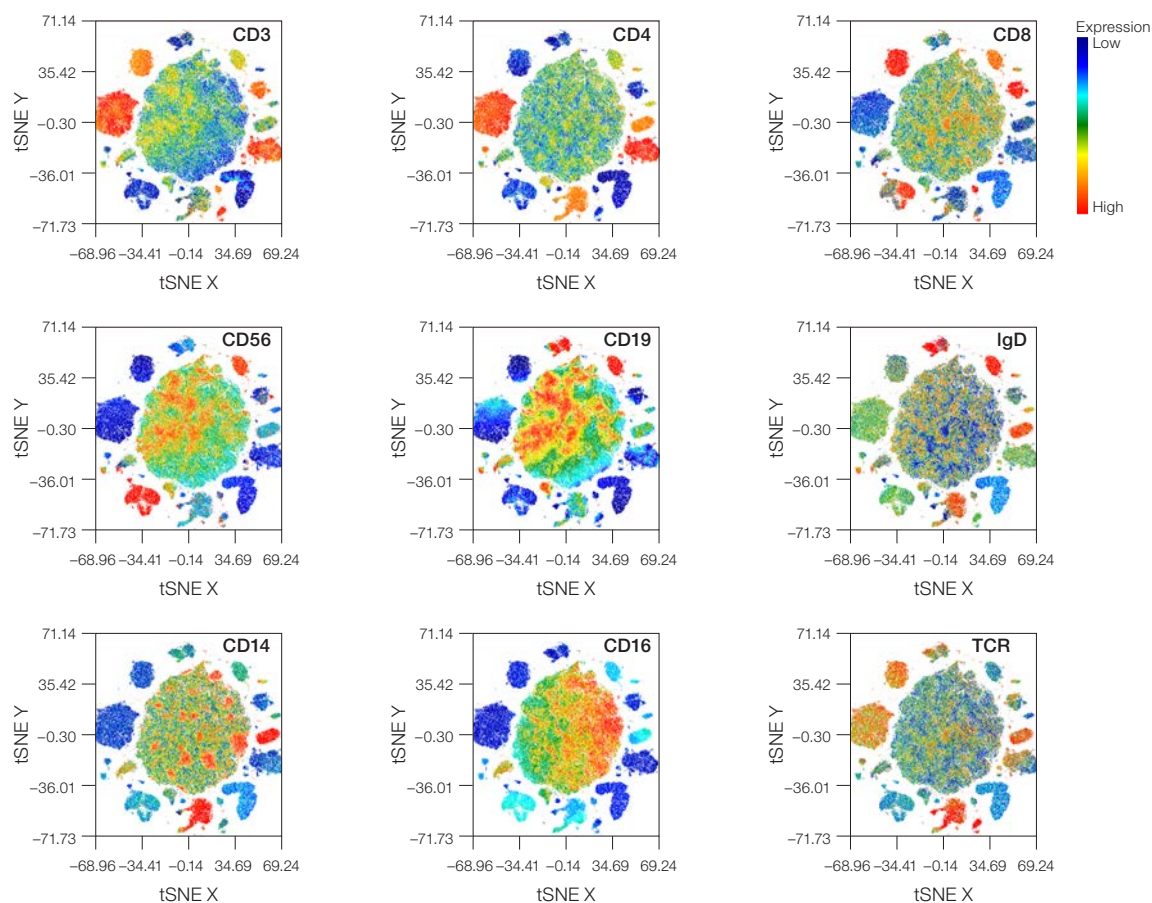


Fig. 44. High-dimensional reduction analysis. The 27-color panel was also visualized as tSNE plots to group together cells with similar staining into clusters or islands. T cell, B cell, NK, and myeloid lineages can be seen as separate islands in the plots. These can be gated and investigated to look at other characteristics such as the activation state using CD45RA, CD45RO, CD27, and CD28. NK, natural killer; tSNE, t-distributed stochastic neighbor embedding.



7. Common Applications and New Technology

So far in this guide, we have covered what flow cytometry involves and how to build experiments and analyze data, but what are the common uses of flow cytometry? This chapter provides examples of flow cytometry applications, such as immunophenotyping and analysis of cellular functions like regulated cell death and cell proliferation. You will learn about novel innovations in flow cytometry, such as the integration of automation.

Immunophenotyping

Flow cytometry is routinely used to identify cell markers, particularly within the immune system. This application is called immunophenotyping, and the need to identify increasing numbers of markers has driven the growth in multiplexing in multicolor flow cytometry.

Immunophenotyping can simply involve identifying a cell by a single marker. More complex immunophenotyping includes the identification of cells using multiple markers, including homing profiles, 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 routinely used in clinical applications to diagnose disease or monitor and evaluate residual disease. A simple example of immunophenotyping using surface staining is shown in Figure 45, where a nine-color staining panel was used to identify major cell subsets in human peripheral blood. Additional information in a more complex panel may include the naïve, memory, or activation status of these lineages (using markers like CD45RA, CD45RO, CD27, CD57, CD62L, and CD69), or their cytokine profile (using markers like IFN- γ , IL-2, IL-17, and IL-9).

It is easy to see how a much larger panel can be quickly built. In fact, in conventional flow cytometry, panels are approaching 30 colors. In full spectrum flow cytometry, panels have surpassed 40 colors and will expand with the release of new fluorescent dyes, including StarBright Dyes. Refer to Chapter 6 for more information on best practices for building multicolor panels.

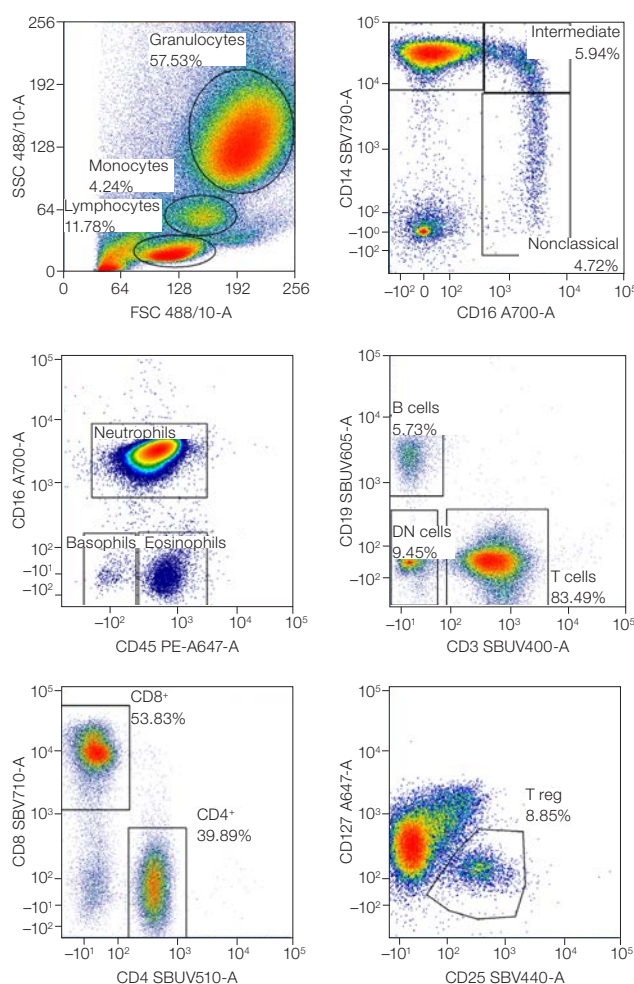


Fig. 45. Immunophenotyping of whole blood. A simple nine-color immunophenotyping panel using CD3, CD4, CD8, CD14, CD16, CD19, CD25, CD45, and CD127 to identify T cell, B cell, granulocyte, and monocyte lineages and subsets. Axxx, Alexa Fluor; FSC, forward scatter; PE, phycoerythrin; SBV, StarBright UltraViolet; SBV, StarBright Violet; SSC, side scatter; Treg, T regulatory.

PRINCIPLES OF THE FLOW CYTOMETER	Cell Sorting Another common use of flow cytometry is cell sorting based on scatter and/or fluorescence properties, either from fluorescent protein expression or from cells stained by fluorescently labeled antibodies. Instruments capable of separating six populations at a time are now available, and many instruments are capable of sorting cells into populations with shared characteristics or into wells containing one cell only. Furthermore, in addition to conventional flow cytometry, full spectrum cell sorters have now been released, allowing the use of novel fluorescent dye combinations, removal of autofluorescence, and easier separation of fluorescent proteins. Sorting of single cells or cell populations allows purification and enrichment for downstream processing in other applications, like PCR or RNA sequencing (RNA-seq), or the selection of cells for culturing. There are also uses in immunological research, physiological research, and protein and cell engineering.
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OPTIMIZING YOUR EXPERIMENTS	Regulated Cell Death Regulated cell death is the overarching term for the many mechanisms by which a cell can undergo programmed death that is not necrosis. It can be in response to many processes, for example development, tissue homeostasis, and host defense. Several of these processes can be measured by flow cytometry, including apoptosis, autophagy, and pyroptosis. Apoptosis is a highly regulated process that plays an important role in embryogenesis, maintaining an organism's size, and eliminating damaged cells. The importance of apoptosis in human health is underscored by the many diseases that result from aberrant apoptosis. Dysregulation of apoptosis has been linked to various cancers, neurological and cardiovascular disorders, and autoimmune diseases. 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, which increases during apoptosis. Annexin V binds to phosphatidylserine and thus annexin V labeled with fluorophores allows apoptosis to be assessed, usually in combination with a viability dye like PI, to distinguish apoptotic from necrotic cells. Healthy cells are negative for both markers, apoptotic cells are positive for annexin V, and necrotic cells are positive for both markers. When Jurkat T cells are treated with staurosporine, they undergo apoptosis, followed by necrosis (Figure 46).
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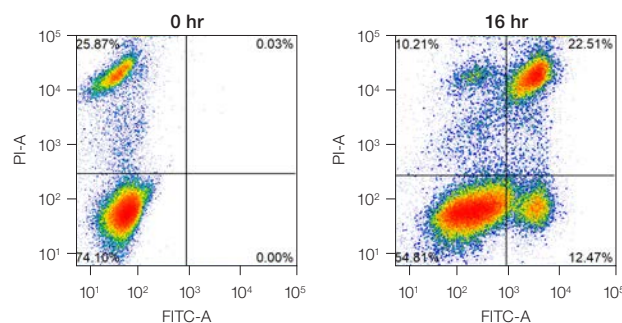


Fig. 46. Annexin V staining to measure apoptosis. Jurkat cells were treated with staurosporine at 1 μ M for 0 hr and 6 hr to induce apoptosis. The cells were then stained with Annexin V FITC and ReadIDrop Propidium Iodide. Apoptotic cells positive for annexin V can be seen in the bottom right quadrant and necrotic positive for both annexin and PI in the top right quadrant. Healthy cells are negative for both stains. FITC, fluorescein isothiocyanate; PI, propidium iodide.

Because PS externalization is a dynamic, reversible process until a cell is committed to apoptosis after mitochondrial outer membrane permeabilization (MOMP), annexin V conjugates are unable to distinguish early from late apoptosis. Polarity-sensitive indicator of viability and apoptosis (pSIVA) probes are biosensors that reversibly bind to PS, and thus turn on and off as PS flips from the outer membrane to the inner membrane. This allows easy comparison of differences in apoptosis rates in response to different experimental treatments in real time.

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 that 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 nonapoptotic 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. Pyroptosis, mediated by caspase-1, can also be measured using FLICA assays (Figure 47).

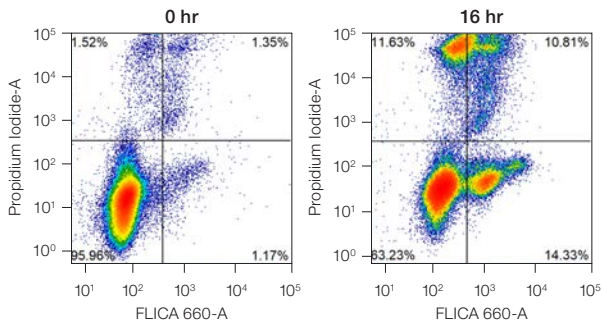


Fig. 47. Caspase-1 staining to measure pyroptosis. Jurkat cells were stained with the Pyroptosis 660 Caspase-1 Kit (#ICT9158) for 2 hr followed by treatment with 10 μ M nigericin for 16 hr to induce pyroptosis as shown. Pyroptosis can be demonstrated by an increase in active caspase-1 detected as an increase in far-red (600 nm) fluorescence. Data analyzed on the ZE5 Cell Analyzer. FLICA, fluorophore labeled inhibitor of caspase assays.

Autophagy plays a critical role in maintaining cellular homeostasis by protein degradation and turnover of damaged cell organelles. The process has a complex dual role, affecting both cell survival and death. It is crucial to the maintenance of a healthy cell, but under certain circumstances autophagy-dependent cell death (ADCDC) can occur. Autophagy can be difficult to detect, often requiring the use of orthogonal techniques for confirmation. It is measured using flow cytometry in two different ways. The first method involves detection of lipidation of LC3-I to LC3-II and LC3-II's subsequent movement from the cytosol to the membrane of autophagosomes. The second method uses cell permeant, aliphatic fluorescent dyes that label autophagosomes and autolysosomes. Signal is often weak, especially in primary cells, but inhibition of lysosomal proteases, by using bafilomycin, for example, provides a way of increasing the signal (Figure 48).

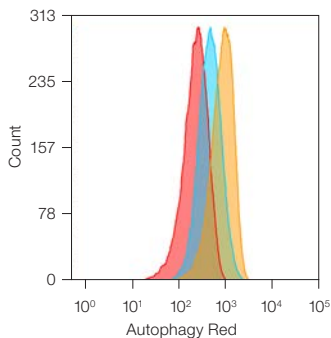


Fig. 48. Autophagy induction in Jurkat cells measured with Autophagy Probe Red. Jurkat cells were either untreated for basal conditions (red), treated with 0.5 μ M rapamycin for 18 hr (blue), or treated with 0.5 μ M rapamycin for 18 hr followed by 10 nM bafilomycin A1 for 2 hr (orange).

Proliferation

Cell proliferation can be measured by flow cytometry using different methods:

- Detecting changes in the forward and side scatter, indicating changes in the cell cycle. This is a simple method but not always the most accurate
- Staining with an antibody against a proliferation marker (Ki67, MCM2, and PCNA are a few examples)
- Using a TUNEL assay. After incubating cells with BrdU, which is incorporated into DNA during the S phase of the cell cycle, BrdU can be detected using fluorescently labeled anti-BrdU antibodies. When combined with a DNA stain, including PI or DAPI, the relative proportion of cells in S phase can be determined
- Using cytoplasmic dyes like CFSE or CytoTrack. 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 (Figure 49). These dyes are nontoxic, do not require cell fixation, and are available in a wide variety of colors. These features make it possible to combine the dyes for immunophenotyping

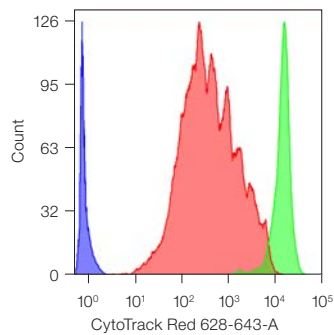


Fig. 49. Measuring proliferation using CytoTrack Assay Kit. Human peripheral blood lymphocytes were stained with CytoTrack Red 628/643 Cell Proliferation Assay Kit (#1351205) and stimulated for 5 days with phytohemagglutinin (PHA). Cells that have proliferated show a reduction in the amount of dye with each cell division. You can see stimulated cells (red), labeled but unstimulated cells (green), and unlabeled cells (blue). Six red peaks corresponding to each division are visible in this experiment. Data were acquired on the ZE5 Cell Analyzer.

Cell Cycle

The proportion of cells within each stage of the cell cycle can be determined using DNA binding dyes that bind in a stoichiometric manner. Common examples include PI, 7-AAD, Hoechst 33342, and DAPI. When using this method, cells in the G2 phase, which have twice as much DNA as cells in G1, will fluoresce twice as brightly. To ensure good staining, the cells should be fixed in cold

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DATA ANALYSIS	70% (v/v) ethanol. However, this can interfere with other staining protocols (see Chapter 8 for advice). Cell cycle analysis is usually measured on a linear scale, unlike most flow cytometry, which uses a logarithmic scale, because the differences in fluorescence are usually smaller. It is important to gate out any doublets from the data and the data can be improved by using a low flow rate on the cytometer. Many flow cytometry software programs offer algorithms to accurately estimate the cell cycle phases.
CONTROLS IN FLOW	Signaling and Phosphoflow In addition to the expression of surface molecules, there is a lot of interest in cellular activation states and measuring signaling cascades. 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 (phosphoflow) using antibodies may differ from normal intracellular staining, and specific experimental controls may be required. For signaling and phosphoflow, 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. Signaling can be measured using dyes that fluoresce in response to changes in calcium. Typically, cells are loaded with calcium indicator dyes, such as indo-1, to determine a baseline level of signaling. They are then stimulated and the change in fluorescence denotes a change in intracellular calcium levels.
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COMMON APPLICATIONS AND NEW TECHNOLOGY	Small Particle Detection An increasing variety of small particles can be studied using flow cytometry. Small particles can include platelets, which are typically 2–3 μm in diameter; bacteria, which can range from 0.3–5 μm; cellular extravesicles, which can be further split into apoptotic bodies, microvesicles; and exosomes, the smallest of which are as little as 50 nm in diameter. Although they do not have the refractive index of exosomes, beads can be used to help set up instrumentation to detect small particles (Figure 50).
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TROUBLESHOOTING	Exosomes are mostly composed of cytoskeletal proteins, mRNA, microRNA, and actin receptors. They are important in crosstalk and regulation of cells because they transfer proteins and RNA between cells. They can be identified by forward and side scatter, but a logarithmic scale is required for adequate separation. Detection of these particles can be problematic because they are often smaller than the wavelength of light used to detect them. Light scatter depends on particle diameter, among other factors, and it is difficult to detect particles smaller than
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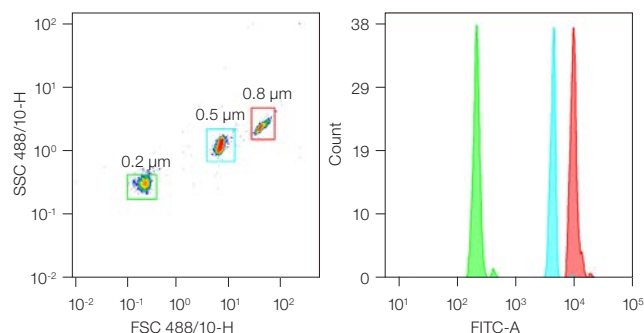


Fig. 50. Small particle detection. Fluorescent calibration beads of 0.2 μm , 0.5 μm , and 0.8 μm (Bangs Labs) were separated on the ZE5 Cell Analyzer using forward scatter and FITC as the detection trigger. FITC, fluorescein isothiocyanate; FSC, forward scatter; SSC, side scatter.

the wavelength. To study small particles, cytometers like the Bio-Rad ZE5 Cell Analyzer have been developed with extra PMT detectors in the forward scatter from shorter wavelength lasers, and the trigger for data collection changed from FSC 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 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, as it may mask signal, and carefully setting the threshold level, so as not to exclude the exosomes. It should be noted that as the particles decrease in size, antigen availability will also decrease, leading to reduced sensitivity or resolution.

Gene Expression and Transfection

Fluorescent proteins are widely used in flow cytometry to determine gene expression and transfection efficiency in live and fixed cells. They are particularly useful when performing cell sorting experiments. They can be 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. Fluorescent proteins can also be photoactivatable, photoswitchable, and suitable for FRET experiments. Initially only available as green fluorescent protein (GFP), there are now over a hundred fluorescent proteins that excite and emit at various wavelengths, making them perfect for multicolor flow cytometry.

Absolute Quantification

Although flow cytometry can quantify marker expression on and in cells, it does not provide information on the cell concentration or absolute quantification. To overcome this, fluorescent beads can be added and counted. If a known concentration of beads is added to your sample,

the number of beads collected will be proportional 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 microliter can be measured.

Particle Internalization

Internalization of particles, cell surface markers, and antigens can occur through phagocytosis. Flow cytometry has proved to be an effective method of quantifying the phagocytosis of fluorescently labeled particles. Dyes that either alter their fluorescent characteristics when internalized or quench surface-bound fluorescence can be used to distinguish between surface and internalized particles.

Fluorescence In Situ Hybridization and RNA Detection

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

Innovations in Cytometry

Flow cytometry has become more accessible to researchers through a reduction in the complexity of instrument setup, true automation, increased sensitivity, and more user-friendly software. Large experiments can now be run and analyzed without the need for specialized training.

These innovations include:

- Artificial intelligence, which has made multiparameter analysis and downstream processing more reliable, removing bias and eliminating errors in manual gating
- Smaller and yet more powerful instrumentation featuring increased numbers of lasers and filters
- Imaging flow cytometry, which uses a CCD camera to capture images of particles as cells pass through the laser. Multiple (spectrally different) images can be captured simultaneously, allowing composites to be made and antigen locations to be determined
- StarBright Dyes, which are high-quality fluorescent dyes with unique spectra that allow larger panels to be built

Mass cytometry (also known as cytometry by time of flight [CyTOF]) is another innovation in cytometry. The technique 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 toward the detector.

Mass cytometry has the ability to detect signals in over 130 channels, significantly increasing multiplexing capability; however, as not all the channels can be currently used, panel size is limited to around 50 markers. Mass cytometry has undergone substantial recent innovations, including the development of Imaging Mass Cytometry. Using this technique, thin tissue slices can be stained and analyzed. A 1 μm laser beam focused on the sample collects the antibodies tagged with the metals and directs them for detection using the CyTOF technology. Using this system, up to 37 markers can be detected and resolved simultaneously.

Mass cytometry allows more markers to be simultaneously identified than regular flow cytometry, and Imaging Mass Cytometry allows more markers to be detected than microscopy. But there are some downsides. The larger the isotope, the longer it takes to pass through the electric field, and the sample acquisition is quite slow. In addition, a specialized analysis software is required and analysis can be problematic and time-consuming. The imaging resolution of Imaging Mass Cytometry is lower than that of microscopy, such that no fine cellular details can be quantified.

A similar technology to Imaging Mass Cytometry is multiplexed ion beam imaging (MIBI) technology. This also uses antibodies tagged with monoisotopic metal tags to visualize tissue sections but does not ablate. It can visualize over 40 markers simultaneously and is able to resolve cellular structures up to 250 nm.

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8. Common Protocols

This chapter brings you trusted protocols to help you jump straight in to your experiments.

Sample Preparation

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

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 blood 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.

However, you 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. Optimizing the isolation of an epitope under investigation via disaggregation, either enzymatic or mechanical, is often a trial-and-error process. Mechanical disaggregation is suitable for loosely bound structures such as adherent cells from culture, bone marrow, and lymphoid tissue. It involves passing a suspension of chopped tissue through a fine-gauge needle several times followed by grinding and sonication as necessary.

Enzymes are used to disrupt protein-protein interactions and the extracellular matrix that holds cells together and are required to release certain cells such as macrophages from spleen and follicular dendritic cells from lymph nodes. 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 these acidic conditions would damage cells without timely neutralization, and cell surface antigens of interest might be lost. As chelators, EDTA and ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA) can remove the divalent cations responsible for maintaining cell function and integrity, but their presence may inhibit certain enzymes. For example, collagenase requires Ca^{2+} for activity.

To study intracellular components like 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.

Protocols for Preparing Cells for Flow Cytometry

Here, we describe methods for suspension and adherent cells and cells derived from specific sources. Protocols for the preparation of human peripheral blood mononuclear, peritoneal macrophage, bone marrow, thymus, and spleen cells are also available.

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.

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

- Bovine serum albumin (BSA)
- 10x Phosphate Buffered Saline (PBS; #BUF036A)

Method

1. Prepare a solution of 1% BSA (w/v) in 1x PBS (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 x g for 5 min at 4°C.

6. Discard the supernatant and resuspend the pellet to a minimum concentration of 1×10^7 cells/ml in cold (4°C) PBS/BSA.

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

- BSA
- 10x PBS

Method

1. Prepare a solution of 1% BSA (w/v) in 1x PBS (PBS/BSA).
2. Decant cells from tissue culture flask into 15 ml conical centrifuge tube(s).
3. Centrifuge at 300–400 x 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 x g for 5 min at room temperature.
6. Discard the supernatant and resuspend the pellet 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

- 1x Accutase solution or 0.25% trypsin
- BSA
- 10x PBS

Method

1. Prepare a solution of 1% BSA (w/v) in 1x PBS (PBS/BSA).
2. Harvest cells by enzymatic release using a solution containing 1x Accutase solution 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 x 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 x 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 (#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

- BSA
- 10x PBS
- Separation media (Histopaque or Ficoll)

Method

1. Prepare a solution of 1% BSA (w/v) in 1x PBS (PBS/BSA).
2. Allow separation media to equilibrate to room temperature.
3. Dilute blood in equal volumes of room-temperature PBS/BSA (for example, add 3 ml of PBS/BSA to 3 ml of blood).
4. Carefully overlay whole blood onto an equal volume of separation media in a 15 ml conical centrifuge tube.
5. Centrifuge at 300–400 x 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.

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- Harvest cells from the serum/separation media interface using a pipet.
- Place harvested cells in a 15 ml conical centrifuge tube.
- Adjust the volume to 10 ml with PBS/BSA.
- Centrifuge at 300–400 x g for 5 min at room temperature.
- Discard the supernatant and resuspend the pellet to a minimum concentration of 1×10^7 cells/ml with cold (4°C) PBS/BSA.

Preparation of Peritoneal Macrophage, Bone Marrow, Thymus, and Spleen Cells

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

Reagents

- Ammonium chloride lysis buffer: 0.16 M ammonium chloride, 0.17 M Tris, pH 7.2
- BSA
- 10x PBS
- Optional: DNase I or EDTA

Method

- Prepare a solution of 1% BSA (w/v) in 1x PBS (PBS/BSA). Optionally, add 25 µg/ml DNase I or 5 mM EDTA to reduce cell aggregates.
- 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 them through a 40 µm cell strainer.
- Centrifuge at 300–400 x g for 5 min at 4°C.
- Discard supernatant and resuspend pellet in 10 ml ammonium chloride lysis buffer.
- Mix and incubate for 2 min at 4°C. Do not exceed this time.
- Centrifuge at 300–400 x g for 5 min at 4°C.
- Discard supernatant and resuspend pellet in 10 ml cold 4°C PBS/BSA.
- Centrifuge at 300–400 x g for 5 min at 4°C.
- Discard supernatant and resuspend pellet to a final volume of 10 ml with cold (4°C) PBS/BSA.
- Count cells using a hemocytometer or an automated cell counter such as the TC20 Automated Cell Counter. Adjust suspension if necessary to give a final concentration of 1×10^7 cells/ml.

Staining of Cells for Flow Cytometry

Direct Immunofluorescence Staining Surface Epitopes of Cells and Blood

This method can be applied when the fluorophore is directly linked to the primary antibody (for example, RPE, FITC, and Alexa Fluor conjugates). RPE conjugates should always be handled in the dark.

Note: Specific steps for blood samples appear in square brackets [].

Reagents

- 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.

- BSA
- Erythrolyse Red Blood Cell Lysing Buffer (#BUF04)
- 10x PBS
- Optional: 0.5% (w/v) paraformaldehyde in PBS

Note: Dissolve paraformaldehyde on a heated stirrer and cool before use.

Method

- Prepare a solution of 1% BSA (w/v) in 1x PBS (PBS/BSA). Prepare a separate solution of 1x PBS.
- Prepare cells appropriately; refer to the Protocols for Preparing 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 buffer. [Whole blood samples may be used undiluted unless the cell count is high, for example in leukemia. Use an appropriate anticoagulant.]
- Aliquot 100 µl of the cell suspension [or whole blood] into as many test tubes as required.
- Add antibody at the vendor-recommended dilution. Mix well and incubate at 4°C for at least 30 min, avoiding direct light.
- Wash cells with 2 ml cold (4°C) PBS/BSA, centrifuge at 300–400 x g for 5 min at 4°C, and discard the resulting supernatant. [To the blood suspension, add 2 ml freshly prepared red cell lysis buffer and mix well. Incubate for 10 min at room temperature. Centrifuge at 300–400 x g for 5 min at room temperature and discard the supernatant. Wash with 2 ml room-temperature PBS/BSA, centrifuge at 300–400 x g for 5 min and discard the supernatant.]

- Resuspend cells in 200 μ l of cold (4°C) PBS (or with 200 μ l of 0.5% paraformaldehyde in PBS if required).
- 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 (streptavidin, for example) must be used to visualize the primary antibody.

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

Reagents

- Anticoagulant

Note: for basic staining, any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. Some instances may require specific anticoagulants.

- BSA
- Erythrolyse Red Blood Cell Lysing Buffer (#BUF04)
- 10x PBS
- Optional: 0.5% (w/v) paraformaldehyde in PBS

Note: Dissolve paraformaldehyde on heated stirrer and cool before use.

Method

- Prepare solutions of 1x PBS and 1% BSA (w/v) in 1x PBS (PBS/BSA). Optionally prepare a solution of 0.5% (w/v) paraformaldehyde in 1x PBS.
Note: Dissolve paraformaldehyde on heated stirrer and cool before use.
- Prepare cells appropriately; refer to the Protocols for Preparing 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 buffer. [Whole blood samples may be used undiluted unless the cell count is high, as in leukemia. Use an appropriate anticoagulant.]
- Aliquot 100 μ l of the cell suspension [or whole blood] into as many test tubes as required.
- Add primary antibody at the vendor-recommended dilution. Mix well and incubate at 4°C for at least 30 min.
- Wash cells with 2 ml of cold (4°C) PBS/BSA, centrifuge at 300–400 x g for 5 min and discard the supernatant. [To the blood suspension, add 2 ml freshly prepared

red cell lysis buffer and mix well. Incubate for 10 min at room temperature. Centrifuge at 300–400 x g for 5 min and discard the supernatant. Wash with 2 ml of room-temperature PBS/BSA, centrifuge at 300–400 g for 5 min and discard the supernatant.]

- 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.
- Centrifuge at 300–400 x g for 5 min at room temperature and discard the supernatant.
- Resuspend cells in 200 μ l of cold (4°C) PBS (or with 200 μ l of 0.5% paraformaldehyde in PBS if required).
- Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

Direct Staining of Intracellular Antigens and Cytokines: Leucoperm Accessory Reagent Method

This cell permeabilization method using Leucoperm Accessory Reagent is required prior to intracellular staining.

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 square brackets [].

Reagents

- 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.

- BSA
- Erythrolyse Red Blood Cell Lysing Buffer (#BUF04)
- Leucoperm Accessory Reagent (#BUF09)
- 10x PBS
- Optional: 0.5% (w/v) paraformaldehyde in PBS

Note: Dissolve paraformaldehyde on heated stirrer and cool before use.

Method

- Prepare solutions of 1x PBS and 1% BSA (w/v) in 1x PBS (PBS/BSA). Optionally prepare a solution of 0.5% (w/v) paraformaldehyde in 1x PBS.

Note: Dissolve paraformaldehyde on heated stirrer and cool before use.

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										- 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 an appropriate anticoagulant.] - Add 100 µl of cell suspension [or whole blood] to the appropriate number of test tubes. - If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light. - Wash cells once in 2 ml cold (4°C) PBS/BSA and discard the supernatant. - 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. - Add 3 ml room-temperature PBS/BSA and centrifuge for 5 min at 300–400 x g at room temperature. [To the blood suspension, add 2 ml freshly prepared 1x Erythrolyse Red Cell Lysing Buffer and mix well. Incubate for 10 min at room temperature. Centrifuge at 300–400 x g for 5 min and discard the supernatant. Wash with 2 ml room-temperature PBS/BSA, centrifuge at 300–400 x g for 5 min at room temperature and discard the supernatant.] - 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. - Wash once in PBS and then resuspend in 200 µl cold (4°C) PBS for immediate analysis (or with 200 µl of 0.5% paraformaldehyde in PBS if required). - Acquire data by flow cytometry. Analyze fixed cells within 24 hr. Direct Immunofluorescence Staining of Intracellular Cytokines in Blood This method describes the staining of intracellular antigens in whole blood using directly conjugated antibodies. This is a rapid and simple approach to the analysis of intracellular cytokines in whole blood. It permits the analysis of small samples and avoids generating artefacts 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 successfully used in this application. Note: Resting cells often require stimulation in vitro prior to the detection of intracellular cytokines. Reagents - BSA - Cell culture medium - Erythrolyse Red Blood Cell Lysing Buffer - Ionomycin - Leucoperm Accessory Reagent - Monensin 10x PBS - Phorbol 12-myristate 13-acetate (PMA) - Optional: 0.5% (w/v) paraformaldehyde in PBS Method - Prepare solutions of 1x PBS and 1% BSA (w/v) in 1x PBS (PBS/BSA). Optionally prepare a solution of 0.5% (w/v) paraformaldehyde in 1x PBS. Note: Dissolve paraformaldehyde on heated stirrer and cool before use. - Aliquot 500 µl of blood into as many tubes as required, including 2 extra control tubes, then add 500 µl of cell culture medium (without any additives) to each sample. - To one tube (the resting population), add monensin to a final concentration of 3 µM. - To another tube (activated cells), add PMA to a final concentration of 10 ng/ml, ionomycin to a final concentration of 2 µM, and monensin to a final concentration of 3 µM. - To the rest of the tubes (experimental samples), add monensin to a final concentration of 3 µM and treat as required by the experiment. - Incubate for 2–4 hr at 37°C in a 5% CO₂ atmosphere. - If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light. - Wash cells once with PBS/BSA and discard supernatant.

9. Add 100 µl Leucoperm Reagent A (cell fixation agent) and incubate for 10 min at 2–8°C.
10. Add 2 ml cold (4°C) PBS/BSA and centrifuge for 5 min at 300–400 g at room temperature.
11. 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.
12. Add 2 ml of freshly prepared Erythrolyse Red Blood Cell Lysing Buffer to the blood suspension and mix well.
13. Incubate for 10 min at room temperature.
14. Centrifuge at 300–400 x g for 5 min and discard the supernatant.
15. Wash once in PBS/BSA, and then resuspend in 200 µl PBS for immediate analysis (or with 200 µl of 0.5% paraformaldehyde in PBS if required).
16. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

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). These are guidelines only and the incubation times may need to be adjusted for different cell types.

Reagents

- 70% ethanol in deionised water (DI)
- Nucleic acid staining solution (1x PBS, 100 µg/ml RNase A)
- 10x PBS
- RediDrop Propidium Iodide (#1351101)

Method

1. Prepare 1x PBS.
2. Prepare cells appropriately; refer to the Protocols for Preparing Cells for Flow Cytometry for further information.
3. Fix cells in 2–5 ml cold (4°C) 70% ethanol. Add dropwise to cell pellet while vortexing. This should ensure fixation of all cells and minimize clumping.
4. Incubate for at least 30 min on ice.

Note: Samples can be left at this stage for several weeks.

5. Centrifuge at 500 x g for 10 min; decant supernatant.
6. Wash twice with 3 ml PBS at 300–400 x g and 4°C for 5 min and discard supernatant.
7. Resuspend cell pellet in 500 µl nucleic acid staining solution. Mix well.
8. Incubate for 30 min at room temperature.
9. Add 1–2 drops of RediDrop Propidium Iodide.
10. Analyze by flow cytometry. The PI should be read on the appropriate channel in the linear scale. Doublets should be gated out using the area versus height or width depending on your instrument.

BrdU Staining of Cells for Cell Cycle Analysis and Apoptosis

BrdU is a thymidine analog readily incorporated into DNA during DNA synthesis and is an accurate method to monitor proliferation and apoptosis. The following protocols 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 DNase I may be applicable if this is required.

Reagents

- BSA
- 2 M HCl containing 0.5% triton X-100
- 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$, pH 8.5
- 10x PBS
- RediDrop Propidium Iodide
- 0.05% (v/v) Tween-20 in PBS/BSA

Method

1. Prepare solutions of 1x PBS and 1% BSA (w/v) in 1x PBS (PBS/BSA).
2. 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.
3. Wash cells twice with PBS/BSA, at 500 x g for 10 min at room temperature, decant supernatant.
4. Resuspend in 2–5 ml cold (4°C) 70% ethanol. Add dropwise to cell pellet while vortexing. Fix for at least 30 min on ice.
5. Centrifuge at 500 x g for 10 min, decant supernatant.
6. Resuspend the pellet in 2 ml of 2 M HCl containing 0.5% Triton X-100. Incubate for 30 min at room temperature (preferably on a rocking platform).

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- Centrifuge at 500 x g for 10 min, decant supernatant. Resuspend in 3 ml of 0.1 M Na₂B₄O₇, pH 8.5, for 2 min at room temperature.
- Centrifuge at 500 x g for 10 min, decant supernatant, and resuspend in room-temperature 0.05% Tween-20 PBS/BSA. Adjust cell concentration to 1 × 10⁷ cells/ml.
- Aliquot 100 µl of the cell suspension into required number of FACS tubes.
- Incubate with antibody at the vendor-recommended dilution overnight at 4°C, avoiding direct light.
- Resuspend in 2 ml room-temperature PBS/BSA. Centrifuge at 500 x g for 10 min at room temperature.
- If a secondary antibody is required, then decant the supernatant, add 100 µl PBS/BSA, and incubate with the secondary antibody at the vendor-recommended dilution for at least 30 min at 4°C.
- Wash with 2 ml PBS/BSA, centrifuge at 500 x g for 10 min.
- Resuspend cells in 1 ml PBS. Add 1–2 drops of ReditDrop Propidium Iodide.
- Analyze by flow cytometry. PI should be read on the appropriate channel in the linear scale. Doublets should be gated out using the area versus height or width depending on your instrument.

Direct Immunofluorescence Staining of Cells with StarBright Dyes
This method provides a general procedure for staining cells in tubes or a 96-well plate with StarBright Dyes. In some cases, specific recommendations are provided on product datasheets, and these methods should always be used in conjunction with the product- and batch-specific information provided with each vial.

Reagents

- BSA
- Human Seroblock (#BUF070A), Mouse Seroblock (#BUF041A), or 10% serum matched to the species of the cells being stained
- 10x PBS
- Optional: Fixation Buffer (#BUF071), Bio-Rad Staining Buffer (#BUF073)

Method

- Prepare solutions of 1x PBS and 1% BSA (w/v) in 1x PBS (PBS/BSA).
- Prepare cells appropriately; refer to [protocol FC2](#) (Preparation of Human Peripheral Blood Mononuclear Cells for Flow Cytometry) for further information. Adjust the cell suspension to a concentration of 1 × 10⁷ cells/ml with cold (4°C) staining buffer. Staining buffer for StarBright Dyes can be PBS/BSA or Bio-Rad Staining Buffer.
- Aliquot 100 µl of the cell suspension into as many tubes (or wells of a 96-well plate) as required.
- Incubate in Fc block (Human or Mouse Seroblock), or 10% serum of the cell species you are using, for 10–30 min.
- Centrifuge at 300–400 x g for 5 min at 4°C and discard the supernatant.
- Add antibody or multiple antibodies at the recommended dilution in 100–200 µl staining buffer and incubate for 1 hr at 4°C or room temperature, avoiding direct light.

Note: StarBright Dyes are available as a 500 µl vial but you may wish to titrate all the antibodies in your experiment for your particular cells, as this may improve the stain index. StarBright Dyes do not require a special buffer when combining with any other StarBright Dye, organic fluorophore, or protein-based fluorophore, including tandem dyes. However, you may need to use recommended staining buffers for certain polymer dyes when combining one or more polymer dyes. Staining cells on ice will result in a lower staining index, so staining at 4°C or room temperature is recommended to improve resolution.

- Wash the cells with 2 ml cold (4°C) staining buffer (200 µl for 96-well plates) and centrifuge at 300–400 x g for 5 min. Discard the supernatant and repeat for a total of three washes.
- Resuspend cells in 200 µl of cold (4°C) PBS or in 200 µl of a fixative such as Fixation Buffer or 2–4% paraformaldehyde. Do not leave cells in fixative for long periods of time. For long-term storage, transfer to PBS and store in a refrigerator, avoiding direct light.
- Acquire data by flow cytometry.



9. Troubleshooting

Troubleshooting Guide

If something does not work, check through the guidelines provided in Tables 9 and 10 to identify and resolve the problem. If there are still difficulties and you have purchased any Bio-Rad reagents, our Technical Services Team (see contact details at bio-rad-antibodies.com/technical) can offer further advice.

Table 9. Cell analysis 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 your antibody is conjugated to a fluorophore. If not, confirm that an appropriate fluorophore-conjugated secondary antibody is being used. 5. Confirm that the secondary antibody is able to bind the antigen. Has it been used successfully with other primary antibodies? 6. Make sure a secondary antibody has the appropriate species reactivity and isotype to recognize your primary antibody. 7. If the fluorophore used is PE- or APC-based, make sure that the product has not been frozen. 8. Is the target antigen present on test tissue? Check the literature for antigen expression and incorporate a positive control of known antigen expression alongside the test material. 9. Does the antibody recognize the antigen in the test species? Check that your antibody cross-reacts with the test species. Not all antibodies will react across species. 10. Confirm that correct laser is being used to excite the fluorophore, and that the correct channel is being used to analyze emissions.
No staining with the PE antibody but the same FITC antibody gives good results	1. The PE conjugate may have been frozen. If so, purchase another vial of antibody. 2. Expired paraformaldehyde may be a problem. Breakdown of paraformaldehyde may release methanol, which will affect staining. Prepare fresh paraformaldehyde.
Nonspecific staining	1. May be due to autofluorescence. Check levels of autofluorescence by including a tube with only cells (without any antibody) in your panel. 2. Certain cells express low-affinity Fc receptors CD16/CD32, which bind whole antibodies via the Fc region. For mouse cells, use Mouse Seroblock FcR (#BUF041A, BUF041B); for human cells, use Human Seroblock (#BUF070A, BUF070B). Alternatively, use serum from the host cell species. 3. May be due to the secondary antibody. Select a secondary antibody that will not cross-react with the target tissue, or stain with the secondary alone as a control. 4. Make sure that sufficient washing steps have been included. 5. Titrate the test antibody carefully. Nonspecific staining may be reduced at lower antibody concentrations. 6. Stain cells known to be negative or look at stained cells in a heterogeneous population that are known to be negative, such as CD3 on monocytes in peripheral blood.

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Problem	Course of Action
Weak staining	1. May be due to overdilution of antibodies. Confirm that antibodies are used at the correct concentration by titrating them before use. 2. Weak staining in indirect staining systems may be due to the prozoning effect, where highly concentrated antibodies may give weak results. Titrate them carefully. 3. May be due to an excessive number of cells. Adjust the cell population to the 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 only. 7. Optimize incubation time and temperature with either a primary or secondary antibody.
Unusual scatter profiles	1. Make sure that cells are as fresh as possible when used. The profile may be showing dead cells and debris. 2. Use a viability dye to clean up the data. 3. Activation methods may affect scatter characteristics of cells. 4. If you are using lysis solution, confirm that this is fresh and has been made 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 the manufacturer's datasheet for the permeabilization reagent.

Table 10. Cell sorting troubleshooting.

High levels of cell death	As the cells are charged, they can be attracted to the charge on the plastic collection tubes. 1. Avoid polystyrene. 2. Precoat the collection tubes with a buffer containing protein. 3. Avoid keeping cells in PBS for long periods of time. 4. Add a protein buffer to the collection tube. 5. Add HEPES if sorting into media. 6. Cells should be stored at an appropriate temperature after collection. Generally, 4°C can be used, but seek literature for your cell type. 7. Reduce the sorting pressure, if possible, to reduce cell stress.
Poor recovery of sorted cells	1. Include a viability dye to remove dead cells. 2. Count the cells prior to sorting to ensure they are not too concentrated (or too diluted). 3. Design proper cytometry panels. Include positive and negative markers, dump channels (if possible), and use best practice techniques on panel design. Use of a spectraviewer or panel building tool may help. 4. Reduce the threshold settings. Note that while doing this makes identification of the cell population easier, whatever the cell sorter ignores (debris, for example) will end up in the collection tube.



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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: a filter that allows 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.

DOUBLETS: two particles that pass together 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 that generates enough signal when it passes through the laser to be recorded as a signal or pulse.

FC RECEPTORS: antibody receptors on certain cells that bind antibodies via their constant region to elicit immune responses.

FIXATION: crosslinking of cellular proteins to preserve them 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 that allows the measurement of properties of individual particles as they pass through a laser.

FLUORESCENCE MINUS ONE CONTROL: specific control in which one fluorophore is removed from the staining panel to account for fluorescence spread.

FLUORESCENT PROTEIN: a protein that can accept light energy and re-emit at longer wavelengths and can be expressed in cells for live marking.

FLUOROPHORE: a fluorescent marker 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 maximal amount of current output by the PMT of the pulse.

HISTOGRAMS: plots that display a single measurement parameter.

HYDRODYNAMIC FOCUSING: a technique in which 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 setup of lasers, optics, and filters within the cytometer.

ISOTYPE CONTROLS: antibodies raised against an antigen not found on the cell being analyzed; used to help determine specific antibody binding.

LASER: a device that emits optically amplified light at a single wavelength.

LONG PASS FILTER: a filter that allows 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 by the most photons.

PARAMETER: the height, area, or width measurement detected in a flow cytometry instrument.

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 (antibodies, for example) to enter the cell for intracellular staining.

APPENDICES	TROUBLESHOOTING	COMMON PROTOCOLS	COMMON APPLICATIONS AND NEW TECHNOLOGY	MULTICOLOR PANEL BUILDING	OPTIMIZING YOUR EXPERIMENTS	CONTROLS IN FLOW	DATA ANALYSIS	PRINCIPLES OF FLUORESCENCE	PRINCIPLES OF THE FLOW CYTOMETER
RESOLUTION: the ability to separate a positive from a negative population. SHORT PASS FILTER: a filter that allows 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 consisting of 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 that allows identification of dead cells through a reduction in cell membrane integrity. WIDTH: the time interval during which the pulse occurs.									

References and Further Reading

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PRINCIPLES OF THE
FLOW CYTOMETERPRINCIPLES OF
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IN FLOWOPTIMIZING YOUR
EXPERIMENTSMULTICOLOR PANEL
BUILDINGCOMMON APPLICATIONS
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COMMON PROTOCOLS

TROUBLESHOOTING

APPENDICES

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