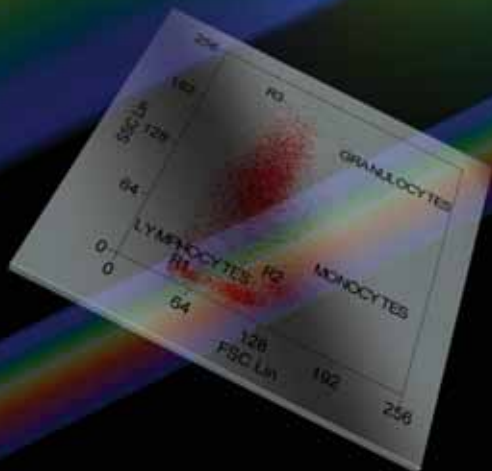
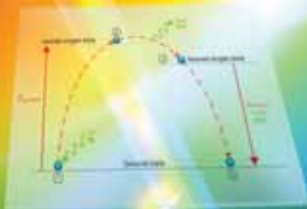


Introduction to Flow Cytometry

- Principles
- Data analysis
- Protocols
- Troubleshooting



By Misha Rahman, Ph.D.

Technical advisors

Andy Lane, Ph.D.

Angie Swindell, M.Sc.

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Your first choice for antibodies!

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Preface

How can I explain what flow cytometry is to someone that knows nothing about it? Well, imagine it to be a lot like visiting a supermarket. You choose the goods you want and take them to the cashier. Usually you have to pile them onto a conveyor. The clerk picks up one item at a time and interrogates it with a laser to read the barcode. Once identified and if sense prevails, similar goods are collected together e.g. fruit and vegetables go into one shopping bag and household goods into another. Now picture in your mind the whole process automated; replace shopping with biological cells; and substitute the barcode with cellular markers – welcome to the world of flow cytometry and cell sorting!

We aim to give you a basic overview of all the important facets of flow cytometry without delving too deeply into the complex mathematics and physics behind it all. For that there are other books (some recommended at the back). Instead, we present a guide that will be invaluable to beginners of flow cytometry or act as a fact-packed synopsis for those of you interested in teaching others about the virtues of this powerful application.

At AbD Serotec, we specialize in flow cytometry reagents and offer the largest range of antibody markers commercially available, together with accessory buffers. Our guiding principle is to manufacture products for use with every known flow cytometry instrument. We offer no bias towards only one machine but are more interested in maximizing scientific performance. Confidence in our reagents is shown by our quality promise that states that if any product does not perform as described on its datasheet we will replace the reagent with a fresh vial or offer you credit.

Please contact us or view our website at **www.ab-direct.com** for our ever-increasing range of flow cytometry reagents. You can see and print out detailed datasheets for every product online. But should you need any further information, just contact our Technical Services Team (see the back cover for your nearest office) and they will be happy to assist.

I hope this booklet will prove useful to you. If you like it, let us know and we'll produce similar guides for the research applications we support.

Morris Gillis
Marketing Manager

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Principles of the flow cytometer

Chapter 1

Fluidics system

One of the fundamentals of flow cytometry is the ability to measure the properties of individual particles. When a sample in solution is injected into a flow cytometer, the particles are randomly distributed in three-dimensional space. The sample must therefore be ordered into a stream of single particles that can be interrogated by the machine's detection system. This process is managed by the fluidics system.

Essentially, the fluidics system consists of a central channel/core through which the sample is injected, enclosed by an outer sheath that contains faster flowing fluid. As the sheath fluid moves, it creates a massive drag effect on the narrowing central chamber. This alters the velocity of the central fluid whose flow front becomes parabolic with greatest velocity at its center and zero velocity at the wall (see Figure 1). The effect creates a single file of particles and is called hydrodynamic focusing.

Under optimal conditions (laminar flow) the fluid in the central chamber will not mix with the sheath fluid.

The flow characteristics of the central fluid can be estimated using Reynolds Number (R_e):

$$R_e = \frac{\rho V D}{\mu}$$

where D = tube diameter, V = mean velocity of fluid, ρ = density of fluid, and μ = viscosity of fluid.

When $R_e < 2300$, flow is always laminar. When $R_e > 2300$, flow can be turbulent, which accelerates diffusion.

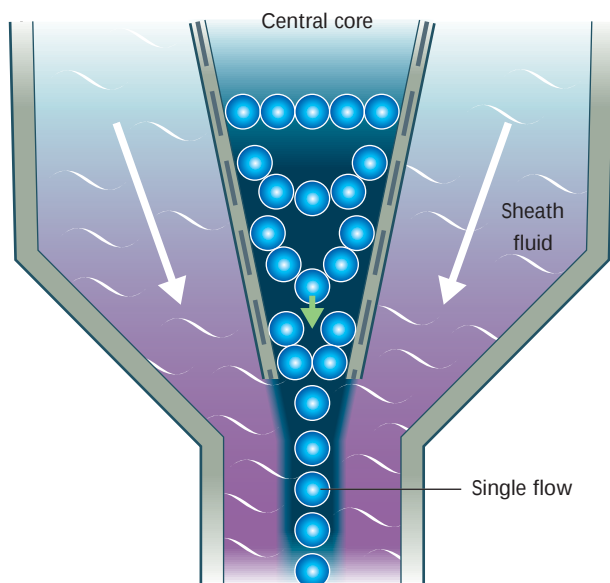


FIGURE 1

Hydrodynamic focusing produces a single stream of particles

Without hydrodynamic focusing the nozzle of the instrument (typically 70 μM) would become blocked, and it would not be possible to analyze one cell at a time.

Optics and detection

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

Lasers produce a single wavelength of light (a laser line) at one or more discreet frequencies (coherent light). Arc lamps tend to be less expensive than lasers and exploit the color emissions of an ignited gas within a sealed tube. However, this produces unstable incoherent light of a mixture of wavelengths, which needs subsequent optical filtering.

Light that is scattered in the forward direction, typically up to 20° offset from the laser beam's axis, is collected by a lens known as the forward scatter channel (FSC). The FSC intensity roughly equates to the particle's size and can also be used to distinguish between cellular debris and living cells. Light measured approximately at a 90° angle to the excitation line is called side scatter. The side scatter channel (SSC) provides information about the granular content within a particle. Both FSC and SSC are unique for every particle, and a combination of the two may be used to differentiate different cell types in a heterogeneous sample.

Fluorescence measurements taken at different wavelengths can provide quantitative and qualitative data about fluorochrome-labeled cell surface receptors or intracellular molecules such as DNA and cytokines. Flow cytometers use separate fluorescence (FL-) channels to detect light emitted. The number of detectors will vary according to the machine and its manufacturer. Detectors are either silicon photodiodes or photomultiplier tubes (PMTs). Silicon photodiodes are usually used to measure forward scatter when the signal is strong. PMTs are more sensitive instruments and are ideal for scatter and fluorescence readings.

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

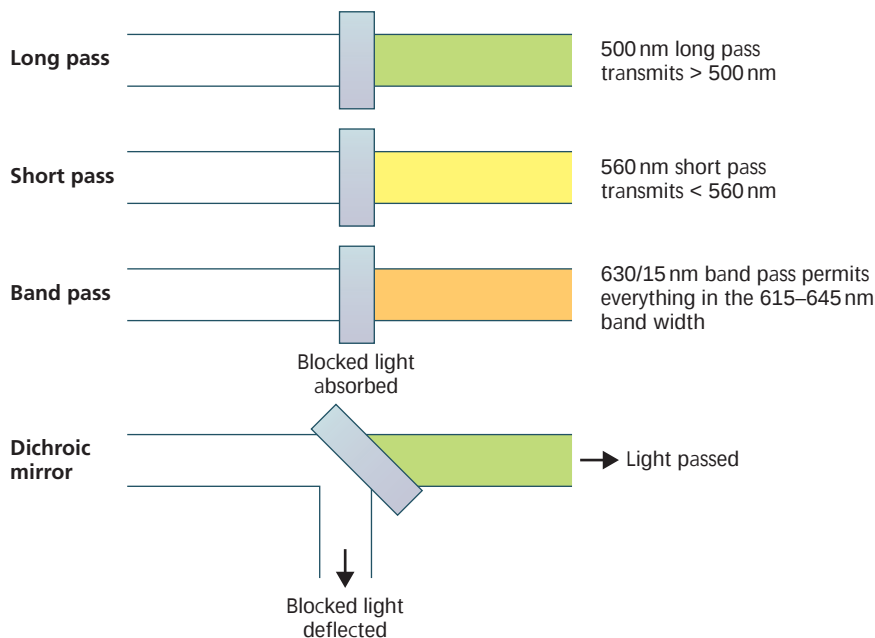


FIGURE 2 Different types of optical filters

When a filter is placed at a 45° angle to the oncoming light it becomes a dichroic filter/mirror. As the name suggests, this type of filter performs two functions, first, to pass specified wavelengths in the forward direction and, second, to deflect blocked light at a 90° angle. To detect multiple signals simultaneously, the precise choice and order of optical filters will be an important consideration (refer to Figure 3).

Signal processing

When light hits a photodetector a small current (a few microamperes) is generated. Its associated voltage has an amplitude proportional to the total number of light photons received by the detector. This voltage is then amplified by a series of linear or logarithmic amplifiers, and by analog to digital convertors (ADCs), into electrical signals large enough (5–10 volts) to be plotted graphically.

Log amplification is normally used for fluorescence studies because it expands weak signals and compresses strong signals, resulting in a distribution that is easy to display on a histogram. Linear scaling is preferable where there is not such a broad range of signals e.g. in DNA analysis.

The measurement from each detector is referred to as a 'parameter' e.g. forward scatter, side scatter or fluorescence. The data acquired in each

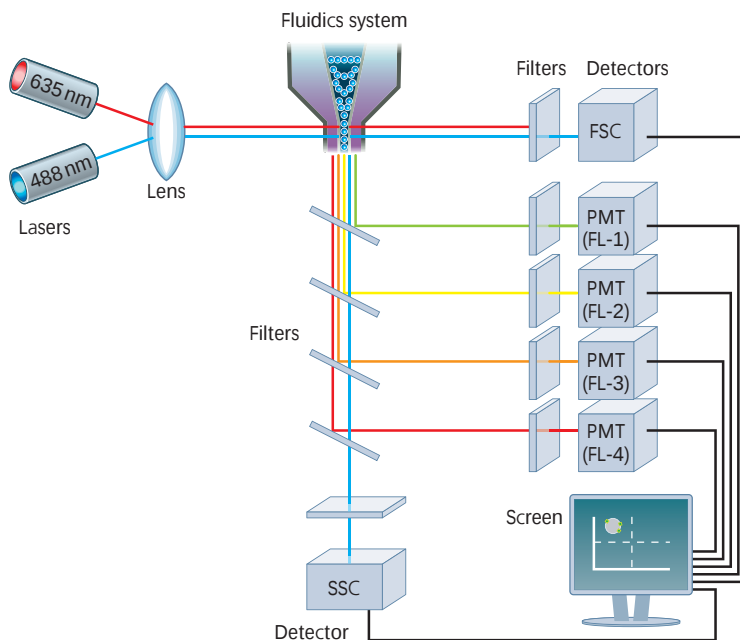


FIGURE 3

Schematic overview of a typical flow cytometer setup

parameter are known as the 'events' and refer to the number of cells displaying the physical feature or marker of interest.

Electrostatic cell sorting

A major application of flow cytometry is to separate cells according to subtype or epitope expression for further biological studies. This process is called cell sorting or FACS™ analysis.

After the sample is hydrodynamically focused, each particle is probed with a beam of light. The scatter and fluorescence signal is compared to the sort criteria set on the instrument. If the particle matches the selection criteria, the fluid stream is charged as it exits the nozzle of the fluidics system. Electrostatic charging actually occurs at a precise moment called the 'break-off point', which describes the instant the droplet containing the particle of interest separates from the stream.

To prevent the break-off point happening at random distances from the nozzle and to maintain consistent droplet sizes, the nozzle is vibrated at high frequency. The droplets eventually pass through a strong electrostatic field, and are deflected left or right based on their charge (Figure 4).

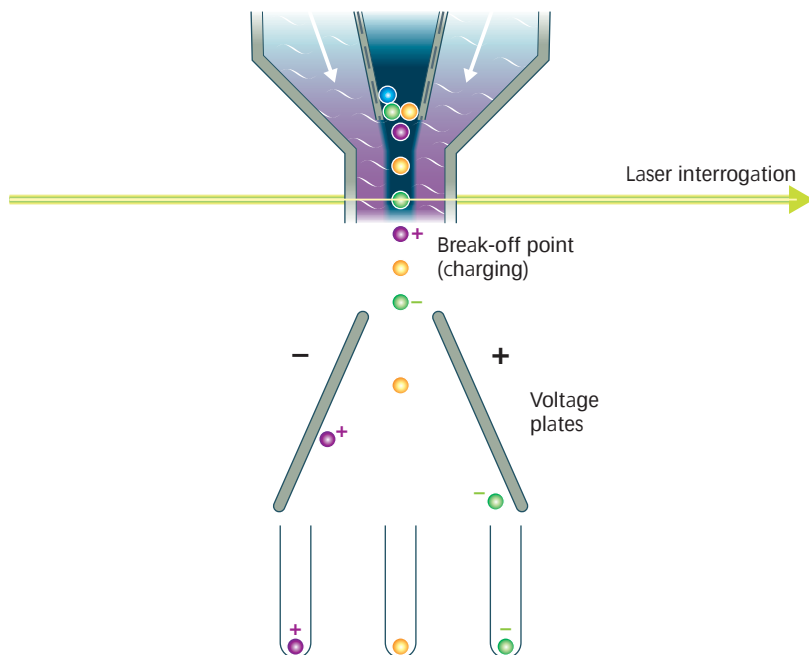


FIGURE 4

Electrostatic flow sorting

The speed of flow sorting depends on several factors including particle size and the rate of droplet formation. A typical nozzle is between 50–70 μM in diameter and, depending on the jet velocity from it, can produce 30,000–100,000 droplets per second, which is ideal for accurate sorting. Higher jet velocities risk the nozzle becoming blocked and will also decrease the purity of the preparation.



Principles of fluorescence

Chapter 2

Fluorochromes and light

Fluorochromes are essentially dyes, which accept light energy (e.g. from a laser) at a given wavelength and re-emit it at a longer wavelength. These two processes are called excitation and emission. The process of emission follows extremely rapidly, commonly in the order of nanoseconds, and is known as fluorescence. Before considering the different types of fluorochrome available for flow cytometry, it is necessary to understand the principles of light absorbance and emission.

Light is a form of electromagnetic energy that travels in waves. These waves have both frequency and length, the latter of which determines the color of light. The light that can be visualized by the human eye represents a narrow wavelength band (380–700 nm) between ultraviolet (UV) and infrared (IR) radiation (Figure 5). Sunlight, for example, contains UV and IR light that, although invisible to the eye, can still be felt as warmth on the skin and measured scientifically using photodetectors. The visible spectrum can further be subdivided according to color, often remembered by the mnemonic ‘ROY G BV’ standing for red, orange, yellow, green, blue and violet. Red light is at the longer wavelength end (lower energy) and violet light at the shorter wavelength end (higher energy).

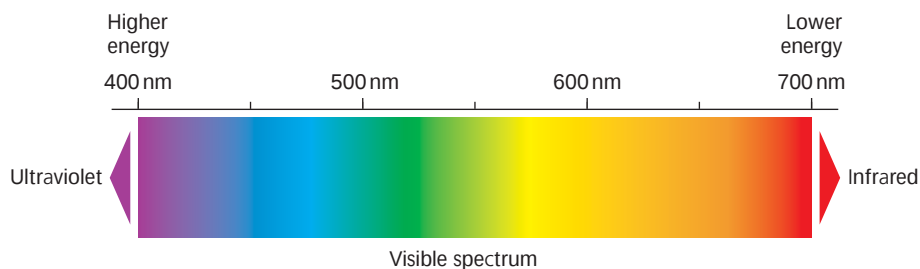


FIGURE 5 The electromagnetic spectrum

Stokes Shift

When light is absorbed by a fluorochrome, its electrons become excited and move from a resting state (1) to a maximal energy level called the ‘excited electronic singlet state’ (2). The amount of energy required will differ for each

fluorochrome and is depicted in Figure 6 as $E_{\text{excitation}}$. This state only lasts for 1–10 nanoseconds because the fluorochrome undergoes internal conformational change and, in doing so, releases some of the absorbed energy as heat. The electrons subsequently fall to a lower, more stable, energy level called the ‘relaxed electronic singlet state’ (3). As electrons steadily move back from here to their ground state they release the remaining energy (E_{emission}) as fluorescence (4).

As E_{emission} contains less energy than was originally put into the fluorochrome it appears as a different color of light to $E_{\text{excitation}}$. Therefore, the emission wavelength of any fluorochrome will always be longer than its excitation wavelength. The difference between $E_{\text{excitation}}$ and E_{emission} is called Stokes Shift and this wavelength value essentially determines how good a fluorochrome is for fluorescence studies. After all, it is imperative that the light produced by emission can be distinguished from the light used for excitation. This difference is easier to detect when fluorescent molecules have a large Stokes Shift.

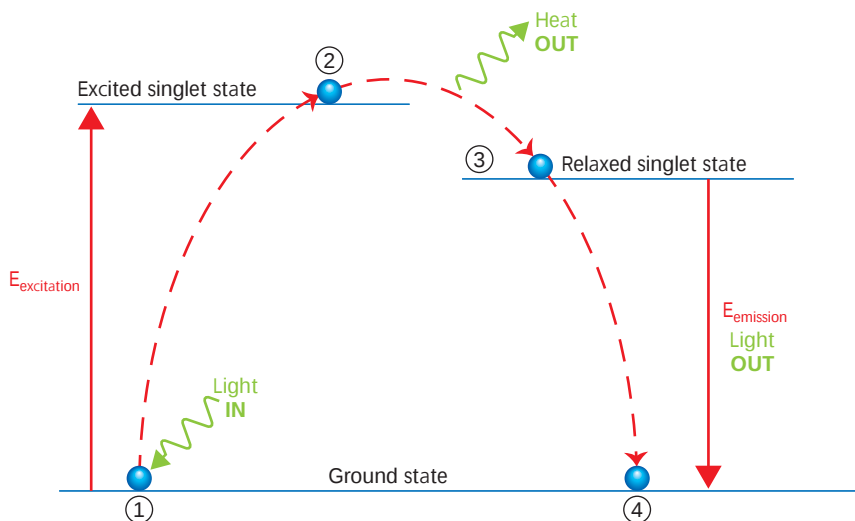


FIGURE 6 **Stokes Shift**

Maximal absorbance and maximal emission

The wavelength of excitation is critical to the total photons of light the fluorochrome will absorb. FITC (fluorescein isothiocyanate), for example, will absorb light within the range 400–550 nm but the closer the wavelength is to 490 nm (its peak or maximum), the greater the absorbance is. In turn, the more photons absorbed, the more intense the fluorescence emission will be.

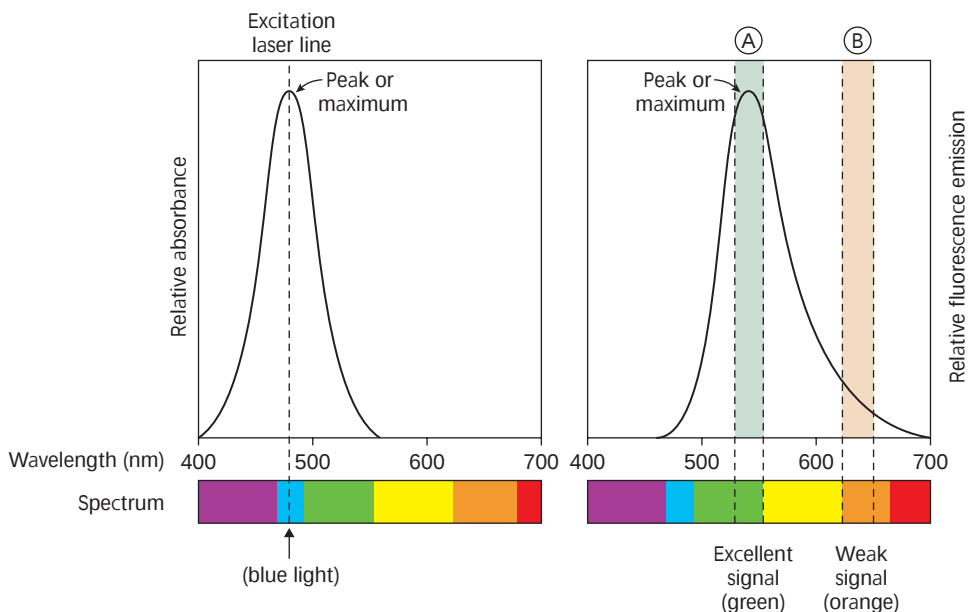


FIGURE 7 **Light absorbance (LEFT) and light emission (RIGHT) of FITC**

These optimal conditions are termed maximal absorbance and maximal emission wavelengths.

Maximal absorbance usually defines the laser spectral line that is used for excitation. In the case of FITC, its maximum falls within the blue spectrum. Therefore, the blue Argon-ion laser is commonly used for this fluorochrome, as it excites at 488 nm, close to FITC's absorbance peak of 490 nm.

FITC emits fluorescence over the range 475–700 nm peaking at 525 nm, which falls in the green spectrum. If filters are used to screen out all light other than that measured at the maximum via channel A (see Figure 7), FITC will appear green. Hence, 'fluorescence color' usually refers to the color of light a fluorochrome emits at its highest stable excited state. However, if FITC fluorescence is detected only via channel B (see Figure 7), it will appear orange and be much weaker in intensity. How the flow cytometer is set up to measure fluorescence will ultimately determine the color of a fluorochrome.

Why use a fluorescent probe?

The purpose of a fluorescent probe, such as a fluorochrome-conjugated antibody, is to directly target an epitope of interest and to allow its biological and biochemical properties to be measured more easily by the flow cytometer. Fluorescent probes are useful in a wide range of applications including: identifying and quantifying distinct populations of cells, cell surface receptors or intracellular organelles; cell sorting; immunophenotyping; calcium influx experiments; determining nucleic acid content; measuring enzyme activity, and for apoptosis studies. By changing the excitation light and using more than one fluorochrome, it is possible to analyze several parameters of the sample at any one time. This forms the basis of multicolor fluorescence studies.

Which fluorochromes are useful for flow cytometry?

There are dozens of fluorescent molecules (fluorochromes) with a potential application in flow cytometry. The list is ever growing but it is not the scope of this booklet to cover them all. Instead, some of the most useful fluorochromes for surface or intracellular epitope detection are described on page 13, including the very latest in fluorescent probe technology – tandem dyes. There is enough variation in the two tables to cover most researchers' needs.

Single dyes:

Some of these single dyes e.g. FITC have been in use for the past 30 years but are now facing competition from alternatives like Alexa Fluor® dyes, which offer the user greater photostability and increased fluorescence.

Tandem dyes:

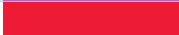
In a tandem dye, a small fluorochrome takes a 'piggy-back' ride on another larger fluorochrome. When the first dye is excited and reaches its maximal singlet state, all its energy transfers to the second dye (an acceptor molecule), located in close proximity. This activates the second fluorochrome, which then produces the fluorescence emission. The process is called FRET (fluorescence resonance energy transfer). It is a clever way to achieve higher Stokes Shifts and, therefore, increase the number of colors that can be analyzed from a single laser wavelength.

The majority of tandem dyes have been manufactured for the standard 488 nm laser, which is found in most flow cytometers. Tandem dyes are very useful for multicolor fluorescence studies especially in combination with single dyes. For example, Alexa Fluor® 488, Phycoerythrin, PerCP-Cy5.5 and PE-Cy7 can all be excited at 488 nm, but will produce green, yellow, purple and infrared emissions respectively, which can be measured using separate detectors.

Single dyes

Dye	Laser excitation line (nm)	Maximal absorbance (nm)	Maximal emission (nm)	Fluorescence color
Alexa Fluor® 405	405, 407	401	421	
Alexa Fluor® 430	405, 407	433	541	
Alexa Fluor® 488	488	495	519	
Alexa Fluor® 633	633, 635, 647	632	647	
Alexa Fluor® 647	633, 635, 647	650	665	
Alexa Fluor® 660	633, 635, 647	663	690	
Alexa Fluor® 680	633, 635, 647	679	702	
Alexa Fluor® 700	633, 635, 647	702	723	Infrared
APC	633, 635, 647	650	661	
FITC	488	490	525	
Pacific Blue™	405, 407	410	455	
PerCP	488	490	675	
Phycoerythrin	488	490, 565	578	

Tandem dyes

Dye	Laser excitation line (nm)	Maximal absorbance (nm)	Maximal emission (nm)	Fluorescence color
APC-Alexa Fluor® 750	633, 635, 647	650	779	Infrared
APC-Cy5.5	633, 635, 647	650	695	
APC-Cy7	633, 635, 647	650	785	Infrared
PerCP-Cy5.5	488	496, 546	695	
PE-Alexa Fluor® 610	488	496, 546	627	
PE-Alexa Fluor® 647	488	496, 546	667	
PE-Alexa Fluor® 680	488	496, 546	702	
PE-Alexa Fluor® 700	488	496, 546	723	Infrared
PE-Alexa Fluor® 750	488	496, 546	779	Infrared
PE-Cy5.5	488	496, 546	695	
PE-Cy5	488	496, 546	667	
PE-Cy7	488	496, 546	785	Infrared
PE-Texas Red®	488	496, 546	615	

Abbreviations

APC	Allophycocyanin
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex

Note. Phycoerythrin (PE) is same as R-Phycoerythrin (RPE).

Fluorescence compensation

One consideration to be aware of when performing multicolor fluorescence studies is the possibility of spectral overlap. When two or more fluorochromes are used during a single experiment there is a chance that their emission profiles will coincide, making measurement of the true fluorescence emitted by each difficult. This can be avoided by using fluorochromes at very different ends of the spectrum e.g. Alexa Fluor® 405 and Phycoerythrin; however, this is not always practical.

Instead, a process called fluorescence compensation is applied during data analysis, which calculates how much interference (as a %) a fluorochrome will have in a channel that was not assigned specifically to measure it. Figure 8 helps to explain the concept.

The graphs show the emission profiles of two imaginary fluorochromes 'A' and 'B' which are being detected in FL-1 and FL-2 channels respectively. Because the emission profiles are so close together, a portion of fluorochrome A spills over into FL-2 (red shade) and conversely, some of fluorochrome B reaches FL-1 (dark blue shade).

To calculate how much compensation needs to be applied to the dataset if both dyes are used simultaneously, some control readings must first be taken. Fluorochrome A should be run through the flow cytometer on its own and the % of its total emission that is detectable in FL-2 (spillover) determined. The procedure should be repeated with fluorochrome B, except that this time FL-1 is spillover.

Suppose the results are:

Spillover Fluorescence		
	FL-1	FL-2
Fluorochrome A	N/A	17%
Fluorochrome B	5%	N/A

This means that when the two fluorochromes are used for a dual-color experiment, the true reading for fluorochrome A in FL-1
= (total fluorescence measured in FL-1) minus (5% of fluorochrome B's total fluorescence)
Similarly, the true reading for fluorochrome B in FL-2
= (total fluorescence measured in FL-2) minus (17% of fluorochrome A's total fluorescence)

Fortunately, modern flow cytometry analytical software applies fluorescence compensation mathematics automatically, which simplifies matters considerably.

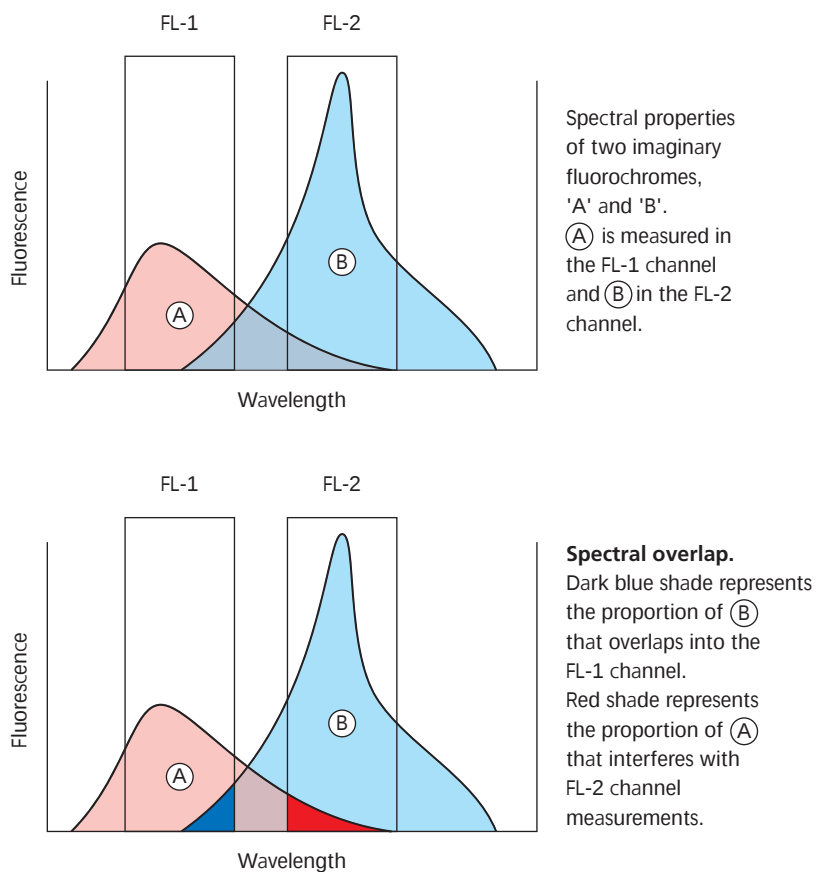


FIGURE 8

Fluorescence compensation



Gates and regions

An important principle of flow cytometry data analysis is to selectively visualize the cells of interest while eliminating results from unwanted particles e.g. dead cells and debris. This procedure is called gating.

Cells have traditionally been gated according to physical characteristics. For instance, subcellular debris and clumps can be distinguished from single cells by size, estimated by forward scatter. Also, dead cells have lower forward scatter and higher side scatter than living cells. Lysed whole blood cell analysis is the most common application of gating, and Figure 9 depicts typical graphs for SSC versus FSC when using large cell numbers. The different physical properties of granulocytes, monocytes and lymphocytes allow them to be distinguished from each other and from cellular contaminants.

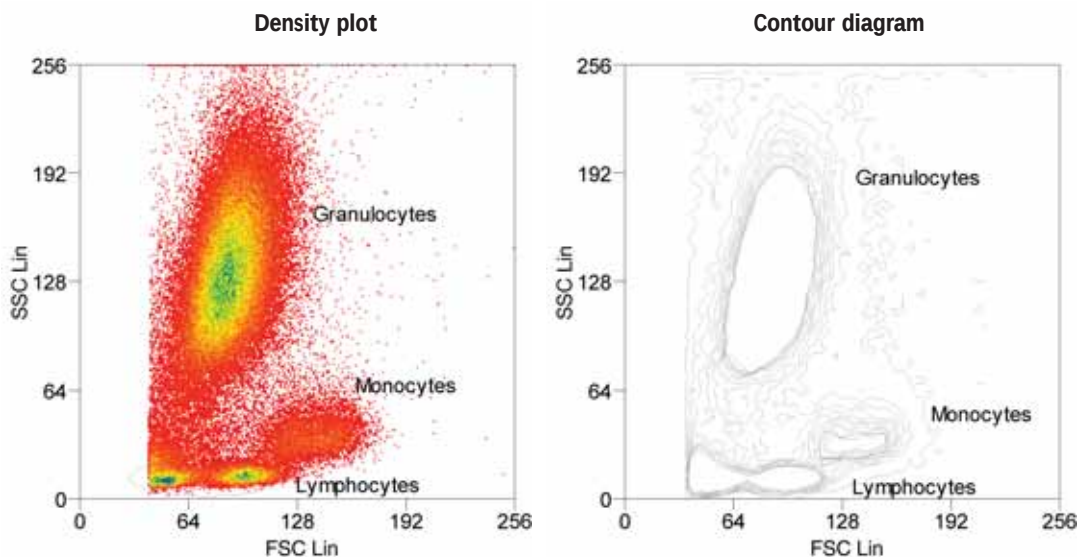


FIGURE 9

Analysis of lysed whole blood using FSC/SSC

Abbreviations used in histograms: Lin = Linear scale Log = Logarithmic scale
Log Comp = Logarithmic scale with compensation applied

On the density plot, each dot or point represents an individual cell that has passed through the instrument. Yellow/green hotspots indicate large numbers of events resulting from discreet populations of cells. The colors give the graph a three-dimensional feel. After a little experience, discerning the various subtypes of blood cells is relatively straightforward.

Contour diagrams are an alternative way to demonstrate the same data. Joined lines represent similar numbers of cells. The graph takes on the appearance of a geographical survey map, which, in principle, closely resembles the density plot. It is a matter of preference but sometimes discreet populations of cells are easier to visualize on contour diagrams e.g. compare monocytes in Figure 9.

Newer gating strategies utilize fluorescence parameters along with scatter parameters. Once again, blood can be used to demonstrate this principle.

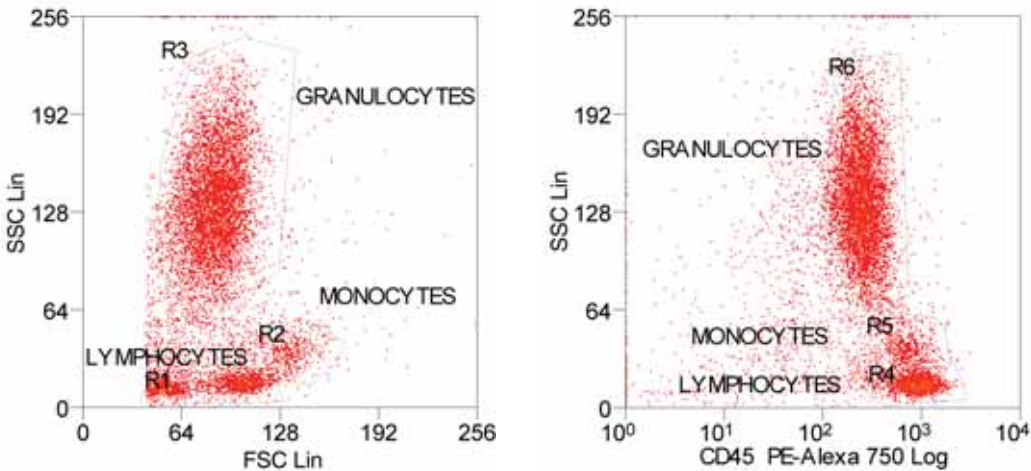


FIGURE 10

Lysed whole blood analysis using scatter and fluorescence

Above on the left is a FSC/SSC plot for human lysed whole blood using smaller numbers of cells than in Figure 9. The lymphocytes, monocytes and granulocytes have been gated as region 1 (R1), region 2 (R2) and region 3 (R3), respectively. 'Region' simply refers to an area drawn on a plot displaying flow cytometry data.

On the right the same cells are now plotted as SSC on the y-axis versus CD45 fluorescence on the x-axis. CD45 is a marker expressed on all white blood cells at varying intensities but is absent on red blood cells. In relative terms, lymphocytes have a low SSC and high CD45 count (R4), granulocytes have a high SSC and low CD45 count (R6), while monocytes are somewhere in between the other two (R5). The major difference between the lymphocytes

gated in R1 and those gated in R4 is the absence of red blood cells in the latter, making it a much purer preparation. This highlights the usefulness of gating strategies that combine a scatter parameter with a fluorescence parameter.

Single-parameter histograms

These are graphs 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.

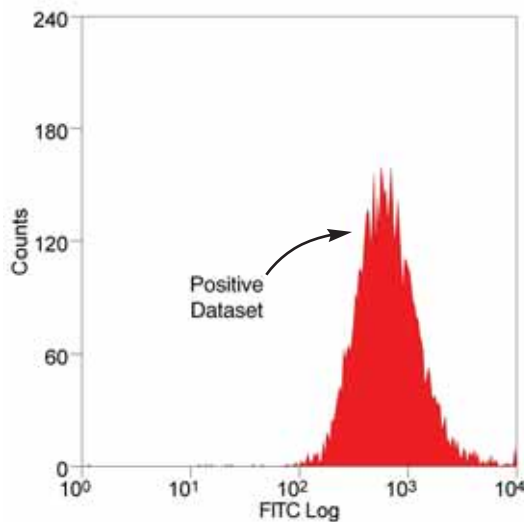


FIGURE 11

A single-parameter histogram

The histogram in Figure 11 looks very basic but is useful for evaluating the total number of cells in a sample that possess the physical properties selected for or which express the marker of interest. Cells with the desired characteristics are known as the positive dataset.

Ideally, flow cytometry will produce a single distinct peak that can be interpreted as the positive dataset. However, in many situations, flow analysis is performed on a mixed population of cells resulting in several peaks on the histogram. In order to identify the positive dataset, flow cytometry should be repeated in the presence of an appropriate negative isotype control (see Figure 12).

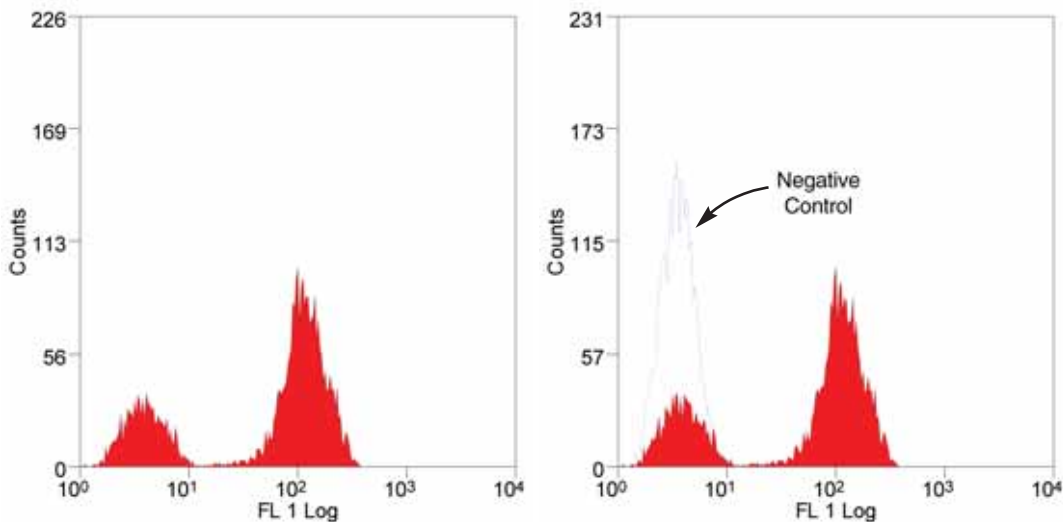


FIGURE 12 **Which is the positive dataset?** LEFT, Using rat anti-mouse F4/80 conjugated to FITC to stain mouse peritoneal macrophages produces two peaks. RIGHT, By running an appropriate isotype control (rat IgG2b negative control conjugated to FITC) and overlaying its image on the histogram (blue outline) the positive dataset is identified as the taller red peak on the right.

Analytical software packages that accompany flow cytometry instruments make measuring the % of positive-staining cells in histograms easy. For example, the F4/80 histogram is shown again below with statistics for R2 and R3 (known on this type of graph as ‘bar regions’).

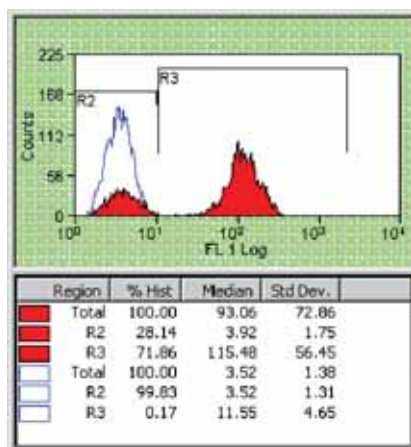


FIGURE 13 **Statistical analysis**

In Figure 13, 99.83% of the negative control (blue outline) is in R2. 28.14% of cells (red shade) 'stain negative' for F4/80 (R2) compared to 71.86% in the positive dataset (R3). Additional statistics about the peaks (median and standard deviation) is also provided automatically here but this will vary with the software. A similar type of analysis will be generated for two-parameter histograms.

Two-parameter histograms

These are graphs that display two measurement parameters, one on the x-axis and one on the y-axis, and the cell count as a density (dot) plot or contour map. The parameters could be SSC, FSC or fluorescence. Some examples of two-parameter histograms were illustrated in Figures 9 and 10.

Another example is the dual-color fluorescence histogram presented below. Lymphocytes were stained with anti-CD3 in the FITC channel (x-axis) and anti-HLA-DR in the PE channel (y-axis). CD3 and HLA-DR are markers for T cells and B cells, respectively.

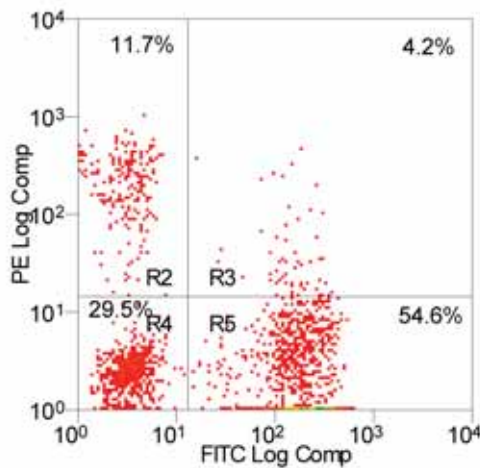


FIGURE 14

Two-parameter (dual-color fluorescence) histogram

In Figure 14, R2 encompasses the PE-labeled B cells – note their positive shift along the PE axis. R5 contains the FITC-labeled T cells (positively shifted along the FITC axis). The top right quadrant contains a few 'activated T cells' (about 4% in this sample) that possess some HLA-DR expression also. As these stain with both antibody markers they are grouped in their own region (R3). R4 contains cells negative for both FITC and PE (no shift).

Currently, flow cytometry can be performed on samples labeled with up to 17 fluorescence markers simultaneously¹. Therefore a single experiment can yield a large set of data for analysis using various two-parameter histograms.

Intracellular antigens

Staining intracellular antigens like cytokines can be difficult because antibody-based probes cannot pass sufficiently through the plasma membrane into the interior of the cell. To improve the situation, cells should first be fixed in suspension and then permeabilized before adding the fluorochrome. This allows probes to access intracellular structures while leaving the morphological scatter characteristics of the cells intact. Many commercial kits are available today that provide the reagents to carry out these crucial steps e.g. LeucopermTM (see Figure 15).

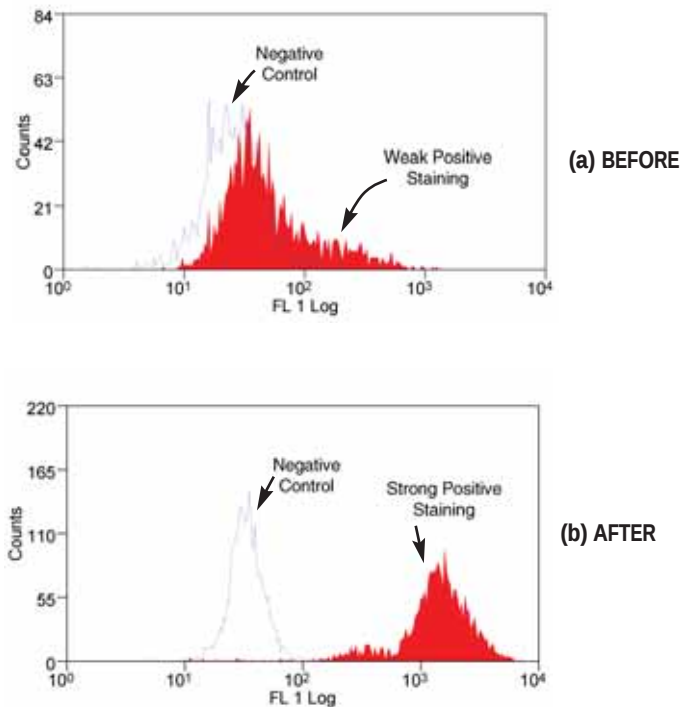


FIGURE 15 LeucopermTM used in conjunction with an antibody that recognizes MOMA-2, an intracellular antigen in mouse macrophages and monocytes. After fixation and permeabilization (b), notice how distinctive the positive dataset becomes.

¹Perfetto, S. *et al* (2004) Seventeen colour flow cytometry: unravelling the immune system. *Nature Reviews Immunology* **4**:648–655

Immunophenotyping

All normal cells express a variety of cell surface markers, dependent on the specific cell type and degree of maturation. However, abnormal growth may interfere with the natural expression of markers resulting in overexpression of some and under-representation of others. Flow cytometry can be used to immunophenotype cells and thereby distinguish between healthy and diseased cells. It is unsurprising that today immunophenotyping is one of the major clinical applications of flow cytometry, and is used to aid the diagnosis of myelomas, lymphomas and leukemias. It can also be used to monitor the effectiveness of clinical treatments.

The differences between the blood profiles of a healthy individual and one suffering from leukemia, for instance, are very dramatic. This can be seen from the FSC v SSC plots in Figure 16. In the healthy person the cell types are clearly defined, whereas blood from a leukemia patient is abnormal and does not follow the classic profile.

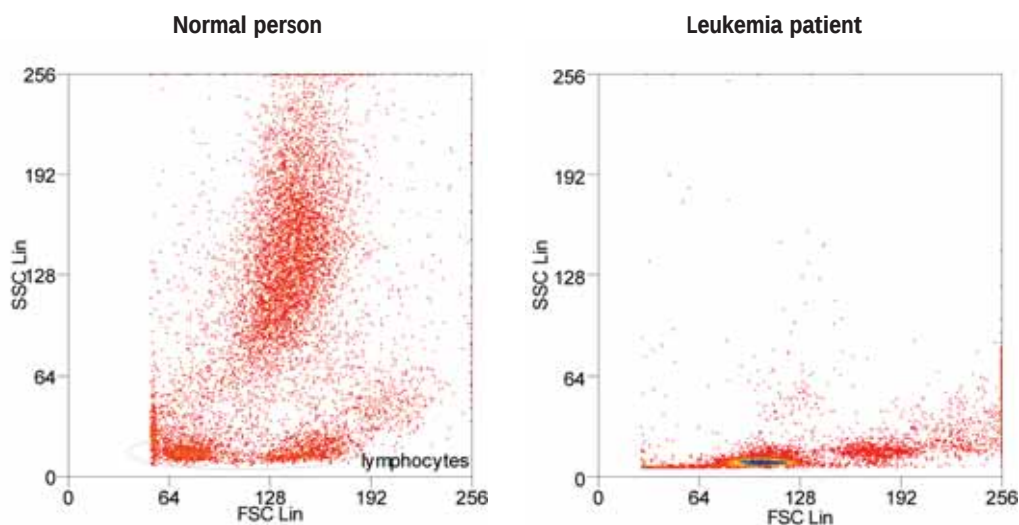


FIGURE 16

Immunophenotyping

Testing the patient's lymphocytes for specific cell surface markers also reveals more about the condition.

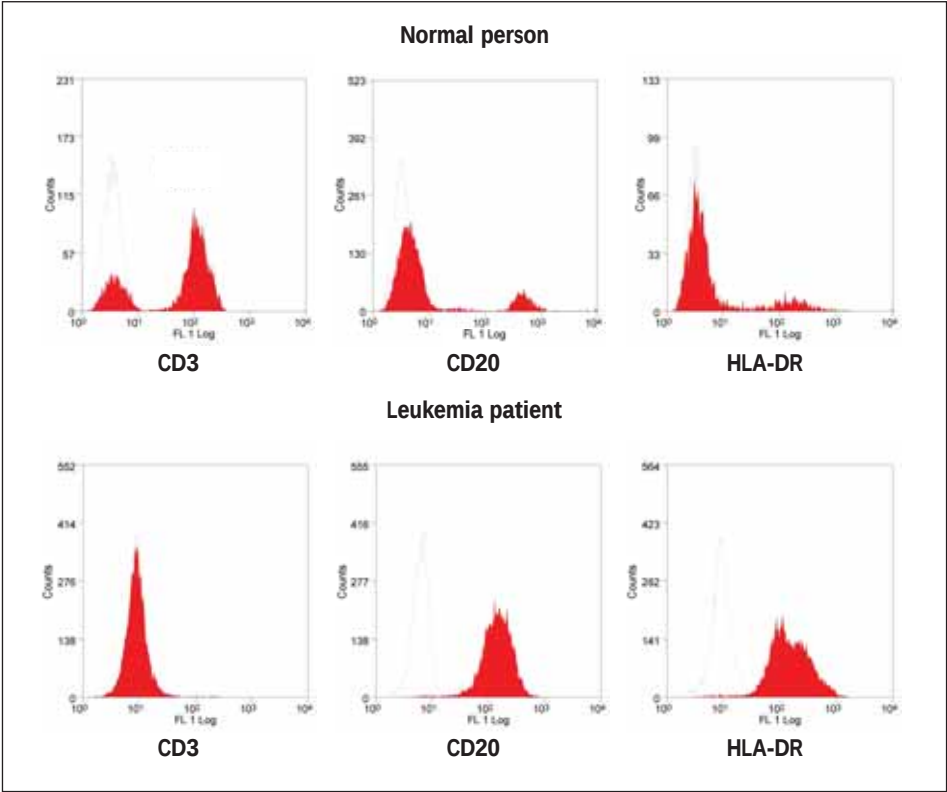


FIGURE 17 Diagnosing leukemia

CD3

A normal person has a significant proportion of CD3-positive lymphocytes. In the patient with leukemia, staining for CD3 is absent.

CD20

In the leukemia patient there are a large number of cells staining positive for CD20. In the healthy person only a few stain positive.

HLA-DR

The leukemia patient is HLA-DR-positive. In the normal person only a small number of cells stain positive.

Being CD3-negative, CD20-positive and HLA-DR-positive, a clinician could diagnose with certainty that this patient is suffering from a B cell lineage leukemia or lymphoma. The precise classification of disease may be determined using further antibodies.



Sample preparation

Single cells must be suspended at a density of 10^5 – 10^7 cells/ml to prevent the narrow bores of the flow cytometer and its tubing from clogging up. The concentration also influences the rate of flow sorting, which typically progresses at 2000–20,000 cells/second. However, higher sort speeds may decrease the purity of the preparation.

Phosphate buffered saline (PBS) is a common suspension buffer and the most straightforward samples for flow cytometry include non-adherent cells from culture, water-borne micro-organisms, bacteria and yeast. Even whole blood is easy to use – red cells are usually removed by a simple lysis step; it is then possible to quickly identify lymphocytes, granulocytes and monocytes by their FSC/SSC characteristics (see page 16).

However, researchers may also wish to analyze cells from solid tissues e.g. liver or tumors. In order to produce single cells, the solid material must be disaggregated. This can be done either mechanically or enzymatically. Mechanical disaggregation is suitable for loosely bound structures e.g. 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 hold cells together. Their action is dependent on factors including pH, temperature and co-factors, so care must be taken when choosing an enzyme. For example, pepsin works optimally between pH 1.5–2.5 but the acidic conditions would damage cells if left unneutralized for too long, and cell surface antigens of interest may be lost. Chelators like EDTA and EGTA can remove divalent cations responsible for maintaining cell function and integrity but their presence may inhibit certain enzymes, for instance, collagenase requires Ca^{2+} for activity. Enzymatic and mechanical disaggregation is often a trial and error process to optimize the isolation of the epitope under investigation.

To study intracellular components e.g. cytokines by flow cytometry, the plasma membrane of the cell must be permeabilized to allow dyes or

EDTA Ethylenediaminetetraacetic acid
EGTA Ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

antibody molecules through while retaining the cell's overall integrity. Low concentrations (up to 0.1%) of non-ionic 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 eventuality here, some standard protocols are given in this chapter.

1 Preparation of cells

(a) Cells stored in liquid nitrogen

- 1 Prepare PBS/BSA buffer (phosphate buffered saline pH 7.4 and 1% BSA).
- 2 Carefully remove cells from liquid nitrogen storage.
- 3 Thaw rapidly using PBS/BSA buffer and place into a 15 ml conical centrifuge tube.
- 4 Centrifuge at 400 *g* for 5 minutes.
- 5 Discard supernatant and resuspend pellet in an appropriate amount of PBS/BSA buffer.

(b) Tissue culture cell lines in suspension

- 1 Prepare PBS/BSA buffer (phosphate buffered saline pH 7.4 and 1% BSA).
- 2 Decant cells from tissue culture flask into 15 ml conical centrifuge tube(s).
- 3 Centrifuge at 400 *g* for 5 minutes.
- 4 Discard supernatant and resuspend pellet in 10 ml of PBS/BSA.
- 5 Centrifuge at 400 *g* for 5 minutes.
- 6 Discard supernatant and resuspend pellet in an appropriate amount of PBS/BSA.

(c) Adherent tissue culture cell lines

- 1 Prepare PBS/BSA buffer (phosphate buffered saline pH 7.4 and 1% BSA).
- 2 Harvest cells by gentle scraping using 2 ml of PBS/BSA buffer.
- 3 Transfer cells to a 15 ml conical tube and add buffer up to 10 ml.
- 4 Centrifuge at 400 *g* for 5 minutes.
- 5 Discard supernatant and resuspend pellet in fresh PBS/BSA (10 ml).
- 6 Centrifuge at 400 *g* for 5 minutes.
- 7 Discard supernatant and resuspend pellet in an appropriate amount of PBS/BSA buffer.

(d) Preparing cells from solid/lymphoid tissues

- 1 Place tissue on a sterile Petri dish. Remove cells by gently perfusing the tissue using a syringe and needle containing approximately 15 ml of PBS/BSA (phosphate buffered saline pH 7.4 and 1% BSA).
- 2 Transfer the cell suspension from the Petri dish into a 15 ml conical centrifuge tube.
- 3 Centrifuge at 400 *g* for 5 minutes.
- 4 Discard the supernatant and resuspend the pellet in PBS/BSA.
- 5 Add 10 ml of ammonium chloride lysis buffer.
- 6 Mix and incubate for 2 minutes. DO NOT EXCEED THIS TIME.
- 7 Centrifuge at 400 *g* for 5 minutes.
- 8 Add 10 ml of PBS/BSA and mix.
- 9 Centrifuge again at 400 *g* for 5 minutes.
- 10 Discard the supernatant and resuspend the pellet to a final volume of 10 ml with PBS/BSA.
- 11 Count cells using a hemocytometer.
- 12 Adjust the cell suspension, if necessary, to give a final count of $0.7\text{--}1.2 \times 10^7$ cells/ml.

2 Direct immunofluorescence staining of cells and blood

This technique is applicable where the fluorochrome is directly linked to the primary antibody e.g. PE, FITC and Alexa Fluor® conjugates.

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

- 1 Prepare cells appropriately (see section 1). Adjust the cell suspension to a concentration of 1×10^6 cells/ml with PBS/BSA buffer (phosphate buffered saline pH 7.4 and 1% BSA).

[Whole blood samples may be used undiluted unless the cell count is high e.g. as in leukemia. EDTA and heparin are preferred anti-coagulants].

- 2 Aliquot 100 µl of cell suspension *[whole blood]* into as many test tubes as required.
- 3 Add antibody at the recommended dilution (see specific datasheets). Mix well and incubate at room temperature for 30 minutes.
- 4 Wash cells with 2 ml of PBS/BSA, centrifuge at 400 *g* for 5 minutes and discard the resulting supernatant.

[To the blood suspension add freshly prepared red cell lysis buffer e.g. 2 ml of AbD Serotec's Erythrolyse and mix well. Incubate for 10 minutes at room temperature. Centrifuge at 400 g for 5 minutes and discard the supernatant].

Resuspend cells in 0.2 ml of PBS/BSA or with 0.2 ml of 0.5% paraformaldehyde in PBS/BSA if required.

Acquire data by flow cytometry. Appropriate standards should always be included e.g. an isotype-matched control sample.

3 Indirect immunofluorescence staining of cells and blood

This technique is applicable where using unconjugated or biotin-conjugated monoclonal and polyclonal antibodies. A secondary reagent must be used to visualize the primary antibody e.g. avidin in the case of biotin.

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

- 1 Prepare cells appropriately (see section 1). Adjust the cell suspension to a concentration of 1×10^6 cells/ml with PBS/BSA buffer (phosphate buffered saline pH 7.4 and 1% BSA).

[Whole blood samples may be used undiluted unless the cell count is high e.g. as in leukemia. EDTA and heparin are preferred anti-coagulants].

- 2 Aliquot 100 μ l of cell suspension [whole blood] into as many test tubes as required.
- 3 Add primary antibody at the recommended dilution (see specific datasheets). Mix well and incubate at room temperature for 30 minutes.
- 4 Add 2 ml of PBS/BSA buffer, centrifuge at 400 g for 5 minutes and discard the resulting supernatant.
- 5 Add an appropriate secondary reagent at the recommended dilution (see specific datasheets). Mix well and incubate at room temperature for 30 minutes.
- 6 Wash cells with 2 ml of PBS/BSA, centrifuge at 400 g for 5 minutes and discard the supernatant.

[To the blood suspension add freshly prepared red cell lysis buffer e.g. 2 ml of AbD Serotec's Erythrolyse and mix well. Incubate for 10 minutes at room temperature. Centrifuge at 400 g for 5 minutes and discard the supernatant].

- 7 Resuspend cells in 0.2 ml of PBS/BSA or with 0.2 ml of 0.5% paraformaldehyde in PBS/BSA if required.
- 8 Acquire data by flow cytometry. Appropriate standards should always be included e.g. an isotype-matched control sample.

4

Staining lambda and kappa chains in whole blood

This method should be used with directly-conjugated dual-color reagents recognizing human kappa and lambda immunoglobulin light chains.

Detection of immunoglobulin expression specifically on B lymphocytes requires a procedure to remove blood serum immunoglobulins that would otherwise cause interference.

- 1 Collect blood in an anti-coagulant e.g. EDTA, heparin or acid-citrate dextrose.
- 2 Aliquot 2–3 ml of whole blood into a 25 ml universal container. Then add 20–25 ml of PBS/BSA (phosphate buffered saline pH 7.4 and 1% BSA), pre-warmed to 37°C, and mix well.
- 3 Centrifuge at 400 *g* for 5 minutes. Carefully aspirate the supernatant taking care not to disturb the cell pellet. Resuspend the pellet in the residual supernatant.
- 4 Repeat the wash (steps 2 and 3) twice.
- 5 Aliquot 100 µl of washed blood into the required number of test tubes. Add antibody at the recommended dilution (see specific datasheet). Mix well and incubate at room temperature for 30 minutes.
- 6 Add a red cell lysis buffer e.g. 2 ml of AbD Serotec's Erythrolyse and mix well. Incubate for 10 minutes at room temperature. Centrifuge at 400 *g* for 5 minutes and discard the supernatant.
- 7 Wash cells with 2 ml of PBS/BSA, centrifuge at 400 *g* for 5 minutes and discard the supernatant.
- 8 Resuspend cells in 0.2 ml of PBS/BSA or with 0.2 ml of 0.5% paraformaldehyde in PBS if required.
- 9 Acquire data by flow cytometry. Appropriate standards should always be included e.g. an isotype-matched control sample.

5

Whole blood protocol for analysis of intracellular cytokines

This is a rapid and simple approach to the analysis of intracellular cytokines by flow cytometry. It permits the analysis of small samples, and avoids any possibility of generating artefactual results during the separation of peripheral blood cells by density gradient centrifugation.

The stimulation conditions described are suitable for IFN gamma, IL-2 and TNF alpha. Different conditions may be needed for other cytokines.

The procedure requires a reagent kit to fix and permeabilize cells. There are several available but we recommend Leucoperm™.

Note. All blood samples must be collected into heparin anti-coagulant. EDTA interferes with the cell stimulation process and, therefore, must be avoided.

- 1 Aliquot 0.5 ml of blood separately into 2 tubes, then add 0.5 ml of cell culture medium (without any additives) to each sample.
- 2 To one tube (the resting population), add monensin to a final concentration of 3 mM.
- 3 To the other tube (activated cells), add PMA, ionomycin and monensin to a final concentration of 10 ng/ml, 2 mM and 3 mM, respectively.
- 4 Incubate for 2–4 hours at 37°C in a 5% CO₂ atmosphere.
- 5 At the end of the incubation period aliquot 100 µl samples into the appropriate number of tubes.
- 6 Add cell surface antibodies at this stage (if needed for your experiment) and incubate for 15 minutes.
- 7 Add 100 µl of LeucopermTM Reagent A per tube and incubate for 15 minutes. This reagent fixes cells in suspension.
- 8 Wash twice with PBS containing 0.1% sodium azide and 1% BSA.
- 9 Add 100 µl of LeucopermTM Reagent B (permeabilizes cells) and the required anti-cytokine antibodies.
- 10 Incubate for 20 minutes.
- 11 Wash twice using the PBS buffer, and analyze by flow cytometry.

6 Direct staining of intracellular antigens

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

- 1 Harvest cells and determine total number present.
- 2 Wash twice in wash buffer (PBS containing 1% BSA and 0.1% sodium azide).
- 3 If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at this stage. Following staining, wash cells once in PBS and discard the supernatant.
- 4 Resuspend cells in LeucopermTM Reagent A (cell fixation agent) using 100 µl per 1×10^6 cells. Incubate for 15 minutes at room temperature.

- 5 Wash once in wash buffer.
- 6 Resuspend cells in Leucoperm™ Reagent B (cell permeabilization agent) using 50 µl per 1×10^6 cells.
- 7 Aliquot 50 µl of cell suspension into the required number of tubes containing directly-conjugated antibodies. Incubate for 30 minutes at room temperature.
- 8 Wash once in wash buffer, and then resuspend in 0.25 ml of 0.5% paraformaldehyde in PBS.
- 9 Store at 4°C until acquisition on the flow cytometer, preferably within 24 hours.

7 Direct staining of intracellular antigens: methanol method

Methanol modification is particularly suitable for the detection of some nuclear antigens, such as PCNA and Ki-67.

Note. Phycoerythrin conjugates are not suitable for the detection of cell surface antigens using this method.

- 1 Harvest cells and determine the total number present.
- 2 Wash twice in wash buffer (PBS containing 1% BSA and 0.1% sodium azide).
3. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at this stage. Following staining, wash cells once in PBS and discard the supernatant.
- 4 Resuspend cells in cold (2–8°C) Leucoperm™ Reagent A using 100 µl per 1×10^6 cells. Incubate for 10 minutes at 2–8°C.
- 5 Add 500 µl of cold absolute methanol, vortex and incubate for 10 minutes at 2–8°C.
6. Wash once in wash buffer.
- 7 Resuspend cells in Leucoperm™ Reagent B using 100 µl per 1×10^6 cells.
- 8 Aliquot 50 µl of cell suspension into the required number of tubes containing directly conjugated antibodies. Incubate for 30 minutes at room temperature.
- 9 Wash once in wash buffer, and resuspend in 0.25 ml of 0.5% paraformaldehyde in PBS.
- 10 Store at 4°C until acquisition on the flow cytometer, preferably within 24 hours.



Troubleshooting

Chapter 5

If something doesn't work check through the following list to resolve the problem. If there are still difficulties and you have purchased an AbD Serotec antibody our Technical Services Team will be happy to offer further advice.

Problem

No staining

Course of action

- 1 Ensure that all antibodies have been stored correctly according to the manufacturer's instructions.
- 2 Ensure that commercial antibodies have not exceeded their date of expiration.
- 3 Ensure that appropriate primary or secondary antibodies have been added.
- 4 Ensure that antibody is conjugated to a fluorochrome. If not, ensure that appropriate fluorochrome-conjugated secondary is being used.
- 5 Ensure that secondary antibody is active – has it been used successfully with other primary antibodies?
- 6 Ensure that correct secondary antibody is being used, which will recognize your primary antibody.
- 7 If the fluorochrome used is Phycoerythrin or Allophycocyanin-based, ensure that the product has not been frozen.
- 8 Is the target antigen present on test tissue? Check literature for antigen expression and incorporate a positive control of known antigen expression alongside test material.
- 9 Does antibody recognize antigen in test species? Check that antibody cross-reacts with species being used. Not all antibodies will cross-react across species.
- 10 Ensure that correct laser is being used to excite fluorochrome, and that correct channel is being used to analyze emissions.

PE antibody does not stain but same FITC antibody gives good results

- 1 PE conjugate may have been frozen. If so, purchase another vial of antibody.
- 2 Paraformaldehyde (PFA) may be a problem. Breakdown of PFA may release methanol, which will affect staining. Make up fresh paraformaldehyde. Cells can be analyzed immediately without fixing.

Non-specific staining

- 1 Non-specific staining may be due to autofluorescence. Solution: check levels of autofluorescence by including a tube of cells only (i.e. without any antibody) into your panel.
- 2 Certain cells express low affinity Fc receptors CD16/CD32, which bind whole antibodies via Fc region. For mouse cells, dilute antibody in SeroBlock FcR (Product Codes BUF041 A/B).
- 3 Non-specific staining may be due to the secondary antibody. Select a secondary antibody that will not cross-react with target tissue.
- 4 Ensure that sufficient washing steps have been included.
- 5 Titrate test antibody carefully. Non-specific staining may be reduced at lower antibody concentrations.

Weak staining

- 1 Weak staining may be due to overdilution of antibodies. Ensure that antibodies are used at the correct concentration by titrating antibodies before use.
- 2 Weak staining in indirect staining systems may be due to prozoning effect, where highly concentrated antibodies may give weak results. Titrate antibodies carefully.
- 3 Weak staining may be due to an excess cell number. Adjust cell population to recommended density.
- 4 Weak staining 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 fluorochrome.
- 6 Weak staining may be seen if using a cross-reacting antibody rather than one specific for the target species.
- 7 Incubation time and temperature with either primary or secondary antibody should be optimized.

Unusual scatter profiles

- 1 Ensure that cells are used as fresh as possible. Profile may be showing dead cells and debris.
- 2 Activation methods may affect scatter characteristics of cells.
- 3 If you are using lysing solution, ensure that this is fresh and has been made up correctly.

Unexpected staining

- 1 Some reagents may affect certain antigens and, therefore, may need reviewing e.g. 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 and, therefore, cell permeabilization methods may be required. Check manufacturer's datasheet for correct permeabilization reagent.

Recommended reading

Flow Cytometry: A Practical Approach, 3rd Edition. (Practical Approach Series).

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Flow Cytometry: Clinical Applications.

Marion G. Macey. Blackwell Scientific Publications, Oxford (1994)

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Alice L. Givan. Wiley (2002)

The Handbook – A Guide to Fluorescent Probes and Labeling Technologies, 10th Edition.

Richard P. Haugland. Invitrogen – Molecular Probes (2005)

Immunophenotyping. Carleton C. Stewart and Janet K.A. Nicholson, Editors.

John Wiley & Sons (2000)

Introduction to Flow Cytometry, First Paperback Edition.

James V. Watson. Cambridge University Press. (2004)

Practical Flow Cytometry, 4th Edition.

Howard M. Shapiro. Wiley Liss (2003)

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