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BASICS OF FLOW CYTOMETRY

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1. INTRODUCTION

The ability to discriminate and quantify distinct populations of cells or cell organelles has become increasingly important with the growing trend to focus biological studies on various cell types. Flow cytometry and laser-activated cell sorting are unique techniques that permit the identification, analysis, and purification of individual cells based on the expression of specific markers. Flow cytometers can be used to analyze DNA content and cell cycle distribution, cellular viability, apoptosis, calcium flux, intracellular pH and membrane potential, expression of cell surface and/or intracellular antigens and markers, fluorescent reporter proteins, and chromosomes. One common approach is to conjugate fluorescent dyes to specific antibodies against antigens expressed in a target cell population. Expression of fluorescent marker proteins injected or transfected into target cells is another popular flow cytometric approach. For this purpose, Green, Yellow, Cyan, and Red fluorescent proteins have been used in flow cytometric studies. Fluorescence-activated cell sorters can be used to identify and isolate viable labeled cells, chromosomes, or cell organelles from complex populations for further studies.

2. COMPONENTS OF A FLOW CYTOMETER

The key components of a typical analytical flow cytometer include fluidics, lasers, optics, electronic detectors, analog-to-digital converters, and pulse processors.

Applications of Flow Cytometry in Stem Cell Research and Tissue Regeneration,
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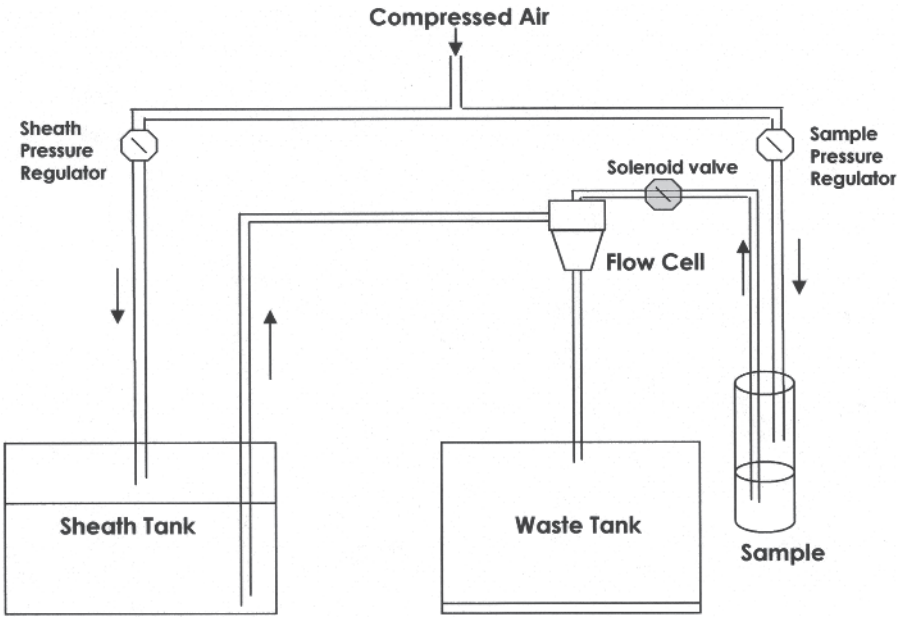

FIGURE 1. Typical flow cytometer fluidics design, showing the sheath and waste tanks, flow cell, sheath, and sample pressure regulators and sample tube.

2.1. Fluidic System

Fluidic systems are designed to rapidly introduce single cells, one at a time, to a point in space where multiple measurements can be made after laser excitation of the individual cells. Cells are aligned much like beads on a string. In general, analytical flow cytometers have a fluidic system such as that shown in Figure 1. The sheath fluid is an isotonic buffer, pumped into the flow cell by applying air pressure using a sheath pressure regulator. By applying higher pressure to the sample than that of the sheath flow, a hydrodynamically focused sample stream is created in the flow cell. A narrow sample stream facilitates the creation of a stream where single cells pass one at a time in single file through a laser beam. The sheath and sample leaving the nozzle are collected in a waste tank.

2.2. Excitation Light Source

The most commonly used excitation light in a flow cytometer is a laser. However, some cytometers use a mercury arc lamp and/or a light-emitting diode (LED) as a light source. Lasers have the advantage of being coherent and monochromatic. Lasers demonstrate a better signal-to-noise ratio than that of noncoherent light sources such as mercury arc or metal halide lamps. Some of the gas lasers used in flow cytometers with their primary excitation wavelengths are argon ion (488 nm), HeNe (633 nm), and HeCd (355 nm). Solid-state lasers that emit at wavelengths of 355, 375, 407, 488, 561, and 633 nm are available commercially.

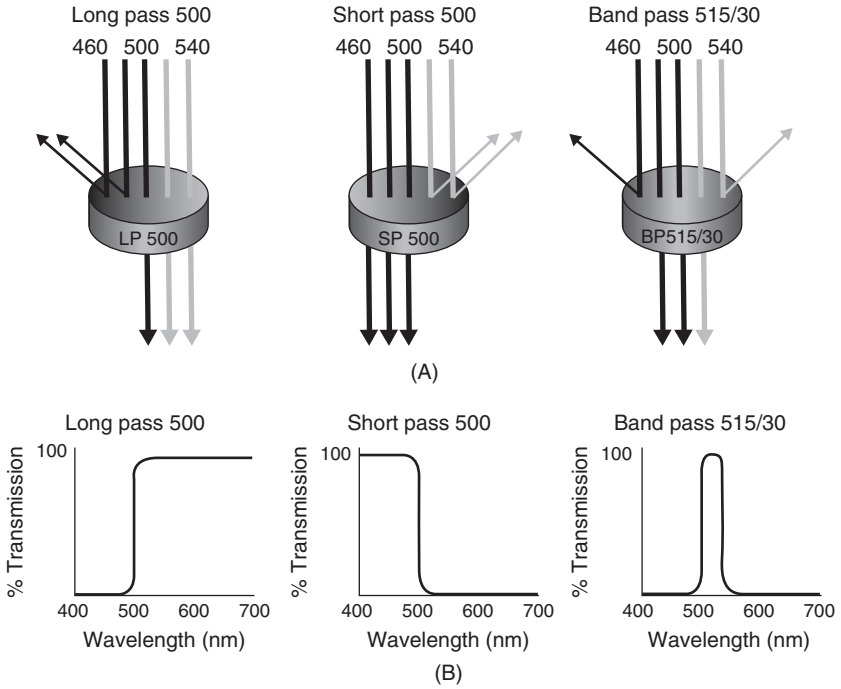

FIGURE 2. (A) Typical optical filters commonly used in a flow cytometer; (B). schematic diagrams of the corresponding transmission curves.

2.3. Optics

Some of the optical components used in flow cytometers are shown in Figure 2A. Combinations of different lens configurations are used to focus the laser beam into either an elliptical or a round beam shape. The excitation optics should withstand milliwatts of laser power. Fluorescence emission optics use primary and secondary dichroic mirrors and long and short pass filters to separate the emitted fluorescence into different wavelengths. Transmission curves for long-pass, short-pass, and bandpass filters are shown in Figure 2B. The emission optics include bandpass filters that are placed in front of each photomultiplier detector (Figure 3).

2.4. Optics Layout

A typical optical layout for a single-laser flow cytometer is shown in Figure 3. The cells moving in single file are illuminated by a laser beam at a point in either a flow cell or within a stream in air. The fluorescence emission from laser excitation or laser light scatter is collected by various detectors after passing through several filters and mirrors (Figure 3). Scattered signals generated in the direction of the incident light beam (small-angle scatter) are collected by a photodiode. Side or 90° scatter and fluorescence signals are collected by various detectors after passing through appropriate optics. The first filter, a beam splitter, will send 8% of the signals to a side-scatter photomultiplier tube via a bandpass filter (488/10), and 90% of the signals toward a 610SP filter. The 610SP filter will pass fluorescent light shorter than 610 nm and reflect the longer wavelengths

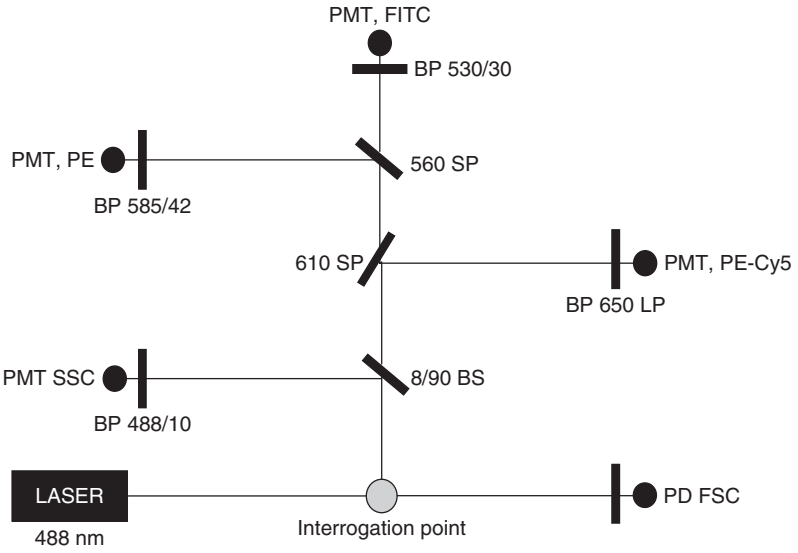

FIGURE 3. Optics layout and ray diagram of a single laser flow cytometer set up to measure FITC (BP: 530/30 nm), PE (BP: 585/42 nm), and Cy5 (BP: 630/22 nm). BS, beamsplitter, PD, photodiode; PMT, photomultiplier tube; BP, bandpass filter; SP, short-pass filter.

toward a bandpass filter (630/22). These red fluorescent signals are collected by the Cy5 detector. Similarly, fluorescent light shorter than 560 nm will pass through the 560SP filter, and green signals will be collected by a fluorescein (FITC) detector after transmission through a 530/30 bandpass filter. Yellow fluorescent signals higher than 560 nm will be reflected and collected after the 585/42 bandpass filter by a phycoerythrin (PE) detector.

2.5. Detectors

The most commonly used detectors in flow cytometers are photomultiplier tubes (PMTs) and photodiodes (PDs). Photodiodes have higher quantum efficiency (>90%) than PMTs (<30% in the green range and 15% in the red range). Typically, PDs are used only for the collection of the stronger forward-scatter signals because of their smaller detection area and high intensity of scattered light compared to the fluorescence signals. Second, the PDs have a lower internal gain (10^{2-4}) compared to that of PMTs (10^8). Fluorescent signals, which are normally weaker than those of the scatter, are collected by PMTs, which offer higher gain and amplification.

2.6. Amplifiers

Electronic amplifiers are used in analog flow cytometers. They are connected to the output of the PMTs. Amplifiers are particularly critical if the signals are weak. If the signals are strong, the PMT will saturate at high voltages, as shown in Figure 4A, and if the signals are weak, the fluorescence signal will plateau at lower PMT voltages (Figure 4B). One has to work at voltage levels that are linear for the range of signals being measured. In such cases the amplifier will be used to enhance the signal intensity.

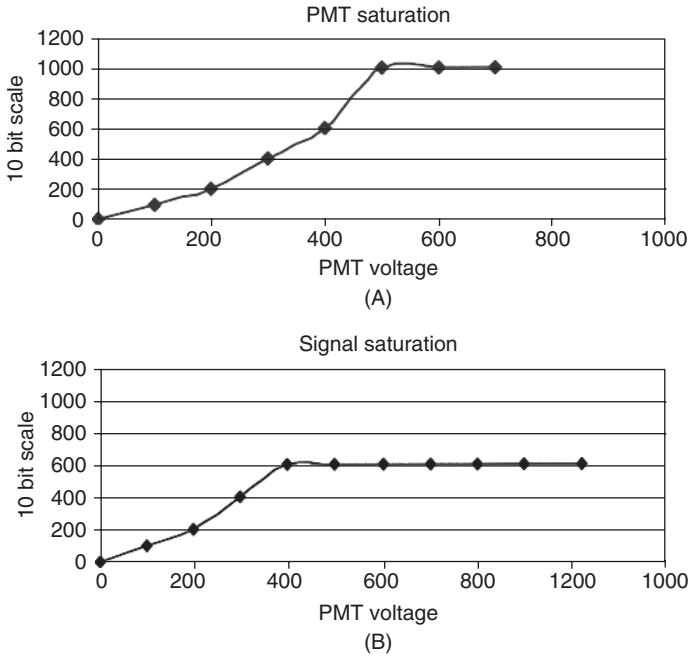

FIGURE 4. (A) PMTs are not linear at very high voltages. Similarly, for weak signals (4B) linearity is lost at lower voltages, due to signal saturation (all the fluorochromes on a cell have been excited and maximum emission has occurred).

2.7. Analog-to-Digital Converters

Analog signals are collected by a detector and digitalized by an analog-to-digital converter (ADC). Digitalization of the analog signals is required to plot the data as histograms, dot plots, contour plots, density plots, or three-dimensional plots. An ADC is also used to eliminate unwanted or noise signals. Digitized data are also used to perform color compensation and to eliminate spectral overlap from different fluorochromes.

2.8. Pulse Processors

The electronic pulse (pulse shape) is different for two cells ($G_0 + G_1$) stuck together, as compared to a single cell in G_2 or M, whereas the total DNA content for both will be equivalent. Two cells stuck together will have a wider pulse width (PW), a lower pulse height (PH), but the same pulse area (PA) as a mitotic cell. To eliminate doublet events from the final analysis, pulse processing is used to measure the pulse area, width, and height of every pulse. When data of pulse area versus pulse width are plotted as shown in Figure 5, single cells will have overlapping area and width signals compared to cell clumps, thus allowing discrimination of single cells from doublets and clumps.

3. HOW THE FLOW CYTOMETER WORKS

For flow cytometric analysis, cells (live or fixed) must be in a single-cell suspension. As cells pass single file through a laser beam (Figure 6), they scatter some of the

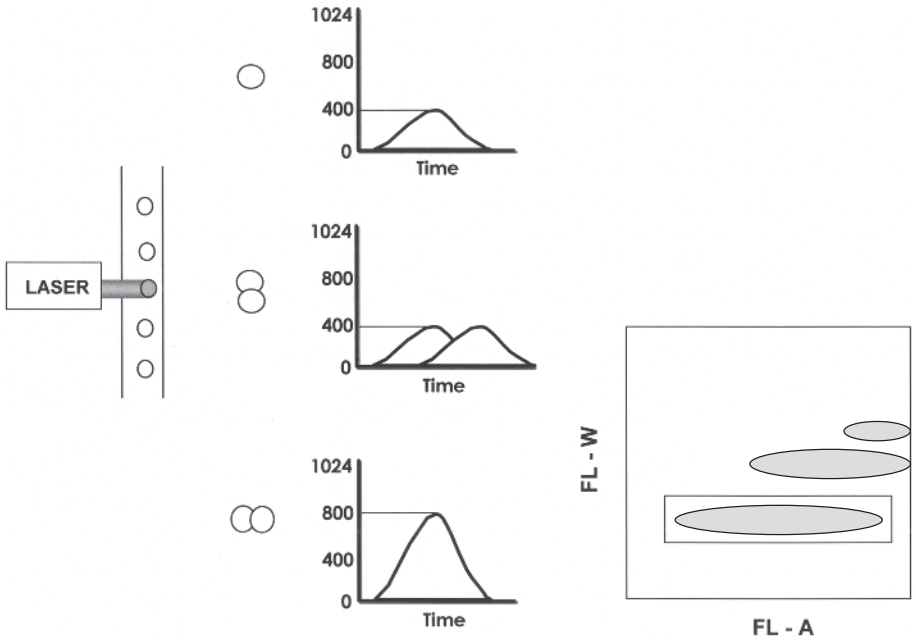

FIGURE 5. The fluorescence signal generated by doublets can have a larger pulse width and larger pulse area than that generated by single cells. Plotting the area and width allows for discrimination of singlets and doublets. The rectangular region set on the dot plot indicates a singlet population.

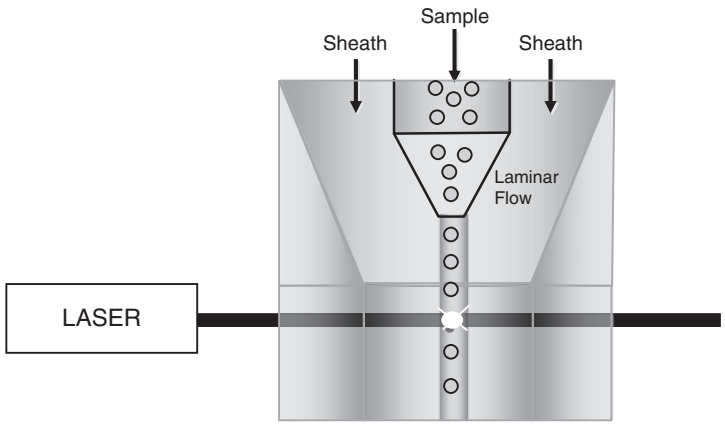

FIGURE 6. The cells are interrogated by the laser beam in the quartz flow cell, where scattered and fluorescent signals are generated simultaneously.

laser light and also emit fluorescence from laser excitation of the fluorochrome used to label the cell. The cytometer typically measures several parameters for each cell simultaneously. The laser beam is focused to the size of a few cell diameters across. The fluidics hydrodynamically focuses the cell stream and, in sorters, breaks the stream into uniform-sized droplets containing individual cells. The electronics quantify the flashes

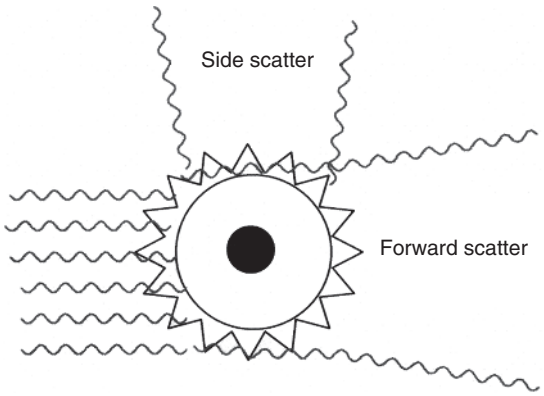

FIGURE 7. Forward-scatter signals are collected around -5° and $+5^\circ$ from the incident light, and side-scatter signals are collected at a 90° angle.

of scattered and fluorescent light, and under computer control, electrically charge those droplets that contain a cell of interest so that they can be deflected into a separate test tube or culture well. A computer records data for thousands of cells per sample and displays the data graphically.

3.1. Forward- and Side-Scatter Signals

Forward-angle light scatter intensity is approximately proportional to cell diameter, whereas side-scatter (orthogonal, right angle, 90°) intensity is approximately proportional to the quantity of granular structures within the cell or cell surface complexity (Figure 7). Light scatter alone is often quite useful to exclude dead cells, cell aggregates, and cell debris from the data. Forward versus side scatter is used to discriminate between lymphocytes, monocytes, and granulocytes in a blood sample.

3.2. Fluorescent Signals

Fluorochromes are used to label specific components (e.g., DNA, antigens, proteins, enzymes) of cells. Fluorochrome-tagged antibodies are used to monitor expression and antigen density of specific surface receptors that enable one to detect discrete subpopulations in a heterogeneous population such as that of bone marrow or a tumor. Intracellular components such as nuclear DNA, RNA, or protein content or enzyme activity can also be reported by use of fluorescent probes (Figure 8). Some common uses of flow cytometry are for quantitation of DNA content for determination of cell cycle phase distribution and aneuploidy, identification of proliferating cells after incorporation of bromodeoxyuridine, determination of specific nucleotide sequences in DNA or mRNA, filamentous actin, and any structure for which an antibody is available. Flow cytometry can also monitor rapid changes in intracellular calcium flux, membrane potential, pH, or transport or efflux of fluorescent dyes in multidrug-resistant and stem cells.

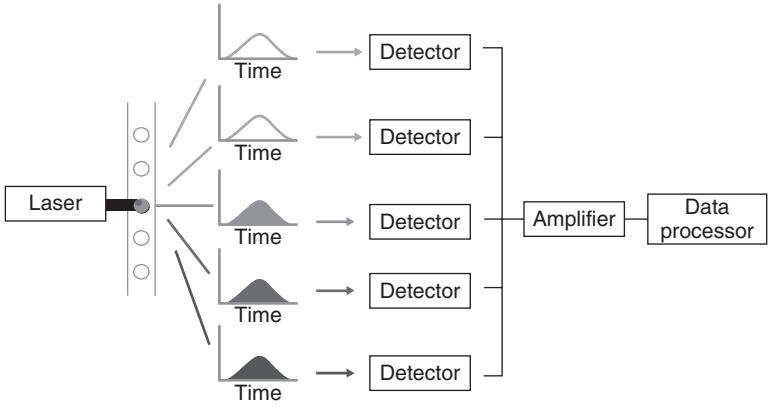

FIGURE 8. Collection of various fluorescence signals. Each cell is labeled with multiple probes, each with a different color of fluorescence. (*See insert for color representation of the figure.*)

3.3. Data Plots

Single-parameter flow cytometry data are presented as frequency distribution histograms showing signal intensity on the x-axis and counts on the y-axis, whereas dual-parameter data are generally presented as dot plots or contour plots showing signal intensity of two different parameters on the x and y axes. The electronic pulse generated by a cell passing through the laser beam is digitalized as shown in Figure 9. If a cell with a smaller amount of DNA is analyzed, a smaller pulse will be generated, and similarly, a big cell with more DNA will create a larger pulse. If pulse height or pulse area is used to plot a frequency histogram, ideally all the small cells should be counted in channel 400, and the larger ones, with twice the fluorescence, should appear in channel 800 (Figure 9A). However, there will be subtle differences in the signal intensity of the cells of the same population and hence, practically, the distribution will appear as a frequency distribution and there will be peaks representing the small and large cell populations (Figure 9B). The x-axis shows the pulse intensity and the y-axis the number of cells per channel. A bivariate plot of dual-parameter data is represented as a dot plot (Figure 9C) or as a contour plot (Figure 9D).

3.4. Linear and Log Scales

If the range of fluorescence intensity in a population is within a decade (e.g., the DNA content of G_0/G_1 and G_2/M cells), the data are plotted on a linear scale (Figure 10A). If the range of fluorescence intensities is greater than one decade, a four- or five-decade log scale is used to facilitate data display. When the difference in intensity between unstained and stained samples cannot be accommodated on a linear scale, a log scale is used (Figure 10B).

3.5. Color Compensation

Emission spectra of commonly used fluorochromes in flow cytometry can be broad and in multicolor flow cytometry, they can often overlap. For example, although the

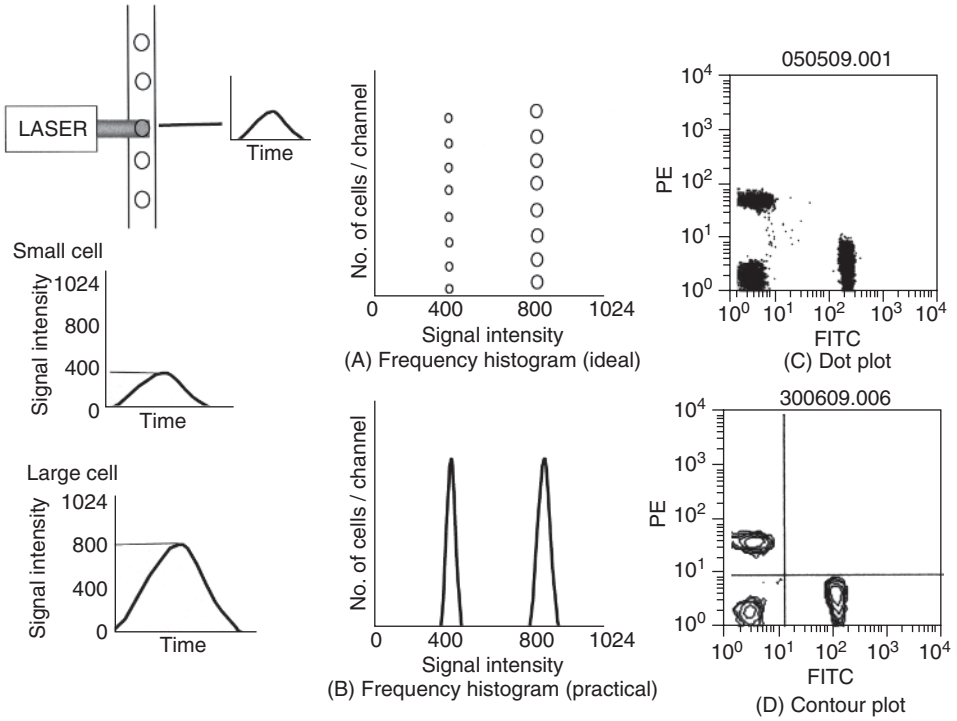

FIGURE 9. Frequency distribution histograms are derived from the pulse height or pulse area of the pulses generated by the passing of cells through a laser beam. (A) Ideally, small (G_0/G_1) cells should fall at channel 400 and bigger ones (G_2/M cells) at 800. (B) However, each population is distributed over a few channels and appears as a peak (a distribution) reflecting small variations in both DNA content and instrumental contributions. Bivariate analysis illustrating two parameters plotted as either (C) a dot plot or (D) a contour plot.

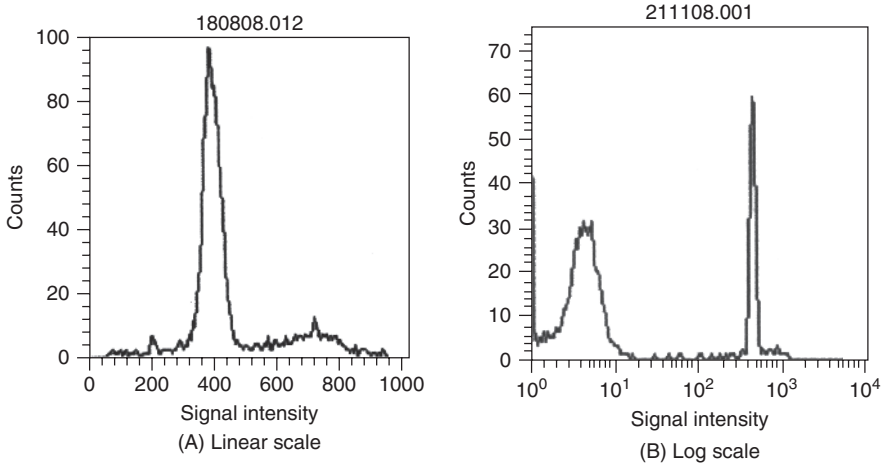

FIGURE 10. Use of linear and log scales in flow cytometry. (A) DNA cell cycle analysis showing G_0/G_1, S-phase, and G_2/M cells on a linear scale. (B) An immunocytometry sample showing the unstained and stained populations. The difference in intensity of the two populations is large, so they are plotted on a log scale.

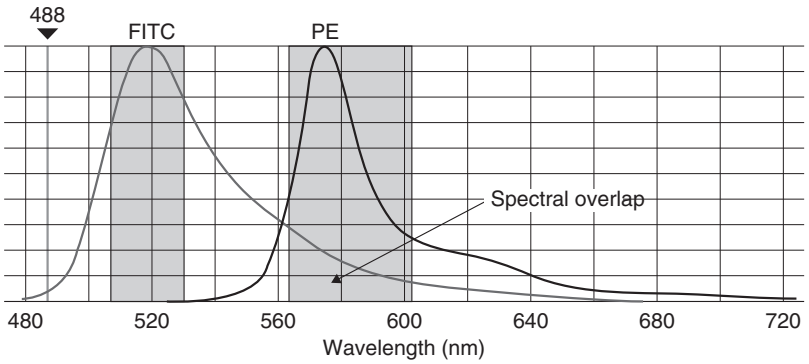

FIGURE 11. Emission spectra of fluorescein and phycoerythrin. The peak of emission of FITC and PE is collected by 530/30 μm and 585/42 nm bandpass filters, respectively.

emission peaks of fluorescein (FITC) and phycoerythrin (PE) are distinctly different, the emission spectrum of FITC overlaps that of PE (Figure 11, spectral overlap indicated by the arrow). Therefore, it is important to remove the overlapping FITC signal in the PE range, particularly while using these two dyes in dual-parametric analysis or colocalization studies. The process of subtracting the overlapping FITC signal in the PE channel is called *color compensation*.

Color compensation is necessary, particularly when acquiring data from multicolor flow cytometric analysis. Generally, the compensation is performed prior to the acquisition; however, digital flow cytometers will allow postacquisition compensation. For example, to perform compensation while using FITC- and PE-stained samples, we first have to run an unstained sample and adjust the PMT voltage such that the population is placed in the lower left quadrant of the dot plot and note the median of this population on FITC and PE channels to set the compensation values of stained samples (Figure 12A). Next, the FITC-stained sample is run and placed in the upper right quadrant (Figure 12B). Color compensation is performed by subtracting the percentage of FITC fluorescence in the PE channel (i.e., PE − % FITC). The median of the unstained and FITC samples must be the same on the PE channel (Figure 12C). Similarly, run the PE sample (Figure 12D) and perform color compensation by subtracting the percentage of PE fluorescence in the FITC channel (i.e., FITC − % PE) to make sure that the median of the unstained and PE stained samples are the same on the FITC channel (Figure 12E). Now the samples stained with both FITC and PE can be run and analyzed.

4. FLUORESCENCE-ACTIVATED CELL SORTING

Fluorescence-activated cell sorting is used to isolate and collect specific cells from a heterogeneous population. Although several other methods are available for cell separation (e.g., magnetic elution), fluorescence-activated cell sorting is used widely because of the high yield and purity of the target population that can be obtained by this procedure (Figure 13).

In all stream-in-air sorters, the mechanism of sorting is based on the formation and deflection of droplets carrying a cell in the electrical field. The nozzle through which

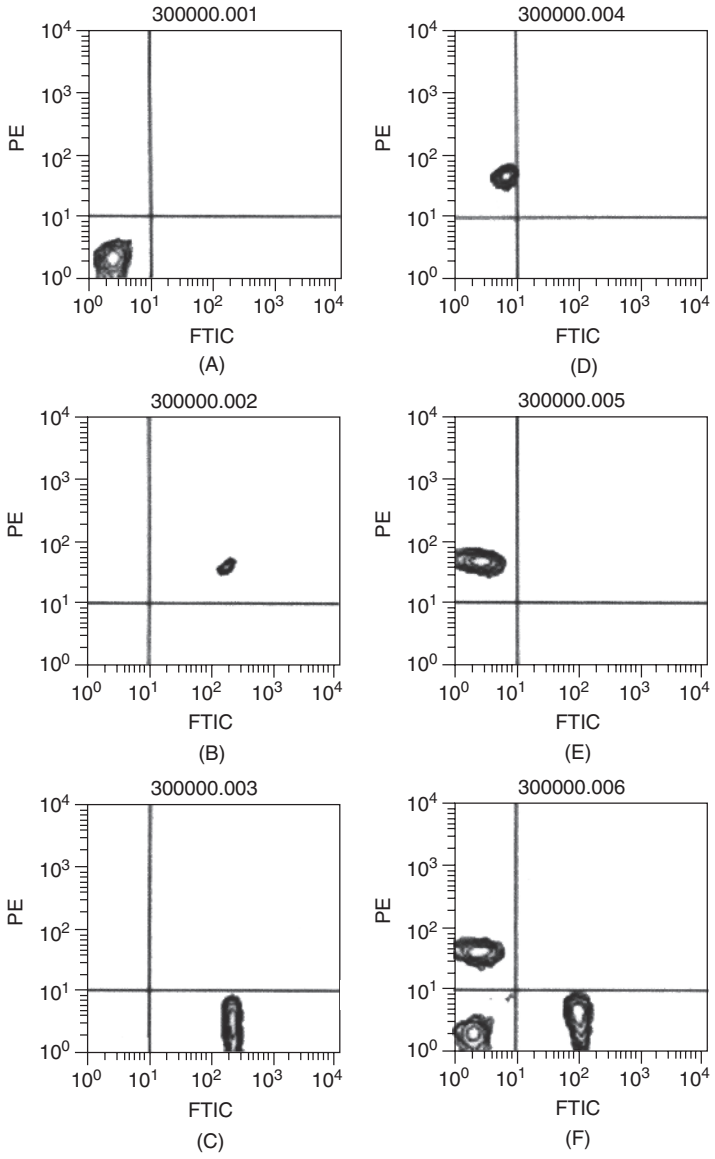

FIGURE 12. Color compensation is performed on dual-labeled (FITC and PE) samples. (A) Unstained sample, (B) FITC-stained sample uncompensated, (C) FITC-stained sample compensated, (D) PE-stained sample uncompensated, (E) PE-stained sample compensated, and (F) an unstained sample was mixed with samples stained with FITC and PE after setting the compensation with single-stained samples.

the cells are passed is about five to seven times bigger than the cell size. The sorter that works at 70 psi with a 70-μm nozzle will be vibrated vertically at a frequency of 20 to 70 kHz. The distance between the point of interrogation and the break-off point is called the *drop delay*. The drop delay setting is critical for accurate sorting. When a target cell signal is acquired and identified by the electronic gate and it reaches the

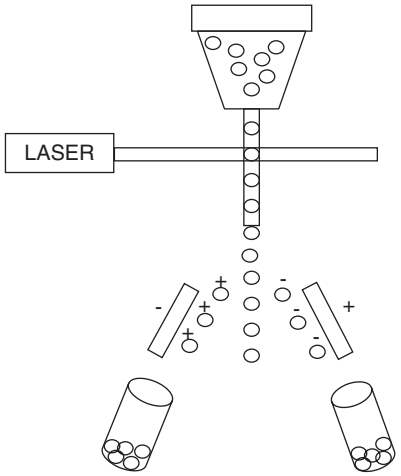

FIGURE 13. Schematic diagram of flow sorting. The charged droplet containing target cells is deflected toward the oppositely charged plate and lands in the appropriate tube.

break-off point, the entire stream is charged and the droplet carrying the target cell breaks off and gets deflected toward the plate that has opposite charge and lands in a collection tube. The nontargeted cells and debris along with empty droplets will be aspirated into the waste tank. The fluidics stability and drop delay can depend on the temperature, humidity, and airflow in the room, and any changes in these ambient parameters will affect sort purity and yield. Osborne discusses cell sorting of stem cells in Chapter 2.

FURTHER READING AND RESOURCES

There are several excellent books available on basics and applications of flow cytometry and cell sorting. The classic and popular book, *Practical Flow Cytometry* (4th edition, Wiley-Liss, New York), by H. M. Shapiro, is an excellent source for historical and applications of cytometry. A free online version of this book is available at http:/probes.invitrogen.com/products/flowcytometry/practicalflowcytometry.html.

Michael G. Ormerod's *Flow Cytometry: A Basic Introduction* (self-published) covers most of the basics and applications of flow cytometry. A free online version of this book is available at http://flowbook.denovosoftware.com/.

The Purdue University flow cytometry Web site (http://www.cyto.purdue.edu) established by Paul Robinson and his team is an excellent resource for information about flow cytometry applications and for seeking advice from fellow cytometrists through its chat room.

For students in the developing countries, flow cytometry workshops are held in India, Turkey, and Kuala Lumpur, Malaysia. Details can be found at http://www.cytometryworkshops.com/and http://www.tcs.res.in/.

The International Society for Advancement of Cytology (ISAC: http://www.isac-net.org) and the Clinical Cytometry Society (http://www.cytometry.org/) post information on meetings, advanced workshops, and other cytometry-related subjects.