

Flow Cytometry

J. Paul Robinson

Purdue University, West Lafayette, Indiana, U.S.A.

INTRODUCTION

Flow cytometry is a technology that has impacted both basic cell biology and clinical medicine in a very significant manner. The essential principle of flow cytometry is that single particles suspended within a stream of liquid are interrogated individually in a very short time as they pass through a light source focused at a very small region. The optical signals generated are mostly spectral bands of light in the visible spectrum, which represent the detection of various chemical or biological components, mostly fluorescence. A key aspect of flow cytometers is that because they can analyze single particles/cells, it is possible to separate particles/cells into populations based upon a statistical difference of any of 10 to 20 variables that can be measured on each particle/cell. Using these statistical analyses, it is possible to separate these populations electronically and identify them using multivariate analysis techniques.

The most common detection system in flow cytometry uses fluorescent molecules that are attached by one means or another to the particle of interest. If the particle is a cell, such as a white blood cell, for example, the fluorescent probe might be membrane bound, cytoplasmic, or attached to nuclear material. It is a common practice to use monoclonal or polyclonal antibodies that recognize specific receptors on cells. By conjugating fluorescent molecules to these antibodies, it is possible to monitor both the location and number of these conjugated antibodies as they bind to cell receptors. Particles of almost any nature can be evaluated by flow cytometry. They can be very small, even below the resolution limits of visible light, because they can be detected by their fluorescent signatures. Similarly, depending on the structure of the flow cell and fluidics, particles as large as several thousand microns can be evaluated.

The key advantage of flow cytometry is that a very large number of particles can be evaluated in a very short time; some systems can run particles at rates approaching 100,000 particles per second while collecting 10 to 20 parameters from each particle. Finally, the principle of cell sorting in flow cytometry allows this technology to separate single particles/cells physically from mixed populations. Thus single particles can be physically placed into a defined location for further analysis and, if necessary,

this process can be performed under sterile conditions. This capability makes flow cytometry a valuable tool for rare event (1:100,000 or even 1:1,000,000) analysis.

In 1983 Shapiro noted that multiparameter flow cytometry was now a reality in the field^[1] because of the availability of commercial instruments. Since that time, the field has expanded well beyond anything that was then considered possible. Today's instruments have the capacity to measure 10–15 spectral bands simultaneously together with a variety of scatter signals. With modern computers it is possible to perform complex multiparametric analyses virtually instantaneously, allowing time to make sorting decisions after measurements are made. The result of this technology is that it is now possible to generate clinical diagnostic information rapidly from complex heterogeneous mixtures of samples such as human blood and to perform this in real time.^[2]

OVERVIEW

Basic Principles

The basic principles of flow cytometry arise from some very old ideas generated early in the 20th century and of course follow the principles of laminar flow defined by Reynolds in the late 19th century. Some 50 years later, Maldavan designed an instrument (although it is not clear that he actually constructed it) that could have identified single cells using a microscope and a photodetector.^[3] In the 1940s Papanicolaou demonstrated that he could identify as cancerous cells from cervical cancer by observing the staining patterns obtained by staining tissues with specifically designed stains.^[4] This suggested several directions of research, primarily using image analysis techniques for the identification of abnormal cells. The limited capability of computers and imaging technology at that time made this quite difficult and resulted in a movement toward single-cell analysis, as opposed to image processing and recognition. It was in the 1960s that Louis Kamensky began the drive to design and build single-cell analyzers. While working at IBM's Watson Labs, Kamensky was interested in using optical character recognition techniques to identify cancer cells. Because of the lack of computation, this became a difficult goal and

in place of image-based technology,^[5] Kamentsky focused on single-cell analysis and the design of a cytometer that measured absorption and scatter and shortly thereafter added the ability to sort cells using fluidic switching.^[6] At the same time, Fulwyler was trying to solve a problem generated by the study of red blood cells using a single-cell analysis system. It had become apparent that a bimodal distribution of red blood cells observed using a Coulter volume detector suggested two different types of red blood cells, contrary to accepted medical understanding. Fulwyler had heard of Richard Sweet's development of high-speed chart recorders using electrostatic drop generation.^[7] Fulwyler visited Sweet's laboratory and essentially utilized this technology to design and build a cell sorter to separate red blood cells.^[8] Ironically, upon completion of the instrument, it took only a few hours to recognize that the supposed bimodal distribution was related to spatial orientation rather than to inherent red blood cell variability (Fulwyler, personal communication). Amazingly, this finding of great significance was never published since it was immediately obvious that sorting of white blood cells was an opportunity not to be missed. The history of the development of cell sorting is well covered by Shapiro.^[9]

Fluidic Systems

Reynolds formulated the relationship for fluid flow as $Re = vdp/\eta$ where Re is the Reynolds number (a dimensionless number), v the average velocity, d the tube diameter, ρ the fluid density, and η a velocity coefficient. Below a Reynolds number of 2300, flow will be laminar, a necessary factor for quality optical measurements in flow cytometry. Maintenance of non-turbulent flow requires careful design of fluidic systems in flow cytometers, particularly the flow cell components. Cells are hydrodynamically focused in a core stream encased within a sheath (Fig. 1). This sheath-flow principle was derived from the work of Moldavan and subsequently Crosland-Taylor,^[10] who designed a system similar to most used today in which an insertion rod (needle) deposits cells within a flowing stream of sheath fluid (usually water or saline), creating a coaxial flow that moves from a larger to a smaller orifice, creating a parabolic velocity profile with a maximum at the center of the profile. The general design of such a system is shown in Fig. 1. Because of the hydrodynamic focusing effect, cells that are injected through the injection tube remain in the center of the core fluid, thus allowing very accurate excitation with subsequent excellent sensitivity and precision of measurement within the flowing stream. There is a small differential pressure between the sheath and the sample (which is the core); the sample is 1 to 2

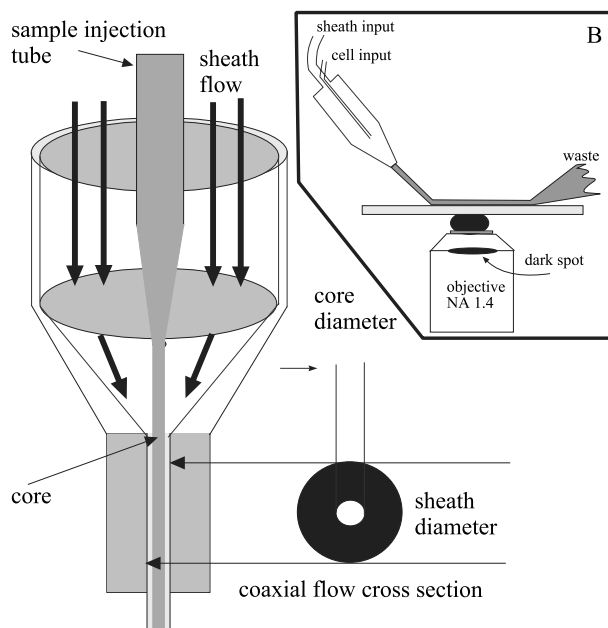


Fig. 1 Shown here is the basic structure of a typical flow cell. Sheath fluid flows through a large area and under pressure is forced into a much smaller orifice. In the center of the cell is an injection tube that injects cells or particles into the center of the flowing stream, forcing the cells to undergo hydrodynamic focusing, which will result in laminar flow if Reynolds number does not exceed 2300. Shown is the coaxial cross section of the sheath and core. B shows an alternative flow cell, an axial flow system typically used in microscope-based flow cytometers. In this system the laminar stream flows across a coverglass to a waste collector on the opposite side.

PSI above the sheath, forcing alignment of cells in single file throughout the core. If the pressure is increased too much, the core diameter will increase, destabilizing the flowing cells and reducing the accuracy and precision of the measurement. If a highly accurate system is required, multiple sheaths can be used to create very stable flow streams, but this is generally not used in commercial systems.

A crucial component of the flow cytometer is the design of the flow cell, in which the fluid flows from a very large area to a very constrained channel. The velocity, which is proportional to the square of the ratio of the larger to smaller diameter, increases significantly within the smaller channel. Within this channel, the velocity profile is parabolic with a maximum at the center of the stream and almost zero at the walls of the vessel. This becomes a crucial issue in flow cytometry when biological specimens are used because these samples contain proteins, and surface binding will eventually increase turbidity and destroy the hydrodynamic nature of the flow. While Reynolds number remains less than 2300, laminar



flow occurs. The acceleration at the core of the vessel is an important aspect of flow cytometers. Since particles are injected into the center of the flowing stream, the highly accelerated central core creates spatial separation of particles within this rather long core stream. This separation creates the ability to analyze the signals from single cells more accurately. Once particles are accurately identified and are spatially separated within the core, it is possible to separate them physically in a process known as particle sorting (discussed later). An alternative system to the one described earlier uses axial flow where cells are shot onto the surface of a microscope objective with a regular nozzle to obtain laminar flow, flow across the objective in a laminar flow, and are extracted from the system on the other side of the objective. This is shown in Fig. 1B and is similar to systems designed by Harald Steen and others. There are several advantages of this system, such as high numerical aperture microscope objectives providing excellent resolution and signal to noise, and the ability to use a regular arc lamp for the light source. This system has extraordinary sensitivity for forward scatter and is the most sensitive system available. It was initially designed to be optimized for very small particles such as microorganisms.

Optical Systems

Most flow cytometers use lasers as excitation sources. In the earliest systems, mercury lamps were used; however, in the late 1960s relatively large water-cooled ion lasers were identified as the most desirable source of coherent light at 488 nm, the best excitation wavelength for fluorescein. These high-cost, large, and inefficient light sources shaped the design of the instruments themselves, making them enormous constructs often taking 60 to 80 sq. feet of floor space and requiring high volume cooling water and high current levels. More recently, however, with the advent of solid-state lasers, the footprint of flow cytometers has been significantly reduced. Further, in the mid-1980s, there was an emerging market for flow cytometers that did not sort. These instruments were known as analyzers and are now commonly referred to as benchtop instruments. This is somewhat of a misnomer, as the third generation of sorters is almost indistinguishable from the benchtop analyzers of the past.

As already indicated, the key to the efficiency and sensitivity of current flow cytometers is the laser-based coherent light source. The chief criterion for selection of a laser is the excitation wavelength. The beam should be

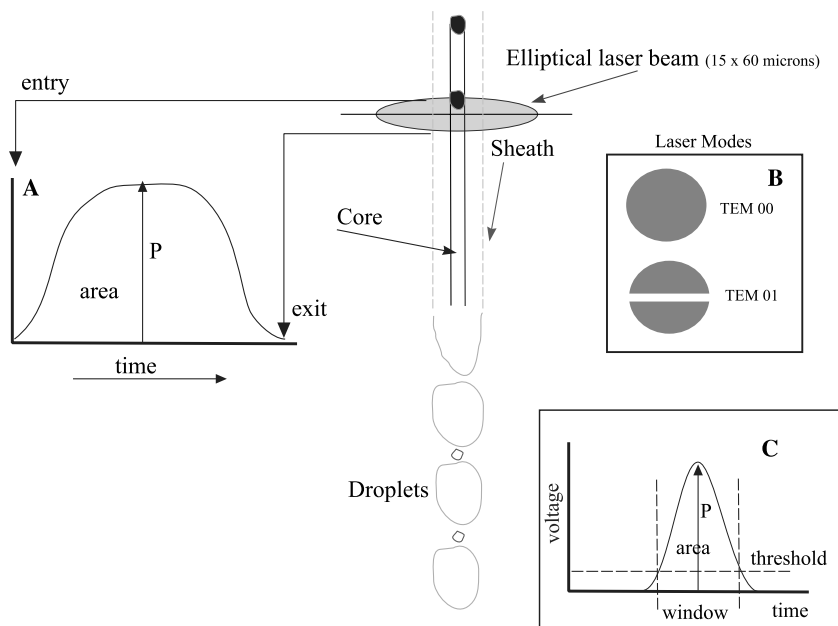


Fig. 2 As cells pass through the interrogation point, they create a pulse that can be characterized as shown above. At the point of entry into the laser beam, the pulse rises to a peak and holds for as long as the cell is in the stream. Once the cell begins to leave the laser beam profile, the signal returns to zero. The maximum signal is the peak, and the time taken for entry and exit of the beam is the time of flight (TOF). It is common to measure the total area under the curve (integral signal) for total fluorescence. Shown in B are the beam profiles most commonly used in flow cytometry. Most desirable is TEM 00; however, it is possible to mix the TEM 00 and the TEM 01 modes. In C are shown the definitions of each component of the signal from a cell passing through an elliptical beam. (View this art in color at www.dekker.com.)



segmented in a transverse emission mode (TEM) of TEM 00, although in some circumstances a mixed TEM 00 and TEM 01 mode does not preclude the usefulness of such a beam mode (Fig. 2A). The excitation source must match the absorption spectra of the fluorochromes of interest. One reason that early systems used large water-cooled argon-ion lasers was that multiple lines could be obtained from these lasers. The argon laser was selected, as it was the only coherent source of excitation satisfactory for the most used fluorochrome in the field—fluorescein. The argon-ion laser could produce lines in the UV (350 nm), deep blue (457 nm), blue (488 nm), and blue-green (514 nm) regions, making this a very useful light source. The light source needs to be focused to a spot and a desired shape. This is accomplished by using a beam-shaping optic to obtain the desired crossed-cylindrical beam shape. For reasons explained in Fig. 2, the most desirable beam shape is an elliptical beam of approximately 15 by 60 microns. This produces a beam with a large, relatively flat cross-section that reduces the variation in intensity of the excitation spot should the particle move around within the excitation area. Reducing the beam even further would have the effect of slit-scanning the traveling particle.

Electronic Systems

Flow cytometers collect a lot of data very quickly. In fact, they are in a class of instruments that push the limits of data collection. For example, it is currently possible to collect at least 11 fluorescent spectral bands simultaneously together with at least two scatter signals on thousands of cells per second, creating a multivariate analysis problem.^[11] The key principle of flow cytometry is that every particle is identified individually and classified into a category or population member according to multivariate analysis solutions. Every particle that passes the interrogation point would be collected on every detector, which would cause a serious overload collection problem. To solve this, a circuit is included called a discriminator, which can be set to exclude signals lower than a preset voltage (Fig. 2C). On many current instruments it is possible to use discriminators on any or all detectors. That is to say, multiple detectors must register a preset signal level or nothing is collected by the data collection system. Once a discriminator setting is satisfied, this detector triggers the entire data collection system and all identified detectors will measure the signal. Frequently, for particles of bacteria to most animal cell size (1–20 microns), a forward-angle light-scatter signal is used to discriminate the presence of a measurable particle. However, it is also useful to use a fluorescence detector if one wishes to detect only particles of a certain level of fluorescence.

The most frequently recognizable detection system in flow cytometers is that of fluorescence. The initial detection system used in the earliest instruments was Coulter volume, based on the original patent of Wallace Coulter,^[12] whereby the principle of impedance changes was transferred from cell-counting instruments to flow cytometers. In addition to impedance, light scatter was also measured. Current systems have taken a rather complex pathway for the measurement of fluorescence.

Linear amplifiers produce signals that are proportional to their inputs, and while it is possible to amplify this signal, most immunofluorescence applications have huge dynamic ranges that are beyond amplification in the linear domain. For this reason, logarithmic amplifiers with scales covering three to five decades are required. This is particularly useful for samples in which some cells exhibit very small amounts of signal, while others have signals four orders of magnitude larger.

Detectors

It has become standard design to utilize a PMT for each spectral wavelength desired. In most pre-1990 instruments, a maximum of four or five spectral bands was collected. However, beginning in the last decade of the 20th century, it became evident that 5–10 spectral signatures were desirable. Each spectral band is collected by a PMT strategically placed within an optical system of which there are many current designs. Figure 4 shows several different optical layouts currently used in commercial systems. It is now evident that the biological requirements are in the range of 10–15 spectral bands. Next-generation systems will include either a vast number of PMTs, avalanche photodiodes, or multichannel PMTs, in addition to high-speed cameras. The disadvantages of the multichannel PMT is that detection sensitivity is reduced and it is not currently possible to adjust the sensitivity of each channel as can be achieved with individual PMTs. The advantage is that the complexity and number of optical components are reduced.

Most cytometers use photomultiplier tubes (PMTs) as detectors for both fluorescence and scatter. The pulse of a particle crossing the excitation beam will depend upon the beam shape, beam intensity, and particle size, as well as the velocity of the particle. Systems running at 10 m/s will cross a 10-micron beam in 1 microsecond, or a 5-micron beam in only 500 ns. The majority of instruments prior to publication of this article were designed around analog detection, rather than digital electronics. Essentially, once the threshold voltage is met (based on the discriminator circuit described earlier) the signal (usually 0–10 volts) is fed into an analog-to-digital converter (ADC) circuit, called a comparator circuit, whose purpose is to identify and signal the presence of a measurable signal that is used



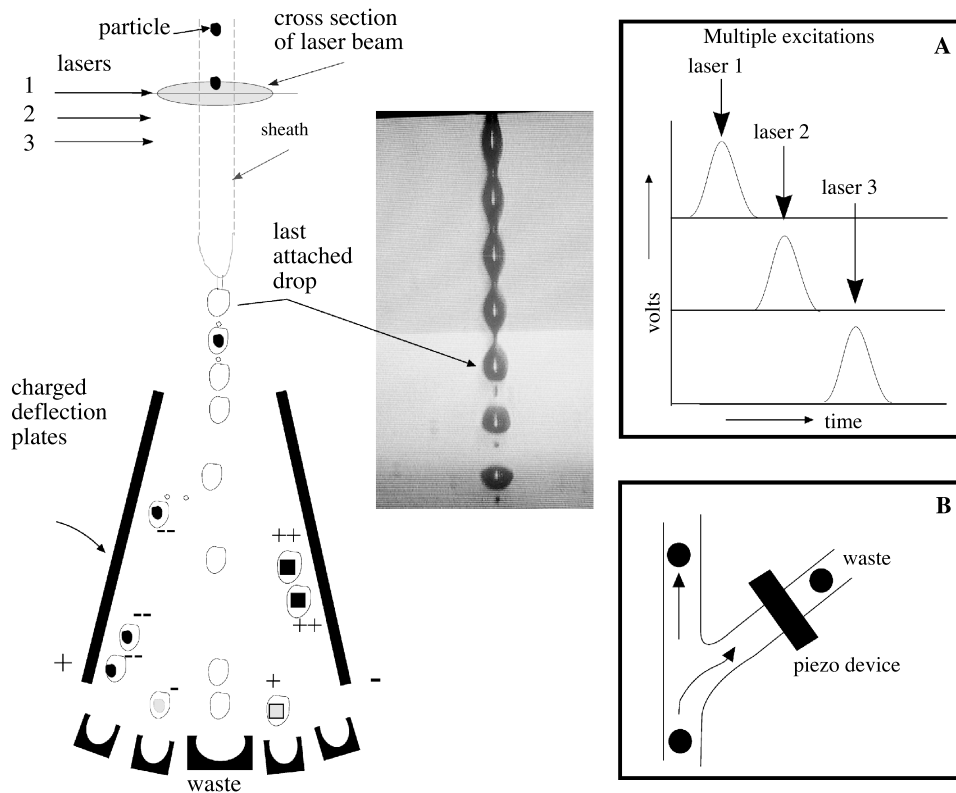


Fig. 3 This figure shows the principle of electrostatic cell sorting based on Sweet's inkjet printer technology. In this figure, a stream of liquid intersects a laser beam (or multiple laser beams 1, 2, 3). The stream is vibrated by a piezo-electric crystal oscillator at frequencies from 10,000 to 300,000 Hz depending upon the orifice size, stream velocity, nature of the stream, and particle size. Typically 30–50,000 Hz is used to create droplets at the same frequency. Once a cell/particle is identified as desirable, a charge is placed on the stream that remains with the last drop (last attached drop) that leaves the stream. Using a computation method, this drop is sorted by being attracted toward a plate almost parallel with the stream and containing opposite charges in the vicinity of 5000 volts. Each droplet containing a desirable particle can be placed into one of several containers (shown is a four-way sorting system). In the center of the figure is a video image of the droplets strobed at the same frequency as the droplet formation. A shows the pulses of 3 different lasers as a particle passes by each beam separated in space. Thus, a particle will pulse from each laser a few microseconds apart. This way, signals from each laser can be individually analyzed. B is an alternative sorting system using fluid switching techniques. In this system the waste stream is blocked momentarily to allow a desired cell to pass into the sorting pathway. (*View this art in color at www.dekker.com.*)

to trigger the rest of the detection systems. This is a binary decision only. Once a decision to collect is made, several measurements for each variable are made, such as peak, integral, and time-of-flight. There are several complications that can cause problems in the detection electronics. For example, if two particles pass the interrogation point at very close intervals, both signals must be aborted if this time is shorter than the reset time for the electronics. Another circuit is required to make this decision.

To further complicate the electronics, many systems use two or more laser beams delayed by a few microseconds. Each particle must be correctly analyzed by each laser, so data from the first beam must be stored while waiting for the same particle to pass the second beam, and so on. If the beam separation is large enough, several cells might be analyzed by the first beam before the first cell passes the

second beam. This rather complex system is not necessary on simpler analysis systems, but it is absolutely necessary on more advanced multilaser cell sorters. In addition, the time taken for all the analysis components is finite, which essentially sets the maximum analysis rate of the flow cytometer. The faster the system, the shorter the dead time must be. For example, to analyze 100,000 cells per second, a dead time of less than 10 microseconds would be necessary. In fact, depending on how many events must actually be analyzed to have 100,000 cells per second, the dead time would need to be considerably shorter.

Spectral Compensation

When a particle or cell contains fluorophores of multiple spectral bands, the identification and analysis become



considerably more complex. For example, a detector with a band pass filter designed to collect fluorescence from FITC (525 nm) and another detector designed to collect signals at 550 nm (PE) will register photons in both detectors. It is impossible to determine which detector is detecting the real photons from FITC. This is not a problem if a single fluorophore is being collected, but when two or more fluorophores with close emission bands are present, it is necessary to identify which fluorophore was the real emitter of the photons. To achieve this, it is necessary to perform spectral compensation whereby a percentage of signal from one detector is subtracted from the other. As the number of fluorophores increases, so too does the complexity of the spectral compensation. A complex set of circuits must be designed that allows for a percentage of each signal to be subtracted from every other detector. Naturally, there are some instances where there is no overlap, but with six or seven detectors competing for signals from the narrow spectral emission range available from a single excitation source, it is absolutely necessary to compensate for spectral overlap. While this can be performed perfectly well in software off-line,^[13] if the goal of the analysis is to sort a certain population of cells physically, the compensation must be performed in real time between the time the cell passes the excitation beam and when the cell reaches the last time available for a sort or abort decision to be made. Compensation in flow cytometry is very complex and requires a large number of controls to establish appropriate compensations setting and photomultiplier voltages. As fluorescent dyes increase in number and spectral proximity, the need for complex spectral compensation circuitry also increases. This is far more complex than anything currently available in image analysis systems.

Cell Sorting

The principle of cell sorting was included in instruments designed by Fulwyler,^[8] Kamensky,^[6] and also Ditrach^[14] in order to analyze a cell of interest definitively. It was Fulwyler, however, who identified the technique developed by Richard Sweet^[7] for electrostatic droplet separation for use in high-speed inkjet printers as the ideal technology for cell sorting. This evolved into the technique of choice for virtually all commercial cell sorters. This is shown in Fig. 2 and also Fig. 3. This idea was implemented into a commercial system by Herzenberg's group^[15] in the early 1970s. As already noted, the initial reason for Fulwyler's implementation was the desire to separate what were apparently two distinct populations of red blood cells that appeared on analysis based on Coulter volume measurements. The principle of electrostatic sorting is based on the ability to first iden-

tify a cell of interest based on measured signals, identify its physical position with a high degree of accuracy, place a charge on the stream at exactly the right time, and then physically collect the sorted cell into a vessel.

The technology of high-speed sorting has been recently well defined by van den Engh,^[16] who discusses in detail the complex issues. In brief, the speed and accuracy of a cell sorter are based on a number of factors. Firstly, despite the initial discussion pointing out that a fully stable laminar flow is required for accurate analysis, for cell sorting the stream must be vibrated by a piezoelectric device to generate droplets. As described by van den Engh, it is necessary to have high-speed electronics and to match the nozzle diameter, sheath pressure, and droplet generation frequency to obtain stable droplet generation and thus high-speed cell sorting. The principle that governs the generation of droplets has been characterized by Kachel;^[17] the wavelength of the undulations is $\lambda = v/f$, where λ = the undulation wavelength, v = the stream velocity, and f = the modulation frequency.

When $\lambda = 4.5d$ (d = exit orifice = stream diameter), the system is optimized for maximum droplet generation. Thus, the optimal generation frequency is given by $f = v/4.5d$. If a system is designed to accommodate this optimal droplet formation, as demonstrated by Pinkel,^[18] the jet velocity is proportional to the square root of the jet pressure. Thus, an optimal system to sort events at 20,000 Hz such that drops are separated by 4.5 stream diameters and flowing at 10 m/s would make each drop 200 microns apart. As the number of sorted drops increases, the diameter must decrease, with the obvious conclusion that the speed of high-speed sorters will eventually be partially regulated by the size of the particle to be sorted and the velocity that the stream can achieve without destroying the sample. This is particularly important for biological particles such as cells.

High-speed sorters are essentially sorters that are designed to operate at sort speeds in excess of 20,000 particles per second. To accomplish this, higher pressures must be placed on the sample stream. When systems exceed 40,000 cells per second, the key issue becomes analysis time—obviously the limiting factor since complex analysis must precede a sorting decision. The maximum speed of droplet formation is therefore not the limiting factor in design of a high-speed flow cytometer. As discussed in van den Engh,^[16] the primary issue is the high pressures that must be used to create very high-speed droplet formation. At droplet frequencies of 250,000/second, the jet pressure must approach 500 PSI, a significantly higher value than can be designed safely in most systems. Thus, if pressures are limited to around 100 PSI, a droplet rate of around 100,000 is closer to the realistic range. This then is the real limitation to current high-speed sorting systems.



Poisson statistics enter the equation at this time as well since it is impossible to predict exactly when any particle is going to pass the interrogation point. This adds uncertainty in the analysis and, as discussed previously, it is crucial to ensure that no measurements take place as two cells try to pass through the interrogation point at or near the same time. Thus there is a relationship between particle concentration and coincidence detection.

Cell sorting has become a very important component of flow cytometry. In particular, the isolation of CD34 human hematopoietic stem cells by flow sorting specifically for transplantation purposes has revolutionized capabilities in transplantation.^[19] Naturally, to perform such a sort, all components of the instrument that come in contact with the cells must be sterile.

Another issue that relates to sorting is the potential dangers involved in sorting certain samples, particularly human samples that may be infected with AIDS, or more commonly, hepatitis virus. This is an area that can cause considerable tension between operators and researchers wanting to sort materials from infected patients. Because aerosols are generated in the normal operation of a flow cytometer, complex biosafety systems must be employed to reduce the potential of infection. There is a significant literature on the dangers posed by both microbes and carcinogenic molecules such as fluorescent dyes that are used to label many cells.^[20]

APPLICATIONS

Clinical Sciences

One of the largest applications of flow cytometry is in the clinical sciences, where the primary measurements are of fluorochrome-conjugated antibodies bound to cellular receptors. This is generally referred to as immunophenotyping since many of the cell types being studied are immune cells such as lymphocytes. In fact, almost every possible human cell has been evaluated by flow cytometry. By far the most significant cell populations are peripheral blood cells such as red blood cells (RBC), white blood cells (lymphocytes, monocytes, and polymorphonuclear leukocytes), and platelets. Each of these populations presents some specific challenge in assay performance, but overall these cells are very amenable to flow analysis. A complex system of receptor identification has been developed within immunology to identify cellular receptors, which are referred to as Cluster of Differentiation (CD) antigens of which at the time of writing there were 166 such classifications. These are based on similarity of antibody binding to specific receptors. Therefore, by conjugating fluorescent molecules to antibodies that recognize specific receptors, a population of

cells binding that antibody and therefore that fluorescent molecule can be identified. With certain clinical syndromes, it is evident that a specific pattern will emerge when identifying which cells bind to certain antibodies. One of the most significant findings in the early 1980s was that the identification of certain subsets of human T cells was important for the monitoring of the clinical status of AIDS patients^[21] (Fig. 5A and B). This significantly increased the utility of flow cytometry and drove the need for simple-to-operate, reliable clinical benchtop analyzers for basic two- and three-color immunofluorescence. These instruments now represent the great majority of flow cytometers in the field.

Cell biology

Some of the earliest studies of cell function investigated neutrophil function by measuring phagocytosis of microorganisms.^[22] This is an excellent example of the value of flow cytometry, which can identify individual cells by their size, structure, or specific identifiers such as cell receptors, and simultaneously measure the nature and number of microorganisms that were internalized via the process of phagocytosis. There are many applications of flow cytometry used for studying unique properties of cells that cannot easily be studied with any other technology. For example, real-time, single-cell production of oxygen radicals is frequently evaluated by using flow cytometry. There are a number of well accepted techniques from the earliest studies^[23,24] to more recent ones whereby both cells and organelles have been studied by flow cytometry.^[25,26] A huge number of applications exist in the field of DNA ploidy research (Fig. 5E). The ability to identify the rate of cell division and to monitor the effect of various therapeutic drugs is of great interest. Studies of the cell cycle by flow cytometry have provided a great deal of information on the nature of cell division and more recently apoptosis.^[27-29]

Microbiology

The study of microbes and their behavior is ideally suited to flow cytometry;^[30] however, there is an apparent disconnect between the capability of flow cytometry to answer microbial-related questions and its use in the field. Early studies quickly focused on the possibilities of developing flow-based assays for such time-consuming assays in the clinical environment as antibiotic resistance. With the growth of resistant organisms, determination of antibiotic resistance would be a desirable measure, but one that is rarely if ever performed outside the hospital environment. Even in the medical microbiology laboratory, it is still considered uneconomic, despite the clear demonstration that both



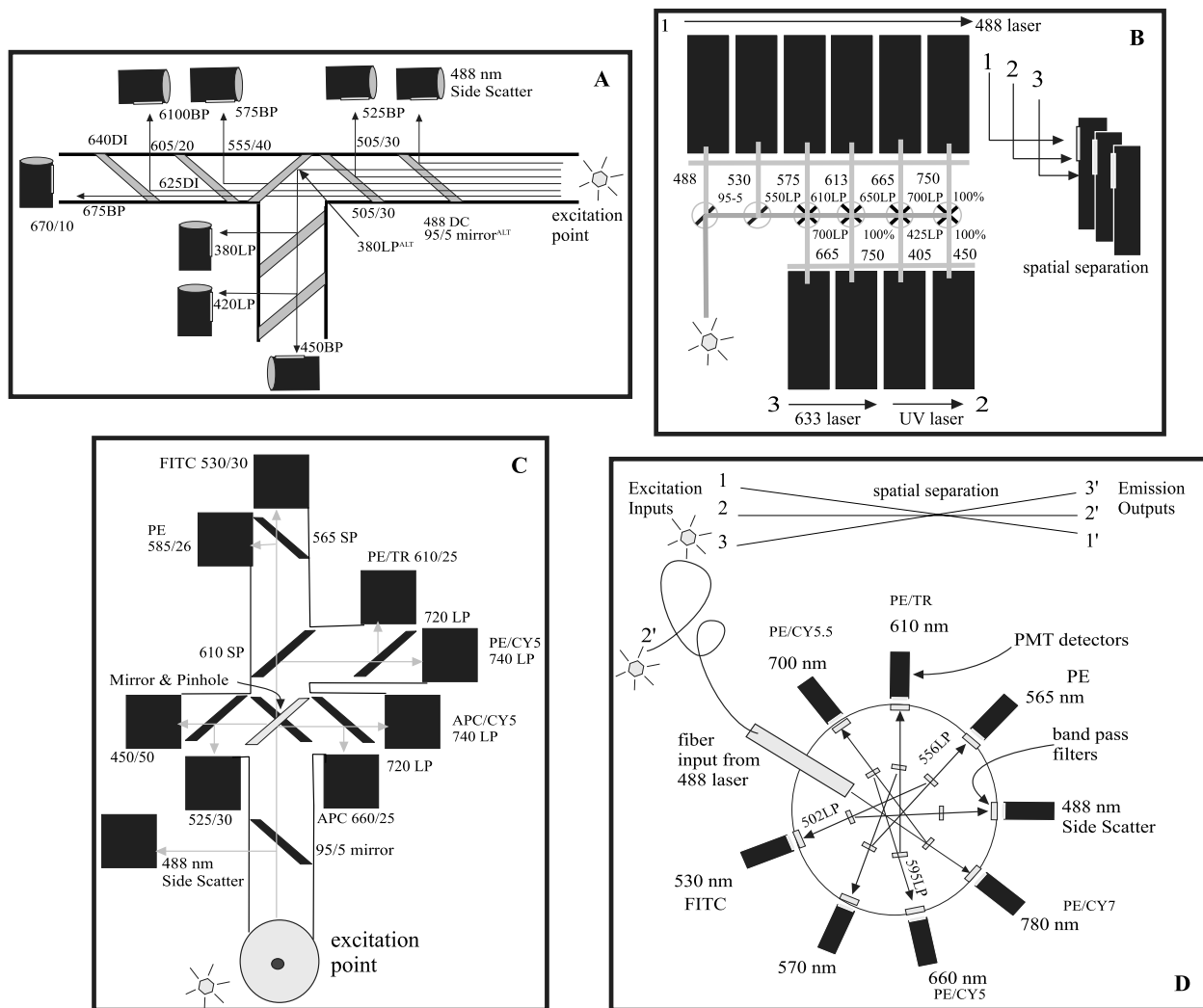


Fig. 4 A–D represent the optical tables of several commercial flow cytometers. A: Beckman-Coulter ALTRA showing the position of 8 PMTs; B shows the Dako-Cytomation CYAN instrument, which has 10 detectors placed in such a way that there are three beams with slightly different trajectories; C shows the Becton-Dickinson Vantage system in a typical configuration showing nine detectors; D is the more recent Becton-Dickinson ARIA system using an innovative PMT array with eight PMTs in a ring, which allows the emission signal to bounce around the ring. There are an additional six detectors on this system (not shown) that come from the first and third lasers (see diagram). In all cases A–D above, each PMT has a narrow bandpass filter immediately in front of the PMT in addition to the dichroic mirrors that are used to direct the various emission spectra.

organism identification and antibiotic sensitivity can be determined within a couple of hours. Unfortunately, the current cost far exceeds the pennies-per-test conditions set by current medical practices. This is definitely one of the potentials for microbiology-specific instruments that should markedly reduce testing costs for such applications (Fig. 5D).

Many studies of microbial kinetics have been performed using flow cytometry, including growth curves, reproduction studies, and metabolic requirements. In addition, exciting new studies are beginning to demon-

strate new opportunities of flow cytometry together with advanced imaging tools for studying growth of microorganisms in complex 3-D environments such as biofilms.^[31]

Plant and Animal Science

Although a great majority of flow cytometry is related to human and laboratory animal systems, there are some excellent examples of studies of plant systems. For example, it was recognized very early in the use of flow

Copyright © Marcel Dekker, Inc. All rights reserved.



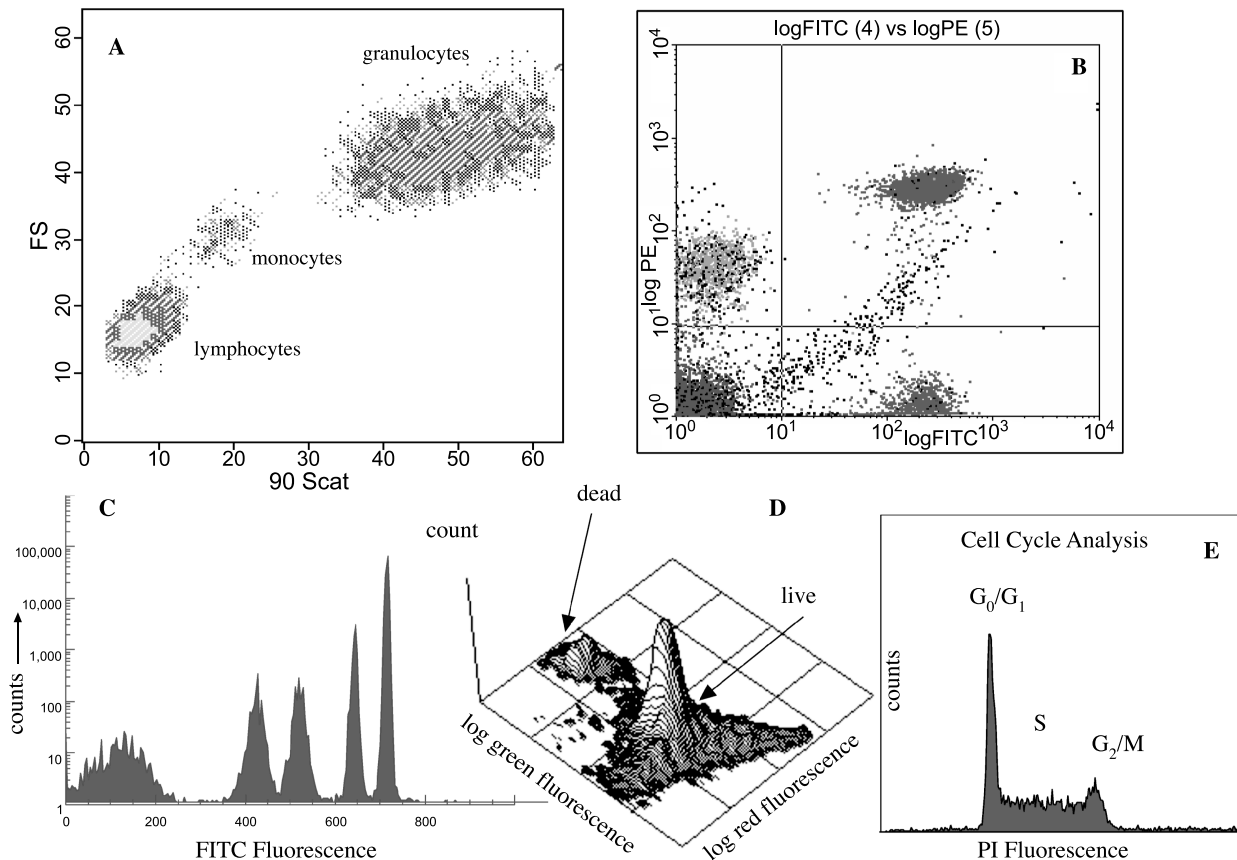


Fig. 5 A. When a cell passes through a laser beam, it scatters light. That light is measured on a detector and the resulting signal can provide information about the cells. Forward angle scatter (FS) is a measure of cell size. Side scatter (90° scat) is a measure of cellular components or granularity. In this dotplot of forward-versus-side scatter, human white blood cells can be differentiated without any other probes. Here is shown the separation of lymphocytes, monocytes, and granulocytes. B. Gating strategies allow identification of populations of cells such as lymphocytes, shown in (A), the fluorescence emission of conjugated antibodies can be further separated to divide the lymphocytes into four distinct populations. In two-parameter space, the populations can easily be divided into four populations: those cells that are double negative, double positive, and single positive for each color. C. Calibration beads with fluorescent molecules attached to their surface are used to create quantitative measures for flow cytometry. This histogram has five peaks, the lowest peak being negative cells and the other four peaks represent four levels of fluorescence. From this histogram a standard curve can be obtained for quantitation of particles being labeled with this probe. D. This isometric display shows a plot of bacteria as observed by flow cytometry. *Pseudomonas aeruginosa* is broth treated with $10 \times$ MIC of the antibiotic Imipenem for two hours and stained with BacLight Live/Dead kit. The log green fluorescence is Syto 9 and the log red fluorescence is PI. Positive PI fluorescence represents damage to the cell membrane, an indication of cell death. E. Propidium Iodide (PI) can also be used to study the cell cycle. In this case, the membrane is slightly damaged to allow penetration of the dye. PI binds to DNA in a stoichiometric manner such that there is a direct relationship between DNA content and PI fluorescence. (View this art in color at www.dekker.com.)

cytometry that cell cycle could be easily analyzed by flow cytometry,^[32] and this stimulated a number of cytometry-related plant-based studies.^[33] Pollen, for example, is perfectly suited to flow cytometry, as are plant chromosomes, even though they are somewhat more difficult to extract. A number of flow sorting experiments performed on plant systems to identify gene expression from transgenic tobacco plants^[34] demonstrated the efficacy of using this technology.

Pharmaceuticals

One of the more recent applications of flow cytometry is high-throughput screening. While there are many technologies that have far greater sample throughput, flow cytometry is one of the few technologies that can identify and analyze individual cells in multiple parameters. Recently, the concept of high-throughput cytometry was introduced, and initial reports suggest the possibility of



Flow Cytometry

achieving as many as 100,000 samples per day,^[35] something that approaches the needs of pharmaceutical manufacturers. The clear advantage of flow cytometry over other technologies, such as imaging cell-culture plates, is that with flow cytometry, a large number of parameters can be analyzed on each and every cell. The disadvantage is that flow cytometry, even with high-speed systems, is very much slower than automated image-processing systems.

Reproductive Medicine

Sperm analysis has proved the value of flow cytometry and especially the cell sorting capacity. There are several approaches to analysis of sperm. One utilizes the ability of DNA dyes such as Hoechst 33342 to bind to sperm DNA without inflicting damage;^[36] another uses antibodies to the H-Y antigen.^[37] The ability of flow cytometry to sort human sperm for sex-selection raises a number of ethical questions. It is clearly well within the means of this technology to sex-select human sperm; although to date there are no published reports of this having been done, the topic is heavily discussed.^[38]

Calibration Issues

Because flow cytometry is defined as a quantitative technology, it is important to have calibration standards. These were primarily developed by Schwartz^[39] and others to allow reproducibility of clinical assays. Schwartz developed the concept of Molecule Equivalents of Soluble Fluorescein (MESF units). Using a mixture of beads with known numbers of fluorescent molecules, it is possible to create a standard curve based on a least-squares regression based on the median fluorescence intensity of each bead population. This value is then converted into MESFs (Fig. 5C) from which comparisons can be made from different instruments or the same instrument on different days. Future instruments will most likely provide data in units such as MESFs rather than "arbitrary fluorescence values," as are frequently observed in present-day publications. It would seem highly desirable to provide more quantitative data for comparison purposes.

CONCLUSIONS

The technology of flow cytometry has made a significant impact on many fields. There are few technologies that can evaluate so many parameters on such small samples in such short time periods. The principle of evaluating each and every cell or particle that passes through the laser beam and then producing a highly correlated data set is

unique to flow cytometry. The combination with multivariate analysis and subsequent ability to separate cells physically by the process of cell sorting gives this technology some unique characteristics. It has been almost 40 years since flow cytometry first demonstrated its importance in medical research. Since that time, well over 60,000 publications have highlighted its usefulness. It was identified as one of the most important technologies in the early 1980s upon the recognition of AIDS. The ability of flow cytometry to identify and quantify the T cell population subsets CD4 and CD8 lymphocytes identified it as a most important technology in the diagnosis and monitoring of AIDS patients. Similarly, the ability of flow cytometry to make complex multivariate analyses of bone marrow to identify the CD34+ cells and subsequently sort and purify them has been a vital resource in transplantation immunology.

ARTICLES OF FURTHER INTEREST

Hematopoietic Stem Cells and Assays, p. 746

Optics, Biomedical, p. 1143

REFERENCES

1. Shapiro, H.M. Multistation multiparameter flow cytometry: A critical review and rationale. *Cytometry* **1983**, *3*, 227–243.
2. Robinson, J.P.; Durack, G.; Kelley, S. An innovation in flow cytometry data collection and analysis producing a correlated multiple sample analysis in a single file. *Cytometry* **1991**, *12*, 82–90.
3. Moldavan, A. Photo-electric technique for the counting of microscopical cells. *Science* **1934**, *80*, 188–189.
4. Papanicolaou, G.N.; Traut, R. The diagnostic value of vaginal smears in carcinoma of the uterus. *Am. J. Obstet. Gynecol.* **1941**, *42*, 193.
5. Kametsky, L.A.; Melamed, M.R.; Derman, H. Spectrophotometer: New instrument for ultrarapid cell analysis. *Science* **1965**, *150*, 630–631.
6. Kametsky, L.A.; Melamed, M.R. Spectrophotometric cell sorter. *Science* **1967**, *156*, 1364–1365.
7. Sweet, R.G. High frequency recording with electrostatically deflected ink jets. *Rev. Sci. Instrum.* **1965**, *36*, 131–136.
8. Fulwyler, M.J. Electronic separation of biological cells by volume. *Science* **1965**, *150*, 910–911.
9. Shapiro, H.M. *Practical Flow Cytometry*, 3rd Ed.; Wiley-Liss: New York, NY, 1994.
10. Crosland-Taylor, P.J. A device for counting small particles suspended in fluid through a tube. *Nature* **1953**, *171*, 37–38.
11. De Rosa, S.C.; Roederer, M. Eleven-color flow cytometry.



- A powerful tool for elucidation of the complex immune system. *Clin. Lab. Med.* **2001**, *21*, pp. vii, 697–712.
12. Coulter, W.H. High speed automatic blood cell counter and cell size analyzer. *Proc. Natl. Electron. Conf.* **1956**, *12*, 1034–1042.
 13. Bagwell, C.B.; Adams, E.G. Fluorescence spectral overlap compensation for any number of flow cytometry parameters. *Ann. N.Y. Acad. Sci.* **1993**, *677*, 167–184.
 14. Dittrich, W.; Gohde, W. Impulsfluorometrie bei Einzelzellen in Suspensionen. *Z. Naturforsch.* **1969**, *24b*, 360.
 15. Bonner, W.A.; Hulett, H.R.; Sweet, R.G.; Herzenberg, L.A. Fluorescence activated cell sorting. *Rev. Sci. Instrum.* **1972**, *43*, 404–409.
 16. van den Engh, G. High Speed Cell Sorting. In *Emerging Tools for Single-Cell Analysis*; Durack, G., Robinson, J.P., Eds.; Wiley-Liss: New York, 2000; 21–48.
 17. Kachel, V.; Kordwig, E.; Glossner, E. Uniform lateral orientation of flat particles in flow through systems caused by flow forces. *J. Histochem. Cytochem.* **1977**, *25*, 774–780.
 18. Pinkel, D.; Stovel, R. Flow Chambers and Sample Handling. In *Flow Cytometry: Instrumentation and Data Analysis*; Dean, P.N., Ed.; Academic Press: New York, 1985.
 19. Andrews, R.G.; Singer, R.G.; Bernstein, I.D. Precursors of colony forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. *J. Exp. Med.* **1989**, *169*, 1721–1731.
 20. Nicholson, J.K.A. Immunophenotyping specimens from HIV-infected persons: Laboratory guidelines from the Centers for Disease Control and Prevention. *Cytometry* **1994**, *18*, 55–59.
 21. Fahey, J.L.; Giorgi, J.; Martínez-Maza, O.; Detels, R.; Mitsuyasu, R.; Taylor, J. Immune pathogenesis of AIDS and related syndromes. *Ann. Inst. Pasteur., Immunol.* **1987**, *138*, 245–252.
 22. Bassoe, C.-F.; Solsvik, J.; Laerum, O.D. Quantitation of Single Cell Phagocytic Capacity by Flow Cytometry. In *Flow Cytometry IV*; Laerum, O.D., Lindmo, T., Thorud, E., Eds.; Universitetsforlaget: Oslo, 1980; 170.
 23. Bass, D.A.; Parce, J.W.; DeChatelet, L.R.; Szejda, P.; Seeds, M.C.; Thomas, M. Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. *J. Immunol.* **1983**, *130*, 1910–1917.
 24. Rothe, G.; Valet, G. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *J. Leukoc. Biol.* **1990**, *47*, 440–448.
 25. Robinson, J.P.; Carter, W.O.; Narayanan, P.K. Oxidative Product Formation Analysis by Flow Cytometry. In *Methods in Cell Biology: Flow Cytometry*; Darzynkiewicz, Z., Robinson, J.P., Crissman, H.A., Eds.; Academic Press: New York, 1994; 437–447.
 26. Li, N.; Ragheb, K.; Lawler, G.; Sturgis, J.; Rajwa, B.; Melendez, J.A.; Robinson, J.P. DPI induces mitochondrial superoxide-mediated apoptosis. *Free Radic. Biol. Med.* **2003**, *34*, 465–477.
 27. Crissman, H.A.; Tobey, R.A. Cell-cycle analysis in 20 minutes. *Science* **1974**, *184*, 1297–1298.
 28. Darzynkiewicz, Z.; Traganos, F.; Sharpless, T.K.; Melamed, M.R. Cell cycle-related changes in nuclear chromatin of stimulated lymphocytes as measured by flow cytometry. *Cancer Res.* **1977**, *37*, 4635–4640.
 29. Ormerod, M.G. Investigating the relationship between the cell cycle and apoptosis using flow cytometry. *J. Immunol. Methods* **2002**, *265*, 73–80.
 30. Steen, H.B. Flow cytometry of bacteria: Glimpses from the past with a view to the future. *J. Microbiol. Methods* **2000**, *42*, 65–74.
 31. Sincock, S.A.; Robinson, J.P. Flow cytometric analysis of microorganisms. *Methods Cell Biol.* **2001**, *64*, 511–537.
 32. Galbraith, D.W.; Harkins, K.R.; Maddox, J.M.; Ayres, N.M.; Sharma, D.P.; Firoozabady, E. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* **1983**, *220*, 1049–1051.
 33. Brown, S.C.; Bergounioux, C. Plant Flow Cytometry. In *Flow Cytometry: Advanced Research and Clinical Application*; Yen, A., Ed.; CRC Press: Boca Raton, 1990; 195–212.
 34. Harkins, K.R.; Jefferson, R.A.; Kavanagh, T.A.; Bevan, M.W.; Galbraith, D.W. Expression of photosynthesis-related gene fusions is restricted by cell type in transgenic plants and in transfected protoplasts. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87*, 816–820.
 35. Kuckuck, F.W.; Edwards, B.S.; Sklar, L.A. High throughput flow cytometry. *Cytometry* **2001**, *44*, 83–90.
 36. Libbus, B.L.; Perreault, S.D.; Johnson, L.A.; Pinkel, D. Incidence of chromosome aberrations in mammalian sperm stained with Hoechst 33342 and UV-laser irradiated during flow sorting. *Mutat. Res.* **1987**, *182*, 265–274.
 37. Peter, A.T.; Jones, P.P.; Robinson, J.P. Fractionation of bovine spermatozoa for sex selection: A rapid immunomagnetic technique to remove spermatozoa that contain the H–Y antigen. *Theriogenology* **1993**, *40*, 1177–1185.
 38. Meseguer, M.; Garrido, N.; Remohi, J.; Simon, C.; Pellicer, A. Gender selection: Ethical, scientific, legal, and practical issues. *J. Assist. Reprod. Genet.* **2002**, *19*, 443–446.
 39. Schwartz, A.; Fernández-Repollet, E. Development of clinical standards for flow cytometry. *Ann. N.Y. Acad. Sci.* **1993**, *677*, 28–39.



Request Permission or Order Reprints Instantly!

Interested in copying and sharing this article? In most cases, U.S. Copyright Law requires that you get permission from the article's rightsholder before using copyrighted content.

All information and materials found in this article, including but not limited to text, trademarks, patents, logos, graphics and images (the "Materials"), are the copyrighted works and other forms of intellectual property of Marcel Dekker, Inc., or its licensors. All rights not expressly granted are reserved.

Get permission to lawfully reproduce and distribute the Materials or order reprints quickly and painlessly. Simply click on the "Request Permission/Order Reprints" link below and follow the instructions. Visit the [U.S. Copyright Office](#) for information on Fair Use limitations of U.S. copyright law. Please refer to The Association of American Publishers' (AAP) website for guidelines on [Fair Use in the Classroom](#).

The Materials are for your personal use only and cannot be reformatted, reposted, resold or distributed by electronic means or otherwise without permission from Marcel Dekker, Inc. Marcel Dekker, Inc. grants you the limited right to display the Materials only on your personal computer or personal wireless device, and to copy and download single copies of such Materials provided that any copyright, trademark or other notice appearing on such Materials is also retained by, displayed, copied or downloaded as part of the Materials and is not removed or obscured, and provided you do not edit, modify, alter or enhance the Materials. Please refer to our [Website User Agreement](#) for more details.

[Request Permission/Order Reprints](#)

Reprints of this article can also be ordered at

<http://www.dekker.com/servlet/product/DOI/101081EEBBE120013923>