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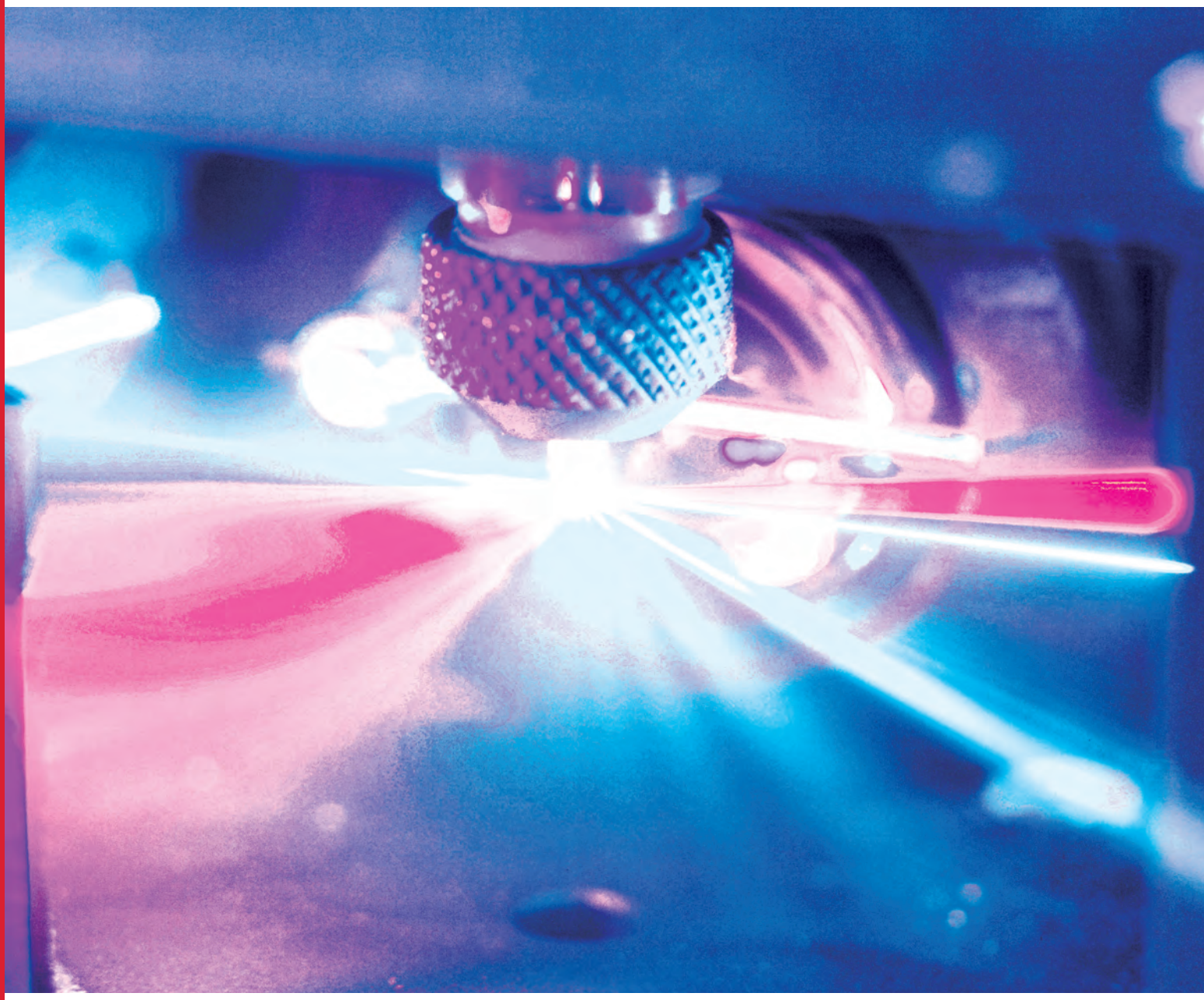
FLOW CYTOMETRY

Educational Guide | 2nd Edition



Guide to Flow Cytometry

EDITOR

Sonja Wulff
Dako ▪ Fort Collins, Colorado, USA

TECHNICAL ADVISORS

Kathleen Martin
Dako ▪ Fort Collins, Colorado, USA

Angela Vandergaw
Dako ▪ Fort Collins, Colorado, USA

CONTRIBUTORS

Thomas Boenisch, MS
Dako ▪ Carpinteria, California, USA

Ian Brotherick, PhD
Dako ▪ Ely, United Kingdom

Terry Hoy, PhD, FRCPath
University of Wales College of Medicine ▪ Cardiff, Wales, UK

Jim Hudson, PhD
Dako ▪ Carpinteria, California, USA

Christina Jespersgaard, PhD
Dako ▪ Glostrup, Denmark

Peter Lopez
Aaron Diamond AIDS Research Center ▪ New York City, New York, USA

Alberto Orfao, MD, PhD
Centro de Investigación del Cancer ▪ Salamanca, Spain

W. Roy Overton, PhD
Dako ▪ Fort Collins, Colorado, USA

Stephen W. Pursley
Dako ▪ Fort Collins, Colorado, USA

John Ransom, PhD
Novasite Pharmaceuticals ▪ San Diego, California, USA

John Sharpe, PhD
Dexcel Limited ▪ Hamilton, New Zealand

Carleton C. Stewart
Roswell Park Cancer Center ▪ Buffalo, New York, USA

Ian Storie
Dako ▪ Glostrup, Denmark

Lars Winther, PhD
Dako ▪ Glostrup, Denmark

Table of Contents

Table of Contents...continued

Foreword: An Introduction to Flow Cytometry

Sonja Wulff, Editor

Flow cytometry is a complex field that draws people from diverse scientific backgrounds. Whether you're an immunologist or aquatic ecologist, researcher or clinician, we hope this guidebook will help you in your journey of discovering the powerful technology and applications of flow cytometry.

Those of you who are already familiar with flow cytometry may want to skip directly to Chapter 1. However, for those of you who would like a little more background before jumping into the language of forward scatter, fluorochromes and FRET, keep reading:

Flow cytometry is the science of measuring physical and chemical properties of live cells or other biological particles as they pass in a fluid, single-cell stream through a measuring apparatus. In the most common scenario, one or more lasers interrogate each particle and, at a minimum, the system measures the degree and direction of scattered light — indicators of the particle's size, shape and structure. If particles have been stained with one or more fluorescent dyes — known as fluorochromes — the light source excites these dyes to provide additional biological information about each particle, such as metabolic activity, DNA content and the presence of specific surface and intracellular markers. Precise optical and electronic elements collect the fluorescent pulses and scattered light, convert them into digital values and send them to a computer for analysis. Some flow cytometers are also equipped to identify and sort user-specified particles into collection vessels. High-performance cell sorters can routinely reach rates of 70,000 cells per second.

The unique power of flow cytometers is that they can rapidly and quantitatively measure multiple simultaneous parameters on individual live cells and then isolate cells of interest. Additionally, the sensitivity and throughput rates achievable by high-performance commercial instruments enable detection of extremely rare populations and events (frequencies below 10^{-6}), such as stem cells, dendritic cells, antigen-specific T cells and genetic transfectants.[1] As a result, applications for flow cytometers continue to grow.

In addition to traditional immunology and pathology applications involving particles such as lymphocytes, macrophages, monocytes and tumor cells, flow cytometers are widely used in conjunction with fluorescence-based protein reporters, such as green fluorescent protein (GFP). In this arena, flow cytometers can monitor both transfection efficiency and protein expression levels.[2-3] They also can detect fluorescence resonance energy transfer (FRET), which provides information about molecular interactions, protein structure and DNA sequence.[4-5]

Interest is growing in the use of flow cytometers to screen cell- or bead-based combinatorial libraries.[6] Increasingly, flow cytometric assays are used to detect molecules that bind to a target protein in vitro or exhibit a particular activity in a cell-based assay. Flow cytometry also enables screening of protein libraries expressed

in cells or displayed on the surface of bacteria or beads. A flow cytometer, for instance, can detect modulation of a signal transduction pathway by a particular small molecule[7-8] and identify proteins with a particular binding specificity, enzymatic activity, expression level and stability.[9-12]

With any application, cells exhibiting desired activity profiles can be sorted into test tubes, multiwell plates or microscope slides. At purity rates greater than 99%, these sorted particles are then available for further studies such as PCR analysis or in situ hybridization,[13] and the cells are fully functional for long-term ex vivo culture, expansion, transplantation or other subsequent applications.[14-15]

Ongoing development efforts in the flow cytometry industry are aimed at automation and laboratory integration. Input/output robotics, pushbutton operation and automated sample preparation will increase throughput rates and make the technology more accessible to a wider user base, as new fluorescent dyes and creative screening approaches expand applications into the proteomic arena. Eventually, software advances will seamlessly network instruments into comprehensive analytical and diagnostic systems, and the industry may marry its technology with imaging and microfluidics.

Obviously, this is only a very superficial discussion of the field of flow cytometry. It is intended merely to set the stage for the rest of the publication, which will introduce you to more details about the technology and applications of flow cytometry. We, at Dako, are honored to be part of your exploration. Enjoy!

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Evolution of Flow Cytometry

Carleton C. Stewart

This book is designed to provide you with the modern applications of flow cytometry and how to perform them. As a preamble, one might wonder how we got to this stage of development. I am indebted to Howard Shapiro's fine interpretation of the historical material presented in his editions of Practical Flow Cytometry[1-2] and recommend them for further reading. Much of what is presented here is a summary of his work and my own experiences. I was fortunate to have trained and worked with many of the scientists and physicians who were a part of the development of modern flow cytometry.

The evolution of flow cytometry can be divided in four distinct phases – the development of the microscope, the development of dye chemistry, the development of electronics and the development of computers. All four of these fields had to reach sufficient maturity — coincident with a global biomedical need — to produce a prototype instrument.

The Microscope

In the 16th Century, Leeuwenhoek is credited with building the first simple microscope, which he used to visualize protozoa and bacteria. He can be considered the father of cytometry. By 1742, Lomonosov described the method for producing dark field illumination and performed light scatter measurements. White light was the sole means of illumination until 1904, when Kohler developed a microscope with an ultraviolet (UV) light source. Essentially, all developments centered on the microscope until 1934, when Moldavan described a photoelectric technique for counting cells flowing through a capillary tube, and flow cytometry was born. In 1938, Caspersson built a crude flow cytometer to measure cell properties in the UV and visible regions. Crosland and Taylor developed a blood cell counter using the sheath flow principle, light scatter and darkfield illumination in 1940.

Dye Chemistry

To visualize prokaryotic and eukaryotic material, stains were sought to enhance their visibility under the microscope. The development of these stains was driven by the absorptive dye chemistry needed in the textile industry after 1850. Malachowski and Romanowski used acidic and basic dyes, which gave rise to the Geimsa, Leishman, MacNeal and Wright stains for identifying parasites in blood cells and for identifying hematopoietic cells. Fluorescent dyes did not appear until the 1880s, when fluorescein was synthesized and used by Paul Ehrlich, who also pioneered the use of mixtures of acidic and basic dyes to resolve the internal structures of leukocytes. DNA dyes were first used in 1900, but the introduction of flow cytometry drove development of several new dyes in the late 1960s and 1970s, and the measurement of DNA content became one of the first major applications of flow cytometry. Dittrich and Gohde first

used ethidium bromide in 1969, Crissman and Steinkamp introduced propidium iodide in 1973, and in 1974, Crissman and Tobey used mithramycin. In 1976, Latt and Stetten introduced the Hoechst dyes, and a year later Stohr introduced DAPI. All of these dyes are commonly used in flow cytometry today.

In parallel, in 1940, Coons, Creech and Jones used antipneumococcal antibodies conjugated with anthracene to detect microorganisms in tissues. By 1950, they were using antibodies conjugated with fluorescein isothiocyanate, or FITC — and immunophenotyping was born. Immunophenotyping allowed for the labeling of specific cell membrane proteins on cells, predominately leukocytes. Instead of relying on morphology, scientists could now identify cells by their unique repertoire of membrane proteins. After Felix Milgrom introduced the process of producing monoclonal antibodies in 1975, it was a simple matter of making monoclonal antibodies and using them to catalog cell features.

Electronics Industry

Meanwhile, development was going on in the electronics industry. By 1945, the photomultiplier tube had been developed to detect photons and convert them to electrical pulses. Amplifiers and analog-to-digital converters were also developed. In 1949, Wallace Coulter patented the first non-optical electronic blood cell counter, and by the 1950s, the realization that automated cytology might be most useful in clinical diagnosis began to permeate academic medical institutions. The ability to electronically count blood cells more accurately and faster than a hemocytometer started the revolution to automate. The first Model A Coulter Counter was introduced in 1957, primarily to count erythrocytes and leukocytes from blood. Four years later, the Model B was introduced. This device could also provide the size distribution of these cells.

Computers

While the Coulter Counter could count the cells, it could not identify what was being counted. An attempt to automate the microscopic identification process was needed. To accomplish this, computers, which had recently been introduced, and the concomitant software were required. Two different groups emerged to tackle this problem. Marylou Ingram, a hematologist at the University of Rochester, and Kendall Preston, a biomedical engineer at Perkin-Elmer, teamed up to develop a process to automate the microscopic identification of leukocytes in stained smears. They built a microscopic-based instrument called the Cytoanalyser to accomplish this. Studies by Mortimer Mendelssohn, Brian Mayall and Judith Prewitt using an automated instrument called CYDAC to extend the image analysis of cells were the first examples of automated image cytometry. The importance of this work as it related to flow cytometry was the introduction of computers and sophisticated computer algorithms.

Modern Flow Cytometry

Up to this point, cells could flow through an orifice and be counted; they could also be stained and identified microscopically in a somewhat automated fashion. The real beginning of modern flow cytometry occurred when Fulwyler at the U.S. Los Alamos National Laboratories built a cell sorter using the Coulter principal to size cells and electrostatic charging of droplets to sort them. Dittrich and Gohde then developed what they called the impulsecytophotometer (ICP), which became known as the "phywe." Cells were introduced into a flowing sheath stream located under a high power microscope objective that provided the optics for scatter measurements and fluorescence detection. Paul Mullaney, also at Los Alamos, introduced multiparameter flow cytometry by combining the measurement of volume, light scatter and fluorescence into a single instrument. Gary Salzman did extensive experiments that resulted in the addition of the ability to measure side scatter. By the mid 1970s, flow cytometers were entering the marketplace, and Leonard Herzenberg at Stanford coined the term, Fluorescence Activated Cell Sorter, or FACS.

In the early 1980s, interest in immunophentoyping grew with the discovery that HIV, which caused a severe immunodeficiency syndrome, or AIDS killed CD4 T cells. Among the first characterized monoclonal antibodies were antibodies to the membrane proteins CD3, CD4 and CD8 expressed by two subsets of these T cells. AIDS began to spread through the populations of the world, and with it, the first major clinical application of immunophenotyping by flow cytometry. The measurement of CD4 T cells using monoclonal antibodies was developed into a test to monitor the progression of AIDS. With this development, and the successful measurement of DNA content, it became clear that flow cytometry would be an important automated approach to cell analysis, and other clinical applications began to appear. These applications included ploidy and S phase fraction measurement in solid tumors, diagnosis and follow-up of hematopoietic malignancy and paroxysmal nocturnal hemoglobinuria, and monitoring of transplant rejection and hematopoietic regeneration. While immunophenotyping was initially directed to the measurement of membrane markers on cells, it became apparent that intracellular markers could also be measured. The ability to identify a cell population by membrane markers and simultaneously determine the cell's function had now become a powerful application of flow cytometry.

Another application pioneered by Larry Deaven and Scott Cram at Los Alamos and Joe Gray and Ger van den Engh at Lawrence Livermore National Laboratory was chromosome staining and sorting for cloning of DNA to produce chromosome-specific sequences. This new application led to the construction of the first high-speed cell sorter for the purpose of sorting each human chromosome. This project, supported by the U.S. Department of Energy, was the first step in the Human Genome Project.

As you can see, since a very slow beginning in the 17th century, the development of dye chemistry, the electronic and computer industries, and the means for producing monoclonal antibodies have all come together to produce the rapid and expanding field of flow cytometry. No other technology has been developed that can rapidly measure the number of correlated properties on a cell-by-cell basis. This book provides a compilation of applications that have been developed to properly utilize this complex technology.

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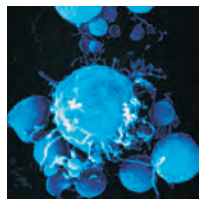


Cell Biology

Sonja Wulff

The cell is the basic building block of all tissues and microorganisms and, as such, deserves attention in this publication. A basic knowledge of cells and their structural components may influence the quality of a cell preparation and, thus, affect fluorescent characteristics. The goal of this chapter is to introduce the key components of the cell that are relevant to the performance and application of flow cytometry. Though nonmammalian cells, including bacteria, yeast, plants, aquatic microorganisms and *Drosophila*, are increasingly the targets of flow cytometric assays, mammalian cells, especially human and murine cells, are most commonly used and, thus, will be the focus of this discussion.

The Nucleus

The nucleus (Figure 1) is a membrane-bound organelle that houses the cell's genetic material. A nuclear membrane surrounds the nucleus and controls movement of materials between the nucleus and the rest of the cell. The cell's genetic material takes the form of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). DNA and RNA differ only in the chemical structure of the sugar groups that make up their backbone. DNA, which is typically double-stranded, has a 5-carbon sugar called deoxyribose, while RNA, which is typically single-stranded, contains a 5-carbon sugar called ribose. The difference in these sugars is a single hydroxyl group (OH^-). Within the nucleus, DNA and proteins are organized into chromosomes.

DNA and RNA are important in cell reproduction, cell death, protein expression and other cellular functions. Consequently, they have proven to be of interest to flow cytometrists, both in research and clinical laboratories. The amount of nucleic acid present in a cell and the general content of that nucleic acid can provide valuable information about cellular activity for research and diagnostic purposes. Scientists also use flow cytometry in sequencing studies, specifically related to chromosome mapping (i.e. identifying a gene and its associated chromosome). With the Human Genome Project, high-speed sorting technology was crucial for isolating individual chromosomes and generating chromosome-specific DNA libraries.

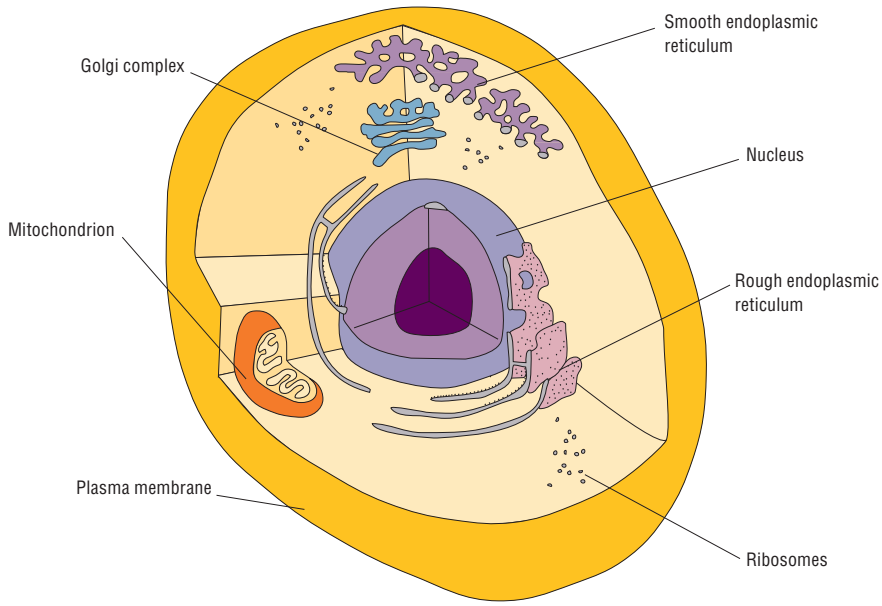


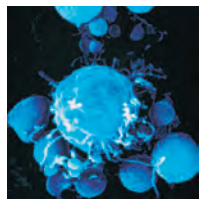
Figure 1. The Cell

The Cytoplasm

The cytoplasm (Figure 1) is the site of most of the cell's housekeeping functions, which are carried out as directed by the nucleus. The appearance of the cytoplasm can vary greatly from cell to cell and, thus, plays a key role in distinguishing different types of cells by microscopy. The cytoplasm also contains several common structures that provide valuable information about the cell and its activity. The focus here will be on structures that are detectable by flow cytometry.

The endoplasmic reticulum is a series of membrane-bound channels that transport secretory products for use in the cell or for export out of the cell. The Golgi apparatus is a stack of membranes that modify, store and route products of the endoplasmic reticulum. Together, the endoplasmic reticulum and Golgi are primarily responsible for the proper sorting of lipids and proteins in cells. They also have critical roles in signal transduction-associated lipid trafficking and a variety of transport-related human diseases. In flow cytometry laboratories, they also can be used in studies related to cholesterol and intracellular expression of immunoregulatory proteins, known as cytokines.

Another cytoplasmic structure of particular interest to flow cytometrists is the mitochondrion. The primary function of mitochondria, which can make up as much as 10% of the cell's volume, is energy production through oxidative phosphorylation and lipid oxidation. They also are involved in apoptosis, intracellular calcium homeostasis, and production of urea, heme and steroids. Their abundance and morphology vary with cell type, reproductive stage and activity level. A variety of flow cytometric probes are available for monitoring mitochondrial morphology and function, which can provide valuable information about metabolic and neurodegenerative disease, drug resistance, fertilization, cell signaling and a number of other topics that are relevant to both clinical and research laboratories.



The Membrane

The entire cell is enclosed by a plasma membrane, which serves as a selective barrier to regulate the cell's chemical composition. The plasma membrane is of particular interest in flow cytometry for a number of reasons. First, it anchors surface proteins, or antigens, that can serve as cell identifiers. These antigens define characteristics about a cell, such as function, lineage and developmental stage. They are so numerous that the scientific world has adopted a classification system based on assigned cluster of differentiation, or CD, numbers. For instance, CD4 is a marker on the surface of helper T cells, while CD56 is a marker on the surface of natural killer (NK) cells. In addition to CD antigens, the cell surface contains countless receptor molecules that can trigger complex signal transduction pathways inside the cell upon binding of specific ligands. Flow cytometers can detect the presence and relative numbers of these receptors and antigens using fluorescently labeled monoclonal antibodies directed against them. Additional protocols exist to assess how close these molecules are to each other and what, if any, interactions they have.

Flow cytometry also can be used to measure membrane potential, or the charge difference across the membrane generated by the relative internal and external concentrations of ions such as potassium, sodium and chloride. Changes in membrane potential play a critical role in many physiological processes, including nerve-impulse propagation, muscle contraction, cell signaling and ion-channel gating. Specific flow cytometric probes are available to directly look at ion channel activity, most commonly for calcium, or Ca2+. Intracellular Ca2+ levels regulate numerous cellular processes, including gene expression, cellular reproduction and motility.

At times in flow cytometry, cell membranes can complicate experiments. In situations where the goal is to detect intracellular proteins, processes and materials, dyes and labeled antibodies must be able to cross the membrane. This precludes the use of certain dyes and fluorochromes and requires special cell preparation.

Cell Preparation

Special consideration must be given to samples destined for the flow cytometer. While an in-depth discussion of these considerations and specific protocols is not possible in this publication, a brief mention of some key concepts follows. For more information on staining and antibody handling, see Chapter 5.

Disaggregation

Single-cell suspensions are required for all flow cytometric assays. As such, certain types of cells (e.g. leukocytes in blood) are ideally suited for flow cytometry. However, this requirement does not preclude flow cytometric analysis of solid tumors or other tissue samples. A number of protocols are available for disagreggating tissue samples into suitable single-cell suspensions (Figure 2). These protocols typically involve either enzymatic digestion (e.g. collagenous) or mechanical chopping and filtering. Cultured cells may also need special treatment — typically gentle trypsin digestion — as many cell lines grow attached to the plastic surface of a culture flask. In all situations, removing debris, dead cells and cell clumps is essential to a successful experiment.

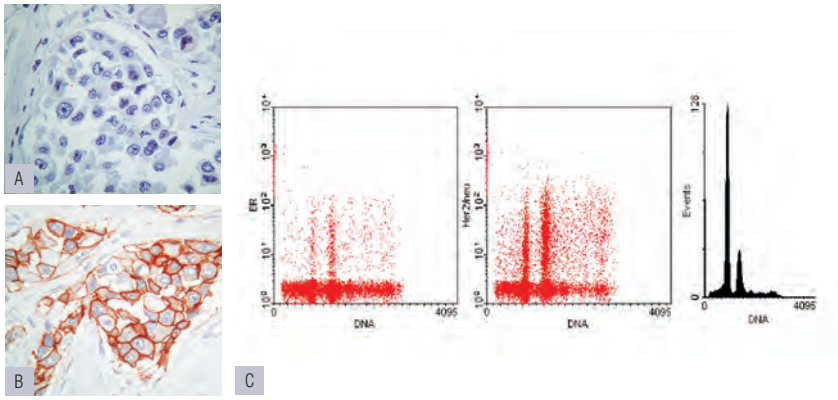


Figure 2. Flow Cytometric Analysis of a Solid Tumor. A breast cancer specimen analyzed by immunohistochemistry was found to be negative for estrogen receptor expression (A) but positive for expression of the HER-2/neu oncogene (B). Upon disaggregation, multiparametric flow cytometry (C) looked at expression of both proteins, along with cell growth parameters. Data courtesy of Mathie P. G. Leers, PhD, Atrium Medical Center, Netherlands

Enrichment

Historically, when dealing with rare populations of interest (e.g. 5% or less), it has been necessary to enrich, or concentrate, these populations to reduce the length of a sort or analysis. Methods of enrichment vary greatly but include centrifugation, density gradients, magnetic particle separation and complement-mediated lysis. With the advent of high-speed flow cytometers, these procedures are no longer necessary for successful rare-event detection. With instruments that can process 100,000 events per second, it has become acceptable not to perform enrichment at all. Eliminating this step boosts laboratory productivity, minimizes potentially harmful cellular manipulations and ensures more accurate rare-population statistics.

Membrane Permeabilization

Though the plasma membrane is impermeable to large molecules, such as antibodies, flow cytometry can still provide a means of detecting intracellular proteins if appropriate cell preparation protocols are followed. Typically, this approach involves cell fixation, typically with formaldehyde, to stabilize the proteins, and subsequent disruption of the membrane with detergents. Fixative and detergent choice, as well as incubation times, will vary depending on the intracellular protein of interest.

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Flow Cytometry Instrumentation

John Sharpe, PhD

The first light-based flow cytometers were little more than a fluid path, an excitation source and a photodetector. Early sorters used technology not dissimilar to that present in ink jet printers. As with many instruments, modern flow cytometers are becoming increasingly sophisticated devices that combine fluidics, optics, electronics and software to enable measurements to be performed on microscopic particles at event rates of many tens of thousands per second. These instruments have taken advantage of many technological developments in other sectors (such as semiconductor and telecommunications) to provide increased accuracy and reliability with significant size reduction.

Flow Cytometer Operation

The prepared sample of particles under investigation begins its journey through the flow cytometer from within a sample vessel (test tube, multiwell plate or similar container). At the desired time for analysis, sample is aspired from this vessel and is transported through tubing to a flow cell or nozzle. At the flow cell, the sample is introduced to the center of a faster flowing carrier fluid and is in turn presented to one or more light sources for excitation (Figure 1). Light scatter and/or fluorescence are captured, spectrally filtered and directed to appropriate photodetectors for conversion to electrical signals. Electronic circuitry is used to process these signals for analysis, classification, sorting and data storage. For sorters, an individual particle is traced through the instrument as it breaks free from the continuous jet into a charged droplet for electrostatic deflection. The droplet passes through an electric field and is ultimately captured in a suitable container for further processing or study, or is disposed of as waste.

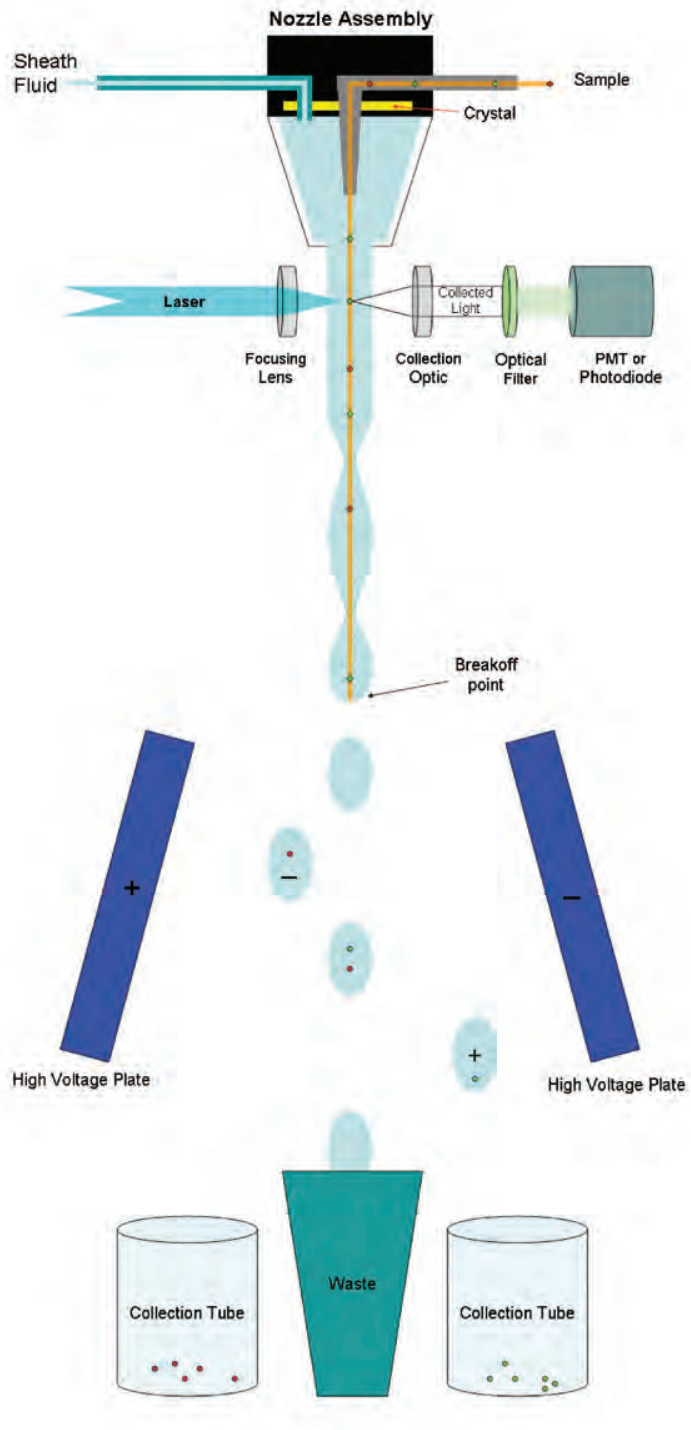

Figure 1. Fluorescence Activated Sorting Flow Cytometer Operation. A suspension of stained cells is presented in single file to an intense light source for measurement of inherent cellular features. On instruments with sort capability, as each cell is characterized, it may be separated from the main population by electrostatic droplet deflection.

Fluidics

The fluidic system of a flow cytometer is used to transport particles from a random three-dimensional sample suspension to an orderly stream of particles traveling past one or more illuminating beams. In sorters these particles are transported in an electrically conductive carrier fluid. The fluidic system often uses air pressure regulation for stable operation and consists of at least one sheath line and a sample line feeding the flow cell.

As the sample enters the flow cell chamber, the outer, faster flowing sheath fluid hydrodynamically focuses this fluid into a narrow core region within the jet and presents a single file of particles to excitation sources (Figure 2). This geometry provides increased positioning accuracy at the laser interrogation point for consistent excitation irradiance and greatly reduced particle blockage of the flow cell. For analysis-only instruments, the flow cell typically has a square or rectangular channel profile with dimensions on the order of a few hundred microns. For sorters, this sheath-sample mix exits the nozzle through a circular orifice (typically 50 µm to 200 µm in diameter) and produces a continuous jet before breaking into droplets. Depending on instrument design, particles are either interrogated when they are in the flow cell, or once they have exited and are contained within the jet in air. After interrogation, the sample-sheath fluid mix is flushed to waste unless particle sorting is desired. To facilitate instrument start-up and cleaning, a vacuum line is usually provided.

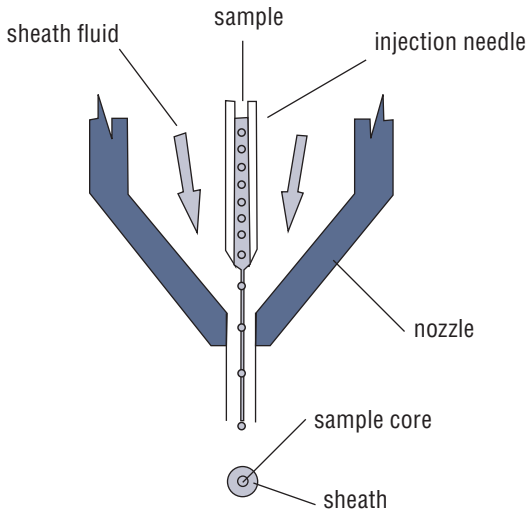

Figure 2. Hydrodynamic Focusing in a Flow Cytometer Nozzle

Optics

Optics are central to flow cytometry for the illumination of stained and unstained particles and for the detection of scatter and fluorescent light signals.

Excitation and Emission

Most flow cytometers utilize one or more laser sources for cell excitation. Emission, in the form of light scattering, occurs when excitation light is absorbed and then re-radiated by the particles under investigation, with no change in wavelength. Scatter is typically strongest in the forward direction with respect to the incident excitation source. Fluorescence occurs when a molecule excited by light of one wavelength returns to a lower state by emitting light of a longer wavelength. The excitation and emission light, being of sufficiently removed wavelengths, can be blocked or differentiated by optical filtering. Several cellular constituents can be measured simultaneously by using several excitation sources and several cell stains that emit at different wavelengths. This key ability to detect fluorescence simultaneously from several different compounds bound to one or many cells has resulted in flow cytometry being a technique of choice for multiparameter fluorescence analysis of cell populations.

Light Collection

In conventional flow cytometers, light is collected by two lenses termed the forward and side collection lenses, depending on their orientation as viewed from the entering laser beam. The forward collection lens gathers scattered light over a region centered on the laser beam axis. Forward scatter can be used to obtain information of particle size. The side scatter lens has a high numerical aperture (NA) for maximum fluorescence collection efficiency and collects light at 90 degrees to the laser beam axis. Side scatter can be used to differentiate particle populations based on morphology.

Optical Filters

Once the fluorescence light from a cell has been captured by the collection optics, the spectral component of interest for each stain must be separated spatially for detection (Figure 3). This separation of wavelengths is achieved using dichroic (45 degree) and emission (normal incidence) filters. Longpass filters permit longer wavelength transmission, while shortpass filters allow shorter wavelength transmission. Bandpass filters only allow a selected wavelength band of interest to be transmitted while blocking unwanted wavelengths.

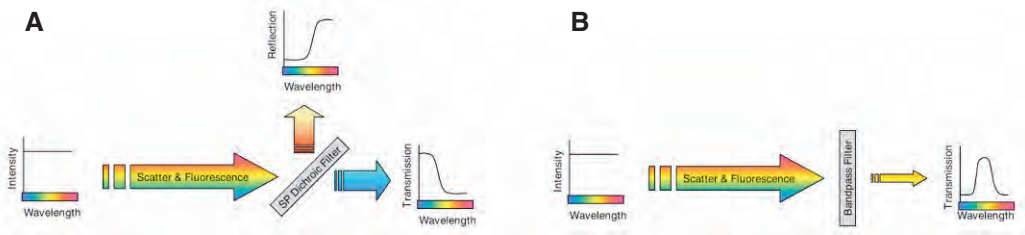

Figure 3. Dichroic filters (A) and bandpass filters (B) provide spatial separation of light.

Detectors

The two main photodetector types used in flow cytometry are silicon photodiodes and photomultiplier tubes (PMTs). Silicon photodiodes are typically used for forward scatter detection where signal levels are high. PMTs have high gain and sensitivity and are, therefore, assigned to side scatter and fluorescence detection.

Electronics

The role of the electronics is to monitor and control the operation of the flow cytometer, from detection of a particle event as it passes through the laser focus, to physical deflection of that particle into a collection flask. As a particle of interest passes through the focus, fluoresces and is detected by a photodetector, an electrical pulse is generated and presented to the signal processing electronics. The instrument is triggered when this signal exceeds a predefined threshold level. The threshold is primarily used to reject non-particle events such as debris or noise from optical and electronic sources.

Pulse characteristics are determined by particle speed and size, width of the illumination beam, and in the case of fluorescence, distribution of fluorochrome within the particle. Various pulse processing modes enable different measurements to be made from the same signal, such as peak height, integral (area), width, skew, etc. (Figure 4). In some instances, linear amplification and analysis of cell fluorescence or scatter is adequate for particle characterization, such as in DNA studies. In many instances, such as immunophenotyping studies, the measurement of both stained and unstained (bright and dimly fluorescent) particles requires measurement over several orders of magnitude. To achieve this broad dynamic range, a variety of analogue, digital or hybrid electronic systems can be used for signal processing. Analogue devices, such as logarithmic amplifiers, provide very large amplification to small signals and near unity amplification for large signals, while digital signal processing devices (DSPs) can be used to digitize pulse parameters. Some instruments use a combination of analogue and digital electronic elements in order to capture advantages of both devices. Real time calculation and decision-making capabilities enable sort functions to be performed at high speed.

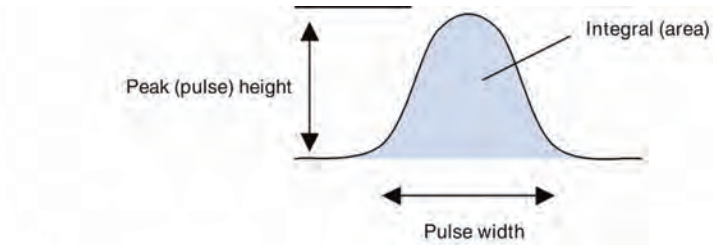

Figure 4. Pulse Measurement Characteristics

Software

Early flow cytometers used an oscilloscope for simple pulse height display and analysis. Today, most instruments use software for instrument control, data acquisition, analysis and display.

Instrument Control

Many instruments provide software control for many or all instrument hardware functions including fluidics, lasers, electronics and sorting. Further, operations such as start-up and shutdown sequences, cleaning cycles, calibration cycles and self-test functions are monitored and regulated by software, so that instrument operation can be simplified and optimized for the user.

Data Acquisition and Analysis

Flow cytometers can be used to provide population-based analysis on a large number of particles in a comparatively short period of time. The data generated form this analysis is usually presented in histogram form or on a dot or contour (bivariate) plot. The frequency histogram is a direct graphical representation of the number of events occurring for each detection channel (i.e. the number of particle events detected as a function of intensity). The dot plot is a two-dimensional extension of the frequency histogram. Each location on the dot plot corresponds to a measured signal at a first detector versus a second detector. Other techniques can be employed to visualize three or more parameters in highly multiplexed parameter analysis applications.

Statistical analysis is performed by graphically, applying regions of interest to data sets. Additional gating schemes can be used to restrict only certain subpopulations of interest to investigate populations with minor variations in structure. These concepts are discussed in more detail in Chapter 6.

Time, Ratios and Computed Parameters

As the level of sophistication of flow cytometry experimentation has increased, so too have the methods by which data analysis can be performed beyond routine fluorescence intensity. Experiments that involve changes in fluorescence intensity over time, such as calcium flux studies, can be performed, displayed and observed in greater detail by using the time parameter combined with fluorescence ratio. Other arithmetic functions can also be applied to extend the capabilities of the flow cytometry technique.

Interpretation of Acquired Data

Flow cytometric analysis, although capable of measurements with high precision, is prone to the introduction of systematic errors. Sources of measurement degradation can include poor sample preparation and staining, nonspecific and nonhomogeneous staining, fluorochrome emission variations with pH and temperature, optical and electronic noise, laser noise and fluidic instability. Any degradation in the accuracy of measurements can be detected rapidly if standard particles are used routinely to monitor and log instrument performance.

Sorting

The flow sorting technique in most widespread use for flow cytometry uses electrostatics to charge and deflect a particle-containing droplet as it passes through a perpendicular electric field. The cell carrier fluid is conductive and forms a jet as it exits the nozzle chamber. A mechanical oscillation is applied through a piezo-electric transducer to the jet to encourage stable droplet formation. Droplets are charged by applying a positive or negative voltage to the carrier fluid stream. If a droplet separates from the stream while such a charge is applied, it will retain a surface charge proportional to the applied voltage. The charged droplet continues its path through an electric field, which is produced by a pair of oppositely charged plates, and is deflected from its original trajectory into one of several collection vessels beneath the apparatus. The droplet charge, volume, mass, density, velocity and the time spent in the electric field will determine the distance it is deflected from its original path when it is collected. A droplet that has no charge with respect to ground will travel an undeflected path. If the charge-inducing voltage on the sample stream can be varied, the number of deflected droplet streams is limited only by the accuracy of the deflection mechanism and the size of, and distance to, the collection vessels. Sorting will be discussed in more detail in Chapter 8.

Automation

Automated introduction of sample to the flow cytometer allows walkaway operation to be performed on large numbers of samples using a variety of formats from single test tubes to multiwell plates. Single tubes are often contained within rectangular grid arrangements or carousel formats to match laboratory equipment used for sample preparation. A large number of samples can be analyzed using multiwell plate feeders that automatically feed samples to the instrument for periods of a day or more.

A number of other routine instrument functions are being automated as well. These include self calibration and self check utilities, start-up and shutdown procedures, biosafety monitoring and intervention, fluorescence compensation and sort monitoring and control.

The electrostatic sorting technique provides a simple and effective means for depositing droplets and, therefore, particles into a variety of collection vessels. When combined with automated positioning of vessels such as multiwell plates, glass slides and other emerging chip and microfluidic devices, this technique can be used to precisely deposit single particles in complex and densely-packed two-dimensional arrangements.

Technical Considerations

Sample Handling

To ensure that sample and, therefore, data integrity is maintained, it is important that the damaging external effects of light exposure, temperature variation and mechanical agitation are minimized to the sample under investigation. Consideration should also be given to the suitable selection of sheath fluid so that cells can be analyzed and/or sorted with minimum disturbance.

Sample contamination can occur when the fluidic lines and other surfaces that come in contact with sample fluid are not cleaned sufficiently or provide a location for carry-over of sample fluid and particles. This issue can be minimized by following appropriate daily cleaning practices and by adhering to instrument replacement and maintenance schedules.

Biosafety

The issue of biosafety is attracting increasing attention in the field of flow cytometry. Risks are similar to those faced in other biological laboratories dealing with hazardous solvents, infectious agents and blood products. The particular challenges with flow cytometry are the high pressures applied to samples and the aerosols that can be associated with sorting. Though standard laboratory safety procedures address these issues in many cases, instrument manufacturers also offer special biosafety accessories, such as custom biosafety cabinets, sort shields and aerosol evacuation pumps.

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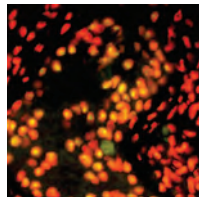


Fluorochromes

W. Roy Overton, PhD

Attaching fluorescent compounds to antibodies, cells, microorganisms and microscopic beads enables scientists and clinicians to gain tremendous amounts of information about the biology and pathology of cells from humans, animals, plants and microbes using fluorescence microscopy or flow cytometry. Following is a discussion of these fluorescent compounds, which are known as fluorochromes.

Physics of Light

Among the properties of many compounds is the ability to absorb one color of light and then emit a different color of light. This causes the compound to appear to be glowing, or fluorescing, and the compound is referred to as a fluorochrome. To better understand how a fluorochrome works, it is necessary to examine this process at the subatomic level. As can be seen in Figure 1, electrons normally spin around the nucleus of an atom at a distance that is referred to as the electron's ground state. If the atom is hit by photons of light that can excite the electron, then the electron will move up to a higher energy state that is farther from the nucleus. The electron is unable to maintain that distance from the nucleus due to the electron's magnetic attraction to the protons in the nucleus and so it quickly drops to a slightly closer distance, called the lowest singlet excited state. Moving to the lowest singlet excited state causes the electron to release a little of the energy that it got from the light, but it releases the energy as heat. The electron then returns to its original ground state, releasing the rest of the energy that it absorbed from the light. This energy is released as light, but since some energy was released as heat, there is less energy in that light. Thus, the emitted light appears to be a different color than the light that excited the atom.

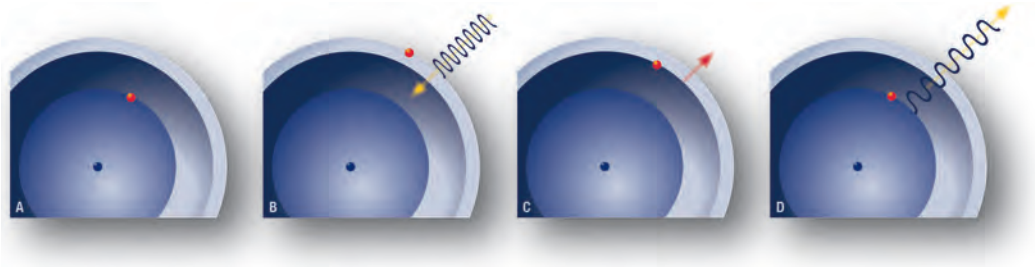


Figure 1. A) Electrons in an atom (red) are normally found at their ground state. B) When an electron is excited by high-energy light, such as ultraviolet light, the electron moves from the ground state to a higher energy state. C) The electron is unable to sustain the higher energy state and quickly drops to the lowest singlet excited state. The energy lost is given off as heat. The amount of energy lost determines the Stokes shift. D) The electron then falls back to its ground state, and energy is emitted as light with a longer wavelength and less energy than the light that excited the electron.

Light travels in waves that determine the color of the light. If the light has a lot of energy, its waves are shorter than a wave of light with less energy (Figure 2). The wavelength of the light determines the color of the light. Very high-energy light, such as ultraviolet (UV) light, has very short wavelengths. The wavelength of UV light is less than 400 nm. Because the wavelength is so short, the human eye is unable to see UV light, but electronic photodetectors are able to detect and measure this invisible light. Visible light has wavelengths from 400 nm to 700 nm, which produce the colors violet, blue, green, yellow, orange and red. Above 700 nm is the infrared range, which is also invisible to the human eye but can be felt as heat or detected by electronic photodetectors.

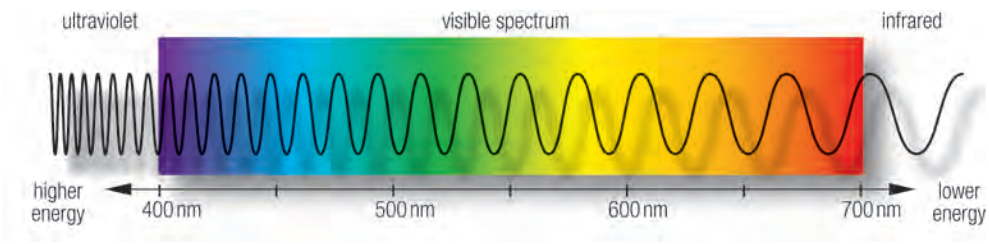
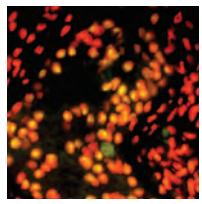


Figure 2. High-energy light has short wavelengths, and low-energy light has long wavelengths.

Excitation and Emission

In flow cytometry, laser light is usually used to excite the fluorochromes. These lasers produce light in the UV and/or visible range. Fluorochromes are selected based on their abilities to fluoresce with the wavelengths of light produced by the lasers. Therefore, if a flow cytometer has only one laser that produces only 488 nm light, then only fluorochromes that are excited by 488 nm light can be used. The chemical properties of the fluorochrome determine whether its electrons can be excited to the higher energy state by a specific wavelength of laser light. If the electrons can be excited to the higher energy state, the chemical properties of the fluorochrome will also determine the amount of energy lost as heat when the electrons drop back down to the lowest singlet excited state and the wavelength of light produced when the electrons return to their ground state.

The difference in the wavelength of the light that excites the electrons and the light that is emitted is called the Stokes shift and is determined by the amount of energy lost as heat. Some fluorochromes have a small Stokes shift, and the excitation and emission wavelengths have almost the same wavelengths, but other fluorescent compounds have large Stokes shifts. For example, the fluorochrome, fluorescein, can be excited by blue-green light and its Stokes shift is only about 25 nm, which means that the light emitted is green. This contrasts with another fluorochrome, phycoerythrin, which can also be excited by blue-green light but has a large Stokes shift. Thus, the light emitted is yellow-orange. In flow cytometry, a laser beam of a single wavelength can be used to excite several fluorochromes with different Stokes shifts and, thereby, produce a variety of fluorescent colors. This is the basis of multicolor flow cytometry. If additional



colors are needed, a second or even third laser with different wavelengths can be used to excite additional fluorochromes.

The electrons of a fluorochrome can be excited by a range of wavelengths of light. For example, the fluorochrome, fluorescein, will fluoresce when hit by light with a wavelength between 430 nm and 520 nm (Figure 3A). However, the closer the excitation wavelength is to 495 nm, the more fluorescence will be produced. This optimal wavelength is called the excitation peak. Similarly, the light produced by fluorochromes has a range of wavelengths. The emission of light from fluorescein, as shown in Figure 3B, ranges from 490 nm to 630 nm, and the emission peak is approximately 520 nm.

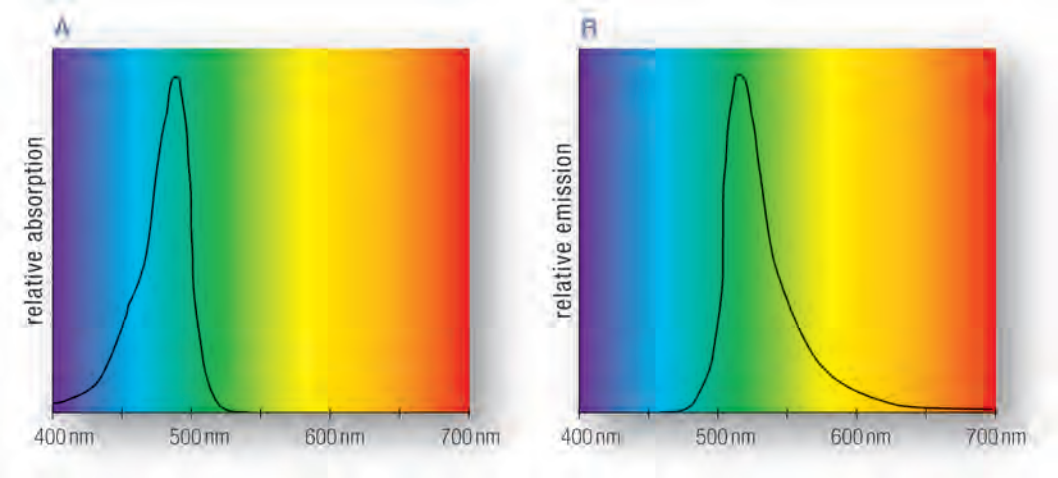


Figure 3. A) Fluorescein excitation. B) Fluorescein emission.

Fluorochrome Selection

Knowing the excitation and emission properties of fluorescent compounds makes it possible to select combinations of fluorochromes that will work together optimally on a specific flow cytometer with specific lasers. However, for a fluorochrome to be useful in a biological application, it must attach to or be contained within a particle of biological significance. Some fluorochromes are useful because they bind to specific chemical structures, such as antibodies (See Chapter 5) or the nucleic acids in DNA or RNA. Fluorochromes that are used most often in flow cytometry are ones that attach in some way to biologically significant molecules and are excitable by the lasers that are commonly found on commercial flow cytometers.

Many fluorochromes can be attached to antibodies, which will then bind to specific chemical structures on or inside of cells. If these chemical structures are unique to a specific type of cell, then the fluorochrome will identify that cell type. This technique of identifying cells using fluorescent antibodies is called immunophenotyping. A list of the fluorochromes used most often in immunophenotyping is shown in Table 1 with their peak excitation and emission wavelengths and the laser wavelengths most often used to excite them on a flow cytometer. Table 2 shows the lasers that can generate the required wavelengths of light to excite the various fluorochromes. Some other common

applications of fluorochromes in flow cytometry include the detection of intracellular calcium, measurement of the relative amount of cellular DNA or RNA, and measurement of transcription levels using a fluorescent protein as a reporter gene. Fluorochromes used for these applications are shown in Table 3.

Table 1. Fluorochromes for Immunophenotyping

Fluorochrome	Excitation Peak (nm)	Emission Peak (nm)	Laser Wavelengths (nm)
AMCA	345	440	334-364, 351-356
Alexa 350	350	445	334-364, 351-356
Marina Blue	365	460	334-364, 351-356, 405, 407
Cascade Blue	395	420	405, 407
Cascade Yellow	400	550	405, 407
Pacific Blue	405	455	405, 407
Alexa 430	435	540	458
Per-CP	490	670	488
FITC	495	520	488
Alexa 488	500	520	488
Alexa 532	532	555	514
TRITC	545	580	568
Alexa 546	560	570	568
Phycoerythrin (PE)	565	575	488, 514, 568
PE-Texas Red	565	615	488, 514
PE-Cy5	565	670	488, 514
PE-Cy5.5	565	695	488, 514
PE-Cy7	565	770	488, 514
Alexa 568	568	605	568
Alexa 594	594	620	568
Texas Red	595	615	568
Alexa 633	630	650	633, 635, 647
Alexa 647	647	670	633, 635, 647
Allophycocyanin (APC)	650	660	633, 635, 647
Cy5	650	665	633, 635, 647
APC-Cy7	650	770	633, 635, 647
Alexa 660	660	690	633, 635, 647
Cy5.5	675	695	633, 635, 647
Alexa 680	680	700	633, 635, 647
Alexa 700	700	720	633, 635, 647

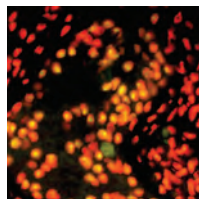


Table 2. Common Laser Wavelengths in Flow Cytometry

Laser	UV	Violet	Blue	Blue-Green	Green	Yellow	Red
Argon	334-364		458	488	514		
Solid-State Violet Laser		405					
Krypton	351-356	407				568	647
Helium-Neon							633
Red Diode							635

Table 3. Fluorochromes for Other Flow Cytometry Applications

Application	Fluorochrome	Excitation Peak (nm)	Emission Peak (nm)	Laser Wavelengths (nm)
Calcium	Indo-1 (calcium)	325	400	334-364, 351-356
Calcium	Indo-1 (no calcium)	345	485	334-364, 351-356
Calcium	Fura Red	485	675	458, 488
Calcium	Fluo-3	500	540	488
DNA Content	Hoechst 33342	355	455	334-364, 351-356
DNA Content	DAPI	360	460	334-364, 351-356, 405, 407
DNA Content	Acridine Orange	495	535	488
DNA Content	Propidium Iodide	305	620	334-364, 351-356
		535	620	488, 514, 568, 633, 647
DNA Content	7-AAD	545	650	488, 514, 568
DNA Content	To-Pro-3	640	655	633, 635, 647
Reporter Gene	eCFP	430	475	458
Reporter Gene	eGFP	495	510	488
Reporter Gene	eYFP	520	535	514
Reporter Gene	Ds-Red	555	585	514, 568
Reporter Gene	HcRed	590	620	568

There are many other chemical and physical properties of fluorochromes that determine where and when these dyes are useful in various biological assays. For example, some of the fluorochromes that bind to DNA, such as Hoechst 33342, can get into living cells, but most DNA-binding fluorochromes cannot get past the cell membrane. The fluorescent dyes that cannot get past a viable cell membrane, such as propidium iodide, are often used to distinguish live from dead or dying cells.

In order to select the best fluorochromes for use in a specific application on a particular flow cytometer, it is necessary to know the laser configuration of the flow cytometer and the physical and chemical characteristics of the fluorochromes that are available for that application.

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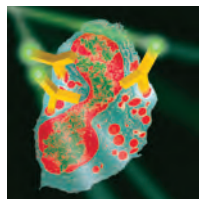


Antibodies

Thomas Boenisch, MS, and Jim Hudson, PhD

Immunoglobulins

The vertebrate immune system has the amazing ability to create millions of different antibodies with different specificities. It is their ability to recognize specific antigens that makes antibodies so useful for fighting disease or as tools for disease diagnosis. The interaction of antibody and antigen is pivotal to immunodiagnostic assays. This chapter will focus on antibodies, their structure and behavior.

Immunoglobulins, the antibody molecules, are delineated into five major classes: immunoglobulin G (IgG), IgA, IgM, IgD and IgE. Of the five classes of immunoglobulins, IgG and IgM will be considered in more detail, as they are by far the most frequently utilized antibodies in immunochemistry. Unless otherwise noted, most of what is described of the IgG structure in this text was learned from studies with human IgG of subclass IgG_1.

The IgG molecule can be separated into two types of protein chains: light (L) and heavy (H) chains (Figure 1). Each IgG molecule is composed of two identical heavy chains (H) and two identical light chains (L). The H chains determine the class and subclass of the molecule. The two light chains are either type kappa or lambda. Whereas in human IgG the overall ratio of kappa to lambda is 2:1, in the subclasses IgG_2 and IgG_4, for example, the ratios are 1:1 and 8:1, respectively. Mice have approximately 95% kappa chains and, therefore, most monoclonal IgG antibodies from this species have kappa chains. Disulfide bridges join the light and heavy chains together.

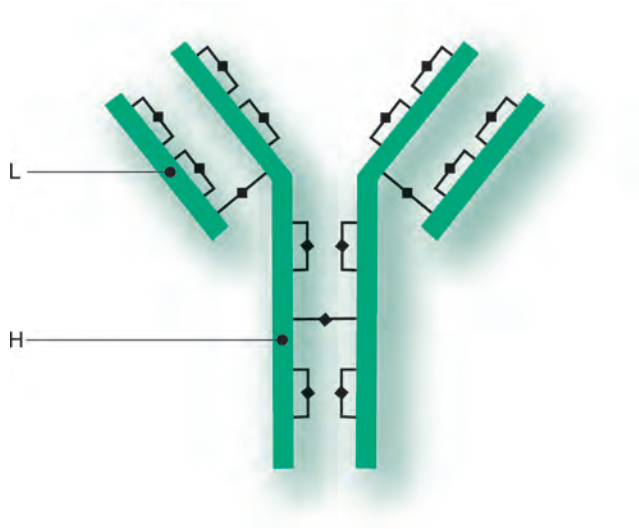


Figure 1. Immunoglobulin Molecule. The molecule is comprised of two identical heavy chains (H) and two identical light chains (L), with inter- and intrachain disulfide bonds for stability.

The IgG molecule can be further divided into so-called domains: the variable domains (V) and the constant domains (C) (Figure 2). Each domain contains 110 to 120 amino acids and one intrachain disulfide bond. The variable domains of the light chain (V_L) and heavy chain (V_H) together form the antigen-combining site. Several hypervariable regions are located within the V_L and V_H domains of the antibody. The regions convey the unique structural idiotype of the antibody molecule. These idiotypic determinants come in close proximity to the specific antigenic epitopes during the binding process. Each antibody clone expresses its own idiotype.

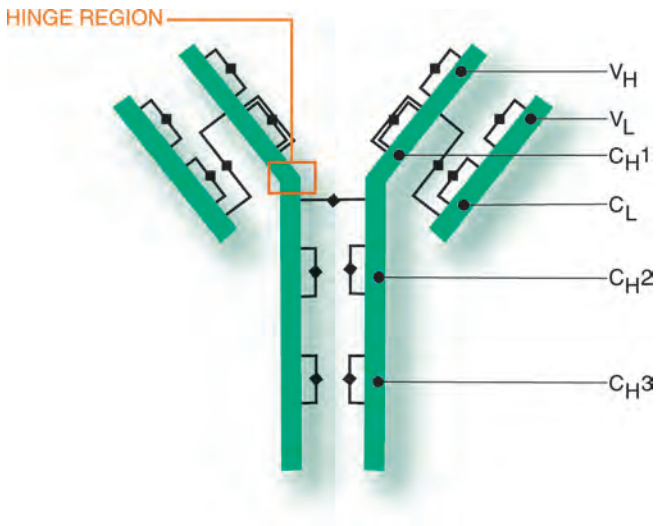
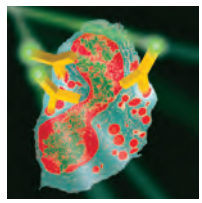


Figure 2. IgG Molecule. The heavy (H) and light (L) chains are composed of variable (V) and constant (C) domains linked by inter- and intrachain disulfide bonds.

Each L chain has one constant domain (C_L) and one variable (V_L) domain. The H chain has three constant domains (C_H1, C_H2 and C_H3) and carries the carboxyl terminal portion of the immunoglobulin. The hinge regions are located between the C_H1 and C_H2 domains of the H chains. Minor differences within these hinge regions contribute to the subclass identities of IgG. The subclasses (or isotypes) are designated by subscripts, as in IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgG_4.

IgM is a pentamer (molecular weight approximately 900 kD) consisting of five subunits of two heavy chains (mu) and two light chains of type kappa or lambda (Figure 3). Each subunit is linked by the J chain. Subclasses of IgM_1 and IgM_2 have been reported.

IgM is the first humoral antibody detectable after immunization. Formation of the primary antibody response proceeds in several major stages. Injected immunogen first reaches equilibrium between extra- and intravascular spaces, then undergoes catabolism resulting in smaller fragments. It finally is eliminated from the intravascular spaces by the newly formed antibodies. The period from the introduction of an immunogen until the first appearance of humoral IgM antibodies is called the latent period and may last approximately one week. Within two weeks, or in response to a second injection, IgG class antibodies usually predominate. IgG is the most abundant antibody in the



hyperimmunized host. While IgM molecules have a relatively short half-life in serum of only four to six days, IgG molecules have a mean survival of approximately three weeks. Unless repeated booster injections with the immunogen are given, the serum antibody level will decrease after this period.

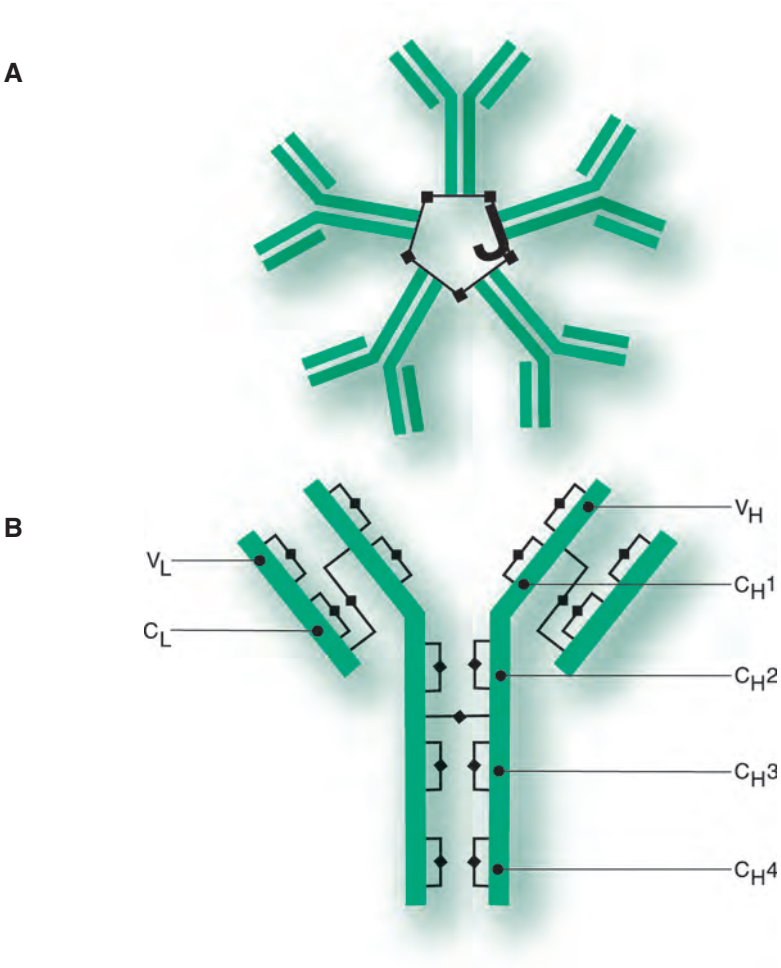


Figure 3. IgM Molecule. A) IgM is made up of five subunits. B) Each subunit contains two heavy chains (H) and two light chains (L) with constant (C) and variable (V) regions.

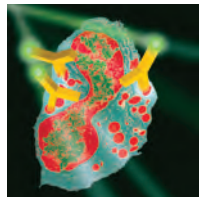
Antibody formation on the molecular level is a complex process and a detailed account of it is beyond the scope of this guidebook. The interested reader is referred to the textbook *Molecular Immunology* by Atassi et al.[1]

Polyclonal Antibodies

Immunogen injected into an animal will result in the production of polyclonal antibodies. These antibodies arise from different B cells within the animal. They recognize different epitopes, (i.e. small regions within the antigen molecule) (Figure 4). The advantage of using these antibodies in immunoassays lies in their ability to saturate the antigen, (i.e. more antibody molecules can bind to the antigen for enhanced detection). However, due to their wider range of specificities there can be more "nonspecific" binding. Depending on the assay design, this can result in higher background staining.

Figure 4. Polyclonal antibodies react to various epitopes on an antigen.

By far, the most frequently used animal for the production of polyclonal antibodies is rabbit, followed by goat, pig, sheep, horse, guinea pig and others. The popularity of rabbits for the production of polyclonal antibodies is attributed primarily to their easy care and maintenance. Many years of selective breeding for favorable immunization response has made the New Zealand White Rabbit the most frequently used animal for the production of polyclonal antibodies.[2] Depending on the immunogenicity of the antigen, doses from 10–200 µg are traditionally administered to generate an immune response in animals. The antigen is most often injected intradermally or subcutaneously, but injections into the footpad muscle or peritoneal cavity are also used. In rabbits, volumes of 0.1–0.5 mL are usually given intradermally and distributed over several sites, with the antigen suspended in an equal volume of adjuvant, such as Complete or Incomplete Freund's Adjuvant. Booster shots, repeated once a month or when decreasing titers are detected, are intended to maintain or increase antibody levels. Blood is most often harvested from the ear (rabbits), the jugular vein (larger animals) or from the heart, sometimes by sacrificing the animal. After the removal of cells from the



blood, polyclonal antibodies can be obtained either in the form of stabilized antisera or as immunoglobulin fractions purified to varying degrees. Precipitation by salts, followed by ion exchange chromatography, serves to remove the bulk of other serum proteins. Affinity chromatography can be used to isolate the antigen-specific antibodies and, thereby, free them of cross-reacting antibodies to other species.

Monoclonal Antibodies

Monoclonal antibodies are the product of an individual B lymphocyte clone. Antibodies from a given clone are immunochemically identical and react with a specific epitope on the antigen against which they are raised (Figure 5). For reasons of economy, mice are currently used almost exclusively for the production of monoclonal antibodies. After an immune response has been achieved, B lymphocytes from spleen or lymph nodes are harvested and fused with non-secreting mouse myeloma cells under specific chemical conditions. While the B lymphocytes convey the specific antibody, myeloma cells bestow upon the hybrid cells (hybridoma) longevity in culture medium. Cells that are not hybridized soon die in culture. The antibody-producing hybrid cells are cultured and tested for desired reactivity. Cells producing antibody to the antigen are selected for propagation in culture medium or by transplantation into the peritoneal cavity of syngeneic mice. Antibodies can be harvested from the resulting ascites fluid. Today most commercial manufacturers use in vitro cell culture systems to produce monocloncal antibodies. Thus, large and, at least theoretically, unlimited quantities of monoclonal antibodies of specific characteristics can be produced.

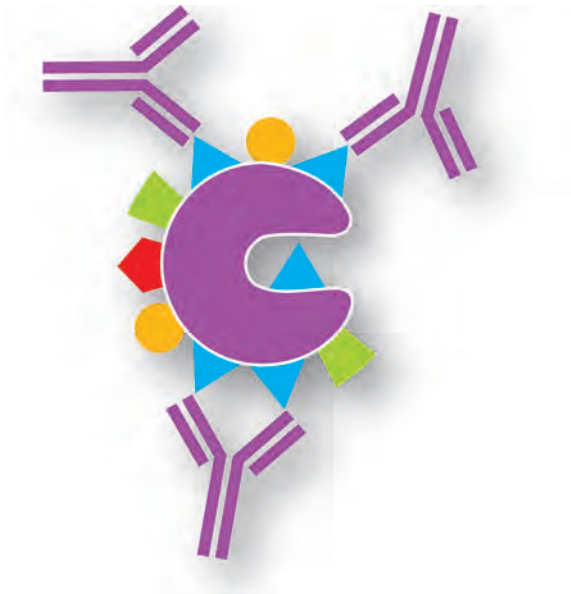


Figure 5. Monoclonal antibodies react with a specific epitope on an antigen.

In immunoassays, monoclonal antibodies have certain advantages over their polyclonal counterparts. These advantages include high homogeneity, high specificity, ease of characterization, and no batch-to-batch or lot-to-lot variability in affinity. However, some

pitfalls in the use of monoclonal antibodies should be noted. All too often, monoclonal antibodies are characterized using fresh or frozen tissue. If the antibody is to be used for formalin-fixed specimens, the targeted epitope must survive fixation. In some cases, polyclonal antibodies that target multiple epitopes within a single antigen have a better chance of detecting that antigen than monoclonal antibodies that are restricted to a single epitope. However, some antigens may have two or more identical epitopes and are described as multivalent.

The targeted epitope must also be unique to a given antigen. Specificity, one of the greatest benefits of monoclonal antibodies, is lost if the antibody recognizes an epitope shared by two or more different antigens (see Antibody Cross-Reactivity).

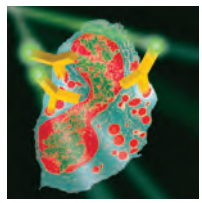
Antibody Affinity

Polyclonal antibodies from hyperimmunized animals not only differ with regard to which epitopes they recognize on a specific antigen, but also in their affinities for the different epitopes of the antigen. The intrinsic affinity of an antibody, or the strength of antibody and antigen interaction, resides in the hyper-variable (HV) region of the antibody molecule. This is the region, or sequence of amino acids, of the molecule that determines specificity. However, to say that the greater the specificity, the stronger the affinity, is probably an oversimplification. Covalent binding between antibody and antigen does not occur. The forces that hold the antibody and antigen together include ionic interactions, hydrogen bonding and van der Waals forces. These are the major contributors to the intrinsic affinity between the antibody and the antigen.

The association constant (Ka) describes the strength of the binding between an antibody and its antigenic determinant. It is a measure of the antibody's affinity. The higher the affinity of the antibody, the lower the concentration of free antigen needed for the available binding sites of the antibody to become saturated (reach equilibrium). The Ka for antibody and antigen binding can range from 10^3 to 10^{10} liters per mole, which is the reciprocal of concentration (moles per liter).

Just as the quantity (titer) of an antibody increases with time during immunization, so does its quality (affinity). This has been called "affinity maturation."[3] Lower doses of immunogen increase the rate of affinity maturation but result in lower titers of antibody, and vice versa.

The time required for the antibody to reach equilibrium or saturation with the cellular antigen is an important factor in determining optimum staining for flow cytometric analysis, sometimes referred to as functional affinity. If equal aliquots of two antibodies (or antisera) of identical titer are incubated for increasing periods of time with the cellular antigen, the antibody that reaches a plateau of maximum staining intensity first is of higher functional affinity. The term "avidity" is synonymous with functional affinity[3] and has also been used to denote the strength of the binding between antibody and its antigen.[4] Frequently, the term avidity has also been used to describe the sum total of all intrinsic affinities found in a polyclonal antibody population.



Because antigen-antibody reactions are reversible, the simple immune complexes formed on the cell may dissociate during the washing cycles used in flow cytometry. Hydrophobicity, the ability of water to force hydrophobic ("water fearing") groups of the complex together, appears to have a stabilizing affect on the antibody-antigen complex. Therefore, the antibody-antigen complex can be influenced by changes in salt concentrations, pH and/or temperature. The ease and degree of dissociation vary from antibody to antibody, and low salt concentrations as well as low temperatures will reduce the likelihood of weak staining due to dissociation of an already formed immune complex. Thus, high affinity antibodies are desirable and have the advantage that during washing, dissociation is less likely to occur than with low affinity antibodies. As mentioned before, a polyclonal population of antibodies contains a more or less continuous spectrum of low to high affinities against several epitopes on a given antigen.

Therefore, after incubation with primary antibodies of this type, excessive washing is unlikely to result in any appreciable loss of staining. On the other hand, monoclonal antibodies are of uniform affinity and, if the affinity is low, loss of staining is likely due to dissociation of the antibody from its epitope. Thus, monoclonal antibodies of high affinity should be selected, if possible. As indicated above, avoid factors that weaken the antigen-antibody bond such as high salt concentrations, high temperature and very low or very high pH during the washing of the cells.

Antibody Cross-Reactivity

The term "cross-reactivity" denotes complex formation between an antibody and two or more antigens or a reaction between an antigen and several different antibodies. Typical examples are when anti-lambda (or anti-kappa) chain antibodies interact with all five Ig classes or when carcinoembryonic antigen (CEA) reacts with antibodies against CEA, blood group antigens and normal cellular proteins. The common denominator in each case is when several antigens share at least one common epitope. Cross-reactivity of antibodies to human antigens that also recognize identical or similar antigens of other species (cross-species cross-reactivity) can be of interest to the researcher and veterinarian because of the scarcity of animal-specific antibodies. To overcome this, two publications reported the results of cross-species reactivity studies using commercially available anti-human polyclonal and monoclonal antibodies.[5-6] It was demonstrated that the majority of animal antigens selected showed strong reactivity with anti-human antibodies.

Antibody Saturation Rates

The size and shape of the antibody molecule and its conjugates affect staining measurements in flow cytometry. This is especially true for intranuclear or cytoplasmic staining. Controlled permeabilization of the cellular membrane is essential to allow the fluorochrome-antibody conjugate penetration into the cytoplasm or nucleus while maintaining cell membrane antigens for optimum extracellular staining.

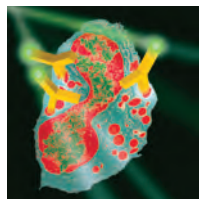
Under ideal conditions, antibodies react with their ligands (antigens) very rapidly; in flow cytometry the conditions are rarely ideal. Depending on cell fixation, antibody concentration, ambient temperature and other variables, antibody incubation times of up to 48 hours may be required for maximum saturation. It is not surprising, therefore, that as flow cytometry has become increasingly useful in clinical diagnostics, the need for shortened processing times has been voiced. Much shorter incubation periods are made feasible by the relatively rapid saturation rates that occur when higher concentrations of high affinity antibodies are used. However, higher concentrations of antibodies can result in high non-specific binding and may require time-consuming wash steps. The formulation of antibody-conjugates must take into account many factors for optimum staining and a timely result.

Once the staining procedure has been determined, with regards to antibody concentration, incubation time, temperature, and washing steps, the results should be stable and reproducible. If variation in any of the above factors is introduced, the staining procedure must be re-evaluated. For example, a new lot of antibody may have a different titer, or the new fluorochrome-antibody conjugate may have a different fluorochrome-to-protein ratio.

Antibody Stability

Antibody stability or degradation can also affect staining. The immunoglobulin fraction of polyclonal antibodies is somewhat less stable than whole antiserum.[7] This reduced stability depends largely on the method of purification, storage and application. Exposure of antibodies to extreme pH, as well as high or very low concentrations of salts, during affinity purification methods tends to decrease their stability more than does exposure to mild conditions such as ion exchange chromatography. Formation of soluble aggregates and subsequently, precipitated polymers are the most frequently resulting changes noted. These changes are probably the result of hydrophobic interaction between the IgG molecules in solution. While the presence of soluble aggregates may enhance their performance as precipitating antibodies, their increased hydrophobicity has been shown to cause increased nonspecific binding.[7] Removal of these aggregates and polymers from IgG fractions is, therefore, important prior to applications for flow cytometry. Just as storage of purified antibodies may augment their hydrophobicity due to aggregation and polymerization, so may their conjugation to other molecules, such as fluorochromes.[8]

Monoclonal antibodies also have been shown to be influenced in their performance by methods of purification and storage.[9] Antibody stability in commercially produced reagents is determined by real-time and real-temperature testing by each manufacturer. Most manufacturers demonstrate stability for a set period of time. While many antibodies may retain activity for a longer period of time, the regulatory requirement for the manufacturer is to certify the period of time that the antibody has been tested. There is no requirement to continue further testing until the antibody loses activity. Furthermore, it is this writer's experience that the conditions for the storage of reagents in the user's



laboratory are frequently not identical to those that prevailed during the shelf life studies. Because of the possibility of adverse storage conditions after the purchase of the product, the manufacturer can only offer a limited liability instead of predicting the actual demise of a reagent.

Antibody Handling

One of the easiest methods to ensure antibody stability is proper handling and storage. In order to achieve optimal performance from the reagents used in flow cytometry, it is imperative to observe certain basic rules for their handling and storage. If properly maintained, most reagents will remain stable for months and even years. The recommendations given by the manufacturer on specification sheets and on vial labels should always be followed.

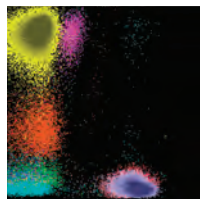
Receiving

Although many commercially produced immunochemicals are guaranteed to be stable for up to several years, more dilute antibodies formulated for few or no washing steps have a shorter shelf life. Upon receipt, antibodies and fluorochrome-antibody conjugates should be promptly stored according to the manufacturer's recommendations. Log in reagents by entering the manufacturer's lot numbers, expiration date, date of receipt and invoice number. These entries provide valuable information for the user to track their use and performance.

Storage

Perhaps the two most important considerations when storing antibodies are the storage container and the temperature. Ideally, storage containers for protein solutions should have negligible protein adsorptivity. Polypropylene, polycarbonate or borosilicate glass are recommended and are used widely. Solutions containing very low concentrations of protein (i.e. less than 10–100 µg/mL) should receive an addition of bulk protein. Generally, 0.1% to 1.0% bovine albumin is used to reduce loss through polymerization and adsorption onto the container. In addition, containers made of tinted material are preferred, as these will prevent quenching of fluorochromes. Container labels also should allow access for inspection.

Probably more than any other factor, observe proper storage temperature as recommended by the manufacturer. Monitor refrigerators and freezers used for storage of immunochemicals for accurate and consistent temperatures. Immunochemical reagents should be stored in equipment with temperature alarm and emergency back-up power systems. Store most "ready to use" antibodies and their conjugates at 2–8° C because freezing and thawing is known to have a deleterious effect on their performance. This also applies to entire kits that contain monoclonal antibodies. Store concentrated protein solutions, such as antisera and immunoglobulin fractions, in aliquots frozen at -20° C or below to prevent cycles of repeated freezing and thawing. Bring frozen protein solutions to room temperature slowly and avoid temperatures above 25° C.



Data Analysis

Stephen W. Pursley

Introduction

Flow cytometers in combination with powerful software programs provide investigators a sophisticated method to rapidly collect and analyze data on large cell populations. Commercial instruments are available that can process upwards of 70,000 particles per second and detect up to nine fluorescent “colors” and two light scatter signals per particle or cell. The capability to analyze multiple parameters simultaneously on a given cell has enabled researchers to better understand complex cellular processes such as oncogenesis, apoptosis or cell cycle regulation within phenotypically defined populations.

Analysis

Today’s software programs provide numerous options for displaying and analyzing flow cytometric data. One option involves histograms where investigators can look at the fluorescence pattern of a population for a single parameter. The histogram shown here (Figure 1) illustrates the phycoerythrin (PE) fluorescence pattern for a sample of 8-peak beads. When analyzed by a flow cytometer, these beads are designed to show eight separate populations with differing fluorescent intensities. The beads were excited by a 488 nm laser and the data was acquired and saved using a logarithmic scale. For many applications, including fluorescent 8-peak beads, data collection on a logarithmic scale is preferred to a linear scale. This is a common practice when working with fluorochromes to account for the broad range of fluorescence exhibited by these compounds.

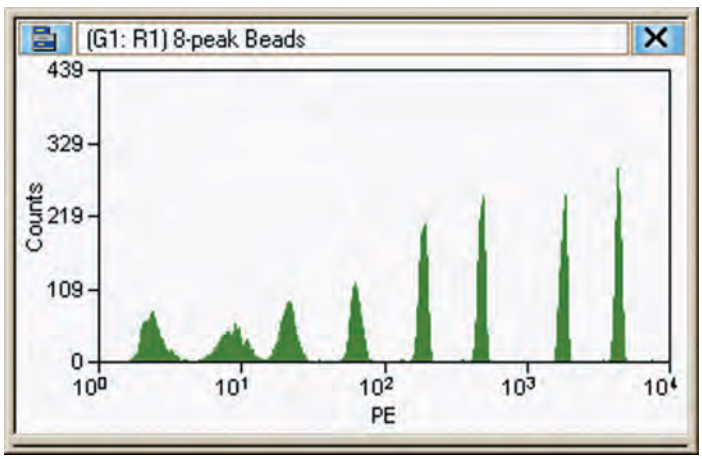


Figure 1. Fluorescence Pattern of 8-Peak Beads for the PE Parameter. The particles are distributed within the histogram based on detected signal intensity. Beads emitting a stronger signal are assigned to the higher channels along the x-axis, while dimmer beads are located in the lower channels.

An overlay constitutes a specialized type of histogram that permits the simultaneous display of parameter data from multiple samples (Figure 2). With this approach, individual samples can be compared based on a particular fluorescent or light scatter parameter, or control data can be overlayed against test samples to distinguish experimental significance. A few applications that benefit from this type of analysis include screening for the expression of specific proteins from reporter gene constructs, identifying cell lines with anomalous gene expression patterns, or monitoring gene expression of a population in response to external factors such as biologically significant compounds.

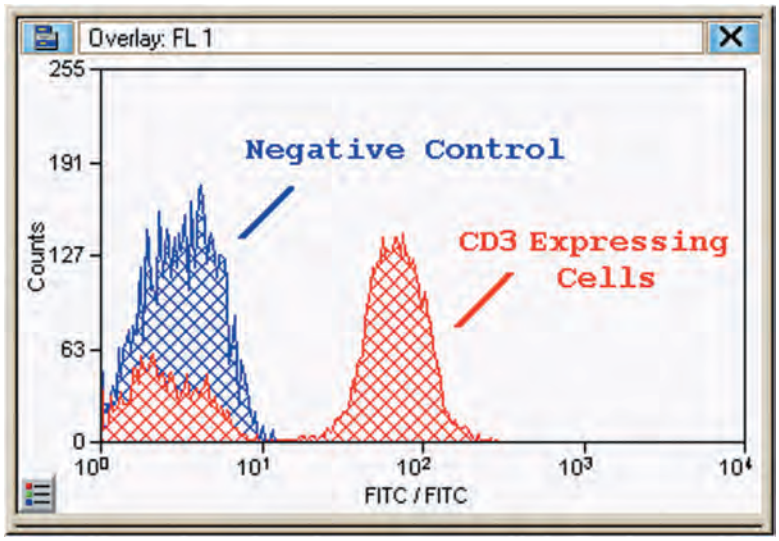
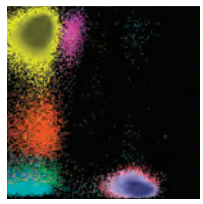


Figure 2. Overlay. Lymphocytes were stained and analyzed using FITC-conjugated anti-CD3 antibodies.

Dot and density plots provide the ability to correlate and assess fluorescent information simultaneously for two parameters. For example, this dot plot of Jurkat (JKT) T cells uses the forward and side scatter light parameters (Figure 3). The forward scatter (FSC) signal results from refracted light in the forward direction as cells pass the laser interrogation point and is usually captured using a photodiode coupled to a notch or bandpass filter configured for the emission spectra of the light source. This parameter has been correlated with particle size, while the side scatter parameter (SSC) tends to reflect cell granularity or internal morphology of the cell or particle.



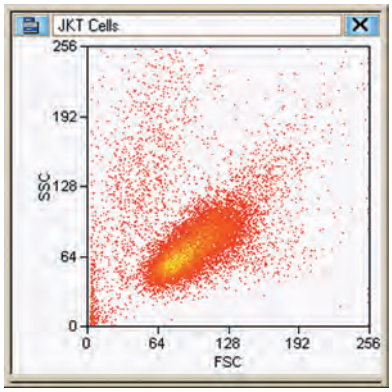


Figure 3. Dot Plot of Jurkat T Cells

Alternate methods of data display are available for two-parameter plots. One option is contouring, which displays the data as a series of lines, similar to what is observed with topographical maps (Figure 4). The contour patterns correlate to the distribution and density levels of cells or particles within the plot and can be used to aid in data analysis or to delineate populations of interest.

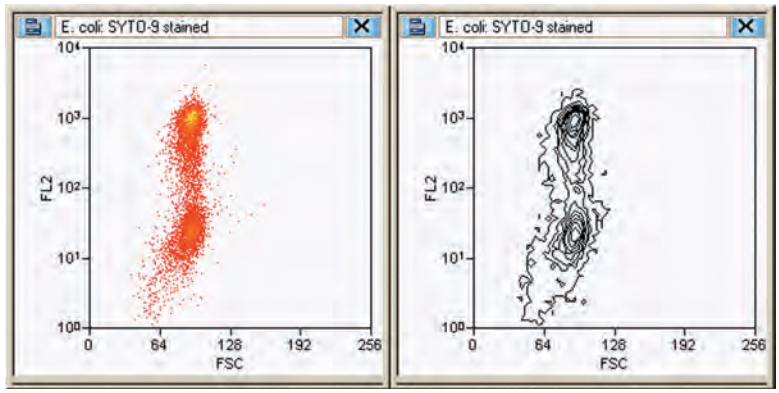


Figure 4. Dot and Contour Plots of *E. coli* Stained with the Nucleic Acid Dye, SYTO-9

Various statistics are available for assessing the data within histograms and dot plots. Common statistics include total counts, population percentages, mean, median, coefficient of variation (CV) and standard deviation. For example, total counts and percentages for specific cell populations can often be used in the clinical diagnosis of disease. In the case of HIV-infected patients, assessment of the T cells expressing the cell-surface proteins CD4 and CD8 can be used to determine a person's immunological status. The CV statistic is traditionally used in conjunction with beads for calibration and daily quality control of the instrument, while mean can be used to quantify the fluorescent intensity of a cell using standard calibration units or Molecules of Equivalent Soluble Fluorochrome (MESF).

Regions can be created within histograms and dot plots to generate statistics on sub-populations. In the case of disease diagnosis, clinicians often rely on the statistics from various lymphocyte subsets to characterize and treat many types of leukemia and lymphoma. Study of these disorders is typically based on identifying those cells containing certain expression patterns of cell-surface proteins. Factors such as total cell count, population percentages, "brightness" or fluorescent differences between populations, or statistical ratios between populations can then be used to analyze and quantitatively assess the data.

Regions can also be used for the creation and application of gates. Gates may involve one or more regions and are used primarily to minimize or eliminate extraneous events within an analysis plot or to isolate specific cells and particles of interest. Some software programs also provide the ability to assign colors to those events that fall within a particular region or gate, thus enabling easy identification of those cells within other histograms or dot plots.

The dot plots in Figure 5 illustrate the pattern of whole blood when analyzed on a cytometer. The plot on the left highlights a few of the major blood cell populations that can be identified using the forward and side scatter parameters. In this experiment, the cells are stained with fluorescently labeled antibodies directed against specific cell-surface antigens, specifically a FITC-conjugated antibody against CD3, a PE-conjugated antibody against CD19, and a PE-Cy5-conjugated antibody against CD45. A gate specific for the lymphocyte population (R1) is created and applied to other dot plots containing experimentally relevant parameters. This allows the identification and analysis of lymphocyte subpopulations expressing various surface proteins. The middle plot identifies those lymphocytes expressing both CD3 and CD45 (R3). Lymphocytes expressing CD45 but not CD3 are located within region R2, while cells expressing neither CD45 nor CD3 appear within region R4. The dot plot on the right uses color gating to highlight those cells expressing either the CD3 or CD19 antigen. Lymphocytes expressing CD3 appear green, those expressing CD19 appear blue, and those expressing neither protein appear magenta. This capacity to simultaneously analyze multiple parameters for a given cell population is one of the great benefits of using flow cytometry.

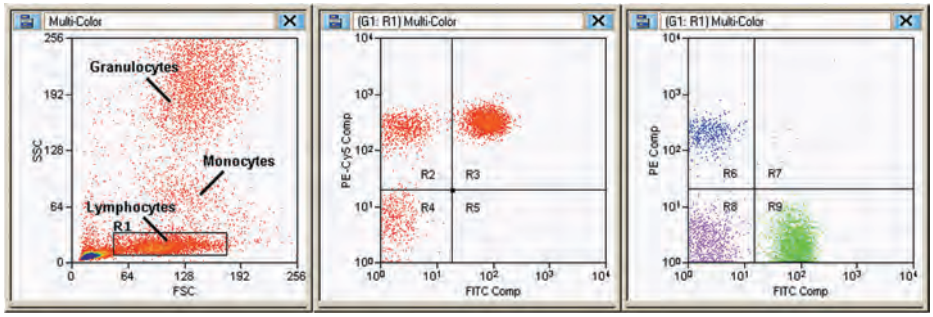
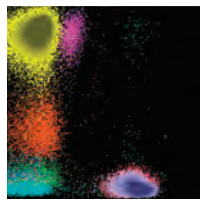


Figure 5. Dot Plot and Gating Scheme to Analyze Lymphocyte Subpopulations. The data is compensated to correct for fluorescence interference between fluorochromes.



Compensation

An important consideration when conducting multicolor experiments is the possibility of fluorescence interference created by dyes or fluorochromes that possess close or overlapping emission spectra. Many flow cytometry programs have algorithms that can mathematically correct for this spectral overlap. In the world of flow cytometry, this process is known as compensation. Compensation refers to specific software or hardware manipulations that mathematically remove fluorescence overlap to simplify multicolor data interpretation and distinguish populations on a dual-parameter histogram. One benefit of this process is that it ultimately enables researchers to better delineate populations based on the expression or non-expression of specific cellular components, such as cell surface antigens. Though many of the issues related to compensation are well beyond the scope of this chapter, compensation currently represents one of the foremost obstacles to conducting multiparameter experiments in flow cytometry.

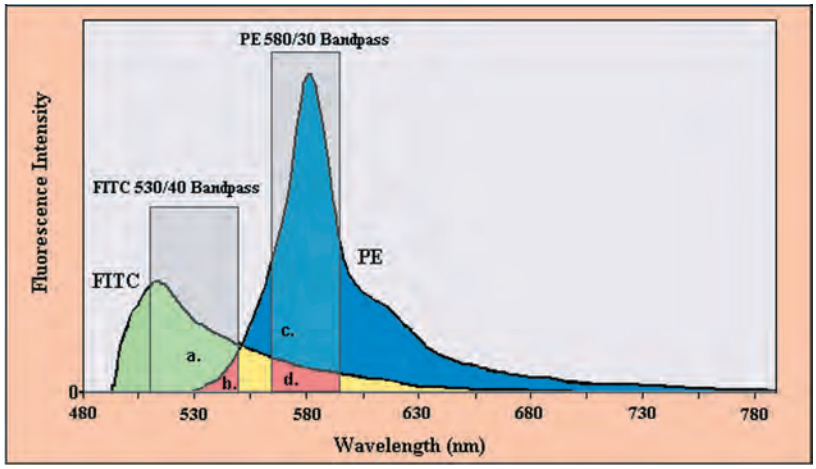


Figure 6. Spectral Emission Graph. Areas **b** and **d** indicate expected fluorescence spillover into the FITC and PE detectors.

How It Works

Dyes such as FITC and PE, which are excited at 488 nm, will fluoresce optimally at 520 and 576 nm, respectively. Filters are typically used to allow photodetectors to respond to a specific fluorochrome, such as FITC or PE. However, because the emission wavelengths of these dyes are sufficiently broad, light from a PE fluorochrome will leak through the filter of the FITC detector and vice versa. Without spectral overlap, a population stained only with FITC would have the same PE signal as an unstained population, and a population stained only with PE would have the same FITC signal as an unstained population. However, as a result of the spectral overlap that occurs between the two signals, the FITC- and PE-positive populations will appear offset, making it difficult to accurately distinguish true populations. For example, a FITC-positive population that is not stained with PE will appear to have some PE staining because of

the spectral contamination from FITC into the PE channel. The degree that populations are shifted with regard to the ideal position represents the spectral overlap from one fluorescence signal into the other. This phenomenon is a result of what is physically observed by the cytometer's photodetectors for each parameter and is illustrated in Figure 7 for a two-color experiment involving FITC and PE.

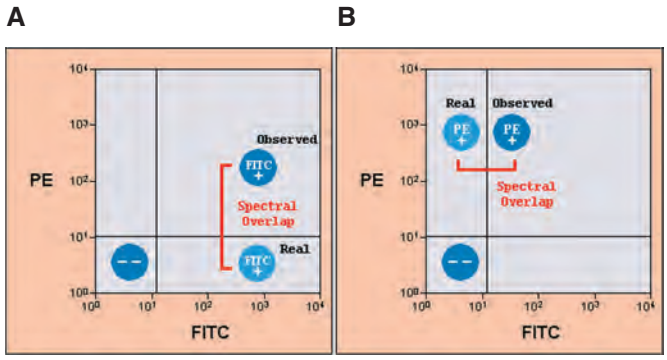


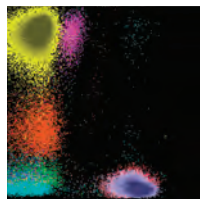
Figure 7. Compensation. This illustration demonstrates the observed fluorescence spillover of FITC into PE (A) and PE ito FITC (B).

Data Storage

An advantage of flow cytometry is the ability to simultaneously detect and analyze multiple parameters. Information collected from most commercial flow cytometers is saved in a standardized file format known as FCS (Flow Cytometry Standard). Adoption of this uniform file format has given researchers the ability to collect data on various instruments, then subsequently analyze that data using a variety of software programs.

Conclusion

While there are numerous technologies and methods available to study cell populations, flow cytometers are distinguished by their capacity to collect and process large amounts of data expeditiously. As new genes are discovered in the post-genomics age, understanding their biological significance within cells, in the context of other genes and gene products, is essential. By having a system to detect, view and analyze the expression patterns of multiple proteins involved in complex biological processes such as apoptosis, oncogenesis or cell division, investigators are able to better understand the possible combinatorial roles specific proteins play in these processes. The genetic activity can then be correlated to morphologic and phenotypic changes in the cells. In the future, the advantages of flow cytometry, including data storage and analysis, should allow this science to retain its prominence in the continued study and understanding of complex cellular processes.



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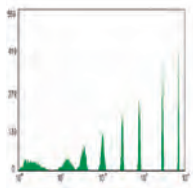


Standardization, Calibration and Quality Control

Ian Storie

Flow cytometry has become an essential tool in the research and clinical diagnostic laboratory. The range of available flow-based diagnostic tests and the number of worldwide laboratories that possess a flow cytometer are ever increasing. Results produced from such laboratories need to be as accurate and reliable as possible, since disease diagnosis, treatment and monitoring of treatment is dependent upon them. How can we be confident in our results and know that our results are comparable with other laboratories performing the same test? Clinical flow cytometrists must always bear in mind the sample they are testing comes from an actual person, and they owe it to that person to ensure the results are as accurate and reliable as possible. One solution is the establishment of methodological standardization both in-house and across laboratories, as well as the implementation of stringent internal and external quality assessment procedures. Research flow cytometrists can benefit from these procedures as well.

Standardization

From the taking of the patient's sample to the issuing of the final results, there are many steps involved, each one having the potential for the introduction of an error. The implementation of standardized protocols for each step of the process can reduce the potential margin for errors to occur. Some organizations have produced written guidelines that suggest optimal techniques to be used for a particular application. However, these guidelines may not in themselves be compulsory, and an individual laboratory can choose not to follow them. Whether a laboratory decides to follow a guideline or not, it should look at each step of the diagnostic process and validate that step to ensure that the results obtained are reliable. The following list, which is by no means complete, is intended to help the reader think about potential variables and where errors may occur.

Pre-Analytical Variables

- Has the sample been taken correctly?
- Is the sample labeled correctly? If not, then all that follows becomes a pointless exercise.
- Is the sample in the correct anticoagulant for the test required?
- Has the sample been stored/transported correctly?
- What is the maximum recommended time allowed between taking the sample and performing the test?

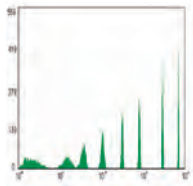
Analytical Variables

Staining

- Is the titre of the antibody correct for the number of cells being added? Using excess antibody is not only a waste of money but can also lead to problems with nonspecific binding. Use insufficient antibody, and the staining will be suboptimal. Antibodies for multicolor applications and intracellular staining also need to be correctly titred.
- Is the most appropriate antibody being used? For example, class 1 antibodies should not be used for CD34 enumeration.[1] Different clones of antibodies can give different results when looking at antigen quantitation.
- Which fluorochrome should be conjugated to each antibody? A general rule of thumb is that an antibody staining a weakly expressed antigen should be conjugated to the brightest fluorochrome.
- Are the antibodies being stored correctly? Some of the tandem fluorochromes are easily broken down by exposure to excess light.
- Are the antibodies within the expiration date supplied by the manufacturer?
- If the test is in kit form, are the manufactures recommendations being adhered to? If not, then is there adequate documentation to validate the changes made?
- How long and at what temperature should the cell-antibody reaction occur?

Lysing

- Has the lyse been made up correctly? What volume, for how long and at what temperature should incubation occur?
- What is the best procedure for the application — no lyse, no wash; lyse, no wash; or lyse, wash?
- After staining and lysing, should a fixative be added? How and for what length of time should the samples be stored before analysis?
- Are there any known conditions or substances that may interfere with the test being performed?



Data Acquisition, Analysis and Interpretation

- Has the instrument been set up correctly?
- What is the best gating strategy for the application? Are an appropriate number of events being acquired to ensure reliable results?
- Are appropriately trained and qualified personnel interpreting the data?

It is impossible to try and answer all the questions posed here. In fact, there are probably as many different answers as questions, which highlights why standardization is required.

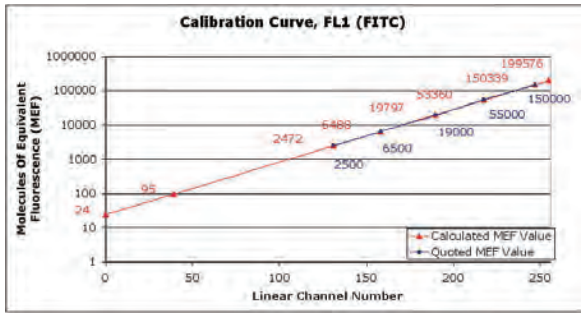
When a laboratory has standardized its protocols, what advantages does this bring? It increases the reliability and consistency of the results produced by the laboratory, resulting in increased confidence from both within and outside the laboratory. It can reduce waste and instances when samples need to be re-stained, thus saving costs. Suitable reference ranges can be produced that are applicable to the test being performed. The limits of the test can be established. The procedure can be documented in the form of "standard operating procedure" for personnel to follow. Training programs can be established for both new and experienced personnel. The education and training of staff is very important, especially in situations where there are multiple staff members who may rotate around other departments or work shifts.

Within Europe, the issues of standardization are being addressed with the formation of the Eurostandards Project. The aim of this project is the production of stabilized reference materials for lymphocyte subset analysis and enumeration, leukemia-lymphoma phenotyping, and CD34 stem cell and low-level leukocyte enumeration. These reference samples are to be used to define and optimize flow cytometric and analysis techniques, resulting in better standardization of these procedures.

Calibration

Calibration of a flow cytometer's response to fluorescence signals is possible using microbeads, and there are many types of beads available.[2] It is important to ensure that the instrument's response to fluorescence signals is both stable and reproducible over time, because the fluorescence signal intensity is used to discriminate between positive and negative populations.

Using a mixture containing a blank and a minimum of four or more microbeads, with each microbead having a predefined level of fluorescence intensity, calibration of the fluorescence scale can be performed. Plotting the mean or median channel number against the predefined fluorescence value (Figure 1) and applying linear regression analysis to these values results in the production of "performance parameters" for each fluorescence channel tested. Table 1 explains the derived performance parameters.



Performance Parameter	Value
r^2	0.9997
Coefficient of Response	65
Detection Threshold	95
Zero Channel	24
Maximum Fluorescence	199576
Log Decades	3.94
Average Residual %	1.73

Figure 1. Calibration Curve and Calculations for FL1 Channel (FITC)

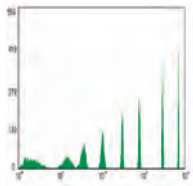
Table 1. Performance Parameters

Average Residual %	The absolute average % that the calculated regression line varies from the actual data points. Indication: The fit of the calibration line and the confidence of the instrument's response across the fluorescence range.
Detection Threshold	The value of the blank bead in units of fluorescence intensity. Indication: The lowest level of fluorochrome that can be detected by the instrument.
Coefficient of Response	The slope of the calculated regression line, determined using a 256-channel scale. Indication: The range of sample space covered by the instrument. For a four-log- decade instrument, the coefficient of response should theoretically be 64. (256/4 = 64).
Zero Channel	The value in units of fluorescence intensity where the calculated regression line intercepts the fluorescence intensity axis in channel zero. Indication: The left-hand boundary of the instrument's sample space.
Maximum Fluorescence	The value in units of fluorescence intensity where the calculated regression line intercepts the fluorescence intensity axis in channel 255. Indication: The right-hand boundary of the instrument's sample space.
Log Decades	Calibrated full-scale or dynamic range of the detector being examined.

Acceptable ranges can be determined for each performance parameter from each fluorescence channel monitored. The results can then be compared to these acceptable ranges, outlying results can be detected, and gradual changes in performance can be monitored using Levey-Jenning plots, which look at mean fluorescence over time.

Quality Control

Quality control in flow cytometry can be divided into two parts — internal quality control and external quality control. External quality control is a means of comparing how well your laboratory performs compared to others. This may be through locally organized "informal" schemes or more national/international "formal" schemes, which are usually overseen by an external authority. Some of these authorities may have the power to initiate systems to close down a laboratory if the laboratory is a consistent poor performer.



Internal Quality Control

Internal quality control consists of a series of activities that are performed by the laboratory to ensure the instrument, reagents and staff are performing within the limits set by the laboratory. These involve the use of microbeads and/or biological controls.

Quality control of the instrument can be divided into three separate procedures.[3] The first procedure is usually carried out once or twice a year by qualified service personnel who check the performance of components such as the lasers, photomultiplier tubes (PMTs), optical filters, and log and linear amplifiers. This may involve re-alignment of the laser and optical system to ensure the best signals with the lowest CVs. With the stream-in-air system found on most cell sorters, the optical alignment needs to be performed daily by the operator.

The second procedure is performed by the operator with each start-up of the instrument. Using appropriate reference microbeads with dedicated plots and instrument settings, parameters such as CVs and mean or median channel number can be recorded. Using Levey-Jenning plots, the data can be monitored for variation and trends. Tolerance limits can be assigned and protocols established to deal with values outside these limits.

The third procedure consists of the calibration of the fluorescence channels. This has already been discussed above.

Immunophenotyping is a very complex process and should be monitored by the use of appropriate control material. Fresh normal samples are ideal, as they mimic exactly the test material (e.g. fresh blood specimens). However, due to biological variation and availability of persons willing to regularly donate samples, it maybe more appropriate to use commercially available preparations. These are preparations of either normal/abnormal cells or cell lines that are fixed to preserve light scatter and antigen staining characteristics. They can either be suspensions of cells with no red blood cells present, known as part process controls, or with red blood cells present, known as full process controls. There are many commercially prepared samples available. Because of their stability, they can be used to monitor day-to-day reproducibility of the test being performed, and lot-to-lot reproducibility of reagents. They can also be used to establish scatter profiles of particular cell populations and demonstrate appropriate antibody binding. Some preparations have predefined percentages and/or absolute numbers of cell subsets present. Full process controls can also be used to check the lysing process. They can also be used for the teaching, training and assessment of staff members to ensure they understand gate positioning as well as the gating procedure being used.

External Quality Control

Samples, either fresh or stabilized, are distributed on a regular basis, and the laboratory is asked to process them as they would any other sample. Results are submitted back to a central agency for analysis, and a report indicating the relative performance of each participating laboratory is issued. Within national/international schemes, anonymity of each laboratory should be maintained. External quality control schemes check the complete process, from the technical side of sample preparation (staining, data analysis, interpretation) as well as the administrative side (Have the results been transcribed accurately?).

The advantages of participation in an external quality control scheme are many. It facilitates direct inter-site comparisons, performance assessment and detailed method assessment, as well as highlighting methods, procedures or reagents that under-perform. Participation in external quality control schemes is also becoming increasingly required for regulation and accreditation. The larger schemes, due to the large number of participants, can generate very robust data that can identify problems early. The data can be viewed as a whole or even subdivided on an individual country basis, if there are enough participants. Participation should also be seen as having a primarily educational role.

Conclusion

As the role of flow cytometry in disease diagnosis and treatment monitoring is becoming more and more important, flow cytometrists need to ensure the results produced are as accurate and as reliable as possible. Standardization of protocols, analysis and data interpretation both within a laboratory and across laboratories will help to ensure the consistency of the results produced and allow better data comparison from multiple sites.

If not already in place, implementation of regular internal quality control procedures should become part of the daily routine of the flow cytometry laboratory. Participation in an external quality control scheme, where available, is strongly advised, if not obligatory. A great deal of data can be generated comparing techniques, methods and reagents to which a new or more experienced laboratory can refer when deciding upon a new technique or changes to an existing technique.

The following web sites contain further information on guidelines and quality control issues:

www.bcshguidlines.com: British Committee for Standards in Haematology.

www.cap.org: U.S. College of American Pathologists.

www.cdc.gov/mmwr: U.S. Centers for Disease Control and Prevention.

www.eurostandards.org: Eurostandards.

www.ewgcca.org: European Working Group of Clinical Cell Analysis.

www.nccls.org: U.S. National Committee for Clinical Laboratory Standards.

www.niaid.nih.gov/daids: U.S. National Institute of Allergy and Infectious Diseases, National Institutes of Health, Division of Acquired Immunodeficiency Syndrome.

www.ukneqasli.org: United Kingdom National External Quality Assessment Scheme for Leucocyte Immunophenotyping.

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2. Schwartz A, Marti GE, Poon R, Gratama JW, Fernández-Repollet E. Standardizing flow cytometry: a classification system of fluorescence standards used for flow cytometry. Cytometry 1998; 33:106-14.
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Flow Sorting

Terry Hoy, PhD, FRCPath

Introduction

Sorting is an elegant use of flow cytometric technology that is attracting new attention from diverse segments of biology and industry. This technology provides the powerful yet unique ability to rapidly isolate pure populations of cells or particles with a desired set of biological characteristics. These populations are then available for morphological or genetic examination, as well as functional assays and therapeutics.

Historically, this technology was developed to facilitate sorting of cells and other particles and it quickly caught on in the study of immunology for purification of lymphocyte subsets. Applications have continued to broaden since then, so that they currently range from ecological monitoring to industrial enzyme production. To mention any of these applications in detail would not be possible here. However, following is a selection of techniques to illustrate the versatility of flow sorting:

- Single-cell cloning of hybridoma cells for the production of monoclonal antibodies
- Sorting progenitor cells in the hunt for pluripotential stem cells
- Purifying different lineages, including stem cells, from bone marrow samples
- Sorting transfected cells with an expression marker, such as green fluorescence protein
- Multiparameter isolation of cells from mixed populations
- Sorting spermatazoa utilizing the difference in DNA content between those bearing the X and Y chromosomes
- Single-cell sorting for clonogenic assays

Principles

Two methods exist for sorting particles by flow cytometry: electrostatic and mechanical. For the electrostatic method (Figure 1), the particles are ejected through a nozzle and forced to break up into a stream of regular droplets by applying a vibration to the nozzle. Droplets containing particles of interest can be deflected from the main stream by applying a charge to those droplets. With the mechanical method (Figure 2), particles of interest are diverted within the flow cell, either by moving a “catcher” tube into the stream or by deflecting them by an acoustic pulse into a fixed tube. The pros and cons of the two methods are listed in Table 1. Most of the discussion that follows applies to both technologies, but the wording is directly appropriate to the more common electrostatic methodology.

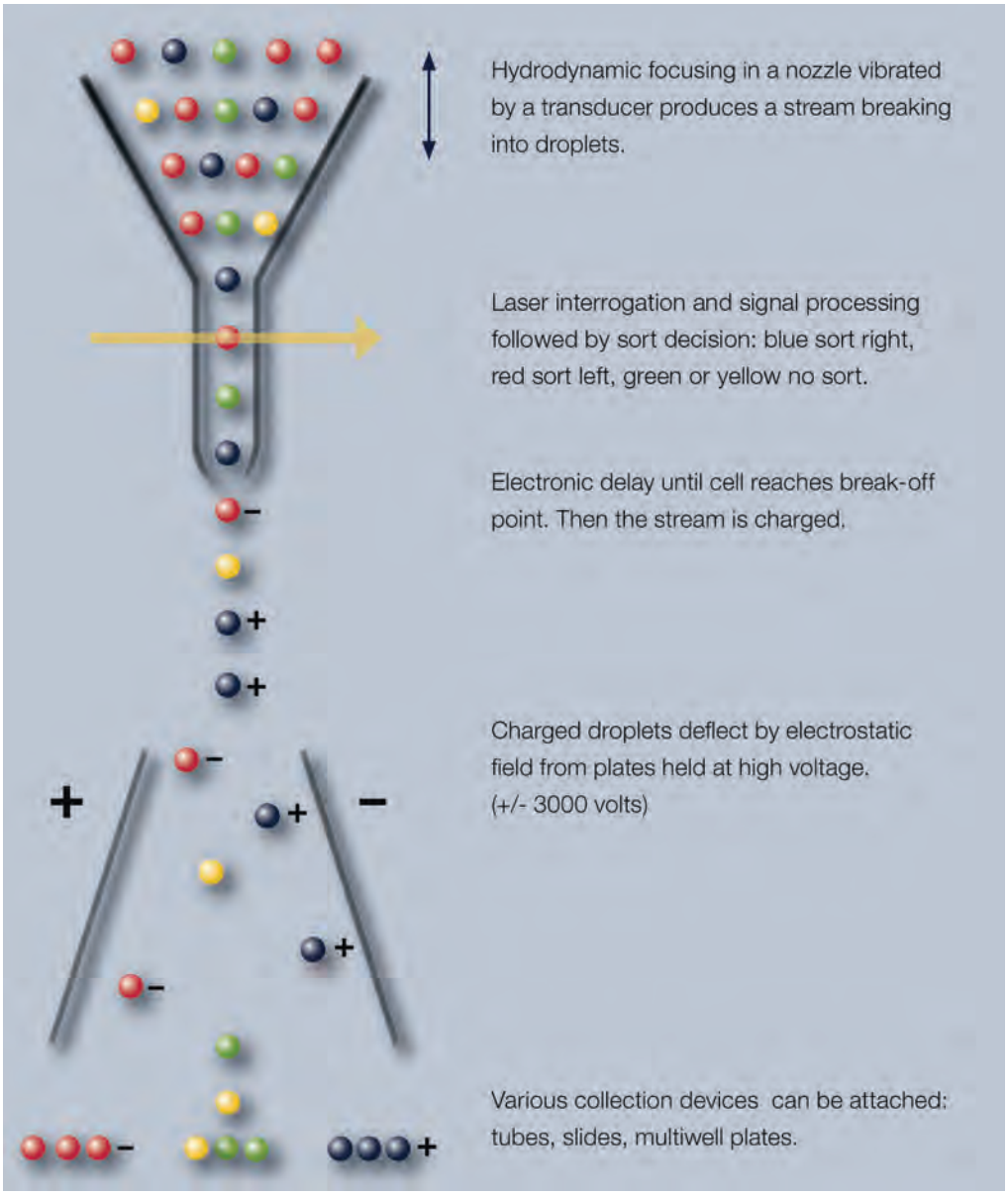


Figure 1. Electrostatic Sorting

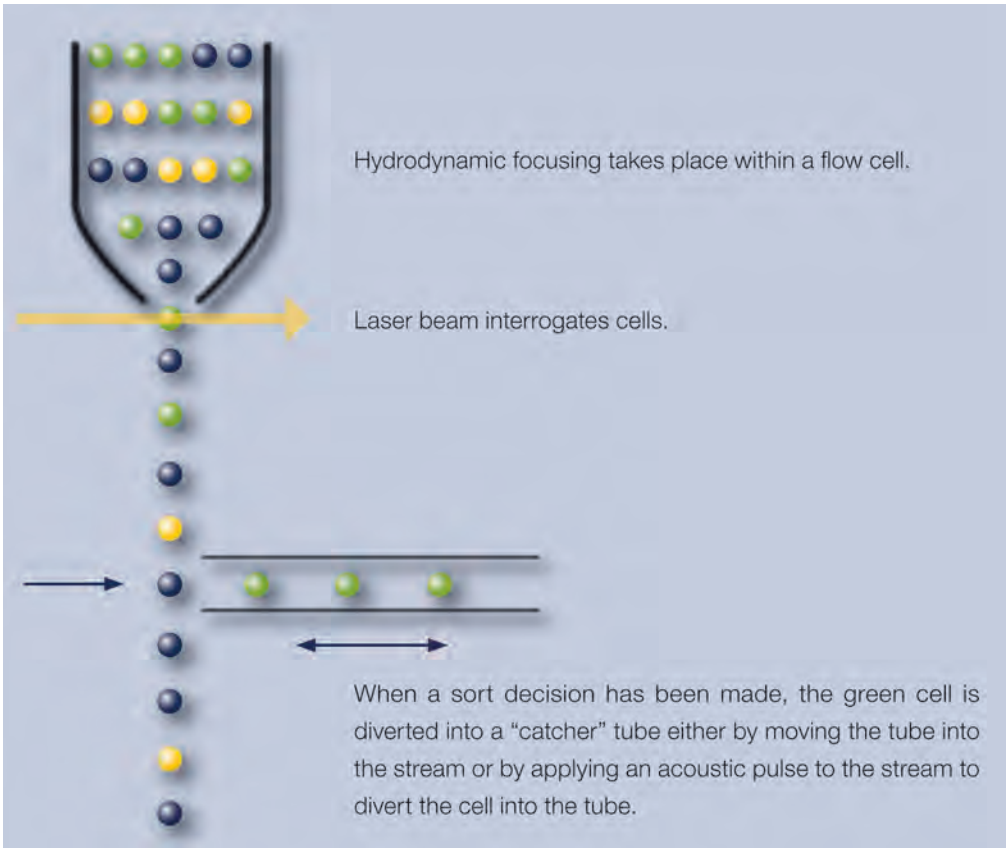


Figure 2. Mechanical Sorting

Table 1. Electrostatic vs. Mechanical Sorting

Electrostatic (Stream in Air)	Mechanical
Sorts up to 100,000 events/second	Processes up to 10,000 events/sec but only sorts up to 300 events/sec
Produces droplets (aerosols)	Fully contained
Prone to blockages	No nozzle to block
At least two sorts – four possible	Only one sort
Sorts at a reasonable concentration	Usually requires a "concentrator"
Multiple receptors available (tubes/slides/multiwell plates)	Not applicable

Yield, Recovery and Purity

Some important parameters used to describe the success of cell sorting are yield, recovery and purity. However, end users often misunderstand these terms.

Yield (Y) is the proportion of sorted particles of interest (S) compared to the total number of particles of interest that could have been recovered from the preparation under ideal conditions (N):

$$Y=S/N$$

Recovery (R) is the proportion of sorted particles of interest (S) compared to the total number of particles of interest satisfying the sort decision (E):

$$R=S/E$$

As will be discussed later, certain sort decisions can be aborted, and in extreme cases, $R<<1$. The operator can control some of these decisions.

Purity (P) is the proportion of sorted particles of interest (S) compared to the total number of particles in the sorted material (T):

$$P=S/T$$

Purity can be, and often is, very high (>98%), but a simple approach to how the material is re-analyzed can often lead to apparently lower purities than are actually achieved.

Enumerating S, N and T requires care if accurate estimates of Y, R and P are to be determined. Counting particles of interest in the sorted material (S) and initial preparation (N) is best approached with a counting chamber, such as a hemocytometer, where the number of particles in a given volume can be determined. However, sorted volumes are often quite small, and estimating the total sorted volume may only be possible as an approximation resulting in errors in S. To determine N, the experimenter must also determine the percentage of positive events. This can be taken from data acquired during the sort or from an independent measurement supplied by the user.

The cell sorter will usually provide the number of particles satisfying a sort decision (E). However, this may not take into account any decisions that may subsequently be aborted. These fall into two categories – hard and soft aborts. Hard aborts are invoked by the firmware, when a second particle arrives at the point of analysis before analysis of the first is complete (i.e. within the dead-time of the electronics which, for the MoFlo (Dako) is 5.5 µsec. The operator has no control over hard aborts. Soft aborts are invoked by the software and can be controlled by the operator. To explain the different situations that might arise, some thought needs to be given to the sorting process, remembering that cells are delivered to the point of analysis in a random sequence and at random time intervals. There is no technology available that allows this randomness to be avoided, so having detected a particle that meets a sort criterion, a decision has to be made about how to proceed, especially if that particle is followed closely by another particle that may or may not satisfy the sort criterion. The fluidic control on early flow

sorters resulted in some uncertainty as to which droplet a particle would be in, and for this reason it was common practice to sort a droplet either side of the most probable position (Figure 3).

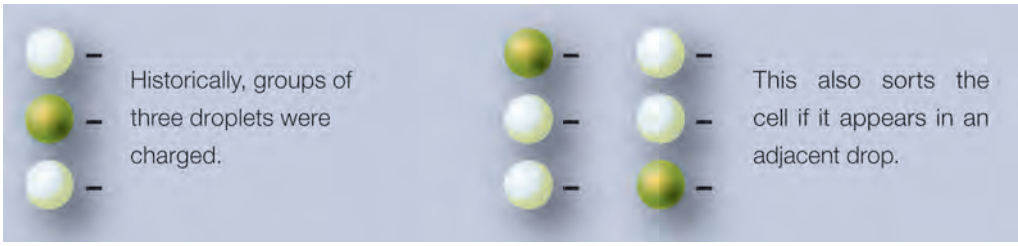


Figure 3. Droplet Charging

The particle following this event may be separated by a few droplets (Figure 4A), which presents no problem but statistically will sometimes be very close (Figure 4B). Obviously, there is a probability that the unwanted particle will be within the sort envelope of the first event, in which case it would be co-sorted. Depending on the requirements for the particular experiment, the sort decision can be aborted, and the charge not applied. If recovery is important, the sort would take place with an increased risk of contamination, but if purity is the prime concern, aborting the decision would cost the desired particle, thereby reducing recovery.

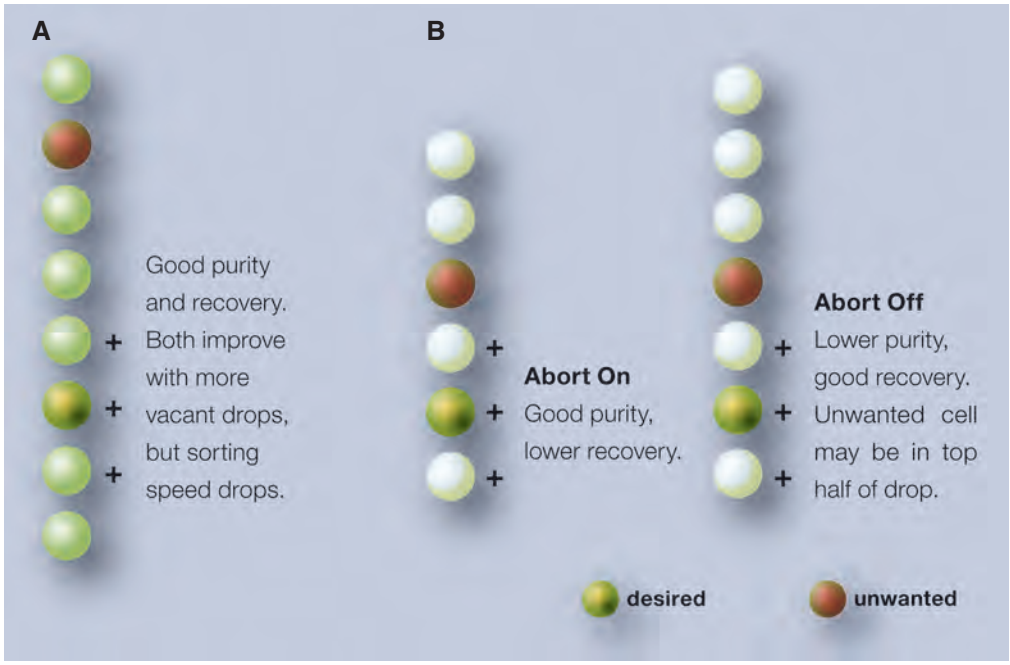


Figure 4. Recovery and Purity

As the technology for controlling fluidics improved, it became more certain as to which droplet a particle would be in, but when the particle lies close to a potential boundary, there will always be an uncertainty.

To handle this situation, modern sorters operate at a much higher resolution (1/16 of a droplet, in the case of the MoFlo) and give a more sophisticated approach to controlling soft aborts. Figure 5 indicates the possibilities. With situation C, the particle is located in the central half of the region between droplet boundaries, and the single droplet can be sorted with confidence. When the particle lies close to a boundary, the situation can be handled by sorting droplet A at the risk of losing a few particles or by sorting A+B, which will improve recovery but increase the overall volume slightly. These are known as one drop and one-two drop sorting: In the former, only A and C would be sorted, and the latter, A+B and C. When single cell cloning is attempted, it is often important to ensure single cells are deposited in every well. Only cells meeting situation C are then sorted, eliminating the possibility of sorting empty droplets. This is referred to as half-droplet sorting, the logic being that the cell lies within the center half of a droplet. When sorting very small populations, the best strategy is to run the sorter as fast as possible without invoking too many hard aborts and disabling all soft aborts to enrich the sample.

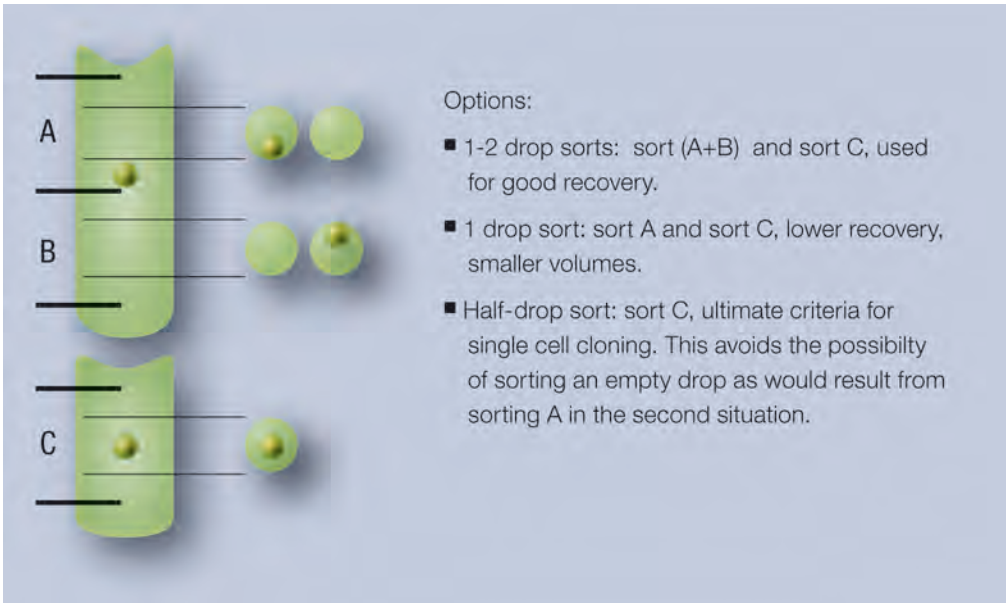


Figure 5. Controlling Soft Aborts

As illustrated in Figure 6, by triggering the system to see only the particles of interest and sorting every time triggering occurs, rapid enrichment is often possible. The enriched material can then be purified by a second “conventional” sort. Colleagues in my laboratory have used this approach for preparing stem cells which, after two passages through the sorter, are still viable and can develop into more mature lineages upon differentiation.

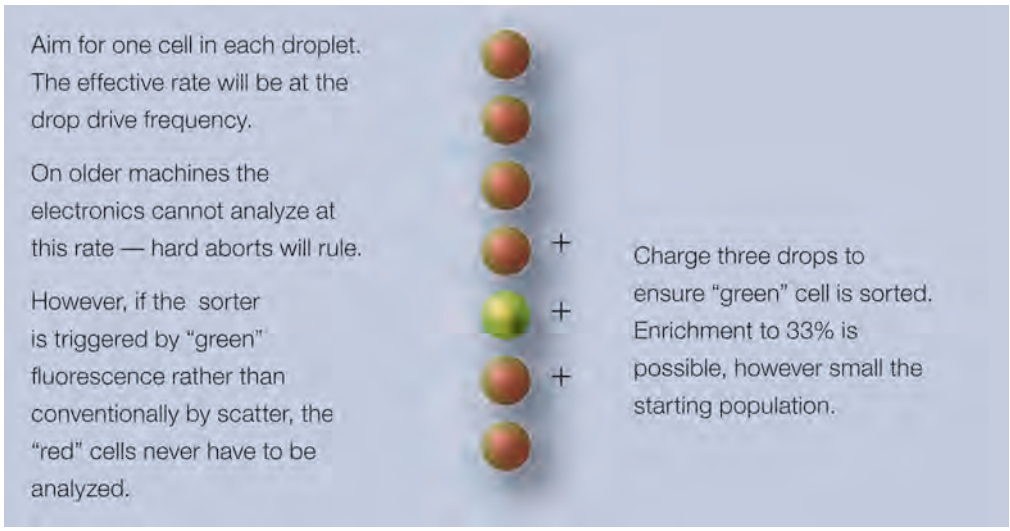


Figure 6. High-Speed Enrichment of Minor Populations

Other possible sources of error arise from the nature of sorted droplets. These droplets will all have identical electrical charges and, therefore, have a tendency to repel one another if sorted into an empty tube. Being extremely small, they evaporate quickly, and cells may be lost as a consequence. Even when sorting into a volume of liquid, the liquid will eventually acquire a charge, causing some droplets to be repelled before coalescing with the liquid. Apparently low recoveries and yields may well result from these losses, especially if small number of particles have been sorted.

It is noticeable that when sorting "hard" particles, such as fluorescent calibration beads, recoveries can be significantly better than when sorting cells. It is worth remembering that after immunofluorescent labelling involving several incubations, centrifugations and washings, the cells are then subjected to:

- Pressures of 60 psi
- Rapid acceleration to 20 m/sec
- Exiting a small orifice
- Returning to atmospheric pressure
- Passing through laser beams
- Charging to a few hundred volts
- Passing through electric field of several Kilovolts/cm
- Hitting a liquid surface still travelling at 20 m/sec

It should, therefore, come as no surprise that not all cells survive this process.

Once again low recoveries and yields will be apparent, but a further phenomenon can affect the calculation of the purity. A single cell may fragment into several pieces, and care must be taken not to count these as part of T. This is best accomplished by slightly increasing the scatter threshold when reanalyzing sorted material. It is also advisable to check any regions used to define the sorting conditions as it is possible that 1) the

fluorescence may have faded or become photobleached during the primary sort, and 2) if "tight" gates have been applied, material that is inside a gate on one pass may be measured as outside on a subsequent pass for statistical reasons. Hence, slightly larger regions are advisable for reanalyzing sorted material.

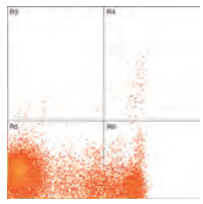
High-Speed Sorting

The maximum rate of sorting particles by flow methods is obviously limited by the formation of droplets. Theory shows that droplet formation is proportional to stream velocity and inversely proportional to stream diameter. Stream diameter is defined by the size of outlet nozzle, which has to be of sufficient diameter to allow the passage of any particles without blockages. For most applications with cells, an orifice of at least 70 µm is used, and the only method of increasing the rate of droplet formation is to increase the stream velocity by increasing the pressure within the nozzle. Older generation sorters operated at about 20 psi and produced about 30,000 droplets/sec with a 70 µm nozzle. The introduction of fluidic systems capable of running at 60 psi has now increased the droplet formation rate to 100,000 per second with a 70 µm nozzle. This equates to 10 µsec per droplet. Although sorting at these speeds is now possible, the number of hard aborts increases significantly. A second problem arises from the density of particles required: A rule of thumb is that a concentration of $n \times 10^6$ particles/mL will flow efficiently at $n \times 10^3$ particles/sec. To approach 100,000 particles/second, a concentration of 100×10^6 particles/mL would be required. For cellular based procedures, this represents a somewhat viscous concentration that becomes prone to forming clumps and is, therefore, not practical to work with. Realistically, few sorters operate above 50,000 events/sec when processing cells. Smaller particles (beads, bacteria, chromosomes) can be processed through smaller nozzles, which will have a higher rate of droplet formation, but hard aborts will eventually become significant.

Before embarking on serious cell sorting it is worth considering how long sorts will take to produce the number of cells required. For typical recovery of a 1% population at 50,000 events/sec, or 1.8×10^8 events/hour, no more than about 10^6 events/hour will be obtained. Although this figure may be sufficient for most clonogenic type assays, it does not represent a large number for typical "bulk" assays or for preparing subcellular material. Longer sorts may provide a solution for some assays using cultured cells, but for animal studies, the amount of starting material may be the limiting parameter. In either case, if antibody-based assays are being utilized, the cost may become prohibitive. Exceptions are, of course, modern molecular biology assays, which can be conducted on very few cells or even single cells.

Viability and Function

Much has been written concerning the viability and function of cells that have been flow sorted, citing extremely bad to very favorable results. In my own experience of over twenty years, there have been few problems that cannot be attributed to other sources. Undoubtedly, there are some cells that will deteriorate rapidly following flow sorting, and single-cell cloning may sometimes produce low efficiencies. The reason for these failures is not always apparent. However, the success of repeat experiments often appears to eliminate the sorting process as the major cause.



Rare-Event Detection

Terry Hoy, PhD, FRCPath

Introduction

Relatively recent advances in the field of flow cytometry have enabled a whole new realm of applications: rare-event detection. With the advent of commercial flow cytometers that can process 100,000 events/second, it is now possible — and practical — to detect subpopulations that occur at very low frequencies, commonly defined as less than 5%. It is also possible to isolate these populations with sorting. Examples of rare-event detection include, but are certainly not limited to:

- Hematopoietic stem cells
- Dendritic cells
- Residual disease detection (tumor cell enumeration)
- Antigen-specific T cells
- Transient transfectants
- Fetal cells in maternal circulation

Definition

The analysis of data acquired by flow cytometric methods can be very simple, utilizing only a basic scatter gate to eliminate unwanted events, or performed on multiparameter measurements involving many gates combined in a logical manner. However, no matter how complex this process is, the final outcome will be a simple binary decision, and an event (or cell) will either meet a pre-defined criterion or not. These decisions can be described in a number of simple formats, such as + or -, 0 or 1, true or false. An important feature of all flow cytometers is that the events arrive at the point of analysis at random, as there is no known way of controlling how and when they are selected from the sample. Having stated that, differential sedimentation can be a problem when analyzing mixtures of particles with different densities. This can be encountered when beads are mixed with cells in an attempt to quantify absolute cell counts, and in these situations, frequent agitation should be applied.

The distribution of any subset of events within a larger parent population will be determined by the binomial distribution. As smaller subsets are considered, a situation is reached when a different type of statistics, called Poisson statistics, can be employed. Poisson statistics become effective when populations of less than about 5%, or 1 in 20, are being analyzed. This was often overlooked in the early days of flow cytometry, and the reason may have been as follows: Traditional detection of immunofluorescense by microscope-based methods suffered from relatively high backgrounds and non-specific staining, which often resulted in about 5% positive events being observed in control samples. As a consequence, experimenters were skeptical about reports of true positive

events of a similar magnitude, and before the emergence of flow cytometric methods, the acceptance of a 5% "cut-off" value had become accepted dogma. However, it soon became apparent that a much "cleaner" analysis was possible with flow cytometric technology, assisted by the simultaneous development of monoclonal antibodies.

As will be demonstrated, counting statistics on populations greater than 5% are reliable when compared to typical biological variations, if more than 5,000 total events are recorded, and this became a second dogmatic value in the early days of flow cytometry. Given the ability of flow cytometry to measure populations smaller than 5%, many experimenters failed to appreciate the need to collect more data as smaller subsets were investigated. By coincidence, the limit set by the dogma discussed above is at the same level at which the Poisson statistics can be used, and we can conveniently make a definition of rare events as below 1 in 20.

Counting Statistics

Consider the general case of enumerating a total of N events, of which R meet a certain criterion (positives). The proportion of positives, P=R/N, will also be the probability of any particular event being observed as positive $0 \leq P \leq 1$, which is clearly related to the random manner in which cells are selected for analysis. As with all statistical distributions, the variance, Var, is a fundamental parameter and, for the binomial, is defined as follows:

$$\mathbf{Var(R) = NP(1-P)}$$

The more familiar standard deviation, SD, is the square root of the variance, and the coefficient of variation (CV) is the SD expressed as a percentage of the population:

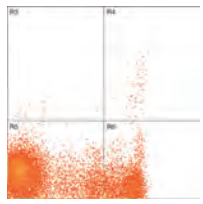
$$\mathbf{CV = (\sqrt{(Var(R))*100)/R}}$$

These simple equations can now be used to examine some practical situations.

Consider a preparation of human peripheral blood mononuclear cells labeled with an antibody to detect B cells. Flow cytometry then indicates that 10% of the cells present are positive for this marker (i.e. P=0.1 and P(1-P)=0.09). If three data sets (list mode files) were collected for 1,000, 5,000 and 10,000 events, we would expect to observe 100, 500 and 1,000 positive cells with variances of 90, 450 and 900, respectively. Expressed as SDs, these would be 9.5, 21.2 and 30 and as CVs 9.5, 4.2 and 3.

Good experimental practice within the biological field usually results in CVs on the order of 5%. Hence, the example above indicates that a list mode file of 5,000 events will provide that level of confidence as expected from the argument relating to dogma: For a sub-population of 10%, a total of 5,000 events would be adequate.

Moving beyond our definition of rare events, now consider a subset of T cells representing only 1% of the total, with P=0.01, P(1-P)=0.0099. Collecting the files as above, we would expect to observe 10, 50 and 100 positive events with variances of 10, 50 and 100 and CVs of 32, 14 and 10. Clearly, 5,000 and 10,000 total events are no longer adequate. Collecting 100,000 events in this situation would result in a CV of 3.2, and an important feature becomes evident: To improve CV by a factor of 10 (32 to 3.2), 100 times more data



is required. As direct consequence of the square root, to improve the CV by X, X^2 events are required in the list mode files. The approximation mentioned above can be used for the rare events defined as populations of less than 5% (1 in 20), when P=0.05 and P(1-P)=0.0475. This simplifies the equation for calculating the variance to Var(R)=NP=NR/N=R (i.e. the number of rare events observed). Hence, the SD=√R and CV=√R*100/R=100/√R. It is obvious that the reliability of the data set is now only dependent on the number of rare events observed. Consequently, for a desired level of reliability (CV), the number of rare events to be counted can be calculated from $R=(100/CV)^2$. To match typical experimental variations of 5%, approximately 400 rare events need to be observed, which at the defined limit of rare events (1 in 20), a list mode file of 8,000 would be required. A useful table can now be constructed from values of desired CV and expected size of subpopulation, showing the total number of events that need to be collected (Table 1).

Table 1. Total Number of Events to Collect

Frequency of Rare Events (1/X)	Desired Coefficient of Variation % (Rare Events Required)			
	30 (11)	10 (100)	5 (400)	3 (1,111)
20	222	2,000	8,000	22,222
50	556	5,000	20,000	55,556
100	1,111	10,000	40,000	111,111
1,000	11,111	100,000	400,000	1,111,111
10,000	111,111	1,000,000	4,000,000	11,111,111
100,000	1,111,111	10,000,000	40,000,000	111,111,111
1,000,000	11,111,111	100,000,000	400,000,000	1,111,111,111

The highest number shown is greater than 10^9, and as the fastest flow cytometers at present can acquire 100,000 events/second, this required amount of data would take sixteen minutes to collect. At a more conventional rate of 25,000 events/sec, this would increase to an hour. Plus, the number of cells required to reach this confidence level could also be a major consideration. However, the time taken to collect data sets usually represents a small fraction of the total experimental time, and as long as the cells are available, a few more seconds spent at the cytometer can be very productive when investigating small populations.

Correcting for Backgrounds

If background counts are appreciable, special care needs to be taken when corrections are performed. This is a consequence of what may appear to be a somewhat strange procedure for those not familiar with statistics. If we have an observed count R with an associated background (control) count of B, the variance will be √R and √B, respectively. However, the variance of R-B is the sum of the individual variances, not the difference.

$$\mathbf{var(R-B) = var(R) + var(B)}$$

In situations where high background counts begin to affect the reliability of data sets, collecting more data will improve the quality. For example, consider test and background counts of 200 and 100 in list mode files of 10,000 events.

The corrected count will be 100 with a variance of 300, SD=17.3, CV=17.3%.

If the data sets are increased to 100,000 events, we would expect 2,000 and 1,000 events, respectively, corrected to 1,000 with a variance of 3,000, SD=54.8 and CV reduced to 5.48%. Once again the importance of collecting sufficient data is obvious.

Applying Confidence Limits

The Poisson distribution becomes skewed as very low numbers are considered and, if these situations are avoided, confidence limits can be applied in the same manner as with Gaussian (continuous) distributions.

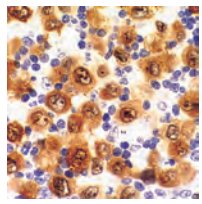
Providing more than about 50 events are observed (after correcting for background counts, if necessary), the standard deviation can be calculated as above and multiplied by 1.64, 1.96 or 2.58 to yield the 90, 95 and 99% confidence limits, respectively, on either side of the determined value. This approach is valuable in clinical situations where, for example, a decision may be made on a flow cytometry-based count of stem cells as to whether sufficient cells are present to provide a successful transplant.

Sorting Rare Events

Some consideration has been given to the amount of time and material that may be needed to acquire reliable data, but the situation is slightly different when sorting cells. In some situations, discrimination between real events and high background events may not always be possible, and high background events will be sorted with the true positives. The approach to analyzing the amount of contaminating material is beyond the scope of this chapter but is essentially the same as outlined above for background correction. When sorting individual cells (cloning), a different concept applies, as time no longer becomes the primary concern. The reason for this is simple. Even at levels as low as 1 per million, a modern high-speed sorter operating at 100,000 events/second will detect a rare event every 10 seconds on average, sufficient to fill a 96-well plate in a few minutes. In these situations, very stringent conditions can be applied to the gating strategies to avoid sorting unwanted material.

The Golden Rules of Rare-Event Analysis

- **Collect sufficient data to justify your results.**
- **CV=100 / √(Rare events observed)**
- **Avoid high background counts if possible.**



Immunophenotyping

Alberto Orfao, MD, PhD

Introduction

Immunophenotyping of biological samples refers to the use of immunological tools (e.g. monoclonal and/or polyclonal antibodies) for the specific detection of antigens, most frequently from proteins, expressed by cells or other particles (e.g. apoptotic bodies) localized either on their surface or inside them. In the past, antibody/antigen reactions on cells were revealed using either enzymatic approaches (immunocytochemistry) or fluorescence (immunofluorescence), both analyzed by microscopy. More recently, flow cytometry has been increasingly used for immunophenotyping purposes. As compared to other techniques, flow cytometry immunophenotyping is more sensitive and simple, and it provides a better way for the simultaneous quantitative assessment of multiple antigens in large numbers of cells (Table 1). Its major disadvantage relates to the need for monodispersed cell suspensions. In addition, the information provided about the morphology and localization of a given molecule inside a cell is limited. For this reason, flow cytometry has had a major impact on the analysis of hematopoietic cells, but its use for the study of cells derived from solid tissues has been rather limited.

Table 1. Major Characteristics of Flow Cytometry as Compared to Conventional Microscopy-Based Immunophenotyping Techniques

	Flow Cytometry	Conventional Microscopy
Simplicity	High	Moderate
Speed	High	Low
Number of Cells Analyzed	High	Low
Absolute Cell Counts	Accurate	Inaccurate
Sensitivity	High	Moderate/Low
Specificity	High	High
Number of Parameters	>10	<3
Information on Antigen Expression	Quantitative	Qualitative
Morphological Parameters	2	>10
Information on Antigen Localization	Limited	Detailed

Applications of Immunophenotyping

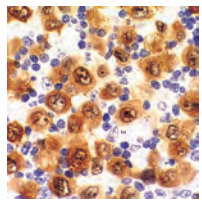
At present, the applications of immunophenotyping by flow cytometry are numerous and almost infinite. In general, staining of cells with antibodies may be used to identify, count and characterize any type of individual cells or subcellular components. In the last two decades, many of the applications of immunophenotyping have shown to be of great clinical utility, mainly in the area of hematology and immunology (Table 2). We will briefly review the currently established and emerging clinical applications of flow cytometry immunophenotyping.

Table 2. Clinical Applications of Flow Cytometry Immunophenotyping

Disease	Diagnosis	Classification	Prognosis and/or Staging	Disease Monitoring
Acute Leukemias	Yes	Yes	Controversial	Yes
Chronic Lymphoproliferative Disorder (CLPD)	Yes	Yes	Yes	Yes
Myelodysplasia (MDS)	To be established	No	To be established	No
Paroxysmal Nocturnal Hemoglobinuria (PNH)	Yes	Yes	No	Yes
Mastocytosis	Yes	Yes	No	Yes
Primary Thrombocytopathies	Yes	Controversial	Yes	No
Primary Immunodeficiencies	Yes	Yes	No	No
HIV Infection	No	Yes	Yes	Yes
Transplantation Outcome	Yes	Yes	Yes	Yes

Hematological Malignancies

Immunophenotyping of hematological malignancies has become one of the most relevant clinical applications of flow cytometry. Its utility initially focused on the characterization of neoplastic cells and classification of disease once diagnosis of leukemia/lymphoma has already been established. During this period, immunophenotyping proved to be of great help in: 1) the distinction between lymphoid and myeloid acute leukemias, especially if equivocal morphology and cytochemistry are found, 2) the subclassification of acute lymphoblastic leukemias, 3) the diagnosis of minimally differentiated, erythroid and megakaryocytic acute myeloblastic leukemia subtypes and 4) the recognition of both T and B cell chronic lymphoproliferative disorders. Progressively, some immunophenotypic markers have been associated with the prognosis of the disease, and the panels were gradually expanded to include assessment of the prognostically unfavorable CD38 and ZAP-70 antigens in B cell chronic lymphocytic leukemia (Figure 1).



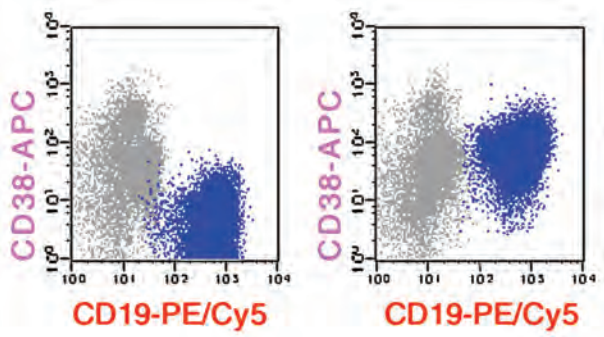


Figure 1. Expression of CD38 on CD19+ peripheral blood neoplastic B cells (blue dots) from patients diagnosed with B cell chronic lymphocytic leukemia (BCLL). In the left panel, a CD38- BCLL case is shown, while CD38+ cells from another BCLL patient are displayed in the right panel.

In the last decade, immunophenotyping of hematological malignancies using multiparameter flow cytometry approaches has proven to be a highly sensitive and specific method for the identification of neoplastic cells, even when present at very low frequencies among a major population of normal hematopoietic cells. This is because neoplastic hematopoietic cells express aberrant phenotypes. Leukemia/lymphoma-associated aberrant phenotypes (e.g. cross-lineage antigen expression, asynchronous-antigen expression, antigen-overexpression, tissue-restricted phenotypes, abnormal maturation pathways) have been reported to be easily detectable in most cases (Table 3). For the unequivocal identification of these aberrant phenotypes, extensive knowledge about the immunophenotypes of normal, reactive and regenerating hematopoietic cells is required.

Table 3. Frequency of Aberrant Phenotypes Detected by Flow Cytometry in Hematological Malignancies

Hematological Malignancy	% of Aberrant Cases
Precursor-B Acute Lymphoblastic Leukemia	> 95%
T Cell Acute Lymphoblastic Leukemia	> 99%
Acute Myeloblastic Leukemia	> 75%
Biphenotypic Acute Leukemia	100%
B Cell Chronic Lymphoproliferative Disorders	> 95%
Chronic T Cell Lymphoproliferative Disorders	NA
NK-Large Granular Lymphoproliferative Disorders	> 40%
Monoclonal Gammopathies	> 90%
Myelodysplastic/Myeloproliferative Disorders	NA

NA: Not systematically analyzed in large series of patients.

The existence of aberrant phenotypes in most hematological malignancies has led to a new era of flow cytometry immunophenotyping, allowing its use also for the detection of minimal disease for staging purposes, for monitoring response to treatment and for predicting relapses. In addition, the possibility of discriminating between normal

and tumoral hematopoietic cells in most patients is progressively pushing the use of flow cytometry immunophenotyping as a first line tool for the diagnostic screening of hematological malignancies. Accordingly, it has been shown that immunophenotyping is of great utility for the establishment of the clonal nature of expansions of lymphocytes in peripheral blood, bone marrow and tissues such as the lymph nodes and the spleen. Diagnosis of B and T cell clonality is typically based on the specific identification of a population of lymphoid cells expressing aberrant phenotypes, clearly different from those present in normal lymphocytes (Figure 2).

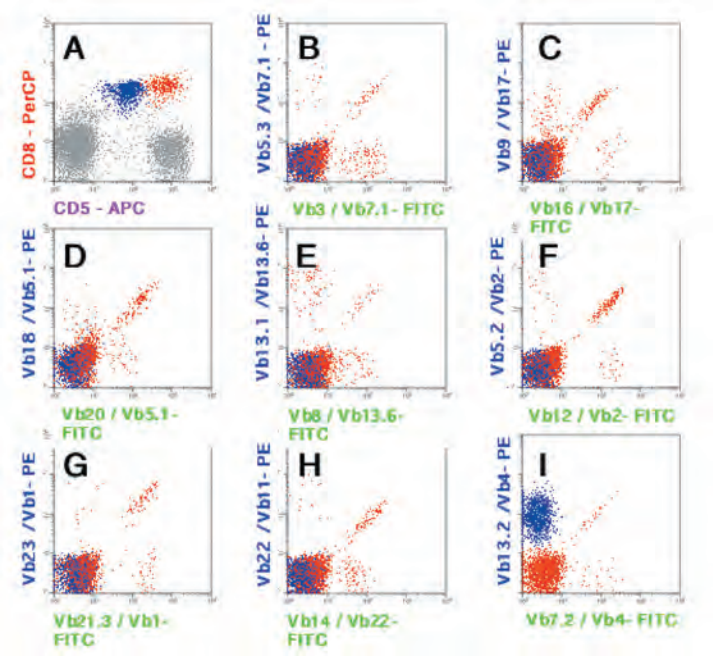
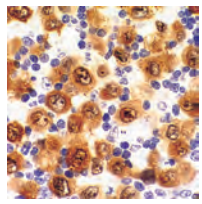


Figure 2. Immunophenotypic detection of T cell clonality in a small population of peripheral blood neoplastic T cells showing an infrequent CD8+/CD5low immunophenotype (blue dots in panel A). Note that all CD8+/CD5low T cells show monoclonal characteristics because they express the same TCRVbeta (TCRVbeta 13.2) (panel I), while normal residual T cells (red dots) are polyclonal and express all TCRVbeta families in relatively small and variable percentages (panels B to I).

Moreover, aberrant phenotypes have been proven to reflect deregulated patterns of gene expression. Within a disease, their detection allows the identification of cases carrying specific genetic abnormalities in which rapid, cost-effective confirmatory molecular studies should be performed. Interestingly, such patterns of aberrant antigen expression might also contribute to the design of new monoclonal antibody directed therapies. In turn, the similarities found between leukemia/lymphoma and normal hematopoietic cells have expanded the utility of flow cytometry to the identification of new subtypes of acute leukemias.

Other Clonal/Genetic Hematological and Immunological Disorders

At present, immunophenotyping is a primary diagnostic tool for the study of individuals suspected of paroxysmal nocturnal hemoglobinuria (PNH), systemic mastocytosis (SM),



primary thrombocytopathies and immunodeficiencies. In all four disease groups, genetic abnormalities carried by the clonal hematopoietic cells are translated into changes on the phenotype and distribution of specific populations of hematopoietic cells. Such phenotypic changes are closely associated with specific underlying genetic abnormalities and can be easily and reproducibly identified by flow cytometry immunophenotyping.

Despite the fact that immunophenotyping does not allow a final diagnosis in most primary immunodeficiencies, it is of great value as a screening tool. For many years, it has been well established that there is an association between the distribution of different populations of peripheral blood lymphocytes, their immunophenotype and the underlying genetic defect. A more detailed knowledge about the exact genetic lesions present in patients with primary immunodeficiencies has largely contributed to expanding the utility of immunophenotyping in the diagnosis of this heterogeneous group of disorders. As an example, the identification of the genetic defect present in patients with X-linked hyper-IgM syndrome has pushed the use of flow cytometry immunophenotyping for the assessment of the ability of in vitro activated T lymphocytes to express CD154.

Transplantation

In the last decade, an important increase in the number of applications of flow cytometry immunophenotyping in transplantation has occurred with regards to both hematopoietic tissues and solid organs. The flow cytometric enumeration of CD34+ hematopoietic progenitors for controlling the quality of biological products obtained for hematopoietic transplantation purposes is the most widely used. At present, it is well established that the total number of CD34+/CD45dim hematopoietic progenitors given in a transplant is the most powerful indicator of the outcome of the graft, and flow cytometry immunophenotyping is the method of choice for counting CD34 cells (Figure 3). More recent studies indicate that the composition of the graft in cell types other than CD34+ progenitors may also influence the outcome of the patient after transplantation. Accordingly, it has been shown that the frequency of severe infections in patients receiving an autologous transplantation is directly associated with the number of CD4+ T cells present in the graft.

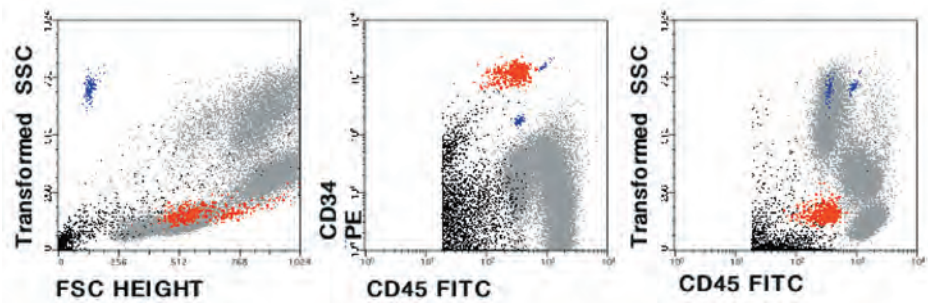


Figure 3. Immunophenotypic identification of CD34+ hematopoietic progenitors and precursor cells (red dots) in mobilized peripheral blood using a combination of light scatter and reactivity for CD34 and CD45 in an erythrocyte-lysed, non-washed stained sample. Blue dots correspond to two populations of internal reference fluorescent beads used to derive absolute cell counts in a single-platform flow cytometry assay.

Another clinically relevant application of immunophenotyping in transplantation is the so-called flow cytometry crossmatch. The goal of this assay is to identify the presence of anti-HLA antibodies, quantify their titer and determine their specificities in the serum of the donor prior to an allogeneic transplant. Although cellular based assays were initially used for the flow cytometry crossmatch, in recent years they can be combined with multiplexed bead arrays that allow routine determination of anti-HLA antibody specificities.

Diagnosis and Monitoring of Infectious Disorders

Staging of individuals infected with the human immunodeficiency virus (HIV) based on the enumeration of the absolute and relative CD4+ T cell counts in peripheral blood, probably represents the most extended and recognized clinical application of flow cytometry. Identification of CD4+ T cells is typically based on staining of peripheral blood samples with an anti-CD4 monoclonal antibody in combination with anti-CD45 and/or anti-CD3 (Figure 4).

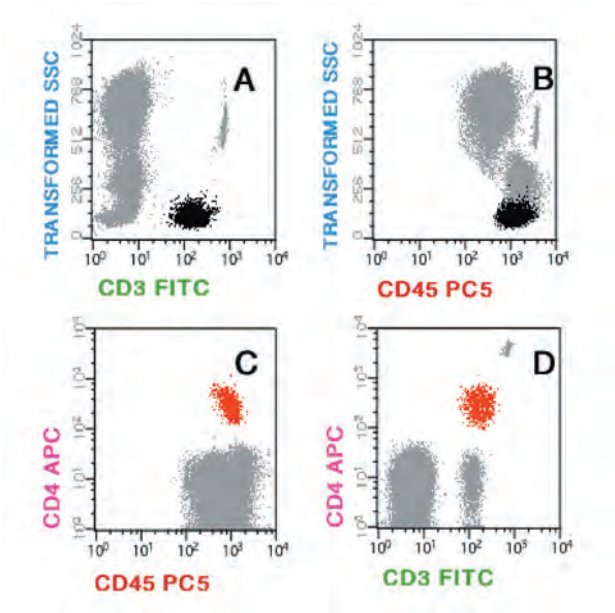
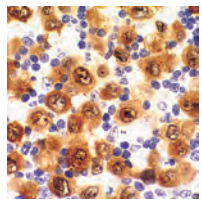


Figure 4. Immunophenotypic identification and enumeration of CD4+ T lymphocytes (red dots) after staining for CD45, CD3 and CD4. The population of gray events on the left of panels A, B and D corresponds to internal reference fluorescent beads used to derive absolute cell counts in a single-platform flow cytometry assay.

Despite the CD4+ T cell depletion, HIV infection induces an anti-viral immune response as reflected by an increased production of cytokines and other soluble proteins, together with modulation of other T cell surface activation-associated proteins, like CD38. For many years, it has been shown that the mean amount of CD38 expressed on the membrane of peripheral blood CD8+ T cells is increased in HIV+ individuals. At present, it is well established that an increase in the expression of CD38 per CD8+ T cell is not only an independent prognostic factor for predicting progression into AIDS and death,



but its quantitation is also of great utility for monitoring response to therapy in HIV-1+ individuals treated with highly active anti-retroviral therapy (HAART).

Although flow cytometry immunophenotyping has not clearly jumped into the diagnosis, staging and monitoring of other infectious diseases, it should be noted that, in this regards, recent results from clinical research studies are encouraging. Especially attractive are the results of the evaluation of viral- and peptide-specific T cell responses in immunodeficient patients suspected of being infected with cytomegalovirus, Epstein-Barr virus and Mycobacterium tuberculosis. In this regard, flow cytometry immunophenotyping allows the evaluation of cytokine responses and the identification of antigen-specific T cells through the use of HLA-multimers loaded with specific viral peptides. Simultaneous enumeration of different T cell subsets and identification of multiple anti-viral peptide-specific serum antibodies using multiplexed flow cytometry immunophenotyping are also promising future clinical applications of immunophenotyping by flow cytometry in the diagnosis of infection.

Quantitation of Fetal Red Cells

Recent advances in flow cytometry have improved the study of red cells. Among other flow cytometric approaches, immunophenotypic detection of fetal red cells has become particularly important, especially for the diagnosis and quantitation of fetomaternal hemorrhage. Recent studies show that flow cytometric identification and enumeration of fetal red cells in maternal blood improve diagnostic accuracy and precision over traditionally used techniques. The flow cytometry-based assays typically use anti-hemoglobin F, anti-i and anti-D antigen monoclonal antibodies. Previous studies have shown a higher sensitivity of flow cytometry for the detection of low numbers of fetal red cells in the maternal blood (sensitivity of between 10^{-2} and 10^{-3}), which translates in clinical practice into a more accurate determination of the needs of Rh immune globulin.

Other Clinical Applications

Apart from those assays used for the clinical applications listed above, flow cytometry immunophenotypic procedures are currently available for a wide variety of other purposes. These include HLA-B27 typing, basophil-activation based allergy tests, measurement of platelet activation, diagnosis and monitoring of autoimmune diseases, identification and quantitation of autoantibodies, identification of multidrug-resistant phenotypes in tumor cells, and quantitative evaluation of soluble proteins (e.g. cytokines).

Information Obtained about Cells in a Sample

Flow cytometry immunophenotyping provides information about the expression of antigens on single cells that have been stained with one or more antibodies. Analysis of stained cells tells us about the presence or absence of reactivity above a certain threshold, the amount of antigen expressed per cell, and the pattern of reactivity observed for that antigen in the whole population of cells in the sample.

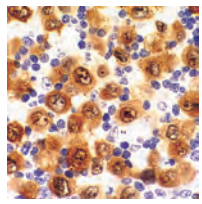
The threshold for positivity depends on the sensitivity of the measurement. In turn, the sensitivity depends on several factors related to the staining reagents, the sample preparation technique and the instrument measurement process (Table 4). Among the former group of variables, the affinity and avidity of the antibody for its antigen, the relationship between the amount of antibody and the volume of sample stained (concentration of the antibody), and the type and amount of fluorochrome conjugated to the antibody are critical variables. With respect to sample preparation, the time, temperature and pH of the solutions employed during incubation of the cells with the antibody, together with the use of additional steps (e.g. cell washings) and/or reagents (e.g. anticoagulant, erythrocyte lysing reagents and solutions containing fixatives) also have a direct impact on the sensitivity of the assay. Regarding the measurement step, the characteristics of the optical system and the intrinsic sensitivity of the flow cytometer used to measure fluorescence emissions associated with an antibody, in addition to the instrument settings employed during cell analysis, are the most important factors that may influence the threshold for positivity. Additionally, the overall amount of antigen present in the sample, the exact combination and sequence of staining used for two or more antibodies, and the speed at which cells are interrogated in front of the laser, may also have an impact on the sensitivity of the fluorescence measurements.

Table 4. Relevant Variables Affecting the Sensitivity of Detection of Antigen Expression by Flow Cytometry Immunophenotyping

Monoclonal Antibody Reagents	Sample Preparation	Fluorescence Measurements
Affinity/Avidity	Time	Chamber of Analysis
Concentration	Temperature	Filters/Lenses
Fluorochrome/Protein Ratio	pH	Fluorescence Detectors
Type of Fluorochrome	Lysing Reagent	Speed of Analysis
Immunoglobulin Isotype	Washing Steps	Size & Shape of Laser Spot
	Autofluorescence	Instrument Settings

The amount of fluorescence emission associated with an antibody is commonly proportional to the number of molecules of antibody bound to the cell, especially if the reagent used has a stable and uniform fluorochrome/protein ratio, in the absence of fluorescence quenching. Under optimally controlled conditions, the number of molecules of a given antibody clone bound per cell also depends on the overall amount of antigen expressed by the cell. Thus, through the use of internal reference standards, it is possible to establish a direct relationship between the amount of a specific fluorescence emission associated with individual cells and the amount of antigen they express. At present, varying units are used for the quantitation of antigen expression, including arbitrary fluorescence intensity channel units, standardized units of molecules equivalent of soluble fluorochrome (MESF), and antibody binding capacity (ABC).

Most cell samples, including cell lines, are heterogeneous in their composition. Typically, they contain more than one cell type or cell subset that can not be clearly discriminated on light scatter grounds. Because of this, characterization of the expression of an



antigen in a specific cell subset most frequently requires simultaneous staining with multiple antibodies. The simultaneous use of two or more antibodies facilitates the unequivocal identification of the cells of interest and then their specific enumeration and/or characterization. Analysis of the pattern of expression of an antigen within a given cell subset of interest is reflected by the mean or median fluorescence obtained for those cells and the corresponding coefficient of variation.

Immunophenotyping Techniques

Immunophenotyping techniques aimed at measuring antigen expression by flow cytometry are diverse. Traditionally, these techniques were divided into two major groups, depending on whether the antigen/antibody reaction is detected with antibody molecules directly coupled to a fluorochrome — known as direct immunofluorescence — or whether it requires the use of a secondary anti-immunoglobulin antibody conjugated to a fluorochrome — known as indirect immunofluorescence. Due to the complexity of performing multiple stains with indirect immunofluorescence methods, the use of indirect immunofluorescence techniques is currently restricted almost exclusively to single antigen stains. Direct immunofluorescence is the preferred method for multicolor antibody/antigen reactions.

Usually, the optimal sample preparation technique depends on the type of specimen and the cells of interest, the antigens and their distribution in the sample, the localization of the antigens in the cell, and the information to be obtained (Table 5).

Table 5. Most Frequently Used Sample Preparation Protocols for Flow Cytometry Immunophenotyping of Cell Surface Antigens

Step	Type of Specimen/Cell		
	Solid Tissues	Blood-Containing Samples	Other Single-Cell Suspensions
1. Disaggregation	Yes	No/Mild[1]	No
2. Staining	Yes	Yes	Yes
3. Red Cell Lysing	No[2]	Yes[3,5]	No
4. Washing[4]	Yes	Yes[5]	Yes

1 Mild mechanical disaggregation may be required for bone marrow.
2 Unless there is contamination of the sample with blood.
3 Lysing of non-nucleated red cells should not be performed in immunophenotypic studies of red cells and platelets; in addition, red cell lysing is not strictly required for the study of white blood cells.
4 Centrifugation/washing steps should be avoided for the calculation of absolute numbers of cells in a sample. In case of detection of antigens present in high amounts in the extracellular medium, washings should be performed prior to staining.
5 In case of searching for the presence of antigens that are also expressed on non-nucleated red cells, these should be lysed, and the red cell membranes washed out prior to the staining.

According to the type of specimen, three major categories are considered: 1) blood containing monodispersed cell specimens, 2) non-nucleated red-cell-free single-cell suspensions, and 3) solid tissues. While the latter specimens require disaggregation of the tissue into single-cell suspensions prior to staining, monoclonal antibodies can be directly

added to sample aliquots of the former two specimens, with two exceptions. In these exceptions, the antigens to be stained are present in high amounts in the extracellular media (e.g. serum samples containing immunoglobulin light chains), or the antigens are expressed on the surface of non-nucleated red cells present in high quantities in blood specimens (e.g. CD55 and CD59). In both conditions, the use of a washing step prior to staining is recommended, and if the sample contains blood, incubation with a red cell lysing agent is also recommended. Alternatively, the concentration of antibody added can be increased. In all other situations, samples derived from blood specimens are preferentially stained first and then lysed and washed.

Depending on the characteristics of the cells, specific changes in the sample preparation techniques may also be required. Accordingly, for the staining of non-nucleated red cells, lysing steps should be eliminated, and quenching reagents (e.g. crystal violet) may be required for optimal immunophenotyping of highly autofluorescent cells. As mentioned above, staining of intracellular antigens also requires modification of the immunofluorescence techniques. For this purpose, appropriate fixation and permeabilization protocols should be used prior to staining.

Finally, the exact type of information desired may also influence the decision on the exact sample preparation protocol to be applied. In fact, if immunophenotyping is used for counting the number of cells present in a given volume of sample, washing steps and solutions that may damage the cells in the sample should be eliminated in order to avoid significant cell loss. In turn, if antigen expression is to be evaluated in MESF or ABC values, special requirements are needed with regard to the monoclonal antibodies and/or control reagents to be applied.

Overall, those variables that influence the decision about the most appropriate sample preparation protocol also determine the most appropriate instrument settings to be applied during data acquisition and the number of events that should be measured in the flow cytometer. In this regard, it is well established that identification of a cell population requires a minimum of between 13 and 15 homogenous events, while reaching an acceptable coefficient of variation (10%) for the enumeration of a cell population requires that a minimum number of 100 cells of interest are analyzed.

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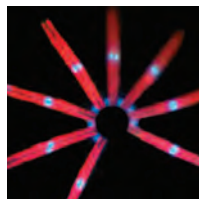


Small-Particle Analysis

John Sharpe, PhD, and Sonja Wulff

Introduction

Flow cytometry instrumentation is a powerful tool for the analysis and identification of complex samples of particles of significantly varying size. However, historically most commercial instruments have been designed with human blood analysis or other similarly sized particles in mind and as such have implemented appropriate fluidics, optics and electronic timing schemes for sample processing and discrimination. Typical particles range in size from a few microns to several tens of microns in diameter. Here, for the purposes of this discussion, we describe small particles as microscopic objects that range from a few microns down to several tens of nanometers, near the detection limit of most flow cytometers. Small-particle analysis is performed in a growing number of areas of application in marine biology, environmental monitoring, industrial microbiology and the food industry, to name a few. Due to a number of unique challenges that exist with small-particle analysis, careful consideration should be given to instrument design, set-up and sample preparation prior to running a sample on a commercial flow cytometer.

Applications

Bacteria

Bacteria, which typically range in size from 1–5 µm, are of particular relevance in any discussion involving flow cytometry and small particles. In the biomedical arena, flow cytometry helps clinicians detect and identify bacterial infections, and it has wide application in research focused on bacterial pathogenesis and bacterial sensitivity to therapeutic agents. Public health agencies also use flow cytometry in epidemiologic studies aimed at detecting and identifying contaminants in drinking water and food.

Outside traditional microbiology, interest is growing in the use of flow cytometric assays to screen combinatorial protein libraries displayed on the surface of bacteria. Bacteria are also being used as vehicles for gene expression studies.

Aquatic Ecology

Aquatic microorganisms, which range in size from 0.02–200 µm in diameter, are gaining scientific relevance as model systems, ecological indicators and sources of novel genetic material. Since these organisms occur naturally in suspension and contain chlorophyll, phycoerythrin and other autofluorescent photosynthetic pigments, they are ideal candidates for flow cytometry. By using flow cytometry to characterize and monitor marine and fresh-water populations, researchers are able to monitor water quality and

global climate change, forecast commercial lobster harvests, detect harmful algal blooms, and provide cultures for use in drug discovery, industrial processing and aquaculture.

Platelets

Platelets, which measure 2–3 µm across, are structures that assist in blood clotting by adhering to other platelets and to damaged epithelium. In flow cytometry, the most common platelet assays involve platelet counting and phenotyping as indicators of impaired bone marrow function and various blood disorders. Less commonly, clinicians and researchers look at platelet activation.

Chromosomes

When chromosomes are suspended in solution and stained with one or more fluorescent DNA dyes, they become small particles available for analysis and sorting on a flow cytometer. Clinical laboratories use flow cytometry to create "flow karyotypes," which provide valuable information about chromosomal abnormalities present in cancer and genetic disease. Chromosomal sorting has proven invaluable in the Human Genome Project, particularly in the production of genetic libraries and subsequent sequencing studies. In fact, the Human Genome Project was the drive behind development of the first true high-speed sorter.

Yeast

Yeast, typically 1 µm in diameter, are common model organisms in genetic studies, and as such, researchers find occasion to analyze and sort them on flow cytometers. The food industry also relies on flow cytometry for rapid yeast counting. Clinical applications include detection of surface antigens in patients with systemic yeast infections.

Microbeads

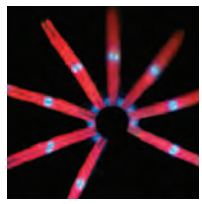
Microbeads, which can be 5 µm or less in diameter, are finding increasing utility in the flow cytometry laboratory. They have long served as calibration particles for instrument set-up and alignment, but they are also being used as carriers for biologically relevant materials, such as peptides and cDNA.

The remainder of this discussion will focus on theory and technical considerations to help optimize these diverse flow cytometry applications.

Theory

Light Scattering

There are a number of light scattering mechanisms that occur in flow cytometric analysis of biological cells and other microscopic particles. In essence, particles that are small



relative to the excitation wavelength uniformly scatter (radiate) energy in all directions, while larger particles tend to produce scatter that peaks in forward and backward directions.

The more predominant scattering mechanism for particles that are smaller than the wavelength of light is termed Rayleigh scattering. This mechanism tends to scatter light at large angles relative to the incident excitation source. As particle dimensions approach the wavelength of light, and forward scatter increases, the effects of Mie scattering become more significant, and as particle dimensions increase further, interference and diffraction mechanisms tend to dominate scatter profiles.

Most flow cytometers employ a number of excitation wavelengths, ranging from approximately 350–650 nm, with a primary excitation source of 488 nm used for scatter measurements. In these instruments, particles with dimensions of a few hundred nanometers (e.g. certain varieties of phytoplankton) produce isotropic Rayleigh scatter behavior, while particles that are a few microns in size (e.g. bacteria) produce substantial forward scatter profiles that follow Mie theory.

Sizing and Morphology

When analyzing numerous subpopulations of differing size and morphology in a single sample, these often subtle inherent differences can be resolved by detecting light intensity differences in forward- and side-scattered excitation light (Figure 1). Typically the intensity of light scattered in the forward (FSC) direction is proportional to particle size while side scatter (SSC) intensity is associated with the presence of structure or granularity of a particle, giving rise to increased reflection to side-angle detectors. A mixture of beads of varying diameter and similar material can be used to demonstrate the effect of Mie scattering to produce a non-linear relationship when displayed on a scatter bivariate plot of FSC versus SSC.

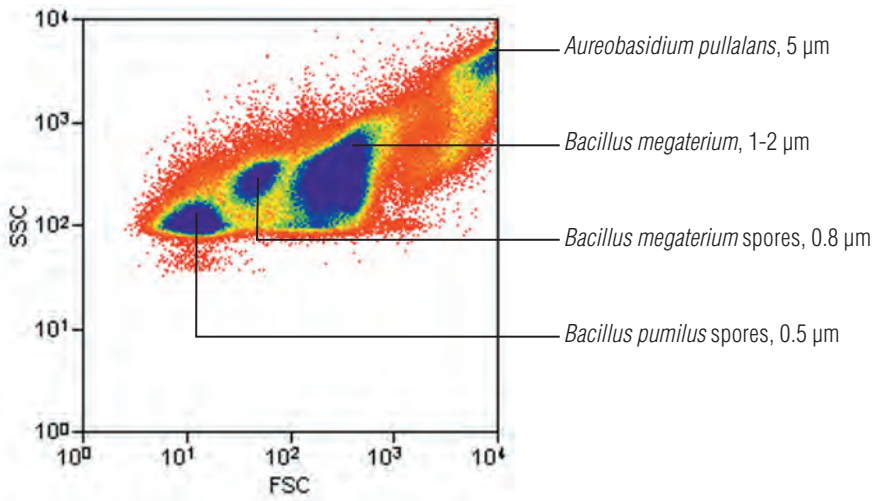


Figure 1. This histogram demonstrates resolution of multiple small species by forward and side scatter. Samples provided by Fei Chen, NASA Jet Propulsion Laboratories. MoFlo® data courtesy of Kathleen Martin, Dako.

Fluorescence

Many of the stains used for large-particle analysis can be applied to small-particle studies. However, care must be taken to ensure appropriate particle preparation and staining protocols for accurate analysis. This is due to a number of constraints that are introduced with smaller particles, primarily related to the difficulty in resolving low-intensity scatter and fluorescence signals from debris and noise. Take bacteria, for instance. The surface area — and, thus, the resulting fluorescence intensity — of a bacterial cell is roughly one-hundredth the surface area of a mammalian cell, and the DNA content is one-thousandth. That means successful flow cytometry assays involving bacteria require particularly bright dyes and highly sensitive instruments.

Small-particle analysis often encompasses DNA stains such as ethidium bromide, propidium iodide, DAPI, TOTO and Hoechst dyes/stains (Figure 2A). Immunofluorescence studies can be performed using fluorochromes, such as FITC and Texas red (Figure 2B). Membrane potential can be examined with cyanine dyes and rhodamine 123.

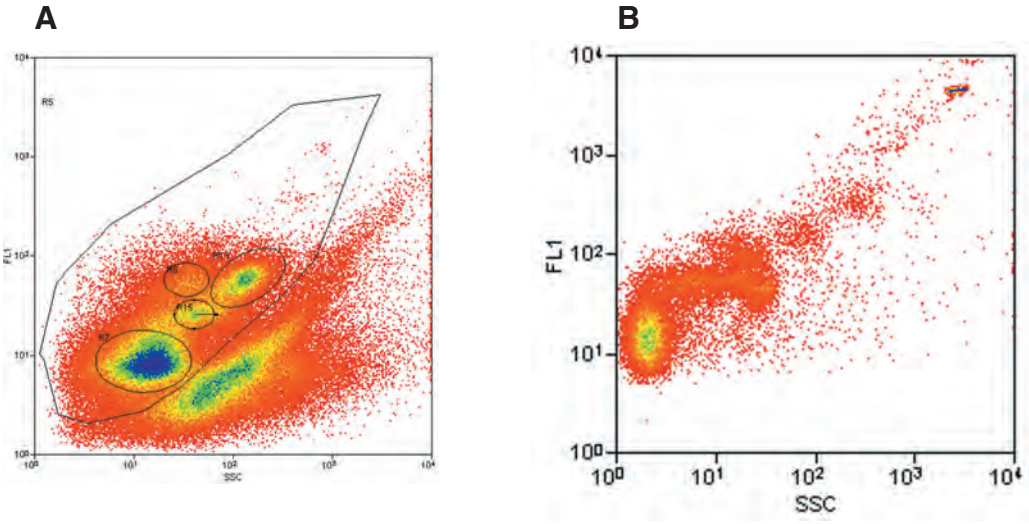
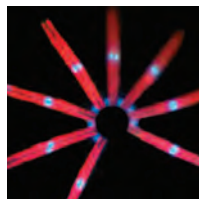


Figure 2. A). This histogram demonstrates discrimination of various high DNA-content bacteria from a transect of the Gulf Stream. MoFlo® data courtesy of Maria A. Guerrero and Christopher D. Sinigalliano, Southeast Environmental Research Center, Florida International University. B) This histogram displays viral populations found in pond water. The sample was stained with SYBR gold fluorescence. MoFlo® data courtesy of Stefan Andreatta, Institute of Zoology and Limnology, University of Innsbruck, Austria.

Autofluorescence

With sufficient excitation source intensity, autofluorescence can be induced in many small particles, such as marine organisms, at a detectable level in one or more detection channels. By using a number of excitation sources covering a broad wavelength range in the visible spectrum and beyond, it is possible to characterize large numbers of subpopulations within a sample. Photosynthetic and other pigments can be exploited using these techniques.



Instrument Optimization

Most commercial flow cytometers can be optimized for small-particle analysis. However, it is important to consider several key areas of the instrument before embarking on such a program.

Detection Geometry

Many instruments employ neutral density filters to attenuate the typically very strong FSC light irradiated from large particles such as whole blood. Removal or reduction of the level of attenuation of such filters can provide increased sensitivity in the FSC channel. Further, the collection angle of the FSC detector may also be adjustable so that a greater proportion of light can be collected. Careful investigation and manipulation of obscuration bars can also yield increased small-particle sensitivity.

Photodetector Type

Often, the FSC channel of a flow cytometer utilizes a photodiode for scattered excitation light. This type of detector performs adequately for resolving blood sample populations because of the strength in scattered light from particles of interest. In contrast, photomultiplier tubes are usually employed in SSC and fluorescence channels where light levels are typically several orders of magnitude lower due to their high gain and excellent signal-to-noise characteristics. Some instruments can be modified to place a photomultiplier in the FSC channel specifically to enhance the sensitivity and performance of small-particle analysis.

Threshold and Noise Floor

Optical alignment, fluidic stability and electronic noise can significantly alter the ultimate detection limit for small particles. When analyzing small particles, it is imperative that the sheath fluid is free of debris; otherwise, the particles of interest will be masked by light scatter from contaminants. For example, if a population of 0.2 µm particles is to be sampled, it is important that sheath fluid is filtered using a pore size of significantly less than 0.2 µm.

An adjustable threshold level is provided on most flow cytometers to differentiate noise from signal in a particular channel of interest, and once this threshold is surpassed, the processing electronics may be triggered (armed) in order to process a detected event. This threshold level is adjustable to allow the user to fine tune the instrument to achieve maximum sensitivity for given conditions and to avoid burdening processing electronics with unwanted signals. This capability can be used to great benefit for particularly small particles, fluorescent particles or samples that contain many subpopulations or significant debris, such as in sea water samples. During instrument setup, the best way to account for background noise, particles or contaminants in each detector channel is to acquire data with no sample flowing (or a known filtered particle-free sample) and

to lower the threshold level until triggered events are observed. The adjusted threshold level for these events represents the minimum signal that the instrument can observe above background, stray light, noise and sheath contaminants.

Triggering

Many flow cytometers provide selectable trigger channel or multiple trigger source capability. The trigger is used in a flow cytometer to time a crossing of the threshold, to ready the processing electronics, and for sorter timing purposes. Often, by simply transferring the trigger channel from FSC (usually a photodiode detector) to SCC (a photomultiplier detector), the particle size detection limit can be reduced greatly due to the inherent noise and gain characteristics of the photomultiplier. Table 1 provides recommendations for various instrument trigger source capabilities.

Table 1. Instrument Trigger Source Capabilities

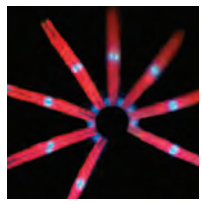
Instrument Capability	Recommendation
Fixed FSC photodiode as only trigger source	Ensure neutral density filter is minimized
Fixed FSC photomultiplier tube as only trigger source	Ensure neutral density filter is minimized.
Selectable trigger source	Shift trigger source to SSC (photomultiplier detector); if particles are fluorescent, shift to appropriate fluorescence channel; consider staining particles for triggering purposes
Multi-trigger source	Use appropriate multiparameter detector scheme with appropriate logic for population sample of interest

Gating

Flow cytometry analysis software can be used to differentiate and resolve populations through multiparameter gating and color-gating schemes. For example, a subpopulation of particles that are seemingly identical from a FSC vs. SSC standpoint can be suitably discriminated by using an appropriate stain-fluorescence channel combination, and then by gating this population in the FSC-SSC bivariate.

Sample Handling

It is important that necessary handling precautions are taken through sample preparation and storage to ensure sample integrity. These precautions should include appropriate temperature and light exposure control measures to ensure sample breakdown is avoided. Particularly where instrument sensitivity has been optimized for small particles, it may be possible to observe debris or other particles that are not otherwise observed on a typical commercial flow cytometer optimized for large-particle analysis.



Conclusion

Flow cytometry provides a powerful tool for small-particle analysis and enumeration across a broad range of applications and fluid sample types. With careful instrument optimization, sample preparation and gating schemes, it is possible to detect and resolve highly complex mixtures of particle populations.

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Special thanks also to Michael Sieracki, J.J. MacIsaac Facility for Aquatic Cytometry, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine, USA, for the image that runs in the top right corner of each page in this chapter. This image depicts a colony of *Thalassionema* phytoplankton.

MHC Multimers

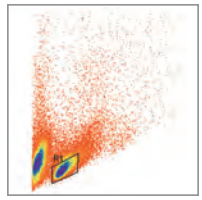
Christina Jespersgaard, PhD, and Lars Winther, PhD

Fluorescently labeled multimers of major histocompatibility complex (MHC) molecules are novel reagents that make it possible to directly identify and enumerate disease- and vaccine-induced T cells in human and animal models by flow cytometric analysis. The antigen specific T cell receptors are recognized by fluorochrome-labeled reagents containing a matching peptide-loaded MHC molecule. This allows the investigator to monitor how the immune system responds to different stimuli. This ability to track specific T cell responses has a wide range of possible applications. For instance, it is of the outmost importance in the development and clinical use of vaccines for cancer; it holds great promise for individualized patient treatment and care in important areas such as infectious medicine, autoimmune disorders and transplantation; and it has tremendous value for basic research aimed at understanding the immune system and disease processes. The analysis of specific T cell receptors is occasionally combined with specific fluorescently labeled antibody staining of cell surface antigens such as CD8, CD4, CD45, CCR7, CD27, CD28 or others to distinguish the functional stage of differentiation of the T cells. Also, specific TCR analysis has been combined with cytokine secretion assays to analyze the functionality of the T cells.

Major Histocompatibility Complex

Vertebrate organisms posses a cluster of genes named MHC.[1] MHC is referred to as the HLA complex in humans and as the H-2 complex in mice. The MHC encoded protein products play a role in the development of both humoral (antibody-mediated) and cell-mediated immune responses. Two distinct classes of MHC encoded molecules present antigen to T cells: the MHC class I and the MHC class II. MHC class I molecules are expressed on nearly all nucleated cells. They are involved in the presentation of endogenous peptide antigens to $CD8^+$ cytotoxic T cells. Examples of endogenous peptide antigens include normal cellular proteins, tumor proteins and viral or bacterial antigens produced within infected cells. MHC class II molecules are expressed on antigen-presenting cells, where they present exogenous antigenic peptides to $CD4^+$ T helper cells. It should be emphasized that a given TCR only recognizes a specific peptide presented in the context of a particular MHC molecule.

Any one individual has a unique set of MHC genes representing maternal and paternal genes, respectively. These encode three distinct types of MHC class I gene products (HLA-A, HLA-B, HLA-C for humans and H-2K, H-2D and H-2L for mice); one set of genes representing the maternal genes and one set of genes representing the paternal genes. In the same manner, any individual has several distinct MHC class II gene products (HLA-DP, HLA-DQ, HLA-DR for humans and H-2IA and H-2IE for mice). The genes are co-dominantly expressed, which means that each individual expresses up to six different class I molecules and up to 12 different class II molecules (due to the dimeric nature of MHC class II molecules). A number of allelic variants of MHC class I and II genes exist, and this is reflected in the variation of the expressed MHC genes among different individuals as well as between different ethnic groups.



MHC Multimers

A given T cell receptor (TCR) binds only to a specific peptide presented in the context of a particular MHC molecule. This has been successfully used to develop technologies that allow detection and isolation of distinct T cell populations in which all of the T cells carry the same specific TCR. However, the very low affinity between the MHC complex and the TCR does not easily permit efficient binding or detection. By the introduction of fluorescence-labeled multimeric MHC reagents, it became possible to detect and quantify the TCRs. Multimeric MHC reagents have an apparent higher affinity, as several MHCs are held close together in the same conjugate. The TCR experiences a local high concentration of peptide-loaded MHC, and the apparent higher binding affinity is a simple consequence of the law of mass action (i.e. The increased binding capacity of a multimer is caused by the increased avidity, which is the sum of the individual affinities of the MHCs.). MHC tetramers introduced by Altman et al[2] used biotin-labeled MHCs bound to fluorescence-labeled streptavidin. The resulting reagent contained up to four MHCs per streptavidin, therefore the name tetramers became common. Figure 1 schematically illustrates an MHC multimer reagent bound to a T cell. In order to avoid sterical interference from the streptavidin, the delicate MHC was enzymatically biotinylated in the heavy chain away from the TCR binding part of the folded MHC. Further technological developments have resulted in polymeric reagents with much higher numbers of MHCs and large numbers of fluorescent labels in order to increase the signal.[3] At this point, more MHC class I than MHC class II products are commercially available, and MHC class I reagents will be the primary focus in this section.

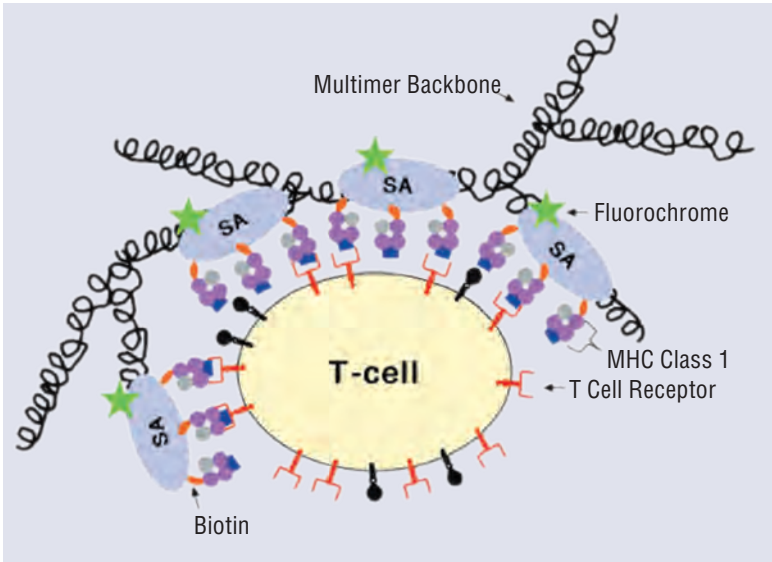


Figure 1. Schematic diagram illustrating MHC multimer binding to T cell receptors on the surface of a T cell. The T cell receptors are recognized solely by a specific peptide presented by the MHC. (SA=streptavidin).

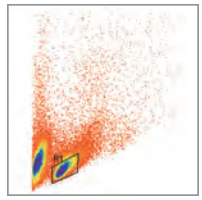
It should be noted that the various fluorescent labels used in flow cytometry and MHC multimer technology are very different in nature. Common fluorescent dyes like fluorescein, coumarins, cyanines, Texas red and the Alexa dye family are all small molecules with pronounced hydrophobic binding properties, which can cause nonspecific binding. These small dyes will quench each other if they are attached too closely in the same conjugate. Fluorescent proteins like PE or APC (240 and 105 kDa, respectively) and their tandem dyes with Cy5 or Cy7 are all large molecules compared to the MHC construct. The large fluorescent molecules can potentially interfere with the delicate MHC complex and the binding to the TCR simply due to their size or localization in the polymeric reagent. Furthermore, any weak and unwanted interaction caused by the conjugate crosslinkers, streptavidins or fluorescent labels will be increased in the polymeric conjugate in the same manner as the wanted MHC–TCR binding is enhanced. Consequently, the ideal multimeric MHC reagent needs to be optimized with respect to the number and position of the peptide-folded MHC construct and the number of the small or large fluorescent labels.

Due to the polymorphism of the immune system, no single set of MHC reagents can recognize all the T cell receptors in a population. The strong dependence of MHC type makes the use of MHC multimers different from other immunological reagents used in flow cytometry. The MHC/peptide/label combination of choice can be purchased directly from several vendors. Some vendors may require a minimum score in a peptide-MHC binding algorithm if the peptide-MHC combination has not been previously prepared. Most MHC multimer stainings of specific TCRs are done on sparse material. Consequently, the reagents quality, stability, consistency and availability of the reagents should be evaluated very carefully, as this can vary between vendors. One should view the MHC multimer reagents with the same or even greater scrutiny than traditional fluorescently-labeled antibody reagents.

Technical Considerations

Unlike many flow cytometry applications, MHC technology is most often used for the detection of specific T cells at very low frequencies.[4] Therefore, MHC multimer staining should be considered a rare-event problem, which is detailed in Chapter 9. In general, a large number of cells are required in order to determine the frequency of MHC multimer-positive cells with statistical confidence. Whenever possible, a minimum of 50,000 $CD8^+$ events should be collected to assure accuracy of the measurement.[5] In addition, an optimization of the temperature, the MHC multimer concentration and the sequence of staining is highly recommended in order to increase the signal-to-noise ratio.

The temperature of choice differs between various protocols and MHC multimer specificities. In general, it has been observed that at lower temperatures, less MHC multimer is required in order to stain specific T cells.[6] The biologic relevance of maximizing the staining intensity must be considered, and it ought to be taken into consideration that MHC multimer staining at 4° C may detect T cells that are of little biologic relevance. Since the conditions that yield the highest percentage of MHC



multimer positive events might not reflect an in vivo situation, care must be taken that the temperature and the concentration of the MHC multimer reagent is optimized according to the required biological relevance of the information.

There are conflicting reports on the role of CD4 and CD8 in TCR binding and activation. However, the sequence of T cell staining is very important since a number of CD8+ antibodies are capable of inhibiting MHC multimer staining.[7] In brief, it was shown with a human T cell line specific for a tumor-associated MHC-peptide complex that three distinct anti-CD8 antibodies were able to completely block the binding of polymeric MHC to TCR. Therefore, the order of incubation events with the reagents used in these staining experiments is critical when an inhibitory antibody is used. Thus, the inhibitory potential of the antibody to MHC multimer binding has to be determined.

As for any other experiments, it is mandatory to use the appropriate controls. A negative control reagent should always be run in parallel to evaluate the nonspecific binding. The negative control reagent should have the same fluorescent label and an identical MHC, but it should be loaded with a nonsense peptide.[8] A positive control must express the TCR with the appropriate specificity for the specific MHC multimer. Sources of positive controls include T cell clones and T cells from an individual known to express the TCR being evaluated. In many cases, these controls will not be readily available, which makes it even more important to include a negative control for the assay.

Gating strategies are designed to enhance the resolution of the MHC multimer-positive events, by increasing the signal-to-noise ratio and reducing the frequency of nonspecific events. This can be done by either exclusion or inclusion gating, by the means of antibody reagents or morphology of the cells studied. Figure 2 gives an example of experimental data generated by MHC multimer reagents and gating.

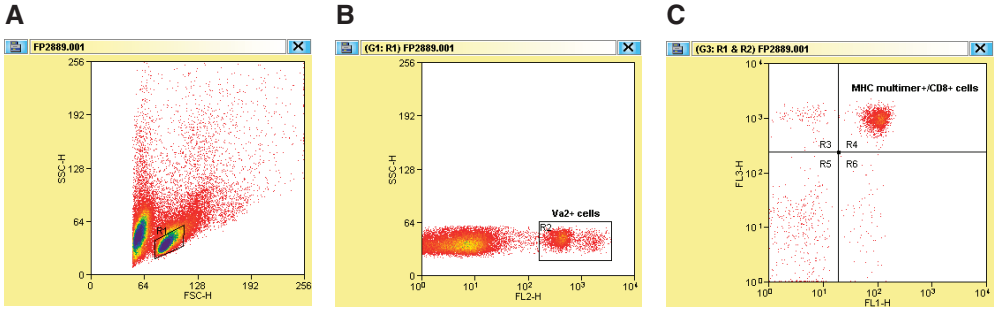


Figure 2. Experimental data generated by MHC Dextramer reagents (Dako) on spleen cells from a transgenic mouse model expressing a distinct TCR. The MHC Dextramer reagent consists of a dextran backbone and mouse H-2Db MHC loaded with the GP33 peptide. A) Ungated data represented by FSC on the x-axis and SSC on the y-axis. Based on morphology, the cells of interest are selected, and debris is excluded. B) Histogram gated on the region selected in A. The x-axis represents anti-Vα2-PE staining, and the region is drawn to include only Vα2+ cells, which represent those cells expressing the transgenic TCR. C) Histogram gated on both A and B, where staining with MHC Dextramer-FITC and anti-CD8-PE-Cy5 is represented on the x- and y-axis, respectively. The upper right quadrant shows the MHC Dextramer +/CD8+ cells. Data courtesy of Drs. Allan Randrup Thomsen and Jan Pravsgaard Christensen, Institute of Medical Microbiology and Immunology, University of Copenhagen, Denmark.

The future of MHC multimer technology holds great promise as a tool to monitor specific immune responses in vaccine and immunotherapy trials and other clinical settings where characterization of T cell responses to specific peptides is determined. Further development of the technology will be helped by the relentless technical improvements of reagents and flow cytometers with respect to multicolor capabilities, sensitivity, speed and ease of use.

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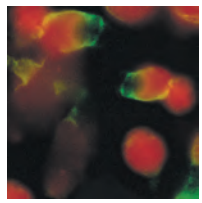


Fluorescent Proteins

Peter Lopez

Introduction

Fluorescent proteins (FPs), of which the green fluorescent protein (GFP) is most often referenced, have become a mainstay in the study of protein localization, protein-protein interaction and gene expression. By rendering a protein of interest natively fluorescent, FPs allow the intracellular localization and quantitation of these proteins without invasive labeling procedures. Since the first introduction of the GFP gene into a foreign cell and its successful fluorescence detection intracellularly,[1] FPs have seen wide use in many fields of biomedical research. They have been extensively cited in scientific publications, as well as the subject of many review articles.[2-4] In this chapter, we will focus on the practical application of fluorescent proteins and their detection using flow cytometry.

GFP is the green-fluorescing component of a naturally occurring bioluminescence complex found in the jellyfish *Aequorea victoria*, which inhabits the waters of the Pacific Northwest USA. In the jellyfish, GFP exists along with the Ca++ activated photoprotein aequorin. Both proteins were first identified in 1962.[5] Aequorin emits blue-green light in response to external stimuli in the laboratory, although the function of its bioluminescence in nature is not yet understood. In the jellyfish, the accessory green fluorescent protein accepts this energy from aequorin and re-emits it as green light.[6] When isolated from the jellyfish photoprotein, wild type GFP (wtGFP) absorbs in the blue and emits in the blue-green (excitation wavelength = 395 nm, emission wavelength = 510 nm). Structurally, wtGFP is a 238 amino acid polypeptide with a molecular weight of 26,900 Daltons. It exhibits a “beta-can” cylindrical structure 30 angstroms wide and 40 angstroms long, which encloses the centrally located chromophore.[7-8]

In order to use FPs for protein localization in other cell types or organisms, the protein of interest is rendered fluorescent by first cloning the cDNA for the protein of interest into a vector to generate a FP fusion protein. In this fusion protein, the FP may be fused either N- or C-terminally to the protein of interest.[8] Another method can be used where the fluorescent protein is co-expressed as a second transcription/translation unit from the same vector expressing the protein of interest. FPs can be also delivered intracellularly using viral transduction.[9] Once expressed in a cell of interest, the FP fusion protein can be observed in a cell or localized in an organism non-invasively by simply providing the proper excitation wavelength for the FP used, and then monitoring the fluorescence. No additional cell processing or fluorescence labeling is needed. Fluorescent proteins have been expressed in many cell types, including yeast,[10] bacteria,[11] virions,[12] and mammalian cells.[13] FPs have also been expressed in entire organisms including zebrafish,[14] plants,[15] fungi,[16] insects[17] and mammals.[18-19]

Although the potential for various applications of wtGFP became readily apparent, a few technical issues remained. In order to deal with these issues, enhancements were made to wtGFP using site-specific mutagenesis. This technique generated variant FPs that

exhibited quicker maturation, varied absorbance/fluorescence spectra and improved quantum yield, or intensity.[20] Modifying the jellyfish protein utilizing human codon optimization generated better expression and tolerance of FPs in mammalian systems.[21] In addition, other FPs (Table 1) were identified from other marine sources, such as the Anthozoan corals, that had desirable features, typically red-shifted fluorescence.[22]

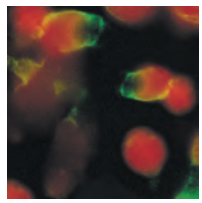
Table 1. Fluorescent Protein Characteristics[21]

Name	Source (Organism)	Emission max (nm)	Characteristics
BFP	*Aequorea victoria*	448	Low quantum yield; UV excitation; rapid photobleaching
ECFP	*Aequorea victoria*	476	Short wavelength excitation
AmCyan1	*Anemonia majano*	486	
wtGFP	*Aequorea victoria*	510	Low quantum yield; poor expression
ZsGreen1	*Zoanthus*	506	
EGFP	*Aequorea victoria*	510	High quantum yield
EYFP	*Aequorea victoria*	529	ECFP partner in FRET applications
ZsYellow1	*Zoanthus*	540	
hrGFP	*Renilla reniformis*	506	Human-codon optimized; low toxicity
HcRed1	*Heteractis crispa*	618	Longest wavelength red emission
Pt. GFP	*Ptilosarcus gurneyi*	509	Brighter than EGFP
PA-GFP	*Aequorea victoria*	510	Photoactivatable; low to high intensity shift
Kaede	*Trachyphyllia geoffroyi*	518/582/627	photoactivatable; green to red shift
AsRed2	*Anemonia sulcata*	592	
DsRed-Express	*Discosoma*	579	Preferred to DsRed2; decreased green emission component

Use in Cytometry

Fluorescent proteins can be either observed within a cell microscopically, or quantitated using flow cytometry. Due to the low background fluorescence and resistance to photobleaching of FPs, cells can be purified based on their expression level by cell sorting for later study using microscopic or molecular biology techniques.

The majority of FPs currently available are readily excited using a 488 nm laser-equipped flow cytometer. Exceptions include EBFP, which requires ultraviolet excitation; ECFP; and AmCyan1, which requires violet excitation. Although an excitation wavelength may not be available near the maximum absorbance of a particular FP, adequate fluorescence levels are usually obtained due to the inherent low background and high quantum yield for many of the FPs. An example is EYFP, which can be readily excited with a 488 nm laser, although this wavelength provides excitation at only about 30% of the peak absorbance of the protein.[23] EGFP detection limits have been reported to be about 100 nM, which translates to about 10,000 intracellular molecules, or 2,000 extracellularly.[24]



Multicolor applications can include detection of multiple FPs respectively in a cell or in a FP-paired fluorescence resonance energy transfer (FRET) application, which is discussed later this in chapter. Also, FP-expressing cells may be labeled using immunofluorescent markers to identify cell phenotype, or they may be labeled for nucleic acid quantitation to determine the protein expression profile related to cell cycle phase. Cells can be fixed in 1.0–4.0% formaldehyde for any of these procedures with good retention of FP intensity over a period of days. Ethanol fixation should not be used unless the GFP fusion protein is anchored intracellularly.[25] A combination of formaldehyde and methanol has also been reported to provide fixation of both transmembrane and soluble proteins while preserving EGFP fluorescence, and is suitable for samples prepared for microscopic evaluation.[26]

Technical Considerations

- When using EGFP with other labels in a multicolor application, select red or far-red emitting fluorochromes to minimize issues of spectral overlap and spectral compensation. An ideal combination is EGFP and APC (or Cy5) when using a dual laser cytometer, or EGFP and PerCP, Cy5/PE, Cy5.5/PE or Cy7/PE on a single 488 nm laser cytometer. Alternatively, choose a red-shifted FP and then use FITC labeling for immunofluorescence.
- Most FPs can be excited well using a 488 nm laser line, common to most flow cytometers. Optical filters must be selected carefully when looking at multiple FPs, or FPs and other fluorescent markers in multicolor experiments. Consider narrow bandwidth (±5–10 nm) optical filters for use in fluorescent protein detection.
- A sub-optimal excitation laser wavelength may only be available on the flow cytometer for a particular FP. This can often be used with satisfactory excitation and fluorescence, since FP fluorescence levels can often be very bright.
- The distribution of FP fluorescence can sometimes span four log decades. Be aware that cells at the highest level of expression may present difficulties related to spectral bleedover compensation when used in multicolor applications. If need be, decrease PMT voltage to bring cells on scale, and then readjust compensation. Also, with certain instruments, laser intensity can be decreased, or a neutral density filter may be used along with the bandpass filter in order to decrease bright FP fluorescence.
- When purifying viable cells for high FP-expression using cell sorting, be aware that the cells with the highest level of expression may not be the best candidates to sort. Verify by backgating before sorting that the cells selected for sorting have the same light scatter characteristics as the lower-expressing cells from the same cell preparation. Cells with decreased forward angle light scatter and increased side scatter are often apoptotic (Figure 2). If possible, use a viability dye such as propidium iodide or Hoechst 33258 to identify live cells.

Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer, or FRET, provides the means for examining the proximity of molecules within the range of 10 to 100 angstroms (Å). In FRET studies, a donor fluorophore is excited optimally, and if a suitably chosen acceptor fluorophore is within the specified distance, the donor fluorescence emission excites the acceptor fluorophore, and the acceptor fluorophore fluorescence is seen. If FRET occurs, donor fluorescence should decrease in intensity, while acceptor fluorescence should increase (Figure 1).

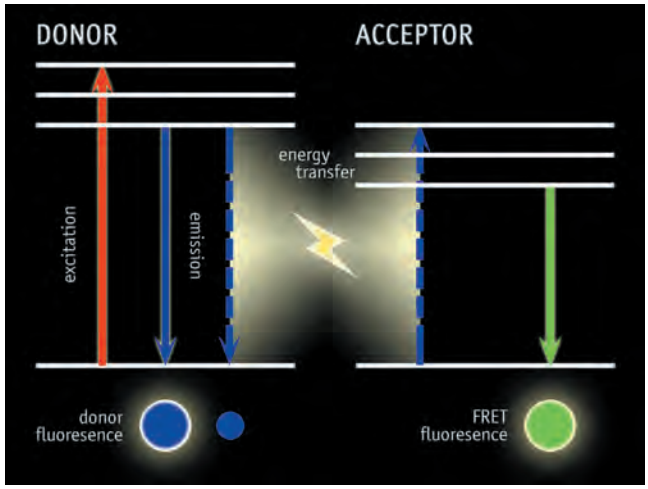
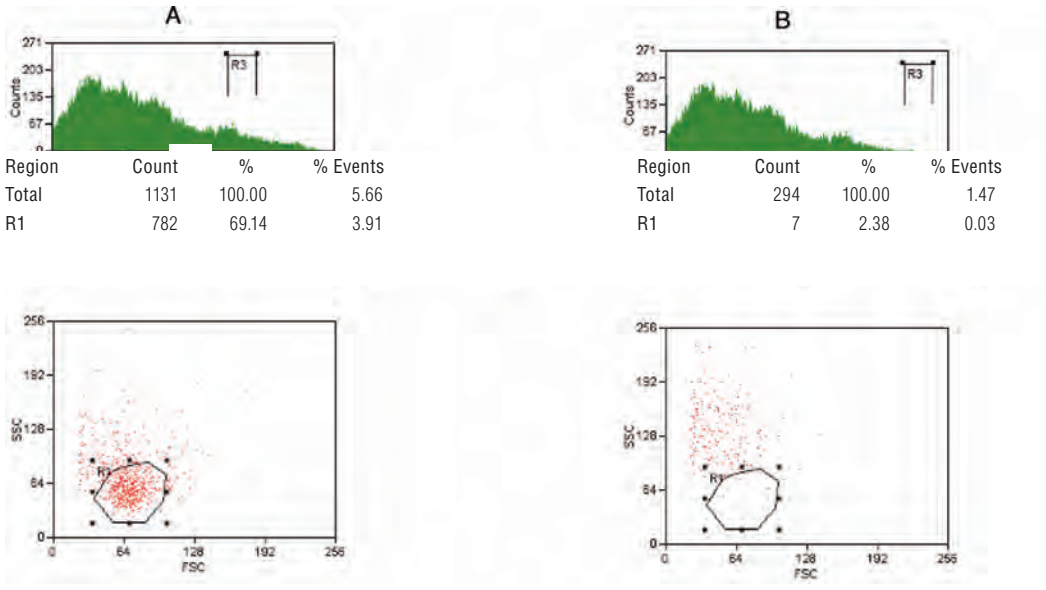


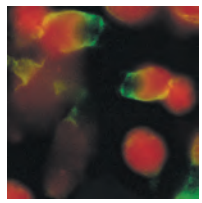
Figure 1. Fluorescence Resonance Energy Transfer



Region	Count	%	% Events
Total	1131	100.00	5.66
R1	782	69.14	3.91

Region	Count	%	% Events
Total	294	100.00	1.47
R1	7	2.38	0.03

Figure 2. A YFP-expressing cell line in Panel A is gated through R3 for positive FP expression, and backgated into the FCS vs. SSC dotplot showing viable cells. In Panel B, the highest-expressing cells (R3 shifted to the right) are outside the gate, in the area typical of apoptotic cells. (MoFlo® High-Performance Cell Sorter, Dako)



FRET systems, such as the bioluminescence aeqourin/wtGFP system in jellyfish or the multi-chromophore phycobiliproteins in blue-green algae,[27] exist in nature. Man-made FRET systems have been widely utilized commercially, marketed as "tandem dyes," and are used for fluorescently labeling antibodies.[28] These tandem-dye labeled antibodies (Cy5/PE or Cy7/APC, for example) are now available as a routinely used flow cytometry reagent, extending the multicolor capabilities in single and multiple laser instruments. Another constructed FRET reagent is the "chameleon" calcium-level probe, which allows observation of cellular calcium changes in vivo without the invasive and sometimes difficult addition of a calcium indicating dye such as Indo-1.[29] The chameleon consists of an energy transfer FP pair, such as CFP and YFP, joined linearly, with calmodulin and M13 separating the two FPs. Calmodulin can bind calcium ions, while M13 binds calmodulin only when calmodulin is bound with calcium ions. In the presence of calcium, this binding between calmodulin and M13 takes place, pulling the attached CFP and YFP into close proximity so that FRET occurs.

FRET has been used as a "molecular yardstick," and is very sensitive to molecular distance. The efficiency of the energy transfer process is inversely proportional to the 6th power of the distance between donor and acceptor.[30] Due to this high sensitivity to molecular distance, careful consideration must be paid in the construction of FP fusion proteins (varying FP fusing N- or C-terminally, for instance). An understanding of potential protein pairing alignment is helpful when designing fusion proteins for FRET studies.

In order to demonstrate in vivo protein/protein proximity and, therefore, implied protein interaction, FP-expressing fusion proteins have been used. Other FRET systems have also been used, including fluorescent labeled antibody pairs bound to their respective antigen epitopes.[31] Although the BFP and EGFP pair has been used for FRET studies,[32] ECFP and EYFP are most often used due to their increased quantum yield, lower tendency to aggregate, and resistance to photobleaching.

In most cases the increase in acceptor fluorescence shows the greater dynamic range, and this acceptor fluorescence increase is scored as FRET, although donor fluorophore quenching during FRET can also be detected as a measure of FRET efficiency.[33] In all cases, donor emission and acceptor excitation spectra must have significant overlap.

An example of a FRET experiment is shown here (Figure 3).

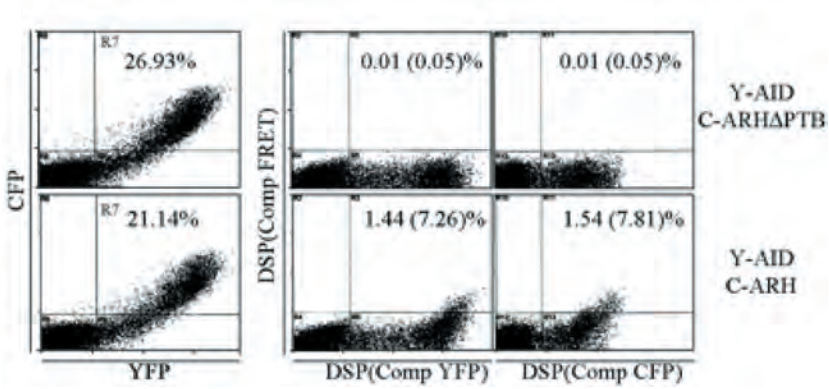
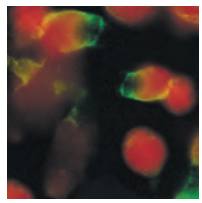


Figure 3. ARH interacts with ABPP in vivo. Panel A illustrates cotransfection of YFP and CFP fusion proteins. YFP-AID (Y-AID), CFP-AHR (C-ARH) and CFP-ARHdeltaPTB (C-ARHdeltaPTB) are fusion proteins expressed in vivo to demonstrate the interaction between the AID portion of the amyloid β protein precursor (AbPP) with the autosomal recessive hypercholesterolemia (ARH) adapter protein. In Panel B, an increase in the intensity of cells on the Y axis indicates FRET between Y-AID and C-ARH, but not between Y-AID and C-ARHdeltaPTB. Cells in the upper right quadrant in plots from Panel B were scored as being positive for FRET, and percentages in parentheses are the fraction of cotransfected cells (R7 in Panel A), which show FRET. (MoFlo® High-Performance Cell Sorter, Dako)

Experimental Protocol for FRET Flow Cytometry

In order to optimally perform a FRET experiment using flow cytometry and control for spectral bleedover issues, several control samples must be included in the experimental protocol. The following suggested protocol illustrates samples required to perform a CFP/YFP FRET analysis. Please note that all samples optimally should be of the same cell type, and single transfectants should use the same fusion proteins as the experimental sample.

1. **A non-fluorescent protein transfectant.** This cell sample is used for initial instrument setup, establishing photomultiplier tube (PMT) settings and determining a live-cell gate using light scatter.
2. **A CFP-only transfectant.** This sample is used to fine tune PMT settings and to monitor spectral bleedover into the YFP and FRET detectors.
3. **A YFP-only transfectant.** This sample is again used to fine tune PMT settings and to monitor spectral bleedover into the CFP and FRET detectors.
4. **A CFP and YFP dual transfectant, where the two proteins are known NOT to interact.** Here dual CFP/YFP expression should be observed without any increase in FRET.
5. **The actual CFP/YFP experimental sample.** FRET can now be reliably detected if present.



Technical Considerations

- Negative cells in CFP and YFP individual control transfectants may have a higher level of fluorescence compared to the mock transfectant and may require lowered PMT voltage.
- It may be necessary to rerun individual CFP and YFP controls to fine-tune PMT voltages and spectral bleedover compensation before analysis of the experimental sample. Mixing these two samples together may be helpful for this task.

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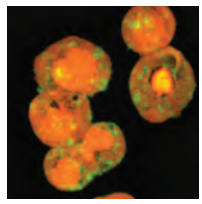
Acknowledgment

Figure 1 was adapted from Siegel RM et al. Measurement of molecular interactions in living cells by fluorescence resonance energy transfer between variants of the green fluorescence protein. Science's STKE 2000; Available at: http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2000/38/pl1



Signal Transduction

John Ransom, PhD

Definition and Overview

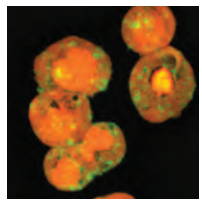
Signal transduction refers to the intracellular biochemical and biophysical processes involved in transmission of extracellular signals from the outside of the cell, across the plasma membrane and into the cell interior. Signal transduction processes serve to translate events in the extracellular environment into a signal that is recognized by molecular systems within the cell so that the cell can respond in some way to the extracellular event. The external signal is typically a chemical, such as a hormone, growth factor or neurotransmitter, but it could also be physical, such as light impinging on the rhodopsin "receptor" located in the retina of the eye or the shear forces acting on pressure sensitive ion channels. Receptors in the cell membrane usually recognize the extracellular signal with a high degree of specificity so that extraneous signals do not randomly activate signal transduction pathways within the cell. Highly specific recognition of certain molecules outside the cell by particular receptors or sensors on the cell leads to activation of unique biochemical and biophysical pathways within the cell. Examples of extracellular triggers, or mediators, are neurotransmitters, such as serotonin, dopamine or acetylcholine; peptide ligands, such as bradykinin or various chemokines; peptide hormones, such as luteinizing hormone-releasing hormone (LHRH); cytokines such as interleukin-2 or interleukin-4; soluble antigens recognized by B lymphocyte-associated immunoglobulin; and major histocompatibility complex-associated antigens recognized by T lymphocytes. Typically, the signaling pathways are composed of a cascade of enzymatically catalyzed events that impact one or more effector molecules in the cell, and the effectors then induce a change in the status of the cell, such as proliferation, cytokine production or contractile events. Intracellular responses are also very diverse and include events such as positive or negative gene regulation, resulting in changes in expression of key proteins, cell migration, initiation or arrest of cell division, or alterations in ion channel properties resulting in changes of membrane potential.

Examples of signal transduction pathways include the G_q, G_s or G_i guanine nucleotide binding protein (GPCR) signaling pathways that result in increased intracellular calcium levels (Ca^{2+}_i), increased cAMP levels or decreased cAMP levels, respectively.[1] In one common example, a GTP-binding protein (Gq) couples to the cytoplasmic side of a G protein-coupled receptor (GPCR) when the GPCR is occupied by a stimulatory ligand (serotonin, 5HT). The G protein catalyzes activation of a phospholipase C enzyme that in turn catalyzes degradation of a specific class of phospholipid from the inner side of the plasma membrane. The two products from the phospholipid activate an intracellular Ca^{2+} ion channel and a protein kinase, resulting in a bifurcation of the signal transduction cascade into Ca^{2+} and phosphoprotein regulated pathways.[2]

Another example of a well described signal transduction pathway is the phosphorylation of tyrosine residues following engagement of intracellular tyrosine kinase enzymes by an activated receptor.[3] This initial event typically initiates a cascade of tyrosine phosphorylation events wherein a series of phosphoproteins are formed by the sequential action of a series of substrate specific tyrosine kinases. A well described example of such a cascade is the MAPK (mitogen-activated protein kinase) cascade that is initiated by several types of extracellular stimuli (receptor ligands, cell stress) and culminates in gene regulation. The initial receptor-mediated event can also lead to assembly of protein complexes via recognition of newly formed, specific phosphotyrosine sequences by other intracellular proteins. The newly recruited molecules may further transmit signaling messages via their own regulated kinase activity. Tyrosine kinase signal transduction cascades can be complex and diverse, involving multiple phosphorylation events, or very simple and direct, involving only one or two phosphorylation events before eliciting a cellular change. The number of receptors known to initiate tyrosine kinase/tyrosine phosphorylation-based signal transduction pathways is very large, and the list includes many receptors for growth factors such as epidermal growth factor receptor (EGFR), fibroblast growth factor (FGF), neurite growth factor (NGF) and the very large collection of cytokines involved in regulation of an immune response, such as the receptors for interleukins-2, -4, -10 and -12 to name just a few.

Finally, signal transduction events that involve changes in membrane permeability to ions via regulation of opening and closing of ion specific channels can be included in the list of signal transduction events suitable for study by flow cytometry. An example of a receptor recognition event that might be expected to lead to alterations in membrane potential is activation of the 5HT3 serotonin receptor, which functions as a ligand-gated Na^+ channel, leading to Na^+ influx and cell depolarization. Such changes in membrane permeability can also lead to depolarization or hyperpolarization of the plasma membrane, which in turn can influence the behavior of other channels in the membrane, such as voltage-gated Ca^{2+} channels. Additionally, voltage-gated channels, such as the L-type Ca^{2+} channel, can be activated by the elevation of extracellular K^+, which leads to instantaneous depolarization of the entire cell, opening of the channel, and a transient influx of Ca^{2+} inside the cell. Changes in membrane potential have been shown to drive a number of cellular responses from muscle cell contraction to neurotransmitter release to proliferation.

These examples of the wide variety of signal transduction events and systems understood to date are not at all inclusive, and there are many other well characterized examples to be found in the literature. It should be understood that the field of signal transduction is very diverse. The study of signal transduction events by flow cytometry is in a state of relative infancy compared to other means of studying signal transduction cascades, but the insights to be gained by the application of flow cytometry to signal transduction events can be very powerful. Like microscopy-based systems, the flow cytometer interrogates individual cells, but unlike microscopy, the analysis of thousands of cells per second by modern flow cytometers enables powerful statistical results and the recognition of very rare events at rates not possible by microscopy-based systems.



Secondly, the flow cytometer is designed to sort, or recover, those cells exhibiting a phenotype of interest to the scientist – a process that is not currently available on microscopy-based systems, with the exception of certain instruments with laser ablation technologies still under development. Thus, in the case of signal transduction events, sorting capabilities allow the investigator to isolate those cells from a population exhibiting the signal transduction response of interest (e.g. Ca^{2+}_i mobilization or altered membrane potential) for further growth or biochemical or molecular analysis. This is a very powerful advantage when trying to understand the molecular or biochemical basis of a particular response, such as when trying to identify an unknown receptor that recognizes a novel "orphan" ligand, or when trying to determine novel proteins involved in a signal transduction pathway. Alternatively, by using molecular biology techniques to insert, or increase, novel proteins with signal transduction potential into cells, sorting those cells based on a particular signal transduction phenotype, and then analyzing the sorted cells for the expression of the candidate molecules, it is possible to quickly understand the role of novel candidate molecules in signaling events.

Measurement of Signal Transduction Events

For practical purposes, measurements of signal transduction events tend to focus on changes in the activity of a single step in the signal transduction cascade. The measurement does not necessarily need to be focused on the final effector molecule in the pathway. For example, the signal transduction pathway that leads to interleukin-2 production by mitogen- or antigen-stimulated T lymphocytes involves several steps between the antigen receptor and Ca^{2+}_i mobilization, and several more downstream of Ca^{2+}_i mobilization that culminate in IL-2 gene transcription and secretion. To successfully produce IL-2, the T cell antigen receptor needs to be activated, and Ca^{2+}_i needs to be mobilized for several hours. Transient Ca^{2+}_i elevations simply lead to abortive activation of the T cell and either cell death or a state of non-responsiveness known as anergy. Yet, despite this sustained temporal requirement for the development of a full response, the study of biochemical and molecular aspects of productive T cell activation has been well served by flow cytometric analysis of relatively transient Ca^{2+}_i mobilization events occurring over just several minutes. Ca^{2+} mobilization is reviewed in the next chapter (Kinetics).

Evaluation of the increase or decrease in phosphotyrosine content of a single intermediate protein in a tyrosine kinase signal transduction cascade, such as the MAPK pathway, which results in gene expression changes that require hours to develop, is an accurate indicator of the degree of activation of the entire pathway. Very good examples of such measurements are illustrated in the studies of Hedley and colleagues,[4-5] where sequence-specific antiphosphotyrosine-specific antibodies were used to detect the relative levels of specific phosphotyrosine content of one or more activated intermediate proteins in the MAPK/ERK and STAT5 signaling cascades. The studies showed that sequence specific antibodies could be used to follow changes in target-specific phosphotyrosine content on a single cell basis by multiparametric flow cytometry and commercially available antibodies. Initially, it was necessary to perform

several experiments to optimize the fixation, permeabililzation and staining conditions. These efforts were necessary to obtain maximal detection of the target with minimal cross-reactivity of the antibodies. Once the assay conditions were optimized, preliminary results were compared and confirmed by standard anti-phosphotyrosine Western blot methods. The systems were then successfully used to evaluate and confirm the activities of several compounds designed to block the signal transduction cascades. This technique offers great potential in many areas of biomedical research, such as monitoring the activities of test compounds on the signal transduction components involved in the aberrant growth regulation of cancer cells.

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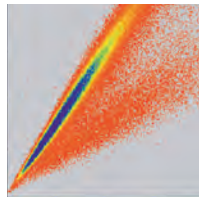


Kinetics

John Ransom, PhD

Definition and Overview

Kinetics, or more precisely, Cellular Response Kinetics, refers to the study of cellular signal transduction events that have relatively transient natures, on the order of a several minutes or less. Typically, once initiated, the response rises to a peak level within seconds, and the peak level may be sustained before declining, or it may begin to decline soon after the peak is attained and at a relatively rapid rate.

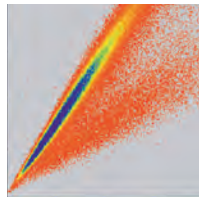
To study kinetic events with a fluorescence-based instrument like a flow cytometer, it is necessary to have a fluorescent probe that faithfully reflects the transient nature of the signal transduction event being studied. An excellent example is the family of probes including indo-1, fluo-4 and fura-2, designed to measure intracellular calcium levels (Ca^{2+}_i) in viable cells.[1] These probes have affinity constants for Ca^{2+} that are slightly higher than the corresponding concentrations of Ca^{2+} in resting cells, exhibit changes in their fluorescent properties when the probe goes from the Ca^{2+}-free to the Ca^{2+} bound state, are non-toxic, and can be easily loaded into viable cells. Thus, the probes will indicate both an increase and a decrease in free cytosolic Ca^{2+} within the viable cell with minimal error in reporting the temporal aspects of the transient changes in Ca^{2+}_i.

In flow cytometry work, kinetic studies are most often typified by the Ca^{2+}_i mobilization responses initiated by a wide variety of receptors that couple to mobilization of a Ca^{2+} store within the cell, or to influx pathways that allow Ca^{2+} to diffuse in from outside of the cell down a very large and inwardly directed Ca^{2+} concentration gradient. As a potentially toxic ion, free Ca^{2+} would be lethal to a cell if it were allowed to exceed normal intracellular concentrations of approximately 100 nM or less for very long. Mammalian cells have developed systems that regulate the free Ca^{2+}_i concentration in the cell by extruding or sequestering the ion away from the cytosol. Along with their other critical cellular functions, certain organelles, such as the mitochondria and endoplasmic reticulum (ER), serve as high capacity storage sites for Ca^{2+} ions. Under the correct conditions, these stores can be transiently released into the cytosol, resulting in a brief pulse of elevated Ca^{2+}_i. This pulse serves to activate other signal transduction machinery, which leads ultimately to an immediate cellular response, such as degranulation or contractility, or changes in gene expression that result in altered cell phenotype and function more slowly. Relaxation of the Ca^{2+}_i signal is achieved by the activation of the various sequestration and extrusion mechanisms operative within the cell that serve to rapidly reduce the concentration of elevated free Ca^{2+} to the non-toxic level of approximately 100 nM. Other events besides Ca^{2+}_i mobilization can be measured by flow cytometry and also show transient kinetic behaviors. These include membrane potential changes, pH_i changes, Na^+_i and K^+_i ion changes.

Measurement of Kinetic Events

This chapter will focus on one example of kinetic events available to all users, Ca^{2+}_i mobilization, but other signal transduction pathways and events can be studied in kinetic mode since the correct fluorescent tools, such as pH and membrane potential sensors, are available. Ca^{2+}_i mobilization can be elicited by a number of different pathways including the action of inositol trisphosphate (IP3) at the IP3 receptor on the ER store or the activation of plasma membrane Ca^{2+} channels. Here, the focus is on activation of the phospholipase C (PLC) pathway, which can occur via activation of Gq type G protein-coupled receptors (GPCRs) in which the Gq protein directly activates PLC or via activation of tyrosine kinase pathways that can also activate PLC activity.[2] The result of both pathways is the rapid hydrolysis of the plasma membrane phospholipid, phosphatidylinositol trisphosphate (PIP2), to yield diacylglycerol (DAG) and IP3. The released IP3 freely diffuses through the cytosol, binds to the IP3 receptor on the ER, which functions as a Ca^{2+} channel, and results in the release of Ca^{2+} from the ER store into the cytosol. The rate of release of Ca^{2+} from the ER is typically sufficient to raise the Ca^{2+}_i from approximately 70-100 nM at rest to peak levels of 200-700 nM. As the initiating signal transduction cascade wanes and the formation of IP3 declines, energy dependent Ca^{2+} pumps on the ER, the mitochondria and the plasma membrane will engage and remove the Ca^{2+} from the cytosol. Typically the Ca^{2+}_i will be returned to resting levels within less than one to two minutes of the initiation of the response. A Ca^{2+} influx pathway (SOCC or ICRAC) is activated when the ER store of Ca^{2+} is reduced, but such a mechanism is beyond the scope of this review and may be studied elsewhere.[3]

Ca^{2+}_i mobilization can be studied on most flow cytometers, as long as the data acquisition software permits continuous acquisition and display of events over time so that a continuous distribution of the status of the cells can be displayed during the time kinetic measurement. The ready availability of fluorescent Ca^{2+}_i-reporting probes that are optimally excited by either visible or ultraviolet wavelength lasers and the ease of loading of these probes into viable cells makes them simple to use. The most accurate dye for common applications is the ratiometric probe indo1, since the emission intensities of the probe measured at two distinct regions of the spectrum will change inversely as the Ca^{2+}_i increases or decreases (Figure 1). By calculating the ratio of the two different emission intensities, any variation in the extent of dye loading amongst different cells is eliminated, and a very narrow range of Ca^{2+}_i is indicated in most resting cell populations. Calculation of the ratio of the fluorescence intensity at two different spectral intensities (e.g. 510 nm/410 nm) will give a value that increases or decreases as the Ca^{2+}_i increases or decreases over time, respectively. The drawback with indo1 is that it requires a UV excitation source, such as a HeCd laser (325 nm), to be excited. Although not ratiometric, several good alternative probes, such as fluo-4 and Oregon Green 488 BAPTA-1, are available and provide nearly as reliable and accurate indices of Ca^{2+}_i. Both are as convenient to measure as standard fluorescein-labeled probes since both are excited by the 488 nm emitting argon lasers available as stock equipment on almost all flow cytometers and both have peak emissions in the 515 nm to 535 nm range. In this case emissions at a single wavelength region are acquired, and the emission intensity increases or decreases as the Ca^{2+}_i increases or decreases, respectively.



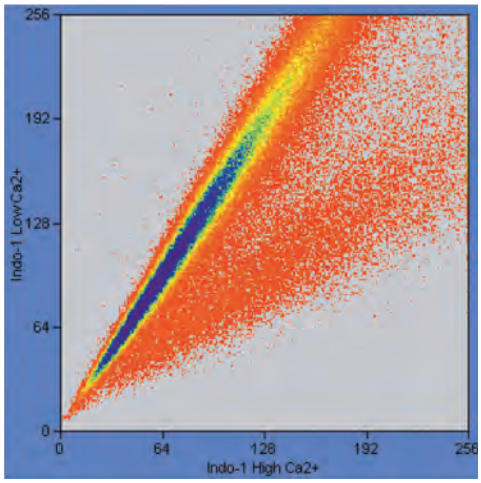


Figure 1. A bivariate distribution plot of cells loaded with indo1 showing the emissions at 400–420 nm (x-axis) and 500–530 nm (y-axis). The upper and lower populations are resting and stimulated cells, respectively. Data was collected on a Dako MoFlo® using Summit Software.

An example of a Ca^{2+}_i response measured by flow cytometry is shown in Figure 2. Here, HEK-293 cells were loaded with indo1 and sampled from a standard tube containing 0.5 mL of the suspension. Carbachol (10 µM) was added to activate the muscarinic receptor constitutively expressed by the cells. The tube contents were gently mixed and placed on the cytometer sample input. The sample was briefly boosted to accelerate transit of the cells to the laser intercept, and the data was acquired for three minutes. Since a GPCR-mediated Ca^{2+} mobilization response can develop within seconds, it is important to minimize the time required to mix the sample and begin analysis. Otherwise, the early phase of the response may not be observed.

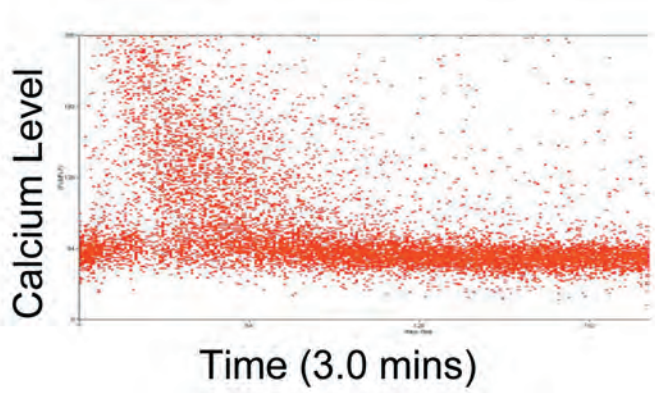
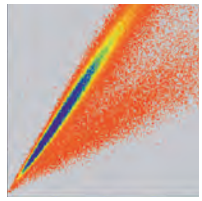


Figure 2. A time kinetic plot of a Ca^{2+}_i mobilization response showing the indo-1 emissions ratio (y-axis) and time (x-axis). Data was collected on a Dako MoFlo® using Summit Software.

Measurement of Ca^{2+}_i Response Kinetics

A standard Ca^{2+} response kinetic experiment can be performed for most mammalian cells using the following techniques, assuming that the cells express a receptor that couples to Ca^{2+} mobilization. The cells should be grown under optimal tissue culture conditions. Cells that are overgrown or starving for nutrients will show blunted responses, or none at all. The cells should be harvested using normal techniques for tissue culture passage, with special efforts not to treat the cells roughly (e.g. pipetting them too vigorously). To load the cells with a Ca^{2+} indicator, the probe should be in the membrane permeant, ester form (e.g. indo1AM, fluo4AM) and prepared in a stock solution of 1 mM in DMSO. Stock solutions should be aliquoted and frozen at –20° C, thawed and used only once, then discarded. The stock solution of probe should be diluted into the cell suspension (1-5 x 10^6 cells/mL) to a final concentration of 1-5 µM. The cells should be in an isotonic buffer such as Hank's Balanced Salts Solution (HBSS) containing Ca^{2+} and Mg^{2+} rather than culture medium. Good loading occurs if the cell mixture is gently rocked on a nutator or rotator for one hour at room temperature, but satisfactory loading also occurs at 37C° for 30–40 minutes with occasional inversion to keep the cells suspended. The best situation is to use the minimal amount of dye to load the cells to obtain the best signal while loading sufficient dye to obtain a good signal.

After dye loading, the cells should be washed once and resuspended in HBSS at 0.5–5 x 10^6 cells/mL for analysis. Virtually all Ca^{2+}_i mobilization events will be apparent at room temperature. Aliquots of cells (0.5–1 mL) are sufficient for most experiments running at normal flow rates. Every experiment should include a baseline measurement where the cells are run for 15–30 seconds with no additions to that sample. This provides an index of the degree of homogeneity of the Ca^{2+}_i levels in the population. Ideally the population distribution will be very tight, but in some cases, distribution of Ca^{2+}_i levels is very diverse, and this can make appreciation of changes in Ca^{2+}_i within the population difficult to resolve and interpret. The second important control is a mock mixing test where the sample tube is removed from the instrument, buffer control is added to the sample, the sample is mixed by gently swirling or flicking the tube, the sample is returned to the instrument and pressure is resumed with a sample boost if necessary. This mock mixing control determines whether the cell population is susceptible to mechanical shear forces, such as mixing and sample pressurization, that can activate pressure sensitive ion flux mechanisms present in some cell lines. If this control is not run, then a ligand can often be misinterpreted as stimulatory when in fact the apparent response was simply due to the false response of the cells to the shear forces encountered during ligand addition, mixing and sample pressurization. If such activities are present, then efforts can be taken to reduce the vigor of the mixing and pressurization steps. If one is quick and clear, these controls can be performed with the same sample that will be exposed to ligand, and a continuous acquistion data file can be obtained from a 1 mL sample over 2 minutes. When the ligand has been added, most receptor-initiated responses with a moderate to high affinity ligand will develop within 5-30 seconds (Figure 2). Peak responses should be seen within 10-60 seconds. This will be followed by a decline phase where the population shows a decreasing number



of cells with elevated Ca^{2+}_i levels, and the average level of the population decreases. The Ca^{2+} ionophore, ionomycin (1-20 µM), serves as an excellent positive control to test whether the cells are properly loaded with reporter dye and whether the flow cytometry system is properly set up.

It is important to note that, unlike microscopic analyses where the response of each cell is evaluated over time, flow cytometry samples individual cells from a large population at very discrete time points. Thus, kinetic flow cytometry data is a continuous sampling of individual cells from a large population at single brief time points throughout the kinetic measurement time span. This example clearly shows the response heterogeneity of the population and the transience of the response.

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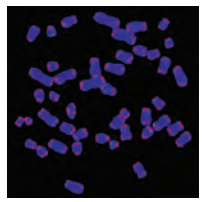


Basic DNA Measurement by Flow Cytometry

Ian Brotherick, PhD

DNA, or deoxyribonucleic acid, is the molecule that carries the genetic information for all living organisms and is the main constituent of chromosomes. DNA is made from a discrete number of chemical building blocks, or bases, which combine to form two strands arranged as a double helix. Existing mainly in the cell nucleus, DNA is important in cell reproduction, cell life and cell death. Consequently, DNA has proved to be of interest to flow cytometrists, both in research and clinical fields.

DNA Probes

In order to measure DNA by flow cytometry, it is first necessary to stain or label the DNA with a fluorescent probe. Unlike those fluorochromes used on labeled antibodies, DNA probes fluoresce more when bound to their target molecule. The base-probe bond is not as strong as that of antibody to antigen, and the DNA probe is, consequently, in equilibrium with the free probe in solution. Changes in probe concentration, by dilution of the sample, for example, can therefore, influence the fluorescence intensity of the DNA due to the change in equilibrium. For this reason, DNA preparations are not washed to remove unbound probe; otherwise the equilibrium will be upset. Fortunately, the unbound probe does not fluoresce and, hence, demonstrates low background fluorescence.

The DNA labeling fluorochrome usually intercalates between the bases in double stranded nucleic acids. However, such fluorochromes can also stain double stranded RNA, which should be eliminated by addition of RNAase to the cell preparation in order to obtain good results. Single stranded nucleic acids do not stain.

The key feature of DNA probes is that they are stoichiometric. This means that the number of molecules of probe bound to DNA is equivalent to the number of molecules of DNA present. Consequently, the amount of emitted light is proportional to the amount of bound probe and, therefore, the amount of DNA.

Generally, the DNA probes belong to the family of chemicals broadly known as the phenanthridiniums (includes propidium iodide and ethidium bromide). They generally excite in the ultraviolet or blue part of the spectrum and show red spectral emission. Table 1 demonstrates the properties of some of the commonly used DNA probes. A more comprehensive list may be found in *Flow Cytometry: First Principles* by A.L.Givan.[1]

Table 1. Common DNA Probes

Probe	Excitation	Emission
Propidium Iodide	536 nm (488 nm laser)	623 nm
DAPI	359 nm (UV laser)	461 nm
DRAQ5	650 nm (488 nm or 633 nm laser)	680 nm
Hoescht	346 nm (UV laser)	460 nm

Terminology

In order to effectively measure DNA, it is first necessary to become familiar with some of the terms employed in DNA analysis.

Cell Cycle

Most cells regenerate to replace dead or damaged cells or to grow. In order to grow, the cell doubles its DNA content. The DNA forms two sets of chromosomes, which line up on spindles within the cell before the cell divides into two equal halves. At one time point in this process, prior to cell division, or mitosis, the cell will contain twice the normal amount of DNA. The normal amount of DNA is referred to as diploid or 2n.

The cell cycle is generally split into three identifiable component parts by the flow cytometrist (Figure 1). These are the G_0G_1, S and G_2M phases of the cell cycle. More correctly, G_0 is a phase where cells are quiescent and not taking part in cell division. G_1 is the phase where cells are gearing up to move through cell division. As both of these components of the cell cycle have 2n DNA, these phases are not distinguishable from each other using solely a DNA probe. S phase is that part of the cell cycle where synthesis of DNA occurs and where DNA staining increases. G_2 and M phases of the cell cycle are where 4n DNA is present, just prior to and during mitosis, respectively. Again, with just a DNA probe, these two phases are not distinguishable from each other by the flow cytometer.

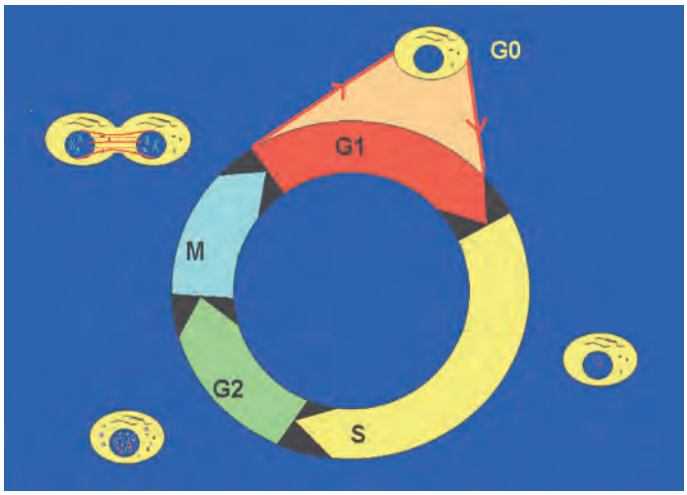
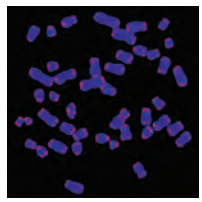


Figure 1. The cell cycle is generally split into three identifiable component parts by the flow cytometrist: G_0G_1, S and G_2M phases of the cell cycle.

This single-parameter histogram (Figure 2) shows fluorescence on a linear scale along the x-axis with number of events up the y-axis. Linear is chosen for fluorescence when examining DNA. This allows the ready determination of DNA index values, or ratios between peaks. Also, in cases where euploid and aneuploid cells are mixed, it aids identification of which G_0G_1 belongs to which G_2M.



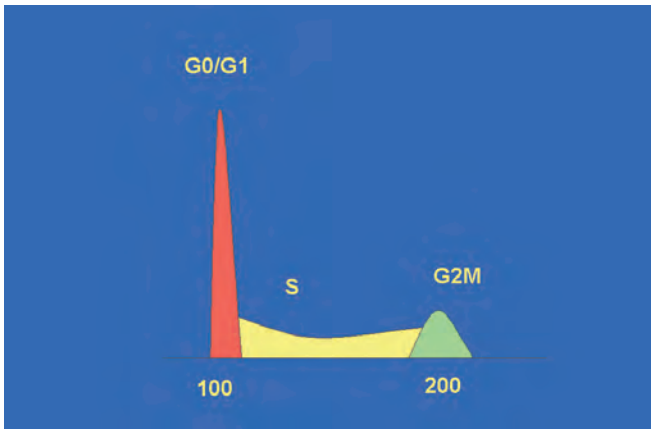


Figure 2. This single-parameter histogram identifies populations in the various stages of cell cycle.

Ploidy

The ploidy of a cell is an indication of the number of chromosomes in that cell. Each species has a different ploidy value. There can also be variations within an individual population due to mutations, natural multiploidy (plants), certain diseases (including cancer) and apoptosis. The flow cytometrist tries to define these different ploidy levels and may use a series of definitions and terms.

1. **Diploid:** The normal (euploid) 2n number of chromosomes. This is the number of chromosomes in a somatic cell for a particular species.
2. **Haploid:** Half the normal 2n number of chromosomes, or 1n. This is the number of chromosomes in a gamete or germ cell (sperm/egg). Again, this is species-dependent.
3. **Hyperdiploid:** Greater than the normal 2n number of chromosomes.
4. **Hypodiploid:** Less than the normal 2n number of chromosomes.
5. **Tetraploid:** Double the normal 2n number of chromosomes, or 4n.
6. **Aneuploid:** An abnormal number of chromosomes.

Sample Preparation

In order to successfully measure DNA by flow cytometry, the following issues must be addressed.

Single-Cell Suspension

Certain cell types naturally lend themselves to flow cytometric analysis, coming in a pre-made solution as a single-cell suspension (e.g. blood). However, this does not preclude the use of flow cytometry to determine DNA measurement from solid tissues. It merely lengthens the procedure necessary in order to obtain a single-cell suspension.

Making single-cell suspensions from solid tissues is usually facilitated by one of two methods — mechanical disaggregation (e.g. Dako Medimachine) or enzymatic disaggregation (collagenase, hyaluronidase). Both methods have their advantages and disadvantages. Mechanical methods are usually quicker and tend not to be so harsh that they strip antigens from the cell membrane. Furthermore, mechanical techniques mean that the cell can be kept cold and, therefore, better preserved. For enzymatic methods to work effectively, temperatures must normally be around 37°C, often for several hours. During this time, cell viability may decrease. Conversely, there may be more shear damage from mechanical techniques. However, mechanical techniques usually process the entire tissue, whereas enzymatic techniques may leave behind undigested tissue, which can potentially contain cells of interest.

DNA Probes

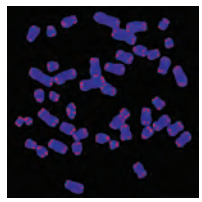
An important point is to check the excitation and emission spectra of the chosen probe (Table 1) and make sure the flow cytometer has the correct laser to excite the probe and the correct filters to detect the emission spectra. Most bench-top instruments and some cell sorters tend not to have powerful UV lasers, effectively reducing the number of probes available for use as DNA probes.

Cell Permeabilization

Also, as most DNA is located in the cell nucleus, it is necessary to get the DNA probe into the nucleus. This may be done in several ways, with varying degrees of harshness, depending on what the final measurement aims are. The treatments usually involve addition of detergents (Saponin, Triton X-100, Nonidet) or alcohol (ethanol, methanol) to the cells. Permeabilizing the cell membrane with, for example, low levels of saponin,[2] enables DNA measurement while maintaining cell surface labelling. Use of Triton X-100 can strip away the cell membrane and cytoplasm, leaving bare nuclei but giving tight DNA peaks. Alcohol permeabilization is also a popular technique, particularly when measuring nuclear antigens in combination with a DNA probe. Some DNA probes actually enter the cell without need for permeabilization (DRAQ5 and Hoescht). Cells have the ability to remove chemicals from their interior by membrane pumps, which are believed to be the cause of multi-drug resistance in cancer patients. For more in-depth discussions on permeabilization, see M. Ormerod's book, *Flow Cytometry*.[3]

DNA Stability

Introducing detergents or alcohol can lead to loss of antigens and more rapid DNA degradation. Introduction of a fixative (e.g. acetone, formaldehyde) helps stabilize the cell. However, fixation can also lead to protein conformational changes and DNA condensation, resulting in reduced antigen and DNA fluorescence profiles.[4]



Doublet Discrimination

One problem that must be overcome when obtaining results for DNA analysis is the exclusion of clumps of cells. On a flow cytometer, two cells stuck together may register as a single event, known as a doublet. If each of those two cells is diploid (2n), seen as one event, they have 4n DNA. In other words, they have the same amount of DNA as a tetraploid cell (G_0G_1), or the same amount as a normal cell that is about to divide (G_2M). To add to the confusion, further peaks may exist for three or more cells stuck together.

The doublet problem is resolved by employing a doublet discrimination gate based on the characteristics of fluorescence height, fluorescence area and signal width. Fluorescence height is the maximum fluorescence given out by each cell as it travels through the laser beam; fluorescence area is the total amount of fluorescence emitted during the same journey; and signal width is the time a cell takes to pass through the laser beam. These characteristics are different for a cell that is about to divide when compared to two cells that are stuck together (doublets shown in Figure 3). A dividing cell does not double its membrane and cytoplasmic size and, therefore, passes through the laser beam more quickly than two cells stuck together. In other words, it has a smaller width signal or a bigger height signal but the same area as two cells stuck together. Also, all of the DNA in the dividing cell is grouped together in one nucleus and consequently gives off a greater intensity of emitted fluorescence, compared with the DNA in two cells that are stuck together. Thus, a doublet, which has two nuclei separated by cytoplasm, emits a lower intensity signal over a longer period. This appears as a greater width signal, lower height signal and the same area. These differences can be seen on histograms of FL2-Area vs. FL2-Width or FL2-Area vs FL2 Height, allowing the generation of gates to exclude doublets from sample analysis for both diploid and aneuploid cells.

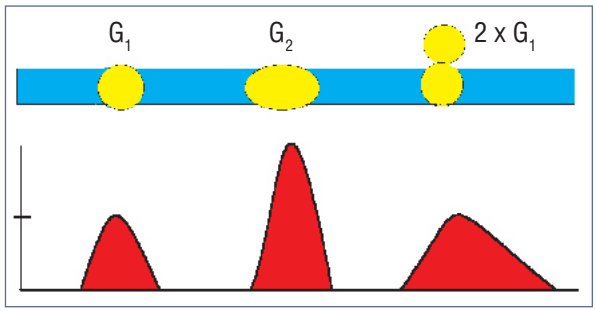


Figure 3. Doublet discrimination is possible based on fluorescence height, fluorescence area and signal width.

Instrument Setup

Instrument setup varies with manufacturer. However, there are some general principles to observe.

1. Select the LIN channel that is most appropriate for the DNA probe.
2. Set the trigger on the channel detecting the DNA probe, as light scatter parameters are not usually of much use for triggering in the case of DNA.
3. Select parameters to enable doublet discrimination.
4. Set a gate to exclude doublets and apply it to the histogram that will display the DNA profile. Displaying an ungated plot of the same may data may also be useful.
5. Make sure the sheath tank is full, as it may help with stability.
6. Make sure the cytometer is clean. Stream disruption will increase the CV.
7. Set a low flow rate and dilute cells to a concentration that is appropriate for the DNA probe solution.
8. Make sure the instrument has been optimized by running routine calibration particles.

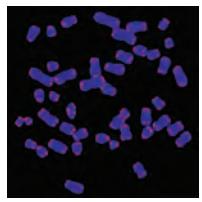
Standardization

Running daily alignment checks and passing standard quality control procedures should mean the instrument is capable of good DNA profiles. Further DNA standards are available and include trout and chicken erythrocyte nuclei as indicators of DNA ploidy. Tumor samples normally contain diploid cells that can be used as an internal standard. For aneuploid cell lines, spiking samples with known diploid cells can help with DNA index determination.[5-6]

Data Analysis and Reporting

Attempts have been made to standardize the generation of DNA flow cytometry results, both in reporting for clinical use and for publication of research data. These attempts have resulted in several articles, based on cell type.[7-11] However, some general guidelines are as follows.

1. Determine the cell ploidy and report the DNA index of all ploidy populations.
2. Report the CV of the main G_0G_1 peak. Generally, less than 3 is good; greater than 8 is poor.
3. When measuring the S-phase fraction (SPF) of a diploid tumor, make a statement as to whether the S-phase was measured on the whole sample, including normal cells, or on the tumor cells alone, gated by tumor-specific antibody.



4. Add a brief comment if necessary to cover any other information that may be helpful to someone looking at the result (e.g. inadequate number of cells, high debris levels, high CV, % background, aggregates and debris).

Not all attempts at DNA measurement will be successful. Methodological, sample and instrument issues may mean some of the DNA information is not suitable for interpretation and use in publications. Generally, a result should be rejected if any of the following apply:

1. CV of the G0G1 peak is greater than 8%.
2. Sample contains less than 10,000 to 20,000 nuclei.
3. Data contains more than 30% debris.
4. Flow rate was too high, as indicated by a broad CV or curved populations on two-parameter plots.
5. G0G1 peak isn't in channel 200 of 1024 or 100 of 512 (i.e. on a suitable scale in a known channel).
6. Fewer than 200 cells in S-phase (if SPF is to be reported).
7. G0G1 to G2M ratio is not between 1.95 and 2.05.

DNA and Beyond

Having mastered simple DNA applications, the cytometrist may incorporate them into more complex protocols. Combinations of surface, cytoplasmic and nuclear markers allow the cytometrist to explore chromosomes, apoptosis, cell doubling times and beyond.

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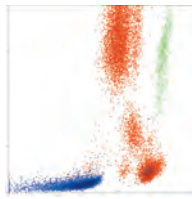


Absolute Cell Counting

Ian Storie

Applications

In the research and especially the clinical setting, there are situations that require not just the percentage positive of a particular cell population but the actual absolute number positive cells. There are three main areas in the clinical setting that require the precise and accurate determination of absolute cell counts.

CD4+ Lymphocyte Enumeration

The absolute CD4+ T lymphocyte count has long been recognized as a useful laboratory tool for the staging of HIV infected patients. It can also be used to assess the likelihood of opportunistic infections, the timing of the administration of preventative treatment and, more recently, for monitoring the effect of new antiviral therapies. Absolute CD4+ T lymphocyte counts of less than 200 cells/μL are generally accepted as being the cut-off point for the laboratory diagnosis of HIV infection. It should be noted that diseases other than HIV can result in a reduced CD4+ T lymphocyte count, and that a diagnosis of HIV infection cannot be made on the CD4+ T lymphocyte count alone.

CD34+ Hematopoietic Progenitor Cell Enumeration

Mobilization, harvesting and transplantation of CD34+ progenitor cells are now well-recognized techniques. By monitoring absolute CD34+ cell levels after growth factor-induced mobilization, it is possible to ensure the maximum number of cells can be collected with the minimum number of time-consuming and expensive harvesting procedures.

Residual White Blood Cell Enumeration

The presence of white blood cells (WBCs) in blood products has been shown to lead to febrile reactions, alloimunization, as well as the transmission of infectious agents (e.g. EBV, CMV and CJD). Many countries have now adopted a policy of filtering all red cell, platelet and fresh plasma products for transfusion to remove the WBCs. Quality control of the procedure involves screening a number of randomly selected filtered units and performing a residual WBC (rWBC) count. In Europe, the upper limit is 1×10^6 WBCs per unit, which equates to a count of around 3.3 WBCs/μL for an average 300 mL unit.

In all the above applications, it is essential to accurately identify the cell population of interest, and this population may be present at very low levels. The development of strategies using multiple gating regions, employing both light scatter and fluorescence parameters, have aided in this process. For example, the use of CD45 (pan leucocyte marker) versus side scatter results in a more reproducible and accurate lymphocyte gate.[1]

Likewise, the use of the ISHAGE gating protocol for CD34 determinations has resulted in more reproducible results.[2]

Methods

There are two flow cytometric methods that can be employed to derive absolute counts.

Dual-Platform Approach

The dual-platform approach employs the use of three separate measurements obtained from two different instrumentation platforms. A flow cytometer is used to determine the cells of interest as a percentage of a "reference" population (e.g. CD3+/CD4+ cells as a percentage of the total lymphocyte population). Then a hematology analyzer provides an absolute white blood cell count (WBC) and the lymphocyte percentage. The absolute CD4+ T Lymphocyte count is, thus, the product of the absolute WBC, the lymphocyte percentage and the CD4+ T lymphocyte percentage.

With the dual-platform approach, it is important to ensure that the cell population of interest, as well as its reference population, can be accurately identified. Different gating strategies have been employed to aid the identification of lymphocytes for CD4+ T lymphocyte enumeration. Initially, light scatter gating using FSC vs. SSC was used. The main drawback of this method is the increased likelihood of non-lymphocyte contamination in the gate, including cell debris, platelets, monocytes and basophils. This method is now not recommended for CD4+ T lymphocyte determinations[3] and has been superseded by the use of light scatter and an immunological marker, for example SSC vs. CD45. Cell debris and platelets are CD45 negative, monocytes have a slightly higher side scatter signal then lymphocytes, and basophils have a dimmer expression of CD45 than normal lymphocytes and, thus, can easily be excluded (Figure 1).

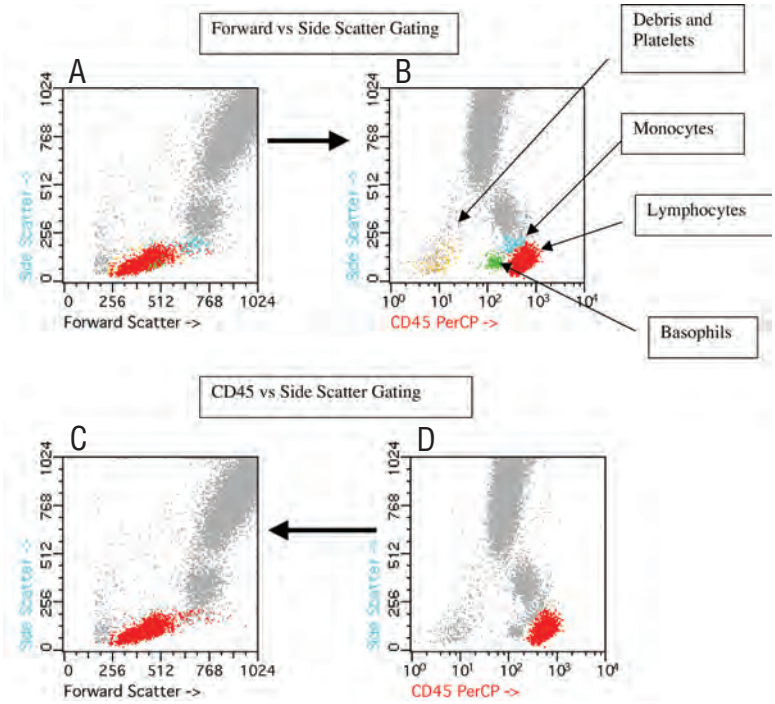


Figure 1. The importance of the correct identification of the reference population in dual-platform determinations. A and B show the potential contamination in a Forward vs Side Scatter gate drawn around the lymphocyte population. This gate includes cell debris and platelets, (yellow), monocytes (cyan) and basophils (green). Note how the basophils (green) sit in the middle of the lymphocyte population on Forward Side Scatter. In C and D, CD45 vs Side Scatter is used to gate the debris, and monocytes and basophils are excluded.

It has been well documented that the dual-platform method has a higher inter-laboratory coefficient of variation (CV) due to the way different hematology analyzers derive the absolute WBC and lymphocyte count, and not to the CD4+ T lymphocyte percentage from the flow cytometer.[4] The variance is seen to increase dramatically with WBC counts less than 0.1×10^9/L.[5] That means this approach cannot be used for rWBC determinations, where very low counts in the order of 0.001×10^9/L may be encountered.

Single-Platform Approach

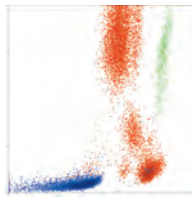
In the single-platform approach, the absolute cell count is derived from the flow cytometer itself, without the need for a hematology analyzer. A very precise, known volume of sample is mixed with relevant antibodies. A gating strategy is employed to accurately identify the cell population of interest and exclude any contaminating cells; identification of a “reference” population is not required. The cell population of interest can then be related back to the original blood volume by a variety of different methods.

- **Microbeads.** The use of microbeads is by far the most common approach to single-platform absolute counting. Microbeads are small fluorescent latex particles with a diameter of 5-10 µm. There are two basic methodologies for using microbeads. The first uses a tube containing a lyophilized pellet of an exact number of microbeads, to which a known volume of sample is accurately added. In the second method, equal volumes of sample and microbeads are accurately added to a tube. In this approach, the microbeads are in a liquid suspension medium of known concentration, which is supplied by the manufacturer. By counting the number of cell events and the number of bead events and knowing the initial concentration of beads, it is possible to determine the absolute cell count using the following formula:

$$\text{Absolute Cell Count (cells/µL)} = \frac{\text{Number of cell events}}{\text{Number of bead events}} \times \text{Concentration of beads (beads/µL)}$$

Using time as an acquisition parameter can give a lot of internal quality control information about the preparation and acquisition of that sample. A histogram plot of time will show if the count rate of the sample has been stable over the acquisition period (Figure 2). Recent studies have demonstrated that the number of beads acquired in a fixed time period is remarkably constant for most flow cytometers.[6] If all the sample preparation pipetting has been performed accurately, then for each tube acquired, the number of beads counted in a fixed time should be constant. It is then possible, for each batch of beads used, to derive a Mean ± 2 Standard Deviation (SD) range of beads counted in a fixed time period. Bead counts outside of this range could be indicative of potential pipetting errors, and the sample should be restained.

- **Volumetric.** With the volumetric method, an exact and reproducible volume of stained sample is passed through the flow cell, and the number of positive cell events can be directly related to the known volume of sample acquired. The exact volume of sample can be measured using either precision syringes or by utilizing two sensing electrodes that monitor the movement of the sample and, hence, sample volume. With volumetric systems, all pipetting steps must be made with great precision. The final dilution of the sample must also be calculated and taken into account.
- **Microfluorimetry.** With this method, blood stained with the relevant antibody is placed into a capillary tube of precise known dimensions. A laser scans the capillary, and the number of positive events is counted. This can be related to the precisely known volume of the capillary.
- **Flow Rate Calibration method.** This is a recently developed method aimed at reducing costs in resource poor countries.[9] The method utilizes the fact that with most bench top flow cytometers, the flow rate of sample through the flow cell is remarkably constant. Hence, the volume of sample acquired in a fixed time period will be constant. An accurately prepared dilution of counting microbeads in lysing reagent can be used to calculate the volume of sample acquired in a defined fixed time period. Accurately prepared stained samples without counting beads



can then be acquired over the same time period. The number of events in the cell population of interest can be expressed per volume acquired, and if the dilution factor for the blood sample is known (i.e. ratio of blood volume to total volume), then the cell count per microlitre of blood can be calculated. A correction factor for the increased viscosity between the tube containing beads plus lysing reagent and the tube containing sample is required. This factor varies for different lysing reagents used.

The use of single-platform absolute counting methods are becoming more popular, especially the use of microbeads. With accurate pipetting techniques, the results can be precise and reliable.

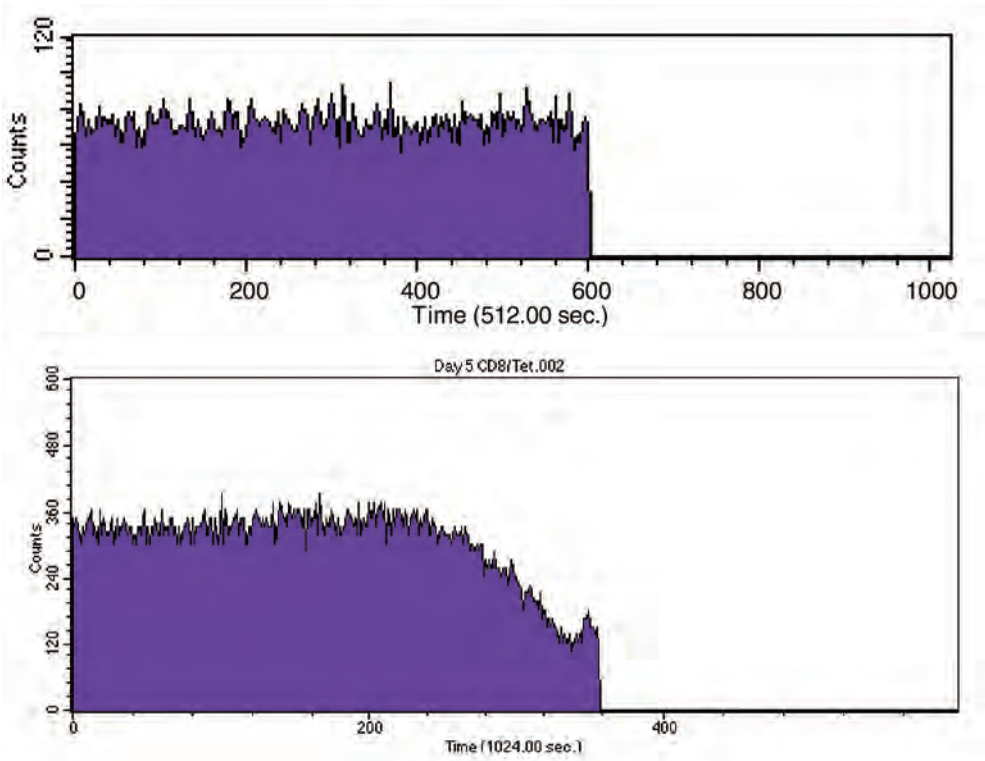


Figure 2. The plots illustrate the value of using time for data acquisition. The first plot shows a normal steady flow rate, indicated by the level appearance of the histogram. The second plot shows that towards the end of the data acquisition period, the sample flow rate began to decrease due to a partial blockage in the flow cell.

Technical Considerations

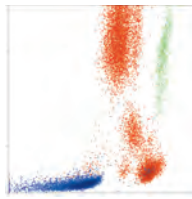
Since the use of microbeads is the most common approach to single-platform absolute counting, the technical considerations discussion will focus on that methodology. Accurate and precise pipetting is perhaps the most important factor in obtaining reliable results with this methodology. It is recommended that a “wet tip” reverse pipetting technique be employed for the dispensing of sample and liquid phase beads. A description of that technique follows:

- Press plunger to the second stop and place tip in the fluid and aspirate the sample. Press the plunger to the first stop dispensing the sample. The pipette tip should contain excess fluid. Repeat this step at least two times.
- The pipette tip is now ready to use. Press plunger to the second stop and place tip in the fluid and aspirate the sample. Carefully remove the pipette tip from the sample and wipe the pipette tip gently with tissue to remove any excess fluid, being careful not to touch the tip orifice and inadvertently removing sample from inside the pipette tip.
- Try to keep the pipette as vertical as possible. Check for the presence of air bubbles in the tip. If air bubbles are seen, then dispense the fluid back into the sample and repeat the sampling procedure.
- Prior to dispensing, place the pipette tip on the tube wall near the top of the sample. Slightly angling the tube and pipette will aid this step. Dispense to the first plunger stop. Residual fluid should be seen in the pipette tip after the fluid has been dispensed. When removing the tip do not touch the walls of the test tube.

The same pipette should be used to dispense the sample and beads. Adopting the same technique for dispensing both sample and beads will ensure more consistent results. Dispensing pipettes should also be well-maintained and regularly checked for accuracy and precision (CVs < 2%).

Following are some additional tips for the use of microbeads that will help to ensure reliable, precise results.

- Ensure the stock microbead vial is at room temperature and has been well mixed before sampling. Harsh vortexing of the stock microbead vial will induce foam formation, which attracts the microbeads, and can also introduce micro-air bubbles into the fluid, which will reduce the expected volume of microbeads pipetted, resulting in increased absolute cell counts.
- Care must be taken not to lose any of the suspending fluid before re-suspension, otherwise the stated microbead concentration becomes invalid.
- It is not advisable to use FSC as the thresholding parameter. Most microbeads have a low FSC signal and can easily be excluded when thresholding on FSC. A fluorescence parameter may be used, for example the one used for CD45. Another option is the use of a ”not” gate, placed in the lower left hand corner of a FSC vs. SSC dot plot. This ensures that all the microbeads are acquired, but debris is excluded (Figure 3).
- To ensure that the microbead-to-cell ratio is maintained, a lyse no-wash procedure should be used.
- A minimum number of 1,000 microbead events should be acquired.
- To ensure that the ratio of microbeads to cells is constant during long acquisition periods, it is best to pause acquisition and gently mix the tube every 7-8 minutes.



- Care must be taken when gating the microbead population to exclude non-microbead events. Some protocols require that all the microbeads should be counted and others only the singlet microbead population. Please refer to the manufacturer's product insert.
- Microbeads should be added to the sample just prior to acquisition to prevent bead loss due to settling, sticking to the tube, or sticking to cells. Some microbead preparations contain detergents to prevent bead aggregation. These can damage cells over prolonged incubation.
- Vortexing of the microbead/sample mixture should be performed with care to decrease foam and micro-air bubble formation.

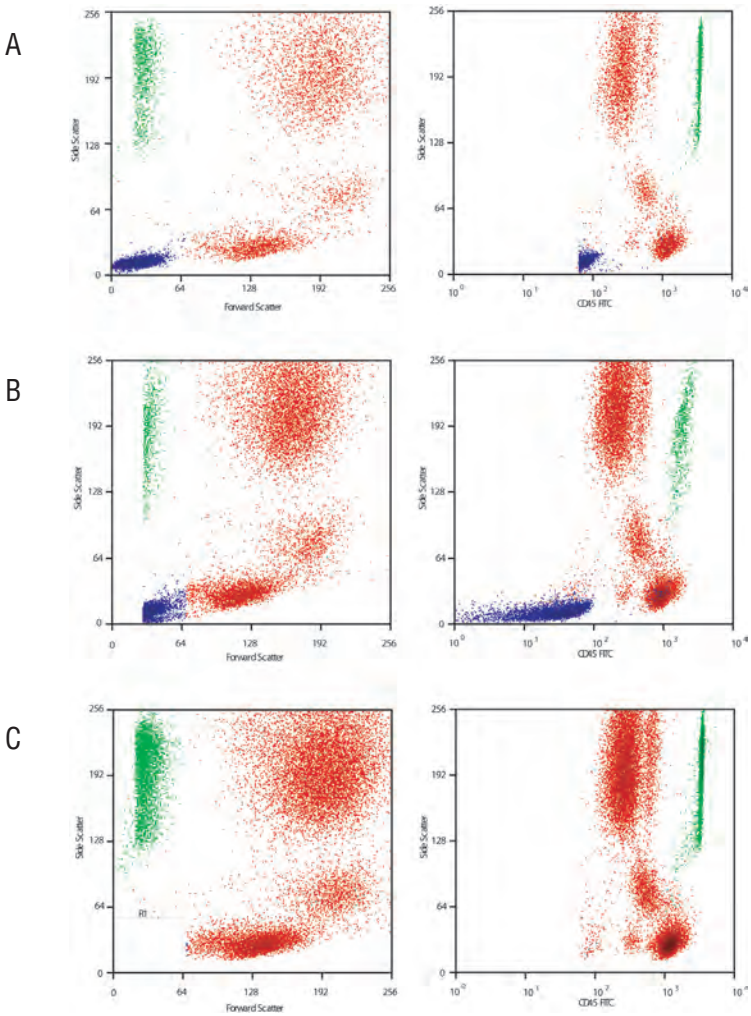


Figure 3. A) Illustrates that care must be taken when using Forward Scatter as the threshold parameter, as the counting beads (green) can be easily eliminated. B) Illustrates that by using a threshold on CD45 the majority of debris (blue) can be eliminated, but the counting beads (green) are left intact. In some samples, the level of debris can still be seen interfering with the lymphocyte/CD34 region. C) Illustrates that using a CD45 threshold and acquiring through a "not gate" removes the majority of the debris that can interfere with the lymphocyte/CD34 populations.

For more detailed information on the practical use of microbeads please see the two articles by Brando and Mandy.[7-8]

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Glossary

Antigen A molecule that is capable of binding to an antibody.

Acquisition The process of recording the intensity of photodetector signals from a particle as it passes through a flow cytometer.

Autofluorescence The light emitted naturally by an unstained, illuminated cell.

Bandpass filter An optical filter that allows passage of a specified range of wavelengths.

Coincidence The appearance of two cells or particles in the laser beam at the same time.

Compensation Hardware or software manipulations that correct for fluorescent interference created by dyes or fluorochromes possessing close or overlapping emission spectra.

Cross-reactivity The ability of an antibody to react with antigens other than the immunogen.

CV The coefficient of variation is the standard deviation of a series of values divided by the mean of those values. It indicates the width of a histogram peak.

Dichroic filter An optical filter that reflects light of certain wavelengths and transmits light of other wavelengths.

Dot plot A two-dimensional diagram that correlates the intensities of two parameters for each cell or particle.

Doublet discrimination The process of eliminating the signal produced by two particles that pass the interrogation point simultaneously.

Drop delay The time between the measurement of signals from a particle and the moment when that particle is about to be trapped in a drop that has broken off from the vibrating stream.

Emission The loss of energy from an excited atom or molecule, in the form of light.

Epitope The structural part of an antigen that reacts with an antibody.

Excitation The energy required to boost an atom or molecule to a more activated state.

FCS format Flow Cytometry Standard is a file format for flow cytometric data storage, such that, theoretically, data acquired on one flow cytometer should be analyzable by software on a different flow cytometer.

Fluorochrome A dye that absorbs light and then emits light of a different wavelength.

Forward scatter (FSC) Light from the illuminating beam that has been bent at a small angle as it passes through a cell.

Gate A restriction placed on flow cytometric data that limits the data to be included in subsequent analyses.

Histogram A one-dimensional diagram that displays single-parameter flow cytometric data.

Immunophenotyping The use of immunological tools for the specific detection of antigens expressed by cells or other particles, localized either on their surface or inside them.

Interrogation point The point in three-dimensional space where the laser beam intersects and illuminates the core of the fluid stream in a flow cytometer.

Longpass filter An optical filter that allows passage of wavelengths that are longer than a specified wavelength.

Monoclonal antibodies Immunochemically identical antibodies produced by one clone of plasma cells that reach with a specific epitope on a given antigen.

Neutral density filter An optical filter used to attenuate light by a desired proportion, independent of wavelength.

Obscuration bar A light-blocking device used to stop unwanted excitation light from entering optical detection path.

Photodetector A device that converts light energy into an electrical signal.

Photomultiplier tube A type of photodetector used to detect relatively weak light signals.

Polyclonal antibodies Immunochemically dissimilar antibodies produced by different cells that react with various epitopes on a given antigen.

Purity The proportion of sorted particles of interest compared to the total number of particles in the sorted material.

Recovery The proportion of sorted particles of interest compared to the total number of particles of interest satisfying the sort decision.

Shortpass filter An optical filter that allows passage of wavelengths that are shorter than a specified wavelength.

Side scatter (SSC) Light that bounces off particles and is deflected 90 degrees.

Threshold An electronic device that tells an analog-to-digital converter to ignore signals below a certain intensity.

Triggering The process by which signals above the threshold intensity are detected and recorded.

Yield The proportion of sorted particles of interest compared to the total number of particles of interest that could have been recovered from the preparation under ideal conditions.

Index

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