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Flow Cytometry: An Overview

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Abstract

Flow cytometry is a technology that provides rapid multi-parametric analysis of single cells in solution. Flow cytometers utilize lasers as light sources to produce both scattered and fluorescent light signals that are read by detectors such as photodiodes or photomultiplier tubes. These signals are converted into electronic signals that are analyzed by a computer and written to a standardized format (.fcs) data file. Cell populations can be analyzed and/or purified based on their fluorescent or light scattering characteristics.

A variety of fluorescent reagents are utilized in flow cytometry. These include, fluorescently conjugated antibodies, DNA binding dyes, viability dyes, ion indicator dyes and fluorescent expression proteins.

Flow cytometry is a powerful tool that has applications in immunology, molecular biology, bacteriology, virology, cancer biology and infectious disease monitoring. It has seen dramatic advances over the last 30 years, allowing unprecedented detail in studies of the immune system and other areas of cell biology.

Keywords

flow cytometry; fluorescence; reagents

INTRODUCTION

Flow cytometry is a technology that rapidly analyzes single cells or particles as they flow past single or multiple lasers while suspended in a buffered salt-based solution. Each particle is analyzed for visible light scatter and one or multiple fluorescence parameters. Visible light scatter is measured in two different directions, the forward direction (Forward Scatter or FSC) which can indicate the relative size of the cell and at 90° (Side Scatter or SSC) which indicates the internal complexity or granularity of the cell. Light scatter is independent of fluorescence. Samples are prepared for fluorescence measurement through transfection and expression of fluorescent proteins (ex. Green Fluorescent Protein, GFP), staining with fluorescent dyes (e.g., Propidium Iodide, DNA) or staining with fluorescently conjugated antibodies (e.g., CD3 FITC).

Flow cytometry is a powerful tool that has applications in multiple disciplines such as immunology, virology, molecular biology, cancer biology and infectious disease monitoring. For example, it is very effective for the study of the immune system and its response to infectious diseases and cancer. It allows for the simultaneous characterization of mixed

populations of cells from blood and bone marrow as well as solid tissues that can be dissociated into single cells such as lymph nodes, spleen, mucosal tissues, solid tumors etc. In addition to analysis of populations of cells, a major application flow cytometry is sorting cells for further analysis. A more detailed look at applications will be discussed later in this unit.

The instrumentation used for flow cytometry has evolved over the last several decades. Multiple laser systems are common as are instruments that are designed for specific purposes, such as systems with 96-well loaders designed for bead analysis, systems that combine microscopy and flow cytometry and systems that combine mass spectrometry and flow cytometry. An overview of current instrumentation platforms will be discussed in this unit.

The increase in available reagents over the last several years has led to explosive growth in the number of parameters used in flow cytometry experiments. There has been a dramatic increase in the fluorochromes used to conjugate monoclonal antibodies, such as tandem dyes and polymer dyes. In addition, there has been an increase in the available fluorescent proteins used for transfection beyond GFP, such as mCherry, mBanana, mOrange, mNeptune, etc. These advances in fluorochromes and instrumentation has led to experiments with the possibility of 30+ parameters.

The final part of a flow cytometry experiment is data analysis. Traditional two parameter histogram (dot plot) gating and analysis is still being used frequently. However, the increase in number of parameters and complexity in experiments is leading to the use of newer cluster data analysis algorithms such a PCA, SPADE and tSNE. These improved methods of data mining allow useful information to be extracted from the high-dimensional data now available from flow cytometry.

INSTRUMENTATION

Traditional Flow Cytometers—Traditional flow cytometers consist of three systems: fluidics, optics and electronics. The fluidics system consists of sheath fluid (usually a buffered saline solution) that is pressurized to deliver and focus the sample to the laser intercept or interrogation point where the sample is analyzed. The optical system consists of excitation optics (lasers) and collection optics (photomultiplier tubes or PMTs and photodiodes) that generate the visible and fluorescent light signals used to analyze the sample. A series of dichroic filters steer the fluorescent light to specific detectors and bandpass filters determine the wavelengths of light that are read so that each individual fluorochrome can be detected and measured. More specifically, dichroic filters are filters that pass light through that is either shorter or longer in wavelength and reflect the remaining light at an angle. For example, a 450 Dichroic Long Pass filter (DLP) lets light that has a longer wavelength than 450 nm through the filter and bounces the shorter wavelengths of light off at an angle to be sent to another detector. Bandpass filters detect a small window of a specific wavelength of light. For example, a 450/50 bandpass filter passes fluorescent light that has a wavelength of 450 nm +/− 25 nm through the filter to be read by the detector. The

electronic system converts the signals from the detectors into digital signals that can be read by a computer.

Multiple laser systems are common with instruments often having 20 parameters (FSC, SSC and 18 fluorescent detectors). There are new instrument platforms being introduced with five or more lasers and 30–50 parameters, but these are less common. The most common lasers used in traditional flow cytometers are 488 nm (blue), 405nm (violet), 532nm (green), 552nm (green), 561 nm (green-yellow), 640 nm (red) and 355 nm (ultraviolet). Additional laser wavelengths are available for specialized applications. In addition, there are instruments that have replaced PMTs with avalanche photodiodes (APD) for fluorescence detection with the aim of increasing sensitivity.

Acoustic Focusing Cytometers—This cytometer uses ultrasonic waves to better focus cells for laser interrogation. This type of acoustic focusing allows for higher sample input and less sample clogging. This cytometer can utilize up to 4 lasers and 14 fluorescence channels.

Cell Sorters—A specific type of traditional flow cytometer is the cell sorter which can purify and collect samples for further analysis. A cell sorter allows the user to select (gate) on a population of cells or particles which is positive (or negative) for the desired parameters and then direct those cells into a collection vessel. The cell sorter separates cells by oscillating the sample stream of liquid at a high frequency to generate drops. The drops are then given either a positive or negative charge and passed through metal deflection plates where they are directed to a specific collection vessel based on their charge. The collection vessels can be tubes, slides or plates (96-well or 384-well are common).

There are two types of cell sorters, quartz cuvette and “jet-in-air” that differ in where the laser interrogation point is located. The quartz cuvette cell sorters have fixed laser alignment and are easier to prepare for a sort. The “jet in air” cell sorters need to have the lasers aligned daily and are more difficult to set up but are more adaptable for small particle detection.

Imaging Cytometers—Imaging flow cytometers (IFC) combine traditional flow cytometry with fluorescence microscopy. This allow for rapid analysis of a sample for morphology and multi-parameter fluorescence at both a single cell and population level (Barteneva, Fasler-Kan, & Vorobjev, 2012). IFC can track protein distributions within individual cells like a confocal or fluorescence microscope but also to process large numbers of cells like a flow cytometer. They are particularly useful in multiple applications such as cell signaling, co-localization studies, cell to cell interactions, DNA damage and repair and any application that needs to be able to coordinate cellular location with fluorescence expression on large populations of cells.

Mass Cytometers—Mass cytometers combine time-of-flight mass spectrometry and flow cytometry. Cells are labeled with heavy metal ion-tagged antibodies (usually from the lanthanide series) instead of fluorescently-tagged antibodies and detected using time-of-flight mass spectrometry. Mass cytometers do not have FSC or SSC light detection which

does not allow for the conventional method of detecting cell aggregates. However other methods such as cell barcoding can be employed for this purpose (Leipold, Newell, & Maecker, 2015). Also, mass cytometry does not have cellular autofluoresce signals and reagents do not have the emission spectral overlap associated with fluorescent labels so compensation is not needed. However, the sample is destroyed during analysis so cell sorting is not possible and the acquisition rate is much lower than a standard flow cytometer (1000 cells/second instead of 10,000 cells/second). Currently, there are commercially available reagents for 40 channels but this number will increase with the introduction of other metal ions such as platinum for conjugation to antibodies (Mei, Leipold, & Maecker, 2016).

Cytometers for Bead Array Analysis—Multiplex bead arrays have become popular for analyzing large amounts of analytes in small sample volumes. Briefly, these assays utilize capture beads with a known amount of fluorescence in a specific channel and a reporter molecule detected by a separate laser to quantify the amount of captured analyte associated with the specific bead. It is essentially the equivalent of 100 ELISA assays.

Small flow cytometers with usually 2 lasers and 96-well loaders have been developed to analyze these assays. These instruments have small footprints and optical bench designs that are optimized to detect and discriminate beads with different amounts of fluorescence along two channels. Instruments have been developed that can detect 100–500 different bead combinations.

Spectral Analyzers—One of the challenges of multi-parameter flow cytometry is compensation (or erasing spectral overlap) between flurochromes. A new type of flow cytometer, the spectral analyzer is specifically designed to address this problem. A spectral analyzer measures the entire fluorescent emission spectra for each fluorochrome in a multicolor sample to create a spectral fingerprint. Then during analysis, each spectra is unmixed to provide a pure signal for each fluorochrome (Sony, 2017). Spectral analysis is starting to replace traditional PMTs as a detection method for high-dimensional flow cytometry.

New Detector Technologies—Photomultiplier tubes (PMTs) remain the standard detector technology for flow cytometry. Their high sensitivity and low backgrounds make them useful for fluorescence technology. However, solid state detectors are starting to appear in some cytometers. Avalanche photodiodes (APDs) are inexpensive, sensitive and highly linear, and are more spectrally responsive in the long red region. Silicon photodiodes (SiPDs) are also a promising option for solid state detectors.

REAGENTS

Small Organic Molecules—Small organic molecules such as fluoroscein (MW=389 D), Alexa Fluor 488 (fluorescein analog), Texas Red (325 D), Alexa Fluor 647 (1464 D), Pacific Blue and Cy5 (762 D) are commonly used for antibody conjugation. They have consistent emission spectra but a small Stokes shift (the difference between excitation wavelength and emission wavelength, approximately 50–100 nm). They are also stable and reasonably easy to conjugate to antibodies. The Alexa Fluor (Thermo Fisher) dyes were designed to be more

resistant to photobleaching and are better reagent choices for samples that will also be used for imaging.

Phycobiliproteins—Phycobiliproteins are large protein molecules derived from cyanobacteria, dinoflagellates, and algae. They are large molecules, for example phycoerythrin (PE) has a molecular weight of 240,000 D. These proteins have large Stokes shifts (75–200 nm) are very stable with consistent emission spectra. Because of their large size, phycobiliproteins are excellent for quantitative flow cytometry since they usually have a 1:1 protein to fluorochrome ratio during conjugation. However, phycobiliproteins are susceptible to photobleaching and are not recommended for applications with long or repeated exposure to excitation sources. Examples of phycobilibroteins are phycoerythrin (PE), allophycocyanin (APC) and peridinin chlorophyll protein (PerCP).

Quantum Dots—Quantum Dots (Qdots) are semiconductor nanocrystals that have tight fluorescence emission spectra associated with the size of the nanocrystal. They are optimally excited with UV or violet lasers but can be minimally excited by multiple lasers. This minimal excitation complicates fluorescence compensation when Qdots are used in multi-parameter experiments. Because of the compensation issues and difficulty in conjugating Qdots to antibodies, these reagents have largely been replaced with the polymer dyes in multi-parameter staining panels.

Polymer Dyes—Polymer dyes consist of polymer chains that collect light signals and can be “tuned” to absorb and emit light at specific wavelengths based on the length of the polymer chain and the attached molecular subunits. These dyes are very stable have similar quantum efficiency to phycobiliproteins with greatly increased photostability. Since polymer dyes can be made to absorb light only at specific wavelengths, they avoid the issues with multiple laser excitation that make Qdot reagents difficult to use in multi-parameter experiments. Examples of these reagents are the Brilliant Violet (BV), Brilliant Ultraviolet (BUV) and Brilliant Blue (BB) reagents.

Tandem Dyes—Tandem dyes chemically couple either phycobiliproteins (PE, APC, PerCP) or polymers dyes (BV421, BUV395) with small organic fluorochromes (Cy3, Cy5, Cy7) to create a dye that uses fluorescence energy transfer (FRET) to increase the available fluorochromes that can be excited with a single laser source. For example, Texas Red has a maximum excitation of 589 nm, and PE has an emission of 585 nm, so by coupling PE to Texas Red, the emission from PE is used to excite Texas Red using FRET allowing PE-TxRed to be excited by either a 488 nm or 532 nm laser. The polymer chain antibodies use the same method to increase available fluorochromes that can be excited by a single laser. Tandem dyes are extremely bright with large Stokes shift values (150–300 nm) which is useful when dealing with low antigen density. However, tandem dyes are less stable than the donor fluorochromes and can differ from lot to lot in their energy transfer efficiency, complicating compensation. Most of the longer Brilliant polymer dyes are also tandems and share these issues.

Metal Conjugates for Mass Cytometry—Antibodies for use in mass cytometry are conjugated to single isotope heavy metal ions in the lanthanide series of elements. There are

currently 35 lanthanide series isotopes commercially available for antibody conjugation. These probes are non-fluorescent and only applicable for mass cytometry. Additional antibody conjugates will become available as soon as other metal elements are evaluated for suitability with this platform.

Fluorescent Proteins—Fluorescent proteins are frequently used as reporter systems for gene expression. The most commonly used is green fluorescent protein (GFP) derived from the jellyfish *Aequorea victoria* (Tsien, 1998). GFP was cloned to generate cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Red fluorescent protein (DsRed) was discovered from mushroom anemone (Mikhail V. Matz, 1999) and then cloned for use in protein expression systems. Next generation monomeric fluorescent proteins (mCherry, mBanana) were cloned from DsRed and have broader excitation and emission spectra. The violet and green/yellow excited fluorescent proteins see especially heavy use in flow cytometry. New fluorescent proteins are being continuously discovered and generated; currently several hundred exist, with excitation and emission spectra ranging from the ultraviolet to near infrared. The presence of many laser wavelengths on modern flow cytometers has dramatically expanded the use of fluorescent proteins in flow cytometry.

Nucleic Acid Dyes—Nucleic acid dyes bind DNA, RNA or both. They are used to quantitate DNA for cell cycle analysis (Propidium Iodide, 7AAD, DyeCycle Violet, DAPI), discriminate chromosomes for sorting (Hoescht 33342, Chromomycin A3), sorting stem cells using side population analysis (Hoescht 33342), cell viability and for sorting bacteria. They can be combined with another marker such as fluorochrome conjugated anti-BrdU to determine proliferation.

Proliferation Dyes—Cell proliferation can be measured by pulsing cells with BrdU (bromodeoxyuridine) and then staining with an antibody against BrdU and a DNA dye. However, this method does not allow for long term proliferation studies. Carboxyfluoroscein succinimidly ester (CFSE) and other similar dyes can be used to follow multiple divisions of proliferating cells. Red and violet excited variants of these dyes are also now available. Each cell is permanently labeled with the dye and the subsequent generations of cells inherit lower amounts of the dye due to the dilution of the dye. These dyes do not affect cell growth or morphology and are suitable for long term proliferation studies.

Viability Dyes—Cell viability can be measured through exclusion of dyes (Propidium iodide, DAPI) or by the binding of a dye to amines within a cell to determine if the cell membrane is intact. The exclusion dyes cannot be fixed are only suitable for cells that are not infectious and will be analyzed immediately. Amine binding dyes such as the Live/Dead reagents (ThermoFisher), Zombie dyes (Biolegend) or Fixable Viablity dyes (BD Biosciences) can be fixed and used for cells that are infectious, cells that need to be stained for internal antigens and cells that need to be stored prior to acquisition.

Calcium Indicator Dyes—Calcium indicator dyes undergo a color shift upon binding to calcium. They are used to indicate cell activation and signaling. The data is expressed as a ratio of the two wavelengths associated with bound and unbound calcium and dye. The most

commonly used dye remains indo-1, an ultraviolet biphasic calcium probe. Blue-green calcium probes including fluo-3 are also available.

APPLICATIONS

Flow cytometry has a wealth of techniques and applications that are suitable for multiple fields of study. In this section, applications are broadly grouped under specific disciplines, however any of these techniques can be used in all fields of study.

IMMUNOLOGY

Immunophenotyping—Immunophenotyping is the most used application in flow cytometry. It utilizes the unique ability of flow cytometry to simultaneously analyze mixed populations of cells for multiple parameters. In its simplest form, an immunophenotyping experiment consists of cells stained with fluorochrome-conjugated antibodies that are targeted against antigens on the cell surface. Most of these antigens are given “cluster of differentiation” numbers or CD numbers by the Human Leukocyte Differentiation Workshops so that a common nomenclature is used to define monoclonal antibodies that are directed against specific cellular antigens. For example, CD3 is “cluster of differentiation number 3” and is used to define the T cell co-receptor that is present on all T cells.

Most immune cells have specific CD markers that define them as a population of cells. These cell markers are called lineage markers and are used to define specific cell populations for additional analysis in each immunophenotyping experiment. Examples are the T cell markers (CD3, CD4, CD8), B cell markers (CD19, CD20), monocyte markers (CD14, CD11b) and NK cell markers (CD56, CD161).

In addition to lineage markers that define populations of cells, other markers are used to characterize each cell population. These markers can include activation markers (CD69, CD25, CD62L), memory markers (CD45RO, CD27), tissue homing markers (α4/β7) and chemokine receptor markers (CCR7, CCR5, CXCR4, CCR6). Often, immunophenotyping experiments also include intracellular markers such as FoxP3 (defines T_{reg} cells), cytokines (IFN-γ, TNF-α, IL-2 define T_H1 cells), proliferation markers (Ki67, CFSE), and antigen specific markers (major histocompatibility or MHC Tetramers). Current instruments and reagents are capable of 28 color immunophenotyping experiments, however, it is more common to have experiments in the 12–15 color range. A sample 15-color T_{reg} cell immunophenotyping panel is shown in Table 1.

Antigen Specific Responses—Antigen specific responses can be measured by stimulating cells with a specific antigen and then looking for cytokine production, proliferation, activation, memory, or antigen recognition through MHC multimers. MHC multimers are MHC monomers (MHC-I or MHC-II) that are usually biotinylated and then bound to a fluorescent streptavidin backbone in groups of 4 (tetramer), 5 (pentamer) or 10 (dextramer). These MHC multimers are “loaded” with the antigen of choice and then used to bind to T cells that recognize the antigen, thus indicating the level of response to a specific antigen. This application is commonly used in vaccine studies.

Intracellular Cytokine Analysis—Intracellular cytokine analysis is performed by treating cells with a protein transport inhibitor (Brefeldin A or Monensin) for 2–12 hours so that any cytokines produced by the cells can accumulate within the cell enabling better detection. Cells can be stimulated with various antigens during this incubation such as peptides from a vaccine to measure immune response.

Following protein transport inhibitor treatment, cells are stained for viability markers and cell surface markers, then fixed and permeabilized for intracellular staining with anti-cytokine antibodies.

Proliferation Analysis—Cell proliferation can be measured by flow cytometry using several different assays and markers. These assays use different methods to target proliferation related events such as incorporation of thymidine analogs (BrdU) into replicating DNA, generational tracking of inheritable permanent dyes (CFSE), and expression of proliferation related antigens (Ki67, PCNA).

The flow cytometry equivalent of the ^{3}H thymidine proliferation assay utilizes the thymidine analogs BrdU or EdU (ethynyl deoxyuridine) to pulse growing cells for 2–6 hours. Following this incubation, the cells are stained for surface markers (optional) and then fixed and permeabilized for staining the incorporated BrdU or EdU. The BrdU procedure utilizes DNase to exposed the BrdU for antibody staining, but the EdU procedure utilizes a copper catalyzed click chemistry to detect the EdU. Both methods are usually counter stained with a DNA binding dye like propidium iodide. In addition, both the BrdU and EdU method are compatible with staining for additional intracellular antigen markers.

CFSE and other similar dyes (CellTrace Violet, FarRed etc) cross the cellular membrane in living cells and bind covalently and permanently to intracellular structures (usually lysine or other amines). The daughter cells of each subsequent generation inherit the dye allowing for long term analysis of proliferation. This technique is very useful when following proliferation resulting from long-term antigen stimulation. An example of CFSE staining is in Figure 1.

Expression of proliferation related antigens can also be used as a marker for proliferation. Ki67 is expressed during cell proliteration (all phases) but not during cell quiescence. PCNA (proliferating cell nuclear antigen) is required for DNA replication. The presence of either Ki67 or PCNA is an indicator of cell proliferation. Ki67, PCNA and BrdU staining on the same cells is shown in Figure 2.

Apoptosis Analysis—Apoptosis, or programed cell death, is a phenomenon that is frequently examined in immunology and other fields of study. It is used to maintain the homeostasis of the immune system by removing cells without triggering an inflammatory response (necrosis). It is the mechanism of death for clonally expanded T cells following an immune response, for self-targeting T cells, for autoreactive B cells, and multiple other cells in the immune system.

The detection of apoptosis by flow cytometry utilizes multiple targets along the cascade of events associated with apoptosis. The translocation of the plasma membrane is targeted by

Annexin V staining, the endonuclease digestion of DNA is targeted by the TUNEL (TdT dUTP Nick End Labeling) assay, the activation of Caspases can be targeted by antibodies and dyes, mitochondrial apoptosis is targeted by dyes that determine mitochondrial membrane potential and chromatin condensation in the nucleus detected by staining with Hoescht 33342.

Annexin V is a phospholipid binding protein that binds to phosphatidylserine when it is translocated to the outer layer of the cellular membrane during apoptosis. A viability exclusion dye (like propidium iodide) should be used when staining with Annexin V to confirm that the binding is happening on the outer surface of the cellular membrane.

TUNEL is a technique that utilizes the ability of terminal deoxynucleotidyl transferase (TdT) to label the ends of DNA breaks associated with apoptosis with dUTP (deoxyuridine triphosphate) or BrdU. The dUTP or BrdU are labeled with a fluorchrome for detection and the cells are counter stained with a DNA dye prior to data acquisition.

The caspase signaling pathway is activated in most cases of apoptosis. This is targeted by using intracellular staining and antibodies that are specific to the active form of caspase 3. There are additional assays that utilize fluorogenic substrates that when exposed to caspase activity are cleaved and then emit fluorescence.

Mitochondrial apoptosis does not always utilize the caspase pathway so different methods are used for detection. Most of these methods examine mitochondria membrane potential such as using the dye JC-1. However, there is an antibody against APO2.7 that is localized on the mitochondrial membrane and only expressed during apoptosis.

MOLECULAR BIOLOGY

Fluorescent Protein Analysis—Fluorescent proteins (GFP, mCherry, YFP, mRuby, etc) are used as markers for protein expression. Typically, cells are transfected with a plasmid that contains a promotor sequence and encodes for a gene of interest along with a fluorescent protein. The expression of the fluorescent protein is used as an indicator for the expression of the gene of interest. More recently, the expression of a split bi- or tri-partied fluorescence complementation linked to other proteins allow detection of RNA–protein and protein–protein interactions. These methodologies revolutionized the detection and isolation of cells where the fluorescence is detected only in response to surrogate (Han et al., 2014). This technology is used for multiple applications, for example *in vivo* tracking of transplanted cells, bacterial or viral infections, and gene knockout in cells to further elucidate gene function.

Cell Cycle Analysis—Cell cycle analysis assays consist of staining DNA with a saturating amount of DNA binding dye. In most cases, the cells are fixed with a 70% ethanol solution which permeabilizes the cells and then stained with the dye (PI, 7AAD, DAPI). However, there are dyes that can enter living cells and stain DNA without harm to the cells such as Hoescht 33342. In this type of analysis, samples are acquired at a low flow rate with linear amplification and then analyzed using ploidy modeling software to determine the cell cycle phases.

Signal Transduction Flow Cytometry—This application uses antibodies made against resting and phosphorylated signaling molecules. The use of these reagents and specialized buffers in staining panels allows for the study of signaling pathways in mixed populations of cells.

RNA Flow Cytometry—RNA flow cytometry combines flow cytometry with fluorescent *in situ* hybridization (FISH) to detect RNA expression along with protein expression. This technique requires staining panel optimization since not all fluorochrome conjugated antibodies will withstand treatment at 40°C for multiple 1 hour incubations. It is a useful technique when antibodies are not available for a target and RNA expression can be used instead.

CELL SORTING

Cell sorting utilizes a flow cytometer with cell sorting capabilities to separate and purify cells or particles for further analysis. Essentially, any cell or particle that can be made fluorescent can be separated by a cell sorter. Cells can be sorted into 96 or 384 well plates, tubes and slides. A few common types of samples are transfected cells expressing a fluorescent protein, stem cells, tumor infiltrating lymphocytes, tumor cells, and white blood cell populations. A major consideration with any cell sort is scaling up the amount of antibody needed for staining large amounts of cells.

OTHER APPLICATIONS

Absolute Cell Counting—Absolute cell counting can be added to any immunophenotyping experiment. The procedure utilizes fluorescent beads of a known concentration that is acquired along with the sample. The sample is analyzed and the gated number of cells for the population of interest is compared with the number of beads acquired in the same sample to generate the number of cells per milliliter.

Quantitative Flow Cytometry—Quantitative flow cytometry uses a bead based standard to generate a staining curve of known fluorescence amounts. Cells are then acquired with the same instrument settings and linear regression analysis is used to calculate the amount of fluorescence on the cells. Depending on the bead system used, this can be expressed as Antibodies Bound per Cell (ABC), Antibody Binding Capacity (ABC) or Molecules of Equivalent Soluble Fluorochrome (MESF). The best fluorochrome for this application is PE which, because of its size, almost always bind to an antibody with a 1:1 Fluorochrome to Protein Ratio. Molecular Equivalent of Soluble Fluorescence (MESF) standards can be used to convert arbitrary fluorescence intensity measurements to number of fluorescent molecules, by generating a standard curve and regression from MESF-bead data in any specific experiment, to quantitate approximate numbers of fluorescent labels on a cell.

Multiplexed Bead Array Assays—Multiplexed bead array assays are sets of beads coated with antibodies against specific soluble proteins or nucleic acids. Each bead has a known amount of fluorescence and a specific target which gives a location for the bead in the matrix. The collection of up to 100 beads are incubated with the sample of interest, treated with a fluorescence reporter and then acquired on a flow cytometer with at least 2

lasers to detect the 2 different fluorochromes. Special software is used to calculate analyte amounts based on fluorescence.

Phagocytosis Assays—Using fluorescently tagged bioparticles or bacteria, it is possible to detect phagocytosis using flow cytometry. The bacteria are labeled with a pH sensitive dye that only fluoresces when exposed to the lower pH of a phagosome, indicating that the bacteria are phagocytosed.

Small Particle Analysis and Sorting—Using flow cytometers with enhanced sensitivity, it is possible to detect and sort exosomes and other sub-micron particles. Analysis of cellular exosomes, viruses and other subcellular particles creates new applications in multiple fields including cancer biology, cancer therapy and vaccine development. This technology is still in its development stages, but techniques and instrumentation are rapidly improving to make this application more accessible in the near future.

DATA ANALYSIS

FCS 3.1 File Standard—The FCS file format was created in 1984 to standardize flow cytometry list mode data files. All flow cytometry data files have the ".fcs" file extension that allows the files to be read by any flow cytometry analysis program. The current fcs file standard is FCS 3.1.

Conventional Flow Cytometry Analysis—Conventional flow cytometry analysis consists of drawing a region around a population of cells (gating) and applying that region to other parameters within the experiment. This allows specific groups of cells to be selected for further analysis of other markers. For example, helper T cells can first be defined by CD3+, CD4+ expression and then analyzed for activation by looking at that population for expression of an activation marker, like CD25 (IL-2Rα) and then IFN-γ cytokine production. An example of gating is in Figure 3.

Multiple commercial computer programs in addition to the instrument provided software are available for analysis of flow cytometry data. The most popular are FlowJo, FCS Express, WinList, Kaluza and WinMDI.

Cell Cycle Analysis—Cell cycle analysis software programs uses ploidy modeling to determine the phase of the cell cycle represented by the DNA histogram. ModFit LT is a program dedicated to this type of analysis. In addition, a cell cycle analysis module is available on FlowJo.

Analysis of High Dimensional Data—Analysis of high dimensional data containing 14 plus parameters using conventional flow gating strategies is cumbersome and time consuming. In addition, it is possible to miss interesting populations of cells because relationships between markers are not easily determined using traditional gating methods. There are multiple new analytical tools that are being used to visualize and analyze this type of data. Examples are SPADE (Spanning-tree progression analysis of density-normalized

events), tSNE (t-Distributed Stochastic Neighbor Embedding), PCA (Principal component analysis), and FLOCK (FLOw clustering without K).

Mathematically, t-SNE is similar to PCA, but it can identify more co-segregating features than PCA, since t-SNE optimizes only the clustering of similar objects with each other, while PCA optimizes both proximity of similar events and separation of dissimilar events. Most of these algorithms require data reduction or down sampling techniques to reduce the complexity of data prior to analysis.

Cytobank is another source for cloud based high dimensional data analysis where users upload data and subscribe to the web- based platform. tSNE is available as plug-in for FlowJo and FCSExpress software.

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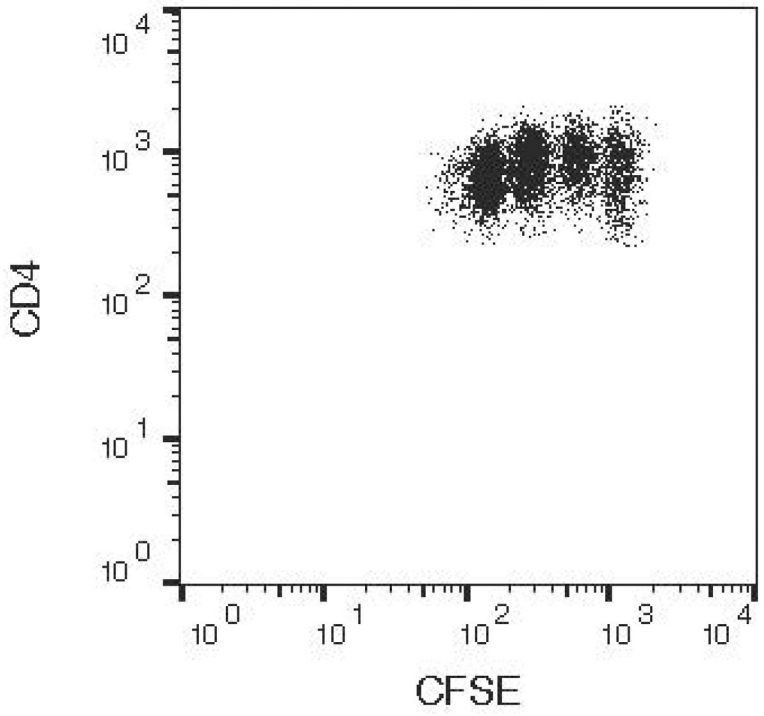

Figure 1.
Example of CFSE staining used for proliferation analysis. Human CD4+ T cells were stained with CFSE and then stimulated for 5 days with an antigen. Each peak of CFSE staining represents one generation of cell division.

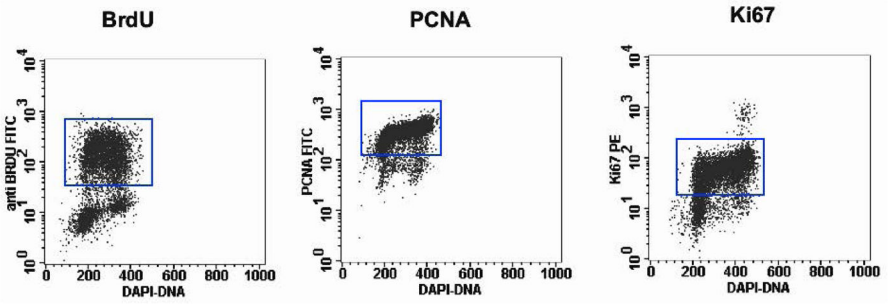

Figure 2.

Example of BrdU, Ki67 and PCNA used to measure proliferation. Cells from H23 lung cancer cell line were fixed and then stained with BrdU, Ki67 or PCNA and DAPI. The BrdU sample was pulsed for 2 hours with BrdU prior to staining. The samples were counterstained with DAPI to indicate cell cycle as well as proliferation. The positive cells are indicated in the rectangular region.

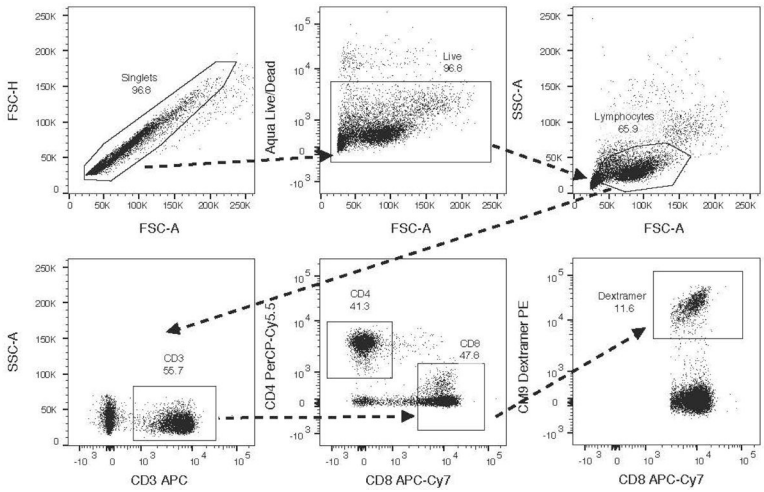

Figure 3.

Example of gating for standard data analysis using FlowJo 10.3. Cells are first gated to remove doublets, for viability, for light scatter and then for specific lineage markers. This example is looking at CM9(SIV-gag) Dextramer staining on CD8 cells in PBMC from a vaccinated *Rhesus macaque*.

Table 1

Example of a 15-color T_{reg} cell staining panel

Laser	Dichroic Filter	Bandpass Filter	Fluorochromes
488 nm	505LP	525/50	CD14 FITC
488 nm	690LP	710/50	CD4 PerCP-Cy5.5
532 nm		575/26	CD127 PE
532 nm	600LP	610/20	TGF-B1 PE-CF594
532 nm	635LP	660/20	HLA-DR PE-Cy5
532 nm	685LP	710/50	
532 nm	755LP	780/60	CD73 PE-Cy7
628 nm		670/30	CD25 APC
628 nm	685LP	730/45	CD3 Ax700
628 nm	755LP	780/60	CD20 APC-Cy7
405 nm		450/50	FoxP3 BV421
405 nm	505LP	525/50	Live/Dead Aqua Dye,
405 nm	557LP	560/20	
405 nm	570LP	585/42	
405 nm	600LP	610/20	CD39 BV605
405 nm	635LP	670/30	CD8 BV650
405 nm	690LP	710/50	IL-10 BV711
405 nm	750LP	780/60	CD45 BV786