

Flow Cytometry – A Basic Overview

Overview

Flow cytometry is a powerful technology for investigating many aspects of cell biology and for isolating cells of interest. Flow cytometry utilizes highly focused, extremely bright beams of light (usually from lasers) to directly reveal aspects of cells - e.g size and granularity - by the way light is scattered or indirectly by attaching fluorescent probes to cell components e.g DNA binding dyes or labeled antibodies. The power of flow cytometry derives from the fact that it quantitatively analyzes individual cells, thus permitting the identification of subpopulations within a sample. The power of single cell analysis is compounded by the ability to measure multiple parameters simultaneously on each individual cell, to do this very fast (in excess of 20,000 cells/second), and to isolate/purify/sort desired subpopulations (up to 4 simultaneously).

History

Flow cytometry of course simply means the measurement of cells while they are flowing through the detector system. Modern flow cytometry began in the late 1940s and early 1950s with Wallace Coulter's invention of the Coulter counter which measured the volume of cells by displacement of an electrolyte and subsequent increase in electrical impedance. Later principles and devices invented principally by Mack Fulwyler, Louis Kamensky, Wolfgang Göhde, and Len Herzenberg's group at Stanford added new detection capabilities - measurement of size and granularity by light scatter and measurement of fluorescence. These devices all used light as the detection system. Len Herzenberg coined the term Fluorescence Activated Cell Sorting - FACS. The term FACS was used and trademarked by one of the manufacturers (B-D) so this class of instrument is now commonly referred to as flow cytometers. Concomitant developments that aided and expanded the utility of flow cytometers were the development of a vast array of fluorescent probes and especially the development of monoclonal antibodies pioneered by Cesar Milstein.

What can Flow Cytometry measure?

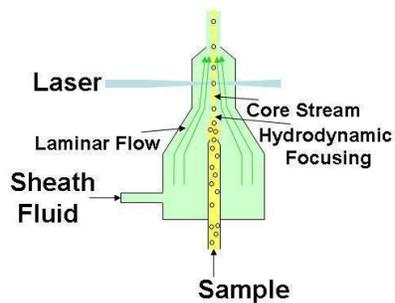
Flow cytometers can measure a diverse set of materials but it is required that the material to be analyzed be particulate. However that does not exclude the ability of a flow cytometer to measure soluble molecules. Virtually any component or function of cells can be measured as long as a fluorescent probe can be made that detects this. Many different types of cells can be analyzed - we have used bacteria, yeast, mammalian cells, and plant cells. Cellular subcomponents can be measured e.g chromosomes. In fact the human genome project started with the sorting of each of the chromosomes to simplify the analysis. Cell functions (e.g intracellular pH i.e. $[H^+]$, $[Na^+]$, $[Zn^+]$, phosphorylation status, membrane potential, DNA content) may be studied using flow cytometers. The development of a host of fluorescent reporter proteins (e.g green fluorescent protein - GFP) allows for easy interrogation of gene expression. To measure soluble molecules the molecules must first be trapped onto a particulate surface and then exposed by binding of fluorescent probes (e.g monoclonal antibodies). Indeed multiplexed assays can be developed that permit the simultaneous measurement of multiple analytes in the same sample.

How does a flow cytometer work (in brief!)

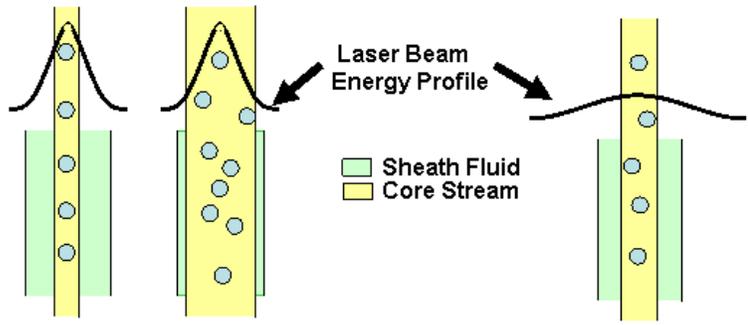
In order for a flow cytometer to work **4 areas** of physics/technologies need to interplay. The first component is the fluidics system of the instrument. Because this is the mechanical component of the instrument most instrument problems are in the **fluidics** system. The heart of the fluidics system on all flow cytometers is the "nozzle". This is the component where cells are injected into the sheath fluid stream, are hydro dynamically focused, and in cuvette-nozzle designs where the cells are illuminated by the laser beams. Requirements for steering and focusing the laser beams and for collecting and detecting the fluorescence and scatter emissions require sophisticated **optics**. The collected light energy must be converted to electrical energy and this in turn converted to digital information. This is the function of the **electronics** of the cytometer. Finally in order for the operator/investigator to interpret what happened within the cytometer the data must be collected by a **computer** and interpreted by software into appropriate figures (histograms).

Fluidics

The fluidics system performs a number of critical functions. The first is to position the cells within the detector laser beam precisely and reproducibly. This is accomplished by using hydrodynamic focusing. The



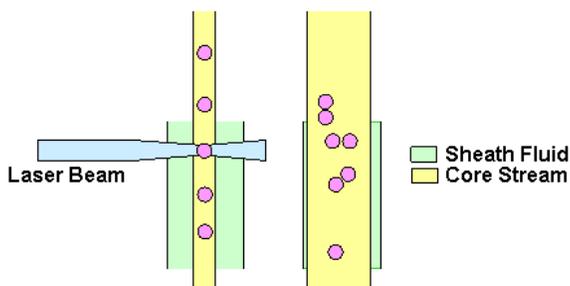
internal shape of the nozzle provides this effect in combination with an accelerating focusing fluid - the sheath fluid (normally simple PBS). The sample particles (cells) in single cell suspension (ideal) are injected into the sheath fluid stream. The figure to the left shows a representation of the major components of a cuvette nozzle design. The sample stream exits the sample injection needle and then via the effect of the hydrodynamic focusing is drawn out into a very small stream called the core stream (or sample stream). The diameter of the core stream is critical for both the accurate positioning of the cells in the laser(s) and for identifying when more than one cell is in the laser (doublets, triplets, etc.). The goal is to have the diameter of the core stream near to the diameter of the particles being analyzed. The diameter of the core stream varies in direct proportion to the pressure applied to the sample tube - less pressure = smaller diameter -- higher pressure = larger diameter. The quality of the data produced will always be better the smaller the core stream diameter is. However, the sample tube pressure also affects the rate at which cells are analyzed. Many users focus only on the event rate forgetting what increasing that by increasing sample pressure will do to the core stream diameter and thus to the quality of the data. While this may be acceptable for many cells up to a point, to obtain higher event rates while maintaining a tight core stream it is better to concentrate the sample and run at the lower sample pressure, if possible. In terms of correct positioning of the cells within the laser, the desire is to have the cells all go through the energy profile of the laser in the same place and, thus, be illuminated by the same number of photons. The energy profile across any diameter of the laser beam is Gaussian as shown. Of course, for many lower power



lasers it is

desirable that this transit point be at the peak of the laser energy distribution (as shown to the lower left). When the core stream is much wider than the cell diameter, cells, which distribute at random in the core stream, will pass through differing laser beam energy levels. This means that cells with the same amount of bound fluorochromes will be measured as having slightly different levels resulting in a measurement error. The problem with precise laser beam localization is maximal when the cross-sectional area of the laser beam is spherical. Most flow cytometers now use lenses to reshape the laser beam to an elliptical cross-section (right most drawing figure above). This flattens and broadens the laser energy profile of the laser beam so that transit across the distribution reduces the positional laser energy that cells off-center experience and, thus, the problem is minimized. It still exists, however, and for applications (e.g. DNA content) where the CVs are critical still requires close attention to the core stream diameter. Flattening the energy profile does, however, have the adverse side effect of reducing the peak photon energy the cells experience and this loss may affect the resolution of the instrument unless the overall energy of the laser can be increased to offset this.

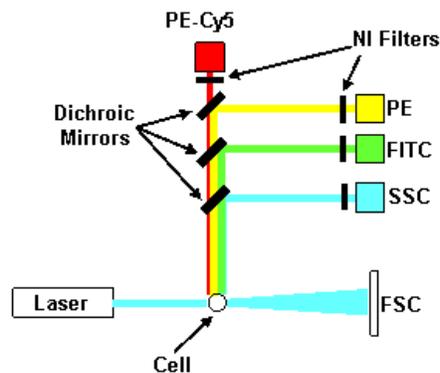
The second major function of the fluidics, that it accomplishes in part, is to deliver single cells to the interrogation laser. Assuming the cells exist as distinct individual cells, the hydrodynamic focusing, particularly when the sample pressure is such to have an appropriate core stream diameter, will put the cells in single file orientation and separate them sufficiently (on average) to have a single cell in the detection laser beam. However, even when cells are fully independent of each other there is a statistical probability (described by the Poisson statistic) of more than a single cell being in the detector.



Identifying with certainty that an event is a single cell is one of the major problems in using flow cytometry especially when analyzing rare events. Other methods are needed to confirm that such events are single cells (e.g. sorting to purify these events). As the sample pressure is increased and the core stream widens the frequency of doublet events will increase. This is shown in the figure to the left. As the core stream widens, cells may now be beside each other from the viewpoint of the laser.

We have at our disposal other methods to help identify when more than one cell is in the detector. In older flow cytometers (e.g. FACSCalibur, FACScan, and MoFlo) coincidence detection circuitry was employed to

attempt to remove these doublet events. When an event triggered the system a time window was initiated and the circuitry looked to see if another event occurred with that window. A second event occurring in the window, while it could be detected, its data would not be resolvable from the first event and in the vast majority of analyses the data from both cells was rejected. Of course, if the cells became close enough together to not even be resolvable within the window time frame, the circuitry failed to detect the event as two cells. In the newer instruments (e.g. CyAn, LSRII, MoFlo XDP, and Reflection) each event independently creates its own analysis window. This results in a much lower loss of data. However, events that are doublets are still not resolved as the doublet still creates only a single pulse and, thus, a single window. Pulse processing analysis allows us to identify many events as doublets and, thus, increase our confidence that events are not doublets - however, there is still a statistical probability that any given event that passes all these tests may not be a single cell. Pulse processing analysis permits use of the fact that for each pulse we may determine the height of the pulse (above threshold), the width of the pulse, and the area under the pulse. These properties have the same relationship for any single cell event regardless of the overall magnitude of the pulse but the relationships will be (may be) different for events that are composed two or more cells. For pulse processing to work best we must present the cells to the laser in the context of a properly sized core stream. When we do so doublets will have their long axis perpendicular to the laser and the event width will be maximized.



Optics

Since flow cytometers use light to make measurements it is no surprise that optical technologies are critical. Optical elements are required to both direct and shape the excitation laser beams but also to collect the light emitted from fluorescent probes. In order to measure and resolve data we must collect data in proper places and colors so that we know which parameter we are measuring. Flow cytometers routinely measure light scattered at two different angles (forward and side). Forward scatter is measured in the same direction (i.e. forward) as the laser beam is moving. Typically the forward scatter light is collected using a photodiode although a PMT (photomultiplier tube) can be used and is preferable for some applications. Forward scatter is typically and proportionally equated with size. However, forward scatter is even more so a function of refraction and, thus, directly inferring size must be done with caution. All other measurements in typical flow cytometers are made at some large angle (e.g. 90° relative to the primary laser beam) and use photomultiplier tubes (PMTs). The light is collected through sophisticated optical elements. In most instruments these are essentially microscope objective lens assemblies. However, the Reflection uses a novel light collection system that employs a parabolic reflector to collect the light. In some machines (e.g.

LSRII and Reflection) the collected light is sent to the detectors via fiber optics. Side scatter signals measure the granularity or internal structure of the cell. Other PMTs are used for the various fluorochrome probes and the color of the light collected determines which probe is being measured. To isolate the color for each probe interference filters are used. Filters designed to work at normal incidence (NI) (i.e. 90° to the incoming light) are placed in front of each detector. A particular color of light is split off from the incoming mixture and directed to the detectors using interference filters designed to work at other angles (e.g. 45°) - called dichroic mirrors. A schematic of a 3-color system is shown.

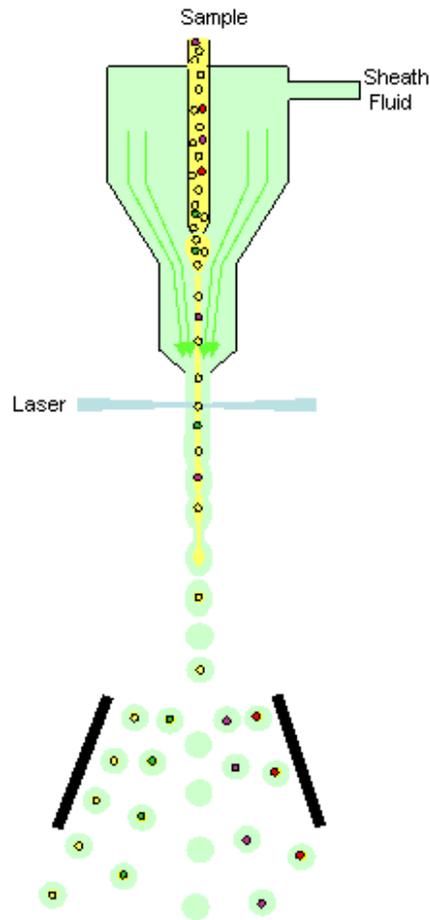
Electronics

The electronics have several functions. The first is to convert the light information to analog electrical information, the second is to convert the analog (current/amp) electrical information to analog (voltage) information, then to convert the analog information to digital information that can be processed by the computer and software. The light enters the PMT and illuminates the photocathode. The absorption of light energy by the photocathode results in the release of photoelectrons from the photocathode. The photoelectrons are directed to the next element in the PMT, the first dynode, via an electrostatic field. Each photoelectron can release multiple electrons from the dynode since the photoelectron has more energy due to the acquisition of kinetic energy as it is repulsed from the photocathode and attracted to the more positively charged dynode. The number of electrons released from the photocathode per captured photon and subsequently from each dynode is a function of the electric potential (voltage) applied to the PMT by the flow operator. Thus, the number of electrons released at each step logarithmically propagates until all electrons are captured by the photoanode resulting in an electric current in the wire connected to the photoanode. This current, at a given PMT voltage, is proportional to the number of photons that entered the PMT. Increasing the PMT voltage will, for the same number of photon generate a current which is higher. The release of photoelectrons from the photocathode can be described statistically and is one place where errors occur in a flow cytometer. As the number of photons and/or their energy level decreases larger errors can occur. This means that if you repeatedly sent the same number of photons of given energy (i.e. wavelength - longer wavelength means lower energy) into the photocathode you will get a range of currents generated at the photoanode. This will increase the width of the fluorescence distribution of a population of cells with this level of fluorescence. A larger number of photons and/or with a higher energy will produce less error and narrower distributions (i.e. CVs). The next step is the conversion of the amperage to a voltage. This is the primary function (in addition to others such as baseline restoration) of the pre-amp. What follows after the pre-amp depends on what instrument one is using. Older designs (e.g. FACScan and FACSCalibur) used linear and logarithmic amplifiers to amplify and resolve signals. (The CyAn also uses log amps but in a slightly different way and in all other regards is like the all digital instruments). Log amps are used to increase the dynamic range so that signals of small and large magnitudes may be displayed on scale within the same histogram - a requirement for quantitative measurements. Log amps are never perfectly logarithmic which creates problems downstream when one uses pure mathematic approaches to deal with spectral overlap between fluorochromes (compensation). The data from the amplifiers then goes to the ADCs (analog to digital converters) where the measured voltage is converted to a digital value. The value is based on the design of the ADC and its bit range (e.g. a 10-bit ADC will convert the lowest to highest voltage

into numbers from 1 to 1024 (i.e. 210). Newer designs (MoFlo XDP, LSRII, and Reflection) do not have amplifiers but send data directly from the pre-amp to high resolution analog to digital converters to generate digital values of sufficient range to enable the use of log lookup tables to derive the logarithmic resolution needed. The electronics must also provide the means to align signals from different detectors, especially from detectors collecting light from additional spatially (and, thus, time) resolved lasers. In droplet sorters the electronics must also provide for the precise generation of droplets, the decision making of what cells are sorted, as well as other control elements that monitor other functions of the sorting process.

Computer/Software

The digital data values are received by the computer and the software translates this information into pictures (histograms - usually either single or dual parameter plots) that we can visually understand. We will not go into detail of the various software packages that are available. Flow cytometry data files mostly conform to a standard - the FCS (flow cytometry standard) file structure (version FCS3.0 is the latest). The file structure permits not only the storage of the raw data from the detectors but also various other pieces of information about the sample and information about how it was collected (e.g. PMT voltages and compensation settings). The FCS file is also referred to as a "listmode file". In an FCS file all the raw data exists as separate pieces of information for every detector for each cell. The file structure allows users to later mix and match how they wish to combine various pieces of information to provide for a logical analysis of the information. One of the more powerful properties of flow software is the ability to generate regions of interest within one histogram and then to gate (restrict) a histogram displaying data for other parameters to only display the subset of information from the region of the first histogram. When analyzing two or more colors of fluorescence the emission from one fluorochrome may get into the detection channel of another fluorochrome. This must be dealt with by a process known as compensation (for a more complete discussion see Compensation in "Application Protocols" and appropriate discussions in "Instrument Protocols"). Algorithms now exist to help you with the proper adjustment of compensation.



Sorting

Sorting is the process where the flow cytometer (not all flow cytometers are sorters) physically separates cells and places them into test tubes. This can be done at high purity and rates, and keep the cells viable and sterile. Most commercial sorters use the principles of electrostatic, droplet sorting. The first part of sorting is, of course, analysis and uses the basic principles described above. Cells of interest are marked in the flow cytometer and this information is used to make the sort decisions. In order to sort, the fluid stream containing the cells must be precisely (and reproducibly over a substantial time frame) broken into droplets - the cells will partition into the droplets. To do this an acoustical energy is supplied to the fluid within the nozzle and this energy travels down the fluid stream and causes the stream to divide into droplets. The sorter's computer can figure out which droplets will contain the cell of interest. When that droplet is the last attached droplet to the fluid stream an electric charge is placed on the stream and maintained until the droplet separates from the stream at which time the charge is reset to zero. Having detached from the stream and, thus, no longer connected to ground, the droplet retains the charge applied to it. The droplet then passes (falls) through a high energy electric field and the droplets are deflected by controlled amounts to fall into properly placed receptacles. The charge applied to the stream and to the droplets may be either positive or negative and, in addition, each may have two amplitudes. Thus, we can sort up to 4 streams and, thus, 4 populations simultaneously. The sorter may also be configured to deposit cells (as few as one per well) into various sized multi-well plates (up to 384 well configurations).