Swayam Course - Analytical Techniques
Week: 7, Module 18 - Flow cytometry: Theory & Concepts
Content Writer - Dr. Janvie Manhas, Senior Resident, Department of Biochemistry, All India Institute of Medical sciences, New Delhi.

Introduction

Flow cytometry is a versatile biomedical technology used for analyzing and characterising cells moving in a single file in a fluid with laminar flow. This tool is very helpful to study the expression of cell surface and intracellular molecules which can delineate different cell types in a heterogeneous cell population. Modern flow cytometers can not only analyse cell size, volume and characteristics but also isolate live cells for cell culture and further pure population studies.

History:

In 1949, *Wallace Coulter* filed a patent for a technology named as, 'means of counting particles suspended in fluid' which led to the development of the coulter counter. He discovered that when cells were made to pass through a constricted path or aperture, the presence or absence of the cell could be calculated as a detectable change in the electrical characteristics of the path which can be measured as particle or cell size. This discovery was one of the many that led to the development of technologies put together in the modern flow cytometer.

Another key principle of laminar flow was utilised in 1953 by *Crosland-Taylor* to design a chamber for optical counting of cells. He observed that when an aqueous suspension of Red blood cells (RBCs) was injected slowly to a faster flowing stream of fluid, cells aligned as a narrow central stream of particles surrounded by the sheath fluid. This was the basis of 'hydrodynamic focusing' the principle concept used in most of the flow cytometers today.

Theory & Principle

Flow cytometry is a biotechnology tool that simultaneously measures, and analyses multiple physical characteristics of **single particles**, such as cells, as they flow in a fluid stream through a beam of light. Interaction with the light source is measured by an electronic detection system as light scatter and fluorescence intensity. Light scatter can be due to size of the cell (forward scatter) or due to internal complexity (side scatter. If fluorochrome is specifically and stoichiometrically bound to a cell surface or intracellular antigen, the fluorescence intensity will represent the amount of that particular antigen in the cell.

The basic principle is to interrogate one cell at a time and determine its biophysical characteristics and similarly it is represented as an individual entity on the data plots.
Interrogation of one cell at a time by the light source or laser is made possible by the concept of hydrodynamic focusing employed in this instrument.

Components of a flow cytometer
The flow cytometer consists of three core components:

1. Fluidics
2. Optics
3. Electronics

FLUIDICS:

The purpose of fluidics system is to transport particles in a fluid stream to the laser beam for interrogation. For optimal illumination, the stream transporting the particles should be positioned in the centre of the laser beam. Only one cell or particle should be interrogated by

the laser beam at a time. To accomplish this, the sample is injected into a stream of sheath fluid within the flow chamber *i.e.,* **flow cell.** The fluidics system should be free of air bubbles and debris and properly pressurized at all times. Cells in suspension are introduced at the Sample injection/introduction port (SIP). A unique droplet containment system prevents sheath fluid from dripping from the SIP.

The introduction of one smoothly flowing stream of fluid (core) into the centre of another smoothly flowing stream (sheath) in such a way that the core becomes "focussed" along a central axis is called **hydrodynamic focussing.** When conditions are right, sample fluid flows in a central core at uniform velocity without mixing with the outer layers of sheath fluid. This is the key concept of **laminar flow.** The sample pressure is always kept greater than the sheath fluid pressure to achieve the laminar flow. This can be achieved by a sample pressure regulator which controls the sample flow rate by changing the sample pressure relative to the sheath pressure. This hydrodynamic focussing helps to keep the cells moving in a file, one after the one such that at a time only on cell is hit and interrogated by the laser/light source.

The primary requirement for all types of flow cytometric analysis is that the cells must be in a single-cell suspension. It is very important to obtain a single cell suspension to avoid clogging up the fluidics system with clumps. Peripheral blood mononuclear cells (PBMCs) isolated from whole blood through Ficoll gradient centrifugation, or RBC lysed whole blood, or non-adherent cultured cells are some routinely used samples for flow cytometric analysis. Adherent cultured cells or cells present in solid organs should first be made into a single cell suspension by using enzymatic digestion (collagenase/trypsin) or mechanical dissociation of the tissue, followed by, mechanical filtration to avoid cell clumps and instrument clogs. The cells are then incubated in test tubes or microtiter plates with unlabeled or fluorescently conjugated antibodies and analyzed through the flow cytometer machine. Live or fixed cell suspensions can be used for analysis.

In our flow cytometry measurements it is desirable to keep variability to a minimum by careful experimental planning, titration of antibodies and making sure we only measure what we want by excluding dead cells and clumps. But as in any biological system, variability exists in our flow data and statistical metrics can be used to quantitate the amount of spread. This is important because the greater the spread, the harder it will be to say that distributions are significantly different. This also helps to judge the quality of the data and how reliable are the interpretations based on that data. One of the best metric used to check the spread of flow data is the coefficient of variation (CV) or coefficient of variance defined as: **(SD/m) × 100**
As CV is expressed as a percentage it is unitless and dimensionless. SD: Standard deviation. CV is very useful to compare results over time, between machines or between labs. Less than 3% is considered as narrow/good CV and greater than 8 as wide/bad CV. Wide CV can be due to improper instrument alignment or bad/degraded sample quality. It is also important to remember that high differential flow rates can also disturb the central sample core and lead to high CV. This is especially important in DNA analysis where to best distinguish between G1 and S phases we have to keep CV of G1 peak as low as possible. Hence, it is always desirable to run the sample for DNA cell cycle analysis at the low differential flow rate on a well aligned flow cytometer.

OPTICS:
The optical system consists of excitation and collection optics. Excitation optics consists of Light amplification by stimulated emission of radiation (LASER), along with filters and mirrors to route the light to the interrogation point in the fluidics stream. Collection optics consists of filters and optical cables that direct the emitted signals to the appropriate photomultiplier tubes (PMT). The detectors or filters are arranged in a well-coordinated layout pattern such that the longest wavelength emitted is collected first followed by shorter wavelengths in a

chronological manner. This ensures that all the emitted wavelengths or signals are properly collected and quantified. This type of layout arrangements also save space and help to make the whole instrumentation compact and less-space consuming. Light detectors can be either photodiodes or photomultiplier tubes (PMT). Photodiodes are used as scatter detectors while PMTs are used for collection of fluorescence signals.

The key optical parameters which are measured by flow cytometer are forward light scatter (FSC), side light scatter (SSC), and fluorescence emission signals.

When the beam of light or laser interrogates the cell flowing in the laminar flow of fluid, three light phenomenon can take place. The first is diffraction, the slight bending of light as it passes around the edge of the cell. The amount of bending depends on the relative size of the cell and continues in the same direction that the light was already traveling in (typically up to 20° offset from the laser beam's axis). This signal is collected by a PMT called the forward scatter channel (FSC), and is commonly used to determine particle size. Usually, bigger particles or cells will produce more forward scattered light than smaller ones, and larger cells will have a stronger forward scatter signal.

The second light phenomenon that happens when a laser hits a single cell in fluid is refraction, bending of a light wave when it enters a medium different from its initial medium measured at a 90° angle to the excitation line. The third is reflection, that is light bouncing off the cell into different directions. Both reflected and refracted light give us a measure of the relative cell granularity and complexity which can be measured in the side scatter channel (SSC). Cells with low granularity and complexity will produce less side scattered light. Similarly, highly granular cells with a high degree of internal complexity (such as neutrophils) will result in a higher side scatter signal. Therefore, by using FSC and SSC, cell populations can often be distinguished based on differences in cell size and granularity. Both FSC and SSC measurements are influenced by multiple factors and also depends on the quality of sample preparation.

Cells can also be separated by fluorescently labelling a specific protein or antigen they express. Fluorophores used for the detection of target antigen can be excited by laser of a particular wavelength. After excitation they emit light of a particular longer wavelength characteristic of that fluorophore. Each type of fluorescent dye or label has its own characteristic excitation and emission spectrum which is important for designing flow cytometry panel of antibodies. There is a wide selection of fluorophores available nowadays; for example, FITC, PerCP, APC, PE, Cy5.5, Alexa Fluors, and more.

It is important to note that the fluorescent signals may also occur from naturally fluorescing substances in the cell such as reduced pyridine nucleotides (NAD(P)H) and oxidized flavins (FAD). This naturally occurring fluorescence seen in unstained controls is termed as autofluorescence. In general, larger granular cells have high levels of autofluorescence due to increased amount of naturally occurring fluorescent compounds. It is often useful to have an unstained control, a positive control and an FMO (Fluorescence Minus One) control. The FMO control will help to identify the correct gating strategy during data analysis.

Another control that can be used is the isotype control. The isotype control ideally should be the exact same isotype, both in terms of species, heavy chain (IgA, IgG, IgD, IgE, or IgM) and light chain (kappa or lambda) class and the same fluorochrome (PE, APC, etc.).
Isotype controls are used as negative controls to help differentiate non-specific background signal from specific antibody signal. Depending upon the isotype of the primary antibody used for detection and the target cell types involved, background signal may be a significant problem in some experiments.

As each fluorochrome has a wide emission spectrum in multi-color flow experiments, there will be spill over of some of the emission spectra of one fluorophore into another. For e.g., the FITC emission spectrum overlaps with that of PE, and some of its light will be transmitted by the PE filter and so enter the PMT for PE. This spectral overlap is corrected by subtracting a fraction of the FITC signal from the PE signal; called as **compensation.** Compensation is the process which corrects the detected "spillover" of the emission of one fluorochrome into the detector designed to collect the emission from another fluorochrome. This can be done with the help of single color or compensation controls. The primary purpose of compensation controls is to allow the measurement of the true fluorescence in the fluorescence channel and eliminate contamination by the spillover.

ELECTRONICS:

As the fluorescent tagged cell or particle passes through the interrogation point and interacts with the laser beam, it creates a pulse of photon emission over time (a peak). This is detected by the PMTs and converted by the electronics component of the flow cytometer to a voltage pulse, typically called an "event". The total pulse height and pulse area is measured by the flow cytometer instrument, and the voltage pulse area correlates directly to the fluorescence intensity for that individual event. These events are assigned particular channel numbers based on its measured signal intensity (pulse area). The higher the fluorescence intensity, the higher the channel number the event is assigned. This signal can be amplified by increasing the voltage running through the PMT which can be increased or decreased by the user as deemed appropriate for the experiment. It is important to note that while setting color compensation all the measurements or ‘acquiring’ of data should be done at same PMT voltages across all tubes.

Data Analysis in Flow cytometry:

In a flow cytometry experiment, every cell that passes through the interrogation point and is detected by multiple lasers, is counted as a distinct event. All types of light scattered or emitted and is detected (forward-scatter, side-scatter, and each different wavelength of fluorescence emission) will be computed in its own unique channel. The data for each event is plotted separately to represent the light detected as signal intensity in each channel for all events.

This data could be visually represented in multiple different ways. Different types of data plots and gating strategy can be utilised to make meaningful interpretations of the data which will help to answer biological questions.

The most common types of data graphs used in flow cytometry include histograms, dot plots, density plots, and contour plots. As multiparametric analysis becomes more complicated, analysis techniques may include higher order plots such as 3 dimensional plots and SPADE trees.

Univariate histogram plots measure only one parameter. Generally, the Y-axis displays the number of events (the cell count) that show a given fluorescence, and the X-axis displays the relative fluorescence intensity detected in a single channel. A large number of events detected at one particular fluorescence will be represented as a peak (or spike) on the histogram. In most experiments, only one unique peak will be produced that can be interpreted as the positive dataset (representing the cells with the desired characteristics of interest).

For bivariate analysis, data is often represented as dot plots or density plots, and contour diagrams. For multiparametric experiments, the relationship between two different markers can be shown and more complex phenotype can be identified. Specific unique populations of interest can be isolated via gating. Gating is an important procedure in flow cytometric data analysis. Gating helps to selectively visualize the cells of interest and eliminate results from unwanted particles such as dead cells and debris.

Scatter plots can be of two types: Dot plots and density plots. These can compare 2 or 3 parameters simultaneously wherein each event is represented as a single point (or dot). The

dot plot is a figure that shows the relationship between multiple variables at once, and the parameters can be any combination of 1) forward scatter (FSC) vs. side scatter (SSC); 2) single color vs. side scatter; and 3) two-color fluorescence plot.

The density plot represents not just expression levels, but the relative number of events (density). On the density plot, each dot or point represents a single cell that has passed through the interrogation point of the flow cytometer. Events with similar intensities cluster together in the same region on the scatter-plot and represent density or closeness of the data. Density plots are an excellent way to analyse the frequency of subpopulations in a heterogenous group.

The characteristic position of different cell populations is determined by different physical properties such as cell size and granularity.
Debris are very small items (such as cellular contaminants) with low FSC and SSC. Debris can be excluded by making changes in the Threshold setting which tells the software to ignore that set of events. This helps to clean up the data and focus on the population of interest.
Some cells can be identified by forward and side-scatter profiles, but fluorescent labelling with a cell-type specific marker provides greater resolution and certainty of cell identity especially in a heterogeneous cell population.
The other way to show the density of flow data is to use a contour plot. Contour plots display the relative frequency of the populations, irrespective of the number of events collected by the flow cytometer. In the contour plot, concentric rings form around populations such that higher the density, the closer the rings. Therefore, this graph takes on the appearance of a geographical altitude map with steeper islands where the density is highest. In a 2% contour plot, two percent of cells fall within each contour line (as defined by the plot). Thus, the outermost line contains 98% of the cells; the second line contains 96% and so on.
Contour lines represent the density, and like the dot plot, the characteristic position of different cell populations is determined by different physical properties such as cell size and granularity.
One disadvantage of contour plots is that information about rare populations is not visible since these diagrams are not good at showing outliers. In a 5% contour plot, 5% of cells would fall outside of the last contour line. In these situations, some data analysis programs will have the option to add outliers. Another strategy is to combine a contour plot with a dot plot, allowing both density estimation and rare event information to be displayed. It is essential to choose the best way to communicate the data as proof of the results one is proposing. Using the right flow figures highlights the strong point of the data and accurately tells the story without confusion.

Flow cytometry is being widely used in both clinical setting as well as research and development. This powerful tool is used for immunophenotyping of a variety of specimens, including whole blood, bone marrow, serous cavity fluids, cerebrospinal fluid, urine, and solid tissues. In our next module we will highlight the biomedical applications of flow cytometry in detail.

Summary:

- Flow cytometry is a versatile tool for rapid, detailed analysis of cells flowing in a single file in a fluid stream.
- The pattern of light scattering by these cells gives information about the biophysical properties of the cell, eg. cell size (forward scatter) and internal complexity (side scatter).
- Fluorescently labelled antibodies can be used to identify cell surface and cytoplasmic antigens.

- Flow cytometry can be used with fluorescently labelled samples to analyse membrane bound as well as intracellular proteins. Special dyes can be used to detect and quantify DNA/ RNA using this technology.
- A routine analytical instrument in clinical labs and research labs, flow cytometer has become an indispensable tool for drug discovery, immunology and cancer research
- There are three keys components of a flow cytometer: Fluidics, Optics & Electronics
- Flow cytometer optics can be composed of a number of lasers and detectors for multiparametric, multicolour high throughput experiments
- Sample in the form of a cell suspension is introduced into a stream created by a sheath of isotonic fluid in laminar flow. This hydrodynamic focussing allows the cells to pass one at a time through an interrogation point.
- At this point, a beam of monochromatic light, usually from a laser, interrogates the single cells.
- Deflected light is given off in all directions and is collected via optics that direct the light to a series of filters and dichroic mirrors to isolate particular wavelength bands.
- If fluorescently labelled cells pass through this light source, the fluorescent molecules get excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit energy at a particular, higher wavelengths. These light signals are detected by photomultiplier tubes and digitized for computer analysis.
- The use of multiple fluorochromes, with different emission wavelengths (or “colors”), allows several cell properties to be measured simultaneously.
- When appropriate controls and gating strategy is used flow cytometry can accurately determine the phenotype and function of cells.