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Flow cytometry: Principle and applications

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Abstract
Flow cytometry is a technique used to detect and measure physical and chemical characteristics of a population of cells or particles. Flow cytometers are widely found in all leading biomedical research institutions and universities where they are used for performing tasks that require analytical precision and high throughput. In addition, flow cytometers have a key role in hospital and medical centers worldwide, where they are widely used for diagnosis as well as research. In the experimental process, a sample containing cells or particles is suspended in a fluid and injected into the flow cytometer instrument. The sample is focused to ideally flow one cell at a time through a laser beam and the light scattered is characteristic to the cells and their components. Cells are often labeled with fluorescent markers so that light is first absorbed and then emitted in a band of wavelengths. Tens of thousands of cells can be quickly examined and the data gathered are processed by a computer. The major diagnostic applications of flow cytometry are ploidy, cell cycle and surface analysis of cancers. They are also of use in the study of surface markers of lymphomas and leukemias which are of diagnostic and prognostic value. Flow cytometry also has been the method of choice for monitoring the progression of AIDS and the response to treatment by measuring CD4 lymphocyte levels in the blood. Light scattering at different angles can distinguish differences in size and internal complexity, whereas light emitted from fluorescently labeled antibodies can identify a wide array of cell surface and cytoplasmic antigens. The above applications makes flow cytometry a powerful tool for detailed analysis of complex populations in a short period of time.

Keywords: Flow cytometry, principle, immunology, applications
Abbreviations: PMT-Photomultiplier tube, CD-Cluster of Differentiation

Introduction
Flow cytometry is an optical to electronic coupling system device that helps in recording how a cell scatters incident light and emits fluorescence. It's a technique used to measure and detect physical and chemical characteristics of a population of cells or particles which is suspended in a fluid and injected in the flow cytometer instrument [Rao *et al.* 2016] [34]. Flow cytometry is derived from two words which are flow meaning cells in motion and cytometry meaning measurement of cells. Flow cytometer helps in quantifying a set of parameters from a particle in suspension [Waller *et al.* 2001] [40]. A sample containing cells or articles is suspended in a fluid and injected into the flow cytometer instrument. A beam of light of a single wavelength is directed to a continuous flow of suspended particles. Cells are often labelled with fluorescent markers so that light is first absorbed and then emitted in a band of wavelengths. Each particle in suspension which on passing by this light beam scatters light which is caught by detectors perpendicular to it [Gunel *et al.* 2015] [2].
These complexes of fluorescent substances conjugated with microscopical particles, when excited, emit light of lower frequency than that from the light source. This emitted light is then caught by detectors and analysed according to the brightness fluctuations of each detector or fluorescence emission [Herzenberg *et al.* 2012] [17]. The end result is formation of real time images of each fluorescent cell and transmission of light [Telford, 2011] [35].
Flow cytometry consists of three components which include optics, electronics and fluidics [Pearlson *et al.* 2014] [32]. Fluidics chamber consists of a flow chamber, optics includes a light source and detectors and electronics includes a digital analog converter that generates fluorescent parameters of size, complexity and signals, a system of amplification of linear or logarithmic signal and a computer for signal analysis [Kurec, 2014] [19].
Flow cytometry is generally used in all leading biomedical research institutions where they are used for performing tasks that require analytical precision and high throughput. They are also used in hospitals and medical centres worldwide. The major diagnostic applications include ploidy, cell cycle and surface analysis of cancers.

They are also used in the study of surface markers of lymphoma and leukemia. Along with this, it has been used for monitoring the progression of AIDS and patient's response to treatment by quantifying CD4 lymphocyte levels in blood. Major clinical applications include assessment of immunodeficiency disorders, haematological abnormalities, circulating tumor cell analysis and intracellular molecular detection [Lim, 2004] [24]. In addition, it's also used for diagnosis of cellular cycle studies, normal or abnormal content of DNA, chromosome analysis, cellular phenotyping and drug development studies [Kurtis *et al.* 2011].

Main Text

Historical Aspects

The first automated cell counter was developed by Moldavan, and consisted of a capillary tube mounted under an optical microscope with an objective provided with a photoelectric detector which recorded the passage of cells stained according to the light change [Moldavan, 1934] [28].

In 1949, Wallace Coulter developed a cell counter based on the voltage change caused by the passage of a particle through the interior of a needle [Carter *et al.* 1964] [6] and Mellors and collaborators used a microfluorimetric scanner for differential cell detection, improving the capacity of detection of technique [Mellors *et al.* 1951] [25].

The evolution of technique was obtained when Crosland-Taylor developed a flow chamber based on the passage of cells in a liquid stream (sheath fluid) through a capillary where samples were detected by a light source [Herzenberg *et al.* 2012] [17].

A year later, Katmentsky built the Rapid Cell Spectrophotometer (RSC), equipment in which the cell suspension passed through a channel in a microscope slide capable of measuring the size and quantity of cellular DNA. This new procedure expanded the capacity of cell count based in DNA quantity, important to study of malignance cells.

In that same year, Katmentsky used spectrophotometry (absorbed light) to measure and quantify the DNA and the multiparametric measurement of dispersed light, introducing the biparametric histograms and the pneumatic sorter [Kamentsky *et al.* 1965] [18].

Principle of flow cytometry: Fluidics

The purpose of fluidics is to transport particles in a fluid stream to the laser beam for interrogation. For this, the sample is injected into a stream of sheath fluid within the flow chamber. The flow chamber in a benchtop cytometer is called a flow cell and that in a stream in air cytometer is called a nozzle tip [Picot *et al.* 2012] [31]. Based on principles relating to laminar flow, the sample core remains separate but coaxial within the sheath fluid. The flow of sheath fluid accelerates the particles and restricts them to centre of sample core. This principle is called Hydrodynamic Focussing. The sample pressure is always greater than the sheath fluid pressure.

The cells are prepared in a monodisperse {single cell} suspension through enzymatic and/or mechanical digestion of cell suspension. It comprises of a pneumatic pump that propels particles and cells in suspension in a continuous flow into a hydrodynamic focussing chamber (flow cell), which due to its conical shape forces the particles or cells into a capillary or nozzle of 250microns diameter producing a single file of cells. The flow chamber consists of a central channel/core through which sample is injected and an outer sheath containing isotonic fluid like 0.9% saline or phosphate buffered saline enclosing the central core.

In benchtop cytometers, the sample stream is pressurized upward through a region of flow cell, particles pass through laser beam while they are still within this flow cell. In stream in air cytometers, the sample stream passes through a small orifice in a nozzle tip before being intersected by light beam in the open air [Pearlson *et al.* 2014] [32].

Increasing the sample pressure increases the flow rate by increasing the width of the sample core. With a wider sample core and more cells entering the stream within a given moment, some cells could pass through the laser beam off centre and intercept the laser beam at less optimal angle. A higher flow rate is generally used for qualitative measurements like immunophenotyping since the cells are less in line the wider core stream, but are acquired more quickly resulting in lesser data resolution. A lower flow rate is generally used where greater resolution is critical, such as DNA analysis.

Optics

On contact with the incident light source at the interrogation point, two phenomena occur, scattering of light and fluorescence. Scattering of light at small angles between 0.5 to5 degrees and travelling along the same axis the laser is travelling is called forward scatter. It's proportional to cell surface area or size. It's mostly a measure of diffracted light. Side scattered light is mostly a measure of refracted light at 90 degree to axis of laser path. It gives an indication about the granularity or internal complexity of cell.

The another phenomenon that takes place is called fluorescence. On excitation of electron by absorption of incident light energy, the electron emits light of longer wavelength as compared to those absorbed and the electron returns to its basal ground state. This transition energy is called fluorescence.

The argon ion laser is commonly used because the 488 nm light that it emits excites more than one fluorochrome. It also provides a monochromatic source of light. The other lasers used are krypton, helium neon and helium cadmium lasers [Telford, 2011] [35]. Mercury arc lamps can also be used although they provide a mixture of wavelengths which needs to be filtered. The diiferent fluorophores used are given in the table:

The specificity of a detector for a particular fluorescent dye is increased by placing a filter in front of the detector. When using different fluorochromes the emission spectra of different fluorochromes may overlap. This phenomenon is called spectral overlap. The mathematical process of correcting the spectral spillover from primary signal in each secondary signal it's measured in is called compensation [Njemini *et al.* 2013] [29]. This spectral band of light is close to the emission peak of fluorescent dye. Such filters are called bandpass filters. Shortpass filters transmit wavelengths of light equal to or shorter than a specified wavelength whereas longpass filters transmit wavelengths of light equal to or greater than a specified wavelength.

Detectors convert photons to electrical impulses. Photodiodes are less sensitive thus used to detect stronger forward scatter signal. Photomultiplier tube is used to detect weaker signals which are generated by side scatter and fluorescence.

Electronics

A voltage pulse is created when a particle enters the laser beam and starts to scatter light or fluoresce. Once the light

signals strike one side of photodetector, they are converted into proportional number of electrons creating a electrical current which on travelling to amplifier gets converted to a voltage pulse. The size of voltage pulse depends on the number of photons detected, the PMT voltage or pre-amplifier gain and amplifier gain. The Analog to Digital converter converts a 0-10V pulse to a digital number. In some analog systems like BD FACSCalibur, BD LSR the voltage pulse is assigned a digital value representing 0-1000 channels. The channel number is transferred to computer via general purpose in/out cable.

Processing and Analysis

The information obtained after the passage of samples in a flow cytometer may be stored in the form of monoparametric histograms wherein fluorescence intensity versus the number of cells is counted, Dot plot wherein the fluorescence intensity of one parameter is compared with that of other parameter and contour plot wherein the denser the concentric ring population the closer the rings are to each other.

Applications of flow cytometry

Flow cytometry is a technique that simultaneously analyses, marks and separates cells of interest from small blood samples [Wood, 2001] [37].

Through this technique, cells can be studied for expression of several surface molecules {immunophenotyping}, in the identification of human naïve T cells by phenotype, function [DeRosa, 2001]. It's a process used to identify cells based on the type of antigens or markers on the surface of the cell. It is a powerful diagnostic tool to differentiate between chronic leukaemias, lymphoma on the basis of cluster of differentiation markers. Considering the example of chronic lymphocytic leukemia in which there is increased production of lymphocytes it can be diagnosed through flow cytometry by typical phenotypic configuration of CD20, CD22, CD23 Positive whereas FMC7 negative.

It is a relevant technique in the quantification and analysis of lymphocyte subpopulations, reticulocyte quantification, chemotherapy and minimal residual disease monitoring. The results obtained are important to check the efficacy of treatment and retreatment.

Flow cytometry assay is used for the evaluation of cancer stem cell analysis. Cancer microenvironment is made up of bulk cells and cancer stem cells [Li *et al.* 2014]. Drug targeted towards cancer stem cell will lead to tumor regression while that targeted towards cancer bulk cell will lead to tumor relapse. Breast cancer stem cells are CD24 negative and CD44 positive. Thus after preparation of single cell suspension and staining with antibodies specific for CD24 and CD44, flow cytometric analysis can help establish the cancer lineage and help in devising appropriate anti-cancer therapy.

It can also be used to check cellular proliferation and in vitro cytotoxicity [Wang, 2002] [36] or permeabilized for the quantification of intracellular components like cytokines, hormones and other molecules, important in the development of basic and applied research too [Freer *et al.* 2013] [15].

The quantification of decreased CD4 T Helper cell count alongwith human immunodeficiency virus {HIV} viral load, remain the gold standard in diagnosis and continue to play a major role in the monitoring of advanced retroviral therapies [Madhu *et al.* 2011] [26].

Flow cytometric analysis of the inhibition of specific cell cycle phase inhibition by the drug under study plays a important role in pharmaceutical industries. The area of research involving drug development will be revolutionized and efficient drug development can be achieved. Also, flow cytometric analysis of apoptic cells can help in regulation of cancers, neurological and cardiovascular disorders and autoimmune diseases [Favaloro *et al.* 2012] [14]. The common features of apoptosis that can be measured by flow cytometryis externalization of phosphatidylserine, a phospholipid found in inner membrane of healthy cells [Demchenko *et al.* 2013] [11]. Annexin V binds to phosphatidylserine and hence annexin V labelled with fluorophores allows apoptosis to be assessed, usually in combination with propidium iodide to distinguish apoptic from necrotic cells.

The use can be extended to detection of parasitic diseases like leishmaniosis or apoptosis of lymphocyte T CD4 by HIV infection [Lecouer *et al.* 2008] [21].

Conclusion

Considering the various merits of flow cytometry in comparison with other techniques like histopathology and immunohistochemistry, flow cytometry has emerged to be the supreme diagnostic tool for disease surveillance. It allows rapid assessment of large number of cells, ability to analyse many samples quickly, multiparametric analysis of cell samples, ability to reanalyse data and high accuracy and reproducibility. Due to its great potential, the use of flow cytometry has been expanded to diverse fields of biological sciences and various health areas and is routinely used in clinical diagnostics, biotechnology and basic and applied research [Errante *et al.* 2016] [13].

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