Swayam Course - Analytical Techniques
Week: 7, Module 19 - Flow cytometry: Applications in Biology & Medicine
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Introduction-

Flow cytometry is one of the cornerstones of medical research, immunology, drug discovery and HIV research. This technique enables the user to rapidly analyse multifactorial data from single cells. The power of this technique lies in the enormous information density of the data which can be extracted very easily and quickly from flow cytometry based assays. This has led to an revolution in the biotechnology market. Flow cytometry is the basis of a huge number of clinical and research assays available in the market today both for diagnosis/prognosis as well as basic research in biology and medicine. The projected market values for flow cytometry and flow cytometry related assays is expected to cross USD 5600 million over the next five years. It has become one of the basic instruments required in every modern hospital or diagnostic facility. All major biomedical universities in the world have a flow cytometer core or central facility which can be assessed for experimental research.
Today, it is one of the most important research techniques to learn and understand for both biomedical researchers and clinicians. In this module, we will summarise the key applications of flow cytometry in biology and medicine.

For a biologist, flow cytometry can help answer critical cell biology questions like quantification of cell proliferation, cell viability, cell cycle analysis, immunophenotyping, cellular protein analysis, membrane potential analysis, enzyme activity, gene expression analysis to name a few. The field of stem cell biology and small particle research has boomed due to the availability of this technique. The discovery of fluorescent proteins and advances in gene cloning techniques helped to make the flow cytometer more accessible and diversified its uses even further.

There are three primary areas in which we can categorise the different applications of flow cytometry:
1.Nucleic acid analysis: DNA/RNA analysis for various assays like cell cycle analysis, cell viability or live/dead analysis, cell death or programmed cell death- apoptosis analysis.
2. Protein analysis: Immunophenotyping by using fluorescently labelled antibodies against the protein/antigen of study. It can be used for both cell surface and intracellular proteins. The lasers interrogate the fluoro-tagged antibodies and give a signal to indicate either presence/absence or abundance of the proteins.
3. Cell function analysis: Study of live cells or cellular processes like cell signalling pathways.

The most commonly used flow cytometric based assay are discussed as below:

1. Cell cycle analysis:
   The eukaryotic cells grow and then duplicate or divide into daughter cells by a process known as cell cycle. Cell cycle consists of two phases, i) Interphase: In this phase cell grows (Gap1, G1) and accumulates energy and metabolites necessary for cell division. The replication of DNA occurs in the synthetic or S phase. Finally when the cell is ready for division (Gap2, G2) it enters the next phase ii) Mitotic phase or M phase: parent cell chromosomes divide as sister chromatids which is followed by a process of division of cytoplasm known as cytokinesis leading to formation of two

daughter cells. Each stage of the cell cycle is tightly regulated by check point proteins known as cyclins. These detect any harm or damage to DNA. If damage can be repaired cell goes into a hibernation phase called Go phase. After repair of DNA, again cell cycles into G1→ S→G2→M and divides. However, if the damage cannot be repaired by the cell, the cell goes onto embrace a type of programmed cell death called apoptosis.

The most common method for cell cycle analysis is to use flow cytometry to measure cellular DNA content. Cells in S, synthetic phase will have more DNA than cells in G1 phase and cells in G2 will have double the DNA as in G1 phase. For this assay, a fluorescent dye that can bind to DNA is utilised and incubated with a single cell suspension of permeabilised or fixed cells. These dyes bind to DNA stoichiometrically, i.e. the amount of fluorescent signal is directly proportional to the amount of DNA and can be used to ascertain which population of cells are in what phase of cell cycle. Different fluorescent dyes can be used for this purpose including propidium iodide (PI), Amino actinomycin D, Hoescht 33342, DAPI (4′,6-diamidino-2-phenylindole), Ethidium Bromide etc. Some of these dyes bind to nucleic acids (i.e. RNA and DNA both), and if cell cycle analysis is to be done using these dyes, another step to treat cell suspension with RNases is added to ensure that only DNA binds to the dye and is measured.

2. Cell viability or Live/Dead analysis:
   Determination of cell viability is a very important and routinely done assay. It is especially critical when evaluating the response to a cytotoxic drug or effect of gene manipulation. Dye exclusion method is a very popular method used for this assay. Live cells with intact plasma membranes will exclude a variety of dyes that penetrate damaged membranes of non-viable cells. One classical example is that of Propidium iodide (PI) dye. PI binds to dsDNA by intercalating between base pairs. PI is excited at 488 nm and with a relatively large stokes shift, emits at 617 nm. Due to the large stokes shift it can be used with other fluorochromes excited at 488 nm like FITC (Fluorescein isothiocyanate) and PE (Phycoerythrin).

3. Apoptosis analysis:
   Apoptosis is the classical form of programmed cell death in which there is regulated cellular break down without induction of any inflammation. It is characterised by cellular shrinkage, loss of mitochondrial membrane integrity, activation of initiator and effector caspases, membrane blebbing and chromatin condensation and DNA fragmentation. Broken down cellular fragments are in membrane bound bodies known as apoptotic bodies and these are ingested and cleared by phagosomes.

Apoptosis leads to loss of phospholipid symmetry on the cell membrane. In the living cells, phosphatidyl serine (PS) is always kept on the inside of the lipid bilayer by a Mg^{2+} ATP dependent translocase enzyme. With the onset of apoptosis, this enzyme fails to function and PS is now present on the external surface of the cell membrane, where it acts as a recognition signal for phagocytes. Flow cytometry makes use of Annexins, which are ubiquitous proteins that bind PS in the presence of calcium to measure apoptosis. Annexin V tagged to FITC is generally used to study early apoptosis by flow cytometry. Annexin and PI can be used together to differentiate between apoptosis and necrosis. PI will only stain necrotic cells as cell membrane integrity will exclude PI from viable and apoptotic cells.

To study the late phase of apoptosis wherein the DNA fragmentation occurs, flow cytometry can be used in an assay called TUNEL assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling). This assay is based on an enzyme, Tdt or Terminal deoxynucleotidyl transferase which attaches deoxynucleotides to the 3'-OH terminus of DNA fragments which are generated in the late apoptosis stage. In this staining technique, the nucleotides attached by Tdt are tagged to a fluorochrome either directly or

through a chemical label that can be indirectly linked to either a fluorescent label or an enzyme.
The most commonly used methods for TUNEL staining are:
1) nucleotide incorporated by Tdt is directly conjugated to a fluorescent dye (usually FITC).
2) Biotin-tagged nucleotides are bound by streptavidin-HRP and detected using a chromogenic HRP substrate, such as DAB, to generate a brown color.
3) Bromo-, digoxygenin-, or FITC-tagged nucleotide that is then bound by an antibody specific for that tag. The antibody is labelled with either a fluorescent dye or an enzyme such as HRP for chromogenic detection. eg BrdU assay which is easily incorporated by Tdt enzyme and gives a brighter detection signal.

In a study by Flesh *et al*, published in *J of Cell Science* in 2001, Flow cytometric analysis of sperm in sub fertile males was done using M540 fluorescence (to check for membrane fluidity) and Yo-Pro 1 fluorescence (for sperm viability). M540 bodies are membrane-bound bodies occurring in semen of sub-fertile men (oligoasthenoteratozoospermic patients). These bodies can be detected by staining with Merocyanine 540, a probe which detects changes in membrane of apoptotic cells.
Yo-Pro-1 (YP1) is a nuclear marker and its relatively large size (630 Da) prevents this dye from penetrating the intact plasma membrane of living cells. Apoptotic processes alter membrane integrity allowing YP1 to enter the dying cells which can be analysed by flow cytometry.

4. Intracellular calcium signalling detection using flow cytometry

Using flow cytometry to understand cellular signalling including calcium signalling inside intact, live cells offers many advantages like rapid analysis of thousands of cells or events with multiparametric analysis of one cell at a time. A number of fluorescent indicators which bind to intracellular calcium can be used for this method of detection, For eg. Fluo-4, Indo1, Quin 2, Fura 2 etc.
Fluo 3 is a long wavelength calcium probe that is almost non-fluorescent in its free ligand form. However on complexing with calcium its fluorescence increases 60-80 times and can be detected flow cytometrically. The long wavelength of the fluorescent signal also minimizes photodamage to the cells. Fluo 3 can also be used for detection of caged calcium which is cleaved by photoirradiation in the UV region.
Fluo 3-AM is an acetoxymethyl ester derivative of Fluo 3 that can be easily loaded into cells by incubation. Excitation of Fluo-3 is maximal at 488 nm and emission occurs at 530 nm. Therefore another advantage is that, this dye can be used to measure the calcium flux response in cells stimulated with an agonist or activating molecule. For eg. Sarcoplasmic/Endoplasmic reticulum Ca^{2+} ATPase or SERCA transfers Ca^{2+} from cytosol of cells to the lumen of sarcoplasmic reticulum with the help of ATP to facilitate muscle relaxation. A drug known as Thapsigarsin was found to be cytotoxic to cancer cells by inducing ER stress and cellular death. Using flow cytometric detection of intracellular calcium signalling it was found out that Thapsigarsin is a non-competitive inhibitor of SERCA and increases intracellular calcium by inhibiting ability of cells to pump Ca^{2+} into ER causing ER stress and cell death.

5. Minimal Residual Disease (MRD) detection using flow cytometry:

Monitoring of minimal residual disease (MRD) or presence of residual malignant cells has become a routine clinical practice for management of all childhood acute lymphoblastic leukemia (ALL) and in many adult ALL patients. Evidence based research has shown that MRD diagnostics is the strongest prognostic factor which allows for risk group triage into different treatment arms for personalised therapy. MRD detection is helpful to make crucial treatment decisions in relapsed ALL cases as well as stem cell transplantation. Kern *et al*., 2003 showed that the presence of leukemia-associated aberrant immunophenotypes (LAIP) on leukemic cells in patients with AML can be used

to flow cytometrically detect and quantify MRD. These LAIP are characteristic of all or a subset of the leukemic cells and are absent or present at very low frequencies in normal bone marrow cells.

6. Clinical and Diagnostic applications of flow cytometry:
   a) Feto-maternal haemorrhage (FMH) detection
      In Rhesus D antigen incompatibility, Rh D-positive fetal cells can sensitize the Rh D-negative mother, resulting in maternal anti-D alloantibody production. These anti-D alloantibodies may lead to hemolytic disease of the newborn (HDN). With the advent of RhIg immunoprophylaxis, the overall risk of Rh alloimmunization and infant mortality from HDN has decreased. The Kleihauer-Betke acid-elution test, is the most widely used biochemical test for quantifying FMH. It relies on the principle that fetal RBCs contain mostly fetal hemoglobin (HbF), which is resistant to acid-elution whereas adult hemoglobin is acid-sensitive. Although the Kleihauer-Betke test is inexpensive and requires no special equipment, it lacks precision and is not accurate in conditions with elevated F-cells. Most adult RBCs do not have any hemoglobin F , however, sometimes few adult red cells can have a small amount of hemoglobin F and are called *F cells*.
      Anti-HbF flow cytometry can be adapted for fetal cell detection, thus giving clinical laboratories a potentially attractive automated alternative for quantifying FMH.
      Higher quantities of hemoglobin F in fetal cells yield a higher fluorescence signal and allows discrimination between fetal cells and adult F cells, thus increasing the sensitivity and specificity of detection.

   b) Paroxysmal nocturnal hemoglobinuria (PNH) diagnosis
      Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal stem cell disorder that leads to intravascular hemolysis due to deficiencies of decay-accelerating factor and membrane-inhibitor of reactive lysis which render red cells susceptible to complement-mediated lysis and thrombotic complications.
      Antibodies to CD55 and CD59 which are specific for decay-accelerating factor and membrane-inhibitor of reactive lysis, respectively, and can be used to make a definitive diagnosis of this condition by flow cytometry.

   c) CD4 counts in HIV/AIDS
      CD4 count measures the number of T Helper cells in blood, along with an HIV viral load test, it helps to assess the status of the immune system in a person who is suffering from human immunodeficiency virus (HIV) infection. CD3 is part of the T cell receptor (TCR) and is expressed on CD8+ Cytotoxic T lymphocytes and CD4+ Helper T lymphocytes. CD4 is expressed on Helper T lymphocytes and monocytes. Using fluorescence detection on a flow cytometer the fraction of total lymphocytes double-positive for CD3 and CD4 (i.e. CD4+ Helper lymphocytes) can be measured. Standards are used to calculate the absolute CD4 counts for HIV/AIDS diagnosis and prognosis.

7. Flourescence Activated Cell Sorting (FACS):
   Cell sorting is based on the physics of droplet formation, first described by Rayleigh in 1879. It states that a stream of fluid which is forced through an orifice, becomes hydrodynamically unstable to break into smaller droplets with smaller surface tension.
   Richard sweet used this principle to invent inkjet printing technology in 1965, which prints with ink that is electrostatically charged and deflected according to the input signal potential. Fulwyler adapted Sweet's principle of electrostatic inkjet droplet deflection and Coulter counter principle wherein cells were measured during passage through a coulter orifice. Fluid stream broke off into droplets which encapsulated

cells. These droplets were charged and deflected into a pre appointed collection vessel after passing between deflection plates mainted at high voltage of opposing polarities. This is how the first cell sorter was born in 1965. Len Herzenberg an immunologist who worked with Milstein during the discovery of monoclonal antibodies, coined the term FACS, flurescence activated cell sorting. He used the fluorescence based flow cytometer designed by Wolfgang Gohde to separate cells stained with fluorescein and rhodamine using an argon laser.
Due to the widespread use of this technology, today flow cytometry and FACS have become synonymous and have revolutionised biomedical research and clinical diagnosis.

8. Cancer Stem Cell biology research
   Cancer stem cells (CSC), is a small subset of cells in a tumor that are responsible for maintaining tumor heterogeneity, invasiveness, metastasis, therapeutic resistance and recurrence. Scientists are trying to isolate and characterize these CSC from different tumors in the hope of finding targets for anti- CSC therapy which would help control metastasis. CSC can be separated or isolated using FACS with the help of CSC markers. In our lab, we isolated and characterized CSC from primary human colorectal cancer tissues and human colorectal cancer cell lines. Characterisation and gene expression analysis of the CSC from high grade and low grade colorectal cancer revealed that gene expression signatures were very different between the two populations. We were able to show that high grade cancers which were more likely to metastasize had high number to CSC as compared to low grade cancers. Flow cytometry is been extensively used for further elucidation of the cancer stem cell biology by scientists around the globe.

9. Single cell analysis using FACS:
   Single-cell analysis allows the study of cellular heterogeneity or cell-to-cell variation within a population (organ, tissue, and cell culture). This technique is indispensable for in-depth analysis of stem cell differentiation, cancer, embryo differentiation and personalised drug development. Scientists are now using the bottom up approach to create complex tissues from single cells using synthetic biology and flow cytometry is the cornerstone technique for such single cell analysis.

10. Imaging flow cytometry:
    Imaging flow cytometry (IFC) is an amalgamation of the high-throughput, multiparameteric capabilities of flow cytometry and morphological and spatial information of microscopy, at a single-cell level.
    Using this advanced technique, multichannel digital images of thousands of individual cells can be captured within minutes. We can program the system to capture several fluorescence channels as well as bright field (transmitted light) and dark field (scattered light) images. This type of analysis is especially useful to study and analyse rare cell types such as circulating tumor cells in blood sample of cancer patients.

Summary:

- Flow cytometry is a powerful technique for analyzing millions of cells quickly. It use has been diversified in may fields of biomedical research and medicine
- Broadly, flow cytometry is used for DNA/RNA/protein analysis and to understand cellular functions.
- Some of the research applications include cell cycle analysis, live/dead analysis using nucleic acid binding dyes like propidium iodide, Apoptosis anlysis using TUNEL assay or Brdu assay.

- Flow cytometry can help in understanding cellular functions such as calcium signaling pathways in health and disease.
- In clinical practice, flow cytometry is widely used for minimum residual detection (MRD) in cancers such as Acute Myeloid leukemia and in hematological diagnosis of disorders like Paroxysmal nocturnal hemoglobinuria (PNH).
- Analysis in CD4 count for diagnosis and prognosis in HIV/AIDS is also done using this technique.
- Flow cytometry was further improved upon by adding the powerful feature of cell extraction or cell sorting. Flourescence activated cell sorting (FACS) is an extremely versatile method which allowed researchers to isolate and study pure sub-populations from a heterogenous parent population.
- Based on the specific fluorescent properties and light scattering patterns of a cell, unique charge is applied to the droplet containing the cell of interest. The charged droplet falls through an electrostatic deflection plate area which diverts the droplet into the appropriate container designated by the software running the sorter instrument.
- FACS is very useful to study cancer stem cell biology and to make key discoveries such as the concept of cancer stem cells in hematological and solid cancers.
- It is also used for single cell analysis
- Further, flow cytometry has been specialised to include imaging of single cells along with morphological and cellular characterisation by combining microscopy and flow cytometry in the form of a high throughput imaging technique called imaging flow cytometry.
- Imaging flow cytometry is being used for detection of rare cell types such as circulating tumor cells to understand metastasis in cancer.
- Flow cytometry technique has a huge potential in biomedical research and medicine. Currently, it is a very important technique to be mastered for any researcher in life sciences.