Flow Cytometry: Basic Principle and Applications in Biotechnology and Pharmacy

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Summary

The use of flow cytometry is extended in each and every area of science. Flow cytometry is a powerful technique for correlating multiple characteristics of a single cell. This qualitative and quantitative technique has made the transition from a research tool to standard clinical testing. The term cytometry refers to the measurement of physical and/or chemical characteristics of cells or other biological samples. Thus this process employs the fluidic flow of a biological sample to identify quickly and accurately the sample characteristics. Sensing is conducted by using an optical sensor where the laser light source beam interacts with each individual cell which will produce light scattering or fluorescence. Flow cytometry uses fluorescence and scattering to analyze a population of cells, organelles, or other similarly sized particles quantitatively. Flow cytometry can examine a multitude of biological parameters, such as particle size, cell type, DNA content, and enzymatic function at up to 10,000 cells per second. With the recent sequencing of a variety of microbial genomes, it is anticipated that flow cytometry will have an increasing role to play in studying the effects of gene expression and mutation on heterogeneity, and in resolving the interactions of genetics and physiology.

Introduction

Flow cytometry is a technique for counting, examining, and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus.

Flow cytometry offers the possibility for this type of specific and detailed analysis of cell populations. Flow cytometric assays have been developed to determine both cellular characteristics such as size, membrane potential, and intracellular pH, and the levels of cellular components such as DNA, protein, surface receptors, and calcium. Measurements that reveal the distribution of these parameters in cell populations are important for biotechnology, because they better describe the population than the average values obtained from traditional techniques.

In flow cytometry, single cells or particles pass through a laser beam in a directed fluid stream. The interaction of the cells with the laser beam – their absorption, scattering, and/or fluorescence – can be monitored for each individual cell. These data can be correlated with different cell characteristics and cell components. Thus, distributed data about a cell population can be obtained easily.

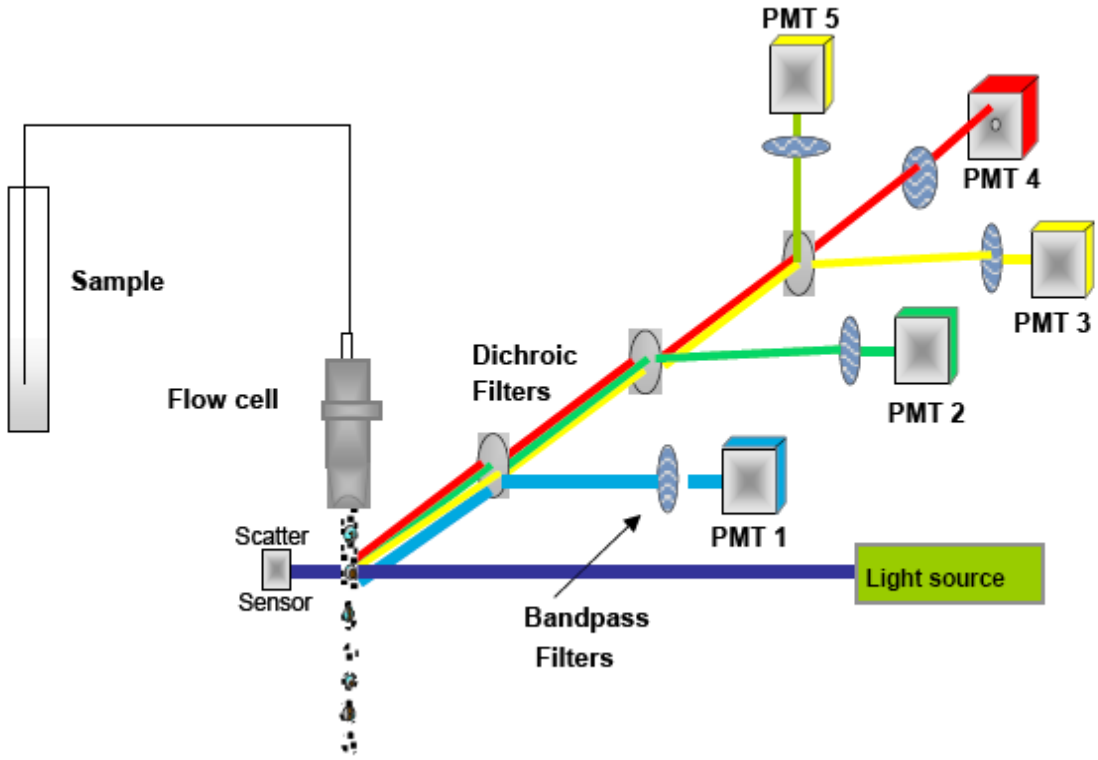

Figure 1 Simplified scheme of flow cytometer (from Murphy 2006).

The key advantage of flow cytometry is that a very large number of particles can be evaluated in a very short time; some systems can run particles at rates approaching 100,000 particles per second while collecting 10 to 20 parameters from each particle. Finally, the principle of cell sorting in flow cytometry allows this technology to separate single particles/cells physically from mixed populations.

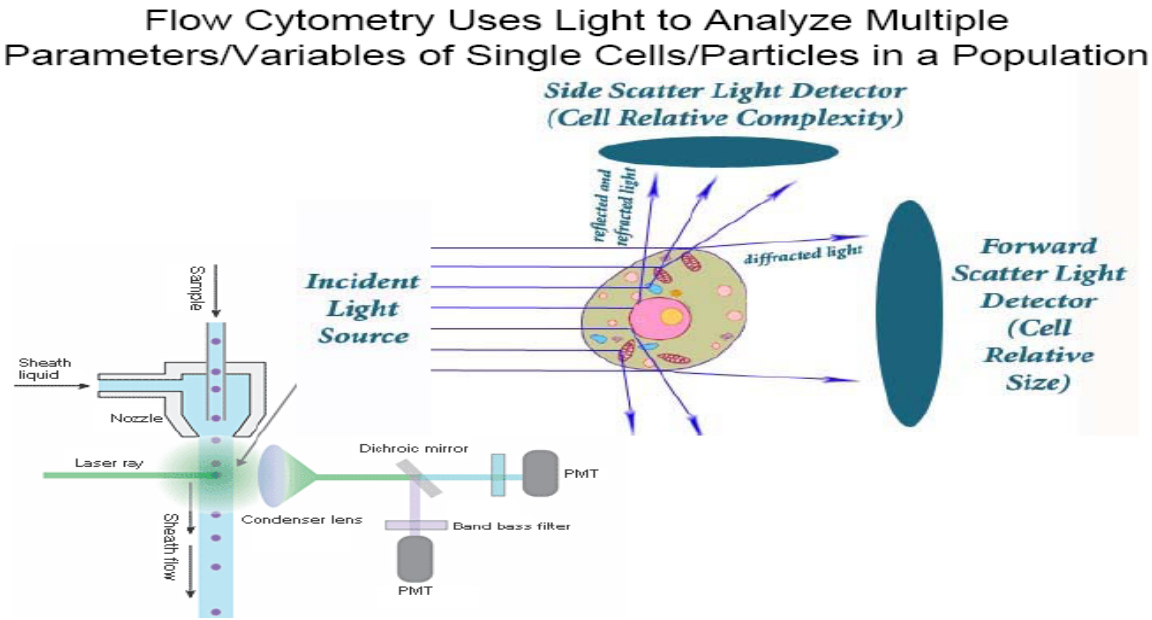

Fig-2 multiple parameter analysis/variables of single cells/particles in a population.

The most common detection system in flow cytometry uses fluorescent molecules that are attached by one means or another to the particle of interest. If the particle is a cell, such as a white blood cell, for example, the fluorescent probe might be membrane bound, cytoplasmic, or attached to nuclear material. It is a common practice to use monoclonal or polyclonal antibodies that recognize specific receptors on cells. By conjugating fluorescent molecules to these antibodies, it is possible to monitor both the location and number of these conjugated antibodies as they bind to cell receptors. Particles of almost any nature can be evaluated by flow cytometry. They can be very small, even below the resolution limits of visible light, because they can be detected by their fluorescent signatures. Similarly, depending on the structure of the flow cell and fluidics, particles as large as several thousand microns can be evaluated.

Flow cytometry is a technology that has impacted both basic cell biology and clinical medicine in a very significant manner. Flow cytometry was first used in medical sciences such as oncology (e.g., for diagnosis of cancer, chromosomal defect diagnosis) and haematology. Medical and clinical applications of flow cytometry still account for the vast majority of publications on this technique, but during the past few years it has also become a valuable tool in biology, pharmacology, toxicology, bacteriology, virology, environmental sciences, and bioprocess monitoring. The recent success of flow cytometry is based on commercially available flow cytometry equipment that is both robust and versatile, together with modern data acquisition and interpretation software, and tremendous successes in the development of various specific staining assays. This review gives a short introduction into the principles of flow cytometry and its application for different areas.

Basic Principle

Basically, a flow cytometer is a fluorescence microscope which analyses moving particles in a suspension. These are excited by a source of light (U.V. or laser) and in turn emit an epi-fluorescence which is filtered through a series of dichroic mirrors. Then, the in-built programme of the equipment converts these signals into a graph plotting the intensity of the epi-fluorescence emitted against the count of cells emitting it at a time given.

Thus, a flow cytometer consists of *fluidics*, *optics* and *electronics*, as it measures cells in suspension that flow in single-file through an illuminated volume where they scatter light and emit a fluorescence that is collected, filtered and converted to digital values for storage on a computer.

Fluidics system

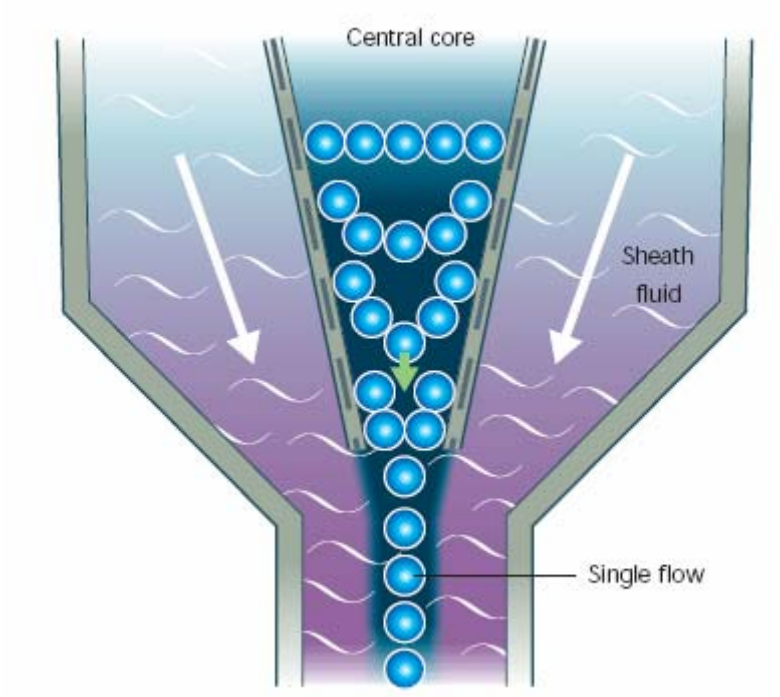

Fig-3 Hydrodynamic focusing produces a single stream of particles.

One of the fundamentals of flow cytometry is the ability to measure the properties of individual particles. When a sample in solution is injected into a flow cytometer, the particles are randomly distributed in three-dimensional space. The sample must therefore be ordered into a stream of single particles that can be interrogated by the machine's detection system. This process is managed by the fluidics system. Essentially, the fluidics system consists of a central channel/core through which the sample is injected, enclosed by an outer sheath that contains faster flowing fluid. As the sheath fluid moves, it creates a massive drag effect on the narrowing central chamber. This alters the velocity of the central fluid whose flow front becomes parabolic with greatest velocity at its centre and zero velocity at the wall. The effect creates a single file of particles and is called **Hydrodynamic focusing**. Also the introduction of a large volume into a small volume in order that it "focuses" along an axis is termed "Hydrodynamic focusing". Under optimal conditions (laminar flow) the fluid in the central chamber will not mix with the sheath fluid. The flow characteristics of the central fluid can be estimated using Reynolds Number.

$$R_e = \frac{pVD}{\mu}$$

Where D = tube diameter,
V = mean velocity of fluid,
p = density of fluid, and
μ = viscosity of fluid.

When Re < 2300, flow is always laminar. When Re > 2300, flow can be turbulent, which accelerates diffusion.

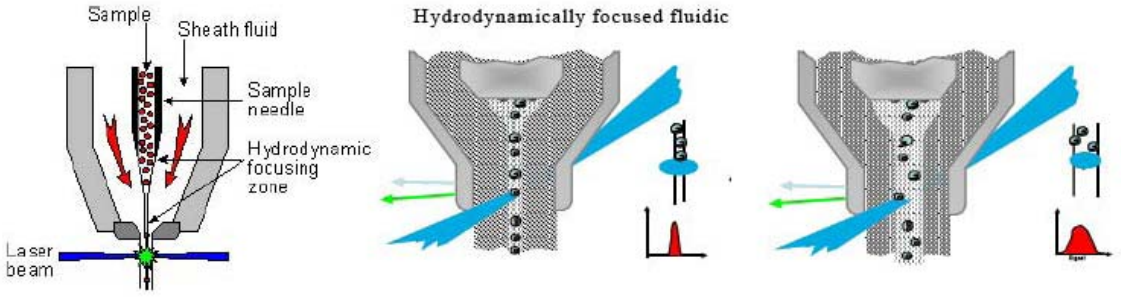

Fig- 4 Basics of fluidics in flow cytometry. From left to right, flow chamber, a hydrodynamically focused fluid and an unfocused fluid where the increse of pressure widens the core and increases turbulance (from Murphy, 2006).

Without hydrodynamic focusing the nozzle of the instrument (typically 70 μM) would become blocked, and it would not be possible to analyze one cell at a time.

Optics and detection

After hydrodynamic focusing, each particle passes through one or more beams of light. Light scattering or fluorescence emission (if the particle is labelled with a fluorochrome) provides information about the particle's properties. The laser and the arc lamp are the most commonly used light sources in modern flow cytometry. Lasers produce a single wavelength of light (a laser line) at one or more discreet frequencies (coherent light). Arc lamps tend to be less expensive than lasers and exploit the colour emissions of an ignited gas within a sealed tube. However, this produces unstable incoherent light of a mixture of wavelengths, which needs subsequent optical filtering. Light that is scattered in the forward direction, typically up to 20º offset from the laser beams axis, is collected by a lens known as the forward scatter channel (FSC). The FSC intensity roughly equates to the particle's size and can also be used to distinguish between cellular debris and living cells. Light measured approximately at a 90º angle to the excitation line is called side scatter. The side scatter channel (SSC) provides information about the granular content within a particle. Both FSC and SSC are unique for every particle, and a combination of the two may be used to differentiate different cell types in a heterogeneous sample. Fluorescence measurements taken at different wavelengths can provide quantitative and qualitative data about fluorochrome-labeled cell surface receptors or intracellular molecules such as DNA and cytokines. Flow cytometers use separate fluorescence channels to detect light emitted. The number of detectors will vary according to the machine and its manufacturer.

Detectors are either silicon photodiodes or photomultiplier tubes (PMTs). Silicon photodiodes are usually used to measure forward scatter when the signal is strong. PMTs are more sensitive instruments and are ideal for scatter and fluorescence readings. Most cytometers use photomultiplier tubes (PMTs) as detectors for both fluorescence and scatter. The pulse of a particle crossing the excitation beam will depend upon the beam shape, beam intensity, and particle size, as well as the velocity of the particle.

The specificity of detection is controlled by optical filters, which block certain wavelengths while transmitting (passing) others. There are three major filter types. 'Long pass' filters allow through light above a cut-off wavelength, 'short pass' permit light below a cut-off

wavelength and 'band pass' transmit light within a specified narrow range of wavelengths (termed a band width). All these filters block light by absorption.

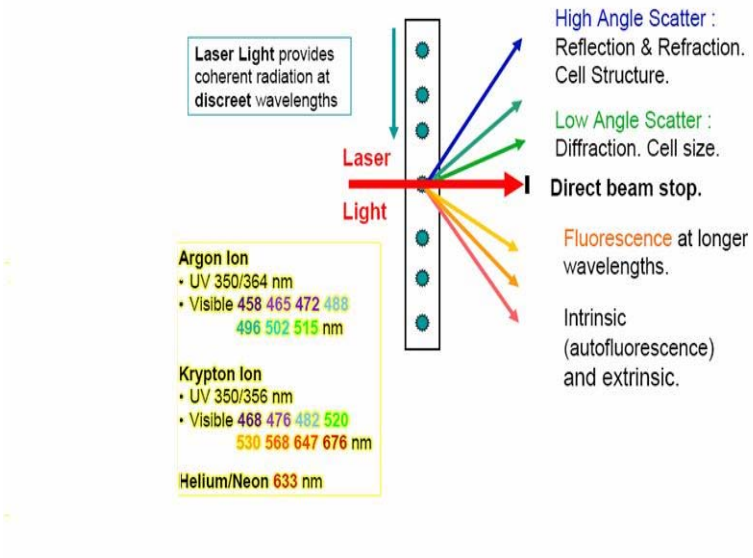

Fig- 5 Light source, Light scatter and Fluorescence

When a filter is placed at a 45º angle to the oncoming light it becomes a dichroic filter/mirror. As the name suggests, this type of filter performs two functions, first, to pass specified wavelengths in the forward direction and, second, to deflect blocked light at a 90º angle. To detect multiple signals simultaneously, the precise choice and order of optical filters will be an important consideration.

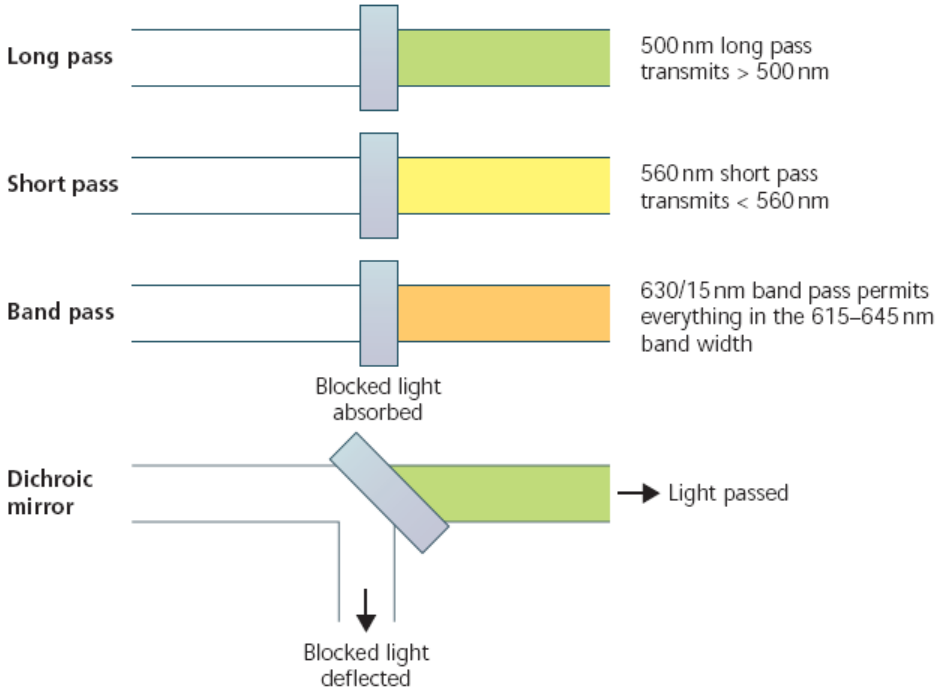

Fig-6 Filters used in Flow cytometer.

Signal processing

When light hits a photo detector a small current (a few microamperes) is generated. Its associated voltage has an amplitude proportional to the total number of light photons received by the detector. This voltage is then amplified by a series of linear or logarithmic amplifiers, and by analog to digital convertors (ADCs), into electrical signals large enough (5–10 volts) to be plotted graphically.

Log amplification is normally used for fluorescence studies because it expands weak signals and compresses strong signals, resulting in a distribution that is easy to display on a histogram. Linear scaling is preferable where there is not such a broad range of signals e.g. in DNA analysis. The measurement from each detector is referred to as a 'parameter' e.g. forward scatter, side scatter or fluorescence. The data acquired in each parameter are known as the 'events' and refer to the number of cells displaying the physical feature or marker of interest.

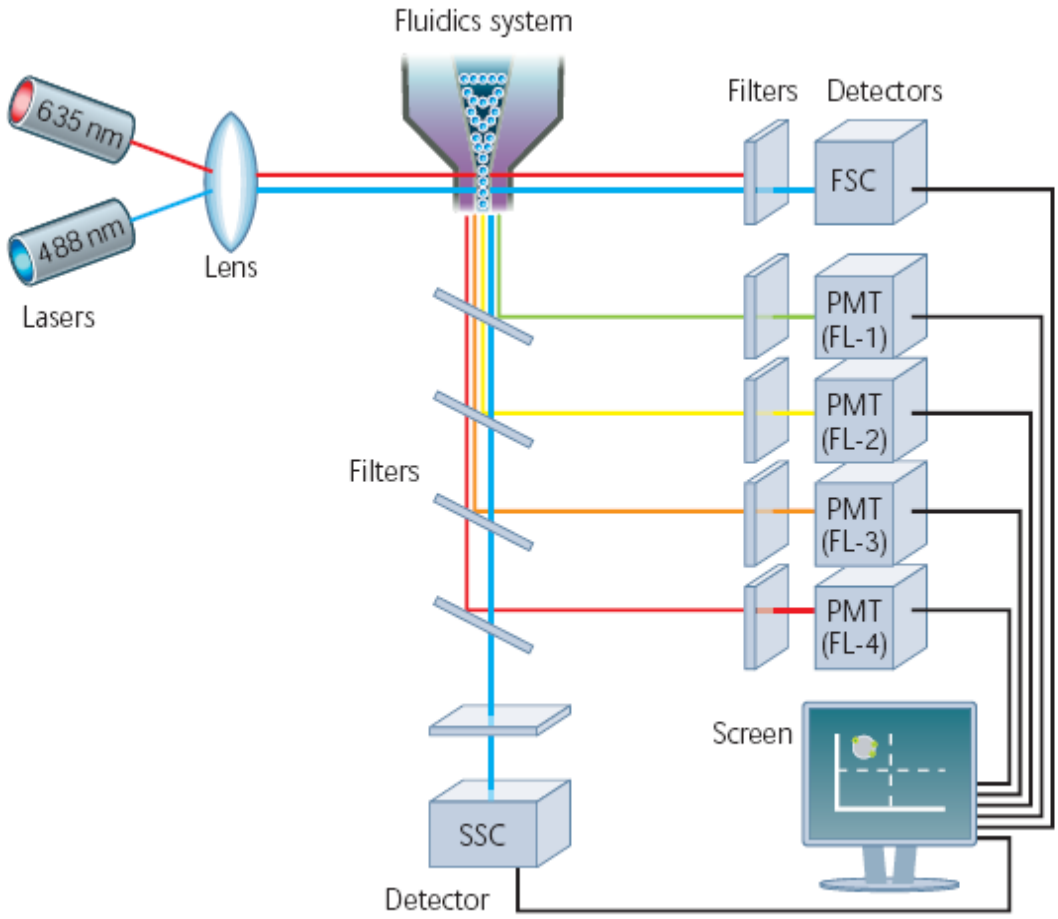

Fig-7 Schematic overview of a typical flow cytometer setup

Applications of flow cytometry

Flow cytometry has various applications in research labs, Clinical labs, and industries. Other applications make use of similar or slightly modified protocols

- Multiplexing immunoassays

  - Functional assays
  - ✓ Oxidative burst
  - ✓ Phagocytosis
  - ✓ Tumour drug resistance

- Multiparameter Immunophenotyping
  - Chronic lymphoid leukemias
  - Immunophenotyping
  - Acute leukemias – Lymphoblastic / Myeloblastic
  - NHL & Plasmacytic neoplasias
  - Hodgkin's disease

- Proliferation antigens
  - Ki67 protein
  - Proliferating cell nuclear antigen
  - p105 antigen
  - Others-4F2,CD25,CD71

- Immunodeficiency
  - HIV/AIDS
  - Primary Immunodeficiency

- Transplantation
  - Allograft dysfunction
  - Anti- CD3 therapy

- Auto antibodies
  - Autoimmune Thrombocytopenia
  - Autoimmune and Alloimmune Neutropenia

- DNA quantitation methodology
- Cytogenetics & other molecular techniques
- DNA ploidy and proliferation fraction
- Measurement of intracellular cytokines
- Signal transduction pathways
- Measuring cellular function

Cellular parameters measurable by flow cytometry.
Intrinsic: size, shape, cytoplasmic granularity, auto fluorescence and pigmentation.
Extrinsic: DNA content, DNA composition, DNA synthesis chromatin structure, RNA, protein, sulphydryl groups, antigens (surface, cytoplaspic & nuclear), lectin binding sites, cytoskeletal components, membrane structure (potential, permeability & fluidity), enzyme activity, endocytosis, surface charge, receptors, bound and free calcium, apoptosis, necrosis, pH, drug kinetics, etc.

The following section discusses major applications of flow cytometry.

1. DNA analysis.

DNA-specific fluorochromes, are generally used for DNA analysis, have important differences against the fluorochromes used for conjugation to antibodies for staining the proteins of cells. In particular, whereas fluorescein, phycoerythrin (PE), and others are fluorescent whether or not they are bound to cells, the DNA fluorochromes fluoresce significantly only when they are bound to their target molecules. In addition, unlike the tight binding of antibody to antigen, DNA fluorochromes are generally in loose equilibrium between their bound and Free states. Therefore procedures for analyzing the DNA content of cells involve sending cells through the flow cytometer without washing them to remove the ``unbound'' fluorochrome. The unbound fluorochrome will not add to background fluorescence because it is hardly fluorescent unless bound to nucleic acid. And washing would, in any case, lower overall specific fluorescence by removing much of the fluorochrome (both bound and unbound) from the cell.

Examples of Nucleic Acid Binding Dyes

Dyes	**Specificities**
Acridine orange	DNA and RNA; metachromatic; permeant to viable cells
Propidium iodide	Double-stranded nucleic acids; impermeant
Thiazole orange	DNA and RNA; Permeant
Ethidium bromide	Double-stranded nucleic acids; impermeant
Chromycin A_3	DNA with GC preference; impermeant
DAPI	DNA with AT preference; slightly permeant to viable cells
Hoechst 33342 and Hoechst 33258	DNA with AT preference; Hoechst 33342 enters viable cells well, Hoechst 33258 less well
7-Aminoactinomycin D (7-AAD)	DNA and RNA; GC preference; impermeant to viable cells
TO-PRO, TO-TO, PO-PO, PO-PRO, YO-YO, and YO-PRO series	DNA and RNA; Impermeant
SYTO series	DNA and RNA; Permeant

Several types of fluorescent stain are available for the analysis of DNA; their characteristics make them suitable for different applications (Table). The most specific stains (e.g., DAPI and the Hoechst dyes, which stain specifically for AT groups on DNA) require the use of a laser with significant ultraviolet (UV) output. Hoechst dyes as well as a newly developed far-red dye called DRAQ5 (alone of all the current DNA-specific stains) also penetrate the outer membrane of living cells and can therefore be used for staining living cells with different DNA content for subsequent sorting for separate culture or functional analysis.

Chromomycin A_3 is specific for the GC bases in DNA and therefore is an appropriate stain for use in conjunction with Hoechst 33258. Propidium iodide, although not very specific (it stains all double-stranded regions of both DNA and RNA by intercalating between the stacked bases of the double helix) and not able to penetrate an intact cell membrane, has the decided advantage of absorbing 488 nm light and then fluorescing at wavelengths above 570 nm. This means that, in the presence of RNase, propidium iodide can be used as a DNA stain in cytometers with low-power argon lasers. Propidium iodide has therefore become the most common DNA fluorochrome for flow analysis.

2. Chromosome sorting

In this case, the particles flowing through the system are individual chromosomes that are released from cells that have been arrested in metaphase. The released chromosomes are stained with a DNA stain (like propidium iodide) and then sent through the flow cytometer. The resulting histograms of fluorescence intensity reveal peaks whose positions along the x-axis are proportional to the amount of DNA in the chromosome and whose areas are proportional to the number of chromosomes with that particular DNA content. Histograms of this type are called flow karyotypes.

Karyotyping is the process of arranging chromosomes into pairs, which helps in detecting structural and numerical abnormalities. Chromosomes are important in understanding the structural and genetic characterization of any species in normal and pathological physiology. Chromosomes contain DNA, which in turn contains all the genetic information needed to carry out all of the biological processes within an organism. The human genome sequencing project benefited immensely with chromosome specific DNA libraries.

DNA measurement using a flow cytometer is done using fluorescent dyes that intercalate into the helical structure in a stoichometric fashion. The amount of fluorescence is directly proportional to the amount of DNA content of the cell. Typically a sample contains about 10000- 20000 cells. To build a chromosome library one microgram of DNA is needed, since human chromosome contains approximately only 65 fg of DNA, millions of sorted chromosomes are required (Ibrahim and van den Engh, 2004). Cytometric chromosomal analysis varies from the total DNA quantization, which also uses DNA specific fluorescent dye. However it yields the entire DNA content of cell as a single intensity value, fluorescent intensity directly proportional to DNA content. Flow cytometry karyotyping uses Propidium Iodide, a DNA binding dye which is excited using an appropriate laser. Each chromosome is of different size and hence the fluorescence intensity varies with the size of the chromosomes. Langlois and Jensen (1979) used two dyes with two different binding affinities for A/T and G/C nucleotides (ATCG- nucleotides are the building blocks of DNA). There will be difference in fluorescence emitted by A-T and C-G bound dye depending on the base pair ratio. This method allows you to analyze tens of thousands of chromosomes per second. The bivariate plot helps the researchers to directly detect abnormalities.

It may be a useful intellectual exercise for readers to ask themselves which technique or techniques would be most appropriate for detecting the following types of chromosome abnormalities: (1) tetraploidy, where the normal chromosome content of cells is exactly doubled because of failure of cytokinesis after mitosis; (2) an inversion in an arm of one particular chromosome; and (3) trisomy (the existence of cells with three instead of two) of one of the small chromosomes. In addition to these limitations, the use of flow cytometry to look for abnormal chromosomes has been confounded by the fact that several human

chromosomes are highly polymorphic, and flow karyotypes, therefore, vary considerably among normal individuals.

3. Cell cycle analysis

Normal cells will have more DNA than the 2C amount appropriate to their species at times when they are preparing for cell division. The cell cycle has been divided into four phases (fig-10). Cells designated as being in the G_0 phase are not cycling at all; cells in G_1 are either just recovering from division or preparing for the initiation of another cycle; cells are said to be in S phase when they are actually in the process of synthesizing new DNA; cells in the G_2 phase are those that have finished DNA synthesis and therefore possess double the normal amount of DNA; and cells in M phase are in mitosis, undergoing the chromosome condensation and organization that occur immediately before cytokinesis (resulting in the production of two daughter cells, each with the 2C amount of DNA). A DNA flow histogram provides a snapshot of the proportion of different kinds of nuclei present at a particular moment. If we look at the DNA content of cells that are cycling (not resting), we will find some nuclei with the 2C amount of DNA (either G_0 or G_1 cells), some nuclei with the 4C amount of DNA (G_2 or M cells), and some nuclei with different amounts of DNA that span the range between these 2C and 4C populations (Fig.16). The traditional method for analyzing cell division involves measuring the amount of DNA being synthesized in a culture by counting the radioactivity incorporated into DNA when the dividing cells are given a 6 h pulse with tritiated thymidine. The DNA histogram resulting from flow cytometric analysis offers an alternative to this technique. By dividing the histogram up with four markers, we can delineate nuclei with the 2C amount of DNA, those with the 4C amount of DNA, and those with amounts of DNA between the two delineated regions and therefore caught in the process of synthesizing DNA.

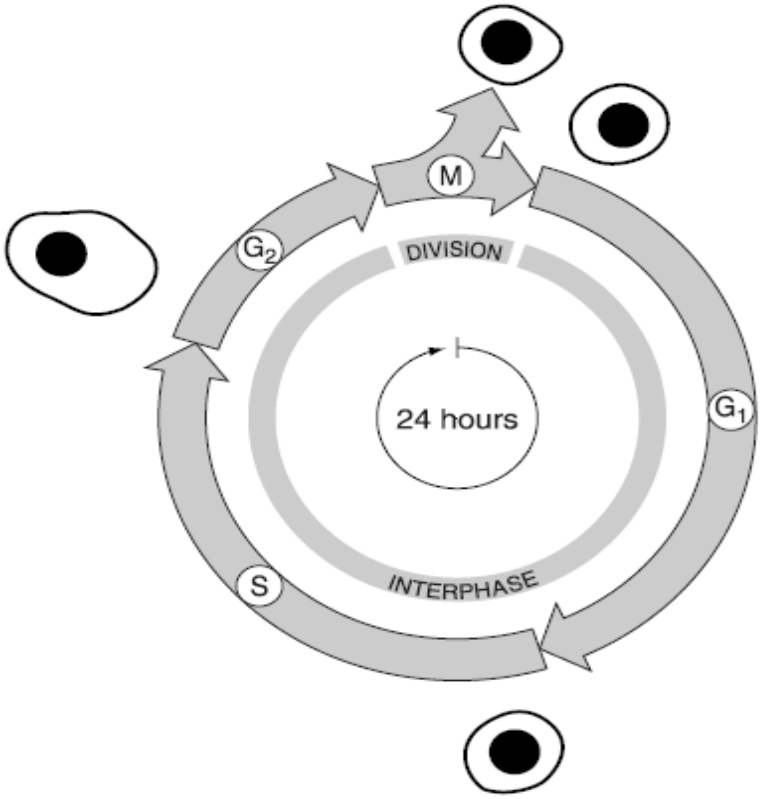

Fig-8 The four successive phases of a typical mammalian cell cycle. From Alberts et al. (1989).

Flow cytometry does, however, offer a more direct way to measure DNA synthesis. Bromodeoxyuridine (sometimes abbreviated BrdU or BUdR or BrdUdr) is a thymidine analog. If cells are pulsed with BrdU, it will be incorporated into the cell's DNA in the place of thymidine. Fluorescein-conjugated monoclonal antibodies with specificity for BrdU are available so that cells that have been pulsed with BrdU for a short period of time (about 30 min) can then be treated to partially denature their DNA, exposing the BrdU within the double helix so that it can be stained with the anti-BrdU antibody. Any cells that have incorporated BrdU during the pulse will then stain fluorescein positive. The clever part of this technique is that the denatured DNA can be stained with propidium iodide at the same time. The red fluorescence axis shows the propidium iodide distribution (proportional to DNA content) with which we have grown familiar; the green fluorescence axis shows which of these nuclei have actually incorporated BrdU during the pulse. As might be expected, the cells in the middle region of the propidium iodide distribution have all incorporated BrdU; but a proportion of the cells at either end of the propidium iodide distribution have also done so. This method, while somewhat time consuming and a bit tricky technically, does allow a flow cytometrist to quantify the proportion of cells in S phase in a way that cannot be done accurately with simple propidium iodide staining.

The bromodeoxyuridine method is, in fact, more comparable than simple propidium iodide staining to the traditional method of measuring cell division by assaying the incorporation of tritiated thymidine. It also provides the ability to distinguish cells that may be blocked in S phase from those that are actually incorporating nucleotides. BrdU staining has also been used to provide information about the kinetics of cell cycles. If we consider cells pulsed for a short period of time with a small amount of BrdU and then killed immediately and stained with both propidium iodide and with a fluorescein-anti-BrdU monoclonal antibody, the fluorescein-antibody should stain equally all cells in S phase (stretching from the 2C peak to the 4C peak). If, however, we wait for some time before killing the cells (and if the BrdU has been used up quickly), then the cells that have incorporated the BrdU (that is, all the cells that were in S phase at the time of the pulse) will have synthesized more DNA, and some of those cells will have progressed into the G_2 or M phase of the cell cycle (or indeed cycled back to G_1). In addition, some new cells will have started to make DNA after the BrdU had been used up, and these cells will now be in S phase but will have DNA that does not contain BrdU. We can estimate the rate of movement of the BrdU-containing cells through S phase and into the G_2 peak by assuming that they are evenly distributed throughout S phase at the time of pulsing and then sampling and staining the cells at one subsequent time. The rate of increase in propidium iodide intensity of the fluorescein-positive nuclei is equivalent to their rate of DNA synthesis and provides us with information about the cycle time of the actively dividing cells. Moreover, the cycle time of the fluorescein-positive cells, in conjunction with the proportion of cells in S phase, can be used to estimate the doubling time of a population of cells.

4. Study of Apoptosis

Cells often die by an active process that is an important part of the maintenance of organismal homeostasis. This process is called apoptosis. Apoptosis or programmed death can prevent the survival of potentially malignant cells with damaged DNA.

Cells that are undergoing apoptosis progress through a series of events. Some of the events are not obligatory and may differ depending on the nature of the apoptotic trigger and the cell type. In any case, several of these apoptotic-associated events can be analyzed by flow

cytometry. One of the events is the flipping and stabilization of phosphatidylserine from the inner surface of the cytoplasmic membrane to the outer surface. On the outer surface, phosphatidylserine appears to identify cells as targets for phagocytosis. Because annexin V binds to phosphatidylserine, staining of intact cells with fluorochrome-conjugated annexin V will detect cells that are in early stages of apoptosis.

Unstained cells are alive and well and are the double negatives; they neither express phosphatidylserine on their surface nor take up propidium iodide through leaky membranes. Cells that stain just with annexin V are apoptotic; they have begun to express phosphatidylserine on their surface, but have not yet gone through the process that leads to permeabilization of their cytoplasmic membrane.

Cells that stain both with propidium iodide and annexin V are necrotic (that is, dead); they take up propidium iodide and also stain with annexin V. With a permeable cell, the flow cytometer cannot tell us whether the annexin V is on the outside of the membrane (because the cells have gone through apoptosis before membrane permeabilization) or on the inside of the membrane (because the cells have died by the necrotic pathway without apoptosis).

Another event associated with apoptosis is the fragmentation of DNA due to endonuclease activation. This fragmentation results in the appearance of increased numbers of ``free ends'' on the DNA molecules in the cell. By incubating fixed and permeabilized cells with the enzyme terminal deoxynucleotidyl transferase (TdT) and with fluorochrome-labeled nucleotides, cells with greater numbers of DNA fragments incorporate more of the fluorescent nucleotides onto their increased number of termini. The resulting increased cellular fluorescence is indicative of an apoptotic cell. Appearance of positive cells in this TdT flow assay correlates well with the classic gel electrophoresis assay for apoptosis, where ``ladders'' of small DNA fragments are indicators of endonuclease activation. In a desperate attempt to find an acronym, this flow assay for apoptosis has been called the TUNEL assay (for Terminal deoxynucleotidyl transferase-mediated dUTP Nick End-Labelling). Propidium iodide staining alone can be used to detect later stages of apoptosis.

5. Study of Necrosis

Unlike apoptosis, which involves the scheduled and active coordination of metabolic processes, necrosis is a passive response to a toxic or injurious environment. Whereas in apoptosis the cell membrane remains intact until very late in the game, permeabilization of the cell membrane is an early event in necrosis. Because propidium iodide is excluded from entering cells by an intact plasma membrane and because it only fluoresces when intercalated between the bases of double-stranded nucleic acid, it will not fluoresce if it is added to a suspension of intact cells. The intact plasma membrane forms a barrier, keeping propidium iodide and nucleic acids apart. It is only when the outer membrane has been breached that the cells will emit red fluorescence. Propidium iodide (or other membrane-impermeant DNA fluorochromes) is therefore a stain (like trypan blue) that can be used to mark necrotic cells (on the reasonable but not necessarily valid assumption that cells with holes in their membranes large enough to allow the penetration of propidium iodide are actually dead according to other viability criteria and vice versa). By this method, cell viability can be monitored in the presence of various cytotoxic conditions.

The only diffculty in using flow cytometry to monitor cell death is that the dead cells have different scatter properties than living cells. In particular, because of their perforated outer

membrane, they have a lower refractive index than living cells and therefore have forward scatter signals of lower intensity. For this reason, it is important not to use a gate or forward scatter threshold when analyzing a population for the proportion of dead and live cells.

The use of membrane-impermeant DNA fluorochromes to mark dead cells has a more routine application in simply allowing the exclusion of dead cells from flow analysis. This is important because dead cells have the habit of staining non-specifically when their broken membranes tend to trap monoclonal antibodies directed against surface antigens. Therefore a cell suspension including many dead cells may show high levels of nonspecific stain. By adding propidium iodide to cells just before analysis, the cells fluorescing red can be excluded from further analysis by gating on red negativity and only the living cells then examined for surface marker staining.

Fixed cells are, in fact, all dead and will therefore all take up propidium iodide even if some were alive and some dead before fixation. Ethidium monoazide offers an alternative to propidium iodide if cells will be fixed before flow analysis. It is a dye that, like propidium iodide, only enters dead cells. It has, however, the added advantage of forming permanent cross-links with DNA when photoactivated. Therefore, cells can be stained for surface proteins, incubated with ethidium monoazide under a desk lamp, washed, and then fixed. At the time of acquisition of data on the flow cytometer, red fluorescence will mark the cells that were dead before fixation.

6. Cell sorting

Cell sorting is a major application of flow cytometry, separating the cells from a heterogeneous population with precisely defined characteristics. Multiple parameters can be measured using flow cytometry, since it has multiple excitation and emission source. It can measure 5-10 parameters simultaneously; namely DNA content, protein content, size, Lipid content, antigenic properties etc. Measuring different parameters of a heterogeneous cell population helps in identifying rare cell group and also gives a multi dimensional representation of individual cells within a population. Electrostatic deflection is used in flow cytometry. The droplet containing cells of interest is charged at the point of illumination by determining the elapsed time between cell sensing and separating. Applying a predetermined charging voltage to the flowing stream containing a heterogeneous cell population is critical, and hence a droplet containing cells of our interest is separated. Once the droplet is charged it gets deflected by the high voltage deflection plates to its corresponding collecting cuvette.

High speed sorting, that requires a pressure of 200psi to generate high stream velocities of 50 (m/sec) permitting droplet generation at frequencies as high as several hundred kHz. But the mammalian cells with their thin membrane could not survive higher pressures. In another interesting application, “Cell Zapping”- unwanted cells are eliminated by a selective process.

7. Detection of microorganisms

Much of the work on microorganisms in flow systems has concerned yeast, algae, and protozoa; although smaller than mammalian cells, these eukaryotes are considerably larger than most bacteria. DNA, RNA, protein, and light scatter measurements have been made on these organisms, and the feasibility of cell cycle analysis has been demonstrated. Bacteria, however, present more acute difficulties. The diameter of bacterial cells is perhaps 1 μm (compared with 10 μm for mammalian blood cells), and therefore the surface area to be

stained (and resulting fluorescence intensity) is 10^2 less than that of a mammalian cell. The DNA content of the E. coli genome is about 10^{-3} times that of a diploid human cell. Hence, bright dyes and sensitive instrumentation are required for studies of bacteria. Nevertheless, reasonable DNA histograms of bacteria can be obtained by flow cytometry. Methods are being developed to investigate cell cycle kinetics, the effects of antibiotics, and the detection and identification of bacteria for clinical investigations.

Now a day, it is also possible to detect virus particles with flow cytometer with latest advancement. Representatives from several different virus families (Baculoviridae, Herpesviridae, Myoviridae, Phycodnaviridae, Picornaviridae, Podoviridae, Retroviridae, and Siphoviridae) are stained using a variety of highly fluorescent nucleic acid specific dyes (SYBR Green I, SYBR Green II, OliGreen, PicoGreen) and examined using a standard flow cytometer equipped with a standard 15 mW argon-ion laser. This rapid and precise assay represents a new and promising tool in the field of virology.

Flow cytometry (FCM) in combination with fluorescent probe technology is a rapid, sensitive, and quantitative technique to detect microorganisms and assess their viability. Quantitative information on the presence and viability of plant pathogenic microorganisms is valuable for risk assessment regarding disease transmission and disease development. FCM has been applied successfully in the fields of food microbiology, veterinary science, and medical research to detect and distinguish between viable and non-viable bacteria.

The brewing industry has long had an interest in applying flow cytometry to the microbiology of the brewing process. Hutter, et al, determined the purity of yeast cultures using immunofluorescence and flow cytometry, as well as using antibodies to discriminate the presence of other organisms in fermentations. More recently, flow cytometry has been applied in the wine industry to quantify wine yeast viability, bacteria viability, and to sort wine yeast populations.

8. Disease Monitoring/Profiling

Leukemia/Lymphoma Phenotyping

Studies of the staining of surface proteins (CD antigens) to determine the phenotypes of the abnormal cells in patients with various leukemias or lymphomas have been useful, revealing much about ways to classify the diseases but also increasing our knowledge of normal immune cell development. In general, immune cells gain and lose various surface proteins in the course of their normal development in the bone marrow until they become the cells (with mature phenotype and function) that are released from the marrow into the peripheral circulation (Fig. 9). Leukemias and lymphomas often involve a block in this development so that cells with immature phenotypes appear in great numbers in the periphery. Alternatively, certain forms of disease may involve rapid expansion of a clone of mature normal cells so that one type of cell predominates in the circulation over all other normal subpopulations. In other cases, cells seem to express abnormal combinations of CD antigens, a condition referred to as lineage infidelity or as lineage promiscuity depending on one's interpretation of the data. These variations in surface protein expression make classification of leukemias and lymphomas a suitable application for flow cytometry, albeit a very complex task. Correlation between classification and predicted clinical course is therefore correspondingly difficult.

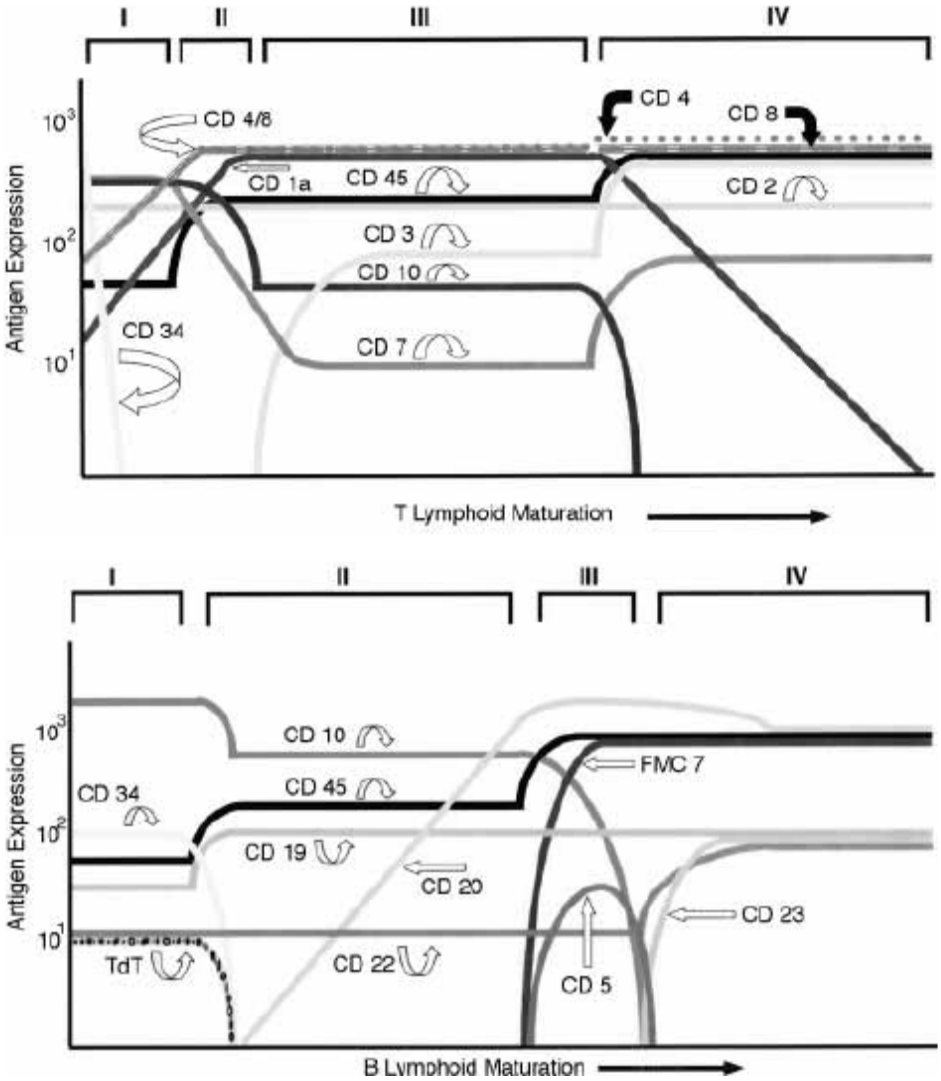

Fig- 9 Surface antigen changes during haematopoiesis. The upper plot is of T-lymphocyte maturation and the lower plot of B cells.

HIV/AIDS

Another condition that involves analysis of peripheral blood leukocytes is AIDS. Early in the natural history of the disease (or at least in the natural history of immunologists' awareness of the disease), it was discovered that one subpopulation of T lymphocytes in particular was destroyed by the HIV virus; the cells destroyed are those that possess the CD4 protein on their surface. It is this CD4 protein that appears to be a receptor involved in virus targeting. Therefore, much of the diagnosis and staging of AIDS involves the enumeration of CD4-positive cells in the peripheral blood (Fig.10). These techniques are for counting CD4-positive cells in connection with AIDS diagnosis and for phenotyping various populations of leukocytes for leukemia/lymphoma diagnosis-can be performed in haematology laboratories by the staining of cells with fluorochrome conjugated monoclonal antibodies followed by the visual identification of different types of white cells and the counting of the fluorescent versus unstained cells under the microscope. Although the microscope has certain very definite advantages over the flow cytometer, two advantages it does not have are those of speed and statistical reliability. Particularly as a result of their work load from the growing number of AIDS patients, haematologists' need for a way to count statistically reliable

numbers of cells from large numbers of patients became increasingly urgent. Flow cytometry is the obvious answer to this need.

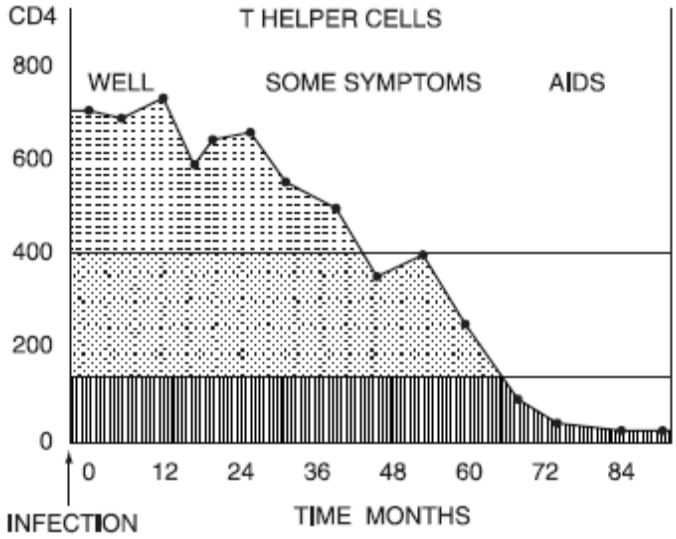

Fig- 10. The number of T-helper (CD4-positive) lymphocytes ($\times 10^3/cm^3$) in peripheral blood of a patient after infection with HIV. Cells <400 are significantly below the normal range; <100 indicates severe risk of clinical AIDS. Courtesy of LeÂonie Walker.

Erythrocytes and Platelets

Although the initial perceived need in the haematology laboratory for a flow cytometer was to aid in the rapid processing of samples from leukemic and HIV-positive patients, the presence of the cytometer has stimulated thought about new haematological applications. Although not yet in routine use for analyzing erythrocytes, flow cytometers have been shown to be useful for looking at red-cell-bound immunoglobulin (as a result of autoimmune disease, sickle cell anaemia, and thalassemia): The bound immunoglobulin on erythrocytes is detected by the use of fluorescent antibodies against human immunoglobulin. The staining of red cells for RNA content with a dye called thiazole orange has made possible the use of flow cytometry to count the reticulocytes (immature erythrocytes) present in blood samples from anaemic patients. Haematologists have also extended the use of flow analysis to platelets-those particles with low forward scatter that usually are ignored in flow cytometric applications because they fall well below the standard forward scatter threshold. Immunoglobulin bound to platelets can be measured with antibodies against human immunoglobulin (as in the detection of immunoglobulin on erythrocytes).

Platelet-associated immunoglobulin (resulting from autoimmune disease) is determined by analyzing the patient's platelets in their natural state. In another clinical situation, antibodies in the serum with specificities for ``foreign'' platelets may develop after pregnancy or transfusions; these can be monitored by flow cytometry if a patient's serum is incubated with a donor's platelets. Recently, the activation state of platelets has also been analyzed. Hyperactive platelets express P-selectin (CD62) on their surface; they have been primed to facilitate coagulation. Although not yet applied in routine diagnosis, research indicates that expression of CD62 may provide prognostic information in myocardial infarction and cardiopulmonary bypass surgery.

Cancer

Dioxin is a compound that is known to damage the immune system which in turn suppresses the response to a tumor in the body. The effect of this compound can be easily and accurately monitored using flow cytometry. Using a mouse as a subject and counting their T cells, we can compare a healthy specimen with one that has been injected with tumor cells. Specifically cytotoxic T-lymphocytes (CTLs) are being examined. The healthy mouse (low level of CTL) and the cancerous mouse (high level of CTL) are used as positive and negative controls in this experiment. When a mouse that was exposed to dioxin 24 hours before being injected with tumor cells is examined, a marked decrease in immune response is observed. In fact, their CTL level is only slightly higher than that of the healthy mouse.

9. Analysis of Protein.

Although many applications of flow cytometry involve the staining of cells for proteins expressed on the outer membrane, cells also have many proteins that are not displayed on their surface. With appropriate procedures, flow cytometry can provide a means to analyze these intracellular proteins. The outer cell membrane is impermeable to large molecules like antibodies; however, if we intentionally fix cells to stabilize proteins and then disrupt the outer membrane, the cells can be stained with fluorochrome-conjugated monoclonal antibodies against intracellular proteins. After time to allow the antibodies to pass through the now-permeabilized membrane, the cells are washed to remove loosely bound antibodies and then are run through the flow cytometer to measure their fluorescence intensity.

This intensity should, under good conditions, be related to the amount of the intracellular protein present. Dead cells have leaky outer membranes; they often show high nonspecific staining because antibodies get through the disrupted membrane and become trapped in the intracellular spaces. Therein lies a conflict in our ability to stain cells for intracellular proteins. Because antibodies of all types are easily trapped in the cytoplasm, there is greater potential for nonspecific staining of permeabilized cells than intact cells. The very procedure that we carry out to give access of the staining antibody to its target (intracellular) antigen actually increases the access of all antibodies to nonspecific targets. To lower this nonspecific background, antibody titers are critical and washing steps are important. Unfortunately, even with low antibody concentrations and careful washing, background fluorescence from isotype-control antibodies is often considerably higher on permeabilized than on intact cells. There is, in addition, a second problem. The procedures used for fixing and permeabilizing cells-to give the staining antibodies access to intracellular proteins-can modify or solubilise some antigens, thus destroying the stainability of the very proteins that are being assayed. To make matters worse, the protocol that works best for one antigen may entirely destroy a different antigen. This should not be surprising after consideration that ``intracellular'' includes proteins of many types and in many different environments. Some intracellular proteins are soluble, some are bound to organelle membranes, and some are in the nucleus. Therefore, methods for staining cells for intracellular proteins cannot be as standard or as dependable as the methods for staining surface proteins. They have to be individually optimized for the cells and the proteins in question.

The general protocol for intracellular staining involves, first, staining the cells for any surface (outer membrane) antigens. Then the surface proteins with their bound antibodies, as well as the intracellular proteins, are fixed gently to stabilize them. The purpose of the fixation is to cross-link the proteins well enough that they are not removed or washed out of the cells after

the cells are permeabilized, but not so well that the intracellular antibody binding sites are masked or destroyed. Although ethanol and methanol can be used for fixation (by themselves or following another fixative), the most common fixative used prior to intracellular staining is formaldehyde. Formaldehyde is generally used at lower concentration and/or for a shorter period of time than for routine fixation of surface-stained cells (where fixation overnight in 1% formaldehyde is the [optional] last step of the procedure before flow cytometric analysis).

Formaldehyde (at 0.5-1.0%) for 10 min is a good suggested concentration and time for cell fixation, but lower or higher concentrations, for shorter or longer periods of time, might be required. This formaldehyde fixation does permeabilize the cytoplasmic membrane a bit (formaldehyde-fixed cells are permeable to small molecules), but proteins are often cross-linked too tightly for staining of intracellular proteins with antibodies. Therefore the fixation step is followed by a permeabilization step. Permeabilizing agents are usually detergents, such as Triton X-100, digitonin, NP40, or saponin, at concentrations of about 0.1%. Combined fixation/permeabilization reagents are also available as proprietary commercial reagents. With luck, the detergent will open up the cell enough so that the now-fixed proteins are accessible to the antibodies used for staining. It is first necessary to maximize the fluorescence intensity of cells that are known to possess the intracellular antigen (the positive control); fixation time and concentration need to be altered in combination with different detergent concentrations to increase the positive staining. It is then necessary to decrease the background staining (using cells stained with isotype-control antibodies) as much as possible; this is done by trying increasing detergent concentrations and washing the cells thoroughly in buffer that contains the detergent. In other words, the goal is to increase the signal-to-noise ratio.

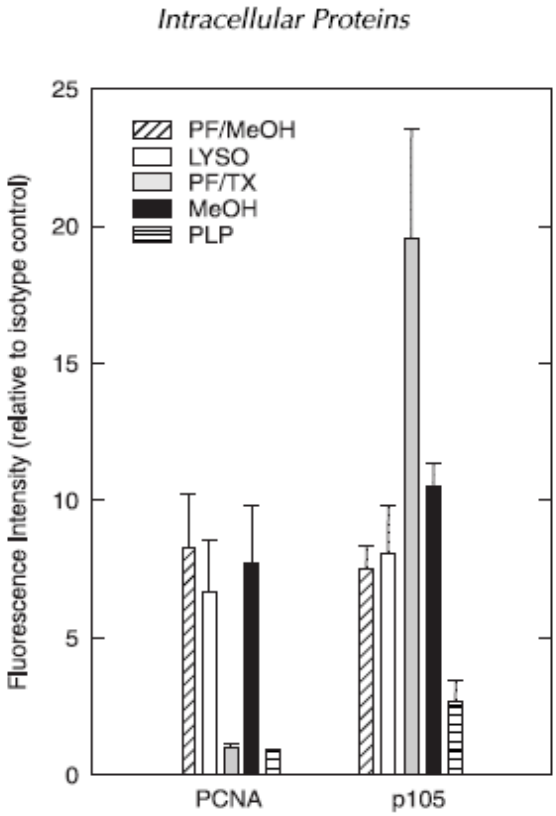

Fig- 11. The effects of different fixation protocols on the relative amounts detected of two different intracellular proteins. Modified from A McNally and KD Bauer as published in Bauer and Jacobberger (1994).

EXAMPLES OF INTRACELLULAR STAINING

From the point of view of a flow cytometer, surface, cytoplasmic, and nuclear proteins are similar. The flow cytometer cannot ascertain the location of the source of fluorescence. In addition, the nuclear membrane has large enough pores that it provides little or no obstacle to staining once the outer, cytoplasmic membrane has been breached. Cells have been stained successfully for nuclear proteins related to proliferation (for example, PCNA, Ki-67, and various cyclins, which will be discussed in the chapter on DNA) and to tumor suppression (for example, p53, c-myc, and the retinoblastoma gene product).

They have also been stained for proteins bound to interior membrane surfaces (e.g., Bcl-2, multidrug resistance protein [MDR] and P-glycoprotein), and many strictly cytosolic proteins have been analyzed (like tubulin, haemoglobin, surface proteins that exist intracellularly at various stages of differentiation, and many cytokines). As an example of one of the more complex biological situations, we can use the staining of cytokines as an illustration. Cytokines are a diverse class of proteins that, in response to cell stimulation, are synthesized and then secreted by leukocytes. For example, when T lymphocytes are stimulated, either non specifically or by immunological triggers, they begin to synthesize interferon-γ in their endoplasmic reticulum, send the proteins to the Golgi apparatus, and then secrete the molecules into the environment for stimulation of neighbouring cells. To stain for intracellular interferon-γ, the usual technique is to stimulate cells with a biological trigger and then to incubate them with an inhibitor (brefeldin A or monensin) for several hours. These inhibitors block the normal secretion of proteins from the Golgi apparatus and thus allow the cytokine concentration to build up in the cell to levels that are detectable. After the incubation period, the cells are stained for any surface antigens of interest, fixed briefly in formaldehyde, permeabilized with saponin, and, finally, stained with a monoclonal antibody against interferon-g.

Fig-12 shows an example of the way in which cells can be stained for a phenotypic surface marker (CD8) as well as the intracellular cytokine, interferon-γ. The flow data indicate that interferon γ is associated, after PMA-ionomycin stimulation, primarily with CD8-negative cells. More of the CD8-negative than the CD8-positive cells have intracellular interferon- γ, and those that have that cytokine have more of it per cell. The tricks in the procedure for staining intracellular cytokines are as much biological as chemical (because the stain is for the end result of a functional process). In addition to a knowledge of how to fix and permeabilize a cell and how to avoid nonspecific staining, we require knowledge of how to trigger the cytokine production, knowledge of the time course of cytokine synthesis with brefeldin A or monensin inhibition so that they build up large amounts of easily detectable cytokines but do not burst from this direct treatment.

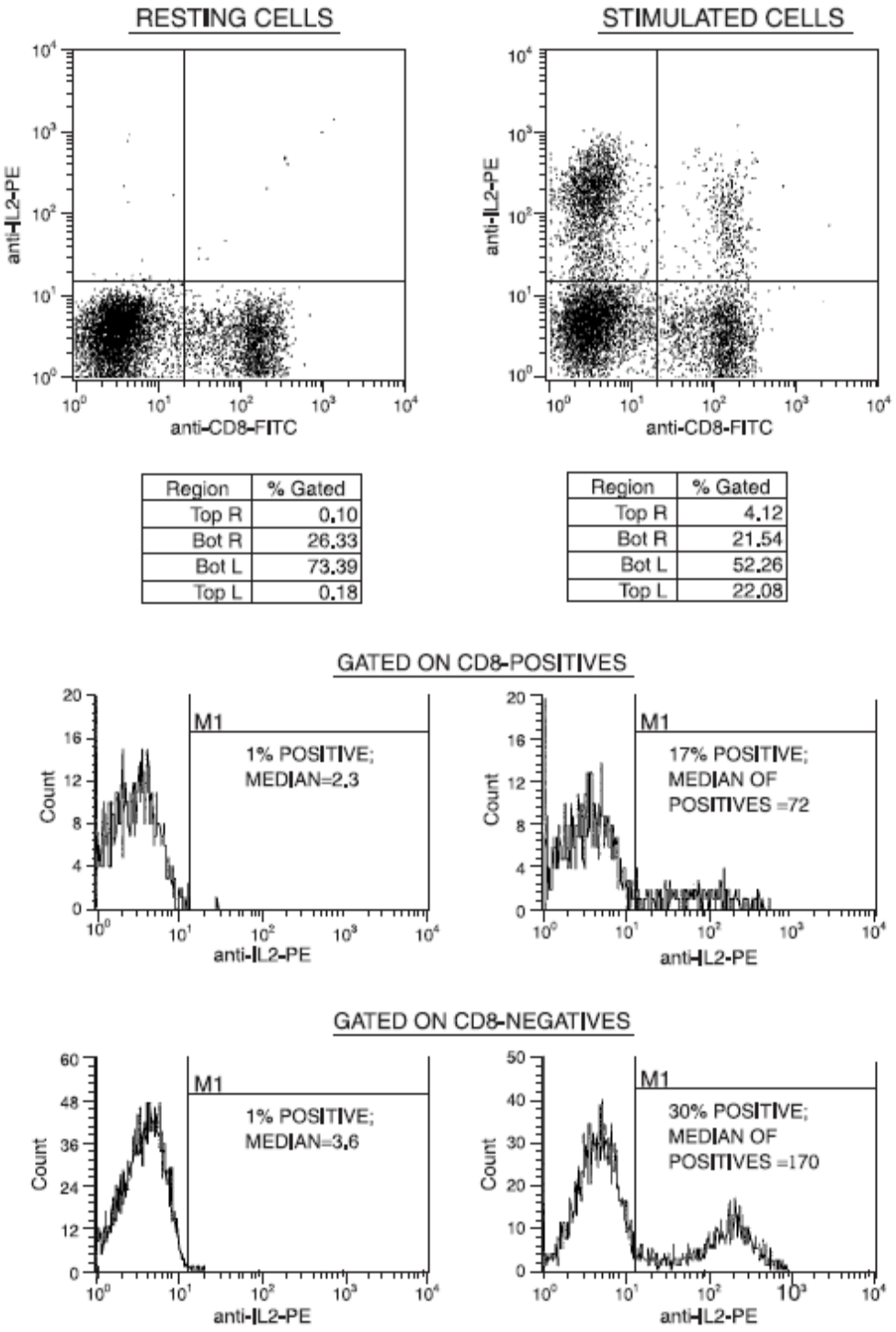

Fig.-12. Dot plots showing the staining of lymphocytes for intracellular interferon-γ in conjunction with an outer membrane stain (against CD8) to phenotype the cytokine-producing cells. Cells were stained for CD8 and then fixed with formaldehyde and permeabilized with saponin. The stimulus was PMA-ionomycin. Data courtesy of Paul Wallace.

Flow Cytometry Analysis of HER2 Expression

Fluorescence staining is a powerful analytical tool used to determine the expression and distribution pattern of a particular protein in cells and tissues. With flow cytometry, analysis

of several protein markers can be performed simultaneously using different types of non overlapping fluorescent dyes, for example to perform population dynamics studies.

The Anti-HER2 Affibody molecule is a highly specific affinity ligand that advantageously can be used for flow cytometry as a robust alternative to antibodies. The Anti-HER2 Affibody molecule is available as a biotin conjugated reagent for staining with streptavidin fluorescent dye or as a fluorescein conjugated reagent, which functions as a convenient one step reagent. The Anti-HER2 Affibody molecule is also available as an unconjugated reagent that is easily coupled to any thiolactivated fluorescent dye.

10. Organ Transplantation

Flow cytometry has been used in solid organ transplantation since 1983. Since that time there have been numerous technological advancements in this field. This method has developed into the most sensitive test available to detect anti-donor antibody. For that reason, many laboratories currently use flow cytometry to crossmatch the donor and the recipient. This technique has proven very useful in predicting graft failure in the initial stages and also rejection of the organ later on. This is done through an analysis of the patient's serum to locate certain antibodies that might react unfavourably with the donor tissue. Specifically, flow cytometry is used to detect alloantibodies that may be present in the recipient's system.

Future Applications

Because of constant changes and advancements, the flow cytometry becomes a powerful tool in the profiling and monitoring of many diseases. The Flowcytometry will be also in the water and food quality control industry. As advancements are made to detect microbial activity the use of flow cytometry will become more common for this application.

Interaction between carbon nanotubes and mammalian cells: characterization by flow cytometry and application

CNT–cell complexes are formed in the presence of a magnetic field. The complexes were analyzed by flow cytometry as a quantitative method for monitoring the physical interactions between CNTs and cells. There is an increase in side scattering signals, where the amplitude is proportional to the amount of CNTs that are associated with cells. Even after the formation of CNT–cell complexes, cell viability is not significantly decreased. The association between CNTs and cells is strong enough to be used for manipulating the complexes and there by conducting cell separation with magnetic force. In addition, the CNT–cell complexes are also utilized to facilitate electroporation. There is a time constant from CNT–cell complexes but not from cells alone, indicating a high level of pore formation in cell membranes. Experimentally, the expression of enhanced green fluorescence protein by using a low electroporation voltage after the formation of CNT–cell complexes. These results suggest that higher transfection efficiency, lower electroporation voltage, and miniaturized setup dimension of electroporation may be accomplished through the CNT strategy outlined herein.

Conclusions

Flow cytometry is a general method for rapidly analyzing large numbers of cells individually using light-scattering, fluorescence, and absorbence measurements. The power of this method lies both in the wide range of cellular parameters that can be determined and in the ability to obtain information on how these parameters are distributed in the cell population. Flow cytometric assays have been developed to determine both cellular characteristics such as size, membrane potential, and intracellular pH, and the levels of cellular components such as DNA, protein, surface receptors, and calcium. Measurements that reveal the distribution of these parameters in cell populations are important for biotechnology, because they better describe the population than the average values obtained from traditional techniques.

Medical and clinical applications of flow cytometry still account for the vast majority of application of this technique, but during the past few years it has also become a valuable tool in biology, pharmacology, toxicology, bacteriology, virology, environmental sciences, and bioprocess monitoring. The recent success of flow cytometry is based on commercially available flow cytometry equipment that is both robust and versatile, together with modern data acquisition and interpretation software, and tremendous successes in the development of various specific staining assays.

Even though, flow cytometer is uneconomical, from application point of view it is cost effective.

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