Bio-medical applications of Flow Cytometry.

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Part I.

Introduction.

I.i) What is Flow Cytometry?

Flow Cytometry (FCM) can be defined as: the measurement of physical and/or chemical characteristics of cells, while they are passing, preferably in single file, through the measuring apparatus in a fluid stream. Such measurements can include optical parameters, such as light-scattering as well as fluorescence from specifically-targeted probes after appropriate excitation, usually by passage through a laser beam.

Advantages of FCM: speed (typically 300 - 3,000 cells or nuclei per second); high degree of statistical precision; multiple parameters can be measured simultaneously; measurements can be made on archival material, as well as on fresh tissue.

Disadvantages of FCM: relative expense (especially if UV laser excitation is required); tissue disaggregation is required, which may cause loss of tissue morphology; a minimum number of cells (usually around 10^5) is required;

I.ii) Brief overview.

Flow Cytometry has been around since the early 1970s, becoming an important clinical and research tool in medicine. The original aims of analytical cytometry were for the automated classification of cancer cells and a great deal of work was carried out in the 1940-60s to achieve these aims. Unfortunately, the problems of automated classification were far too difficult for the capabilities of either the available technology, or the understanding of biology at that time. Presently however, flow cytometry has become a well used and well understood science.

I.iii) Historical context.

Quantitative cytology and therefore Flow Cytometry, began with Caspersson *et.al.* during the 1930-'50s, which together with the work of others pioneered the use of absorption measurements for the study of individual cellular components. In 1956 Coulter developed his instrument for electronic cell counting and sizing, measured on a single cell basis as they flowed past a measuring point in a conductive liquid. Later, in 1965 Kamentsky *et.al.* described the first two-parameter flow cytometer, which was used to determine cell nucleic acid content and size, while in the same year Fulwyler described the first cell sorter. By 1967 Van Dilla *et.al.* were preparing highly purified (>95%) human granulocytes and lymphocytes. However, it is only recently that technological advances (eg. monoclonal antibodies, powerful and cheap computers) have brought flow cytometry into routine use, enhancing greatly the diagnosis and management of various diseases, as well as providing new understanding of their pathogenesis.

I.iv) Basic principles of Flow Cytometry.

The instrument operates by causing cells in a fluid stream (usually PBS) to pass in single file through a beam of light, usually generated by a laser, or in some cases by an arc-lamp. This is achieved by means of hydrodynamic focusing, where the cell suspension is introduced into the centre of an accelerating "sheath" of flowing fluid. The photons of light are scattered from the cells in all directions, but usually are collected in the "forward-angle" (ie. along the axis of the incident beam) and "right-angle" (otherwise known as "90^{o}-, side-, or orthogonal-scatter", ie. perpendicular to the beam) directions, together with fluorescent light emitted by specifically-targeted fluorochromes used to stain the cells, are separated into constitutive wavelengths by a series of filters and mirrors. This separated light is directed on to individual detectors (usually photo-multiplier tubes [PMTs], or photo-diodes) which generate electrical impulses, or analogue signals, proportional to the intensity of the light, which then is converted to a digital signal for collection, display and subsequent analysis by a computer.

I.v) Measured parameters.

Forward angle light scatter: this signal pulse is caused by refraction and diffraction from the cell, thus being related to the size and shape of the individual cells, but is not an absolute measurement of either. It can be used so to discriminate cell sub-populations, eg. lymphocytes from monocytes in blood.

Right angle (90^{o}, side, orthogonal) light scatter: this signal pulse is caused by reflection and refraction from the cell contents, being related to the complexity, or "granularity" of the cell. Again it can be utilised to discriminate sub-populations, eg. lymphocytes from granulocytes.

Fluorescence: cells may be labelled with fluorochromes which can be linked to antibodies (used to label specific cell surface, or cytoplasmic markers, eg. antigens), or stained with fluorescent membrane, cytoplasmic, or nuclear dyes. Light is absorbed by the fluorochrome and re-emitted at a lower energy of a longer wavelength. By this means, differentiation of cell types, presence of receptors and antigens, viability, membrane potential, pH, enzyme activity and nucleic acids may be achieved.

I.vi) Fluorescent probes.

The choice of suitable fluorochromes, or combinations thereof, is determined by a number of factors, including:-

(a) The available excitation light wavelength(s). Lasers can emit at a number of specific lines, determined by the type of laser in use and related to the plasma tube gas. The most common lasers for FCM are argon ion, which are run most often at 488nm. (they have emission lines also at: 515, 457, 465, 472, 496 and 501nm.), but additionally can emit in the U.V. at 351 and 363nm. for use with U.V.-excited probes, but such lasers can be expensive. Other lasers used in FCM include helium-neon (633, 543, 594 and 611nm.) and helium-cadmium (441 and 325nm.).

(b) The emission wavelengths of the fluorochromes and the available detection filter systems. Fluorescent light emitted from the stained cells is collected by lenses, then separated into its constituent wavelengths by combinations of dichroic mirror and optical filter (short-, long- and band-pass) systems, for direction to specific detectors. With multiple colour fluorescence measurements, any spectral overlap between

emissions of the different fluorochromes can be subtracted using electronic compensation circuitry.

(c) With antibodies: is the fluorochrome available conjugated directly to the antibody in question, or conjugated to a second reagent which can bind specifically to the primary antibody, as part of an indirect assay? Such second indirect reagents include antibodies which recognise only the primary antibody, or another molecule conjugated to it, such as binding of a streptavidin/avidin second reagent to a biotinylated primary antibody. Direct assays are faster, whereas indirect assays result in brighter fluorescence (staining is amplified because more fluorochrome molecules can be linked per target molecule) and thus may be preferable for low density markers.

(d) The location of the target: eg. on the cell surface, or within the cytoplasm. It may be difficult for some larger (reagent) molecules to penetrate the cell membrane.

(e) The method of any cell fixation and/or permeabilisation. Fixation kills the cells, rendering potentially bio-hazardous material safer to handle and enables stained cells to be stored in a stable condition. However, this prevents any determination of cellular viability (eg. using PI) and hence exclusion of dead cells from analysis, of course! Permeabilisation enables reagents to enter cells, for intra-cellular staining. Methods include use of: aldehydes (eg. paraformaldehyde, glutaraldehyde), which cross-link stained surface antigens (often better for immuno-fluorescence applications) and permeabilise the membrane; alcohols (eg. ethanol, methanol), employed more often for DNA analysis; combinations of these two methods; nuclear extraction with hypotonic media plus non-ionic detergents (eg. Triton X-100, NP-40), generally used also for DNA analysis.

(f) The specific characteristic(s) of the cell which it is desired to measure.

For example:-

- Fluorochromes in common use for covalent conjugation to antibodies, lectins and ligands include: fluorescein isothiocyanate (FITC), phycoerithrin (PE), allophycocyanin (APC), tetramethylrhodamine (TRITC), Texas red.
- DNA and/or RNA: Propidium Iodide (PI), Ethidium Bromide (EB), Acridine Orange (AO), 7-Amino-Actinomycin-D (7AAD), Mithramycin, Chromomycin, Thiazole Orange, TOTO, YOYO, DAPI, Hoechst 33342 and 33258.
- Total protein: FITC, TRITC, etc.
- Viability: PI, 7AAD, etc.
- Calcium flux: Indo-1, Fura-2, Fluo-3, etc.
- Membrane potential: DiOC5, DiOC6, etc.
- Intracellular pH: BCECF, SNARF, SNAFL, etc.
- Enzyme activity: fluorescein diacetate (FDA), NMA, DDF, etc.

I.vii) Monoclonal antibodies (Mabs).

Since Kohler and Milstein (1975) introduced a method for generating hybrid cell clones which could produce mono-specific immuno-globulins in hight titres, multitudes of antibodies have been produced. These have been classified into groups, based on their cellular reactivity, which have been termed Clusters of Differentiation, or CDs. New CDs are being added and existing classifications redefined, by international workshops. Examples of some common clinical CD applications include:-

- T-cells: CD3, 4, 8, 2.
- B-cells: CD19, 20.
- Others: CD11b (MAC-1: adhesion glycoprotein), CD54 (ICAM-1 intercellular adhesion molecule), CD45 (LCA), CD16 (FcR-III), etc.

Part II.

Clinical applications of Flow Cytometry.

A wide variety of clinical assays have been adapted for analysis by FCM, the number and variety of which are increasing constantly. Examples of the major categories are described below.

II.i) Immuno-phenotyping.

Surface and cytoplasmic marker analysis: These represent the most common type of clinical flow cytometric assay and may be performed using either direct, or indirect immuno-fluorescence techniques (see above). These usually involve staining lymphoid, or myeloid cells with monoclonal antibodies, often for the enumeration of lymphocyte subsets (eg. T-helper/suppressor ratios, disappearing subsets in AIDS patients, etc.), detection of activated subsets, phenotyping leukaemias and lymphomas. Cytoplasmic marker assays also require fixation, or permeabilisation of the cells, to allow penetration of the antibody to the target.

T-cell subset analysis. This is a major area of clinical work with immuno-fluorescence FCM, with much of the early work involved with the determination of B- to T-cell ratios and/or helper/inducer T-cells to cytotoxic/suppressor T-cells in various disorders of immune function, such as auto-immune diseases and immuno-deficiency syndromes. With AIDS patients, the proportion and counting of CD4-positive T-cells in peripheral blood is useful in following and predicting the clinical course of HIV infection. Electronic gating on light scatter to identify lymphocytes for the purposes of immuno-fluorescence measurement on lysed whole blood, together with the use of *back-gating* to confirm the purity of that gate and the fraction of the lymphocytes included, forms the basis of the clinical guidelines and standards issued by the NCCLS, CDC and DAIDS regulatory authorities here. Modern routine use of three-colour fluorescence analysis, using CD45/CD3/CD4 allows for the specific identification of lymphocytes in a single tube, with the addition of CD8 as a fourth colour enables all of the major T-cell subsets thus to be determined.

Lymphocyte activation. This can be of importance for clinical applications such as tissue typing and organ matching for transplantation, evaluation of cellular immune response, assessement of auto-immune disease processes' activity and monitoring of transplant recipients for early signs of rejection. There are a number of successive events, associated with T-cell activation, which can be monitored by FCM, including: Calcium ion flux; pH change; inactivation of a glycoprotein pump; oxidative metabolism changes;

membrane potential changes; appearance of activation antigens (CD69, CD98 [4F2], CD25 [IL2R], CD71 [TfR], HLA-DR); insulin receptors; RNA synthesis; DNA synthesis and finally, cell division.

Leukaemia phenotyping. Multi-parameter FCM can be applied to the determination of myeloid leukocyte differentiation patterns in normal and leukaemic bone marrow. Early leukocyte precursors, identified by their light scatter, CD34 and CD38 antigen expression, show little variation. However, bone marrow from acute myeloid leukaemia (AML) patients show patterns which are not indentical, but can be characterised by the appearance of leukaemic cells in regions where normal marrow have few, or no cells. Analysis of changes in expression of CD34, CD11b and CD15 antigens with myeloid maturation show abnormal patterns of progression in AML patients compared with normal marrow, but again no two patients show the same pattern.

II.ii) DNA content analysis.

Another major flow cytometric assay, usually to determine the ploidy of malignancies, or analysis of the cell division cycle to examine growth rate (eg. S-phase fraction, doubling time, mitotic index, etc.). Precision is required because the amount of (eg.) DNA is constant for most cells and small measured variations can have major biological significance. This can be assessed from calculation of the Coefficient of Variation (CV=Standard Deviation/Mean) for data histogram peaks. Adverse influences can include debris and cell doublets (ie. two G0/G1 cells stuck together have the same total DNA content as a single G2/M cell!). Doublet discrimination (electronic "pulse processing") can be used to eliminate spurious data which may arise from cell doublets, by comparison of peak (height or width) and integral (ie. area) values of detected fluorescence signal pulses. Cell permeabilisation (eg. using hypotonic media and non-ionic detergents such as Triton X-100, or NP40), or fixation (eg. using alcohols, Paraformaldehyde, or Glutaraldehyde) prior to staining usually is necessary in order to allow dye molecules (eg. PI, 7AAD) to pass through the cell membrane and enter cells.

Analysis of the **Cell Division Cycle**: Deconvolution of DNA data histograms:-
[Gap 0 --> Gap 1 (2c) --> Synthesis --> Gap 2 (4c) --> Mitosis --> etc.]

Most methods now employ increasingly sophisticated and automated computer software packages (eg. CellFit, ModFit, MultiCycle, etc.) for rapid DNA Cell Cycle analysis. Problems encountered during computer analysis of the DNA Cell Cycle include interference from cell debris and from cell aggregates, separations of G1/G0-phases from early S-phase and of G2/M-phases from late S-phase. Assumptions commonly made by analysis software models are that G1- and G2-phases have Gaussian distributions and S-phase has a polynomial distribution.

Bromodeoxyuridine (BrdUrd, Budr, BrUdR, BrdU, etc.). This is not actually a nucleic acid stain, but a Thymidine nucleotide analogue, often used in conjunction with DNA dyes (after incorporation of BrdU during S-phase, which then is labelled usually with fluorescent antibodies) to stain total DNA and to highlight the S-phase fraction. BrdU substitution into DNA during synthesis also quenches subsequent Hoechst dye

fluorescence and when used in combination with total DNA (eg. PI) staining, can allow multiple successive rounds of cell cycle phases to be resolved and measured.

Ploidy. The significance of this can be considerable. Often tumours showing aneuploidy and/or high S-phase fraction, may have significantly poorer clinical prognosis. Measurement of ploidy requires that DNA staining be stoichiometric and allows the DNA Index (DI=ratio of mean or modal fluorescence channel numbers of G0/G1 peaks in the distributions from test and reference cells) to be determined, revealing any cells with abnormal DNA karyotypes (ie. aneuploid).

Analysis of Apoptosis ("programmed cell death"). In apoptosis, but not necrosis, activation of a nuclease results in DNA fragmentation and this low molecular weight DNA is lost after cell permeabilisation, producing a hypodiploid peak when labelled with DNA-specific fluorochromes (eg. PI). Apoptotic cells further can be resolved from necrotic cells by pre-treatment with a dye such as 7AAD, which will penetrate the compromised membrane of necrotic cells and stain their DNA, prior to treatment with Hoechst dye. DNA fragmentation can be detected directly using nick-translation or terminal deoxynucleotidyl transferase (TdT) assays, in which fluorescently-labelled dUTP is incorporated into DNA at the sites of strand breaks.

Chromosome analysis, sorting and Flow Karyotyping. Chromosomes can be labelled with either a single dye (eg. PI) for monovariant analysis, or dual dyes (eg. Hoechst and Chromomycin A3, see above) for bivariant analysis and subsequently sorted on the basis of their Flow Karyotype (eg. 18 out of the 24 human chromosome types can be resolved now). This can be used for such potential applications as: investigation of inherited and other genetic disorders, the generation of chromosome libraries, the purification of chromosomes for research, molecular biology and possible gene therapy.

Tumours (or any soft tissue). Preparation of single-cell suspensions from tissues may be minimal, involving only hypotonic lysis of erythrocytes (eg. leukaemias), or a few passes through a syringe may be sufficient (eg. lymph node cells). Some solid tumours may require combinations of dissection (to remove connective tissue), scraping minced tissue through a wire screen and enzymatic treatment (eg. cells from breast, head, neck, lung, liver, testis, prostate, etc.). These are analysed using FCM usually for surface marker and DNA content studies, but also for determination of drug sensitivity by means of glutathione measurements, functional probes and antibodies to examine the cellular drug efflux pumps.

Archival material (eg. clinical specimens stored in paraffin wax blocks). These may require careful dewaxing and rehydration during preparation, prior to DNA content analysis.

Cell cycle-dependence of antigen expression. This can be determined using combined analysis of DNA, after prior (for cytoplasmic antigens), or subsequent (for surface antigens) permeabilisation and staining for other markers.

II.iii) Haematology.

Blood cell counting, sizing and differentials. Initially, this had to be performed manually, but the appearance of the Coulter Counter in the 1960s enabled red cell counting and sizing, as well as white cell counting (after selective lysis of red cells) to be accomplished rapidly, using fewer less highly-trained operators, with greater accuracy and precision. Using FCM, three-part (lymphocyte/monocyte/granulocyte) differential counts based on light scatter, and later, five-part differential counts based upon fluorescence staining to discriminate eosinophils and basophils from neutrophils, lead to rapid improvements in the technology. Major areas of application include: blood banking, detection of maternal/foetal haemorrhage in Rh-incompatible pregnancies, detection and quantification of specific cell-bound antibodies to platelets as well as other cell types in autoimmune cytopenias.

Functional and viability studies. Neutrophil function can be assessed using FCM of appropriate parameters, such as tetrazolium dye reduction; dichlorofluorescein oxidation; dihydrorhodamine 123; membrane potential probes; AO, or orthogonal light scatter measurement of degranulation. Phagocytosis assays can employ fluorescent microspheres or bacteria. Integrity and viability of stored platelets may be determined by dye exclusion or membrane potential measurements. Platelet activation can be determined from measurements of cytoplasmic Ca^{++}, activation antigens, or bound fibrinogen, while anti-platelet antibodies can be detected with fluorescent anti-immuno-globulins. Thrombopoiesis, as indicated by increased numbers of reticulated platelets in blood, can be detected by staining of immature platelets with thiazole orange and is the result of megakaryocyte activity in the bone marrow. The isolation of haematopoietic stem cells from bone marrow has received a consdiderable amount of effort, in connection with marrow transplantation for leukaemia treatment and multi-colour FCM (of eg. CD34, CD45/R/RO, CD33, HLA-DR, glycoprotein pump activity measured by rhodamine-123 retention) has been used to provide further detail on normal blood cell development.

Reticulocyte enumeration. These RNA-containing immature cells normally comprise only about one percent of circulating red blood cells and visual methods for manual enumeration can be tedious, as well as often inaccurate and unreliable. The first approaches to reticulocyte counting by FCM were based on the use of tricyclic RNA dyes (eg. AO), but this could be tricky because of high background fluorescence levels. An alternative method was based upon the identification of surface transferrin receptors, but more recent methods have utilised dyes (eg. thiazole orange) which increase fluorescence by up to several thousand-fold upon binding to RNA. These stain DNA also in nucleated cells, but in reticulocytes DNA should be absent and thus not present a problem. Some dedicated FCM-based counters also measure RNA using auramine-O fluorescence. Since reticulocytes lose RNA over time, less-mature cells will contain higher RNA levels, which enables a fluorescence-based *reticulocyte maturity index* (RMI) to be calculated. This has been found to be predictive of successful engraftment following marrow transplantation and of marrow recovery after intensive chemotherapy.

Organ transplantation. Studies have shown that cross-matching by FCM, where recipient serum is mixed with donor cells and anti-donor antibodies are detected by staining cells (eg. separated T-cells, which do not bear surface immuno-globulins normally) with a fluorescent anti-human immuno-globulin reagent, can be more sensitive than conventional methods, especially in high-risk recipients (eg. where grafts have failed previously). Cross-matches using two-colour FCM are used commonly now to identify T, B, or other specific donor cell types. The use of FCM for diagnosis of graft rejection has been reported, with analysis of lymphocyte activation and DNA synthesis in T-cell subpopulations (specifically: increases in S/G2/M-phase cells) being involved.

II.iv) Sperm analysis.

Assessement of sperm quality can involve correlation of microscopic observations of sperm mobility with measurements of dye exclusion and mitochondrial membrane potential (eg. using Rhodamine 123). Fertility potential can be related to chromatin integrity and measured by determination of chromatin structure, based on the principle that abnormal sperm chromatin has a greater susceptibility to physical induction of partial denaturation *in situ* (eg. from heat or acid treatment), can be made by measuring the metachromatic shift from green fluorescence of Acridine Orange (AO intercalated into dsDNA) to red (AO associated with ssDNA) and was found to be the most reliable. This technique has been termed the Sperm Chromatin Structure Assay (SCSA). Alternative techniques for investigation of infertility have involved detection and quantification of antibodies bound to sperm, as well as the DNA content of testicular aspirates and biopsies (sperm are detected readily by their haploid DNA content), while progress of the acrosomal reaction has been monitored with fluorescent antibodies and lectins. Since sperm become haploid (1c DNA content) during maturation, this process can be investigated. Spermatogenesis has been examined and the genotoxic effects of various agents described (eg. rat, mouse). Small differences in the relative DNA content of X- and Y-bearing sperm (eg. bovine), as well as X- and Y-chromosome fluorescent labelling, has been utilised for the separation by Flow-Sorting of female- and male-yielding sperm populations. However, the ethical questions surrounding the potential application in humans will not be considered here!

II.v) Clinical microbiology.

This has been regarded as an area in which FCM looks very promising (eg. for diagnostic bacteriology), but this has been only of limited success because of instrument sensitivity limitations and the sheer number of bacterial types involved (eg. in a urine specimen). Approaches which have been used to identify bacteria include: light scatter; 16S ribosomal RNA probes; DNA staining (eg. Hoechst 33342 + chromomycin-A3) on the basis of nucleotide base composition, or using ethidium bromide (EB); fluorogenic enzyme substrates (eg. FDA); membrane potential (eg. rhodamine-123, DiIC1). An FCM "Gram stain" can be performed by adding EDTA to some samples, which can permeabilise the outer membrane and allow penetration of an otherwise excluded dye. Sensitivity of micro-organisms to therapeutic antibiotics has been investigated also using FCM.

II.vi) Quality control and assurance.

For clinical work especially, it is vital that data obtained from FCM instruments be precise, accurate, consistent and reproducible. Instruments can be aligned optically and calibrated either with synthetic standard particles (eg. latex micro-beads), or with biological material (eg. cells, stained nuclei). Beads can be obtained in a range of precise sizes (narrow CVs) and with specific fluorescence emission ranges, for use as optical alignment standards. Reference standards for immunofluorescence measurements can employ beads with known numbers of bound fluorochrome molecules. Compensation standards are usually beads with mixtures of dyes, used to set up the instrument to compensate for fluorescence spectral overlap. Binding standards are beads which bind known numbers of antibodies and can serve as calibration standards for quantitative immuno-fluorescence measurements.

For DNA analysis, standardisation and quality control (Q/C) can be ensured with suitable DNA standard cells (eg. Chick RBCs, Trout RBCs), run either externally (ie. separately), or internally (ie. added to the test cells).

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