Analysis of Nucleic Acids using Flow Cytometry.

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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) History.

Early use of FCM for measurements of DNA: modified Feulgen technique - Van Dilla et.al. (in use by 1969); Ethylene Bromide (EB) - Dittrich and Goehde (1969); Propidium Iodide (PI) - Crissman and Steinkamp (1973), used in conjunction with Fluorescein Isothiocyanate (FITC) to label protein; Continuing evolution and development of new dyes: eg. AO, CA3, Hoechst, DAPI, 7AAD, TOTO, TOPRO, YOYO, YOPRO, etc.

I.iii) Applications.

Analysis of the **Cell Division Cycle**:-

[Gap 0 --> Gap 1 (2c) --> Synthesis --> Gap 2 (4c) --> Mitosis --> etc.]

Analysis of Kinetics: S-phase fraction (SPF); Doubling Time; Mitotic Index.

Measurement of DNA content: ploidy; DNA Index (DI); mini-chromosomes.

Analysis of Apoptosis ("programmed cell death"): hypodiploid DNA peak; enzyme (eg. TdT) assays; etc..

Use in combination with simultaneous analysis for other markers (eg. Antigens).

Fluorescence-Activated Cell Sorting (FACS) of eg. viable cells and chromosomes.

I.iv) Precision.

Required because the amount of eg. DNA is constant for most cells and small measured variations can have major biological significance. 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!).

I.v) Significance.

Can be considerable: eg. for the clinician, often tumours showing aneuploidy and/or high S-phase fraction, may have significantly poorer clinical prognosis.

II) **Methodology.**

II.i) Sample preparation.

"Garbage In - Garbage Out" principle applies! Cell preparation is crucial.

Simplest for homogenous cell suspensions.

Large amounts of debris, eg. from minced tissue or paraffin block preparations, can cause instrument blockages, as well as interfering with subsequent analysis of data. Often this can be overcome with modern computer data analysis software models.

II.ii) Cell Permeabilisation and Fixation.

Usually necessary in order to allow dye molecules to pass through the cell membrane and enter cells. *Permeabilisation:* eg. using hypotonic media and non-ionic detergents (eg. Triton X-100, NP40). *Fixation:* eg. using alcohols; Paraformaldehyde; Glutaraldehyde. Suggested fixation is with cold 70% Ethanol.

II.iii) Reagents, Dyes and Stains.

Propidium Iodide (PI) - used most commonly. Intercalates into ds-DNA and RNA, so RNAse must be added to samples for DNA measurement. Excess dye is added to ensure stoichiometry. Excitation with 488nm. Argon laser line, emission above 590nm. Can be used in combination with simultaneous FITC labelling, but emission spectrum overlaps Phyco-Erythrin (PE) emission (at 570-600nm.).

7-Amino-Actinomycin-D (7AAD) - relatively new dye. Excitation at 580nm. and emission at 660nm. so can be used in combination with FITC and PE labelling, but yields poorer quality data (higher CVs).

Acridine Orange (AO) - Intercalates into DNA, which then emits at 530nm. and binds electrostatically with RNA, which then emits at 640nm. enabling simultaneous DNA/RNA measurement. A disadvantage is that it stains tubing in the instrument!

Hoechst dyes: 33342 and 33258 - Bind electrostatically with DNA, preferentially to AT-rich (33342), or GC-rich (33258) regions. Excitation at 360nm.(ie. requiring a UV laser!) and emission at 460nm.. Can be used with live cells, eg. for Flow-Sorting while retaining cell viability and function.

Chromomycin-A3 - Complexes with DNA, preferentially to GC-rich regions. Excitation with 457nm. Argon laser line, emission at 555nm.. Can be used together with Hoechst dye (33342) for bivariate analysis of chromosomes, to enhance resolution on the grounds of base composition as well as DNA content: "Flow Karyotyping".

4'-6'-diaminido-2-phenylindole (DAPI) - Again, AT-specific and requiring UV-excitation, but known for yielding very high resolution data.

Cyanine dyes: Thiazole Orange, TOTO, YOYO, etc. - Range of newer dyes, with excitation by the Argon laser 488nm. line and emissions at 533 - 509nm. bands, depending on the specific dye.

Bromodeoxyuridine (BrdUrd, Budr, BrUdR, BrdU, etc.) - 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.

II.iv) Running DNA samples on the Flow Cytometer.

Alignment: instrument optics must be aligned precisely (suitable fluorescent particles can be used) to ensure high precision measurements.

Quality Control (Q/C.): standardisation and 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).

Gating: electronic "gates" on light scatter can be used to eliminate unwanted material (eg. debris) from the instrument's fluorescence measurements.

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.

Flow Sorting: *Viable cells:* Hoechst dyes can be used to label DNA in these, which then can be sorted on the basis of their DNA staining. *Chromosomes:* these 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: the generation of chromosome libraries, the purification of chromosomes for further research, molecular biology and possible gene therapy.

III) **Types of samples.**

The following are examples of sample types which can be stained successfully and analysed using FCM to give useful DNA data.

III.i) Cell culture. Single cell suspension cultures. eg. cell lines (eg. HeLa, HL-60, etc.); cultured cells from tumours, ascites, blood and bone marrow; hybridomas; genetically transfected cell lines; etc..

III.ii) 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.).

III.iii) Blood components. Preparation may include density gradient centrifugation (eg. Ficoll), red cells lysis and fixation, but it must be remembered that changes may occur in cell subset percentages and/or ratios due to the preparatory process

and any delays in preparation (eg. lymphocytes, monocytes, granulocytes, macrophages, bone marrow, joint fluids, CSF, lung exudates, etc.).

III.iv) Animal cells. Types which have been investigated using DNA analysis with FCM include: parasite-infected cells; mini-chromosomes in schistosome parasites; liver (eg. DNA ploidy levels can increase from 2N to 4N and 8N in older rats); hair follicles; fibroblasts; fish blood and fry; etc..

III.v) Chromosome preparations. Mitotic chromosomes can be isolated from plant and animal cells (eg. cell cultures, peripheral blood lymphocytes, amniocytes, bone marrow, somatic cells hybrids, etc.) with most, if not all, being resolved rapidly and quantitatively (eg. human, mouse, Chinese hamster, pig, etc.). Metaphase-arresting agents (eg. colcemid) may be used to enhance the fraction of mitotic cells, but mitotic indices as low as 5% have yielded usable samples. Isolation protocols include disruption of the plasma membrane, chromosome stabilisation using isolation buffers (eg. magnesium sulphate, polyamine) and DNA labelling with one or more fluorochromes.

III.vi) Sperm. Since these 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, may be utilised for the separation by Flow-Sorting of female- and male-yielding sperm populations. Fertility potential can be related to chromatin integrity and measured using the Sperm Chromatin Structure Assay (SCSA).

III.vii) Archival material. eg. clinical specimens stored in paraffin wax blocks, which require careful dewaxing and rehydration during preparation.

III.viii) Plant material. Analysis and especially sorting of such cells, which may be large (eg. protoplasts), may require some modification to FCM instruments. Other plant material which can be examined using FCM include pollen, subcellular organelles (eg. chloroplasts, mitochondria) and chromosomes, as well as measurement of fluorescence from chlorophyll and other natural pigments.

III.ix) Microbial cells. DNA analysis, plus detection and counting of bacteria, yeasts, algae, fungi and protozoa. For analysis of Gram-negative bacteria, the outer cell membrane requires permeabilisation for successful staining. Applications include fermentation process monitoring and quality control, (eg. to detect wild yeast infections in breweries), as well as food science and industrial microbiology (eg. purification of subpopulations with desired metabolic characteristics, following gene transfer).

III.x) Pharmacology and toxicology. For eg. analysis of interactions between drugs and cells' metabolism; drug uptake (eg. methotrexate); drug resistance; antibiotic sensitivity testing of micro-organisms, etc..

III.xi) Ecological monitoring. For eg. analysis of fresh and sea water quality, contamination and pollution.

IV) **Deconvolution of DNA Cell Cycle data histograms.**

IV.i) Methods. Most now employ increasingly sophisticated and automated computer software packages (eg. CellFit, ModFit, MultiCycle, etc.) for rapid DNA Cell Cycle analysis.

IV.ii) 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.

IV.iii) Assumptions. Commonly made by analysis software models: that G1- and G2-phases have Gaussian distributions and S-phase has a polynomial distribution.

V) **Specific examples of DNA analysis.**

V.i) Simple determination of the Cell Cycle. With many modern computer software packages, analysis of simple DNA data generally involves initial identification of the data histogram peak ranges representing G0/G1 and G2/M populations, as well as the internal DNA standard (if any) and the boundaries of any debris component. The next step is the selection of a suitable mathematical model appropriate to handle this category of data. The choice of a model is dependent upon such considerations as the origin of the sample (eg. fresh, frozen, paraffin wax block, etc.), the amount of background debris, the presence of aggregates, the ploidy status (eg. diploid, aneuploid, tetraploid, etc.), whether the sample has a diploid S-phase and/or a visible G2/M-phase population, plus any overlapping cell cycles and/or synchronised cell cycles. This is followed usually by calculation of initial results estimates from a rapid first pass analysis of the raw data and if all appears to be well, the selected model is executed and a full final analysis is performed. The resulting output is displayed usually as a histogram showing the fitted data, together with the associated analysis statistics. Some software may be capable of doing all of this automatically: scaling the data; finding peak positions; assigning ranges to the peaks; determining an appropriate model to use; performing the analysis and displaying the results, with the option of reversion to a manual mode should this be unsuccessful, or the data unsuitable for automated analysis.

V.ii) Analysis of Kinetics with BrdU. Incorporation of BrdU into the DNA of S-phase cells and its detection with fluorescent antibodies, allows the S-phase fraction to be visualised directly and that cohort of cells to be followed through the cell cycle with time, by sampling at various time points after BrdU labelling of the cells. This allows all of their cell cycle parameters to be determined, as well as S-phase cells to be distinguished readily from those in G1 and G2-phases.

Analysis of simultaneous quenching of subsequent Hoechst dye fluorescence resulting from BrdU incorporation, together with total DNA content from eg. PI, can enable the cell cycle phases for each of up to three successive cycles to be followed and their growth fraction, lag-time, compartment exit rate, compartment duration and compartment arrest to be assessed.

V.iii) 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).

V.iv) Analysis of Apoptosis. 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.

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

VI) **Advanced applications.**

VI.i) Fluorescence In-Situ Hybridisation (FISH): the application of specifically-synthesised and fluorescently-labelled probes (either DNA, RNA, or oligonucleotides) to target nucleic acids, leading to hybridisation and complementary binding in the specific locations where nucleotide sequences correspond. This enables the presence, absence, or location of specific target sequences to be detected. These can be visualised directly, as well as very elegantly, if analysed and viewed using a high resolution Confocal Fluorescence Microscope.

VI.ii) Sperm viability.

The separation of X- and Y-chromosome-bearing sperm would be of immense practical consequences for domestic animal breeders, but problems can arise from the levels of condensation of DNA in sperm heads and from the variety of sperm head shapes, many of them asymmetric, which can cause artifacts in measurements. Also, there have been doubts raised as to the desirability of insemination with sperm stained with eg. Hoechst fluorochromes. 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). 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.

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