Origins of...

Flow cytometry and applications in oncology

Walter Giaretti

Flow cytometry dates back to the synthesis of dyes in the late 1800s and to the fundamental work done in Stockholm by T Caspersson and colleagues.¹⁻⁴ They demonstrated, in 1938, that DNA content, measured by ultraviolet and visible light absorption in unstained cells, doubled during the cell cycle.⁴ Feulgen and Papanicolaou staining techniques were then in their infancy, and a few fluorescent dyes had been proposed for detection of cancer cells in cytology. However, it was not until 1950, when Coons and Kaplan reported on the improved detection of antigens using fluorescence antibody methods,⁵ that it was realised that fluorescence measurements could offer more advantages than absorption. Since then, fluorescein remains the most common label for quantitative immunofluorescence analysis, and now for routine flow cytometric applications in haematology and immunology.

In 1956 there was a breakthrough in flow cytometry when an apparatus was built by WH Coulter in which blood cells, in saline suspensions, passed one by one through a small orifice and were detected by changes of electrical impedance at the orifice.⁶ This instrument was the progenitor of the modern flow cytometers developed almost simultaneously in United States and Europe between 1965 and 1970.¹⁻³ Concomitant driving forces at the origin of such new instruments were the established principle of Caspersson and colleagues that cancer cells had higher DNA content than normal cells, the Coon's principle of cell recognition by immunofluorescent antibodies, and the immense increase in the use of computers in the early 1960s.

Flow cytometry was based mainly on light scattering and fluorescence and the first applications were measurement of nuclear DNA (using stoichiometric DNA stains) and cell surface antigens (using immunofluorescence). Methodologies and applications since then have increased tremendously.¹⁻³

Basic characteristics of instrumentation

The evolution of the instrumentation was not as fast as for the methods and applications. Early and most recent flow cytometers are quite similar, except for the fact that they have become increasingly computer controlled. Powerful general purpose computers assist today in optimising nearly all measurement conditions and allow automatic data storage, list-mode acquisition, graphic representations,

and often automatic analysis and final reports. Earlier instruments required manual intervention of skilled people at many steps of the measuring process.

The heart of a flow cytometer is a chamber in which the cell suspension fluid, commonly coaxial to an external fluid (sheath fluid), is driven to reach a laminar flow so that the cells are hydrodynamically focused to the centre of the stream. Incident light sources were originally arc lamps; the vast majority of flow cytometers today use laser light. One of the first flow cytometers designed to measure DNA content was based on a mercury arc lamp that provided good ultraviolet illumination. This choice remains most suitable after cell or nuclei staining with 4',6-diamidino-2-phenylindole-2HCl (DAPI).^{7,8} Arc lamp based flow cytometers commonly have an optical scheme similar to that of a fluorescence microscope under epi-illumination conditions to keep the illumination homogeneous at the focus through which the cells flow. These instruments use oil immersion optics with a numerical aperture of about 1.3 and are insensitive to the defocusing effect of the liquid stream or the shape and orientation of the cells in the flow. Most recent laser based flow cytometers offer the advantage of measuring scatter and absorption as well as fluorescence. In particular, intensities of scattered light in the forward and perpendicular directions, that correlate with size and internal structure of the cells or cell subunits, are commonly measured. Fluorescent light intensity of five or more colours can be measured simultaneously. Fluorescence emission at 90° with respect to the incident light is measured through an optical system of lenses, filters, and dichroic mirrors. Light signals are converted by photomultipliers into electronic signals which are amplified for analysis of area, peak, and duration. Each analogue parameter is then converted into an integer (analogue-digital conversion).

Monoparametric and multiparametric data are supervised in real time by a dedicated microcomputer, which also controls cell "gating" and "sorting". These two important properties are based on a predefined set of threshold criteria applied to the acquired real-time parameters to select and physically separate specific cell subpopulations. Cell sorting, in particular, is commonly obtained in laser based systems by breaking the liquid stream into droplets containing single cells, by selectively

Laboratory of
Biophysics and
Cytometry, National
Institute for Cancer
Research, Genoa, Italy

Correspondence to:
Dr W Giaretti,
Laboratory of Biophysics and
Cytometry, National
Institute for Cancer Research
(1st), Largo R Beni n. 10,
16132, Genova, Italy.

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charging the droplets with negative and positive charges according to the "sorting" criteria, and by deflecting them into separate cell collectors. Sorting rates of 5000 cells per second with a purity up to 99% can be obtained. Suitable cell viability after cell sorting for subculturing and functional studies have been reported.

Early and late applications

The earliest flow cytometric applications—measurement of DNA and surface antigens in haematology and immunology—remain the most common applications. However, the flow cytometric measurements of cell components other than DNA and the recent important sector of applications of cell function measurements are becoming increasingly important. In general, flow cytometers can potentially provide rapid, sensitive, and quantitative cytochemical measurements of any cell component that is specifically stained by specific fluorochromes and monoclonal antibodies. In addition, they may exploit properties of autofluorescence, scattering, and absorption. A great potential of flow cytometry is the simultaneous acquisition of multiple parameters corresponding to different cell compartments of the same cells. Suspensions of cells may enable—for example, simultaneous measurement of nuclear DNA, RNA, and cytoplasmic or nuclear proteins, as well as membrane antigens. The main aim of acquiring multiple parameters in flow cytometry is optimally to characterise heterogeneous cell populations. Cell sorting, in addition, enables purification and enrichment of homogenous cell populations which can then be investigated by other techniques.

Measurement of DNA content in human cancer

Benign and malignant human neoplasms often contain subclones with abnormal DNA content. Models of DNA content aneuploidisation and evolution have been proposed,^{9–10} but the precise mechanisms are poorly understood. Protocols for specific DNA content measurement in nuclei or cell suspensions by flow cytometry have been extensively reviewed.^{1–3 8 11 12} The DNA index (DI) that represents the degree of DNA aneuploidy relative to DNA diploid cells is a common parameter extracted from DNA histograms.¹³ Quality controls are extremely important; a recent consensus conference aimed to evaluate critical quality controls and clinical usefulness of flow cytometry measurement of DNA content in bladder cancer,¹⁴ breast cancer,¹⁵ colorectal cancer,¹⁶ neoplastic haematopathology,¹⁷ and prostate cancer.¹⁸

Before and during this consensus conference, several North American and European groups examined published studies. Controversies, such as those concerning the use of paraffin wax embedded material, according to the method of Hedley *et al*,¹⁹ were analysed. Overall, the methodological adequacy of most published studies (adequate criteria for patient and clinical parameters, adequate flow cytometric guidelines) was considered critical. Ret-

rospective studies of paraffin wax embedded material did not provide enough useful data because of the poor quality of measurements. New and more strict guidelines were necessary, using fresh-frozen material in large prospective studies and multiparameter flow cytometry and these were developed by the various groups.^{14–18} Overall, more significant biological insight and clinically useful information appeared necessary before more widespread application of DNA content flow cytometry could be considered appropriate or warranted.

To achieve a better understanding of the biological significance of DNA aneuploidy, several studies have recently tried to investigate flow cytometric DNA content in combination with molecular biology analysis of oncogenes and tumour suppressor genes.^{20–26}

Evaluation of proliferation, cell cycle kinetics, and apoptosis in human cancer

The fraction of cells in the cell cycle S phase (SPF) is a common parameter extracted from DNA histograms obtained by flow cytometry. SPF values may be evaluated according to different cell cycle models.^{27–28} For DNA aneuploid tumours, SPF may be evaluated relative to the DNA aneuploid cells. However, for DNA diploid tumours, SPF is commonly and imprecisely evaluated from total number of cells. The clinical usefulness of SPF was addressed by the same consensus conference referred to previously.^{14–18} Overall, it was found that a large body of literature supported an important association between SPF values and increased risk of recurrence and mortality for patients with cancer at the various sites examined. However, methodological problems for assessing SPF, because of the type of analysis, paraffin wax embedded material, and especially in DNA diploid and DNA aneuploid near-diploid subclones, were clearly recognised. Multiparameter flow cytometric analysis, including coupling with potentially relevant proliferation and differentiation associated antigens was recommended.^{14–18}

A relevant multiparameter flow cytometric analysis of proliferation was introduced after the development of a monoclonal antibody against bromodeoxyuridine (BrdUrd).^{29–32} Recent studies have demonstrated the feasibility of making routine flow cytometric measurements of detailed cell kinetics in human cancer, including the potential doubling time (Tpot) after administration of BrdUrd solutions to cancer patients.^{33–35} Tpot evaluation³⁶ appears promising in predicting local control in head and neck squamous cell carcinomas and to select radiotherapy regimens.³⁷ Tpot includes information about the quiescent cell cycle compartment but does not take into account the cell loss factor. Cell loss information in cancer, due to exfoliation, metastasis, necrosis, and apoptosis³⁸ would be most valuable for a better understanding of tumour biology and for optimising therapies. The usefulness of flow cytometry for the assessment of apoptosis in human cancer needs to be evaluated. Several flow cytometric techniques have been proposed.^{39–41} It is likely that the combination

of flow cytometry and image cytometry using the "Tunel" apoptosis method^{39, 42} and others may be relevant. Similarly, image and flow cytometry coupling may be relevant in the future with use of histological type specific proliferation and differentiation markers.

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