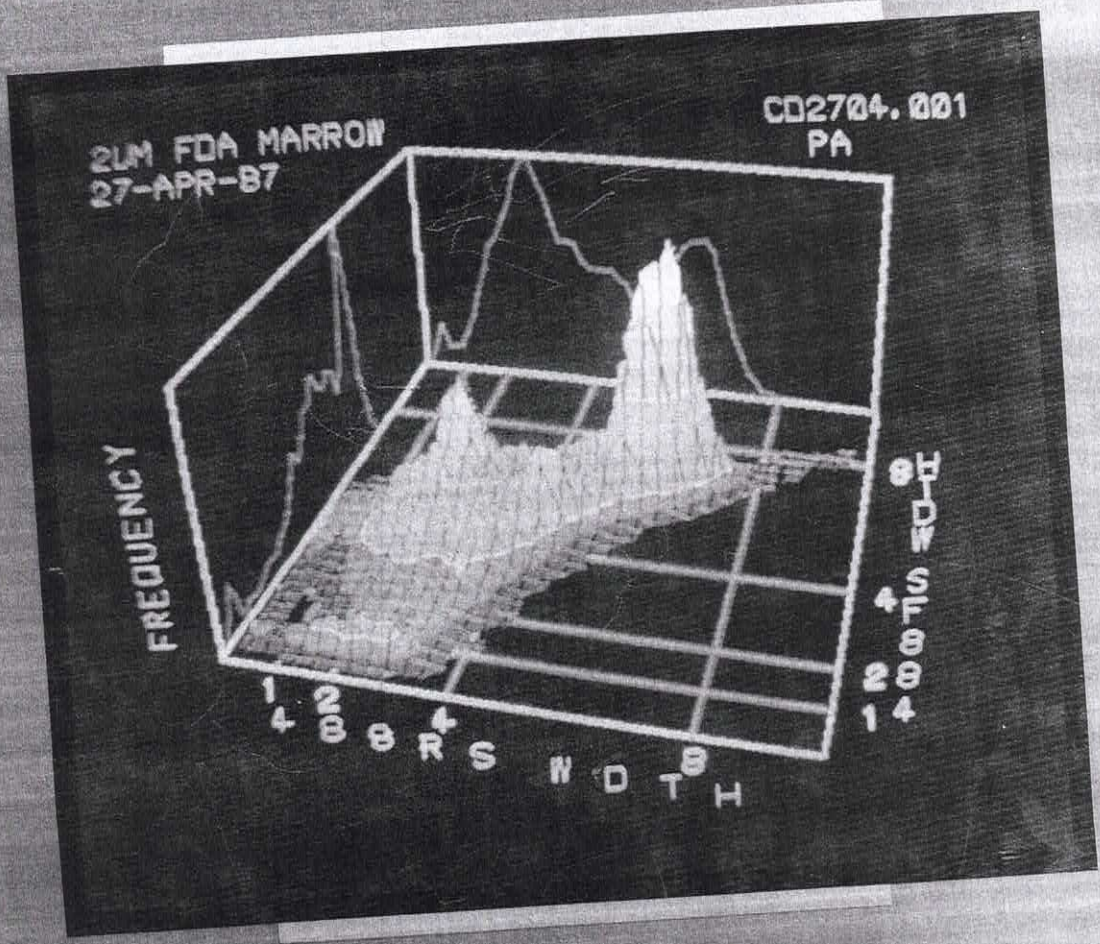
Introduction to Flow Cytometry



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Introduction

Classical histological methods of investigating cellular pathology involve characterizing morphological features using light absorbing dyes and fluorescent probes. The first category of stains gives rise to different colours in different subcellular constituents due to differential binding and hence differential absorption of transmitted light. Staining was used increasingly in microscopy after Virchow's work with various pigments including those from blood (Virchow, 1847) and the most extensively used example is the combination of haemotoxylin (introduced by Waldeyer, 1863) and eosin. The former is a basophillic blue dye which binds to nuclear components and the latter is acidophillic which binds to cytoplasmic constituents. Heamotoxylin appears blue to the human eye as it absorbs red light and eosin appears yellow/orange due to blue light absorption. Without the aid of this type of differential stain combination the histopathologist would hardly exist as details of the unstained cell are essentially invisible.

Immunoperoxidase staining of specific molecules using monoclonal antibodies (Köhler and Milstein, 1975) is an extension of this type of approach with the deposition of brown/black granules which absorb light of all wavelengths at a site where the antibody binds to its target molecule. This method, of course, is used in conjunction with other stain combinations to identify the site at which the antibody binds. With the orange/blue combination of eosin plus haemotoxylin as a counterstain for the immunoperoxidase we can locate the molecule of interest as being nuclear, cell surface or cytoplasmic.

Fluorescent antibody probes, introduced years ago (Coons, Creech and Jones, 1941; Coons and Kaplan, 1950), can also be used in this type of morphological study where different molecules or classes of molecule can be identified by using two different antibodies coupled to different fluorochromes which emit light at different wavelengths. One example is the use of fluorescein and rhodamine which are both excited by blue/green light and which emit fluorescence in the green/yellow and red wavelength bands respectively. Another example using fluorescence is the combination with propidium iodide which stains DNA and fluorescein isothiocyanate which stains proteins. Under the fluorescence microscope the nucleus is red and the cytoplasm is green. It is very difficult to combine fluorescence with absorption staining, which relies on light transmission, due to the different light intensities involved and differential absorption of wavelengths

which are necessary for fluorochrome excitation. Fluorescence methods now tend to rely exclusively on epi-excitation techniques.

Classical techniques are excellent for qualitative studies but it is very difficult to obtain quantitative information from individual cells by eye using the fluorescence microscope. It is possible to answer the question 'what proportion of the population is labelled with a given probe' by either immunoperoxidase or fluorescence techniques. However, it is virtually impossible to obtain reliable information about the quantity of that probe in, or on, a given cell except to score this as high, medium or low. Usually, however, all we can do is to give a score of positive or negative. The normal human eye has excellent wavelength discrimination (colour) but an almost total inability to quantitate objectively at a given wavelength.

Future developments in pathology and cellular physiology will include the precise quantitation of specific molecules in both normal and abnormal cells. These molecules may differ both qualitatively and quantitatively in diseased cells and with the advent of monoclonal antibody technology and oligonucleotide hybridization (Southern, 1975; Thomas, 1980) we have the capacity to discriminate very precisely between different molecular species at the DNA, RNA and protein levels. There will, however, be many pathological states which are due to quantitative changes of a given molecule or molecules and classical microscope techniques in conjunction with the human eye are not capable of making this type of discrimination reliably.

Flow cytometry is an investigational technique which is able to make multiple objective simultaneous measurements at the single cell level at rates of up to 5000 cells per second. The quantitative aspects of the technology take their origins from the work of Caspersson and colleagues in the 1930s where stained images were projected onto a wall and the amount of light absorbed in different areas of the images was quantitated with primitive photodetectors. Nucleic acid metabolism in *Drosophila melanogaster* salivary gland chromosomes was studied by banding pattern changes using this method (Caspersson and Schultz, 1938).

Apart from their analytical capability many instruments have the additional feature of electrostatic cell sorting which places individual cells with predetermined characteristics in a test tube for subsequent morphological identification or biological manipulation. The technology has a number of advantages and disadvantages. The former includes objective quantitation of specific molecules, statistical precision, multi-parametric cross correlated data analysis, distributional information and hence subset identification, dynamic measurements, sensitivity, speed and the generation of a vast amount of data. The disadvantages include loss of 'geographical' information from solid tissues as a single cell suspension is mandatory, absence of a direct visual record and the generation of vast amounts of data. The last of these is included under both headings as this is a two-edged sword. Data have to be converted to information. There is no merit in being the proud custodian of 40 000 MgBytes of data (the size of our data base on 1 December 1989) if the numbers are random and hence have no meaning. The

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conversion of data, particularly multi-parameter data, into information is a science in its own right and presents considerable problems.

It is pertinent at this point to ask why we should wish to make measurements on an individual cell basis and at such rapid rates. The answers are really quite simple. If we take a sample of tissue, homogenize it and perform a given assay we obtain a grand average for that sample. Let us suppose that the answer is 100 units. However, we do not know if half the cells in the sample have zero units and the other half has 200 units or if all cells have exactly 100 units each. The answer is 100 units for each scenario. Individual cell analysis, by whatever means, is the only method of resolving this problem and hence of obtaining reliable data in heterogeneous populations. That in itself is justification irrespective of the other advantages that flow cytometry offers which importantly includes statistical precision. It is possible to use a fluorescence microscope to determine the proportion of fluorescently labelled cells in a population but the precision of manual counting is highly dependent on the proportion of labelled cells and the number of cells you are prepared to count. If the labelled fraction constitutes only 5% of the population and you count a total of 200 you will, on average, score 10 positive cells. However, due to statistical factors this could be anything between 3 and 17 cells which gives a range of 1.5% to 8.5% at the 95% confidence interval. Hence, the ability to analyse and count large numbers of cells very rapidly has major advantages particularly for analysis of minority subsets. However, the various flow cytometry techniques should not be regarded as being able entirely to replace existing methods, they should be regarded as an adjunct although 'classical' techniques just cannot compete with the speed of flow technology. Moreover, there are some things you can do using flow cytometry that just could not be done in any other way.

The technique relies upon measuring both scattered light and fluorescence from suitably stained constituents in individual cells in the population. The stained cells are streamed single file in fluid suspension through the focus of a high-intensity light source. As each cell passes through the focus a flash of scattered and/or fluorescent light is emitted. This is collected by lens systems and filtered before reaching a photodetector which may be either a photomultiplier or a sold-state device. The photodetector quantitatively converts the light flash into an electronic signal which is digitized by an analogue-to-digital converter into a whole number (integer) which is then stored electronically.

The first commercial flow system that actually worked was the Coulter counter where impedence changes were measured as cells passed through a narrow capillary orifice (Coulter, 1956). This type of approach was extended in the early 1960s by Kamentsky with measurements of DNA by UV absorption and size by violet light scattering in attempts to automate cervical cytology (Kamentsky, Melamed and Derman, 1965). Kamentsky and Melamed (1967) also adapted their instrument as a fluidic cell sorter but, it was Fulwyler (1965) at Los Alamos who produced the first cell sorter using electrostatically charged droplets, a development of Sweet's invention for ink-jet writing (1965). Volume measurements were

able to be made with sufficient precision to sort normal white cells with a very high degree of purity (van Dilla, Fulwyler and Boone, 1967). Fluorescence measurements were introduced by van Dilla, Trujillo, Mullaney and Coulter, by Dittrich and Gohde and by Hulett, Bonner, Barrett and Herzenberg all in 1969. Since then the uses of flow cytometry have been expanding at an alarming rate and during the late 1960s and early 1970s major developments took place in fluorescence activated cell sorting at Stanford University (Hulett *et al.*, 1969; Bonner *et al.*, 1972; Herzenberg, Sweet and Herzenberg, 1976).

At first sight these instruments appear complex but many of the basic principles on which they operate were discovered centuries ago. Because of the fundamental importance in understanding the technology some of these principles of physics will be considered. I am well aware that most biologists tend to cringe at the mention of physics. However, there is nothing in the sections which contain some physics and technology that would cause any difficulty for a reasonable intelligent 16- or 17-year-old studying first-year sixth-form physics for advanced level GCSE examinations. Furthermore, the quantity of physics is strictly limited, with no 'fancy' mathematics, and an attempt has been made to relate the relevant concepts from physics directly to the technology, and the technology to the biology.

This book aims to describe the technology and some of its applications and potential applications for students of pathology, medicine and cell biology, laboratory technicians and postgraduates who, hopefully, will have recourse to use the various techniques on a routine basis within the next decade. It is not intended for highly experienced users intimately involved with the technology who should know everything in this book already. The initial intention was a series of notes for users of the instruments in the MRC Clinical Oncology Unit at Cambridge so that I would not have to say the same things over and over again each time a new user wished to take advantage of the systems. The notes got bigger with time and I decided to put them into book form as a general introduction to the subject.

To a large extent the book is based on the author's experience of designing, building, developing and working with the Cambridge MRC dual-laser multiparameter instrument in a highly interdisciplinary, varied and stimulating biological environment. A large proportion of the assays and results used as examples are drawn from our data base accumulated over the past 15 years. This does not mean that some of the assays cited as examples are unique to our instrument; they are not, it's just that it was easier to illustrate with what I know best. As a consequence there is a considerable bias towards the interests of our group and collaborators; however, even if you are not interested in what we have been doing the examples serve to illustrate some of the power, potential and problems associated with the technology.