5 Cytometry and Pathology

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Introduction

Flow cytometry is a complex field that draws people from diverse scientific backgrounds. It is a technology that simultaneously measures and analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. Some flow cytometers are also equipped to identify and sort user-specified particles into collection vessels. High-performance cell sorters can routinely sort at rates of 70,000 cells per second. The strength of flow cytometers is that they can rapidly and quantitatively measure simultaneously multiple parameters of individual live cells and then isolate cells of interest. Additionally, the sensitivity and throughput rates achievable by high-performance commercial instruments enable the detection of extremely rare populations and events (frequencies below 10^{-6}), such as stem cells, dendritic cells, antigen-specific T cells, and genetic transfectants [1]. As a result, applications for flow cytometers continue to increase. In addition to traditional immunology and pathology applications involving particles such as lymphocytes, macrophages, monocytes, and tumor cells, flow cytometers are widely used in conjunction with fluorescence-based protein reporters, such as green fluorescent protein. In this context, flow cytometers can monitor both transfection efficiency and protein expression levels [2, 3]. In addition, interest is growing in the use of flow cytometers to screen cell- or bead-based combinatorial libraries [4]. Therefore, flow cytometry also enables the screening of protein libraries expressed in cells or displayed on the surface of bacteria or beads. A flow cytometer, for instance, can detect modulation of a signal transduction pathway by a particular small molecule and identify proteins with a particular binding specificity, enzymatic activity, expression level, and stability. Ongoing development efforts in the flow cytometry industry are aimed at automation and laboratory integration. Input/output robotics, pushbutton operations, and automated sample preparation will increase throughput rates and make the technology more accessible to a wider user base, as new fluorescent dyes and creative screening approaches expand applications into the proteomic field. Eventually, software advances will seamlessly network instruments into comprehensive analytical and

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diagnostic systems, and the industry may marry its technology with imaging and microfluidics.

Scope

In this chapter, the main aspects of flow cytometry as applied to pathology are described: a brief history of cytometry, the characteristics of cells suitable for flow cytometry, methods used to identify cell phenotypes, cell sorting, cell cycle analyses, and data analyses. This chapter can be read as a self-contained brief of the field of flow cytometry.

Historical Background

The evolution of flow cytometry can be divided into four distinct phases:

1. The development of microscopy
2. The development of dye chemistry
3. The development of electronics
4. The development of computers

All these produced instruments coincident with global biomedical need.

In the sixteenth century, Leeuwenhoek built the first microscope to visualize protozoa and bacteria and can thus be considered the father of cytometry. By 1742, Lomonosov had described the method for producing dark-field illumination and performed light scatter measurements. Light was the sole means of illumination until 1904, when Kolher developed a microscope with an ultraviolet (UV) light source. Essentially, all further developments centered on the microscope until 1934, when Moldavan [5] described a photoelectric technique for counting cells flowing through a capillary tube, and flow cytometry was born. In 1938, Caspersson built a rudimentary flow cytometer to measure cell properties in the ultraviolet and visible regions. Crosland and Tylor developed a blood cell counter using the sheath flow principle, light scatter, and dark-field illumination in 1940. Following work by various researchers in subsequent decades to develop instruments to count particles in suspension [6–9], Kamentsky and Melamed [10, 11] implemented a design in 1965 and 1967 to produce a microscope-based flow cytometer for detecting light signals to distinguish abnormal cells in a cervical sample. Following this, the work of Fulwyler [12], Dittrich and Göhde [13], Van Dilla et al. [14], and Hulett et al. [15] led to significant changes and resulted in a cytometer that largely resembled those of today. Like modern cytometers, a flow cytometer in 1969 in no way resembled a microscope but was still based on Moldavan's prototype and on the Kamentsky instrument in that it illuminated cells as they progressed in single file in front of a beam of light, and it used photodetectors to detect the signals that came from the cells [15–18]. Even today, the definition of a flow cytometer involves an instrument that illuminates cells as they flow individually in front of a light source and then detects and correlates the signals from those cells as a result of that illumination.

Stains were required to enhance the visibility of prokaryotic and eukaryotic material under the microscope. The development of these stains was driven by the absorptive dye chemistry needed in the textile industry after 1850. Malachowski and Romanowsky [19, 20] used acidic and basic dyes, which gave rise to the Giemsa, Leishman, MacNeal, and Wright stains used for identifying parasites in blood cells and hematopoietic cells. Fluorescent dyes did not appear until the 1880s, when Paul Ehrlich [21] synthesized and used fluorescein. He also pioneered the use of mixtures of acidic and basic dyes to resolve the internal structure of leukocytes. DNA dyes were first used in 1900, but the introduction of flow cytometry drove the development of several new dyes in the late 1960s and 1970s, and the measurement of DNA content became one of the first major applications of flow cytometry. Dittrich and Göhde [22] first used ethidium bromide in 1969, Crissman and Steinkamp [23] introduced propidium iodide in 1973, and Crissman and Tobey [24] used mithramycin in 1974. In 1976, Latt and Stetten [25] introduced the Hoechst dyes, and a year later Stöhr et al. [26] used 4',6-

diamidino-2-phenylindole) (DAPI). All of these dyes are commonly used in modern flow cytometry. In parallel, in 1940, Coons et al. [27] used antipneumococcal antibodies conjugated with anthracene to detect microorganisms in tissues. By 1950, they were using antibodies conjugated with fluorescein isothiocyanate (FITC), and immunophenotyping was born. Immunophenotyping allowed for the labeling of specific cell membrane proteins on cells, predominately leukocytes. Instead of relying on morphology, scientists could now identify cells by their unique repertoire of membrane proteins. In 1974, Kano et al. [28] introduced the process of producing monoclonal antibodies.

Meanwhile, development was ongoing in the electronics industry. By 1945, the photomultiplier tube (PMT) had been developed to detect photons and convert them to electrical pulses. Amplifiers and analog-to-digital converters were also developed. In 1949, Wallace Coulter [29] patented the first non-optical electronic blood cell counter; by the 1950s, the realization that automated cytology might be most useful in clinical diagnosis began to permeate academic medical institutions. The ability to electronically count blood cells more accurately and faster than with a hemocytometer started the revolution towards automation. The first Model A Coulter counter was introduced in 1957, primarily to count erythrocytes and leukocytes from blood; Model B was introduced 4 years later. This device could also provide the size distribution of these cells. The disadvantage of the Coulter Counter was that it could not identify what was being counted. This led to the need for an automated microscopic identification process. To accomplish this, computers, which had recently been introduced, and the concomitant software, were required. Two different groups emerged to tackle this problem. Marylou Ingram (a hematologist at the University of Rochester) and Kendall Preston [30] (a biomedical engineer at Perkin–Elmer) together built the first cytoanalyzer, a microscope-based instrument, to automate the microscopic identification of leukocytes in stained smears. At the same time, another group, Mortimer Mendelssohn, and Judith Prewitt [31], began studies to extend the image analysis of cells using an automated instrument called a "CYDAC". This was the first example of automated image cytometry. Cells could now flow through an orifice and be counted; they could also be stained and identified microscopically in a somewhat automated fashion.

Modern flow cytometry began when Fulwyler [32], at the US Los Alamos National Laboratories, built a cell sorter using the main Coulter cell size and the electrostatic charging of the droplets to order. Then, Dittrich and Göhde [22] developed the impulse cytophotometer (ICP), also known as the "Phywe." Cells were introduced into a flowing sheath stream located under a high-power microscope objective that provided measures of scatter and fluorescence detection. Mullaney and Dean [33] introduced multiparametric flow cytometry, combining the measurement of volume, light scatter, and fluorescence in a single instrument. By the mid-1970s, flow cytometers had entered the market, and Leonard Herzenberg [34] coined the term "fluorescence activated cell sorter," or "FACS," revolutionizing immunology and cancer biology. In the early 1980s, interest in immunophenotyping grew with the discovery of AIDS. The first monoclonal antibodies identified membrane proteins such as CD3, CD4, and CD8 expressed by T lymphocytes. The first major clinical application of immunophenotyping using flow cytometry was in the treatment of AIDS. With this development, and the ability to measure DNA content, flow cytometry became the important and primary approach to automated cell analysis in clinical applications. These applications included ploidy and S-phase fraction measurement in solid tumors, diagnosis and follow-up of hematopoietic malignancies and paroxysmal nocturnal hemoglobinuria (PNH), and monitoring of transplant rejection and hematopoietic regeneration. Whereas immunophenotyping initially focused on the measurement of membrane markers on the cells, researchers soon found that intracellular markers could also be measured. With its ability

to identify a cell population from membrane markers and simultaneously determine the function of the cell, the power of flow cytometry was quickly realized. Another application launched by Stubblefield et al. [35] in Los Alamos and van den Engh et al. [36] at the Lawrence Livermore National Laboratory was chromosome staining and sorting for DNA cloning to produce chromosome-specific sequences. This new application led to the construction of the first high-speed cell sorter to classify each human chromosome. This project, supported by the US Department of Energy, was the first step in the Human Genome Project. Hence, since a very slow start in the seventeenth century, the development of chemical dyes, the electronics industry, the computer, and the production of monoclonal antibodies have all come together to produce the rapidly expanding field of flow cytometry. No other technology has been developed that can quickly measure the related properties of basic cells.

Techniques

The word "cytometer" itself is derived from two Greek words, "kυτοζ," meaning container, receptacle, or body (taken in modern formations to mean cell), and "μετρον," meaning measure. Cytometers measure particles. "Particle" can be used as a more general term for any of the objects flowing through a flow cytometer. "Event" is used to indicate anything that has been interpreted by the instrument, correctly or incorrectly, to be a single particle. For example, if the cytometer is not quick enough, two particles close together may actually be detected as one event. Because most of the particles sent through cytometers and detected as events are in fact single cells, the words are used somewhat interchangeably.

Flow Cytometry and Particles/Events

Because flow cytometry is a technique for the analysis of individual particles, a flow cytometrist must begin by obtaining a suspension of particles. Historically, the particles analyzed by flow cytometry were often cells from blood; they are ideally suited for this technique because they exist as single cells and require no manipulation before cytometric analysis. Cultured cells or cell lines have also proven suitable, although adherent cells require some treatment to remove them from the surface on which they are grown. More recently, bacteria [37, 38], sperm [39, 40], and plankton [41] have been analyzed. Flow techniques have also been used to analyze individual particles that are not cells (e.g., viruses [42], nuclei [43], chromosomes [44], DNA fragments [45], and latex beads [46]). In addition, cells that do not occur as single particles can be made suitable for flow cytometric analysis using mechanical disruption or enzymatic digestion; tissues can be disaggregated into individual cells, and these can be run through a flow cytometer. The advantage of a method that analyzes single cells is that cells can be scanned rapidly (500 to >5000 per second), and the individual characteristics of a large number of cells can be enumerated, correlated, and summarized. The disadvantage of a single-cell technique is that cells that do not occur as individual particles must be disaggregated; when tissues are disaggregated for analysis, some of the characteristics of the individual cells can be altered and all information about tissue architecture and cell distribution is lost. Therefore, when the cell suspension used for cytometric analyses derives from cell culture or fresh biopsy, careful evaluation of the resulting data is necessary.

Immunophenotyping

Immunophenotyping of biological samples refers to the use of immunological tools (e.g., monoclonal and/or polyclonal antibodies) for the specific detection of antigens, most frequently from proteins, expressed by cells localized either on their surface or inside them. Flow cytometry is a sensitive technique currently employed by pathologists, especially for quantitative and qualitative evaluation of hematopoietic cells [46].

In a clinical setting, the main purpose of flow cytometric analysis is to identify abnormal cells by defining their immunophenotypic characteristics. To accomplish this task, multiparameter analysis represents a reliable approach that integrates the information provided by forward scatter (FSC) and side scatter (SSC) with multiple fluorescence parameters. Thus, the cell size and internal complexity as well as the simultaneous expression of three to four or more different cell antigens are evaluated and used to outline distinct cell populations [47]. Importantly, multiparameter analysis permits the detection of abnormal cells that occur at a very low frequency in the context of normal populations, such as in the case of minimal residual disease [48]. Multicolor flow cytometry is currently used by many laboratories for the analysis of leukemia and lymphoma, although technology is moving rapidly towards more complex types of analysis, employing six or more fluorescence parameters. In multicolor analysis, a widely used strategy for the gating of lymphocytes is based on CD45 expression and SSC characteristics [49]. This approach permits the analysis of aged samples with a better lymphocyte recovery than FCS vs. SSC gating. Therefore, gating via CD45/SSC represents the strategy of choice for immunophenotyping blood lymphocyte subsets, for example, monitoring CD4 counts in patients with AIDS. The staging of human immunodeficiency virus (HIV) infection based on the enumeration of the absolute and relative CD4+ T-cell counts in peripheral blood probably represents the most extended and recognized clinical application of flow cytometry. Gating via CD45/SSC strategy is also being applied successfully in the analysis of many lymphoid malignancies detected in blood, bone marrow, and lymph nodes [50]. Specific immunophenotypic profiles have been established for several lymphoid neoplasms [51–53]. The abnormal profiles are compared with the pattern of cell marker expression normally seen in the organ/tissue being examined, and a diagnostic interpretation is made. While the normal reference ranges for differentiation antigens expressed by lymphoid cells from peripheral blood, bone marrow, and lymph nodes are well established, immunophenotyping is also currently a primary diagnostic tool for the study of individuals suspected of PNH, systemic mastocytosis (SM), primary thrombocytopathies, and immunodeficiencies. In all four disease groups, genetic abnormalities carried by the clonal hematopoietic cells are translated into changes in the phenotype and distribution of specific populations of hematopoietic cells. Such phenotypic changes are closely associated with specific underlying genetic abnormalities and can be easily and reproducibly identified with flow cytometry immunophenotyping. Although immunophenotyping does not allow a final diagnosis of most primary immunodeficiencies, it has great value as a screening tool. It is well established for that an association exists between the distribution of different populations of peripheral blood lymphocytes, their immunophenotype, and the underlying genetic defect. More detailed knowledge of the exact genetic lesions present in patients with primary immune deficiencies has largely contributed to expanding the utility of immunophenotyping in the diagnosis of this heterogeneous group of disorders.

Over the last decade, the application of flow cytometry immunophenotyping in transplantation has increased in terms of both hematopoietic tissues and solid organs. The most widely used example is the flow cytometry enumeration of CD34+ hematopoietic progenitors to control the quality of biological products obtained for hematopoietic transplantation. It is well established that the total number of CD34+CD45dim hematopoietic progenitors given in a transplant is the most powerful indicator of the outcome of the graft, and flow cytometry immunophenotyping is the method of choice for counting CD34 cells. Another clinically relevant application of immunophenotyping in transplantation is the so-called flow cytometry crossmatch. The goal of this assay is to identify the presence of anti-human leukocyte antigen (HLA) antibodies, quantify their titer, and determine their specificities in the serum of the donor prior to an allogenic transplant. Although flow

cytometry cross-match initially used cellular-based assays, in recent years they have been combined with multiplexed bead arrays that allow routine determination of anti-HLA antibody specificities. The use of cytometry for immunophenotyping has the advantage of quick multiparametric analysis of a very large number of cells (20,000–50,000) and a better statistical representation of the population of interest. The choice of the appropriate antibody combinations, gating strategy, and multiparametric analysis plays a key role in diagnosis. Cytometric analysis offers another advantage over other techniques: dual and triple markers can be applied to detect co-expression of two or three antigens on the same cell. Flow cytometry also has the ability to precisely detect immunoglobulin light chain expression and therefore assess the monoclonality of lymphoid populations. However, its major disadvantage lies in its need for mono-dispersed cell suspensions: fresh specimens are required to maintain viability and avoid loss of antigenicity through tissue fixation. Samples must be immediately suspended in chilled nutritional medium after surgical excision, followed by processing of fresh tissue within minutes or only a few hours. In addition, the information provided about the morphology and localization of a given molecule inside a cell is limited. On the other hand, immunohistochemistry (IHC) on paraffin-embedded tissue is limited by poor antigen preservation and difficulties in defining antigens that are restricted to cell surfaces; these are lost through fixation and include the majority of lymphoid markers. Sufficient antibodies have been developed for the identification of B- and T-cell populations on paraffin-embedded tissue. However, immunoglobulin light chains are not reliably demonstrated on cell surfaces by this method [54]. It has been reported that IHC does not usually provide clear evidence of surface light chain restriction due to the large amount of background immunoglobulin in the interstitial spaces of tissues, which obscures the relatively weak monoclonal immunoglobulins on the surface of B cells. However, flow cytometry is also more sensitive than molecular techniques (reverse transcription polymerase chain reaction [RT-PCR], quantitative RT-PCR [Q-RT-PCR]) as there is no possibility of sample contamination by non-neoplastic cells. In addition, complementary DNA (c-DNA) arrays are costly and tedious sophisticated lengthy tests that can only be performed in specialized laboratories and requires significant experience and a special software system. The possibilities for immunophenotyping by flow cytometry are almost infinite in both clinical and research applications. In general, cells stained with antibodies may be used to identify, count, and characterize any type of individual cell or subcellular component. Many of the applications for immunophenotyping are of great clinical utility, mainly in the area of hematology and immunology.

Procedures

Immunophenotyping techniques measure antigen expression by flow cytometry. Traditionally, these techniques have been divided into two major methods: (1) direct immunofluorescence staining and (2) indirect immunofluorescence staining. The first is obtained when the antigen/antibody reaction is detected with an antibody directly coupled to a fluorochrome; the second requires the use of a secondary anti-immunoglobulin antibody conjugated to a fluorochrome. Given the complexity of performing multiple stains with indirect immunofluorescence methods, the use of these techniques is currently restricted almost exclusively to single antigen stains. Direct immunofluorescence is the preferred method for multicolor antibody/antigen reactions. Usually, the optimal sample preparation technique depends on the type of specimen and the cells of interest, the antigens and their distribution in the sample, the localization of the antigens in the cell, and the information to be obtained. The main steps to obtaining direct staining for immunophenotyping are as follows:

- Disaggregation of solid tissue or detachment of cells derived from cell culture
- Washing with PBS
- Adding the antibody on pellet

- Incubation, usually for 30 min at 4 °C in the dark
- Washing with PBS
- Acquisition and analysis with flow cytometry

Depending on the characteristics of the cells, specific changes in sample preparation techniques may also be required. For example, for the staining of non-nucleated red cells, the lysing steps should be eliminated, and quenching reagents (e.g., crystal violet) may be required for optimal immunophenotyping of highly autofluorescent cells. Another technique that requires modification of the immunofluorescence protocols is the staining of intracellular antigens: appropriate fixation and permeabilization protocols should be used prior to staining.

Finally, the type of information desired may also affect the exact sample preparation protocol to be applied. In fact, if immunophenotyping is used to count the number of cells present in a given volume of sample, washing steps and solutions that may damage the sample cells should be eliminated to avoid significant cell loss. Overall, variables that determine the most appropriate sample preparation protocol also determine the most appropriate instrument settings during data acquisition and the number of events that should be measured in the flow cytometer. It is well established that identification of a cell population requires a minimum of between 13 and 15 homogenous events, while reaching an acceptable coefficient of variation (10 %) for the enumeration of a cell population requires that a minimum of 100 cells of interest are analyzed. Table 5.1 shows the main variables that can affect the detection of antigen expression by flow cytometry.

DNA Measurement

In recent years, flow cytometry has been recognized as a useful, rapid, and novel method to determine—efficiently, reproducibly, and at lower costs per sample—the relative nuclear DNA content and ploidy level of cells. This technology has also been used to isolate cell population with different DNA content. On the consequence, after immunofluorescence, DNA analysis is the second most important application of flow cytometry. DNA measurement is very important because we obtain two main information: (1) ploidy, relevant in tumors, and (2) distribution of cells in phases of cell cycle. DNA is a molecule that carries most of the genetic instructions used in the growth, development, functioning and reproduction of all known living organisms. It is composed of bases that form two strands arranged as double helix. Being mainly involved in cell life in terms of reproduction, growth and death and tumor development, DNA study has aroused enormous interest to research and clinical cytometrists. To perform DNA analyses, fluorescent probes such as propidium iodide, DRAQ5 (a Vybrant DyeCycle compound) or the bis-benzimidazole, Hoechst 33342, can be used. Usually, fluorescent signal is proportional to the DNA amount in the nucleus identifying strong gains or losses in DNA content. Interestingly, it is the study of abnormal DNA content, defined as "DNA content aneuploidy," especially in tumors; although, some benign conditions may be aneuploid [55–60]. In several types of tumors, DNA aneuplody is correlated with a bad prognosis, but it may be associated with improved survival in rhabdo myosarcoma, neuroblastoma, multiple myeloma,

Table 5.1 List of the main variables in analyzing antigen expression in immunophenotyping

Monoclonal antibody reagents	Sample preparation	Fluorescence measurements
Affinity	Time	Nozzle size
Avidity	Temperature	Fluorescence detectors
Concentration	pH	Speed of analyses
Fluorochrome	Lysing solution	Instrument settings
Immunoglobulin isotype	Washing steps/autofluorescence	Compensation

and childhood acute lymphoblastic leukemia (ALL) [61–64]. For example, in ALL, hypodiploid tumors, myedisplastic syndromes, DNA hypodiploid content lead to a poor prognosis. On the contrary, DNA hyperdiploid content in ALL have a better prognosis [64]. Regarding hematologic tumors, data are discordant on the independent prognostic value of DNA content analyses.

DNA Probes

Before we focus on the measurement of DNA content, we provide a small prompt on the cell cycle. It is usually subdivided in three phases: the G_0G_1, S, and G_2M phases of the cell cycle, as shown in Fig. 5.1.

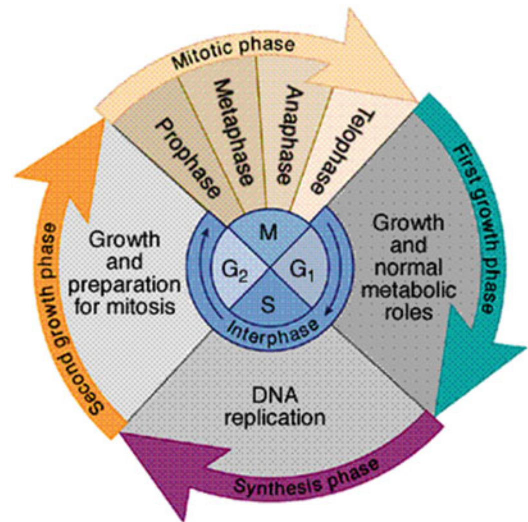

Fig. 5.1 Main phases of the cell cycle

G_0 (gap) is a phase in which cells are quiescent and not undergoing cell division.

G_1 is the phase in which cells are preparing to move through cell division and start to synthesize the factors involved in S phase. Both in G_0 and G_1, the DNA content is 2n. Therefore, both phases are indistinguishable from each other using a single DNA probe.

S (synthesis) is the phase in which the synthesis of DNA occurs. Therefore the DNA content ranges from 2n to 4n.

G_2 is the phase where the cells start to synthesize all components involved in M phase.

M (mitosis) is the phase in which it has the physical division of the mother cell with the formation of two daughter cells. Again, these two phases are indistinguishable from each other by the flow cytometer with a single DNA probe because the DNS content is 4n.

In order to measure the DNA content by flow cytometry, it is necessary to stain the nucleic acids using specific fluorescent probes. The bond between DNA and probe is not as strong as that of the antibody with its antigen. Therefore, it is important to establish the specific concentration of probes that must be used for DNA staining in cytometry, and samples are not washed after staining also because the unbound dye does not emit fluorescence. There are two main classes of DNA probes: (1) intercalating agents and (2) agents binding specifically to DNA bases. Intercalating agents usually intercalate between the bases in double-stranded nucleic acids, DNA or RNA. Therefore, these probes can also stain RNA and not only DNA. On the consequence, RNA must be eliminated using RNase that is added to staining solution. The advantage of these dyes is they are stoichiometric. Hence, the number of molecules of dye bound to DNA is equivalent to the number of DNA molecules. As a result, the amount of emitted light is proportional to the amount of bound dye that, in turn, is equivalent to DNA amount. Usually, intecalating probes belong to the family of phenanthridiniums and include propidium iodide and ethidium bromide. They are excite with ultraviolet or blue laser and emit in red spectrum. The Table 5.2 shows the properties of some mainly used DNA probes. The most widely used dye is propidium iodide (PI), which emits in red spectrum and is excited at 488 nm with blue laser. Unfortunately, the PI shows two disadvantages. First, PI stains both the DNA and double-stranded RNA. Therefore, the cells must be treated with RNase. Second, it is excluded by the plasma membrane. On the consequence, the cells must be fixed and, then, permeabilized. Subsequently, the probe

can be added to cell preparation. Appropriate protocols for PI staining are found in Ormerod [65] and Darzynkiewicz [66]. For fixation, usually, 70 % ethanol is used or cells are suspended in a buffer containing a detergent [67]. The Fig. 5.2 shows a typical DNA histogram obtained using PI staining. The stromal population of normal diploid cells is usually not cycling. To analyze the DNA content, the fluorescence must be read using a linear scale. This strategy allows to determine quickly DNA index values. The second class of DNA probes includes dyes that bind preferentially to adenine-thymine (A-T) regions of DNA. This stain binds into the minor groove of DNA and exhibits distinct fluorescence emission spectra that are dependent on dye: base pair ratios. DAPI, either DRAQ5 (a Vybrant DyeCycle compound) or the bis-benzimidazole, Hoechst 3334,2 belong to this class of probes. DAPI and Hoechst3342 show an emission at 358 nm and 346 nm, respectively. Their emission is 461 nm. Therefore it is necessary an ultraviolet (UV) or violet laser. DRAQ5 probe is excited with 488 or 633 nm laser and its emission is 680 nm. In addition, DAPI is used for cells that must be fixed and permeabilized, whereas Hoechst33342 and DRAQ5 are vital probes, thus the cells must not be fixed. Independently of the dye used, it is necessary to perform preliminary assays to evaluate and determine the correct and specific times of incubation and dye concentration to obtain a satisfactory DNA histogram. Times and concentration are specific for each type of cells. In clinic diagnosis, DNA is extracted starting from sections of 50 μm thick of paraffin embedded tissues. The paraffin is removed, and the nuclei are extracted by treatment with pepsin [68]. The quality of the histograms can be very good, and depends on especially the way in which the tissue was initially handled. A strategy commonly used is to incubate tissue sections at 80 °C before treating with pepsin [69].

Table 5.2 Main DNA probes used in cytometry

Probe	Maximum excitation (nm)	Laser (nm)	Maximum emission (nm)	Features
Propidium Iodide	535	Blue (488)	623	Intercalating agent—not vital dye
Hoechst 33342	350	UV (355)	461	Bind strongly to A-T-rich regions in DNA—vital dye
DAPI	358	UV (355)	461	Bind strongly to A-T-rich regions in DNA—not vital dye
DRAQ5	650	Blue (488) or red (633)	680	Bind strongly to A-T-rich regions in DNA—vital dye

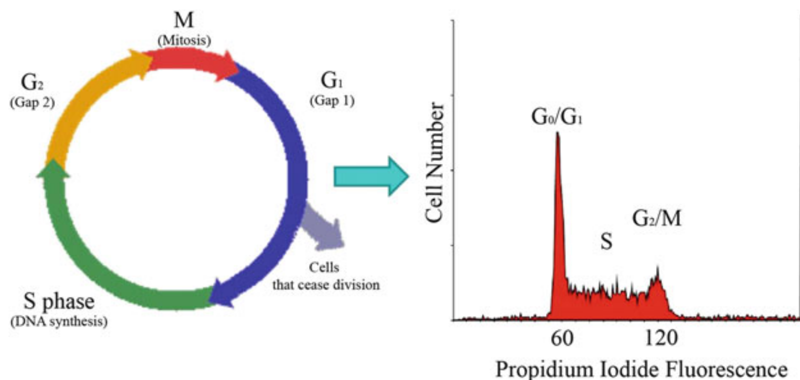

Fig. 5.2 This single-parameter histogram shows fluorescence on a linear scale along the *x*-axis, with number of events up the *y*-axis

Ploidy

The ploidy of a cell is an indication of the number of chromosomes in that cell. Each species has a different ploidy value. There can also be variations within an individual population because of mutations, natural multiploidy (plants), certain diseases, and apoptosis. The number of chromosomes in a tumor is frequently greater than $2n$ (hyperdiploid) and sometimes less (hypodiploid). An abnormal number of chromosomes is called aneuploidy and is reflected by a change in the amount of DNA. The flow cytometrist tries to define these different ploidy levels and may use a series of definitions and terms, as reported in Table 5.3.

Therefore, when aneuploidy is measured as a change in DNA content, as opposed to a change in the number of chromosomes, it should be referred to as DNA aneuploidy. The DNA content of a tumor may be expressed as the DNA index (DI), defined as the ratio between the DNA content of a tumor cell and that of a normal diploid cell; Fig. 5.3 provides an example.

The quality of a DNA histogram is estimated from the width of the peak of DNA from cells in G1 of the cycle. This is measured by the coefficient of variation (CV) across the peak and is calculated from the standard deviation (SD).

The smaller the CV of the peaks in the DNA histogram, the more accurate the measurement of ploidy and the better the estimation of the percentage of cells in the different compartments of the cell cycle. It is essential that any unnecessary broadening of the peaks because of misalignment of the instrument should be eliminated. It is possible to obtain CVs as low as 1 %, although the best CV may be closer to 2 % in aneuploid tumors and cultured cells because of the heterogeneity of the DNA content. The number of clumps and the amount of debris present are also important factors. The key elements in obtaining a high-quality histogram are sample preparation, instrument alignment, and data analysis. The object of sample preparation is to obtain single cells (or nuclei) with the minimum of debris and clumps. When staining fixed cells with propidium iodide, sufficient time must be allowed for the RNase to remove all double-stranded RNA. If the cells have been fixed, leaving them overnight at 4 °C will often improve the histogram. If the cell (or nuclei) concentration is high, there should be sufficient dye present to maintain stoichiometric binding. The performance of the instrument should be checked daily using fluorescent beads of known CV, which can be purchased from several manufacturers. The CV and the peak channel number for a standard set of conditions (laser power, PMT voltage, and gain) should be recorded. If these fall outside predetermined limits (e.g., 2 % CV), action should be taken to restore the instrument's performance. Check that the flow rate has not been accidentally set too high; check there is no partial blockage of the flow cell; and, if possible (with a conventional cell sorter), realign the instrument.

If realignment is not possible (most bench-top instruments), call the service engineer. Any perturbation of the sample stream in the cytometer will increase the CV, so the concentration of cells or nuclei should be kept high (between 5×10^5 and 2×10^6/ml) and the flow rate low. The DNA histogram should be displayed on a linear scale.

Table 5.3 Definitions to indicate the ploidy value

Definitions	DNA content
Diploid	The normal (euploid) $2n$ number of chromosomes. This is the number of chromosomes in a somatic cell for a particular species
Haploid	Half the normal $2n$ number of chromosomes, or $1n$. This is the number of chromosomes in a gamete or germ cell (sperm/egg). Again, this is species dependent
Hyperdiploid	More than the normal $2n$ number of chromosomes
Hypodiploid	Less than the normal $2n$ number of chromosomes
Tetraploid	Double the normal $2n$ number of chromosomes, or $4n$
Aneuploid	An abnormal number of chromosomes

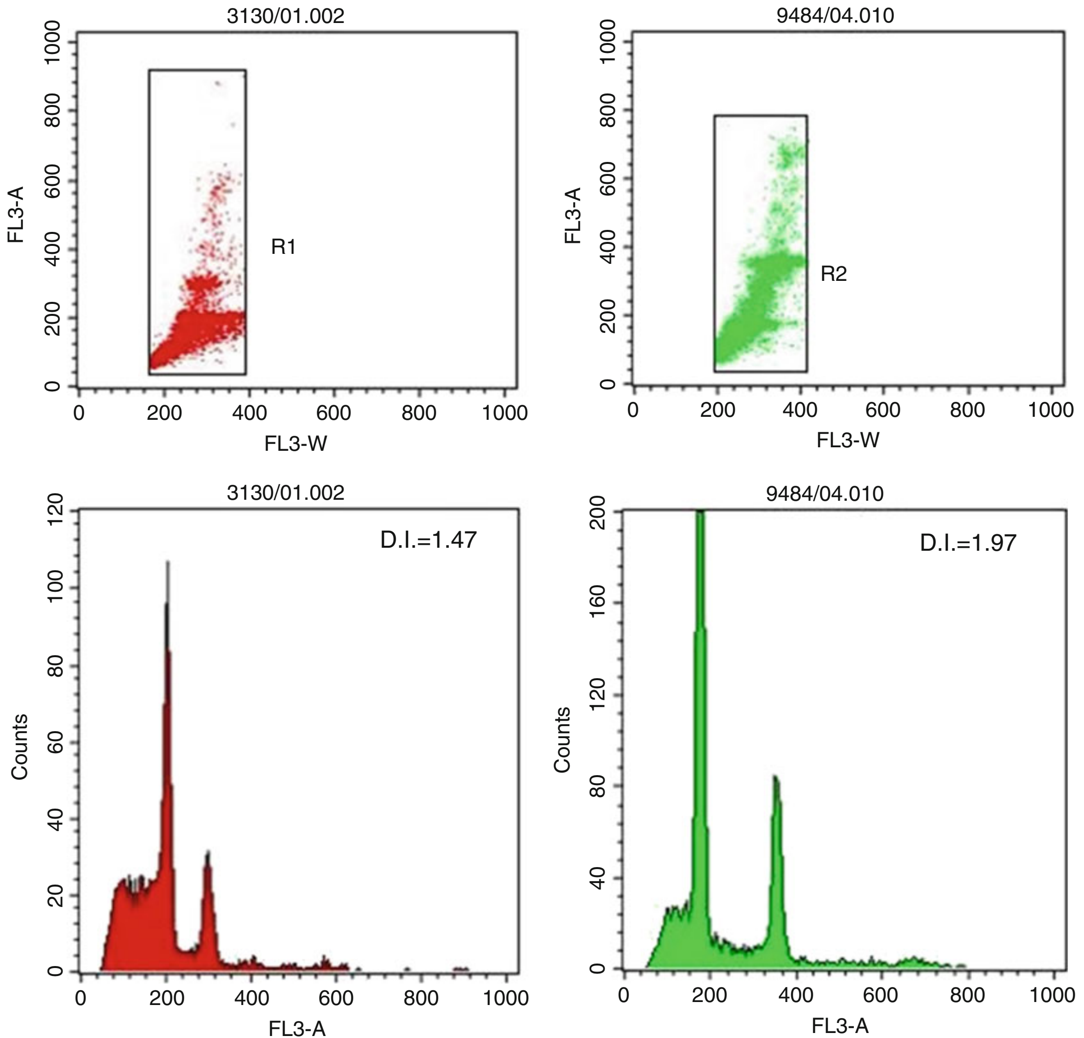

Fig. 5.3 DNA histograms from two endometrial carcinomas. Cells stained with propidium iodide (PI). DNA index (DI) values indicate samples with aneuploidy. Specifically, DNA content is hyperdiploid

Double Discrimination

One problem that must be overcome when obtaining results for DNA analysis is the exclusion of clumps of cells. On a flow cytometer, two cells stuck together may register as a single event, known as a doublet. If each of those two cells is diploid ($2n$), seen as one event, they have 4n DNA. In other words, they have the same amount of DNA as a tetraploid cell (G0G1) or a normal cell that is about to divide (G2M). To add to the confusion, further peaks may exist for three or more cells stuck together. The doublet problem is resolved by employing a doublet discrimination gate based on the characteristics of fluorescence height, fluorescence area, and signal width. Fluorescence height is the maximum fluorescence given out by each cell as it travels through the laser beam; fluorescence area is the total amount of fluorescence emitted during the same journey; and signal width is the time a cell takes to pass through the laser beam. These characteristics differ between a cell that is about to divide and two cells that are stuck together.

A dividing cell does not double its membrane and cytoplasmic size and therefore passes through the laser beam more quickly than two cells stuck together. In other words, it has a smaller width signal or a bigger height signal but the same area as two cells stuck together. Also, all of the DNA in the dividing cell is grouped together in one nucleus and consequently gives off a greater intensity of emitted fluorescence than the DNA in two cells that are stuck together. Thus, a doublet, which has two nuclei separated by cytoplasm, emits a lower-intensity signal over a longer period. This appears as a greater width signal and lower height signal, with the same area. These differences can be seen on histograms of FL3-Area vs. FL3-Width (as shown in Fig. 5.3) or FL3-Area vs. FL3 Height, allowing the generation of gates to exclude doublets from sample analysis for both diploid and aneuploid cells.

Data Analysis and Reporting of the Cell Cycle Phases

There is obviously considerable overlap between early S phase and G1 and between late S phase and G2M because of the broadening of the distribution caused by variability in the staining of the cells as well as instrumental variability. The problem with analysis of a DNA histogram is finding a model to reliably estimate the extent of the overlap. The variety of approaches for modeling the cell cycle phases have been summarized by Rabinovitch [70]. The most rigorous algorithm is probably the polynomial method of Dean and Jett [71]. Few algorithms will handle every histogram, particularly if the data are noisy, the CV large, or the cell cycle severely distorted. The numbers generated should not be blindly accepted but instead used in conjunction with the original DNA histogram. Note also that the numbers produced by the computer program are only estimates.

The two most commonly used commercial DNA analysis software packages are ModFit (Verity Software) and Multicycle (Phoenix Flow Systems). Figure 5.4 shows a computer analysis of some DNA histograms produced with ModFit.

Some general guidelines are as follows:

1. Determine the cell ploidy and report the DNA index of all ploidy populations.
2. Report the CV of the main G0G1 peak. Generally, less than three is good and more than eight is poor.
3. When measuring the S-phase fraction (SPF) of a diploid tumor, make a statement as to whether the S phase was measured on the

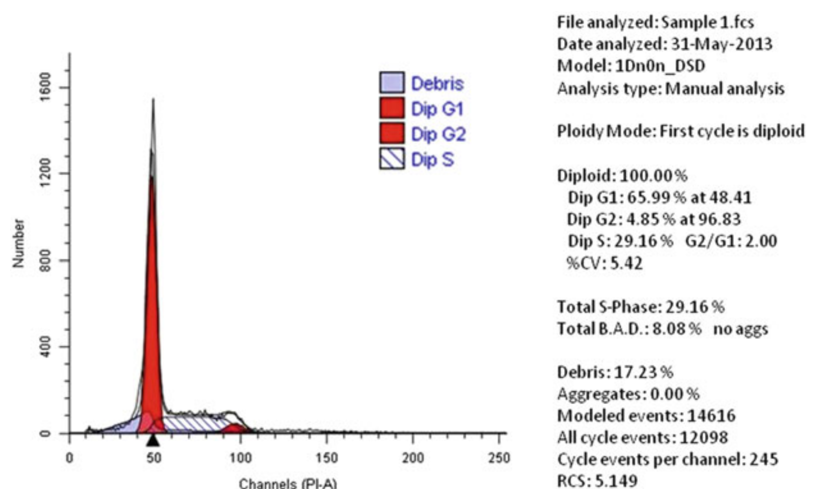

Fig. 5.4 Cell cycle analysis using ModFit software

whole sample, including normal cells, or on the tumor cells alone, gated by tumor-specific antibody.
4. Add a brief comment if necessary to cover any other information that may be helpful to someone looking at the result (e.g., inadequate number of cells, high debris levels, high CV, % background, aggregates, and debris).

Generally, a result should be rejected if any of the following apply:

1. CV of the G0G1 peak is greater than 8 %.
2. Sample contains fewer than 10,000–20,000 nuclei.
3. Data contain more than 30 % debris.
4. Flow rate was too high, as indicated by a broad CV or curved populations on two-parameter plots.
5. G0G1 peak is not in channel 200 of 1024 or 100 of 512 (i.e., on a suitable scale in a known channel).
6. Fewer than 200 cells in S phase (if SPF is to be reported).
7. G0G1 to G2M ratio is not between 1.95 and 2.05.

Instrument Setup for Cell Cycle Analyses

The instrument settings are also critical to obtaining a good histogram. Instrument setup varies with manufacturer. However, some general principles to observe are as follows:

1. Select the LIN channel that is most appropriate for the DNA probe.
2. Set the trigger on the channel detecting the DNA probe, as light scatter parameters are not usually of much use for triggering in the case of DNA.
3. Select parameters to enable doublet discrimination.
4. Set a gate to exclude doublets and apply it to the histogram that will display the DNA profile. Displaying an ungated plot of the same may data may also be useful.
5. Make sure the sheath tank is full, as it may help with stability.
6. Make sure the cytometer is clean. Stream disruption will increase the CV.
7. Set a low flow rate and dilute cells to a concentration that is appropriate for the DNA probe solution.
8. Make sure the instrument has been optimized by running routine calibration particles.

Flow Cytometric Cell Sorting

Flow sorting is a process that allows the physical separation of a cell or particle of interest from a heterogeneous population. Sorting is an elegant use of flow cytometric technology that is attracting new attention from the diverse fields of biology and industry.

This technology provides the powerful yet unique ability to rapidly isolate pure populations of cells or particles with a desired set of biological characteristics. These populations are then available for morphological or genetic examination as well as functional assays and therapeutics. The main applications of flow sorting are as follows:

1. Single-cell cloning of hybridoma cells for the production of monoclonal antibodies
2. Isolating and purifying stem and/or progenitor cells
3. Sorting transfected cells with an expression marker, such as green fluorescence protein
4. Multiparameter isolation of cells from mixed populations
5. Sorting spermatozoa utilizing the difference in DNA content between those bearing the X and Y chromosomes
6. Single-cell sorting for clonogenic assays

In this context, although flow sorters are primarily used to sort mammalian cells, it is more accurate to refer to particle sorting given that flow sorters have also been used to sort yeast

[72], bacteria [73], and phytoplankton [74]. Flow sorting is the only practical way of isolating large numbers of specific chromosomes from humans, other primates [75], or plant species [76], and flow sorters proved invaluable during the human genome sequencing project [75] and more recently in the production of chromosome paints [76]. In addition, as the newer scientific fields of genomics and proteomics have evolved, flow sorting has become important in, for example, sorting large numbers of specific subsets of cells for microarray analysis [77]. At the other end of the scale, single particles may also be sorted into individual wells of a plate for cloning [78] or for PCR analysis [79]. Therefore, the applications of flow sorting are range widely, and a flow sorter, or access to one, is an invaluable resource.

Two methods exist for sorting particles by flow cytometry: electrostatic and mechanical. For the electrostatic method, the particles are passed in a stream of fluid out through a narrow orifice, at which point they pass through a laser beam and are analyzed in the same way as in a standard flow cytometer. A vibration is passed to the sample stream, which causes it to break into droplets at a stable break-off point. If a particle of interest passes through the laser beam, it is identified; when it reaches the droplet of the break-off point, an electric charge (positive or negative) is applied to the stream. As the droplet leaves the stream, it passes through deflection plates carrying a high voltage, and the droplet will be attracted to one of these plates, depending on the charge it was given. Uncharged droplets pass through un-deflected, and deflected droplets are collected in tubes. Thus, two or more different populations of particles can be sorted from the one sample. Most of the high-speed cell sorters use the electrostatic deflection of droplets method.

With the mechanical method, particles of interest are diverted within the flow cell, either by moving a "catcher" tube into the stream or by deflecting them with an acoustic pulse into a fixed tube. Briefly, the catcher tube is located in the upper portion of the flow cell and moves into the stream to collect the particles. When particles pass through the laser beam, the system determines whether each cell belongs to the selected population defined by boundaries in the cytogram. If the particle is identified as being of interest, it is captured by the catcher tube and collected into a tube or into a concentration module; otherwise it is dispatched to the waste tank.

The main drawback of mechanical sorting is the possibility to sort only one population of cells at a slow speed. Nevertheless, no aerosol is involved, which means it is safe to sort samples that have been treated with toxic substances such as radioactive compounds.

The advantage of electrostatic sorting is the ability to sort two subpopulations of cells at a high speed. However, it generates aerosols, so it is not appropriate to sort samples that have been treated with toxic substances. The high pressure generated in electrostatic sorting can also damage the sorted cells.

For precise sorting it is very important to adjust several parameters, including the following:

- The nozzle vibration conditioned by the drop drive frequency (ddf; the number of drops formed per second) and its amplitude level, the particle rate, i.e., the speed, which influences the distance between each cell.
- The dead time: time taken by the instrument to measure a particle's signal and reset to measure the next particle (i.e., time necessary to analyze one particle).
- The drop delay: distance between the laser beam interception of the cell and the break-off point, the point where the stream beaks into droplets.

In addition, other important parameters used to describe the success of cell sorting are yield, recovery, and purity. Yield is the proportion of sorted particles of interest compared with the total number of particles of interest that could have been recovered under ideal conditions. Recovery is the proportion of sorted particles of interest compared with the total number of particles of interest satisfying the sort decision.

Purity is the proportion of sorted particles of interest compared with the total number of

particles in the sorted material. Purity can be, and often is, very high (>98 %), but a simple approach to how the material is re-analyzed can often lead to apparently lower purities than are actually achieved.

Sample Preparation for Cell Sorting and Setup

Sample preparation prior to sorting is important; in fact, successful sorting depends almost entirely on the state of the input sample. It is a prerequisite for flow cytometry that cells or particles be in a monodispersed suspension. This is relatively easy when the cells used are in a natural suspension (e.g., blood cells or suspension-cultured cells) but more problematic when using cells from adherent cultures or from solid tissue. However, several methods for preparing samples for flow sorting are well established. The main steps for preparing samples from suspension cells are as follows:

1. Take cells directly from the flask into 50-ml conical tubes and centrifuge at 800 g for 6 min.
2. Discard the supernatant and re-suspend in medium (cell culture medium or PBS with 1 % bovine serum albumin).
3. Centrifuge again at 800 g and discard supernatant. Count cells and re-suspend at an appropriate concentration, which will vary with sorter used. The final suspension medium will depend on the cell types to be sorted. In general, a low protein concentration is recommended because this will lead to less cell clumping.

In addition, samples from adherent cells should be prepared as follows:

1. Harvest cells using trypsin or versene. Transfer cells to 50-ml conical tubes and centrifuge at 400 g for 5 min.
2. Discard the supernatant and re-suspend in medium (cell culture medium or PBS with 1 % bovine serum albumin).
3. Centrifuge again at 800 g and discard supernatant. Re-suspend the cells in a small volume of medium and aspirate up and down through a pipette several times to help disaggregate clumps. Count cells and re-suspend at an appropriate concentration. In practice, adherent cells tend to be larger, and a lower concentration is recommended. It is always better to keep the concentration high prior to sorting and dilute to an appropriate concentration immediately prior to a sort.

Finally, the main steps for preparing cells from solid tissue are as follows:

1. Place tissue in a sterile Petri dish. Tease tissue apart using a needle and scalpel or alternatively use an automated system such as a MediMachine (Consults, Italy) [80]. In addition, enzymatic disaggregation (e.g., collagenase or dispase) may also help free single cells.
2. Decant cells into a 50-ml conical tube and centrifuge at 800 g for 6 min.
3. Discard the supernatant and re-suspend in medium (cell culture medium or PBS with 1 % bovine serum albumin).
4. Centrifuge again at 800 g and discard supernatant. Re-suspend the cells in a small volume of medium and count cells as above.

All preparations may be filtered through sterile nylon mesh prior to sorting; a range of pore sizes from 20 to 70 μm will be suitable for most cell types encountered.

Another central concern that must be considered has to do with procedures for sorting setup. It is important to ensure all fluidics lines are cleaned and/or replaced regularly. If a sort is aseptic (i.e., one in which cells will be required to be re-cultured or transplanted), a sterilization procedure will be needed. In general, this involves running 70 % ethanol through all fluidics lines for 30–60 min before flushing with distilled water (30 min) and finally sterile sheath fluid (at least 30 min before commencing a sort). All areas where the cells can potentially be in contact with the atmosphere (i.e., the sample line

and sort chamber) should also be cleaned with ethanol prior to a sort. To check the sterility of a flow sorter, it is useful to periodically remove fluid from key locations (sheath tank, nozzle, and sample line) and put this into culture in an appropriate medium; cultures should remain sterile for at least 7 days.

The next consideration is nozzle size. The cell type will influence the size of orifice used. A general rule of thumb is that for blockage-free sorting and coherent side streams, a cell should be no more than one-fifth the diameter of the nozzle. In practice, this means that small round cells such as lymphocytes would require a 70-μm nozzle, whereas many cultured adherent cell lines and primary cells such as keratinocytes would require a 100-μm nozzle.

The third consideration is the laser alignment. The laser or lasers must hit the stream in parallel and the beams must be focused correctly.

Alignment may be checked using fluorescent beads that are excited by a particular wavelength of light and have a broad emission spectrum; it is also important to monitor the sensitivity of the sorter using multipeak beads with a variety of fluorescence intensities. The number of lasers used and the fluorochromes to be detected will also vary and need to be determined before the flow sorter is prepared. Once aligned, the frequency and amplitude of the drop must be found to obtain a stable break-off point. The latter will be found at several harmonic resonance frequencies, and the ability to find the most stable of these comes with operator experience. Many modern sorters can automatically detect movement of the break-off point and change the amplitude accordingly. The principles of sorting are relatively straightforward, but successful sorting is very much a result of experience; a basic grounding in and understanding of lasers, fluidics, computing, and biology are essential if a flow sorter is to be used to its full potential.

Data Analyses

Numerous software options are available for the analysis of flow cytometric data. One possibility involves histograms that show fluorescence patterns of a population for a single parameter, as represented in Fig. 5.5.

The histogram shows the FITC fluorescence pattern for collagen II of human primary chondrocytes. First, at the moment of analysis, the researcher must always perform a dot plot for physical parameters, defining a gate that delimits the cell population of interest. The gate is important because it excludes events such as cell debris that can affect the analysis.

The operator can then undertake different analyses independent of software. In this case, as reported in Table 5.4, the percentage of cells, the percentage of gated cells, and the geometric mean were obtained.

For many applications, such as phenotypic characterization, data collection on a logarithmic scale is preferred to a linear scale. An overlay constitutes a specialized type of histogram that permits the simultaneous display of parameter

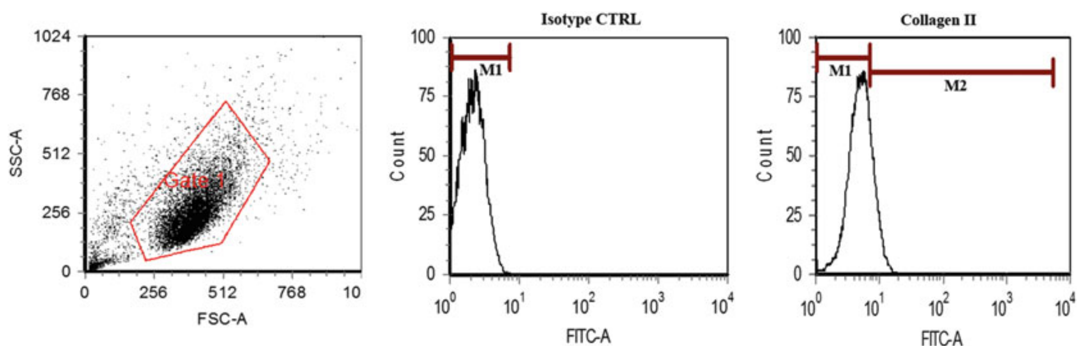

Fig. 5.5 Dot plot for physical parameters and histograms for isotype control and collagen II

Table 5.4 Analyses of cells expressing collagen II

Histogram#	Filename	Parameter	Marker	#Events	% of all cells	% of gated cells	Geometric mean
1	chondro-41	FITC-A	M1	7840	66.7	79.71	4.4
1	chondro-41	FITC-A	M2	2148	18.27	21.84	8.9

data from multiple samples, as reported in Fig. 5.6.

With this approach, individual samples can be compared based on a specific fluorescent or light scatter parameter, or control data can be overlayed against test samples to distinguish experimental significance. This type of analysis is performed to screen for the expression of specific proteins from reporter gene constructs, identifying cell lines with anomalous gene expression patterns, or monitoring gene expression of a population in response to external factors such as biologically significant compounds.

Alternate methods of data display are available for two-parameter plots. One option is contouring, which displays the data as a series of lines, similar to that observed with topographical maps. The contour patterns correlate to the distribution and density levels of cells or particles within the plot and can be used to aid in data analysis or to delineate populations of interest.

Various statistics are available for assessing the data within histograms and dot plots. Common statistics include total counts, population percentages, mean, median, CV, and SD. For example, total counts and percentages for specific cell populations can often be used in the clinical diagnosis of disease. In the case of HIV-infected patients, assessment of the T cells expressing the cell-surface proteins CD4 and CD8 can be used to determine a patient's immunological status. The CV statistic is traditionally used in conjunction with beads for calibration and daily quality control of the instrument, while the mean can be used to quantify the fluorescent intensity of a cell using standard calibration units or molecules of equivalent soluble fluorochrome (MESF). Regions can be created within histograms and dot plots to generate statistics on subpopulations. In the case of disease diagnosis, clinicians often rely on the statistics from various lymphocyte subsets to characterize and treat many types of leukemia and lymphoma. Study of these disorders is typically based on identifying those cells containing certain expression patterns of cell-surface proteins. Factors such as total cell count, population percentages, "brightness" or fluorescent differences between populations, or statistical ratios between populations can then be used to analyze and quantitatively assess the data.

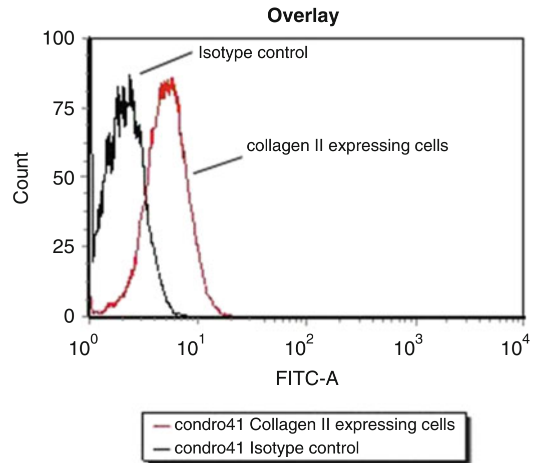

Fig. 5.6 Overlay. Primary chondrocytes were stained and analyzed using fluorescein isothiocyanate (FITC)-conjugated anti-collagen II antibody

Regions can also be used for the creation and application of gates. Gates may involve one or more regions and are used primarily to minimize or eliminate extraneous events within an analysis plot or to isolate specific cells and particles of interest. Some software programs also provide the ability to assign colors to those events that fall within a particular region or gate, thus enabling easy identification of those cells within other histograms or dot plots.

Compensation

Another important consideration when conducting multicolor experiments is the possibility of fluorescence interference created by dyes or fluorochromes that possess close or overlapping emission spectra. In flow cytometry, this process is known as compensation. Compensation refers to specific software or hardware manipulations that mathematically remove fluorescence overlap to simplify multicolor data interpretation and distinguish populations on a dual-parameter histogram.

One benefit of this process is that it ultimately enables researchers to better delineate populations based on the expression or non-expression of specific cellular components, such as cell-surface antigens. Compensation currently represents one of the foremost obstacles to conducting multiparameter experiments in flow cytometry. Therefore, the experience of operator is also very important.

Conclusions

In summary, flow cytometry is an interesting tool for characterization of cells, ploidy level of tissues, nuclear DNA content, division frequency (through the detailed analysis of cell cycles), and phenotypic characterization. Flow cytometers are distinguished by their capacity to collect and process large amounts of data expeditiously. With a system to detect, view, and analyze the expression patterns of multiple proteins involved in complex biological processes such as apoptosis, oncogenesis, or cell division, investigators are better able to understand the possible combinatorial roles that specific proteins play in these processes and to provide data for diagnosis and ongoing patient treatment.

The advantages of flow cytometry, including data storage and analysis, ensure the prominence of this science in the continued study and understanding of complex cellular processes.

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