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Chapter 29
Flow cytometry and fluorescence activated cell sorting (FACS)

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The demands of immunological investigations played an important role in the early development of flow cytometry and particularly in fluorescence activated cell sorting (FACS). Applications of flow cytometry in immunologically related research are still expanding, and the requirements of such work continue to be a major source of innovations in flow cytometry. With the routine application of multiple immunofluorescence, FACS techniques are breaking new ground in the characterization and functional study of small subpopulations of cells.

Flow cytometric techniques can be applied only to cells in suspension. As such, they are complementary to microscopy, which provides information about tissue architecture and cell localization as well as data on the distribution of cellular fluorescence. These capabilities are important in assessing the immune status of an individual, in monitoring cell surface antigen modulation and in discriminating intracellular from surface fluorescence, information which is not available from usual flow cytometric measurements. With microscopy, however, the quantitative measurements and follow-up functional assays that are routine in flow cytometry are difficult or impossible.

The analytical power of flow cytometry comes from making rapid quantitative, multiparameter measurements on large numbers of cells so that the characteristics of cell populations or of their component subpopulations can be defined. Quantification is important because a population is often identified on the basis of the amount of a particular marker, rather than simply on its presence or absence. Multiparameter analysis, combining measurements of intrinsic cell properties, like light scatter, with quantitative assessment of features such as cell surface immunofluorescence, makes it possible to evaluate discrete cell populations even in complex mixtures. Large numbers of cells must be examined in order to characterize rare subpopulations or to select rare cells. Cell sorting criteria based on any combination of the measured properties make it possible to isolate defined populations of viable cells for functional studies, to select particular cells for expansion in culture, and to isolate cells for morphological examination or other forms of secondary analysis.

Orientation of the chapter

In this chapter, the authors focus on cell sorting instruments and their use for analysis and sorting of viable immunofluorescent stained cells. General information on flow cytometry and sorting can be found in various books [1], conference proceedings [2], review articles [3,4] and the journal *Cytometry*. More detail and theoretical background in many of the topics discussed in this chapter can be found in a recent review by some of the present authors [5].

How a cell sorter works

The main components of a 'typical' cell sorter are illustrated in Fig. 29.1. Cells in suspension pass from a reservoir into the centre of the nozzle, where they are surrounded by cell-free sheath fluid which confines the cells to the centre of a liquid jet. As cells pass through the focussed laser beam they scatter laser light, and fluorescent molecules that they contain or carry are excited and fluoresce. A forward light scatter detector collects light scattered out of the laser beam at relatively small angles and converts it to an electronic signal. Fluorescent light is collected by a lens and the different colours (generally produced by different dyes) are directed to separate photomultiplier (PMT) detectors by a dichroic reflector. Optical filters are

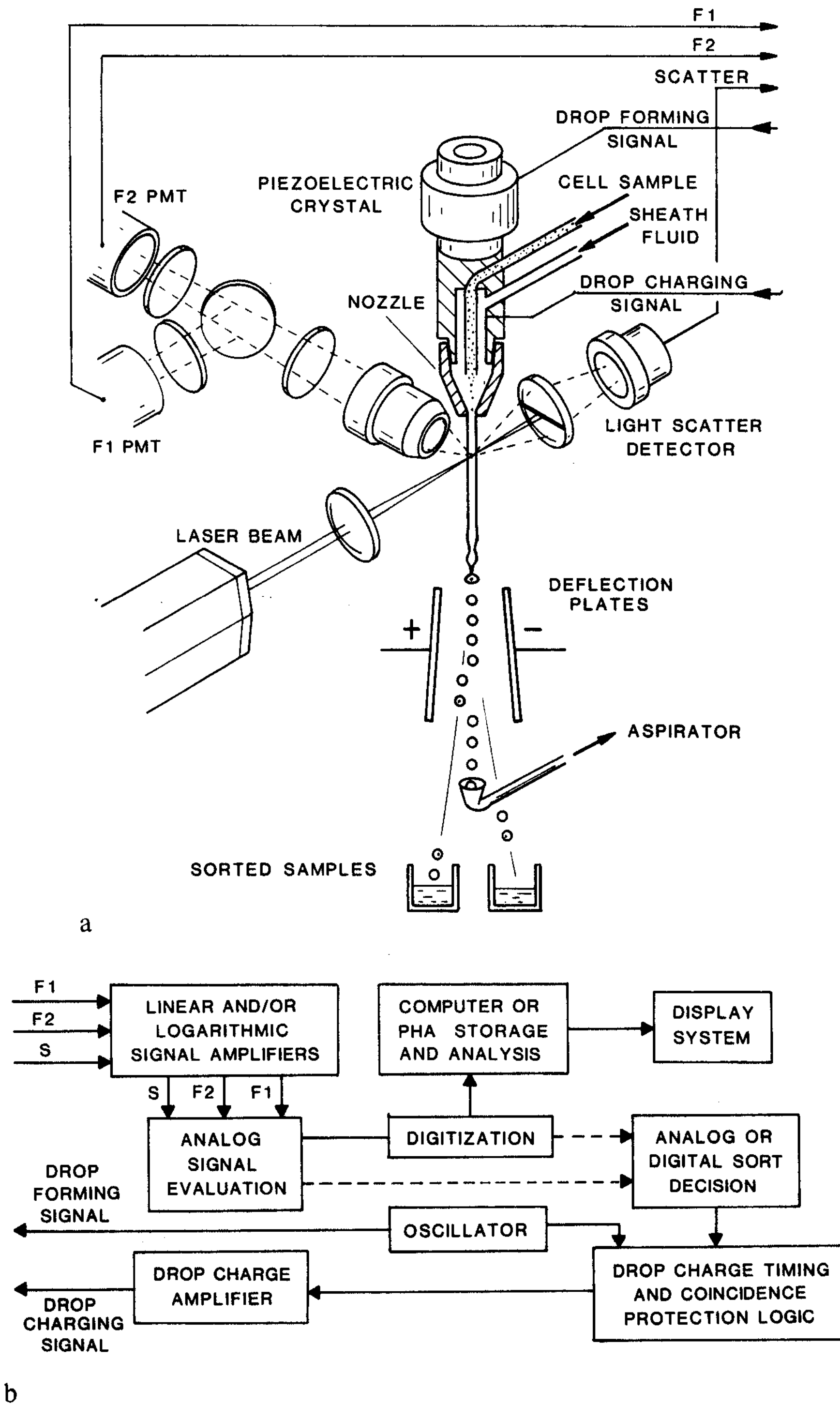


Fig. 29.1. Generalized cell sorter diagram. The functions of the components in (a) the mechanical and optical systems and (b) the signal processing and sorting electronics are described in the text.

used to reject scattered laser light and to further define the acceptance wavelengths for each PMT detector.

Proceeding to the signal processing electronics (Fig. 29.1b), the light scatter and fluorescence detector signals are amplified and evaluated. The signal levels are digitized and fed to a computer or pulse height analyser for storage and analysis. Cell frequency data in one or two of the scatter/fluorescence measurement dimensions can be displayed on a CRT screen or on a hard copy plotter to assist the investigator in visualizing and interpreting the cell population measurements.

For sorting, the nozzle assembly is vibrated by an oscillator-driven piezoelectric crystal at a frequency near the natural drop break-up frequency of the jet. This stabilizes the drop formation at that frequency, resulting in uniform drop size and a well defined time delay between detection of a cell at the laser beam intersection and incorporation of the cell into a free drop.

If a cell is to be sorted, a potential in the 100 V range is applied to the fluid inside the nozzle. Since the fluid is conductive, drops that break from the jet while the voltage is applied will carry a corresponding electric charge. The timing and duration of the applied voltage are chosen to charge one or more drops which will contain the desired cell. Two populations of cells can be sorted simultaneously by applying a positive charge to drops containing one population and a negative charge to drops containing the other. The train of drops passes between two deflection plates charged at plus and minus several thousand volts. The electric field between the plates deflects the charged drops away from the uncharged ones. The undeflected drops are removed by a central aspirator, and the deflected drops are collected in appropriate receptacles. The aspirator is very effective in preventing formation of aerosols by the undeflected drops [6]. This can be important when handling biohazard materials.

Sorting decisions are usually made by combining independent 'windows' on the signals so that a cell is selected for sorting if its light scatter signal falls within a selected range and each of its fluorescence signals falls within the range selected on that channel. In two dimensions (e.g. light scatter vs. green fluorescence) the selection criteria can be visualized as a rectangular (or polygonal) box such that cells whose signals correspond to a point in the box are to be sorted, and those outside the box are not.

Once the decision about the preferred disposition of the cell has been made (i.e. left sort, right sort or no sort) the electronics must produce the drop-charging pulse at the appropriate time. It is also usually desirable to protect the purity of sorted fractions by examining situations in which a cell that fulfils the sorting criteria is close to a cell which does not ('coincidences') so that sorting of any drop that might contain the wrong cell can be suppressed.

As the cell populations under investigation and the questions being asked about them become more complex, it becomes important to increase the array of measurements made on each cell. A number of systems have been produced using two or three lasers to excite several dyes [7–11]. Use of such systems may require three or four fluorescence detection channels and computerized collection and monitoring of the data and sorting. Also light scatter measurements in angular ranges other than the moderate forward angles mentioned above have proved useful in analysing complex populations. These additions are discussed in more detail below.

Light scatter analysis

Light scatter signals yield extremely valuable information in flow analysis and sorting. They provide reliable detection of all cell-sized objects regardless of their fluorescence and thus are generally used to initiate analysis and sort timing. Light scatter measurements also give some information on relative cell size [12], allow live–dead discrimination in some populations [13,14], and provide useful cell type discrimination in mixed populations [4,13–15]. The basic principles and theoretical considerations of light scatter measurements in flow cytometry have been reviewed extensively by Salzman [16,17].

Some general guidelines derived from theoretical calculations and from measurements on real cells may be summarized as follows.

1 Light scatter intensities for cells are highest at very small angles and are several orders of magnitude lower at large angles.

2 Light scatter is most proportional to overall cell size and least sensitive to internal structure at the smallest angles.

3 Light scatter over a range of moderate forward angles (e.g. 2–15°) shows more sensitivity to cell structure and provides good live–dead discrimination among lymphocytes.

4 At large angles in the 90° range, fine internal structure and granularity of the cell significantly affect the scatter signals.

5 Back angles (near 180°) show weak signals that seem to be similar in information content to the stronger signals at corresponding forward angles, although this may be an artifact caused by reflections of the bright small-angle scattered light.

6 Light scatter from non-spherical cells (e.g. avian erythrocytes) is affected by cell orientation, which can

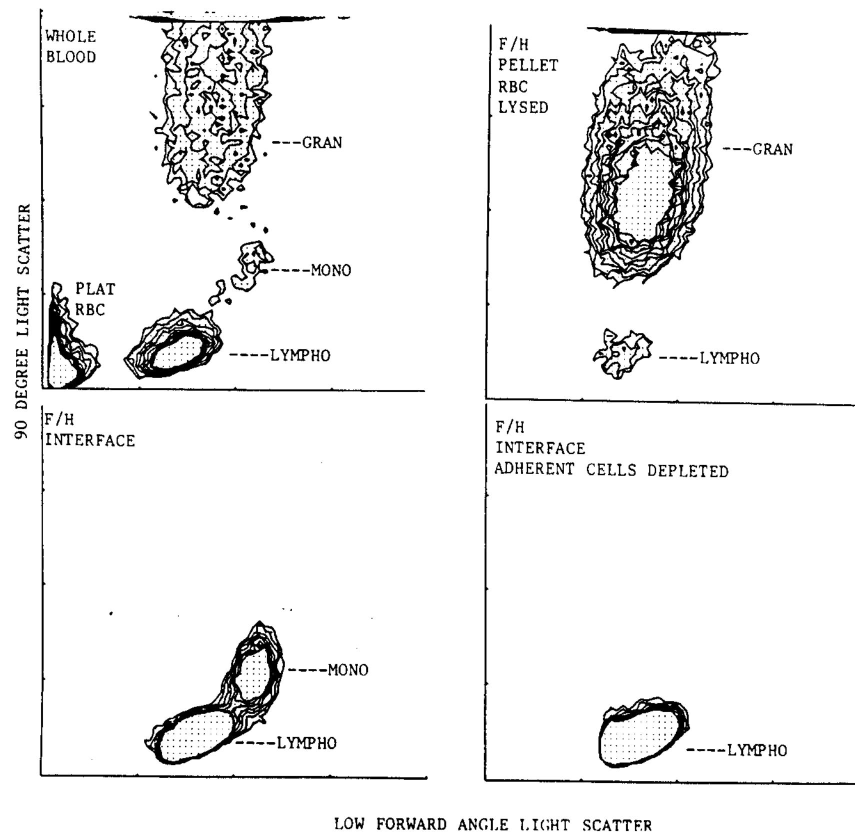


Fig. 29.2. Discrimination of cell types in human blood by two angles of light scatter. Human peripheral blood was collected and the majority of erythrocytes (RBC) were lysed by hypotonic lysis using distilled water (panel A). The majority of platelets (PLAT) were removed by centrifugation. The granulocyte (GRAN) (panel B) and mononuclear cell (containing lymphocytes (LYMPHO) and monocytes (MONO)—panel C) enriched populations were obtained from the interface and pellet of a Ficoll–Hypaque gradient, respectively. Monocytes were removed from the mononuclear cell fraction by adherence on a tissue culture-treated plastic flask (Falcon Plastics, Oxnard, CA).

Cells were analysed using a FACS 440 system (Becton Dickinson FACS Division, Sunnyvale, CA) equipped with an argon-ion laser (488 nm; 200 mW) (Spectra Physics, Mountain View, CA). Data were collected in list mode files by using a Consort 40 (PDP 11/23) computer system (Becton Dickinson FACS Division, Sunnyvale, CA). A linear measure of forward light scatter at 488 nm with an acceptance angle from about 2.5 to 12 degrees is shown on the horizontal dimension. The vertical axis plots linear measurement of '90' degree light scatter with an acceptance range from about 70 to 110 degrees. Contour levels were chosen to enclose areas with greater than 10–100 cells, at intervals of 10 cells.

result in striking differences in light scatter measurements among essentially identical cells [18].

One of the most common applications of light scatter measurements is the discrimination of different cell types in a heterogeneous population of haematopoietic cells. An example of how the major leucocyte populations in whole human blood, without any prior preparation, can be identified by flow cytometry solely on the basis of forward (3–12°) and 90° (about 70–110° range) light scatter properties is presented in Fig. 29.2. Lymphocytes, erythrocytes, monocytes and granulocytes are clearly discriminated. As is evident from the contour plots in the figure, resolution of these major subpopulations requires correlated measurement of *both* forward angle and 90° light scatter. Although the discrimination of lymphocytes from monocytes can be achieved reasonably well using only forward angle light scatter, the resolution is greatly improved using both signals. Platelets show less forward and 90° light scatter than nucleated leucocytes.

Monocytes, lymphocytes and granulocytes can also be separated using a variety of biophysical methods, such as density gradient centrifugation using Ficoll–Hypaque or Percoll, and adherence to solid surfaces.

As shown in Fig. 29.2, when the blood sample was centrifuged in a Ficoll–Hypaque gradient, the interface contained both the monocyte and lymphocyte populations (i.e. mononuclear cells), whereas the granulocytes and erythrocytes were present predominantly in the Ficoll–Hypaque pellet. Erythrocytes can be eliminated easily by hypotonic lysis [19]. Subsequent incubation of the mononuclear cells on a plastic, tissue-culture-treated dish in the presence of serum allowed the removal of monocytes by adherence. In many circumstances, it is desirable to use these methods as preparative steps to enrich the cell type of interest, prior to immunofluorescent staining and flow cytometric analysis.

Light scatter standards are useful in monitoring system stability and in detecting problems in cell samples. For example, 1–2 μm diameter plastic microspheres (Polysciences, Inc., Warrington, PA Cat. No. 15702 or 9847) provide uniform, stable samples for signal standardization. The intensity of light scatter of these microspheres as a function of the angle of detection is quite different from that of cells. Hence, small changes in detector geometry or small differences between instruments may result in substantial differences in the ratio of cell to microsphere scatter.

Measurement of fluorescence

Fluorescence excitation sources, filters and detectors

The functions of the fluorescence measuring system in a flow cytometer are (1) to yield fluorescence signals proportional to the amount of each dye on a cell, (2) to obtain sufficient signal for accurate evaluation of each dye, and (3) to minimize noise and extraneous signals, including those due to other dyes in use. Signal-to-dye proportionality can be degraded if the laser illumination is not sufficiently uniform over the part of the jet through which cells pass. This can be a serious problem if the sample fluid flow rate is too high (e.g. above 1 μl/s in most systems). Proportionality can also be lost if there is non-linearity in the electronics (due, for example, to photomultiplier tube or amplifier saturation).

The principal excitation sources used in flow cytometry are argon-ion lasers, krypton-ion lasers, tunable dye lasers and mercury arc-lamps. Sensitive measurements of immunofluorescence require intense light sources well matched to the dyes being measured if they are to be made over the short time intervals required for high cell rate analysis and sorting. In sorting systems operating at several thousand cells per second the excitation source spot size should be small to minimize the frequency of situations in which more than one cell is in the beam at one time. These requirements for high intensity and a small spot size make arc-lamp sources marginal or inadequate for sorting systems. Adequate amounts of fluorescence can be obtained in non-sorting arc-lamp systems by illuminating a larger area and by passing the cells through the beam more slowly.

Flow cell type non-sorting systems can also be designed with large numerical aperture collection lenses (e.g. N.A. 1.2) which collect several times as much light as the N.A. 0.6–0.75 lenses that are used in free jet sorting systems.

Fluorescence excitation and emission spectra of some important dyes for immunofluorescence work are shown in Fig. 29.3. The figure also shows the laser wavelength normally used to excite each dye. For all of the dyes except rhodamine the excitation efficiency at the laser wavelength is over half of the peak excitation efficiency. The use of these dyes, except for rhodamine, are discussed below under 'Dyes for immunofluorescence'.

To obtain fluorescence signals from a particular dye the authors use optical filtering which is optimized to pass fluorescent light from one dye and to discriminate against light from all other sources, including the exciting laser beam, cell autofluorescence, stray room light and, of course, light from other dyes and excitation sources in multi-fluorescence systems. Imageing the jet on to a mask with apertures passing light only from the region around each laser/jet intersection helps to minimize stray light acceptance and avoid interference in two-laser systems (see Fig. 29.4).

Since laser light scattered by a cell is normally several orders of magnitude brighter than immunofluorescence, fluorescence filters must have an attenuation approaching 10^6 at the laser wavelength. Fluorescence of the filters themselves is much less of a problem with interference filters than with coloured glass or gel absorption filters, but the latter can be used if they are kept well away from the detectors. The sensitivity of a detector to cell autofluorescence and to other dyes can be minimized by passing a narrow spectral band near the emission peak of the dye of interest, but narrowing the band also decreases collection efficiency somewhat for that dye. The optimal choice of filtering depends on the details of the system, but the authors have found that interference or interference-plus-absorption filters with a band-width of about 30 nm are generally good for single dye measurements and also adaptable to multi-dye work. 'Six-cavity' design filters can have 50% transmission at a wavelength as close as 20 nm to the exciting laser and >80% transmission in the central region of the band with $< 10^{-6}$ transmission at the laser wavelength. Depending on the wavelength difference between the laser line and the dye emission peak the authors have obtained filters (most recently

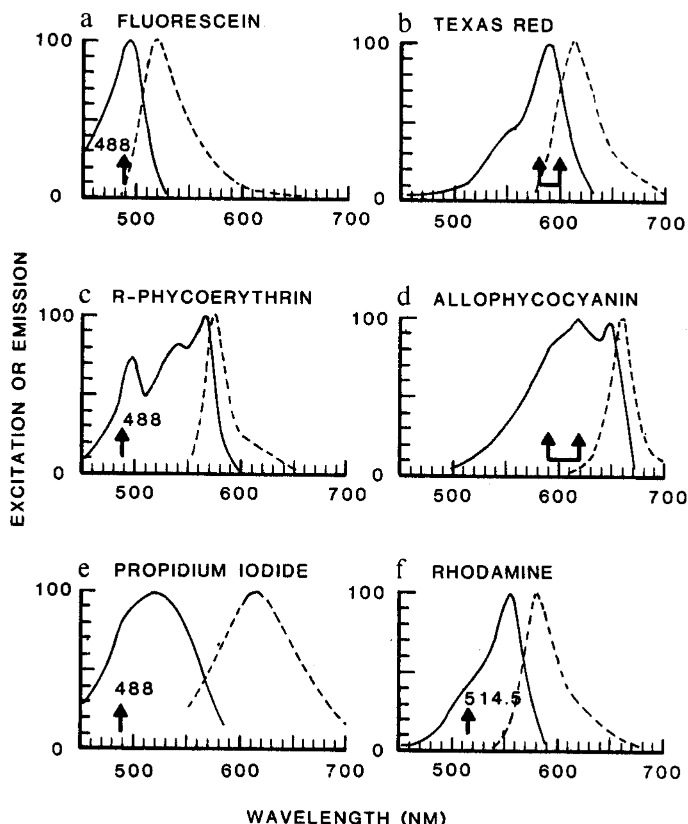


Fig. 29.3. Excitation and emission spectra of dyes used in flow cytometric immunofluorescence studies. Excitation spectra (solid lines) and emission spectra (broken lines) were taken with a SPEX Fluorolog instrument and are uncorrected. The vertical scale is in arbitrary units. The arrows in panels a, c, e and f mark the argon-ion laser lines normally used to excite the dyes. The arrowed ranges in panels b and d mark the best range for excitation using a tunable dye laser. R-phycoerythrin (c) was from the red alga *Gastroclonium coulteri*, and the allophycocyanin (d) was from a cyanobacterium.

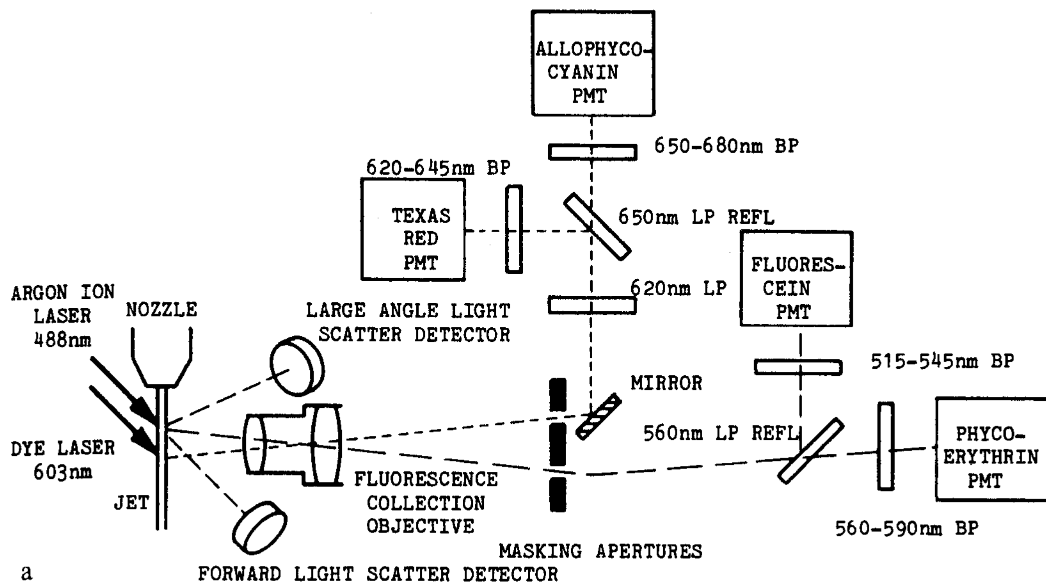
from Becton Dickinson, FACS Division, Sunnyvale, CA) which transmit 35–55% of the total dye emission. Dichroic reflector interference filters which reflect some wavelengths and pass others are useful for efficient separation of signals when two dyes are excited by one laser.

Filters can be tested for blocking of scattered laser light by running non-fluorescent particles such as 2 μm diameter polyvinyltoluene microspheres (Duke Scientific Co., Palo Alto, CA, Cat. No. 115). Comparing fluorescence detector signals with neutral-density filters and with the colour filters alternately in the filter holder gives an estimate of the blocking of the laser wavelength by the colour filters.

Photomultiplier tubes (PMTs) are used for low level fluorescence detection because they offer very-high-gain/very-low-noise amplification of the photoelectron signal produced by the fluorescent light. They also have good linearity over a wide range of signal levels. The spectral sensitivity of a PMT depends on the photocathode material. In the visible range, quantum efficiency usually decreases at increasing wavelengths, so it is important to use PMTs that have good red sensitivity if dyes emitting much beyond 600 nm are to be used.

Measurement systems for immunofluorescence

The optical systems used for fluorescence measurements can be simple or fairly complex. Fig. 29.4 illustrates two-laser, two-scatter, and four-colour immunofluorescence systems, which are the most complex configurations used by the authors. Other single and multiple fluorescence measurements can be carried out using appropriately simplified versions of these systems. In the system as illustrated in Fig. 29.4a, an argon-ion 488 nm laser beam excites both fluorescein and phyco-erythrin. Light collected by the objective is imaged on to one aperture and divided by a dichroic reflector. Band-pass filters define the final acceptance wavelengths for each detector. The intersection of the 600 nm dye laser beam with the jet is imaged on to a second aperture, and fluorescences from Texas red and from allophycocyanin are divided by another dichroic reflector, band-pass filtered and



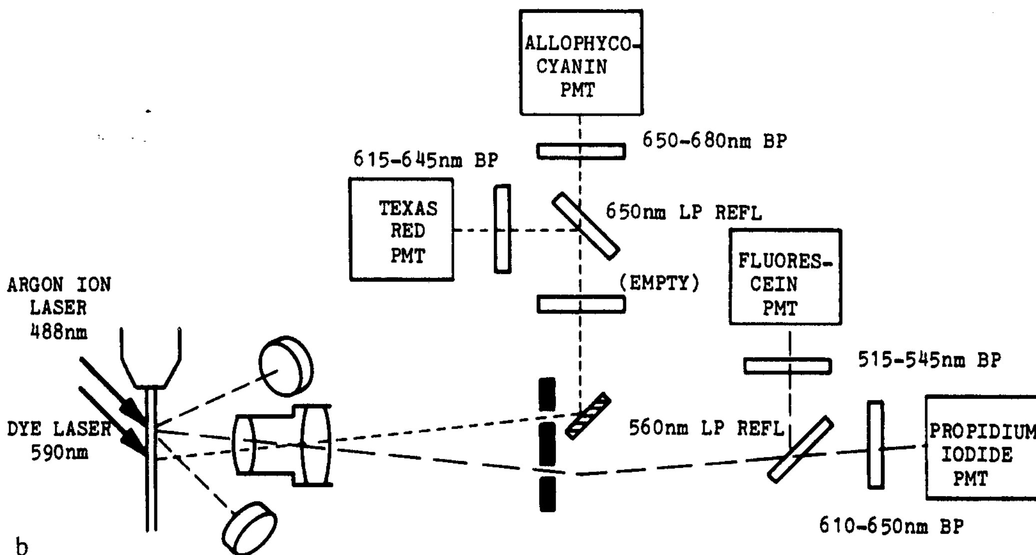


Fig. 29.4. Optical systems used for measurements on immunofluorescent stained cells. (a) Shows a two-laser/four-immunofluorescence system for measurements of fluorescein, phycoerythrin, Texas red and allophycocyanin. This system or appropriate simplifications of it are also used for two-colour immunofluorescence with fluorescein and phycoerythrin or (with the dye laser wavelength at about 615 nm) for three-colour immunofluorescence with fluorescein, phycoerythrin and allophycocyanin.

(b) Illustrates the system arranged for two-laser/three-immunofluorescence (fluorescein, Texas red and allophycocyanin) with propidium iodide detection for dead cell exclusion. This arrangement is also used for fluorescein, Texas red two-colour immunofluorescence with propidium iodide.

Optical filters are designated by their 50% transmission wavelength(s) and their type: LP, long wavelength passing filter, BP, band-pass filter, LP REFL, long wavelength passing dichroic reflector. Some components in these diagrams have been rotated from their actual orientations to allow the drawing to be made in one plane.

detected. The two one-laser/two-detector subsystems are basically independent systems in one machine.

Spectral overlap correction when one laser excites two dyes

In the systems shown in Fig. 29.4, two dyes are excited by a single laser beam. The filters in the appropriate subsystem are selected to make each detector much more sensitive to one dye than to the other, but, in general, each detector has some sensitivity for both dyes. Since the relation between the signal components on each detector is linear (e.g. doubling the fluorescein emission doubles the fluorescein-dependent signal on both fluorescein and phyco-erythrin detectors), linear combinations of the two detector signals can be constructed that represent each of the dyes alone [20]. The transformation to obtain two signals that are each dependent on only one dye can be performed simply by making analogue combinations of the two detector signals in the fluorescence amplifiers (a process sometimes referred to as 'compensation'). The amounts of compensation necessary are determined by running samples containing only one dye or the other and adjusting to zero the output for the 'wrong' dye. This adjustment is best done using a linear amplifier output even if a logarithmic amplifier is used for actual data collection. (The mixing must be done before, not after logarithmic amplification!) Changes in PMT voltage will require readjustment of the compensation. The resulting signals can be processed like direct detector signals. For analytical purposes the transformation described above could be performed after-the-fact by processing recorded list mode data, but the authors find analogue real-time correction to be preferable since the signals they see, which are used to define sorting conditions, correspond directly to the final recorded data.

Fluorescence standards, quantification and system calibration

When aligning and tuning up a cell sorter it is very convenient to run a sample of uniform particles which give signals on each light scatter and fluorescence detector. Uniformity of the particles in light scatter properties and in fluorescence makes it easier to find optimum adjustment positions and helps to detect problems in the flow system or electronics that degrade measurement quality. In multiple fluorescence conditions the authors often use a mixture of two types of plastic microspheres as a tuning and calibration sample (e.g. Polysciences, Inc., Warrington, PA, Cat. No. 15702 and 15703). These are kept frozen in sterile 2 ml aliquots at 2×10^6/ml of each type of microsphere.

In order to make valid comparisons from one experiment to another it is useful to have standard calibration conditions so that a particular signal level corresponds to a constant amount of dye per cell. This can be accomplished by adjusting the signals from stable calibration particles to a standard output level. As long as the excitation wavelength and the emission filters and detectors are not changed, it is not necessary for the fluorescence spectrum of the calibration particles to match that of the relevant cell labelling dye.

True standardization that will allow valid comparisons of staining levels from one machine or laboratory to another is best accomplished with particles whose fluorescence spectrum is the same as that of the dye being standardized. At present there is no generally accepted set of stable reference particles with spectra matching immunofluorescence labels, but such a set would be quite useful. The authors have produced usable standards for fluorescein, Texas red, phyco-erythrin and allophycocyanin by labelling non-fluorescent microspheres (Polysciences, Inc., Warrington, PA, Cat. No. 8226) non-covalently with the appropriate fluorochrome-conjugated avidin. This can be accomplished simply by incubating the microspheres with an optimal amount of fluorochrome-conjugated avidin for 30–60 min at room temperature followed by extensive washing of the microspheres. Conjugated microspheres can be stored in frozen aliquots. (The aliquots should be thawed at room temperature.)

Autofluorescence

Many types of fluorescence measurements are possible using flow cytometry. The inherent fluorescence of a cell, often referred to as 'autofluorescence', usually appears as a background signal against which other specific fluorescence must be measured. In many cases cell autofluorescence rather than instrument sensitivity sets the real limit on our ability to estimate the amount of reagent on a cell. Autofluorescence is a function of cell size, cell type, excitation wavelength and emission detection range. The autofluorescent molecules are normal cell constituents like flavins [21] and cytochromes which tend to be found in greater quantity in larger cells. Cultured cells tend to be more autofluorescent than corresponding 'fresh' cells, and dead tissue culture cells can show enough greater autofluorescence than live cells for this to be used as a criterion of non-viability [22]. Cell samples such as spleen and bone marrow include not only lymphocytes and other low autofluorescence cells but also a variable-sized fraction of much brighter cells which can interfere with efforts to define and characterize small subpopulations of cells by immunofluorescence.

The effects of autofluorescence can be minimized by

using brighter reagents and by optimizing optical filtering to match the dye of interest. Also, changing to longer wavelength excitation tends to decrease autofluorescence. The authors often observe better separation between stained and unstained cells with Texas red and dye laser excitation (590 or 600 nm) than with fluorescein and 488 nm excitation.

Dyes, reagents and staining for single and multiple immunofluorescence

Dyes for immunofluorescence

Most fluorescence measurements are obtained from cells stained with specific fluorescent reagents. There are a number of probes that allow fluorescence measurements of cellular DNA content, RNA content, protein, membrane potential, membrane fluidity, intracellular pH and other properties [23–25]. In this chapter, the authors address primarily the use of cell surface immunofluorescence, i.e. measurements on cells using fluorochrome-labelled antibodies or other ligands such as avidin.

Several characteristics are important in a dye to be used for immunofluorescence.

1 It must absorb strongly at wavelengths for which a good excitation source is available.

2 Since signal brightness is often the limiting factor in immunofluorescence measurements, the dye should have a high quantum efficiency (ratio of emitted to absorbed light).

3 A large Stokes shift (i.e. a large wavelength difference between the exciting and emitted light) is desirable to permit easy discrimination of fluorescent light from background due to the excitation light source.

4 It must be possible to couple the dye to an antibody or other specificity-conferring molecule without disrupting that specificity or the dye fluorescence.

The spectral and biochemical properties of the dyes used for immunofluorescence studies are summarized in Table 29.1 and Fig. 29.3. The majority of immunofluorescence studies in the past have been limited to the use of a single fluorochrome, usually fluorescein. This was because fluorescein isothiocyanate could be easily coupled covalently to antibodies without loss of antibody activity or excessive dye quenching. Fluorescein also shows good quantum yield (about 0.5 on protein) and can be excited efficiently with the 488 nm light from an argon-ion laser. Other immunofluorescence studies employed antibodies conjugated to rhodamine dyes. Tetramethylrhodamine-conjugated antibodies can be excited using the 514.5 nm argon-ion laser line; however, this results in suboptimal excitation of the dye. The mercury arc lamps commonly used in fluorescence microscopy and in some flow cytometers are well matched to excite rhodamine. X-RITC (Research Organics, Cleveland, OH) and Texas red (Molecular Probes, Junction City, OR) are newer rhodamine derivatives which demonstrate longer wavelengths of excitation and emission than the parent compound. Either dye can be excited by a krypton-ion laser (568 nm line) or, more effectively, by a tunable dye laser. The major limitation of X-RITC is its hydrophobic nature, which often results in insolubility of protein conjugates. Texas red is more soluble and has been used most successfully as an avidin conjugate [25]. Although some direct monoclonal

Table 29.1. Biochemical and spectral properties of dyes for immunofluorescence

Dye	Molecular weight (approx.)	Extinction coefficient	Absorption maxima	Emission maximum	Quantum yield
B-phyco-erythrin	240 000	2 410 000	545,565	575	0.98
R-phyco-erythrin	240 000	1 960 000	495,545,565	578	0.82
C-phycocyanin	224 000	1 690 000	620	650	0.51
Allophycocyanin	104 000	700 000	650	660	0.68
R-phycocyanin	103 000	760 000	555,618	634	0.7
Fluorescein isothiocyanate	390	80 000	495	525	0.5
Tetramethyl-rhodamine	444		555	582	
X-RITC	547		582	601	
Texas red	625	84 000	596	615	

A majority of the data in this table are from data sheets of Molecular Probes, Inc., Junction City, OR. Some data are from the authors' own measurements.

antibody conjugates have been produced [25], the authors have not generally found theirs to be satisfactory. At high coupling ratios this dye also demonstrates significant quenching of fluorescence.

A family of natural fluorochromes derived from photosynthetic cyanobacteria and red algae, known as phycobiliproteins [27,28], have been recently applied to immunofluorescent labelling [29] (see also Chapter 31 in this handbook). The principal types of phycobiliproteins are phyco-erythrin, phycocyanin and allophycocyanin. Each whole phycobiliprotein molecule is a complex of several polypeptides with open-chain tetrapyrrole chromophores attached. Different organisms have different forms of these molecules, resulting in somewhat different spectral properties. These fluorochromes have high quantum yields and can be coupled to antibodies using bi-functional cross-linking agents such as SPDP [29] or SMPB (R.R. Hardy, personal communication; both reagents are available from Pierce Chemical Co., Rockford, IL) and do not impair antibody activity.

As immunofluorescent labels, phycobiliproteins have several advantages:

(1) they can be excited efficiently over a wide range of wavelengths (see excitation spectra in Fig. 29.3, panels c and d);
(2) each molecule carries many chromophores yielding high extinction coefficients, and the quantum yields are high;
(3) the Stokes shift can be large, making it easy to discriminate exciting from emitted light;
(4) phycobiliproteins are highly soluble in water;
(5) their fluorescence is essentially pH independent and not easily quenched by biological materials.

Methods for immunofluorescent staining: single colour and general considerations

Numerous immunofluorescence assays have been developed using conventional antisera and monoclonal antibodies. In assays on cells, antibody directed against the surface marker of interest is allowed to react with the cells, and then the amount of bound antibody is determined. Immunofluorescence assays can be either direct (in which the primary antibody is fluorescent labelled) or indirect (in which the primary antibody is revealed by the binding of a second fluorescent labelled reagent). For optimal staining specificity it is important to remove aggregated material from reagents before use. This can be accomplished, for example, by centrifugation for 10 min at 20 000–50 000 *g* in a microfuge or airfuge. The methods for direct and indirect immunofluorescence used in the authors' laboratories are presented in Table 29.2. A list of the necessary controls and the relative advantages and disadvantages of direct versus indirect immunofluorescence are also presented. Additionally, a description of several different systems used for indirect immunofluorescence are described in Table 29.3. (See also Chapter 23 in this handbook.)

Several modifications of the standard methods have been developed to increase sensitivity. For example, in a three-stage indirect immunofluorescence system, cells are labelled with a mouse monoclonal antibody, washed, incubated with FITC goat anti-mouse IgG serum, washed, incubated with FITC $F(ab')_2$ rabbit anti-goat IgG serum, washed, and examined. This has permitted detection of low density antigens which could not be detected using a conventional two-stage indirect immunofluorescence method [30]. The use of multiple-step indirect immunofluorescence procedures using combinations of any of the method systems listed in Table 29.3 is limited primarily by the increased non-specific staining resulting from the use of multiple reagents.

Some cells carry Fc receptors which can bind antibody reagents 'non-specifically'. For such cells it is desirable to use $F(ab')_2$ fragments or compare staining to that with isotype-matched control antibodies. Among haematopoietic cells, monocytes, macrophages and granulocytes show the most Fc binding. Although B lymphocytes, NK cells and a minor population of T lymphocytes also possess Fc receptors, they are either less avid or fewer in number than on the myeloid-derived cell populations. In the authors' experience with non-specific binding of mouse antibodies to mouse and human leucocytes, IgG2 > IgG1 > IgM in non-specific binding. For indirect immunofluorescence, affinity purified $F(ab')_2$ fluorochrome-conjugated second antibodies against the Ig of several species are readily available. If non-$F(ab')_2$ second antibody is used, goat antibodies are less reactive with human and mouse Fc receptors than rabbit antibodies [31]. Finally, these commercial goat or rabbit anti-Ig sera, even if affinity purified, often cross-react with immunoglobulins of other species and, therefore, must be absorbed appropriately if necessary.

A final technical consideration is the handling and storage of the stained cells prior to analysis. With fresh, viable sample preparations, it is necessary to prevent loss of the surface antigens by modulation or catabolism and to prevent cell death. This can be accomplished best by suspending the cell pellet in a nutrient-containing medium with a low concentration of serum or serum proteins which is supplemented with sodium azide (0.1%) to inhibit antigen modulation. The samples should generally be held at 4 °C until analysis, to prevent modulation of cell surface antigen.

An alternative and in some cases preferable method is to fix the cells after immunofluorescence staining.

Table 29.2. Immunofluorescence techniques

Direct immunofluorescence	
Procedure	Cells + optimal amount of fluorochrome-conjugated antibody—incubate for 15 min at 4 °C; wash cells twice; analyse
Necessary controls	Unstained cells; cells stained with fluorochrome-conjugated non-reactive antibody of the same isotype as the experimental antibody
Advantages	Simple and fast; allows quantification of binding sites
Disadvantages	Requires that all antibodies be directly conjugated to fluorochrome; less sensitive than indirect immunofluorescence
Indirect immunofluorescence	
Procedure	Cells + optimal amount of unlabelled antibody—incubate for 15 min at 4 °C; wash cells twice; add optimal amount of fluorochrome-conjugated second reagent—incubate for 15 min at 4 °C; wash cells twice; analyse
Necessary controls	Unstained cells; cells stained only with fluorochrome-conjugated second reagent; cells stained with a non-reactive antibody of the same Ig isotype as the experimental antibody + fluorochrome-conjugated second reagent.
Advantages	Increases sensitivity of detection; does not require that all antibodies be conjugated directly to fluorochrome
Disadvantages	Lengthy procedure; may increase non-specific background staining

All steps should be carried out at 4 °C in medium containing 0.1% sodium azide to inhibit modulation and capping of surface antigens.

Stained leucocytes can be fixed in a solution of 2% paraformaldehyde in normal saline (pH 7.4) or phosphate-buffered saline, as described previously [32]. The forward angle and 90° light scatter, Coulter volume and immunofluorescence of human and murine leucocytes fixed in this manner were essentially identical to those of the fresh, unfixed cells. Fixed cells could be stored in the fixative at 4 °C in the dark for at least two weeks before analysis. After an extended period of storage (e.g. several weeks), the autofluorescence of the fixed cells increased noticeably.

Methods for multiple colour immunofluorescence

The requirements for designing and performing multicolour immunofluorescence assays are summarized in Table 29.4. Until recently the use of multicolour immunofluorescence was limited largely by the small number of suitable fluorochromes. New dyes like Texas red and the phycobiliproteins are overcoming this problem. Texas red and fluorescein are becoming a commonly used pair for two-colour immunofluorescence. Since the excitation spectra of these dyes are quite different, two lasers are needed, an argon-ion laser (488 nm) for excitation of fluorescein and a dye laser (tuned near 590 nm) or a krypton-ion laser (568 nm) for excitation of Texas red. More recently, it has been shown that the combination of fluorescein and phyco-erythrin (R or B type) is advantageous in that both dyes can be excited using a single argon-ion laser (488 nm) or a mercury arc light source (485 nm). Signal adjustments are necessary to correct for the small amount of fluorescein emission reaching the phyco-erythrin detector and vice versa. Details of this technique have been presented previously [11]. Single laser two-colour immunofluorescence with fluorescein and phyco-erythrin provides a very workable system, but the spectral overlap adjustments must be set correctly for reliable data interpretation. The near total independence of fluorescein and Texas red signals in a two-laser system means that no adjustments are necessary, even when measuring a small

Table 29.3. Systems for indirect immunofluorescence

Primary antibody	Fluorochrome-conjugated reagent	Comments
Unconjugated	Second antibody hetero-antisera (e.g. FITC goat anti-mouse IgG serum)	Advantage: very sensitive system Disadvantage: batch-to-batch variation, requires absorption, and/or affinity chromotography to avoid high background staining
	Monoclonal antibody (e.g. FITC mouse anti-rat κ monoclonal antibody)	Advantage: batch-to-batch consistency; generally higher specificity; absorption not required in preparation; low background staining Disadvantage: less sensitive since monoclonal antibody recognizes only few sites on the primary antibody compared to conventional polyvalent sera
Unconjugated	Protein A	Advantage: batch-to-batch consistency Disadvantage: less sensitive than other indirect methods; requires that the primary antibody bind to protein A (e.g. protein A binds strongly to mouse IgG2 antibodies, but only weakly to IgG1 or IgM); will bind to cytophilic Ig in sample causing high background staining
Biotin-conjugated	Avidin	Advantage: high avidity of binding between biotin and avidin; sensitive method of detection. Disadvantage: requires that primary antibody be biotin conjugated; avidin may bind non-specifically to monocytes and lymphocytes
Hapten-conjugated (e.g. ars, TNP, etc)	Anti-hapten antibody heteroantisera (e.g. rabbit anti-monoclonal antibody) (e.g. mouse anti-TNP monoclonal antibody)	Advantage: sensitive system; low background Disadvantage: requires that primary antibodies be hapten conjugated

Table 29.4. Two-colour or multicolour immunofluorescence assays

1 Requires that the two (or more) antibodies be conjugated with two (or more) fluorochromes which: can be excited by a single light wavelength and distinguished by special emission; or can be excited by different excitation wavelengths and the emissions distinguished either by spectral differences or by spacial and time differences.

Necessary controls: for single excitation, dual emission systems, samples stained individually with each fluorochrome must be analysed to ensure that the emission of one dye is detected optimally in the appropriate detector system and not in the detector system of the other fluorochrome (e.g. fluorescence from FITC dye is detected in the green fluorescence channel and not in the red fluorescence channel used for phyco-erythrin).

2 Requires that each fluorochrome-conjugated antibody does not interfere with the specificity of the antibody reaction or fluorochrome characteristics of the other reagents in the assay.

Necessary controls: requires that each fluorochrome-conjugated antibody be analysed individually. The fluorescence emission profile (e.g. fluorescence histogram) of the single stained sample should be qualitatively and quantitatively identical to the results using the reagent in combination with all other reagents in the assay.

3 Can be performed using directly fluorochrome-conjugated monoclonal antibodies or with a combination of the techniques listed in Table 29.3 above, provided the appropriate controls are performed to ensure that there is no interference between reagents.

4 Commonly used multicolour dye combinations for immunofluorescence flow cytometry:

Dyes	Excitation light source	Emission (nm)	Application
Fluorescein/Texas red	Argon ion laser Krypton or dye laser	515–545 >600	Two-colour surface fluorescence
Fluorescein/phyco-erythrin	Argon or mercury arc	515–545 560–590	Two-colour surface fluorescence
Fluorescein Phyco-erythrin Texas red or allophycocyanin	Argon Dye	515–545 560–590 515–545	Three-colour surface fluorescence
Fluorescein/propidium iodide	Argon or mercury arc	>620	Surface fluorescence and DNA content

The 488 nm argon laser line or the 485 nm band of mercury arc lamp are used for excitation of fluorescein, phyco-erythrin, and/or propidium iodide in multicolour analysis. For excitation of Texas red, the 568 nm light from a krypton ion laser or 600 nm light from a tunable dye laser are used.

amount of one dye on a cell carrying a large amount of the other. A summary of the several multicolour dye combinations used in the authors' laboratories are presented in Fig. 29.4.

With the currently available fluorochromes and two-laser excitation systems the authors have found three-colour and four-colour immunofluorescence to be both possible and valuable. Technical details of the reagents and instrumentation are described elsewhere [9,11]. The combination of fluorescein, phyco-erythrin, Texas red and allophycocyanin provides four possible immunofluorescences. Measurements of the correlated expression of multiple cell surface antigens and biophysical properties now makes it feasible to define small, discrete subsets of leucocytes. Moreover, by cell sorting it is possible to isolate these small subpopulations for functional studies. A final cautionary note, however, is that the use of multiple fluorochromes does require that appropriate controls be included in the experimental designs (see Table 29.4).

Propidium iodide labelling of dead cells

Propidium iodide (PI) is a dye that quantitatively stains cellular DNA and double stranded RNA [33]. PI is excluded efficiently from intact plasma mem-

branes (i.e. living cells), but readily penetrates dead cells. Therefore it can be used as an indicator to discriminate living and dead cells in a sample so that dead cells can be excluded from analysis. This has been particularly useful in work involving rare cells or small subpopulations of cells [34], but it is also useful in routine analyses. For this application about 1 μg/ml PI (a much lower concentration than that used in saturation staining for DNA quantification) is added in the final wash medium after immunofluorescence staining. Several minutes' exposure to the dye results in distinct labelling of the dead cells with no detectable effect on live cells. Excitation and emission spectra of PI-labelled dead cells are shown in Fig. 29.3e. PI excites well at 488 nm but emits fluorescence mostly above 600 nm. These characteristics make it possible to measure PI along with fluorescein and/or Texas red or phyco-erythrin. Combining PI staining and light scatter criteria, it is possible to 'gate' the incoming data to assure that further analysis is carried out only on live cells.

Collection, display and analysis of flow cytometric data

Linear and logarithmic fluorescence measurement

The choice between linear and logarithmic amplification depends on the range of signal levels to be measured and on the types of distributions expected from the measurements. Logarithmic amplifiers are particularly valuable for immunofluorescence measurements where we may have (in a single sample) cells with only their autofluorescence, stained cells a few times as bright and stained cells hundreds of times as bright. A logarithmic amplifier gives good resolution over the whole range. In addition, stained cell populations in logarithmic displays tend to have a symmetrical shape, which is helpful in choosing break points between populations for analysis and sorting.

Linear amplifiers are appropriate for signals that vary over a relatively small range and/or represent biologically linear processes. Measurements of forward light scatter or cellular DNA content fall in this category. For large angle light scatter measurements the authors have found that the wide range of signal levels makes logarithmic amplification desirable.

One useful aspect of logarithmic displays for immunofluorescent stained cell populations is that the shape of the distribution in the display is not affected by changes in the photomultiplier tube voltage (i.e. gain) or by use of brighter or less bright reagents. These changes merely move the whole population up or down on the display scale. Thus, once a logarithmic amplifier is adjusted to give the desired channel resolution (e.g. so many channels per decade), there are no day-to-day or sample-to-sample adjustments to be made.

In multiple immunofluorescence work, linear/linear two-dimensional displays tend to be very difficult to interpret while log/log displays are much easier. This difference is illustrated in Fig. 29.5, which shows data from one cell sample displayed in four ways. In both the dot display and the contour plot formats the log/log version shows the cell populations more clearly.

While the authors originally began to use logarithmic amplifiers for immunofluorescence data just to handle the wide range of observed signals without continual changes in amplifier gain, they have concluded that logarithmic processing is in fact more appropriate biologically in many cases. The authors' observations are that immunofluorescence distributions often look approximately 'normal' in logarithmic display. This is what would be expected if the amount of the antigen on the surface were controlled by the multiplicative effects of a series of synthesis and transport rates. In such cases any statistical tests that assume normal distributions are better performed on the logarithmic data [35].

Data storage

The usual way to record flow cytometric data is to compile digitized measurements on a statistically adequate number of cells into a data file. The actual methods of data storage and manipulation will depend on the particular instrument and computer system used. There are many different commercially available and customized systems that have been developed. Although each has different features, in general data are stored as histograms, correlated two-dimensional arrays or as lists with a digital value for each measurement on each cell (list mode). In situations where only single parameter data are of interest, histograms provide the most efficient storage. If correlated data of only two parameters are needed, e.g. forward angle light scatter versus green fluorescence, two-dimensional arrays and list mode storage are roughly comparable in efficiency. For three- or more dimensional data, list mode storage is clearly necessary. While list mode data require considerable storage space, the operation of the FACS can be more efficient and fewer cells are needed since immediate data analysis decisions are not required while the sample is being run. The experiment can be 'recreated' and the correlation between multiple independent parameters examined on the computer at a later time.

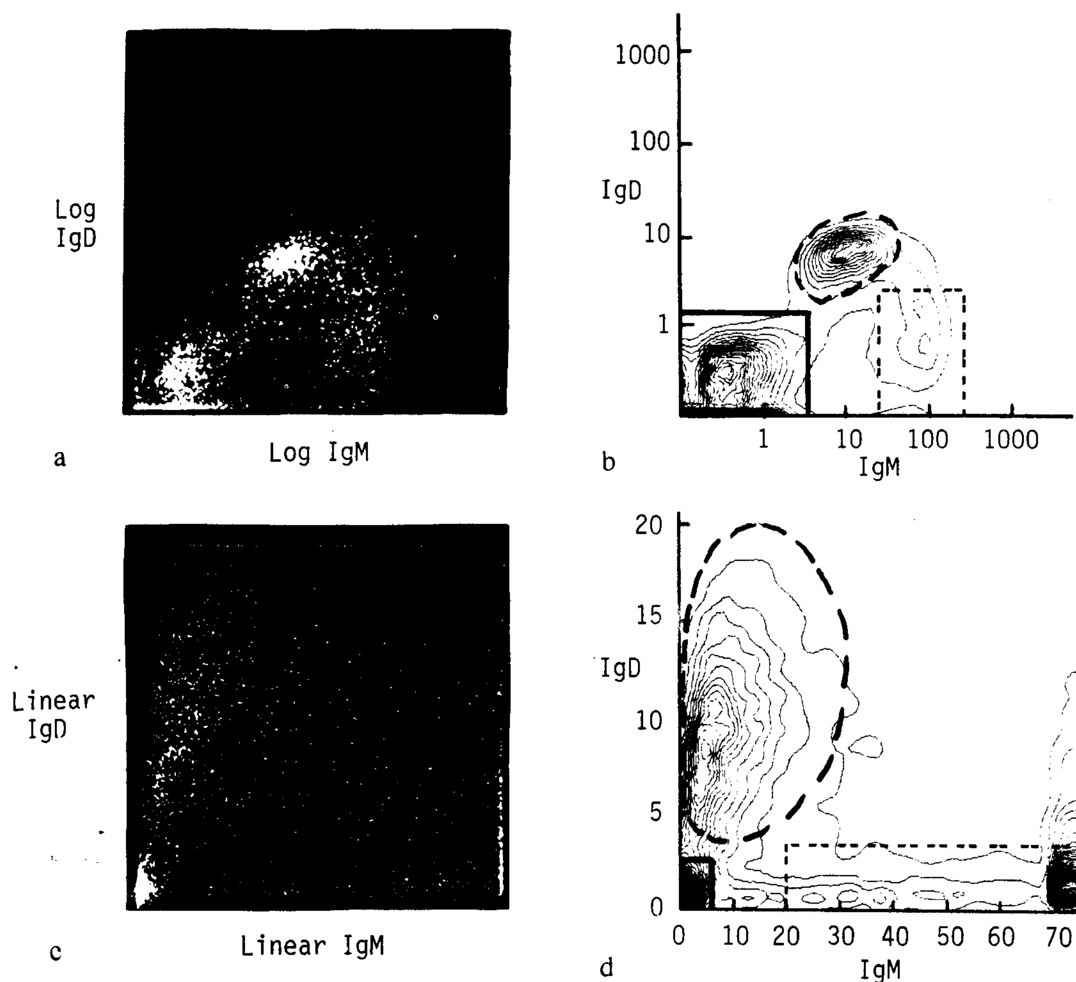


Fig. 29.5. Logarithmic vs. linear dot displays and contour maps of dual immunofluorescence data. Mouse spleen cells were stained with fluorescein-conjugated antibody to IgM and biotin-conjugated antibody to IgD, which was revealed by a second step of Texas red avidin. Measurements were made with a two-laser FACS. All of the fluorescence data were light scatter gated on the main lymphocyte population. In panels (a) and (c), each dot represents the measurements on one cell. Panels (b) and (d) show contour plots of the logarithmic and linear data, respectively, with constant 'vertical' spacing between contours. The rectangular boxes and broken lines enclose corresponding regions (i.e. the same cell populations) in the two displays.

Analysis of one-dimensional data

For presentation of single parameter data the usual display is a graph of the histogram referred to above. In these plots, relative amount of light scatter or fluorescence (represented by the 'channel number', 'fluorescein units', etc.) is presented on the x axis, and the frequency of cells on the y axis. The scale of the x axis is either linear or logarithmic, depending on the amplification chosen when the experiment was performed. In the example shown in Fig. 29.6a, human peripheral blood mononuclear cells were stained with fluorescein-conjugated antibody to Leu 11a (a natural killer cell associated antigen, Fc receptor) [36]. An isotype matched fluorescein-conjugated protein was used for control staining. Cells were analysed on a FACS 440 system with argon-ion laser excitation (200 mW at 488 nm). The log fluorescence histogram of the lymphocytes is presented. The x axis is represented as 256 channels which span 5.3 decades (i.e. 48 channels represent a factor of 10 in signal level). The histogram of the anti-Leu 11a stained cells (solid line) is superim-

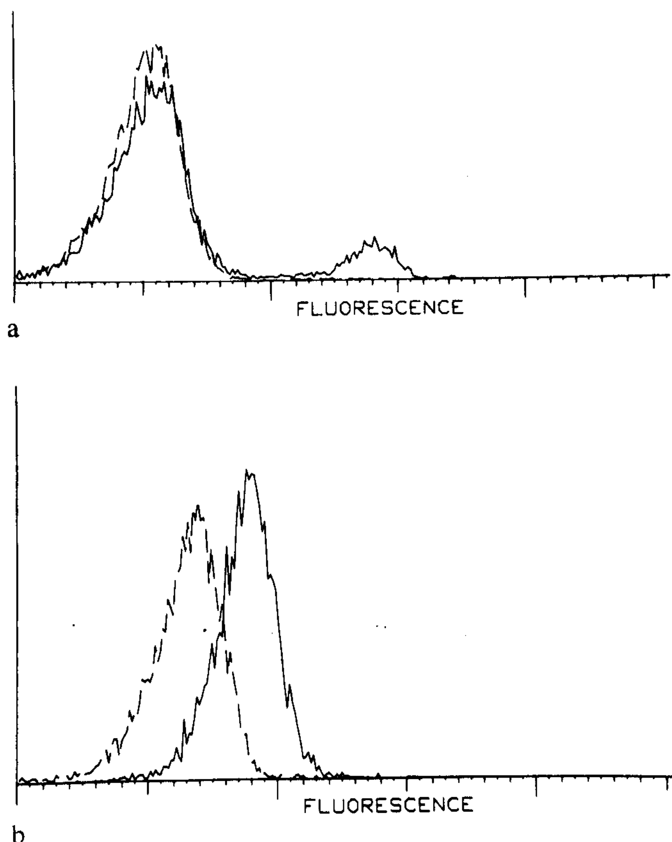


Fig. 29.6. Histogram data for quantitative analysis. In (a) human blood mononuclear cells were stained with anti-Leu 11 monoclonal antibody (solid line) or a matched (IgG1) myeloma protein (broken line), followed by FITC goat anti-mouse IgG sera. In (b) a cloned human T cell line, HPB-ALL, was stained with anti-Leu 1 monoclonal antibody (solid line) or an IgG2a myeloma protein (broken line), followed by FITC goat anti-mouse IgG sera.

Cells were analysed using a FACS 440 system. In each histogram, the Y axis represents relative number of cells, and the X axis is divided into 256 channels, spanning 5.3 decades, representing the amount of fluorescence.

posed on the histogram of the control stained cells (broken line).

It is evident that the profile for the anti-Leu 11a stained cells is bimodal, indicating the presence of two discrete populations. The next question then is how to extract a number or a few numbers which convey the basic features of the staining distribution. In this case the fractions of 'positive' and 'negative' cells are appropriate. If the populations are not totally distinct, other criteria must be included to enumerate positive and negative populations. Therefore, in any publication specifying 'percentage positive cells', it is important that the actual criteria used for these calculations be fully documented. There are several ways to select reasonable division points for estimating the frequencies. For example, it is common to use the intersection of the control and stained histograms as the marker. Another method is to set the dividing point so that a predefined 'background' of 'negative control' cells (typically 1–5%) is in the positive zone.

When there is significant non-specific staining in the control samples and/or if the antigen of interest is present in small amounts on the cells, the distinction between positive and negative cells becomes ambiguous. An example of this situation is shown in Fig. 29.6b. Here a cloned, cultured human tumour cell line, HPB-ALL, has been stained with antibody to Leu 1. The histograms of the negative control and anti-Leu 1 stained cells are superimposed. Examination of the histogram indicates that the distribution is not bimodal. In this example, there is no indication that two qualitatively distinct populations (i.e. antigen positive and antigen negative) exist. Rather, since this is a cloned cell line, the more likely interpretation is that all cells express the antigen, but that only a few of these cells express sufficient quantities of antigen to be detected as 'positive' by direct immunofluorescence. In such a case 'percentage positive' is not an appropriate way to characterize the staining pattern. Mean stained signal level minus mean control signal level would be better.

Other statistical properties of cell distributions, such as median, variance or skew, are sometimes useful in characterizing cell populations. Again, the criteria and statistical methods should be specified in any publication of the data. Means calculated on

logarithmic data are geometric means with respect to the original data. In making certain comparisons, such as average cell fluorescence by FACS and by bulk fluorometry, means of linear FACS data are appropriate. However, for characterizing a cell population that appears relatively symmetrical in logarithmic display, the mean of the logarithmic data is probably more representative of the typical cell in the population.

Data display in two dimensions

The major limitation to the use of one-parameter histograms is that this type of data presentation does not display the correlated relationship of two independent parameters. When the relationship between two measured quantities is important, dot plots, isometric displays or contour maps can be used. In a dot plot display each dot represents the x and y coordinates of one cell that has been analysed. On many flow cytometers, it is possible to observe a dot display on an oscilloscope during data acquisition. This is quite useful in monitoring the performance of the cytometer during operation. The disadvantage of using dot plots for quantitative data analysis is that all cells that fall into a particular location will be represented only by a single dot. Therefore it is not possible to assess visually the quantitative distribution of the various cell populations. Isometric displays, viewed in perspective, make it easy to visualize the two-dimensional histogram, but with most available software it is difficult to manipulate the data for gating and statistical evaluation. In their studies the authors prefer to use contour maps. These are similar to topographic maps, with increasing density of cells in a region represented by higher contour levels. Contour levels can be chosen in a variety of ways, ranging from methods which take uniform steps in cell frequency to methods which derive the contour levels from the data itself. Four contouring methods are illustrated for a single data set in Fig. 29.7. The authors have found that the method of Fig. 29.7b, which places contours so that a fixed fraction of the cells is found in the area between adjacent contours, is particularly useful as a starting point for data analysis. Details of the definitions and meaning of the different display methods are covered in Chapter 30.

Multidimensional data

In most of the authors' experiments, data from more than two parameters are collected. Typically, correlated data from two light scatter detectors and two fluorescence detectors are collected for 10 000–30 000 cells and stored in list mode data files. The actual number of cells collected is dictated by the frequencies of the cell populations of interest. If low frequency populations are to be examined, more cells must be analysed to provide statistically adequate representation of these populations.

It is difficult to display or visualize more than two parameter data. However, in many instances it is possible to subdivide the measurements into one or two parameter sets which can be analysed sequentially. Fig. 29.2, shows that lymphocytes, monocytes and granulocytes can be identified in human blood on the basis of forward-angle and 90° light scatter measurements. If this were part of a two-scatter, two-fluorescence data set, the authors would define light scatter 'gates' enclosing each of the cell populations and search the list mode data file for the set of cell data within each scatter gate. Two-colour fluorescence contour maps would then be generated representing the cells in each set. Analysis becomes more complex when the data cannot be initially subdivided into one- or two-parameter groups. For example, analysis of three- or four-colour immunofluorescence data often requires several gated two-dimensional displays to visualize fluorescence data from each light scatter defined cell population.

Cell sorting

Drop formation

In order to charge the appropriate drop to sort a particular cell, the time delay between its arrival at the laser beam and its incorporation into a free drop must be known and stable. Stabilization of the break-up of the liquid jet into droplets is accomplished by imposing vibrations at a frequency near the natural drop formation frequency of the jet. For typical jet velocities of 8–10 m/s the drop formation frequency is about 40 kHz for a 50 μm diameter jet and 20 kHz for a 100 μm jet. The vibration frequency defines the number of drops produced per second, setting an upper limit on the number of theoretically separable events. In practice, the number of cells per second that can be sorted with good efficiency is only a fraction of the drop formation rate.

Number of drops per sort

Initial sorting decisions are made on a cell-by-cell basis, but execution of sorting requires drop-by-drop charging. In the standard sorting mode any drop which might contain a desired cell and cannot contain an unwanted cell is sorted. (On commercial cell sorters this mode has been called coincidence 'out' or 'normal' by Becton Dickinson, 'anticoincidence on' by Coulter

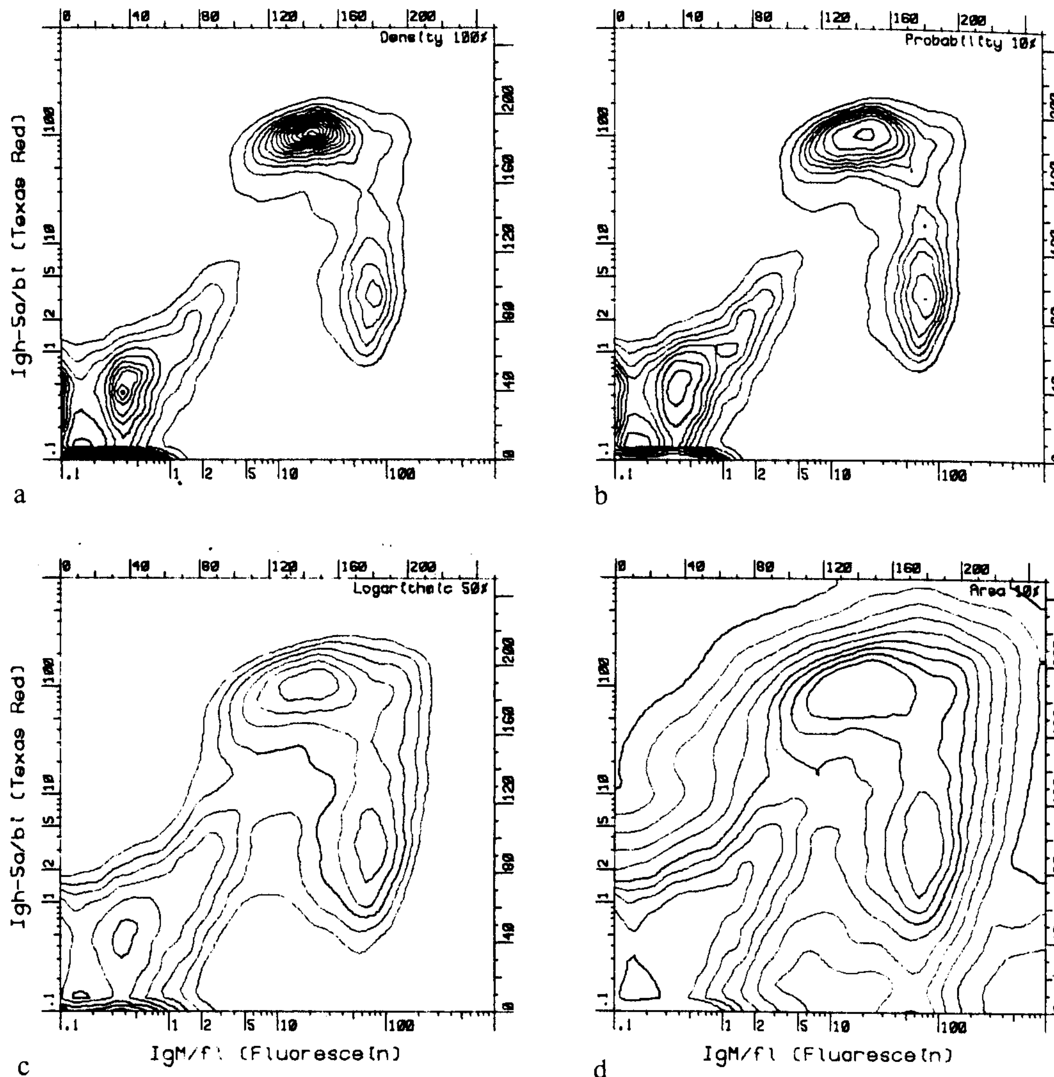


Fig. 29.7. Four different contouring algorithms applied to a single dual immunofluorescence data set. Mouse spleen cells were stained for IgM and IgD and measured on a two-laser FACS. The first method gives the ordinary uniform contour spacing (a). In probability contouring (b) the region between adjacent contour levels includes a constant fraction of the cells (10% in this case). In the logarithmic spacing display (c) each contour level is twice as high as the previous one. The equal area contours (d) are defined to give equal parts of the area of the final two-dimensional display (10% in this case) to the region between each successive pair of contour lines.

and 'charge gate' by Ortho.) In practice, several factors make it difficult to predict in precisely which drop a cell will be found so that more than one drop must be assigned as a possible location for each cell.

Some commercial sorters have a less efficient but sometimes useful sorting mode (called coincidence 'in' or 'full deflection envelope' by Becton Dickinson and 'anticoincidence' by Ortho) which assures that the full number of requested drops is sorted with each cell. In this mode unwanted cells are excluded as in normal sorting, and each group of sorted drops definitely contains a wanted cell. This is useful when accurate counts of the number of cells actually sorted are needed, such as in fixed count replicate cultures or in estimating cloning efficiency.

The factors limiting how well cells can be assigned to

drops are (1) measurement uncertainty in setting the average delay between detection of a cell and break-off of the drop containing it, (2) changes in the jet and break-off conditions over time, and (3) the effect of the cells themselves on the drop forming conditions. The amount of uncertainty produced by cells is greater for larger cells and less for larger jet diameter or higher piezoelectric vibration amplitude.

To maintain good recovery of sorted cells it is necessary that a desired cell always be found within the range of drops assigned to it, and conversely high purity of sorted fractions requires that no unwanted cells be found outside their assigned drops. Loss of desired cells due to proximity of unwanted cells is minimized by assigning the smallest number of drops that will definitely contain a particular cell. In practice, when the authors sort lymphocytes using a 60–80 μm diameter jet driven at 25–35 kHz, they find that as few as 1.5 drops can be assigned per cell. This gives only ±0.25 drop cycle latitude for error in the cell-to-drop assignments, but with care proper conditions can be maintained for hours at a time. (Assigning 1.5 drops means that cells detected during half of the oscillator cycle are safely assigned to a single drop, while cells in the other half cycle might appear in either of two drops, both of which must be assigned to the cell. Thus an average of 1.5 drops is assigned.) Two drop sorts should be adequate for most cell types and sorting conditions.

Cell flow rate and coincidence losses in sorting

In large-scale sorting experiments, a high cell flow rate is desirable to minimize the time needed to obtain the necessary number of sorted cells. This is particularly desirable in live cell work where cell viability and staining uniformity may decrease over time. The problem is that at higher cell flow rates the fraction of desired cells lost due to proximity of unwanted cells ('coincidences') increases, thus diminishing the returns.

Theoretical loss rates in sorting are calculated and graphed in reference 5. The choice of a cell flow rate depends somewhat on the availability of extra cells to be sorted. Actual recoveries may be significantly below theoretical estimates if there is considerable clumping of cells in the sample. The important variables in estimating sorting efficiency are cell flow rate and the ratio of the drop formation rate to the number of drops assigned per sort. Higher sorting rates become allowable if fewer drops are sorted. In general, the authors tend to use cell flow rates of about 30% of the ratio of drops-per-second to drops-per-sort. This gives an estimated 74% recovery and a cell collection rate 60% of the theoretical maximum. To cover all cell losses including cells remaining in the tubes, cells used to set up the initial sorting conditions, etc., the authors try to start with a sample twice as large as would be needed to supply the required sorted cells at 100% efficiency.

Sorting procedure details

The following suggestions and comments are designed to help prevent some common problems in sorting and to clarify procedures.

Maintaining cell viability

If the system sheath fluid is normal saline, cells can become unhappy during the time they are held in the sort collecting vessel. In such cases the collecting vessel should be pre-stocked with buffered medium containing extra protein (e.g. serum) so that good conditions can be maintained as cells and saline are added. It is also helpful to mix the liquid in the collection vessel occasionally. For more sensitive cells the authors use RPMI (without pH indicator dye) as sheath fluid. Some cell types, such as mouse splenocytes, retain best viability when both the cell supply tube and the sorted fractions are kept ice cold.

Charge build-up in collection vessels

Since the deflected drops are electrically charged, a significant potential can build up on insulated collectors to the point of repelling newly sorted drops away from the vessel and even attracting oppositely charged drops from the other side in two-fraction sorting. Normally only plastic vessels are good enough insulators to hold much charge. A grounded wire in contact with the liquid in the vessel or use of glass collection tubes will solve the problem.

Selecting the optimum sort delay setting

In order to sort at 1.5 drops per cell it is necessary to set the delay between cell detection and drop charging quite accurately. The authors estimate the optimum delay by sorting easily identified microspheres at several delay settings near an initial estimate and counting the number of microspheres actually recovered. Sorting about 50 single drop deflections into a pool on a slide allows a good estimate of the recovery rate. The optimum delay can be found by fitting the counts at various delay settings using the model that the recovery should be 100% at the best delay setting, and it should fall linearly to 0% at delays one drop cycle longer or shorter than the optimum.

Sterile (aseptic) sorting

Sterile sorting can be carried out routinely with no great difficulty. The authors filter the sheath fluid with an in-line 0.22 μm filter (Millipore Corp., Bedford, MA). Before a sterile sort the authors replace a disposable 0.45 μm filter which is in the sheath line between the 0.22 μm filter and the nozzle. Ethanol (70%) is pumped through the 0.45 μm filter to fill the filter, the sheath line, the nozzle and the sample line. After 30 min the normal sheath fluid is used to flush out the ethanol. The flush is continued until the drop break-off is stable (about 5 min).

Since collection vessels may be open to the air for many minutes during a sort, it is best to keep the surrounding areas clean and avoid air turbulence in the vicinity.

Reanalysis of sorted fractions

It is good practice to evaluate the purity of sorted fractions by reanalysing a small portion of each fraction. Special care is required in cleaning the input lines leading to the nozzle since cells tend to remain after long sorts, and the sample for reanalysis is usually at low concentration. A good test is to run a 'sample' of cell-free medium to see that very few extraneous cells will be included in the reanalysis.

Rare cell selection and isolation

The FACS can be a very powerful tool in the identification and isolation of rare cells. The applications include enrichment of fetal cells from maternal blood [37], selection of hybridoma variants producing different classes of immunoglobulin from the parent line [34,38], isolation of cells incorporating and expressing a new gene in DNA transformation [39] and selection of cells with increased gene copy number [40,41, and Chapter 90 in this handbook].

Multiparameter gating is very useful in eliminating artifacts due to dead cells, debris, etc. which would otherwise greatly outnumber the rare cells being sought. In selection of cells from tissue culture populations, most of the artifacts would not grow in subsequent culture, but their elimination in the analysis makes it possible to identify more accurately the regions in which rare cells should be sought and to make better estimates of the frequency of rare cells.

With cell populations growing in culture the selection process can include several cycles of sorting for enrichment with intervening growth periods. FACS direct cloning of selected rare cells is usually effective when they have been enriched to a frequency in the range of 0.1–0.01% of the total.

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Some material from reference 5 has been used or adapted for use in this article: the authors thank Academic Press, Inc., for permission to reproduce Figs. 29.1a and b, 29.3, 29.5 and 29.7, and to adapt various of the text from *Methods in Enzymology*, Vol. 108 [5].

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