Chapter 47
Flow Cytometry Instrumentation and Measurements

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This chapter is intended to provide functional descriptions of how flow cytometers work and how they are used with emphasis on aspects that are most relevant to immunological work. We hope that it will serve the reader at three levels: (1) providing background to aid in interpreting and critically evaluating published flow cytometry results, (2) assisting in evaluating the utility of flow cytometry in research projects, and (3) supporting researchers in planning and carrying out flow cytometry experiments. The chapter includes details of some procedures and analysis methods that have not been included in readily available references.

Fundamentals of the instrumentation

Flow cytometry and fluorescence-activated cell sorting (FACS) are in use in a wide array of fields ranging from oceanography to clinical patient monitoring. In particular, they have become powerful and versatile tools for cellular and molecular biomedical research. This power and versatility derive from the multiple independent and quantitative measurements that can be made on large numbers of cells and from the ability to isolate cells based on any combination of the measured properties.

Flow cytometers vary in particular aspects of their design and operation, but the most relevant features can be discussed with reference to Figure 47.1. Cell-free sheath fluid forms a continuous flow converging into an enclosed observation chamber or (as shown in Fig. 47.1) through an orifice to form a cylindrical jet. This flow intersects one or more laser beams. The cell sample is injected into the center of the converging sheath flow, forming a small central core in which the cells are carried. Cells occur randomly within the core fluid, but their concentration and the volume flow rate of the sample are normally adjusted to make it unlikely that two cells will cross a laser beam at the same time.

As a cell passes through a laser beam, light is scattered in all directions in amounts that depend on the size and structure of the cell. Fluorescent molecules in or on the cell are excited and emit light whose spectrum is defined by the nature of those molecules. Scattered and fluorescent light is collected by lenses, commonly one near the laser beam axis for forward light scatter, and another orthogonal to both the flow and laser beam axes to collect fluorescence and orthogonal light scatter. Dichroic reflectors divide the collected light into wavelength ranges that are directed to an array of detectors. Bandpass filters are usually placed in front of each detector to limit further the range of light detected. The detectors convert the light into electrical signals that are amplified and evaluated to yield a set of numbers characterizing the cell. Computerized records of these measurements constitute the primary data in flow analysis. In some cases, it is desirable to exclude some of the measured events, such as those due to dead cells or debris, from the data collection by setting criteria comparable to sorting selection criteria and recording data only from events with the desired characteristics. If the cells are not to be sorted, they pass on to waste.

If cells are to be sorted, the analytical signals from each cell are compared to specified criteria to determine whether the cell should be sorted or not. The unique power and flexibility of flow sorting stems from its ability to use quantitative comparisons in multiple measurement dimensions to reach a sorting decision for each individual cell. During cell sorting, a continuous oscillatory signal is applied to the nozzle to induce stable uniform drop formation. After measurement, the cells continue along the jet and are incorporated into drops. If we want to sort a cell, a voltage is applied to the jet, giving a charge to the appropriate drop as it breaks from the stream. The drops fall between charged plates that pull charged drops away from the central uncharged drops. The central stream is removed as waste, and the deflected drops are collected in appropriate receptacles.

Flow analysis elements

This section describes certain components and aspects of FACS instrumentation that should be understood by anyone designing FACS experiments or making detailed analyses of FACS measurements.

Excitation sources and excitation optics

Flow cytometry requires high radiance excitation sources since we need to obtain maximal fluorescence signals from cell-sized objects, particularly for immunofluorescence, and we need cell/beam transit times of only a few microseconds to analyze thousands of cells per second with few overlapping events. Mercury arc lamps are used in some systems, but they require much more sophisticated optics than laser-based systems, and their use for immunofluorescence and in multiple beam systems is limited. In particular, arc lamps require high numerical aperture lenses to bring adequate excitation to the sample and must use emission filters to block all light except the region of interest, while lasers are usually operated in single line mode (no excitation filters required) and can be focussed to high intensity with simple lenses. Arc lamp based cytometers often provide excellent quality DNA analysis, but commercial droplet sorters all use laser illumination. In what follows we will assume laser illumination, but much of it also applies to arc lamp systems.

The choice of beam-shaping optics and laser power is influenced by several factors and tradeoffs. The profile along the cell flow path should be short to minimize opportunities for two cells to be in the beam at once, but we also want high enough intensity over a long enough time to allow sensitive measurement without excessive dye saturation (see "Dye saturation and photobleaching" later in this chapter). In the direction across the flow path, we want the beam to be sufficiently broad to provide uniform illumination of cells following slightly different paths in the

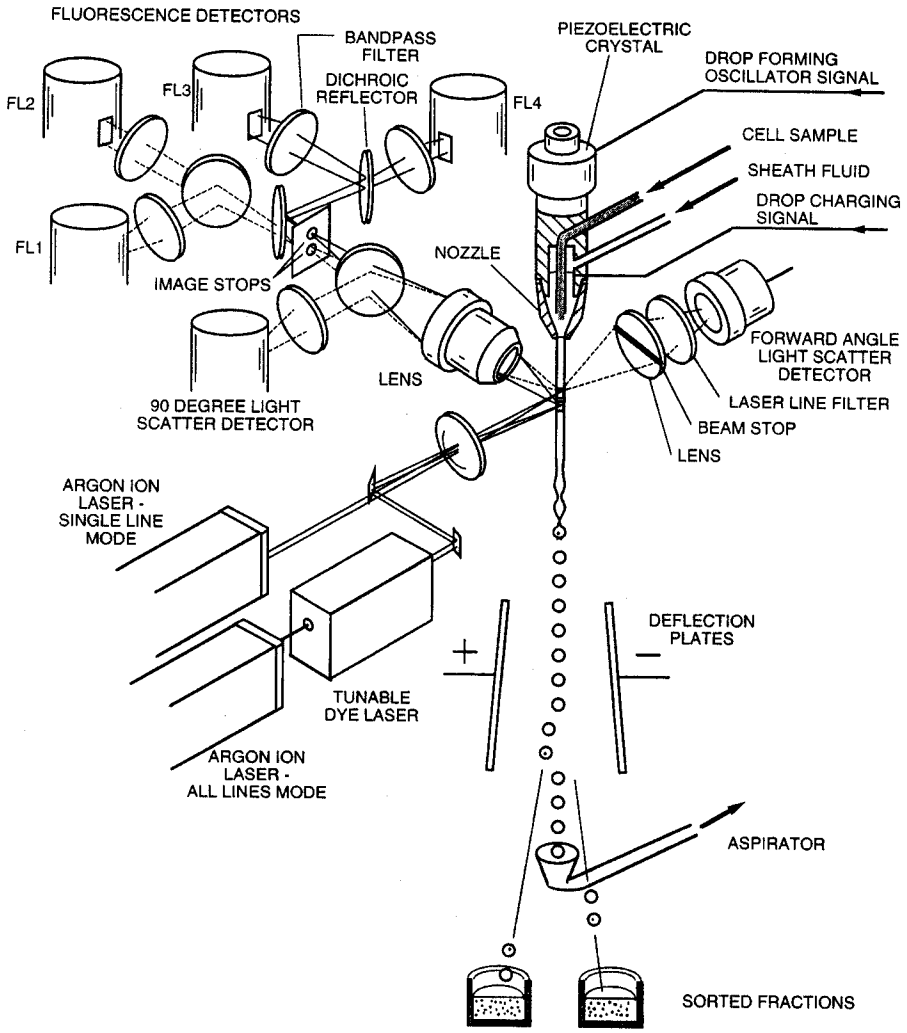

Fig. 47.1. *Diagram of a two laser, four color fluorescence jet-in-air cell sorter (mechanical, optical, fluidic, and electrical transduction elements).*

stream, but making the beam too broad wastes most of the power outside the region where cells occur. Using crossed cylindrical lenses or a combination of spherical and cylindrical optics, we can control the vertical and horizontal beam profiles independently. Typical beam heights are in the 20 to 40 micron range, with widths in the 40 to 100 micron range.

Flow cells and free jets, sample flow, and light collection

Enclosed flow cells have significant advantages over free jets for making measurements on cells, but free jet measurement contributes to the ease and efficiency of cell sorting. The laser light reflected and refracted by a cylindrical jet must be blocked out of less light scatter detectors and physically or optically rejected from fluorescence detectors. The flat walls of flow cells scatter much less light from the illumination source, minimizing the need for blocking. Flow cells also lend themselves to use of high numerical aperture immersion optics for fluorescence light collection which can provide on the order of five times the light collection efficiency that is typical of free jet optics. On the other hand, droplet-based cell sorting with flow cell measurement involves greater delays between detection and drop formation and changes in velocity going from flow cell to jet. This makes it more difficult to predice which drop a cell will be found in. Also, the fluid velocity goes to zero along the walls of a flow cell leading to velocity—and, therefore, timing—differences between centered and off-center cell paths. These considerations make it easier to define and accurately reproduce sorting conditions in free jet measurement systems.

Image stops

Image stops (see Fig. 47.1) limit the region from which light can originate and still reach the detectors. They help to minimize room light sensitivity and acceptance of fluorescence light from anything other than the intended laser-cell intersection. Effective use of image stops requires the use of good imaging optics (such as microscope objectives) for light collection. If a fluorescence collection lens is used which provides efficient light collection but not good imaging, not all the collected light will pass through a small image stop. The average signal level may be fine, but measurement uniformity will be degraded if the light loss differs for particles following different paths through the beam. This problem will occur in any case where image stops are too small to pass the full range of light coming from the intended source. The effective source position of different cells is most variable when

the sample flow rate is high, so this is the conditon where degradation of measurement quality will be most marked.

Image stops are particularly useful in multi-laser systems. When several fluorescence signals are derived from a single laser, only the optical filters provide separation among the different dye emissions. For signals excited by different lasers, image stops and time-gated signal evaluation provide additional discrimination so that dyes with overlap or even near identity in their emission spectra may be measured independently, as long as their excitation characteristics are sufficiently distinct.

Optical filters

The work of separating collected light into different wavelength components falls on optical filters. Interference filters are used for most purposes, but absorption longpass filters are useful in some cases. Interference filters are constructed by evaporative coating of many thin layers onto a substrate material. The principle behind these filters can be illustrated by the single layer antireflection coating in which a substrate is coated with a layer of transparent material 1/4 wavelength in thickness. Light reflected from the air/coating interface will be 1/2 wavelength out of phase with light reflected from the coating/substrate interface, resulting in destructive interference. If the index of refraction of the coating is appropriate, the two reflected components will be of equal intensity and cancel completely, giving essentially no reflection and 100% transmission. At wavelengths for which the coating thickness does not correspond to 1/4 wavelength, there will be reflection and less than 100% transmission. By careful adjustment of materials and coating layer number and thickness, interference filters can be made with complex but precisely specified transmission and reflection properties. For fluorescence detection, the filters must block scattered laser light to about one part per million. Current standard fluorescence filters are usually "six cavity" bandpass designs with a passband 20 to 40 nm in width, which can begin as close as one passband width above the laser wavelength (such as 30 nm from the laser wavelength to the beginning of the passband for a 30 nm bandpass filter). (These values and other filter characteristics quoted in this section are drawn from data sheets by Omega Optical, Inc., Brattleboro, Vermont, USA.) The passband center wavelength and width are chosen to optimize acceptance of emission from a particular dye with minimum background light sensitivity and minimum sensitivity to other dyes used in the system.

Dichroic reflectors are interference filters designed to be used at an angle with respect to the incident light, so that both the transmitted and reflected light can be detected. The are usually designed to be used at 45 degrees and to have broad transmission and reflection regions separated by a steep transition (such as change from 10% to 80% transmission over 17 nm in wavelength at 500 nm).

As an interference filter is tilted in relation to the incoming light, its transmission/reflection properties change. The main effect is to move transitions to shorter wavelengths (such as a 500 nm transition at normal incidence will move to about 463 nm at 45 degrees), but the details are affected by the polarization of the light in relation to the filter plane. Using a 45-degree dichroic reflector at other angles will give altered transmission/reflection properties. Correspondingly, normal incidence filters can be "tuned" somewhat by tilting. This angular dependence of interference filter properties can become a problem if a filter is used where collected light is strongly converging or diverging, or where stray light may come onto the filter at an angle.

Colored glass filters and other absorbance filters can be useful where long wavelength pass/short wavelength block characteristics are needed. Tilting these filters affects their characteristics only slightly, but they tend to produce a lot of secondary fluorescence when illuminated by scattered laser light, so they should be located well away from fluorescence detectors.

Light detection

After we've gone to all the trouble to generate, collect, and filter fluorescent light, it turns out that only a small fraction of the photons produce any useful electronic signal that we can process and evaluate. Photomultiplier tubes (PMTs) employ the the photoelectric effect, in which a photon of light is absorbed and its energy frees an electron from the surface of the absorber. Good PMTs of the normal types have photon conversion efficiencies in the 10 to 20% range, sometimes over 20% in blue wavelengths and always below 10% (and declining with increasing wavelength) in the far red. This lower far red sensitivity means that more fluorescent light is needed from a far red dye than from a mid-spectrum emitting dye to produce the same photoelectron signal. Thus, while there are advantages to using longer wavelength-emitting dyes to avoid autofluorescence background signals and to gain more options in multiple fluorescence work, there are also diminishing returns in the absolute signal levels measured. The photoelectrons generated at the photocathode of a PMT are accelerated by a voltage difference and impact onto an electron multiplying electrode ("dynode") where several secondary electrons are emitted for each incoming photoelectron. These electrons are in turn accelerated to another dynode, and so on, until after 9 to 11 of such steps, a final amplified current signal is collected at the anode. The resulting high gain and low noise make it possible to detect signals from even a single photoelectron in optimized systems. PMTs are used for all high sensitivity fluorescence detection in flow cytometry and are usually used for large angle light scatter as well.

In the commonly used Hamamatsu R928 and similar PMTs, the original photoelectrons are amplified over a sequence of nine dynodes resulting in a total gain of 10^4 (at about 400V) to 10^7 (1000V). Because of the sequential amplification, the gain goes up much faster than the applied voltage, showing a power relationship. Gain changes we have measured for R928 family PMTs yield an exponent of about 7.4, (i.e., (gain2/gain1 = (voltage2/voltage1)$^{7.4}$).

The other light detectors used in flow cytometry are silicon photodiodes, in which the energy from one absorbed photon creates one electron-hole pair. The current produced by collecting the electrons and holes to different electrodes provides an output proportional to the light input which is linear over a wide range. The production of electron-hole pairs from incoming photons is commonly over 70% efficient, but there is no internal amplification, leading to higher effective noise levels than for PMTs. Photodiodes are, however, compact, robust, inexpensive, and highly linear for detecting medium to large light signals, so they are often used for forward light scatter measurements.

Analog electronics and digitization

The current signals from PMTs or photodiodes are usually converted to voltage levels in a preamplification stage. Analog

fluorescence compensation circuitry may be used at this point to subtract spectral overlaps and obtain single dye signals as described below (in the section on "Fluorescence compensation"). The resulting signals are then amplified by either linear or logarithmic amplifiers, evaluated, and the result digitized.

Threshold triggering and cell detection

Since cells pass through laser beams in a few microseconds and appear at average intervals of hundreds of microseconds to several milliseconds, most of the time is spent waiting for a cell to appear. When a cell does appear, we want to obtain measurements on all of the signal channels. Since some signals may be essentially zero for certain cells, we normally rely on a single signal that is always well above background for cells of interest to trigger the analysis system. Thus we obtain a count of all cells of interest, even if they have essentially zero signal on some channels. The threshold level is normally user-adjustable, so that whenever the chosen signal rises above that level, an evaluation is forced on all measurement channels. In multi-laser systems, delay electronics are used to assure that each signal is measured when the cell is crossing the appropriate laser beam.

Light scatter signals are most commonly used for threshold triggering, since they readily distinguish all cell sized objects from background signal fluctuations. A fluorescence trigger signal is often used for cellular DNA/cell cycle analysis to exclude cell sized but non-nucleated debris. Fluorescence triggering may also be useful when the cells we want to analyze are a small fraction of the total sample, such as for analysis of nucleated cells in whole blood. In that case we stain with one reagent that marks only the cells of interest and use its signal for triggering. Other fluorescent reagents in other colors can be used to subdivide the population as desired.

Pulse height

The simplest evaluation of the magnitude of a signal is its pulse height, the maximum level reached during the cell's transit through the beam. It is relatively easy to maintain low noise conditions and achieve 4 decade dynamic range (10,000:1, for example, 10 V full scale signal with no more than 1 mV noise) in signals used for pulse height analysis. However, differences in cell size and shape result in small differences in the shapes of signal pulses, so that a given amount of dye on a small cell will give a slightly higher pulse height than the same amount of dye spread over a larger cell. In cell cycle analysis, this effect may result in G2+M pulse heights that are a little less than twice the G1 pulse heights.

Pulse area

In cases where cell size is a significant variable (as in cell cycle analysis as mentioned under "pulse height" above), the pulse area (integrated signal) may be preferable to pulse height, since the area is more linearly related to dye content. However, the additional electronic manipulation to control active integration of the pulse makes it difficult to maintain very low noise levels required for the 4-decade signal range that is typically available in pulse height measurements. We have seen effective dynamic ranges of more like 3 decades for pulse area. The integration must be performed on a linear signal input, but the result may be log-amplified for evaluation and digitization.

Relative pulse width

The pulse width is obtained by evaluating the time a signal spends above a defined level. When the transition level is defined as a fixed fraction of the pulse height, the resulting relative pulse width is independent of the signal amplitude and is a function only of the laser beam profile, the flow velocity, and the distribution of signal source in or on the cell. The measurement works well, however, only when the signal is strong enough to provide a smooth, well-defined pulse shape. Either a light scatter signal or a fluorescence signal can be used as the source for pulse width evaluation, but the selected signal should have relatively high levels for all cells of interest. Since the laser profile and flow velocity are constant in a particular run, small differences in relative pulse width between cells usually correspond to differences in cell size (or nuclear size when a DNA stain is the signal source). Unfortunately, the circuitry in some instruments does not set the transition level at a fixed fraction of the pulse height, resulting in width measurements that vary with both the signal amplitude and the cell size.

Detecting abnormal events

Cytometers may include circuitry to detect abnormal signals such as when a new threshold event occurs while a previous cell is still being processed or when a pulse has more than one peak.. Excluding events that are not derived from normal single cells is particularly important when rare cell populations are to be evaluated. For normal single cells, the relative pulse width and the relation between pulse height and pulse area show limited variation, so events giving widths outside the normal range will be due to multiple cells or non-cellular objects and can be excluded from analysis.

Linear and logarithmic amplifiers

Linear amplifiers typically provide an array of gains to match the incoming cell signal levels to the instrument's evaluation and display electronics. If the gain setting is too low, the signals will occupy only a small part of the available range. If the gain is too high, the larger signals will be "offscale" and not properly evaluated. The need to select an appropriate gain is largely avoided by the use of logarithmic amplifiers with at least 4-decade range, since both high and low signals can be accomodated on a single scale. The main problem with logarithmic amplifiers is that they are only approximately logarithmic, making it risky to rely on them for accurate comparisons of different signal levels. Linear amplifiers, if they are working properly, should be accurately linear. Conditions for deciding whether linear or logarithmic presentation is more appropriate are discussed in Chapter 50 [1].

Analog-to-digital conversion

In order to store, display, and analyze our signal evaluations, they must be digitized. The digitized results must have enough resolution to support the required analyses over the appropriate dynamic range of the signals. For logarithmic data, digital channel steps correspond to a constant ratio between the level represented by one channel and the next. Ten bit digitization (1024 channels) of 4-decade log data gives channel step ratios of about 1%. This is adequate for the normal uses of logarithmic signals. (Eight bit data give 4% channel steps, etc.) Digitized linear data has constant signal level steps and therefore variable step ratios at

different channel numbers. Very high resolution measurements usually have limited dynamic range and can be accomodated in the parts of a linear scale where the step ratio is small (such as 10 bit linear data above 1/4 of full scale gives step ratios of 0.4% or less).

Light scatter measurements

Whenever a cell passes through a laser beam, light is scattered in all directions in a pattern related to the size, shape, and structure of the cell. The complexity of the pattern, however, prevents us from extracting specific cellular parameters from a few light scatter measurements. In particular, the pattern may be strongly orientation-dependent, so that the same cell rotated and passed through the system again could give significantly different measurements. On the other hand, light scatter measurements can be very useful in distinguishing different cell types and in distinguishing single cells from other objects in the sample.

Light scatter is commonly used for four purposes in flow cytometry:

1. Light scatter provides reliable detection of the presence of a cell in the sensing volume independent of any fluorescent label. Therefore, a light scatter signal is the usual source for initiating measurement on all the signal channels.
2. Among cells with different structures, light scatter signals provide only a rough indicator of cell size. For cells of similar structure, both forward light scatter and orthogonal light scatter signals tend to be monotonic with cell size, and can provide a useful indicator of relative size. However, even in that case, it is usually not possible to establish a true calibration of signal versus size.
3. Orthogonal light scatter, particularly in relation to forward light scatter, can be used to distinguish different types of cells on the basis of their internal structure and granularity differences.
4. Dead cells and cellular debris tend to have higher orthogonal scatter than live cells. On many instruments, dead cells show lower forward light scatter than the corresponding live cells, but this depends on the exact geometry of the forward light scatter detection. Taken together, forward and orthogonal light scatter can be extremely useful in excluding dead cells and debris, particularly when it is not feasible to incorporate dead cell exclusion by propidium iodide fluorescence (see the section on "Dead cell exclusion using propidium iodide" in this chapter).

Even in multi-laser systems, light scatter is normally measured only at one wavelength, but there are some reports showing that, at least in particular cases, light scatter at two different wavelengths can help to discriminate different cell types [2, 3, 4].

Fluorescence measurements

Characteristics of fluorescence

In a fluorescent process, a molecule absorbs a photon of light and moves into an excited state from which it can emit a photon and return to the ground state. Since some energy is lost to internal molecular transitions in this process, the emitted light generally has a longer wavelength (i.e., lower energy per photon) than that absorbed. The excitation efficiency varies with wavelength depending on the match between photon energy and the energy levels of the molecule. The wavelength difference between excitation and emission (called the Stokes Shift) makes it possible to measure fluorescence with high sensitivity and low background as long as the excitation beam includes essentially no light in the wavelengths used for fluorescence detection.

Because of the different energy levels available in the excited and ground states, there is some variation in the emitted photon energies, resulting in fluorescence output over a range of wavelengths. The probability that any particular excited molecule will emit at a particular wavelength corresponds to the observable emission spectrum of the dye. For optimal detection sensitivity, we want to accept as much of this light as possible, while minimizing light from extraneous sources, including laser light, emission of other dyes, and autofluorescence. If we want to use several dyes excited by a single source, their emission spectra must be well separated to allow relatively independent measurements.

Another relevant factor is the quantum efficiency for fluorescence, the fraction of excited molecules that return to the ground state by emitting light rather than through a nonradiative path. Good dyes for immunofluorescence have quantum efficiencies in the 30 to 90% range. The quantum efficiency, however, can be reduced by the proximity of molecules or parts of molecules that increase the rate of nonradiative de-excitation, leading to quenching of fluorescence. For example, fluorescein conjugated to an antibody may have lower quantum efficiency than free fluorescein. Quenching can occur even when the interacting molecules are the same, such as when several fluorescein molecules are conjugated to one antibody molecule. The observed fluorescence of such an antibody reagent will not correspond to the amount of fluorescein measured by absorbance.

A popular way to obtain emission well removed from the excitation wavelength is to take advantage of *resonance energy transfer*. In energy transfer conjugates like the phycoerythrin/cyanine dye conjugate PE-CY5, the donor component (such as PE) absorbs light and enters its excited state, but the excitation is rapidly passed to the acceptor (CY5) which emits with its characteristic (longer wavelength) spectrum. The amount of overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor determine the rate and efficiency of the transfer. Since the donor molecule is excited for only a short time, it is unlikely to emit its own fluorescence directly (i.e., it is effectively quenched by the presence of the acceptor). Thus we observe only a small amount of donor fluorescence and a large amount of acceptor emission. The phycobiliproteins like PE are themselves natural energy transfer conjugates containing two different chromophores in a protein complex.

Sources of background signal in fluorescence measurement

Background signal in a fluorescence measurement includes sources synchronized with the cell/laser transit and continuous or unsynchronized sources. The synchronized sources include autofluorescence, discussed at length below, secondary fluorescence due to light scatter from the cell, excitation light leakage through the emission filters, and crosstalk between signals in the evaluation electronics.

Other background sources include Raman scatter, PMT dark current, room light, free dye in the sample, laser fluctuations, and noise in the preamplifier/compensation/ampifier electronics. Raman scatter is inelastic scattering of light by, in our case, water molecules. It shows up in typical phycoerythrin measurements,

since the Raman emission from 488 nm is at about 592 nm in the normal detection band for PE. PMT dark current is usually not a problem, since the real light background is almost always larger. Free dye in the sample stream can be a serious problem, producing variable levels of background signal depending on the sample flow rate. Most fluorescence evaluation circuitry contains restoration for average continuous background signals, but too much continuous loading of the detectors can exceed the restoration range and lead to invalid rather than simply noisy measurements.

Non-fluorescent microspheres can be useful for testing different optical filters and evaluating the background signal levels on different measurement channels. This is discussed below in the section on "Standards, sensitivity and signal-to-background evaluation."

Dye saturation and photobleaching

Considering that typical fluorescence lifetimes are a few nanoseconds, while cells spend several microseconds crossing a flow cytometer beam, it should be possible to excite a single molecule hundreds of times if the beam intensity is high enough. This in fact happens routinely, but the process is subject to diminishing returns that limit the utility of higher laser powers. For example, if the laser intensity is such that the average time taken to excite a dye molecule is the same as the average excited state lifetime (typically 2 to 15 nanoseconds depending on the dye), 50% of the molecules will be in the excited state at any given instant, and the emission rate will be 50% of its maximum possible value. (The emission rate is proportional to the number of molecules in the excited state, since each excited molecule has a constant probability of emitting a photon in the nest time interval.) Doubling the laser intensity would take these values up to 67%, an increase of only 1/3, but autofluorescence, scatter-induced fluorescence, etc., would be doubled or nearly doubled, diminishing the signal-to-background ratio [5, 6]. Also, some excited molecules do not return to the ordinary ground state, so that the number of active dye molecules decreases as the cell crosses the beam. This dye loss may be permanent ("bleaching") or simply temporary, but long compared the the laser transit time [5, 7].

The net result is that for strongly absorbing dyes, particularly those subject to rapid loss, the excitation intensity giving optimal signal with minimal background may be quite low. When several dyes are excited by a single laser beam, the choice of excitation intensity involves making a compromise between the different optimal values. For several dyes commonly used for immunofluorescence at 488 nm excitation, the observed order of saturation/loss sensitivity from low to high (and optimum excitation intensity from high to low) is fluorescein, PE-CY5, PE, and PerCP. Thus, in a system optimized for fluorescein and phycoerythrin, the laser intensity might be set at a level lower than the optimum for fluorescein, but higher than the optimum for phycoerythrin.

Dye saturation/loss effects can be easily checked by measuring a labeled cell sample at several laser powers and comparing the light scatter signal levels (which always scale with beam intensity) to the fluorescence measurements. The other, and perhaps more relevant, comparison is between the fluorescence measurements and the signals from unstained cells under the same conditions. The method described in Chapter 50 of this volume [1] should be useful in evaluating fluorescence signal versus background as a function of laser power.

Dead cell exclusion using propidium iodide

Dead cells often give aberrant staining patterns that interfere with data interpretation. Propidium iodide (PI) is widely used for identifying dead cells. It is a small molecule which has low fluorescence in solution and much higher fluorescence when bound to nucleic acids. Intact mammalian cells exclude PI very effectively, so that measurements on live cells are not affected by its presence at low concentration (such as 0.5 microgram/ml). When cells lose membrane integrity, PI enters and gives strong nuclear fluorescence, which can be used to eliminate dead cells from consideration.

Propidium iodide has broad excitation and emission spectra, which can be advantageous in its use as a dead cell marker. In a system set up to measure phycoerythrin, PE-CY5, Texas Red, and/or allophycocyanin, PI signals will appear on all these channels. Thus if any one of these channels is available but not needed for immunofluorescence, it can be used as a PI viability gating channel. In fact, if one of the dyes is being used for positive exclusion of unwanted cells (see Chapter 49 of this volume [8]), PI exclusion may simply be included on the same channel. In systems where PI positive cells will show up as positively labeled on several measurement channels, dead cell exclusion must be carried out before other staining can be analyzed.

Fluorescence compensation

As noted above (under "Characteristics of fluorescence"), fluorescent molecules are excited with variable efficiency over a range of wavelengths, and they emit light over a range of wavelengths. Therefore, when cells are labeled with several dyes that may be excited with a single source, a fluorescence measurement in any wavelength range will consist of the sum of contributions from each dye. We select optical filters so that one detector is optimally matched to each dye (accepting as much light from that dye as possible, while minimizing acceptance of emission from the other dyes or from background sources). What we really want, however, is measurements that are proportional to the amounts of each dye individually. It turns out that such individual dye estimates can be constructed as linear combinations of the primary signals, a process referred to as "fluorescence compensation."

The compensation coefficients needed to subtract out the signal contributions due to spectral overlap are obtained by measuring samples, each labeled with only one of the dyes. The compensation should be adjusted by comparing cell populations that are as equivalent as possible except for the level of one dye. The objective is then to make these populations indistinguishable on all fluorescence outputs except the one intended to measure that dye. Often this is most readily accomplished by mixing unstained and single dye-stained samples and adjusting so that their median signal levels are equal on the compensated dimensions. For n fluorescence detectors and n dyes, this is equivalent to solving n linear equations in n unknowns.

The traditional way to implement fluorescence compensation has been to generate analog combinations of signals before the main amplification and signal evaluation stages in the cytometer electronics. This has been dictated by a desire to maintain 4-decade dynamic range using logarithmic amplifiers and by the need to present prompt fluorescence compensated signals to aid the investigator in monitoring the results during data collection

and cell sorting. For analog fluorescence compensation to work well, the shapes of pulses on the different channels must be well matched. Otherwise subtracted pulses that should go to zero level will not do so along the full length of the pulse, resulting in peak evaluation of the residual signal. In some cases, adjustments in the preamplifier/compensation circuitry can be used to optimize the pulse shape matching. This analog approach does not work multiple laser situations where one dye is excited by more than one of the lasers, and it becomes complex when three or four dyes are excited by a single laser. It is now feasible to digitize the primary signals with enough accuracy and dynamic range to allow compensation calculations and logarithmic transformation to be done digitally, and future instruments will probably take that direction. This digital approach has been used in the Coulter XL analyzer (Coulter Corporation, Miami, FL, USA) in which compensation and logarithmic evaluation are done in the immediate data stream, so that user displays reflect the transformations.

In data analysis, linear tranformations equivalent to fluorescence compensation can also be useful, usually in situations where stored fluorescence data are uncompensated or under-compensated. In multiple laser systems, a dye which has significant excitation efficiency with more than one laser may produce unwanted signal contributions that cannot be corrected by ordinary fluorescence compensation. For example, we have difficulties using PE-CY5 with 488 nm excitation in the same system with allophycocyanin excited at 600 nm. The CY5 is excited through the PE at 488 nm and is excited directly by the dye laser at 600 nm; the allophycocyanin is excited efficiently at 600 nm, but will excite a little bit at 488 nm; and both dyes emit in the 660 to 670 nm range. The two separately-timed fluorescence measurements will each have contributions from both dyes. Analysis of data from singly stained cells of each type provides the subtraction coefficients that can be used to correct the mixed dye results. In cases where autofluorescence is well correlated with a light scatter signal, the light scatter data may be used to subtract estimated autofluorescence on a cell-by-cell basis.

When highly stable fluorescent microspheres are used to reestablish standard instrument conditions (see "Standard particles and their use" below), we have found that compensations based on some dyes, particularly fluorescein, phycoerythrin, and Texas Red, can be set automatically by a computer routine monitoring the microsphere peak positions [9]. On the other hand, allophycocyanin and PE-CY5 show variations by lot or source that normally require matched single dye samples for accurate compensation.

Autofluorescence

Autofluorescence refers to cell-derived fluorescence that is not due to intentionally added dye. For many immunofluorescence measurements, autofluorescence sets the lower limit of reagent sensitivity. (Actually, it is the cell-to-cell variability in autofluorescence that is limiting, since a truly constant autofluorescence could be simply subtracted.) We distinguish three types of autofluorscence which might be termed ordinary, specific, and induced.

Ordinary autofluorescence is that due to molecules found in most cells, although the amounts may vary considerably, with cell type or metabolic state. With current instrument designs and good optical filters, the autofluorescence of lymphocytes is in the range of 1000 fluorescein molecule equivalents (according to Abe Schwartz of Flow Cytometry Standards Corp., San Juan, Puerto Rico as quoted in reference [10], p.239). Much of the blue-excited green autofluorescence seen in mammalian cells is due to flavin nucleotides [11, 12], while UV-excited blue emission is due to pyridine nucleotides. The former have been used to distinguish neutrophils [3] and eosinophils [13] from other cell types, while the latter have been used for analysis of redox state via NADH fluorescence [14]. In cultured cells the levels of autofluorescence can vary with the state of the culture, usually being minimal in exponential growth and increasing when growth-limiting conditions develop.

Specific autofluorescence refers to molecules that are characteristic of particular types of cells. Photosynthetic pigments are outstanding examples, since some leakage in photosynthetic energy capture appears as fluorescence. Chlorophyll is easily detected in green algae, and phycobiliproteins are found in cyanobacteria. Running a pond water or hot spring sample in your flow cytometer, you should be able to distinguish several types of organisms by the pattern of their yellow-versus-red autofluorescence.

Induced autofluorescence is most commonly due to fixation procedures. For lymphoid cell immunofluorescence, paraformaldehyde fixation procedures have been developed which give minimal increases in autofluorescence. Autofluorescence does increase if samples are held too long before analysis, however.

For sensitive measurements of immunofluorescence on lymphocytes or for any measurements on some highly autofluorescent cells, it may be desirable to use longer wavelength excitation and emission. Autofluorescences tend to be relatively high for the blue-excited green and yellow emission conditions used to measure fluorescein and phycoerythrin. Autofluorescence is much less of a problem with dye laser or HeNe laser excitation. In our current experience, the best signal to autofluorescence is often observed using dye laser excitation of allophycocyanin. When measuring fluorescein or phycoerythrin, but not both, on high autofluorescence cells, autofluorescence compensation may be useful [15]. The two requirements are (1) that the autofluorescence of unstained cells shows good correlation between the green and yellow signals, and (2) that the autofluorescence yellow/green ratio is distinctly different from that of the dye you are using (check by running stained and unstained cells). For example, for fluorescein staining, you can treat the autofluorescence like a yellow-emitting dye and use the yellow-to-green compensation to subtract the autofluorescence from the green signal (corrected unstained cells should have minimal compensated green signal). The net compensated green signal for stained cells will then be essentially due to fluorescein alone. Autofluorescence correction using two lasers, one exciting both reagent fluorescence and autofluorescence and the other exciting mostly autofluorescence, has also been reported [16].

In some cases, particularly if the cell sample is a homogeneous cultured population, a light scatter signal will be well correlated with autofluorescence, indicating that differences in both signals are mostly related to cell size. The light scatter signal can then be used to estimate and subtract out the autofluorescence contribution for stained cells. If this is not possible directly on the instrument, it may still be useful in the data analysis process to produce an autofluorescence-corrected version of the reagent staining distribution.

Another possibility is to suppress autofluorescence by adding a reagent which enters cells, quenches autofluorescence, and does

not itself emit fluorescence. Crystal violet has been used for this purpose and is reported to be compatible with measurements of cell surface immunofluorescence [17].

Standards, sensitivity, and signal-to-background evaluation

Standardization samples play an important role in obtaining accurate and reproducible results in flow cytometry. Their purpose is in general to assure that data taken at different times or on different instruments can be compared reliably.

Standard particles and their use

These are used to optimize adjustments in the optical system, to evaluate operating conditions, and to reestablish previously defined signal levels as closely as possible. The critical attributes are good uniformity (which makes it easy to see deviations from optimum alignment and minimizes the number of events that must be used to evaluate signal uniformity and measure peak position accurately), availability of a reasonable signal level on all measurement channels, and stability. Polystyrene microspheres are available (multidye particles designed for multi-laser excitation from Spherotech, Inc., Libertyville, IL, USA, others from Polysciences, Inc., Warrington, PA, USA) which meet these criteria quite well.

Electronics testing

Suites of microspheres with known relative fluorescence levels can be used to provide an overall check on amplifier performance. Inclusion of a "blank" minimally fluorescent particle gives an estimate of the minimum detectable signal level. Sets of four or five fluorescent particles and one blank are available from Flow Cytometry Standards Corp. (FCSC; San Juan, Puerto Rico) and from Spherotech. The FCSC sets use the same dyes as are used in flow cytometry reagents, and their fluorescence levels are specified in terms of equivalent dye molecules. This is helpful in maintaining a consistent reference for different lots of microspheres and in giving a rough indication of the relation between signal level and reagent antibody molecules bound. Variabilities in dye/antibody conjugation ratios and in actual dye quantum efficiencies in various reagents, however, prevent accurate antibody molecule evaluation even when the dye/antibody ratio is known. The Spherotech particle sets include multidye polystyrene microspheres like those mentioned in the previous paragraph.

To evaluate a logarithmic amplifier, the microsphere fluorescences are measured, and the signal level for each particle peak is determined. Plotting the log of the reference level for each peak against the measured channel number for that peak should yield a straight line whose slope corresponds to the log characteristic (such as channels per decade) of the system. A straight line supports the conclusion that the amplifier is reasonably logarithmic, although it is possible that deviations are present that will not be detected in a four or five point check.

A pair of microsphere samples, preferably with a fluorescence signal ratio of about 2:1, can be used to test linear amplifiers for linearity and zero signal offset. Multilevel microsphere sets can also be used. Data sets are taken at different PMT voltages, and the peak positions are found for each particle type. The peak position for one particle is plotted against the peak positon for the other. If the linearity is good and there is no zero signal offset, the points will fall on a straight line through the origin with a slope equal to the signal ratio between the particles [18].

Fluorescence compensation

The emission spectrum of the sample used to adjust fluorescence compensation and the dye spectrum of the test samples must match quite accurately to assure correct compensation. This accurate matching is not usually attained by coupling the same dye to plastic particles and to the cell staining reagents. We have found this to be true even for PE, where it would seem that the large protein would insulate the chromophores from other influences. Thus the only really accurate way to set compensation is to use actual reagent labeled cells. We have, however, found that once correct fluorescence compensation has been established on an instrument, we can reestablish equivalent conditions from day to day using stable polystyrene microspheres and reset compensations accurately enough for most routine applications. Single dye labeled control samples are still recommended to check the settings and to optimize them for high precision work.

Reagent binding per cell

Antibody-couples microspheres are available (from Flow Cytometry Standards Corp. and Biocytex, Marseille, France) that are designed to bind a specified number of antibody molecules. These particles can be labeled with the same reagents used to stain cells. To the extent that the binding is really the same for different antibodies and dye conjugates, the signal levels measured for the antibody labeled microspheres provide calibration points for numbers of antibody molecules bound to stained cells analyzed under the same conditons. They should also be very nearly equivalent to stained cells for adjusting fluorescence compensation.

Evaluating background, sensitivity, and signal-to-background in fluorescence measurements

Non-fluorescent particles can be used to estimate the minimum detectable fluorescence signal on each measurement channel. Ideally, the 90 degree light scatter of the particles should be similar to that of the cells used in biological measurements. In that case, scatter-induced fluorescence will be appropriately accounted for in the total background signal measurement. Measurements on these particles can be compared to the signal distribution obtained for unstained cells to estimate the actual autofluorescence of the cells.

For measuring the minimum detectable signal, the distribution of measurements for an appropriate background sample (non-fluorescent microspheres, unstained cells, etc. as required for the particular evaluation) is analyzed to determine how much added fluorescence would be required to produce a signal that could be reliably distinguished from background. A systematic way to evaluate this is described in Chapter 50 [1]. The result is a reference sensitivity or minimum detectable signal estimate corresponding essentially to two SDs in the signal distribution.

Calibrated microspheres or standard stained cells can be used to provide reference fluorescence signal levels. The signal to background ratio is then just the ratio of median signal minus median background divided by the minimum detectable signal.

Cell sorting

The background and details of droplet sorting are discussed in several references including an historical 1976 *Scientific American* article [19] and chapters in multi-author books [20, 21]. In this

section, we treat some of the theoretical and practical aspects as they relate to immunological applications. Droplet sorting is presented in detail, since most of the considerations are common to all instruments using that technology. Figure 47.1 illustrates the non-electronic components of a droplet sorter. Non-droplet sorters are mentioned briefly, since they are more variable in operational principles and have fewer user adjustable controls.

Drop generation and drop charging

Liquid jets of the type relevant for flow cytometry break up under the influence of surface tension into droplets which are energetically favored, since they have a smaller surface area than the cylindrical jet. The drops form at a spacing of roughly 4.5 times the jet diameter (**d**) [20, 22]. The jet velocity divided by this drop spacing corresponds to a frequency in drops per second. Applying a small oscillatory signal to the jet near that frequency will regularize the drop formation to yield uniform drops separating from the jet at a uniform distance from the nozzle. This distance will vary with the amplitude of the applied oscillation. It is possible to form drops with spacing from $\pi \cdot \mathbf{d}$ to well over five **d**, but spacings of 4 to 5 **d** are typical for cell sorting. Higher frequencies are desirable, since they give more drops and better recovery of selected cells in cell sorting, but it is also important to stay within the region of optimal stability of droplet formation. Increasing the jet velocity allows higher frequency operation in proportion the velocity, but the fluid pressures required increase more than linearly with velocity.

To charge a drop for sorting, a potential in the range of 100 volts is applied to the fluid in the nozzle. A drop breaking from the stream with the potential on will acquire a corresponding charge. The deflection plates are charged at + and − several thousand volts. Charged drops falling through the electric field between the plates are deflected in proportion to their charge-to-mass ratio. For a particular drop charging potential and deflection plate voltage, larger drops have a lower charge-to-mass ratio than smaller drops and will be deflected at a smaller angle.

The fringing electric field of the deflection plates at the droplet break point will induce a charge on the drops if the break point is not a zero potential point in the field. In some systems, this interaction is used to adjust the path of the "undeflected" drops by changing the relative potential of the deflection plates. A strong electric field at the droplet break point can, however, result in unintentional charging and deflection of droplets when the drop formation is disturbed by the passage of large cell aggregates (possibly producing serious contamination of rare cell sorts). We have found it convenient to minimize this effect by placing a grounded wire loop around the drop stream near the droplet break point.

Ideally, all deflected droplets will carry the same charge and follow the same trajectory to the collecting vessel. However, whenever adjacent drops are to be charged, our ability to charge the second drop is affected by the nearby charge on the first. To actually get the same charge on the second drop, we must apply a somewhat higher voltage than to the first. Likewise, a small voltage should be applied to the first following undeflected drop to counter the inductive effect of a previous charged drop or drops and leave that drop truly uncharged. On instruments with drop charging adjustments, these may be used to optimize the uniformity of deflected drop paths.

Sort decisions and sort timing

Sort decision criteria are commonly specified by the same methods used in gated data analysis. These include regions in one measurement dimension and/or rectangles, polygons, ellipses, etc., specified on a display of two data dimensions. Combinations of the regions are defined for "left" and "right" sorts, and drops containing cells meeting the specified conditions are charged and deflected. Droplet sorting systems normally provide for two charged fractions (positive and negative) and an uncharged waste stream, although systems have been demonstrated using different charging levels to sort four or more fractions [23].

Since there is considerable delay between cell measurement and incorporation of the cell in a drop (250–1000 microseconds for typical jet-in-air analysis), cell sorting electronics must include provision for tracking several cells between the analysis point and the drop charging point.

Establishing and reproducing optimal sorting conditions

It does not seem to be widely appreciated how easy it is to carry out routine and consistent cell sorting with jet-in-air systems when good sorting conditions are properly defined and carefully reproduced. Therefore, we provide a description of the process in some detail. The critical factors to be defined and reproduced are jet velocity, drop frequency, and position of the droplet break point.

Establishing good sorting conditions

The jet velocity is related to the pressure in the sheath fluid reservoir, but the relationship is not exact due to variability in the pressure drop between the reservoir and the nozzle and due to changes in fluid viscosity with temperature. Velocity (**V**) is roughly related to the square root of the pressure, so the range of velocities is limited for reasonable pressures: 0.8 atmosphere overpressure yields a velocity of about 10 m/sec. Higher pressures (within the allowable range for your instrument) allow higher drop frequencies and higher sorting rates with shorter illumination times for the cells. The drop frequency is chosen to provide stable drop formation at moderate oscillation amplitude. This should occur in the range of frequency $\mathbf{f} = \mathbf{V}/4.5\ \mathbf{d}$. The actual oscillation amplitude used should be high enough to provide stable drops, but not so high as to affect any of the desired measurements. The light scatter signals are particularly susceptible to jet vibration interference. (Sample-specific conditions used for a FACStar Plus with 80 micron nozzle orifice: velocity 10.5 m/sec, frequency 30 kHz ($\mathbf{f/V} = 4.4\ \mathbf{d}$), laser to free drop distance 17 drop spaces.)

Having selected a workable set of drop formation conditions, they are recorded in terms of instrument supported measurements, typically the drop frequency, the laser-to-first-free-drop distance, and the drop spacing measured over a range of perhaps 10 drops.

Determining the appropriate sort delay setting

Run test particles that can be easily identified on a microscope (fluorescent plastic microspheres, fixed cells, etc.), and adjust drop charging to obtain uniform deflected drop trajectories. Select single drop sorting, and sort the test particles onto a microscope slide (fixed numbers in the range of 50, if possible) at a series of sort delay settings, using coarse steps in the delay setting to find the right region, and then fine steps across that area

if necessary. It is often helpful to sort onto microscope slides that have been smeared with protein solution (such as serum) and allowed to dry. The location of sorted drops is then easy to see, and the protein prevents the formation of large salt crystals as the droplets dry, thereby making it easier to identify the sorted particles. The maximum recovery of sorted particles (which should be near 100%) identifies the correct delay setting. Remember to set the number of deflected drops back to the usual value if you are not normally doing single drop sorting.

Reproducing the sorting conditions

The frequency is set to the previously established value. Setting the reservoir pressure to a previous value will roughly reestablish the jet velocity, but direct measurement and fine adjustment are necessary to make it exact. The velocity measurement is done by measuring the distance between drops (as the average over, such as a 10 drop interval). Since the frequency is set to match the standard value, reproducing the jet velocity reduces to measuring the drop spacing and adjusting the reservoir pressure to bring that spacing to the standard value. Then the vibration amplitude is adjusted to bring the drop break point to the standard distance from the laser intersection. At this point all the factors are matched, and the sort delay setting previously determined will be correct. If different nozzle orifice sizes are to be used, appropriate drop frequencies and other conditions must be established for each, but once this has been done, test sorting should only be required when overall sorting conditions are changed.

Sorting rate, coincidence losses, and selection of the number of drops per sort

There is always some uncertainty in assigning cells to their appropriate drops due to limited accuracy in defining the initial sorting conditions, to drift in the sorting conditions over time, and to the effects of the cells themselves on the drop formation process. Therefore, one or more adjacent drops may be sorted to assure that the cell is deflected. On the other hand, conflicts in drop assignment between wanted and unwanted cells increase dramatically as more drops are assigned per cell, so we generally want to sort as few drops as possible while maintaining high assurance that each cell will be found in its assigned drop or drops. For routine sorting, assigning two drops per cell provides + or − 1/2 drop latitude before contamination or unexpected losses occur. Some instruments provide a mode in which one drop is sorted if the cell is assigned to the "center" of that drop, and a second drop is added if the cell is near the "edge." Another form allows single drop sorting of desired cells but provides greater than one drop exclusion of unwanted cells to assure purity. Sorting fewer drops also increases the cell concentration in the sorted fractions (such as an 80 micron diameter 10 m/sec jet and 2 drop sorting at 30 kHz yields a cell concentration of about 300,000/ml; with 3 drops this would be 200,000/ml).

Since cells appear randomly in the stream, some wanted and unwanted cells will be too close together to be separated. The frequency of such "coincidence" problems depend strongly on the cell flow rate, on the drop frequency, and on the number of drops assigned per cell. In normal sorting, drops that could possibly contain an unwanted cell are not sorted, resulting in loss of some desired cells. For multiple drop sorting, a mode in which a cell is sorted only if all drops assigned to it are unconflicted provides assurance that each sorting event will yield a cell, but at high flow rates a lot of desired cells will be lost. Such a mode is appropriate for fixed count sorting, including cell cloning. A mode in which any unconflicted desired drop is sorted will provide a higher yield of desired cells without loss of purity, but the cell count will not be exact. As a rule-of-thumb, the tradeoff between increasing cell rate and increasing fraction of losses results in a maximum/optimum cell run rate of about 0.3 (drop frequency)/(drops/cell). For 2 drop sorting at 30 kHz, this is 4500 cells/sec or about 15,000,000/hr. With coincidence losses, we would expect to obtain the desired cells from about 10,000,000 cells per hour. When high cell rate and maximal recovery of selected cells are more important than purity of the sorted fraction, an "enrighment" sort mode which ignores coincidence effects and sorts all selected drops can be useful.

To allow sorting at higher rates, instruments have been constructed to operate at up to 200 kHz with nozzle pressures of 200 psi [24]. Commercial instruments are being adapted to operate at 100 kHz and 50 to 60 psi. For mammalian cells specifically investigated, including human stem cells, these conditions are compatible with good cell viability as measured by dye exclusion methods and give results in functional studies that are the same as found with cells sorted at lower pressure (personal communication, Dennis Sasaki, SyStemix, Inc., Palo Alto, CA USA). The brief laser transit times in these instruments may lead to somewhat lower sensitivity for immunofluorescence.

Cell aggregation is a major cause of poor recovery of cells, since only single cells can be properly measured and sorted. A majority of the cells in a sample can easily be contained in aggregates that make up only a few percent of the detected objects.

Reanalysis of sorted fractions

Whenever possible, sorted cell fractions should be reanalyzed to assess the actual purity of the sorted population. It is especially critical, however, to be sure that residual cells from the original sample are removed from the apparatus, since the sorted fractions are typically at rather low cell concentration. We change sample filters and flush the sample line with 70% ethanol to kill any residual cells. Cell-free fluid is then run as a sample until the background rate is acceptably low. Then a small aliquot of the sorted fraction is run and data collected for it. Propidium iodide may be added (see "Dead cell exclusion using propidium iodide" on page 47.6) to mark both dead cells in the reanalysis sample and ethanol-killed residual cells.

Optimizing cell viability in sorting

Cell handling should be oriented toward keeping the cells in a single cell suspension and otherwise as happy as possible. Media with high protein, etc., can be used. For most staining procedures, cells are cooled to minimize changes in the staining pattern. The sheath fluid should be at least buffered saline. Tissue culture media without pH indicator dye or protein can be used as sheath fluid, but media formulated for CO2 buffering should have additional buffering with, e.g., HEPES. Extra buffering may also be needed for media used to pre-fill sorting receptacles such as cloning trays, but for that use, optimal protein/serum should be included. Since sheath fluid should not contain protein, it may be desirable to stock collection receptacles with serum or high serum media and mix every few minutes to distribute the cells.

Sterile sorting

In our experience, sterile sorting can be carried out routinely and reliably and even alternated with non-sterile analysis or sorting with 1–2 hours setup time. In the sheath fluid line, we use a 0.22 micron filter followed by a disposable 0.45 micron filter. Ethanol (70%) is injected between the filters to fill the 0.45 micron filter, the rest of the sheath fluid line, the nozzle, sample lines, etc. After 20 to 30 minutes, normal sheath fluid is used to flush the system for several minutes. Since collection receptacles may be open for for extended periods, it is good to minimize dust and air turbulence in the area. When allowable for the sample we include basic antibiotics in the collection media.

Cell cloning and fixed count sorting

Flow sorting is a very useful technique for efficient provision of single cell clones. Light scatter gating can usually be used to increase assurance that cell doublets are excluded and that each culture is derived from a single cell. Rare cells can be selected and directly cloned, and even in the case of simple viable cell cloning, sorting single cells is very preferable to limiting dilution methods. The technique is also applicable to isolating cells with specified properties for single cell PCR and sequencing. This work should be done in a sorting mode which assures that all selected drops are sorted.

Enclosed flow sorters

Instruments have been produced using several different principles for cell sorting in an enclosed system. This can be a desirable property when biohazardous samples are to be sorted. Cell rates are limited to a few hundred per second, and some systems maintain continuous flow to the collecting receptacle, yielding low cell concentrations.

Bulk enrichment and flow cytometry

When larger numbers of purified cells are needed than can be conveniently obtained by flow sorting, bulk selection methods can often be combined advantageously with flow cytometry (see Chapter 51 [25]). Enriching a rare cell population in bulk and flow sorting, the resulting sample may yield sufficient numbers of highly purified cells in an acceptable sorting time. Alternatively, flow analysis may simply be used to provide a quantitative assessment of the populations produced by a bulk separation method.

Rare cell isolation

Techniques for isolating rare cells are treated in Chapter 52 [26], but we would like to emphasize one critical point in the design of flow sorting experiments. For the best possible exclusion of unwanted cells, debris, etc. in a rare cell sort, design the sample staining protocol, so that the cells of interest have minimal signal in at least one of the fluorescence measurements in which they differ from the bulk of the cells in the sample. Selecting cells of interest to be positive for all the fluorescent stains is an invitation to include fluorescent debris, non-specifically staining dead cells, pairs of cells whose sum produces the positive pattern, and "passenger" cells whose fluorescence added to that of an actual cell of interest still gives signals in the selected pattern.

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