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CHAPTER 22

Analysis and separation using the fluorescence activated cell sorter (FACS)

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General Description

The fluorescence activated cell sorter (FACS) is used both for analysis and separation of cells in suspension [1–4]. It rapidly measures the size and fluorescence of individual cells as they flow, single file in a stream past a laser illumination system coupled with a set of highly sensitive detectors. The measurements, collected and displayed, create a set of analytical profiles of the cell population for each parameter and for pairs of parameters. These profiles provide the basis for definition and separation of sub-populations.

The most recent version of the FACS (FACS-II) [4, 5] has three simultaneously operating detectors: one for low angle light scatter, which essentially measures cell size; one for fluorescence; and one which can be used either for large angle light scatter or for fluorescence (to allow measurement of two fluorochromes, e.g. fluorescein (green) and rhodamine (red), associated with same cell). With this system, it is possible to determine the frequency distribution of cells of different sizes within a population, the distribution of cells with various amounts of two different colours of cell associated fluorescence (surface or internal) or the frequency of cells showing different amounts of large angle light scatter. Furthermore, by combining parameters (gating), the system can determine the fluorescence intensity distribution in each colour of cells within selected size ranges, the rhodamine fluorescence distribution of cells within selected size and fluorescein fluorescence ranges, etc. All three parameters can be used simultaneously in any combination.

The triads of parameters can also be used to separate cells which fall within specified ranges in each. For example, the FACS can isolate sub-populations of cells within a selected size range which also fall within selected fluorescence ranges in each colour, e.g. a sub-population of cells which are small, dull green and bright red fluorescent. Two such sub-populations are collected at a time while the rest of the cells, debris, cell aggregates pass into a waste container. Between 10^7 and 10^9 cells may be processed per hour, the number processed depending on the frequency of the desired cells in the overall population, and the desired yield and purity of the isolated sub-populations. The separation procedure does not impair viability of the cells and may be carried out under sterile conditions. Therefore, the functional activities of isolated cells are readily measurable.

FACS analyses and separations have found a wide range of applications in cell biology. These include: measurement of DNA content per cell to identify hybrid cells [6] or the stages of the cell mitotic cycle in a population [7, 8]; measurement of DNA content of individual chromosomes in suspension for karyotyping and sorting of individual chromosomes [9]; viability determinations using fluorescent dye exclusion or fluorochromasia with fluorescein diacetate [10] (see pp. 22.7–22.9 and section III); estimations of frequency of virus infected cells using immunofluorescence of fixed cells [11]; and even identification and separation of algae in natural mixtures [12]. By far the largest number of FACS studies to date, however, have involved the identification, isolation and functional characterization of sub-populations of lymphocytes carrying surface antigens detectable with fluorescein (or rhodamine) conjugated antibodies.

In this chapter, we will concentrate mainly on methods developed (usually within our own laboratory) in the course of studies of the immune system, drawing specific examples from these studies as required. As the reader will soon recognize, however, the methods described here in an immunological context generally will require only minor modification to become applicable in other disciplines, such as cell genetics, neurobiology, developmental biology, microbiology, etc.

How FACS works

The schematic diagram in Fig. 22.1 shows the essential components of the FACS analytic and sorting assembly. Cells leave the reservoir (not shown) and are injected under slight pressure into the centre of a stream of cell-free sheath fluid which is being expressed through a nozzle. A short distance below the nozzle, cells (still centred in the stream) pass through the detection zone where they are illuminated by a laser beam. A detector for light scattered at a small forward angle (typically 1°–13°) and two detectors (only one is shown) for fluorescent light emitted at two wavelengths register the light from each cell (or particle). These detectors translate the optical signals to electrical pulses which are amplified, processed and stored for display and analysis. The cells continue down the stream and are sorted, or, in the case of analysis, discarded.

When the FACS is used for sorting (rather than analysis only), the stream is subjected to a low intensity vertical vibration at 40 000 cycles per

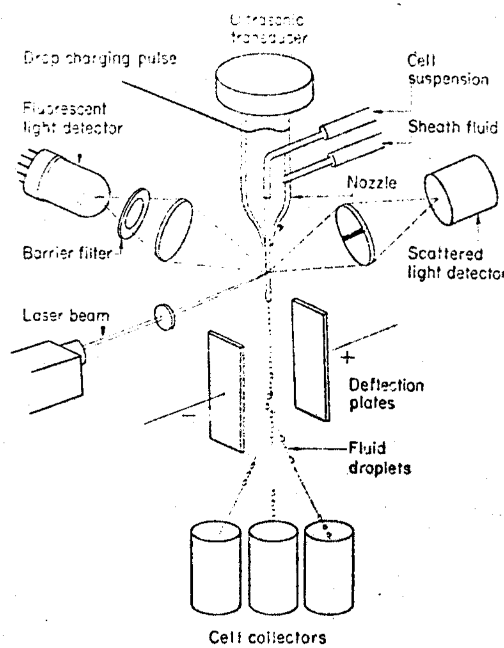


FIG. 22.1. Schematic diagram of the FACS-I. FACS-II has a second fluorescence detector.

second. This causes it to break into droplets (40 000/second) a short but accurately fixed distance below the detection point. Under these conditions, cells which have passed the detectors continue down the stream, reach its tip and become encased in droplets. Each droplet is approximately a thousand times the volume of a cell. Usually only 1/6th of the droplets contain a cell.

During the time it takes for a cell to go from detectors to stream tip, its fluorescence and scatter measurements are processed and compared with preset parameter levels defining criteria for inclusion (sorting) into each of two sub-populations. If the inclusion criteria are met, and if no undesired (coincident) cells are present in the stream in close proximity to the desired cell, then an electrical charge is applied to the stream at precisely the time the desired cell reaches the stream tip so that the droplet containing the cell will be charged (negative or positive, depending on whether the deflection is to be left or right). In practice, two or even three droplets are charged to insure deflection of the desired cell, and the coincidence exclusion distance between desired and undesired cells is adjusted accordingly.

The charged (and uncharged) droplets broken from the stream continue to fall directly downward

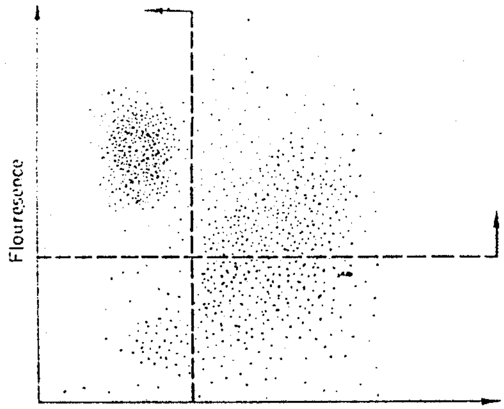


FIG. 22.2. Simulated 'dot-plot'. The scatter and fluorescence measurements taken for each cell in the population determine the co-ordinates of dots displayed on an oscilloscope screen. An imaginary population (created for demonstration purposes) is shown in the figure. It has a brightly fluorescent, small-sized sub-population (upper left quadrant). The dashed lines represent the location of scatter and fluorescence threshold (gates) which isolate the population (see Fig. 22.4).

from the nozzle until they pass through a constant electrical field created by a set of charged 'deflecting plates'. In the field, the charged droplets are deflected towards the left or right collection vessel. All uncharged droplets continue directly downwards and fall into the centre discard vessel.

How FACS displays analysis data

Two parameter displays: dot-plots and isometric displays

FACS systems provide several options for collecting and displaying the fluorescence and light scatter (size) measurements taken for each cell as it passes the detector. One of these systems, a 'dot-plot' display, is generated during the analytic run and displays pairs of parameter measurements for each cell. As the cell is examined by the detectors, a dot appears on a screen at co-ordinates determined by the magnitude of one parameter (e.g. size) on the horizontal axis and the magnitude of a second parameter (e.g. green fluorescence) on the vertical axis (see Fig. 22.2). A third parameter may be used to restrict (gate) data displayed on the 'dot-plot' for signals from cells within a selected range, e.g. to obtain a size versus green fluorescence dot-plot of bright red fluorescent cells.

Dot recording is generally terminated at an arbitrary point, when a sufficient number of dots are marked to allow a pattern to be recognized and before too many dots are recorded to obscure its visibility. Alternatively, recording is terminated after a preset number of cells is analysed. A permanent record of the analysis can be obtained by photographing the screen.

The dot-plot allows visualization of the correlation between parameters and hence of sub-populations in which parameters are correlated. Thus, in the figure shown, a sub-population of small bright cells is apparent in the upper left-hand quadrant. It should be emphasized, however, that 'dot-plot' is only a semiquantitative indicator of sub-population density. Because only one dot can be recorded at each pair of co-ordinates, a large sub-population in a narrow size and fluorescence range will appear deceptively as a small spot.

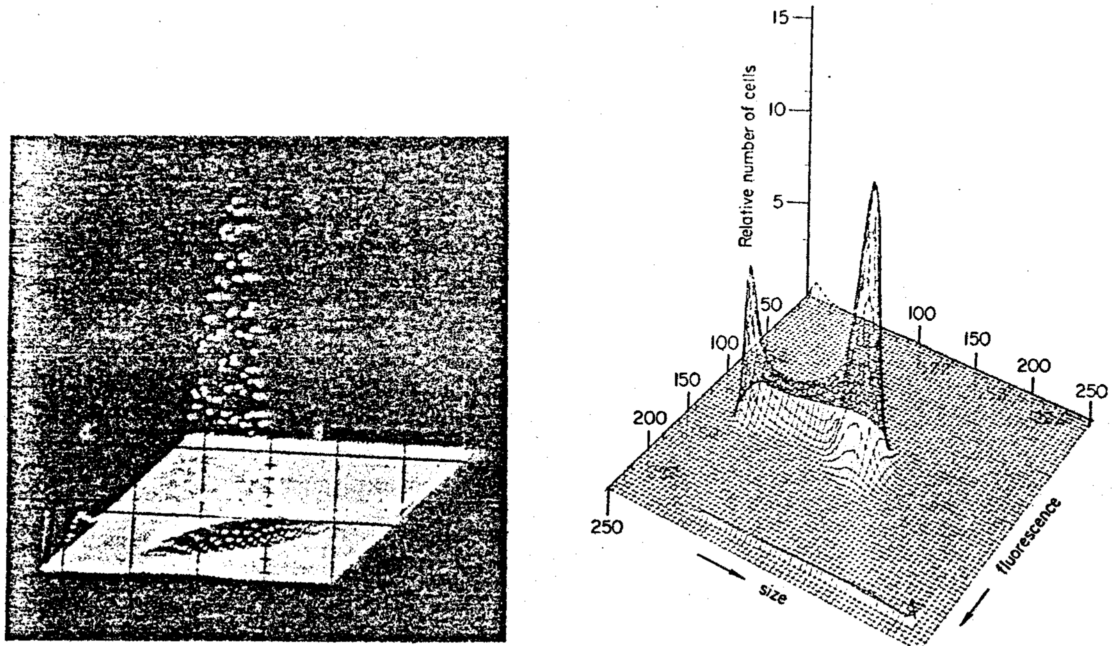
The isometric display provides a quantitatively accurate substitute for the dot-plot. It uses the oscilloscope screen to display a two-dimensional histogram. Two FACS parameters, e.g. size and fluorescence, are used for the X and Y axes. The number of cells for each pair of co-ordinate values is represented on the axis (height). An angular view is presented so that sub-populations are seen as peaks (see Fig. 22.3a). Like the dot-plot, the FACS 3-D display is photographed to provide a permanent record. Fig. 22.3b shows a computer-generated and drawn perspective display. It differs from the internal FACS isometric display in that the dots are suppressed and lines are drawn to connect the points. (Note: The cell populations analysed in Figs. 22.3a and 22.3b are different.)

Dot-plots and 3-D displays can be obtained for an entire cell population or for sub-populations expressing specified levels of a third undisplayed parameter (see later section on gated analysis).

The definition of sub-populations on the basis of correlated parameter expression can also be (and usually is) accomplished without 3-D displays. The methods used for these analyses are described in the sections which follow.

Histograms

Most of the FACS analysis data collected (and published) on the size and fluorescence of cell population is presented in the form of one dimensional histograms in which the horizontal axis shows units of fluorescence or light scatter (size) per cell and the vertical axis shows frequency of cells. Thus



(a) (b)

FIG. 22.3. 3-D displays. (a) *Isometric display*: The population analysed consists of glutaraldehyde-fixed sheep erythrocytes with covalently bound fluorescein residues. This photograph of data displayed on a FACS-II oscilloscope was kindly provided by Dr D. Mason, Oxford University. (b) *Perspective display*: The population analysed consists of glutaraldehyde-fixed chicken erythrocytes (CRBC) rendered fluorescent by the fixation. This display was generated on a PDP-11 (Digital Equipment Corporation) computer interfaced with the FACS.

each point on the analytical histogram shows the frequency of cells in the population displaying the amount of fluorescence (or scatter) indicated on the horizontal axis. Interpretation of such histograms is essentially straightforward; however, because they are basic both to analyses and separations, it is perhaps worthwhile to consider in some detail how the historgams are generated and used.

Separate detectors are used for light scatter and for each fluorescence colour. The amount of fluorescent or scattered light received by each detector as a (laser illuminated) cell passes is translated proportionally into an electrical (analogue) pulse. The pulses (from each cell) are processed simultaneously by parallel systems which convert analogue pulses to numerical (digitized) values proportional to pulse size.

The numerical values thus obtained are then simultaneously transmitted to the FACS logic circuitry where they are tested against externally chosen numerical boundaries (gates) for each parameter to determine whether the set of values is to be included in the data set or excluded (gated out). These same testing systems are used during separation to determine whether a cell is to be discarded or deflected into one or the other collection vessel.

If the set of values from a given cell is to be included in the data set, the numerical values are transmitted to the pulse height analyser (PHA) for tallying. Values from two detectors (parameters) can be tallied simultaneously by the PHA during a single analytic run. The third parameter values are used for gating only.

The PHA uses a series of electronic counters (channels) to keep a cumulative count, for each parameter, of the number of times a given numerical value (or group of numerical values) appears. To do this it classifies each value for a parameter, as belonging either within a chosen range or as greater than the upper limit of the range. Those values within the range are further classified as belonging within one of a series of numerical intervals (approximately 250) which equally divide the range. (The size of the range, hence of the intervals, is determined by selection of a PHA gain setting.)

As analysis proceeds, the PHA tallies the number of values received in each interval in channels assigned to correspond to the intervals. All pulses above the range are scored in the last several channels of the series. The numbers in the PHA channels at the end of the analytic run constitute the raw data from which the histograms are constructed.

The format of the histograms displayed by different FACS installations may vary depending on the computer support available and the preferences of individual investigators. The most common format displays PHA channel number on the horizontal axis and number of cells per channel on the vertical. Scaling the vertical axis gives frequency in the overall population of cells scored in each channel. Scaling the horizontal axis gives units of light scatter, units of fluorescein or units of rhodamine per cell. These fluorescence units are directly proportional to the amount of fluorescence expressed per cell and, in general, directly proportional to the number of molecules of fluorochrome bound. Regardless of format, however, all histograms currently used display the distribution of cells as a function of linear increase in parameter (size, fluorescence).

There is some variation between FACS systems with respect to display of data in the last several channels. Since all signals greater than the preset range are accumulated in these channels, an artificial peak may appear at the end of a distribution. Some displays omit this peak, although it can be taken into account when the data is scaled to show frequencies.

The preset range is controlled by the gain setting on the FACS. When the horizontal axis is given in terms of units of fluorescence or scatter, the gain setting is normalized and histograms taken at different gain settings may be compared directly. When the horizontal axis is given in terms of channel number, direct comparison is possible only between histograms taken at the same gain. To compare histograms taken at different gains, channel number must be corrected by multiplying by the ratio of the gains, e.g. channel 30 at gain 20 is equal to channel 75 at gain 50.

How the histogram displays are used: gated analysis and separation

FACS histograms serve two key functions: (1) as primary analytic data, they provide a quantitative basis for the definition of sub-populations, by displaying the frequency of cells in the population as a function of parameter expression, the histograms allow visualization of clustering of cells within a restricted parameter range and estimation of the mean or modal amount of parameter expressed per cell in the sub-population; and (2) as operation data, the histograms provide an index for establishing FACS thresholds either for separation or analysis of cells expressing one parameter in a sub-population defined on the basis of one (or two) other parameters. This latter function, usually called 'gating', stands as one of the most powerful capabilities of the FACS.

To illustrate the principles of gating, we will return to the hypothetical cell suspension whose size and scatter distributions are shown on the dot-plot in Fig. 22.2. A small, bright sub-population appears in the upper left quadrant of the dot-plot. This sub-population can be isolated (sorted) or its frequency in the overall population determined by properly setting the FACS size and fluorescence gates (thresholds). The procedure would run essentially as follows.

First, un-gated fluorescence and scatter histograms, such as those simulated in Figs 22.4a and 22.4b, are obtained for the overall population. In the case we have chosen to illustrate, neither of these histograms shows an obvious small or bright sub-population. This is consistent with the dot-plot data which indicates that the desired sub-population of small bright cells represents only a small fraction of an overall population in which size and fluorescence are poorly correlated in general.

The absence of a definite peak or shoulder in the histograms means that the gates for isolation or frequency estimation of the sub-population will have to be set arbitrarily: the dot-plot, however, can be used as a guide. Either scatter or fluorescence gates could be set first in this case with the same result. We will choose scatter gates and set them as indicated Fig. 22.4b to include (roughly 10 per cent of) the smallest cells in the population.

With the scatter gate set, the population is re-analysed to obtain a second fluorescence histogram showing the fluorescence distribution of cells within the selected size range (see Fig. 22.4c). This histogram reveals the presence of two distinct sub-populations amongst the small cells, one with very little cell associated fluorescence (dull) and the other with substantial amounts of fluorescence (bright). Setting upper and lower fluorescence gates to include

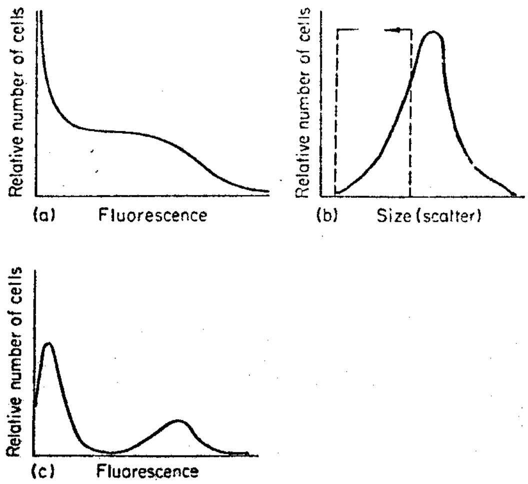


FIG. 22.4. Simulated gated analysis. The imaginary population shown in the dot-plot in Fig. 22.2 was 'analysed' for this figure to reveal a bright fluorescent, small-sized sub-population. All cells in the imaginary population are viable. (a) Ungated fluorescence histogram. (b) Ungated scatter histogram showing scatter gate used to exclude signals from large- and medium-sized cells. (c) Scatter-gated fluorescence histogram showing the fluorescence distribution of small-sized cells.

the cells under the 'bright' peak readies the FACS to specifically separate the small bright population and discard all cells not falling within the selected criteria. The actual separation then requires setting up collection vessels and switching on the droplet deflector (see later section).

The frequency of sub-populations in the overall population can be determined directly by integration of the histograms. Thus, in the example chosen, integrating between the points at which the scatter gates are set in the scatter histogram in Fig. 22.4b gives the frequency of 'small' cells in the overall population; integrating between the fluorescence gates indicated on the scatter-gated fluorescence histogram in Fig. 22.4c gives the frequency of 'bright' cells in the 'small-size' population; and multiplying the frequencies then gives frequency of the small, bright cells in the overall population.

The use of scatter-gating in fluorescence analysis actually extends beyond the type of sub-population analysis described above. Virtually all fluorescence histograms obtained with the FACS are scatter-gated, because appropriately setting the scatter gate eliminates fluorescent signals from dead cells (see pp. 22.8–22.10). Thus, in a real experiment, the fluorescence distribution shown in Fig. 22.4b would have been obtained after the scatter gate location for exclusion of dead cells had been determined and the lower gate set to allow only those fluorescent signals associated with live cells to be plotted in the fluorescence histogram.

Standardization of the FACS

Standard fluorescent particle suspension

In many laboratories (including our own), chicken erythrocytes (CRBC) rendered fluorescent by glutaraldehyde fixation are used for scatter and fluorescence standardization of the FACS. The fixed CRBC have a characteristic bimodal FACS light scatter profile (histogram) well separated from the scatter profile for most mammalian nucleated cells. Furthermore, the fixative renders the CRBC fluorescent. They develop a uniform amount of fluorescence per cell with broad excitation and emission spectra spanning the range required for both fluorescein and rhodamine. The ready availability of CRBC, the ease of fixation and the long-term stability of the fixed cells recommends them for use as a standard.

We routinely fix the CRBC by making a suspension in 1 per cent glutaraldehyde in phosphate buffered saline (pH 7) for 48 hours at room temperature. Fluorescence increases and then stabilizes during this period. The suspension is then washed, and resuspended in distilled water and aliquots are frozen. For use, thawed aliquots are washed and resuspended at 2×10^7 cells/ml.

The fixed frozen CRBC are stable for years. A working suspension kept in saline in the refrigerator is stable for at least a week.

Routine alignment and adjustment to a reproducible standard

A standard suspension of fluorescent particles, such as the glutaraldehyde fixed CRBC described above, is used for standardization. At the beginning of an analysis or separation, the FACS is 'fine tuned' to obtain the sharpest peaks (lowest variance) for fluorescence and scatter with the standard. The peak (modal) positions for scatter and fluorescence intensity are then adjusted to correspond to standard values (channel numbers) on the PHA. At intervals thereafter (as analysis or separation proceeds), the CRBC standard is rerun and the FACS readjusted if necessary to return to the original peak channel and sharpness.

This standardization procedure allows accurate comparison between experiments run on the same machine at different times. In addition, once standards are compared, it allows comparison between experiments run at different FACS installations.

Proportionality of pulse height to fluorescence intensity

When the FACS is properly set up for fluorescence analysis using mono disperse particles, the peak position (modal channel number) is proportional to actual particle fluorescent intensity. Linearity of response can be tested readily using a CRBC standard by taking advantage of coincident passage of two and three (and more) CRBC through the detection zone. This gives signals two and three times as great as the single cell signals and can be seen as smaller peaks following the main peak on the fluorescence histogram. If these smaller peaks are not at positions corresponding to 2x and 3x, some component(s) of the amplifier system is being saturated. This can be remedied by decreasing the photo multiplier (PMT) voltage or the laser intensity, or both.

When unknown cell populations are analysed, linearity is tested by changing amplifier gain and seeing if the peak(s) change proportionally. The remedy for non-linearity of response is again to decrease signals fed into the amplifier by decreasing PMT voltage and/or laser intensity. A frequent artifact due to amplifier saturation with particularly bright cells is a peak appearing on the PHA display at about channel 180 (using a 256 channel range).

Light scatter analysis

Size determination

FACS uses the amount of light scattered at a small forward angle (1°–13°) as an index of cell size. This measure is mainly due to diffraction but it not strictly proportional to cell size because it is influenced by such cell properties as asymmetry, reflectivity, refractive index, granularity, and nuclear size. Nevertheless, there is a strong positive correlation between cell size as measured by the FACS scatter detector and cell size as measured by sedimentation at unit gravity [13] or with the Coulter counter [14].

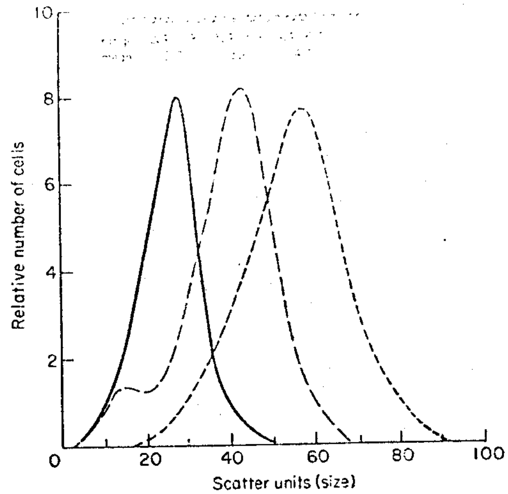


FIG. 22.5. Correlation between cell size measured by light scatter and unit gravity sedimentation. Thymocytes were separated into three fractions (small, medium and large) containing approximately 45, 45 and 10 per cent, respectively, of total population. A scatter histogram is shown for 10 000 cells in each fraction. Mean sedimentation rates and range are indicated in the figure for each fraction above its scatter histogram.

Data in Fig. 22.5 shows FACS scatter histograms for three different-sized thymocyte sub-populations separated by unit gravity (1 *g*) sedimentation. For each sub-population, mean sedimentation velocity, which is proportional to mean cell volume, is shown above the histogram. The modal scatter for each population correlates well with this value. Similar results were obtained with spleen, lymph node and bone marrow cells separated by unit gravity sedimentation.

Light scattering, then, can be used empirically as a relative index of cell volume (size); however, care must be taken in interpreting results from size analyses using light scattering, since for some types of cells the scatter signal may be considerably more dependent on the non-volume related cell properties noted above. With this reservation in mind, we use the terms 'size' and 'scatter' interchangeably in this text.

Viability determination

The FACS light scatter signals provide a convenient means of distinguishing live from dead cells. The scatter profile (histogram) of murine spleen cells (depleted of erythrocytes) shows a minor peak

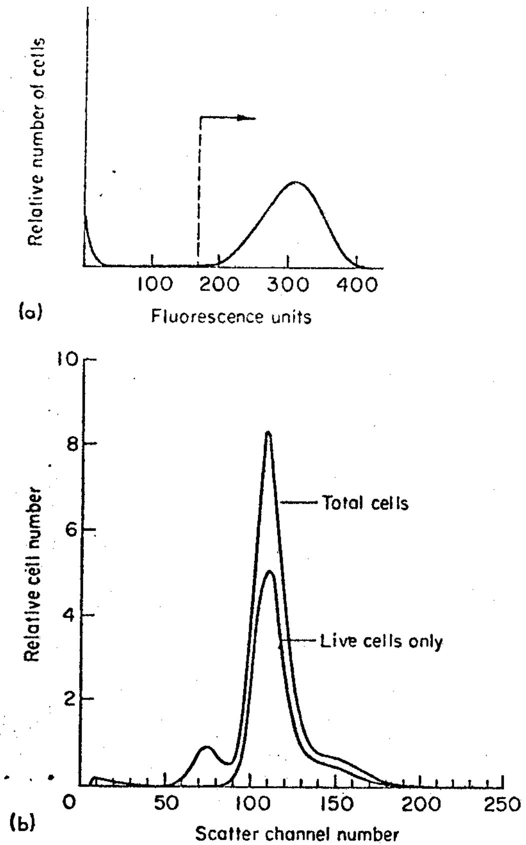


FIG. 22.6. Difference in light scatter profiles for live and dead cells. Live cells retain fluorescein after incubation with fluorescein diacetate (FDA); dead cells do not. (a) Fluorescence histogram of FDA incubated spleen cells showing fluorescence gate used to exclude non-fluorescent (dead) cells; (b) Scatter histograms of fluorescence-gated (live) population and total (ungated) population.

representing cells with less scatter and a major peak of cells which scatter more light. Experiments using the fluorogenic compound fluorescein diacetate (FDA) and a gated analysis show that the minor peak is entirely composed of dead cells and the major peak represents nearly all live cells [26].

FDA is non-fluorescent and rapidly penetrates cell membranes. Inside cells it is hydrolysed by esterases to yield free fluorescein which is highly fluorescent. Free fluorescein cannot pass intact membranes but readily leaks out of the damaged membranes of dead and dying cells. Thus following brief FDA incubation, viable cells become intensely fluorescent while dead cells do not [10].

Using gated analyses (described earlier) we can examine the scatter profiles of all the cells and of only the fluorescent (live) cells after FDA incubation. These profiles are shown in Fig. 22.6. The fluorescent cells are all included in the major peak. Thus the minor peak represents only dead cells.

This distinction between live and dead cell scatter profiles occurs clearly in all lymphoid populations and with most other cells to varying degrees of clarity using the FACS. Size alone cannot be the only basis for such a clear resolution by scattering of live from dead with lymphocytes. Refractility, perhaps related to the difference in appearances of live and dead cells with phase contrast microscopy, may play a major role. Whatever the physical explanation, the empirical observation is extremely useful when working with the FACS. We have also seen a number of cases where cells judged live by trypan-blue exclusion were dead by functional and scatter criteria suggesting that FACS scatter and fluorescein retention may be the best available criteria for cell viability.

The characteristic 'small' appearance of dead cells in the scatter histogram is of enormous practical significance for FACS fluorescence analysis and separations especially when fluorescent labelled antibodies are used to detect surface markers on sub-populations. Dead cells tend to non-specifically adsorb the fluorescent reagent. Therefore, without the ability to exclude dead cells from consideration, a substantial number of falsely positive cells would be introduced into the fluorescence distribution. This has led (as indicated earlier) to the standard practice of scatter gating all fluorescence distributions so that only live cells are included.

There are of course, some exceptions to this rule. First, although the procedure works well for lymphocytes and many other cells, it does not work for some mixtures of large and small cells since very large dead cells can give the same scattering signal as live small cells. Secondly, a relatively large dead cell peak can substantially overlap the live cell peak so that the gating threshold will be difficult to set without excluding a large fraction of the live cells. Proper practice dictates that these exceptions be recorded in published observations.

Cytotoxicity measurements using light scatter and FDA criteria

The FACS can be used to determine the percentage of cells killed by a treatment such as antibody plus complement in a cytotoxicity assay. The fraction of viable cells can be estimated by light scattering, but FDA staining also can be used for this purpose.

Straightforward use of scatter histograms to determine relative proportions of live and dead cells (per cent viability) in a treated suspension is not sufficiently accurate for this assay because dead cells can disintegrate past recognition by the FACS. Use of gated scatter or FDA fluorescence analysis to obtain an absolute count of residual live cells per unit volume of suspension is also inaccurate because FACS flow rate variations prevent sampling of a set volume of the suspension. These problems can be overcome, however, by preparing FACS-countable particles which are easily distinguished from cells. If a standard number of particles are added to a standard volume of the suspension, the ratio of live cells to particles can be used to calculate the absolute number of residual live cells per unit volume of suspension.

Either live cell scatter or FDA fluorescence analysis, which is somewhat less convenient but more accurate, has been used for these determinations. In both cases, glutaraldehyde-fixed chicken erythrocytes (CRBC) were used as particles since the CRBC scatter histogram does not overlap most mammalian live cell scatter profiles; and CRBC fluorescence, which occurs as the result of fixation, is stable, uniform and considerably duller than FDA live cell fluorescence.

The procedure for determining live cell to particle ratios is also essentially the same in both cases. It utilizes a set of counters provided with the FACS which tally signals received from separate gated populations. To set up the analysis, a control mixture of particles and live cells is used to obtain a histogram showing the boundaries of each population. From the histogram, gates are established to create two windows, one which includes all live cells and the other all particles. Counters are then set, matched to each of the windows, so that the number of live cells will be counted on one until a fixed number of particles (generally 10 000) is counted on the other.

With the FACS set this way, a series of samples can be rapidly analysed. If 10 000 particles are counted, the percentage of live cells in each sample can be determined by dividing the number on the cell counter by 100.

Immunofluorescence analysis

General considerations

Immunofluorescence staining techniques are currently used in many areas of biological research to demonstrate the presence of a particular cell type in a suspension, to recognize (or count) different types of cells or to show that a particular determinant is found on cell surface membranes (Chapter 15).

The dependence on fluorescence microscopy for visualization of stained cells, however, severely restricts the application of these techniques. Visual observation, although frequently adequate, is slow, subjective and at best semi-quantitative. Thus the development of the FACS analytic system, which offers greater sensitivity, quantitation, statistical accuracy and speed markedly extends the range of conventional immunofluorescence studies.

But the FACS should not be considered just a mechanical–electronic substitute for the fluorescence microscope. The real value of the instrument lies in its innovative features: The parameter gated analysis and sorting allow qualitatively new applications of immunofluorescent staining techniques toward the solution of problems previously unapproachable with these techniques.

The ability to analyse gated populations means that fluorescence or size distributions can be obtained for sub-populations within a single cell suspension. Thus gated analysis allows comparison of fluorescence distributions of small versus large cells, comparison of size distributions of bright or dull cells, and, by these comparisons, identification of sub-populations even though the overall size or fluorescence distribution does not indicate the presence of a sub-population. Similarly, using two fluorescence parameters (colours), gated analysis allows determination of the distribution of cells carrying different amounts of one surface determinant among a sub-population of cells carrying a second determinant. Combining all three parameters then, it becomes possible to define a sub-population by size and the amount of two determinants carried on the cell surface; for example, a sub-population of medium sized cells which carry a large amount of one determinant and a small amount of a second.

The ability to measure the amount of determinant (fluorescence) per cell and to determine modal amounts of fluorescence for sub-populations opens up new applications for immunofluorescence methods in addition to sub-population definition. For example, FACS enables study of the genetic control of the quantity of a surface determinant expressed per cell. Similarly it allows study of quantitative expression of cell surface determinants during development or re-expression after removal.

In the following sections these principles are illustrated with a series of FACS analyses of surface determinants found on murine lymphocytes. Hopefully these examples will provide the FACS user with a guide to the instrument's strengths and weaknesses. In addition, they should provide a useful base for the investigator not directly involved in FACS utilization but desirous of interpreting FACS studies appearing in the literature.

Immunofluorescent staining

Fluorescent staining methods fall into two general categories: 'direct' staining, where fluorochromes such as fluorescein or rhodamine are conjugated to the antibody which binds to the cell surface determinant; and 'indirect' staining, where the antibody which binds to the surface determinant is not fluoresceinated and its binding is demonstrated by the binding of a second-step fluoresceinated antibody reagent specific for determinants on the non-fluorescent first-step antibody. This latter procedure amplifies the stain because several molecules of second-step fluoresceinated antibody generally bind to one molecule of first-step reagent.

The presence of two different surface determinants on a single cell is demonstrable either by direct or indirect staining as long as the antibody reagents used to detect each are conjugated with fluorochromes which emit at different wavelengths (i.e. show different coloured fluorescence). Fluorescein (green) and rhodamine (red–orange) are commonly used for this purpose.

Methods for raising antibodies to immunoglobulins and other cell surface determinants, for preparation of specific second-step reagents and for conjugation of fluorochromes to antibodies are discussed in other chapters of this volume (see Chapter 15). We shall limit ourselves here to a description of the staining protocol used in our laboratory, and the special precautions we have found necessary when using immunofluorescence as a quantitative technique with a highly sensitive instrument.

In our usual staining protocol, 5×10^6 to 2×10^7 cells are pelleted in a conical tube and resuspended in 100 μl of medium containing the staining reagent at an appropriate dilution. The suspension is incubated for 15–30 min then underlaid with a cushion of fetal calf serum (FCS), pelleted and washed once in buffered saline. A second (or third) step follows the same procedure. Stained cells are resuspended finally in 1 ml of cold medium, generally with sodium azide and analysed on the FACS.

Conditions and reagents used for staining are obviously dictated by the type of cells to be stained and the determinants to be detected; however, certain general procedures are applicable in nearly all cases.

(1) Antibody reagents should be made as specific as possible, preferably by adsorption and elution from solid immunoadsorbants. Absorption of reagent with cells which do not bind the reagent is sometimes helpful.

(2) Fluorescein or rhodamine conjugated reagents should be stored at 4° C (unfrozen) under sterile conditions to prevent formation of aggregated materials which tend to bind to cells non-specifically.

(3) Just prior to staining, *all* reagents, whether conjugated or not, should be clarified by centrifugation at 100 000 *g* for 30 min to remove aggregated material; because even small amounts of aggregated material can interfere with analyses. (Beckman Airfuge, Beckman Instruments, Palo Alto, California, is convenient for clarifying small volumes of reagent.)

(4) The percentage of viable cells in the suspension to be stained should be as high as possible to minimize the number of dead cells with non-specifically bound fluorescence. (Although the FACS can 'gate out' dead cells, the gating becomes less efficient as the ratio of dead to live cells increases.)

(5) Both first- and second-step reagents should be titrated and then used, whenever possible, at saturation staining concentrations.

Ideally the number and 'brightness' of positive cells in the population used for titration should be equal to the number and 'brightness' of positive cells in the populations to be tested. This is because the amount of antibody bound generally depends more on the ratio of antibody amount to determinant amount than on the absolute amount of antibody used. Under most circumstances, antibody and cell concentrations can be established with sufficient leeway to allow for variations in positive cell number or brightness; however, it is well to bear in mind that small numbers of bright cells or large numbers of dull cells can appear brighter if a large, dull sub-population is depleted.

(6) The staining temperature and medium should

be chosen according to the stability of the determinant on the cell surface during the staining incubations. Some surface determinants, particularly immunoglobulins, tend to be rapidly sloughed when incubated with antibody, unless the incubation is carried out for short times at 0° C. Incorporation of 0·1–0·2 per cent sodium azide in the staining and washing media prevents this sloughing and does not appear to interfere with staining.

Staining with fluorescent beads

Rather than directly conjugating fluorescein or rhodamine to the second-step (or first-step) antibody, the fluorescent compound can be incorporated into plastic beads about a thirtieth the diameter of a small lymphocyte [14]. Purified antibody can be conjugated to the beads and the bead–antibody conjugate used to replace the soluble fluoresceinated antibody in an ordinary staining protocol. Since many fluorochrome residues can be incorporated in each bead, the sensitivity of antibody–bead conjugate staining can greatly exceed sensitivity with soluble conjugates. Even one bead per cell can be seen. This method is currently being developed [15].

Indirect immunofluorescent staining of suspensions containing immunoglobulin-bearing cells

The staining of lymphocyte suspensions containing a significant number of (Ig bearing) B cells presents a special problem when allogeneic antibodies (e.g. mouse antimouse antibody with mouse B cells) are used as first-step reagents. A second-step reagent which reacts with all mouse immunoglobulins will stain cells to which the first-step antibody has bound, but it will also stain all B cells in the suspension because they naturally carry IgD and/or IgM surface immunoglobulins. Therefore the second-step antibody must be specific for the first-step antibody and unreactive with native surface immunoglobulin.

Three methods have been developed to solve this problem: (1) the first-step antibody can be haptenated, e.g. with dinitrophenyl (DNP) or benzene arsonate (ars), and used with an antihapten second-step reagent [16]; (2) the first-step antibodies can carry different immunoglobulin allotypes from the B cells in suspension and be used with an anti-allotype second-step (see Chapter 12); and (3) the first-step antibodies can be prepared to consist mainly of IgG immunoglobulins and used with a specific anti-IgG second-step reagent. This latter method, which depends on the fact that very few B cells (less than 10 per cent) carry IgG molecules, is useful only when the frequency of cells stained by the first-step antibody is high.

Examples of immunofluorescence analysis

Frequency estimation: immunoglobulin-bearing (B) cells in mouse spleen

The FACS histograms in Fig. 22.7 show the analytical sequence for estimating the frequency of live immunoglobulin bearing cells in spleen. In this analysis, rabbit anti mouse-immunoglobulin antiserum (R anti Ig) which reacts with all mouse immunoglobulin classes was used for the first-step staining reagent. Fluorescein conjugated goat anti rabbit immunoglobulin (FG anti RIg) was used for the second step.

The scatter histogram shown in Fig 22.11a (p. 22.16) was obtained first. This enabled the setting of a scatter gate to exclude fluorescence signals from dead cells. The position of the scatter gate was established from the position of the valley between the first and second scatter peaks.

The two fluorescence histograms in Fig. 22.7 show the fluorescence distribution of live cells. The fluorescence histogram (Fig. 22.7b) is a negative control histogram. It establishes the extent of indirect (second-step) staining when the R anti MIg first-step reagent is replaced by normal rabbit serum. All fluorescence signals obtained from this control population may be due to small amounts of cell-associated fluorochrome (i.e. non-specific staining). It should be noted, however, that live cells never exposed to fluoresceinated reagents also give signals picked up by the FACS fluorescence detectors, especially if the amplification settings (gain and photo multiplier voltage) are high. Such signals may be due to endogenous fluorescence or to small amounts of scattered light which pass the fluorescence detector filters. The origin of negative control fluorescence becomes important mainly when specific staining is weak and the histograms from control and stained samples overlap making it difficult to establish a threshold (gate) for positivity.

The histogram obtained with stained (R anti MIg first-step) is presented in Fig. 22.7a. The distribution shows a minimum of two populations of cells. One population falls clearly within the limits defined by the negative control curve and thus has not bound

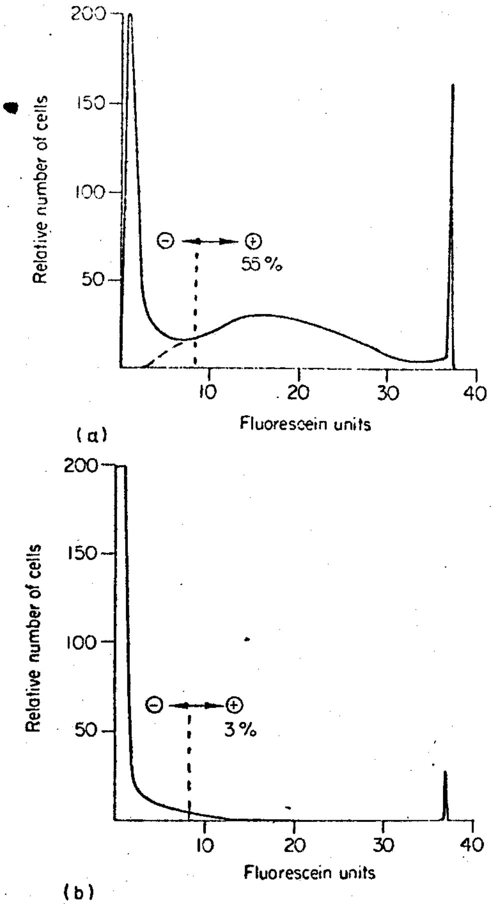


FIG. 22.7. One colour fluorescence analysis: immunoglobulin bearing (B) cells in mouse spleen. Histograms in panels a and b were obtained with scatter gates set to include only live cells in the fluorescence analysis (see Fig. 22.11a). (a) Mouse spleen cells stained with R anti MIg followed by FG anti RIg. Fifty-five per cent of cells are above the threshold (gate) for positivity. (b) Staining is the same as panel with normal rabbit serum replacing the first-step antiserum. Three per cent of cells exceed the chosen threshold.

the first step reagent, i.e. is Ig negative. The second population has significantly more cell-associated fluorochrome (second-step reagent) per cell; thus it binds the first-step R anti MIg and therefore carries surface Ig.

The fluorescence distribution of Ig-positive cells is broad and flat, indicating a wide variation in the amount of surface Ig per cell and the absence of a major sub-population defined solely by this amount.

The lack of a deep valley between the Ig-negative and Ig-positive cells indicates that the separation between the two populations is incomplete (i.e. the distributions partially overlap). The dotted line extending toward the origin indicates the probable distribution of positive cells in the overlap region. The actual curve obtained in this region is a summation of the frequencies of Ig negative (non-stained or non-specifically stained) cells and Ig-positive cells with small amounts of surface immunoglobulin

Exact estimation of the frequency of positive cells with a distribution such as the one shown is not possible without knowledge of the shape of the positive cell profile unless either an arbitrary threshold for minimum number of Ig molecules is assumed or an independant parameter such as B cell function is measured. When the populations of negative and positive cells are cleanly resolved, integration of each curve gives an exact estimate of frequency. For the distribution obtained here a reasonable estimate can be made by setting a threshold (gate) at the point indicated in the figure (Fluorescein Units (Fl-U)) and integrating to determine the frequency of cells above the threshold. Good practice dictates that thresholds for positivity be indicated in published figures from which frequency estimates are obtained, especially when negative and positive cell distributions overlap significantly.

Kinetic studies: removal and reappearance of surface immunoglobulin on B cells

Immunoglobulin is removed (stripped) from B cell surfaces when the cells are incubated at 37° C for 1 hour in medium containing a suitable concentration of anti-immunoglobulin antibody (e.g. R anti MIg). (Including sodium azide in the medium prevents this process.) The immunoglobulin reappears when stripped cells are washed and cultured in growth medium for 24 hours. The series of histograms in Fig. 22.8 shows the reappearance of surface Ig as a function of time [17].

Cells were stained indirectly for surface Ig with the same reagents used in the previous section. The first histogram in the figure shows the distribution of Ig-positive cells prior to stripping. The second histogram shows the population just after stripping when virtually all the surface Ig is gone.

The next histograms show that starting at 3 hours after stripping, the number of cells stained and the amount of Ig per cell increases until, by 24 hours the population has regained its normal (pre-stripping) surface Ig distribution. The sharp peak at the high

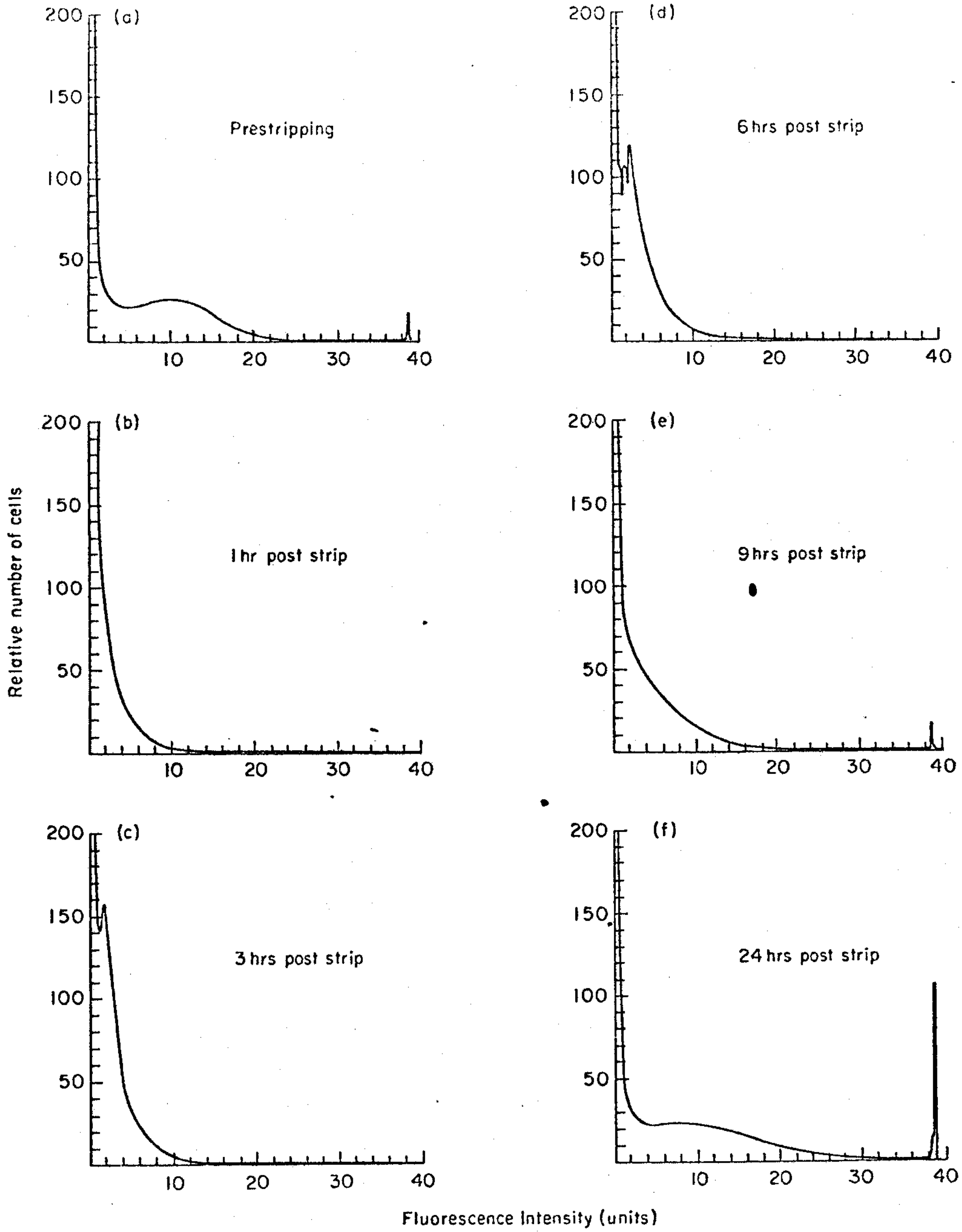


FIG. 22.8. Kinetics of re-expression of surface Ig after stripping with anti Ig. Splenic lymphocytes were reacted with rabbit anti mouse Ig and placed in culture medium (RPMI-1640) at 37° C for various time periods. The original distribution of Ig on these cells is shown in the first curve (prestripping). The cells for this curve were placed in azide immediately after treatment with rabbit anti mouse Ig (i.e. not allowed to strip) and then stained with ^{F}goat anti rabbit Ig. For subsequent curves equivalent numbers of cells were removed from the cultures at 1 hour, 3 hours, 6 hours, 9 hours and 24 hours, placed in azide containing medium and restained with rabbit anti mouse Ig followed by ^{F}goat anti rabbit (M. Loken, unpublished).

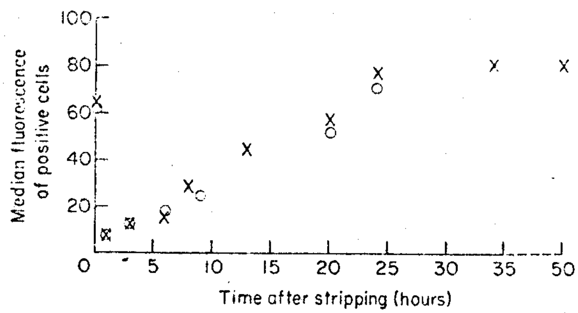


FIG. 22.9. Kinetics of re-expression of immunoglobulin on mouse spleen cells. The median fluorescence of stained cells (see Fig. 22.8) was determined for each time point after the stripping process (two identical experiments are shown). The data points at 0 time were obtained from samples prior to the stripping process.

end of the distribution (38 Fl-U) is a histogram artifact. It represents the number of cells with fluorescence signals greater than 37 Fl-U.

The data from the histograms in Fig. 22.8 are reduced to a single curve (see Fig. 22.9) showing the increase in the median fluorescence of Ig positives as a function of time after stripping.

Quantitative expression of surface determinants: genetic typing of individuals for alleles which control quantitative expression of ThB

Adult splenic B cells from BALB/c and SJL mice carry ThB antigenic determinants [20]. FACS analyses, however, show that the amount of ThB expressed per cell is markedly lower in BALB/c spleen than in SJL spleen (see Fig. 22.10). (BALB/c × SJL)F_1 hybrids express an intermediate ThB level clearly distinguishable from either parental strain. Backcrosses of the F_1 hybrids to each of the parental strains yield an equal number of progeny expressing the hybrid level and progeny expressing the level of the parental strain used in the cross, i.e. a 1:1 segregation for the trait. Thus these analyses showed that a single locus, ThB, with two alleles ThB^{hi} and ThB^{lo}, controls expression of this determinant on B cells [21].

Two-colour fluorescence analysis: Ia determinants on Ig bearing (B) cells

Loci mapping within the I region of the mouse major histocompatibility complex (H-2) control a series of cell surface (Ia) determinants selectively expressed on different types of lymphocytes [18]. B cell Ia determinants (Ia-1, Ia-3, and Ia-5) are easily detectable with conventional serologic methods, i.e. immunofluorescence or cytotoxicity. At least some T cell Ia determinants are also detectable with the FACS (K. Okumura *et al*, personal communication); however, as the double staining analyses presented in this section show, under usual staining and analytic conditions, detectable fluorescent staining with anti Ia is restricted to cells which also stain with anti Ig and vice versa. Thus with respect to the Ia determinants detected under these conditions, the

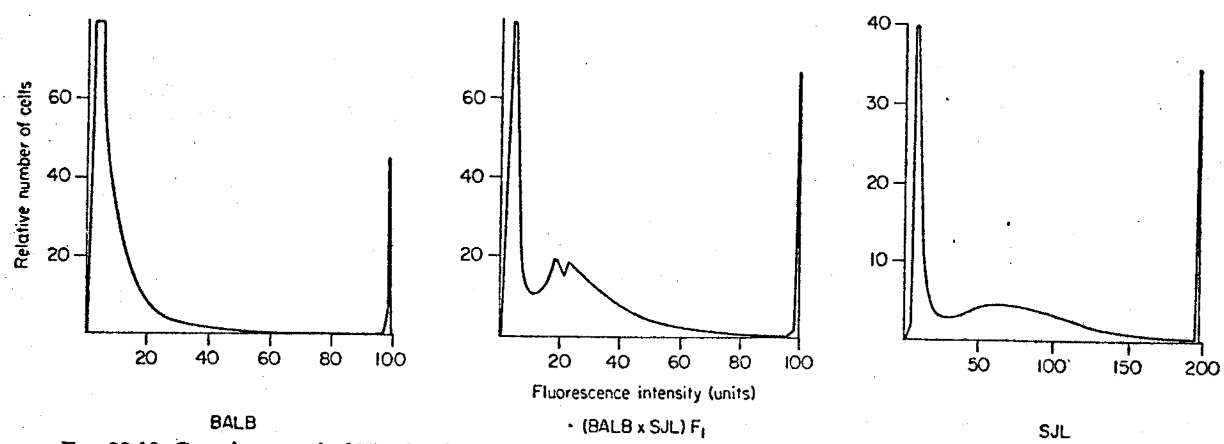


FIG. 22.10. Genetic control of ThB levels on splenic B cells. FACS profiles showing fluorescence intensity distributions of SJL, (BALB/C × SJL)F_1 and BALB/C spleen cells stained with goat anti ThB followed by fluorescein-conjugated rabbit anti goat Ig. In each panel, roughly half the cells show more than 10 units of fluorescence intensity, i.e. are ThB positive.

same lymphocyte sub-population, namely B cells, carries surface Ia and surface Ig determinants.

Double staining for Ia and Ig presents a major problem because mouse antisera are used as first-step reagents for Ia staining. Thus the second-step reagent for the Ia stain must distinguish between native surface Ig and the exogenous (first-step) antibody bound to Ia. Similarly, the reagent used for surface Ig staining must specifically bind to native surface Ig. Haptenating the mouse anti Ia (see p. 22.11) is not feasible because of the difficulties in purifying the antibody and the small amounts of antiserum available.

A simpler example could have been chosen to illustrate the principles of two-colour fluorescence analysis. We have opted for complexity here instead in order to provide a vehicle for consideration of the interpretative limitations of immunofluorescent staining FACS analysis along with description of a basic analytical method.

There are relatively few complications in interpreting data from two-colour fluorescence analyses where antibodies used to detect the determinants do not react either with each other or with irrelevant surface immunoglobulin (or other) determinants on cells present in the population being stained. For example, analysis of spleen cells stained directly with fluoresceinated rabbit anti mouse-IgD (^{F}R anti-MIgD) and rhodaminated goat anti mouse-IgM (^{R}G anti MIgM) will give a clearcut estimate of the frequency of cells bearing IgD only, IgM only or IgM and IgD together. It will also show the relative amounts of IgD and IgM per cell on double stained cells and thus give a measure of the extent of correlation in the quantitative expression of these determinants on the cell surface [19]. As long as the reagents used are specific, other surface determinants, including irrelevant immunoglobulins such as IgG or IgA, will not interfere with the analysis.

The picture becomes decidedly more complicated when there is a potential for interaction between the reagents themselves. If a fluoresceinated rabbit anti sheep immunoglobulin reagent (^{F}R anti SIg) which cross-reacts with goat immunoglobulins is used as a second-step reagent to detect binding of sheep antibodies to a surface determinant, it usually cannot be used in a double-staining experiment where rhodaminated goat antibody is used to detect a second determinant. Similarly, if ^{F}R anti MIg is used to stain mouse Ig-bearing (B) cells in a spleen cell suspension, it is difficult to use a mouse alloantiserum to detect a second surface determinant. In both cases, double-stained cells could be due either to direct binding of each antibody to its own determinant on the cell surface or to binding of one antibody to its own determinant and binding of the second antibody to the first.

Fortunately, first- and second-step antibody concentrations in many such cases can be adjusted to avoid these problems. Controls where normal serum is substituted for the cross-reactive antiserum indicate the extent of spurious (non-specific) double staining, and thus establish the limits of sensitivity of the analysis. With the FACS, the maximum amount of non-specifically bound immunoglobulin can be determined and gates set to exclude all cells below this threshold. These analytic conditions are not ideal and dictate severe caution in data interpretation. Nevertheless, if the magnitude of the difference between non-specific and specific staining is sufficient, useful data can be collected which would otherwise be unobtainable.

The problem, framed earlier in this section in terms of the expression of Ia antigens on B cells, translates operationally into the following questions: What fraction of mouse spleen cells carry both Ia and Ig surface determinants (i.e. double stain for Ig and Ia); what fraction carry neither; and what fraction carries each of these determinants alone? Reagent selection in this case is difficult because Ia determinants are detected with allo-antibodies raised in one mouse strain against lymphocyte surface determinants present in another. Thus the binding of the mouse anti Ia antibody (which logistic considerations dictate must be used as an unmodified first-step reagent), must be specifically detectable (1) in the presence of mouse B cells carrying a large amount of native surface Ig; and (2) in the presence of a second staining reagent which binds mouse Ig (and thereby stains Ig-bearing cells).

The potential for interaction between these reagents appears to preclude a succesful experiment; however, by choosing an anti Ia antiserum containing mainly IgG anti-Ia antibodies (or by removing IgM antibody from the antiserum), the difficulties can be overcome. A ^{F}R anti MIgG can then be used as a specific second-step reagent to stain cells which have bound the anti Ia (see Fig 22.11b) because very few IgG bearing cells are found in spleen, and the concentration of the ^{F}R anti MIgG can be adjusted so that the direct staining of this small number of cells is considerably duller than the indirect staining of the Ia-bearing cells. The negative control curve in

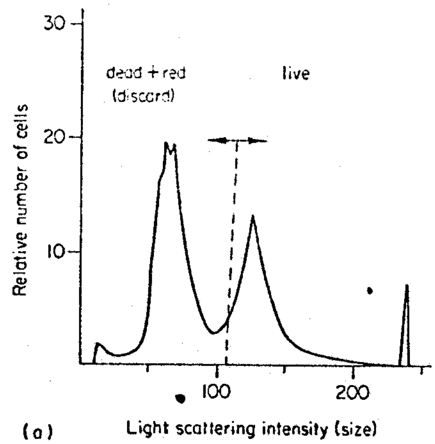
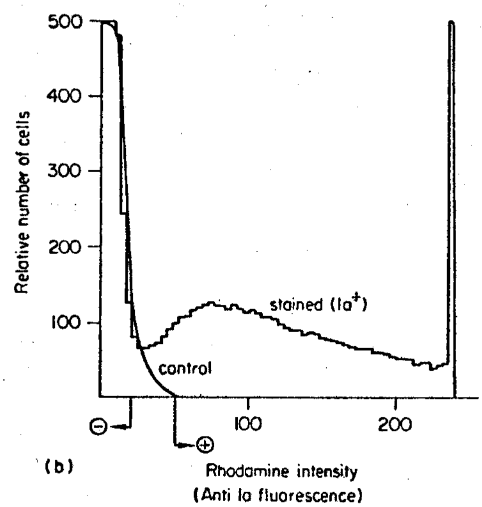
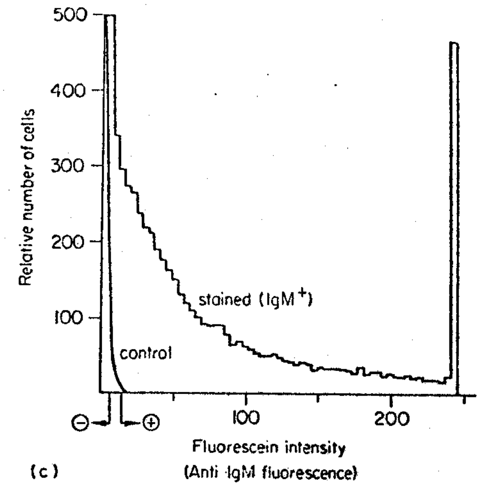


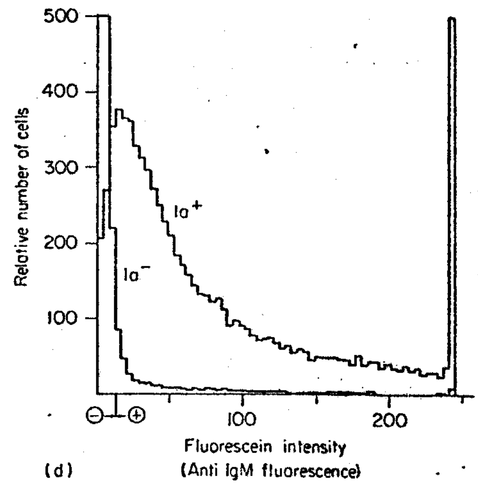
FIG. 22.11. Two-colour fluorescence analysis: determination of frequencies of spleen cells carrying surface IgM, Ia or IgM and Ia together. (a) Scatter histogram of total spleen cell population. Live cells fall to right of indicated gate. Erythrocytes (red) and dead cells fall to left of gate.



(b) Anti Ia (rhodamine) fluorescence histogram of total live cells. For gated analysis shown in panel d, cells with > 50 units of fluorescence (i.e. to right of the upper gate), are taken as positive and cells with <20 units are taken as negative. Cells with > 20 but <50 are ignored because of possible ambiguity in classifications. 'Stained' cells are stained with mouse anti Ia followed by ᴿGoat anti mouse IgG. 'Control' cells are stained with normal mouse serum followed by ᴿGoat anti mouse IgG. All histograms are truncated at 500 on the y axis to enable visibility of curve structure at low y values.



(c) Anti IgM (fluorescein) fluorescence histogram of total live cells. For gated analysis shown in panel e, negative cells = <7 units, positive cells = > 12 units (see legend for previous panel). 'Stained' cells are stained with ᶠgoat anti MIgM. Control cells are unstained.



(d) Anti IgM fluorescence histograms of Ia^+ and Ia^- cells (see legend for panel b). Gate position of 12 as indicated is used for integration to determine frequencies of positive and negative cells.

Fig. 22.11b shows the extent of staining with the second-step reagent in the absence of anti Ia.

To stain Ig-bearing (B) cells, a ᶠR anti MIgM can be used as a specific 'second colour' (green) direct staining reagent when cells are stained red for Ia as indicated above. The anti IgM will stain nearly all

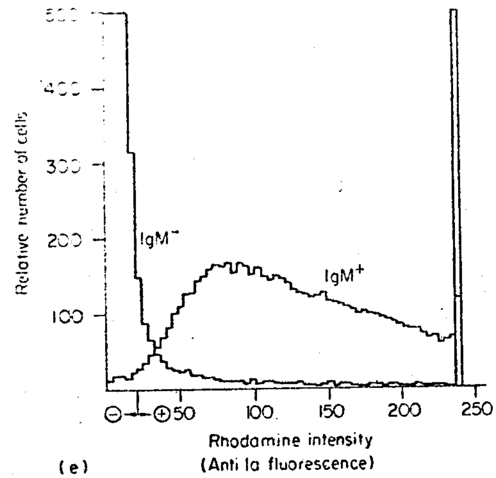


FIG 22.11. (e) Anti Ia fluorescence histograms of IgM^+ and IgM^- cells (see legend for panel c). Gate position of 20 is used for integration to determine frequencies of positive and negative cells.

Ig-bearing cells in spleen, including most IgG-bearing cells because these often carry surface IgM (see Fig. 22.11c). The cell bound anti IgM can bind spurious (non anti Ia) IgM from the anti Ia antiserum; however, such binding will not give rise to double-stained cells because the ^{R}R anti MIgG used as second step with the anti Ia antiserum will not bind to IgM.

With these reagents, only those cells with surface Ia determinants and native IgM determinants will be detectable as double-stained cells. Cells with one or the other determinant but not both will stain singly with the appropriate colour. The dot-plot presented in Fig. 22.12 shows the correlation between anti Ia and anti IgM staining of live cells.

Gated analyses are used as follows to determine the frequencies of doubly, singly and non-stained live cells in the suspension. All analyses are scatter-gated to exclude dead cells (Fig. 22.11a). From the fluorescence distributions for all live cells (Figs 22.11b and 11c), fluorescence gates are chosen for each colour to distinguish stained and unstained populations. In each case, an upper and lower fluorescence gate is chosen, leaving a 'window' between the positive and negative populations in the region where these populations overlap. Choosing the location of the gates for the anti Ia (red) stained distribution is essentially straightforward (Fig. 22.11b). Choosing the location of the gates for the anti IgM (green) stained distribution is based on comparison with the negative control (unstained) distribution. Previous analytic and functional studies with this staining reagent have shown that the gates are appropriately located.

The four sub-populations defined by the gates are then successively analysed by passage of the original stained population through the FACS under each of the gated conditions. Thus histograms are obtained

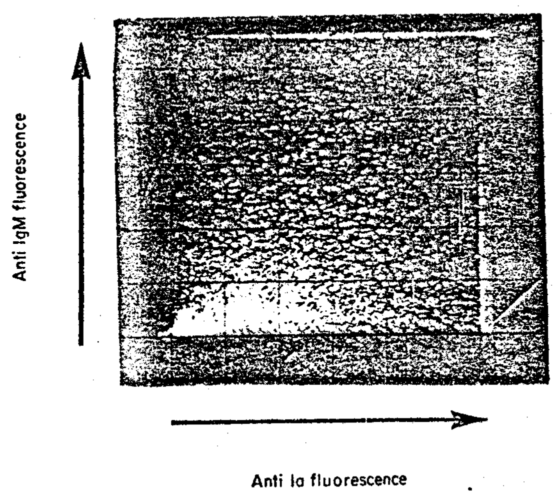


FIG. 22.12. Dot-plot of two-colour fluorescence analysis. Dots representing cells carrying IgM only lie along the y axis, dots for cells carrying Ia only lie along x axis, dots for cells carrying both are located within the square according to how much of each determinant they carry. Staining details are given in legend for Fig. 22.11. For further details on dot-plot, see p. 22.3.

showing the fluorescence distribution of cells stained with the opposite colour in each sub-population, i.e. the red (Ia) positive sub-population is analysed to determine the distribution of green (IgM) positive cells among the red positive cells, etc. Frequencies are then determined from the gated histograms by integration using the lower of the established gates to define positive and negative cells in each colour.

The histograms obtained from analysis of the gated populations are shown in Figs 22.11d and 11e. They show that nearly all stained cells are double stained. (IgM^+, $Ia^- \cong 1$ per cent; IgM^-, $Ia^+ \cong 2$ per cent). Thus, in general, spleen cells which carry Ig surface determinants also carry Ia determinants.

The use of a 'window' between positive and negative populations prevents contamination of each sub-population with overlapping cells from the other. Conclusions drawn from 'windowed' analyses or separations, however, should be tempered with the reservation that cells falling within the window and hence not included in the analysis could be a unique sub-population expressing a small amount of determinant rather than the overlapping part of the unwanted sub-population. If this third sub-population exists, it could be qualitatively different with respect to expression of the determinants being analysed. This reservation is important and should be considered whenever a 'window' covers a substantial portion of the population. It applies to scatter-gated as well as fluorescence-gated populations.

FACS cell separation

General considerations

The principles of separation follow from the principles of gated analysis. Any population on which a gated analysis can be performed can also be isolated merely by indicating to the FACS that cells within the gates are to be deflected into a collection vessel (and by turning on the sorter assembly). In fact, the gated population need not be analysable to be sorted, since analysis generally requires a minimum of a few per cent of stained cells in the preparation. Regardless of their frequency, cells which fall within gates established for one, two or three FACS parameters can be deflected, collected and then re-analysed, examined visually, or tested for function.

The FACS allows two sub-populations to be collected simultaneously. Cells which do not meet the gated criteria established for either of these populations are discarded together with dead cells and those cells which arrived at the detectors too close to each other to be separated clearly from one another ('coincidences').

Cell recovery

Cell recoveries of deflected cells run between 40 and 100 per cent depending on the initial frequency of the desired population and the rate of processing through the FACS. (Major losses can occur in concentration of cells from the collection tubes. Due attention to use of siliconized tubes, adequate centrifugation and suitable suspending media is important.) Viability and functionality in isolated populations, at least for lymphocytes, are generally equal to or better than the original population because dead cells are discarded. Cell clumping must be avoided or much loss will occur on the in-line filters.

Processing rate

Processing rates for sorting generally vary between 10^7 and 10^9 cells per hour. Yields per unit time vary according to the frequency of desired cells and the flow rate. The usual sorting rate is about 2×10^7 per hour. This rate allows an average of about one cell per six droplets and thus causes less than 10 per cent discards due to 'coincidence'.

When the frequency of desired cells is low (<5 per cent), it is often more convenient to increase the flow rate and turn off the coincidence circuitry. The separated population will then be markedly enriched for the desired cells although it will also contain 'passenger' (coincident) cells which do not necessarily meet the gating criteria but are close enough to desired cells in the stream to be included in the deflected droplets along with the desired cells. The enriched population can be further purified by sorting again, this time at a lower flow rate and with the coincidence circuitry activated.

Collection of isolated populations

Isolated populations are usually collected in 15 ml conical plastic or siliconed centrifuge tubes kept immersed in ice during the separating. Generally, 1–2 ml of fetal calf serum is placed in the tube at the beginning of the separation. Modifications of this procedure, however, are often made to suit experimental needs. If sterility of isolated populations is required, the entire FACS assembly with which cells come into contact, including collection vessels, can be sterilized and kept aseptic throughout the run.

Collection of cells which occur in low frequencies and thus represent rare separation events, sometimes becomes difficult because the droplets tend to dry on the large tubes usually used for collection vessels. To overcome this problem, small plastic petri dishes with a hole cut in the cover to allow droplet entry can be positioned such that deflected droplets fall directly into medium covering the bottom of the petri dish. The wells of microtitre plates are also useful collection vessels for small numbers of cells.

A static electric charge often builds up on plastic collection vessels. A wire bringing a charge to the well opposite to that of the collected droplets helps prevent losses of droplets by electrical repulsion.

If the isolated cells are to be collected only for microscopic examination, the usual collection vessels can be replaced by a microscope slide on which two small glass cylinders have been cemented. (The slide must have a slot between the cylinders to allow passage of the central discard stream.) Cells can be collected directly into the cylinders often containing a drop of serum (or fixative). The slide is then centrifuged in a Cytocentrifuge (Shandon Instruments), the cylinders removed, and the slide processed and examined directly under the microscope. This procedure has been used effectively for microscope counting of fluorescent stained cells where the frequency of stained cells in the original suspension was less than 0·1 per cent.

Estimation of contamination in isolated populations

To estimate the frequency of undesired cells in the isolated population, i.e. contamination, a sample of isolated cells is generally analysed on the FACS at the end of the separation run. The extent of contamination can also be estimated by fluorescence microscopy provided the FACS fluorescence threshold was set above the visual threshold.

Assuming that the FACS separation assembly is operating properly, the contamination of the isolated population will be a function of the resolution between sub-populations in the original suspension. If the subpopulations do not overlap, or if an appropriate window is set between them, detectable contamination of isolated populations can frequently be avoided. Partial nozzle clogging causing aberrant droplet deflection is often a source of contamination; therefore debris-free and monodisperse-cell suspensions give better separations. The FACS operator can detect partial clogging by monitoring the scatter signals continuously and by watching for changes in the droplet streams.

Functional testing of isolated populations

Fluorescence activated cell sorting is applicable to a wide variety of cell types used in biological studies, Each study dictates its own rules for handling separated cells, for relating functional activities to the starting population, for reporting data, etc. We have, however, established the following general procedures for testing lymphocyte function which may be useful for other types of studies in other laboratories.

(1) *Controls*. Ideally, in each separation experiment the following controls are included: unstained cells, stained unseparated cells and, when the original population is separated into two sub-populations which include all (or nearly all) of the original population, a reconstituted mixture of the two separated populations in proportion to their frequencies in the original population. Thus if functional activity is not recovered in one of the separated populations, its point of loss in the procedure can be identified.

(2) *Cell dosages*. In most cases, the activity of a given number of isolated cells should be equal to the activity of that number of cells in the unseparated suspension. Therefore, in *in vivo* function tests, the number of separated cells injected is usually adjusted in proportion to the frequency of that type of cell in the unseparated population. For example, if 10^7 unseparated cells are to be injected to demonstrate a desired activity, and if the unseparated population contains 10 per cent of cells bearing the stained determinant, then 10^7 separated cells will be injected. If, however, the separated cells are significantly contaminated with unstained cells, then the number of stained separated cells injected will be maintained in proportion to the number of stained cells in the original population. Matching cell doses in this way allows easy calculation of enrichment factors and recoveries of functional activities.

This same principle is useful in *in vitro* function testing, although it sometimes must be sacrificed to the requirement for a constant number of cells per test dish. Whenever possible, a suitable irrelevant cell type is used as 'filler' to make up the difference in cell number in dishes containing separated cells.

Examples

Most of the data presented in the analysis section of this chapter was collected in preparation for separation and functional testing of stained cell populations. Presentation of the results from these or similar functional studies would introduce very little additional information relevant to methods of FACS utilization, save to indicate that immunofluorescent staining and FACS separation do not interfere with biological function (at least in the cases thus far tested). On the other hand, presentation of such separation data would require extensive descriptions of the biological systems used in order to provide adequate background for interpretation. We therefore refer the reader to several FACS separation studies reported from other laboratories [22–24] and our own [25–27].

Acknowledgment

The development of the FACS and the methods for its utilization represent the interactive efforts of numerous engineers, scientists and technicians in our own and other laboratories throughout the world. Bernard Shoor and his associates at Bectin-Dickinson Electronics Laboratory (Mountain View, California) and Richard Sweet and his staff in the Genetics Department, Stanford University, deserve special mention for their intensive and effective commitment to making the FACS a practical instrument for widespread use in biological and clinical research.

For the preparation of this manuscript, we are deeply indebted to our daughter, Berri Herzenberg. Without her patient and devoted services as voluntary secretary during our sabbatical year, this chapter could not have been completed for inclusion in this edition of the Handbook.

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