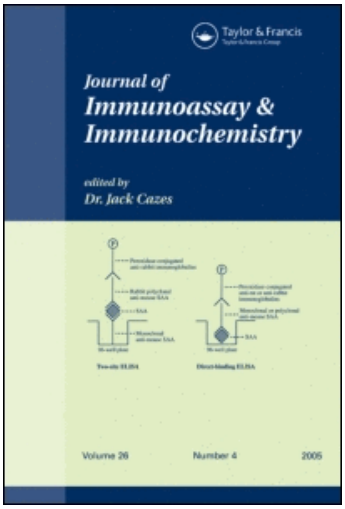

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Multiparameter Flow Cytometry

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Multiparameter Flow Cytometry

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I. INTRODUCTION

Immunophenotyping is an important method for identifying cells, and there is no single field for which it is applied more often than in hematology. Although immunohistochemistry or fluorochrome-labeled antibodies are often used for microscopic identification of cells, flow cytometry has the advantage of identifying cell subsets more rapidly by using multiple antibodies simultaneously, and these subsets can be sorted for further characterization. Microscopic analysis is the method of choice if morphological information is desired, but flow cytometry is the choice for identifying and quantifying cell subsets and evaluating their frequency in a heterogeneous population.

Even though polyclonal antibodies are still used occasionally, monoclonal antibodies (MAbs) to epitopes on membrane or internal antigens are the reagents used for identification. We now know that any given protein is often found on several different kinds of cells, and one antibody cannot be used to identify any particular cell lineage. Instead, each cell subpopulation has a very unique repertoire of proteins, and a mixture of antibodies to them can be used for explicit identification. When the function of the protein is known, additional information about the cell is obtained.

In this chapter, I will describe the methods for labeling cells with up to four antibodies simultaneously. This number is chosen because it is possible to measure all of them with a single laser. With human lymphocytes as the example, we now recognize over 80 subpopulations and, although these subpopulations are not all mutually exclusive, they do represent specific functional subsets that interact together to produce the hematopoietic system and exemplify the power of flow cytometry for resolving them.

II. ANTIBODIES USED FOR FLOW CYTOMETRY

A. CD Nomenclature

Any protein antigen contains hundreds of unique sites (epitopes) that initiate the formation of antibodies to them by the B-cell-derived plasma cell clone. Each clone produces a monoclonal antibody that binds specifically to one epitope on the protein antigen. As development of

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monoclonal antibodies to cell membrane proteins on hematopoietic cells increased in the early 1980s, it became apparent that many different investigators were producing antibodies to the same antigens, albeit to different epitopes. This resulted in the formation of the Leukocyte Differentiation Antigen Work Shop the function of which has been to explicitly define the binding specificity of antibodies to cell membrane proteins.

Because the function of these proteins was usually not known, the clusters of differentiation (CD) nomenclature was developed, and any antibody that bound to a particular protein epitope has been assigned a CD number. In this way, antibodies from different laboratories or suppliers to different epitopes on the same cell membrane antigen are clearly identified. There are currently 78 defined antigens with CD numbers. A complete listing of them and their properties can be found in *Leukocyte Typing IV: White Cell Differentiation Antigens* (Knapp et al., 1989). A very brief summary of the most commonly used antibodies for immunophenotyping is found in Table 1.

Table 1 CD Antibodies in Common Use[a]

Antibody	Target cell population
CD1	Immature T cells
CD2	T cells, NK cells
CD3	All mature T cells
CD4	Helper/inducer T cells
CD5	T cells, B cell subset
CD7	T cells, immature progenitor cells
CD8	Cytotoxic/suppressor T cells, NK-cell subset
CD10	Common acute lymphocytic leukemia antigen (CALLA)
CD11 family	CD11a, LFA-1α, leukocyte function associated antigen, all leukocytes
	CD11b, CR3 receptor associated with C3bi complement receptor [MAC1] NK cells, myeloid cells
	CD11c, myeloid cells, NK cells, hairy cell leukemia
CD13	Myeloid cells
CD14	Monocytes
CD15	Myeloid cells
CD16	FcRIII, myeloid cells, NK cells, CD8 T-cell subset
CD18	β-Chain for CD11 family, all leukocytes
CD19	Immature and mature B cells
CD20	Mature B cells
CD22	Immature and mature B cells
CD26	T-cell subset
CD32	FcRII receptor, myeloid cells, B cells
CD33	Immature myeloid progenitor cells and myeloid monocytic cells
CD34	Hematopoetic stem cells and progenitor cells
CD38	Progenitor cells, NK cells, band T-cell subsets, plasma cells
CD41 family	Megakaryocytes and platelets
CD42 family	Megakaryocytes and platelets
CD45	Common leukocyte antigen
CD56	NK cells, cytotoxic T cells, immature progenitor cells, plasma cells
CD57	NK cells, T-cell subset
CD61	Megakaryocytes and platelets
CD64	FcRI receptor, monocytes
CD71	Transferin receptor

[a]For extensive description of each antibody, see Knapp et al. (1989).

B. Primary Antibodies

A complete discussion of immunoglobulin structure and function may be found in Chapter 1 of this book. Virtually all MAbs used for immunophenotyping are of the IgG1, IgG2a, or IgG2b isotype and subclass, obtained from the mouse or rat. The light chains are either kappa or lambda. Currently, these MAbs are directed to epitopes on either murine, rat, or human cells. There is a paucity in the availability of MAbs directed to epitopes on hematopoietic cells from other species.

Often a biotinylated version of the primary antibody can be obtained and counterstained with a fluorochrome conjugated to avidin. The avidins are also available with all the commonly used fluorochromes conjugated to them. In this situation avidin, available with any fluorochrome, is used as a second reagent. Streptavidin exhibits considerably less nonspecific binding than avidin, and it should always be used. Finally, primary antibodies can also be obtained directly conjugated with a fluorochrome.

C. Secondary Antibodies

When a primary antibody is unavailable as a direct conjugate, it can be labeled using a second polyclonal antibody. These second antibodies are available with virtually any fluorochrome conjugated to them. Two kinds of second antibody reagents are available, those that are isotype-specific and those that are not. For example, the label on a second reagent might read "FITC–goat antimouse IgG $(F(ab)'_2$, heavy and light chain-specific, affinity purified." This reagent is not specific for mouse IgG because it contains antibodies to mouse light chains that are also found on all the other isotypes. This label actually means that purified mouse IgG containing all subclasses was used to immunize a goat. The goat antiserum was purified using an affinity column to which was bound mouse IgG. The $F(ab)'_2$ fragment was then prepared and labeled with fluorescein isothiocyanate (FITC). Only if the label indicates "Ig (isotype and subclass) heavy chain-specific" would the reagent have been specific for the indicated isotype and subclass.

III. FLUOROCHROMES USED TO LABEL ANTIBODIES

Although there are many fluorochromes that can be used for conjugation to antibodies, only fluorescein isothiocyanate (FITC), phycoerythrin (PE), and the tandem conjugates will be discussed. A summary of their properties is shown in Table 2.

There are two kinds of tandem complexes, the first is PerCP (Stewart and Stewart, 1993; Koka and Song, 1977; Rechtenwald et al., 1990). This fluorochrome is extracted and purified from marine dinoflagellates and consists of a peridinim component that protects the chlorophyll component from photolysis. Energy transfer occurs through a series of photosensitive molecules;

Table 2 Fluorochromes Used for Flow Cytometry

Fluorochrome	Ex (nm)	Em (nm)	Stability
FITC	495	525	Excellent
PE	488	578	Excellent
PE-TR	488	613	Good
PE-Cy5	488	670	Poor
PerCP	476	672	Poor

accordingly, it can be excited at 488 nm and emit at 672 nm, with virtually no intervening fluorescence emission. This fluorochrome, however, has a low fluorescence yield, and it is unstable at high excitation power.

The second are tandem complexes of phycoerythrin to which either Texas red (TR) or Cy5 have been complexed and act as energy receptor molecules (Titus et al., 1993; Southwick et al., 1990; Waggoner et al., 1993). When the PE is excited, its energy is transferred to the acceptor molecule (TR or Cy5) that emits its photons. The excitation band width for TR is not ideally matched to PE; hence, there is a high percentage (about 20–30%) of PE photons that escape to produce a lower Texas red fluorescence and significant PE fluorescence. The latter is corrected by compensation (described later).

The PE–Cy5 tandem is a much better construct because the Cy5 excitation band is optimal for accepting PE energy and there is negligible PE emission (< 3%). This fluorochrome is very bright, often exceeding that of PE alone. This otherwise ideal fluorochrome, however, is light-sensitive, and the Cy5 is slowly degraded. The photodegradation can be solved by storing the fluorochrome in the dark and minimizing its exposure to light (especially long-wavelength visible light) during sample preparation and storage.

A more serious problem with this fluorochrome is its specific binding to monocytic lineage cells. The binding is so specific that its conjugation to protein (e.g., albumin or avidin) can by itself be used to identify them. Although this binding is not understood, the molecule may mimic some yet to be discovered ligand.

Often the epitope frequency on cells is very low; consequently, they are poorly resolved. This problem can often be overcome by selecting the appropriate fluorochrome. The relative order to fluorochrome brightness is PE–Cy5 > PE > PE–TR > FITC > PerCP. Thus, the brightest fluorochromes should always be conjugated to antibodies that stain cells with the lowest epitope frequency. With the most sensitive flow cytometers, epitope frequencies as low as 500 per cell can often be resolved.

IV. STAINING CELLS

There are three basic ways to stain cells, and it is the appropriate combination of these three methods that provides the strategy for staining cells with multiple antibodies. Although strategies can be designed for labeling cells with any number of antibodies, we will consider up to four antibodies. The three basic reactions for staining cells are (1) a primary antibody followed by a fluorochrome conjugated to a second antibody (FL–Ab), (2) a biotinylated primary antibody (B–Ab) followed by a fluorochrome-labeled avidin (FL–Av), and (3) a fluorochrome conjugated to the primary antibody.

A. Blocking

Although not often done, it is important to block nonspecific and Fc receptor binding before staining cells with an antibody. This requirement increases in importance as the number of MAbs used simultaneously increases and when cells other than T cells are being stained. Table 3 shows the Fc receptor (FcR) repertoire for human leukocytes. T cells are the only cells that have no Fc receptors (except for a small $CD8^+$ T-cell subset that expresses FcRIII). When formed as a single panel of three antibodies, virtually all leukocyte classes can be resolved. Basophils, neutrophils, monocytes, and eosinophils each exhibit their own unique Fc receptor repertoire, and all four populations can be resolved from one another based on this repertoire in combination with forward scatter (FSC) and side scatter (SSC) profiles.

Table 3 Fc Receptors on Human Leukocytes

Cell type	FcRI CD64	FcRII CD32	FcRIII CD16
Neutrophils	Absent	High	High
Eosinophils	Absent	High	Low
Basophils	High	High	High
Monocytes	High	High	Low
NK cells	Absent	Absent	High
B cells	Absent	Intermediate	Absent
T cells	Absent	Absent	Absent[a]

[a]$CD8^+$ subset expresses this receptor.

B. Single-Antibody Immunophenotyping

1. The Indirect Antibody Method

The indirect second-antibody method for staining cells is the one most often used, and it also produces the most artifactual data, because two different antibodies are involved. The basic procedure is outlined in Fig. 1. Because MAbs are almost always intact immunoglobulins and because they are produced by malignant cells, their structure is abnormal, causing them to bind both specifically to Fc receptors and nonspecifically in an unpredictable manner; each MAb behaves differently, regardless of its subclass (Stewart, 1990a,b).

The second antibody can be obtained with virtually any fluorochrome conjugated to it. Since the $F(ab)'_2$ fragment lacks the Fc portion, it should always be used for staining the primary antibody. Second antibodies are almost always polyclonal.

Inappropriate binding can be reduced by blocking the cells first before staining with the MAb. This is easily accomplished by incubating the cells first with 2000 μg IgG per milliliter of cells at a density of no more than 20×10^6/ml. Purified IgG derived from the species from which the second antibody was derived should be used. This IgG is least likely to cross-react with the second antibody, because both are derived from the same species. Any cross-reactivity and effectiveness of the block should be tested by including an isotype control antibody test. Serum, although often used, is not an effective block because the IgG concentration is too low.

2. Use of Biotinylated or Directly Labeled Monoclonal Antibodies

A schematic representation of the staining procedure is shown in Fig. 2. When an MAb that has been directly conjugated with a fluorochrome is used, the avidin step is not required. Since a second antibody with specificity for the murine MAb is not required, the blocking IgG can be mouse IgG. This may be preferable, since it is likely to behave most like the MAbs.

3. Signal Amplification

An approach for detecting cells with low epitope frequency is to use an amplification system. This can be done by using a combination of a second antibody and avidin. Although there are several strategies that can be designed, the one shown in Fig. 3 is easily done. It is imperative that an isotype control antibody is substituted for the primary antibody so that inappropriate staining is detected. As long as the signal increase is greater than the noise with each cycle, specific epitope expression is detected. However, if poor quality reagents are used, this amplification system completely fails.

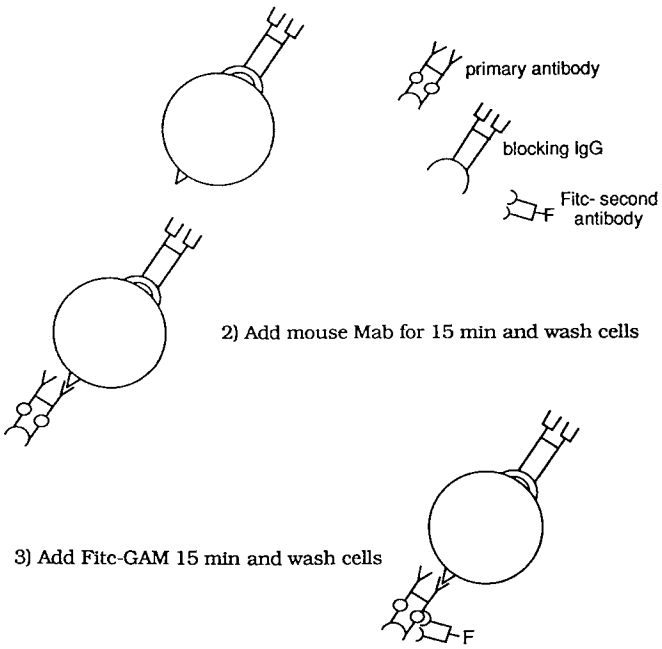
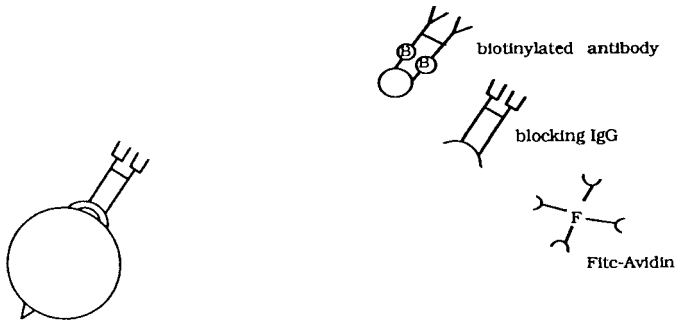


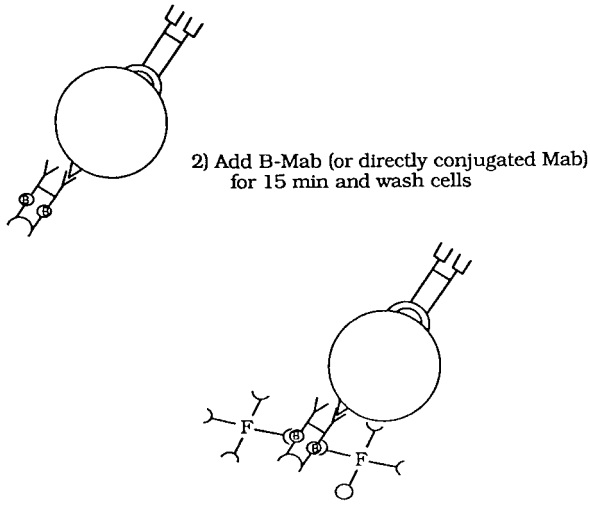
Figure 1 Staining cells with a monoclonal antibody and a second antibody: In this example, a mouse (or rat) primary monoclonal antibody and goat antimouse (or antirat) second antibody, conjugated with FITC (or any other fluorochrome), are shown. The second antibody can also be from any species and the blocking antibody should be from that same species. Antibodies are diluted in PBS containing 0.1% sodium azide. A wash consists of adding 3 ml of PBS and centrifuging the cells for 3 min at 2000 × g. The supernatant is decanted, the tube blotted, and the cells are resuspended in the residual buffer. If erythrocytes are to be lysed, the following lysing reagent is recommended: 4.13 g ammonium chloride, 0.5 g potassium bicarbonate, 0.0185 g tetra sodium EDTA, 500 ml double-distilled water. This reagent must be prepared daily because the ammonium chloride is not stable in aqueous solution; failure to do this will result in poor lysis on subsequent days. When cells are fixed, 2% ultrapure formaldehyde (Polysciences, Warrington, CT) is added to the resuspended pellet at the end of the procedure.

D. Two-Color Immunophenotyping

With use of the basic reactions, several strategies for combining two antibodies can be designed. A common strategy is described in Fig. 4. When combining the reaction consisting of a primary and secondary antibody with either of the other two reactions, the indirect-staining reaction must always be performed first. The next step is to block the cells by mouse IgG so that any uncombined binding sites on the second antimouse IgG reagent are completely blocked before adding any other primary antibody (biotinylated or directly conjugated). Failure to strictly adhere to this rule will result in artifactual data, because the subsequent murine MAb will combine with unfilled sites on the second-step antibody, causing them to become inappropriately labeled.



1) Block with 10 μg mouse IgG per 10^6 (or less) cells per 50 μl for 10 min



3) Add Fitc-Avidin for 15 min and wash cells
(step not required for directly conjugated Mab)

Figure 2 Staining cells with a biotinylated MAb and avidin or a directly conjugated MAb: This procedure is nearly the same as described for Fig. 1, except that a second antibody is not used. Accordingly, the blocking IgG can be from the same species from which the monoclonal antibody was derived. A biotinylated antibody is useful because streptavidin can be obtained conjugated with virtually any fluorochrome. If a directly conjugated antibody is used, the need for step 3 is eliminated. Unfortunately, the selection of directly conjugated monoclonal antibodies with the various fluorochromes is limited.

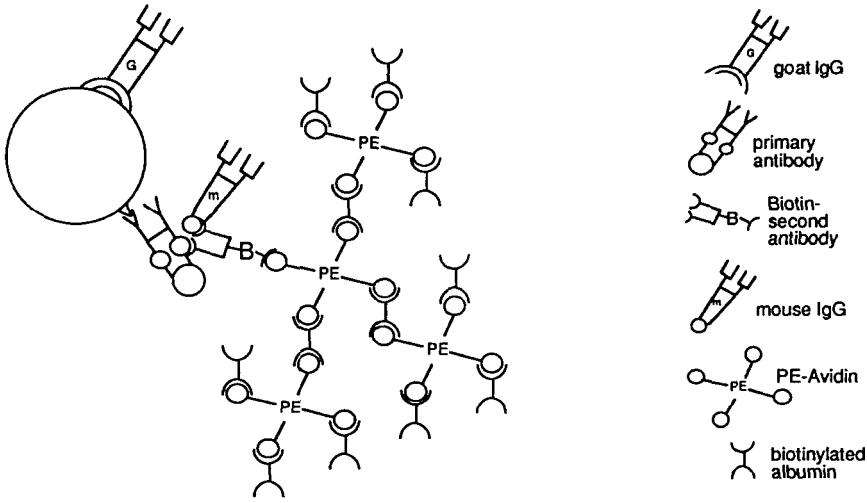


Figure 3 Amplification cascade for detecting cells with a low epitope frequency. The steps for performing this amplification strategy are to:

1. Block with 10 μg goat Ig 10 min (to block FcR and nonspecific binding)
2. Add primary MAb for 15 min and wash
3. Add biotin GAM $F(ab)'_2$ for 15 min and wash
4. Add 10 μg mouse Ig for 10 min to block unoccupied antibody-binding sites
5. Add PE–avidin to bind to biotin for 15 min and wash
6. Add biotinylated albumin for 15 min and wash
7. Repeat steps 5 and 6 as many times as necessary

It is very important to substitute an isotype control antibody in step 2 as a separate control. Amplification is effective for as many cycles as the signal improves at a more rapid rate than noise.

E. Three-Color Immunophenotyping

The three-color method is simply an extension of the two-color strategies, except a third antibody (one must be directly conjugated with a fluorochrome, the other could be biotinylated) is added. Three-antibody staining can be any combination of the three basic reactions, but if using the indirect second-antibody method, it must be performed first and the second-step antibody must be appropriately blocked, as described in the foregoing Sec. D, before adding the other two antibodies.

F. Four-Color Immunophenotyping

It is possible to stain cells simultaneously with antibodies labeled with all four of the fluorochromes summarized in Table 2 and to detect them with a single laser flow cytometer. Various combination strategies are possible, depending on the availability of reagents. The easiest combination to use consists of three directly conjugated MAbs combined with a biotinylated MAb, or four directly conjugated MAbs. The latter strategy is limited by a paucity of reagents. Since directly labeled antibodies with the third-color fluorochromes may not be available, the indirect second-antibody reaction combined with a biotinylated antibody in combination with

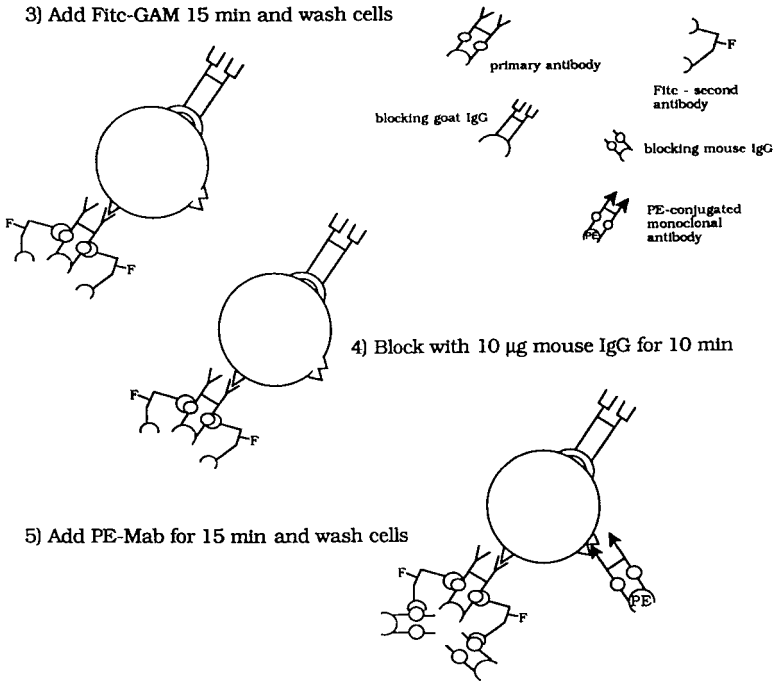


Figure 4 Staining cells with two monoclonal antibodies and a second antibody: There are several strategies for staining cells with two antibodies by combining the single-color procedures. The one shown here combines an unconjugated MAb that requires a second antibody (FITC) with a directly conjugated (PE) MAb. The first three steps are identical with those shown in Fig. 1. Before another mouse MAb can be added, however, uncombined sites on the FITC–second antibody *must* be blocked with mouse IgG. This will ensure that the PE–MAb will bind only to the epitope and not to the FITC–antimouse IgG second antibody.

FITC–MAb and PE–MAb can also be used. The indirect steps must be performed first, as described earlier, because unbound reaction sites must be blocked before adding any of the other MAbs.

V. DEAD CELL DISCRIMINATION

Dead cells are the single most troublesome and most overlooked problem in immunophenotyping. Immunoglobulins stick nonspecifically to dead cells, and no amount of blocking can overcome the problem. Superimposed on this problem is a variation in the degree to which different MAbs bind nonspecifically to both live and dead cells. Accordingly, dead cell discrimination should always be performed.

One method is to add either propidium iodide (PI) (Sasaki et al., 1986) or 7-amino-

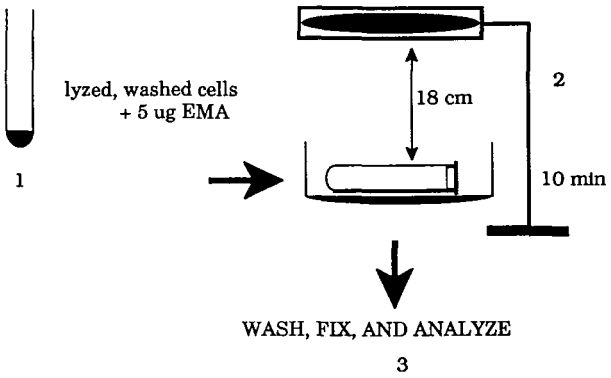
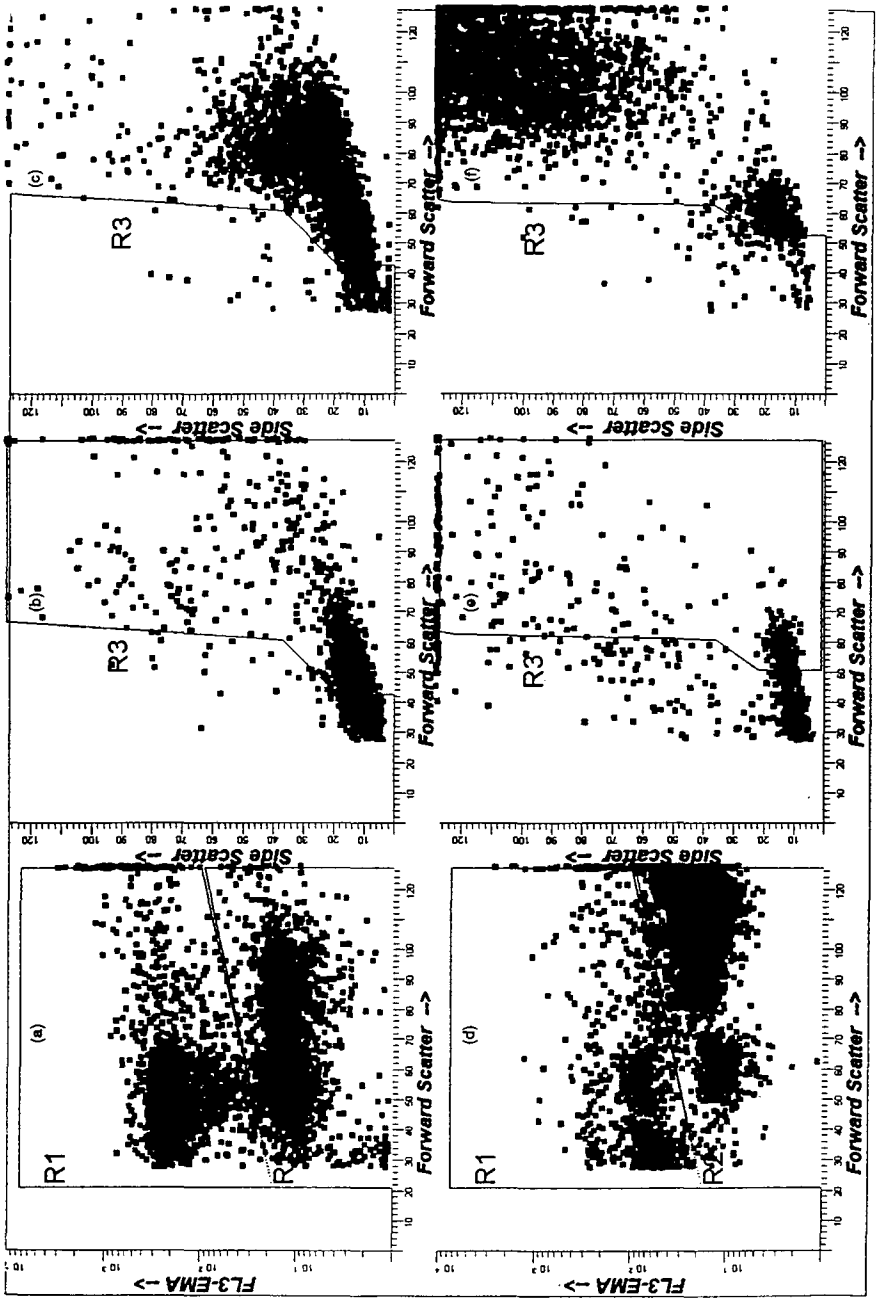


Figure 5 Staining for live or dead cell discrimination: Ethidium monoazide (EMA) is first added to the cell suspension at a final concentration of 5 μg/ml. The suspension is exposed to fluorescent light for 10 min and the EMA is covalently linked to DNA through the photoactivated azide group. The dye is then washed out, and the cells are fixed with ultrapure formaldehyde.

actinomycin D (7AAD) (Schmid et al., 1992) to all tubes, and gate on the viable cells that exclude the dyes. The one disadvantage to these procedures is that unfixed cells must be used because, if they are fixed in the presence of either dye, they become stained and if the dye is washed out, it comes off the DNA. Analyzing unfixed human cells can present a health hazard. Because PI has a broad emission spectrum, cells dimly stained with PE–MAb cannot be resolved from those stained with the PI, leading to artifactual data, and only FITC–MAb can be used in combination with PI. This problem is not as troublesome with 7AAD because its emission spectrum is less broad and farther into the red region, and it can be used with both FITC- and PE-labeled antibodies.

Another reagent and strategy that can be used to resolve dead cells and that provides the opportunity to fix the preparation, is use of ethidium monoazide (EMA) (Riedy et al., 1991). The EMA enters the dead cells, but not live ones and, after exposure to light, the photoactivated azide group irreversibly binds to DNA, providing an assessment of live and dead cells after fixation. The procedure is schematically shown in Fig. 5. This dye can be used in combination with FITC- and PE-conjugated antibodies, but when a third- and fourth-color antibody is included, viability must be assessed separately. As shown in Fig. 6, dead cells are resolved from live cells by locating each population in the FSC vs EMA display. A region is created around the live cells that includes the highest frequency of live cells and the lowest frequency of dead cells. This gate is then used to acquire (or analyze) the data. If conditions cannot be found to include more than 90% viable cells, the sample should not be analyzed because the results are likely to be artifactual. This is especially true for any subsets that have a frequency less than the fraction of dead cells in the gated region.

Figure 6 Determining regions for live or dead cell discrimination: When FSC vs EMA, (a,d) is displayed, dead cells (R1) are resolved from live cells (R2). The FSC vs SSC of dead (b,e) and live cells (c,f) are then displayed, and region R3 is set to obtain 90% of the live cells. When this is done for cells illustrated in the top row, only 71% of cells are viable, because a significant proportion of dead cells overlap the live cells. In contrast, for the bottom row, when 90% of the live cells are in region R3, the viability is 95%.



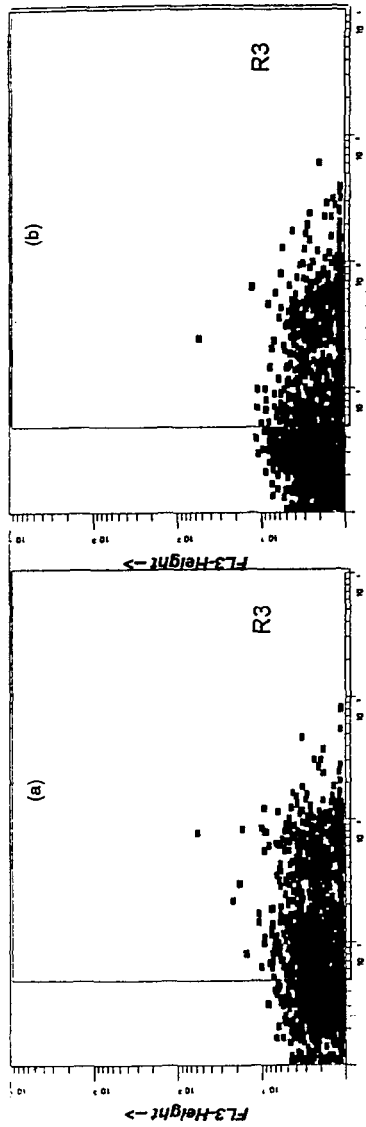


Figure 7 Determining the antibody titer: Serial ½ or ⅓ dilutions of the antibody are made and used to stain the target population. In this example, FITC-CD15 was used to stain human blood. At high concentration (a), positive cells are clearly resolved, but the negative cells have bound antibody so they are shifted to the right, past the marker set for the isotype control. The mean channel fluorescence of positive (352) to negative cells is 2.75. In (b), the correct concentration has been used, and mean channel fluorescence of positive cells (368) is more than those in (a) because the dim cells no longer contribute to the calculation, and the signal noise ratio (3.83) is improved. This method of titering is most important because the markers set for positive cells using the isotype control would be incorrect for the concentration of CD15 shown in (a).

VI. COMBINING ANTIBODIES TO RESOLVE SPECIFIC POPULATIONS

A. Antibody Titer

There are two factors that determine the optimal amount of antibody to use. The first, and most important, factor is the concentration of antibody and, the second, is the concentration of epitopes. The latter factor is dependent on the concentration of cells and the number of epitopes per cell. The standard concentration for all antibody-staining reactions in flow cytometry is an amount of antibody per 10^7 cells per milliliter. Usually, only 0.5×10^6–1×10^6 cells are used in 50–100 μl of volume.

Use of the correct antibody titer for staining cells is most important for minimizing nonspecific binding (noise), while maximizing the specific binding (signal). Because some MAbs produce more noise than others, it is important to maximize the signal/noise ratio for each MAb to be used, which is accomplished by determining the mean fluorescence intensity for a series of antibody dilutions. A typical series is shown in Fig. 7 for an antibody that has a high amount of noise. At high concentration, the antibody stains all cells to some degree, causing them to shift to the right. The signal/noise ratio of the mean channel fluorescence for each population is determined. As the antibody is diluted, the populations shift to the left, and the signal/noise increases to a maximum. Further dilution eventually results in no further shift of the negative cells, but positive cellular fluorescence and the signal/noise continues to decrease, reflecting nonsaturation conditions. The optimal antibody titer is the concentration that produces the maximum signal/noise ratio.

Virtually all good quality antibodies achieve saturation at concentrations between 0.1 and 1 $\mu g/10^6$ cells per 100 μl. If more than this amount is required, the preparation is likely to contain degraded aggregated antibody molecules or other proteins, and consideration to seeking a better reagent should be made.

B. Panel Verification

When combining antibodies for immunophenotyping, they must not produce artifactual data and, since each antibody will dilute the others, their concentrations must be appropriately adjusted. To verify that an antibody combination works well together, cells are stained with each antibody separately and then with them combined. After acquiring the data, the histograms for each antibody run separately are overlaid with the histograms for the corresponding color in the combination. There should be no appreciable difference between the two. For this to be achieved, it is most important that the samples are correctly compensated when acquiring the data. This simple test will also verify that the proper amounts of each antibody in the combination have been used.

VII. QUALITY CONTROL FOR MULTIPARAMETER FLOW CYTOMETRY

It is very important that the instrument is properly adjusted for collecting flow cytometry data and that verification is performed daily. The purpose of microspheres is for optical alignment and for obtaining preliminary instrument adjustments. Because the index of refraction of microspheres is different from cells (Salzman, 1990), cells should always be used for the final instrument adjustments. Even though it is customary to use cells from each experiment to make these final adjustments, this practice is not recommended. Instead lymphocytes from any convenient source should be used, because these cells provide the least cellular autofluorescence and the least

amount of nonspecific binding of antibodies. It is also common to setup the instrument using the isotype control. This practice is also not recommended, because it often covers up the problem of nonspecific binding. Once an instrument has been setup, using lymphocytes, virtually all other cells can be run, without changing any of the instrument settings. This is most important, because once an instrument setting is changed, all other instrument settings must be reverified if high-quality data are to be obtained.

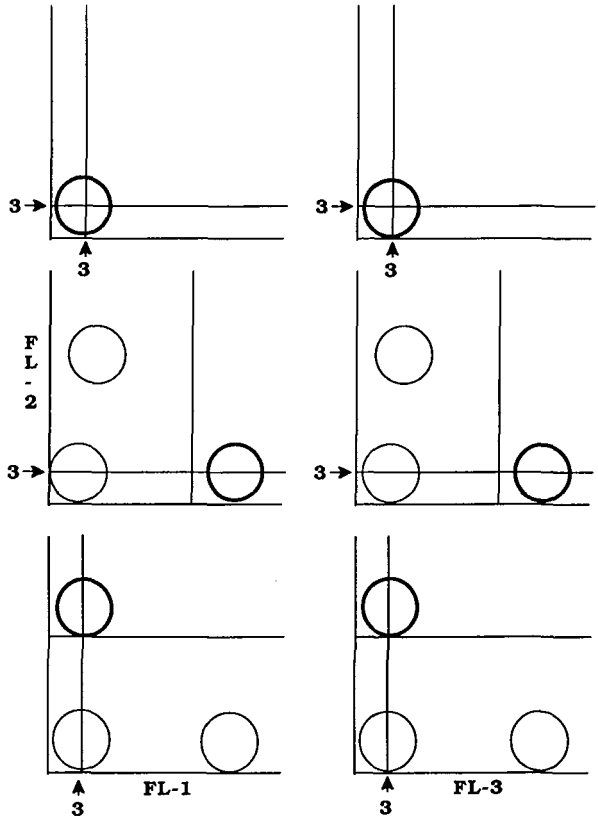


Figure 8 Adjusting compensation: Cells are used that have been stained separately with an MAb that is very bright. They may then be mixed together or run separately to adjust compensation. The first step (top row), is to set the intersection of the quadrant marker along the x and y axis at 3-log–fluorescence units and adjust the high voltage so the approximate center of the unstained cells is at their intersection. The next step (middle row) is to set the y marker on 3 and the x marker at a position so that only the FL-1 (left) or FL-3 (right) positive cells are evaluated. The compensation is adjusted so that half the cells are on either side of the y marker. The final step (last row) is to set the x marker on 3 and the y marker at a position so that the FL-2 positive cells in both views are evaluated. The compensation is adjusted so that half the cells are on either side of the x marker. If a fourth color is also used, the same procedure is followed.

A. Initial Procedures

The first step in instrument verification is to evaluate the alignment (some instruments do not require alignment, and this step may be omitted). To perform this task, target regions are established (and saved) for the position of the microspheres in each parameter. Each day the instrument is aligned so that the microspheres are in the target channels. Failure to achieve positioning of microspheres in their target channels for alignment alone, when using previous instrument settings, is indicative of an instrument problem. The cause of this problem should be sought in lieu of changing instrument settings which may "cover up" the problem.

The coefficient of variation (CV) of the microspheres can also be used to verify the instrument's performance. After establishing the best CV that can be achieved, a threshold, based on experience, can be established beyond which the instrument fails to meet performance standards. When this occurs, corrective action needs to be initiated.

The next step is to set the FSC, SSC, and fluorescence detectors appropriately. With use of the FSC versus SSC view, a previously saved target region for lymphocytes is displayed, and the instrument adjusted so the lymphocytes are circumscribed by the target region; very little change in instrument settings should be required. Nevertheless, fixed cells change their optical properties over time and, depending on when they are used relative to when they were fixed, significant differences can be seen. Next, the high voltages are adjusted so that the *median fluorescence intensity* for each fluorescence detector is in the desired channel.

B. Compensation

When performing multicolor immunofluorescence, it is necessary to compensate for the overlapping emission spectra of the various fluorochromes. This can be accomplished by either electronic instrument compensation or by software compensation. Although FITC- and PE-labeled microspheres are available for compensating these two fluorochromes, none are available for use with the third-color reagents, accordingly the user must prepare their own compensation standards. This is accomplished by staining cells with each antibody conjugated with the specific fluorochrome in a separate sample. It is very important that antibodies to epitopes in the highest frequency are chosen for each color, because cells that stain brighter than those used for compensation will not be properly compensated. A suggested procedure for adjusting instrument compensation is outlined in Fig. 8.

When software compensation is employed, it is only necessary to create files containing each compensation standard and one containing unstained autofluorescence cells with all the instrument compensation settings turned off. Computer software for this compensation has been described (Bagwell and Adams, 1993), and can be obtained from Verity Software House.

VIII. DATA ANALYSIS

The strategy for analyzing data when more than one color of fluorescence is measured is different from that established for a single color. In multiparameter flow cytometry, each parameter can be envisioned as a dimension in space and the bivariate display as a window to see into this multidimensional space. The FSC, SSC, and each color represent the parameters shown in Fig. 9, and the number of bivariate windows increase geometrically with each parameter. This provides increasingly higher resolution of the subsets of cells, because each subset is resolved in higher-dimensional space, but it also increases the complexity of the data analysis. Currently, available software is generally limited to analyzing data of no more than five or six parameters, but new approaches are now in development.

We recommend using bivariate dot–plot displays, because every acquired event is displayed

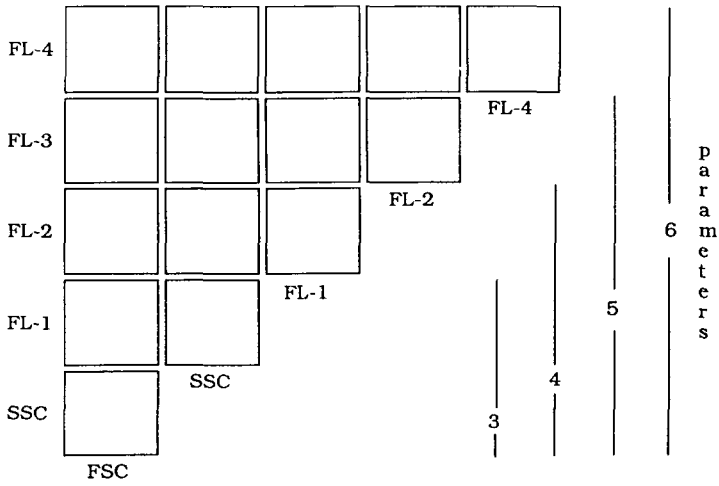


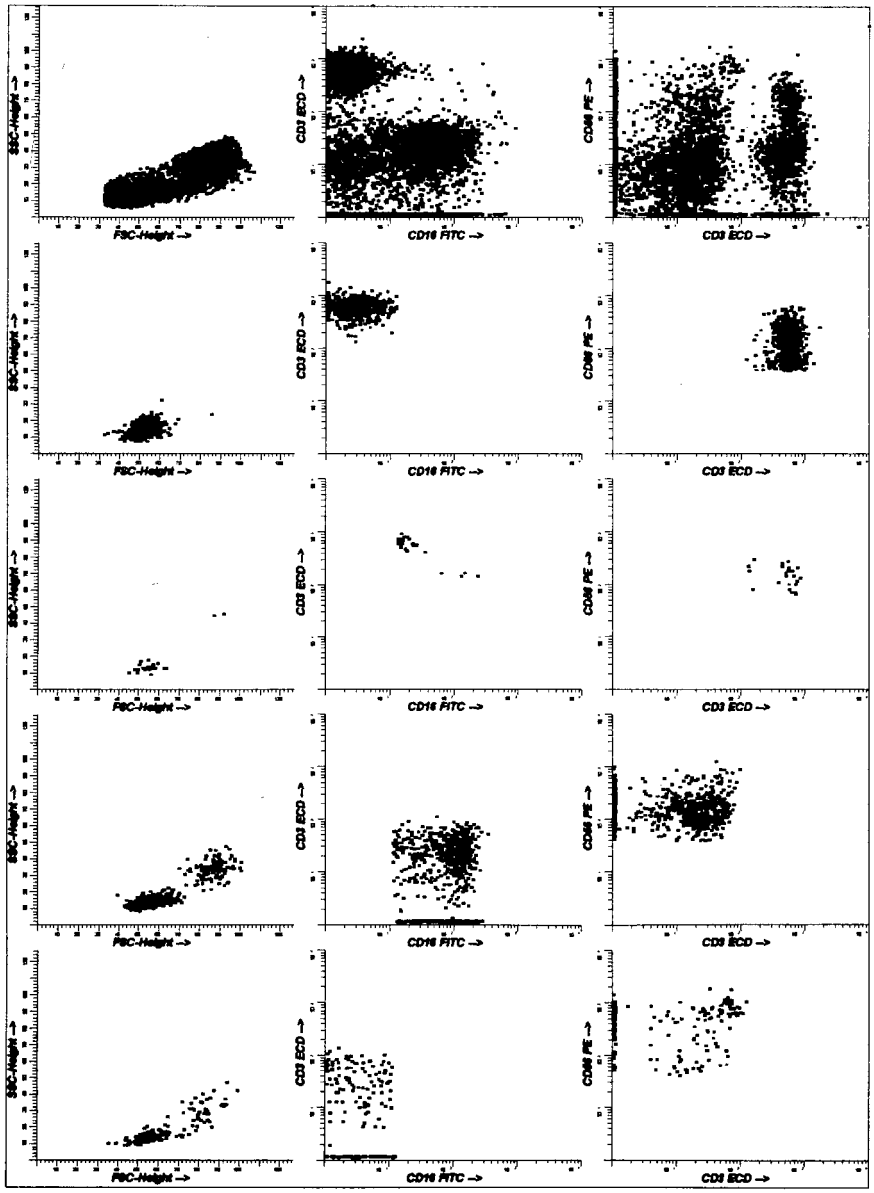
Figure 9 Combinations of parameters for viewing up to six sets of parametric data: A strategy for viewing all combinations of bivariate plots up to six parameters is shown. The parameters FSC, SSC, and FL-1 would likely be measured for cells stained with a single antibody. Here, three bivariate displays would allow all possible views. If two antibodies were used, three additional displays are required for a total of six. The number of views increases to 10 for three antibodies and 15 for four antibodies.

as a dot. This produces a plot containing discrete clusters of dots, representing the cell subsets that have been resolved. As shown in Fig. 10, subsets containing a high frequency of cells produce dense clusters, whereas those containing few cells produce thin clusters. Contour plots, although useful for viewing cells in high frequency, are not useful for viewing clusters containing a low cell frequency.

The most common strategy for analysis has been to use quadrant markers. For two-antibody combinations, the display is divided into four quadrant regions, and four populations of cells (+−, ++, −−, and −+) can be explicitly resolved. This technique, however, fails when more than two MAbs are used, because the correlation for the third (or more) color is lost. Furthermore, software programs that do not provide for many regions are not useful in analyzing more than three-antibody data.

One approach to analyzing complex multiparameter data is to use Boolean logic. For three

Figure 10 Four-color immunophenotyping: Human blood was stained with CD16 FITC, CD56 PE, CD3 ECD, and CD45 TC. Compensation standards were prepared by staining human blood with CD45 conjugated with each color. Data were acquired using a mononuclear cell gate without instrument compensation; compensation was performed in software. The views in the first row show all the data. For clarity, fewer populations are shown in this figure than can actually be resolved. Those shown illustrate the ability of the pattern recognition approach to resolve both very large and very small populations. While the regions are not shown, a region was first drawn around CD45 bright cells representing the lymphocytes to exclude monocytes and non granular granulocytes. This region was combined using boolean algebra with regions drawn in the view that best resolved the cluster representing a unique population. The second and third rows show cytotoxic T cells that are CD3+CD56+CD16− and CD3+CD56+CD16+. Note there are very few cells in the latter population. The fourth and fifth rows show CD3−CD56+CD16+ and



CD3−CD56+CD16− NK cells. While the great majority of them are CD16+, a substantial fraction are CD16−; note also the large proportion of large NK cells that are often excluded from analysis when a FSC vs SSC gate is used for data acquisition. Using multiple markers, cell populations representing less than one in one hundred cells can easily be resolved (for example row 3 contains only 0.3% of the total cells).

MAb combinations, eight separate populations can be explicitly resolved (+++, ++−, +−+, +−−, −++, −+−, −−+, and −−−). The regions are defined by using the isotype control to determine positive from negative cell subsets, and the shape of the region is defined using the compensation standard. This latter approach is most important, because high-intensity fluorochromes produce increased cellular fluorescence heterogeneity, causing them to produce elliptically shaped clusters when properly compensated, and this requires nonrectilinear-shaped regions.

Although this same approach can be applied to four-color (or more) data, and 16 separate populations can be resolved, it is easier to produce a template containing the regions that define each of cells that are resolved in multidimensional space. An example is shown in Fig. 10, where the position for each cluster of cells is shown in 7 of the 15 possible bivariate views.

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