Chapter 49
FACS Analysis of Leukocytes

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The development and selection of individual reagents and the choice of reagent combinations are as crucial to a successful FACS experiment as is the proper use of the instrumentation. In this chapter we focus on the use of antibody reagents to cell surface markers for the evaluation of leukocyte populations. We discuss general considerations for staining cells and titrating reagents. Emphasis is placed on multiparameter analysis using two or more antibody reagents. We show examples from single laser (488 nm) and dual laser (488 and 600 nm) instruments, but these are general in nature and apply to other instrument configurations. The issues presented here will aid any immunologist who is defining new lymphocyte populations, evaluating new monoclonal antibodies, or investigating new experimental paradigms.

Preparing and staining cells for flow cytometry

Cell preparation

A single cell suspension which can pass freely through the flow system is essential for any flow cytometry analysis or sorting experiment. There are numerous methods for preparing leukocytes from blood or whole tissue which yield single cell suspensions free of aggregates and debris and with good cell viability (for example, see Chapter 51 and references [1, 2]). A few points are considered here.

In general, cells should be maintained in a nutrient-supplemented, protein-containing medium for staining and analysis. Such a medium affords better long-term viability than simpler media such as PBS-BSA. Media without protein (such as plain PBS) or buffer (such as saline) should not be used, since cell viability can be negatively affected. Also, recovery of cells after centrifugation steps is significantly enhanced by the presence of protein (1% or greater). We generally prefer a modified RPMI-1640 (with L-glutamine) which is commercially prepared without biotin, riboflavin, phenol red, and bicarbonate. Biotin is avoided since it will interfere with avidin second-step reagents, and the riboflavin and phenol red are eliminated because they can increase apparent cell autofluorescence. The pH is buffered at 7.2 with 10 mM HEPES, and the protein source is usually heat-inactivated 3% newborn calf serum or serum albumin. For staining, 0.02% sodium azide is added, and the cells are kept on ice to avoid modulation of the cell surface antigens (such as capping or sloughing). Note that the presence of azide generally has no effect on viability as long as cells are maintained at room temperature or colder.

Cellular aggregates and clumps (such as arising from preparations of mouse spleen) can be reduced by passing the suspension through nylon mesh before staining. For sorting experiments, we sometimes also pass the cells through a 25-gauge syringe needle and nylon mesh after staining. Other techniques which may reduce clumping of cells include adding EDTA (1 mM) to reduce macrophage aggregates, and digesting the very sticky extracellular DNA (released by dead cells) with DNAse prior to staining. The DNAse is particularly effective in reducing clumping when tissue preparations exhibit considerable cell death. It is also often useful to further process the cells by density gradient centrifugation with a medium such as Ficoll. This procedure also results in a preparation with very high viability by exclusion of dead cells.

Staining

Staining cells with antibodies for FACS analysis is relatively straightforward, in principle. Direct (one-step) and indirect (two or more steps) methods are outlined in Table 49.1. In general, cells are added to an appropriate cocktail of optimally titrated antibodies, and then incubated on ice for sufficient time to allow the antibodies to bind to the cells. For a cocktail of antibody reagents containing only direct fluorochrome-antibody conjugates, the cells are then washed and processed for flow analysis. If some indirect stains are used (i.e., conjugated avidin to reveal biotinylated antibodies, or fluorochrome-conjugated anti-immunoglobulin to reveal unconjugated antibodies), further incubation with the second step reagents and washings are needed.

Fluorochrome-labeled monoclonal antibodies are the reagents of choice for most multiparameter cell surface analysis. The specificity, availability, and reliability of monoclonal antibodies makes them preferred over polyclonal antibodies for quantitative antigen measurements. They can be used as direct (one-step) or indirect (two-step) reagents. Polyclonal antibodies and indirect staining methods can yield higher fluorescence signals, but the reagents are often less specific than monoclonal reagents. This can result in an undesirable decrease in the signal to background ratio.

One-step reagents, where the monoclonal antibody is directly coupled to a fluorochrome, are significantly easier to use than two-step reagents for a number of reasons, but principally because the staining time is reduced and the number of necessary controls is fewer. While substantial effort may be required to prepare several direct conjugates, the widespread commercial availability of direct conjugates specific for leukocyte antigens from humans, mice, and other species, has made them the favored class of reagents. For most purposes, the direct reagents provide adequate fluorescence signal. Moreover, it becomes impractical, if not impossible, to use indirect reagents for doing three (or more) color analyses.

Two-step staining is advantageous when a single fluorescent reagent (such as a rat anti-mouse kappa) is needed to reveal multiple unlabeled first step reagents, such as in the screening of new monoclonal antibodies. In multiparameter analysis, the second step must react specifically with only one first-step reagent (in each sample). With each additional fluorescence parameter, it becomes increasingly difficult to obtain second-step reagents that bind to only one first step reagent. For example, analysis of human

Table 49-1. Antibody staining techniques

Direct (one-step) multi-color staining	
Procedure	Add cells to a cocktail of optimally titrated fluorochrome-conjugated antibody reagents Incubate for 15–20 minutes on ice; wash cells 2 or 3 times Resuspend with 1 μg/ml propidium iodide (PI) if appropriate Analyze
Fluorochromes	Argon laser (488 nm): FITC, PE, Cy5-PE, PerCP, PI Dye laser (595–610 nm): Cy5, APC, PI
Controls	Unstained cells Isotype controls with the same fluorochromes Cells stained individually with each fluorochrome for compensation *Each reagent must not interfere with the specificity of fluorescence characteristic of the other reagents in the cocktail*
Advantages	Simple and fast
Disadvantages	All antibodies must be directly conjugated to fluorochromes, requiring extensive one-time preparations or large library of reagents May be less sensitive than indirect staining
Combination direct and indirect (two-step) multi-color staining	
Procedure	Add cells to a cocktail of optimally titrated first-step reagents Incubate for 15–20 minutes on ice, wash cells 3 times Add optimal amount of fluorochrome-conjugated second-step reagent(s), and any fluorochrome-conjugated first-stp reagent(s) Incubate for 15–20 minutes on ice, wash cells 3 times Resuspend with propidium iodide (PI) if appropriate Analyze
Fluorochromes	Same as above, plus TR-Avidin with the dye laser (600 nm).
Controls	Unstained cells Cells stained only with the second-step reagent(s) Isotype controls with the same fluorochromes and first-step reagents *Each reagent must not interfere with the specificity or fluorescence characteristic of the other reagents in the cocktail* *Second-step must be specific for only one first-step reagent*
Advantages	Second-step increases sensitivity All antibodies do not have to be coupled to a fluorochrome
Disadvantages	Longer procedure May increase non-specific staining More difficult to evaluate reagent compatibility.

Note: This table is adapted from an earlier edition of this Handbook [12].

lymphocytes with mouse monoclonal antibodies could be done with unlabeled first step reagents and fluorochrome-labeled rat anti-mouse Ig second step reagents. However, as the number of first step reagents increases, it becomes increasingly difficult to construct a combination of isotype and allotype-specific second step reagents which are specific for only one first step reagent. Also, second step reagents which are compatible with a combination of first step reagents used for one analysis (such as T cells) may not be suitable for the first step reagents used in a second analysis (such as B cells).

One approach to overcome reagent incompatibility is to increase the number of staining steps to three or four. This has particular utility for screening new hybridomas. After a single first step is added (such as hybridoma supernatants), the cells are washed and then stained with a second step reagent designed to reveal the first (such as anti-mouse kappa). After washing, the cells are stained with direct or indirect stains to identify the leukocyte populations. Since the revealing reagent (anti-mouse kappa) has already been added and the excess washed away, the identifying reagents used in the third step will not be bound by the second step reagent (and this is why the last step reagent cannot be added prior to the revealing step!). Obviously, this multi-step procedure can be quite time consuming.

In contrast to the anti-Ig reagents, two-step systems in which the first step antibody is chemically modified and the fluorochrome-conjugated second-step is specific for the modification are compatible with multiparameter analysis. Biotin-avidin is the most commonly used such system, although hapten-anti-hapten systems are also used. Small molecules like biotin, digoxigenin, or other haptens are readily coupled to the primary antibody, and the second-step reagents such as avidin or anti-digoxigenin antibody react with high affinity and fine specificity.

It is preferable to use certain fluorochromes as the second step reagent. Texas Red (TR) Avidin is best used as a second-step reagent in the dual laser (488 nm and 600 nm excitation) instrument, because direct conjugates of TR tend to have a very high nonspecific staining (whereas the TR-Avidin does not). Also, phycoerythrin (PE) avidin, and especially the tandem Cy5-PE-avidin, are often a good choice for a second step color in the single laser (488 nm excitation) instruments, since the preparation of direct conjugates of these fluorochromes is more difficult and not commonly done in most labs.

Plates versus tubes: washing cells

Most of the experiments encountered in immunology involve multiple stains (such as 6 to 12 combinations of conjugated antibodies) with multiple reagents (2 to 4 conjugates per stain) performed on multiple samples (8 to 32). Thus, it is quite common to have more than one hundred samples (i.e., tubes) in a single experiment. We find it highly efficient to stain and wash the cells in microtiter plates (flexible, U-bottom) rather than individual FACS tubes. There are fewer physical units to handle, typically one plate versus 48 tubes, and reagent addition is facilitated by use of a multi-channel pipette. There is no need to physically resuspend each pellet with a multi-channel pipette after the washes, since the flexible U-bottom plate allows the pellet to be loosened simply by rubbing the underside of the plate after each wash. When the staining is complete, cells are readily resuspended and transferred to tubes using a 12-place multi-channel pipette.

Some care must be given to the layout of cells and stains in a plate. An example is given in Figure 49.1. Wells receiving the same reagents may be placed next to each other, but wells containing different reagents should be separated by one empty well. This precaution guards against accidental spilling of reagents between adjacent wells. (I.e., contamination of reagents between samples is typically a much more severe problem than contamination of cells, since most samples have similar cell compositions. However, if cell contamination would be a problem, then all

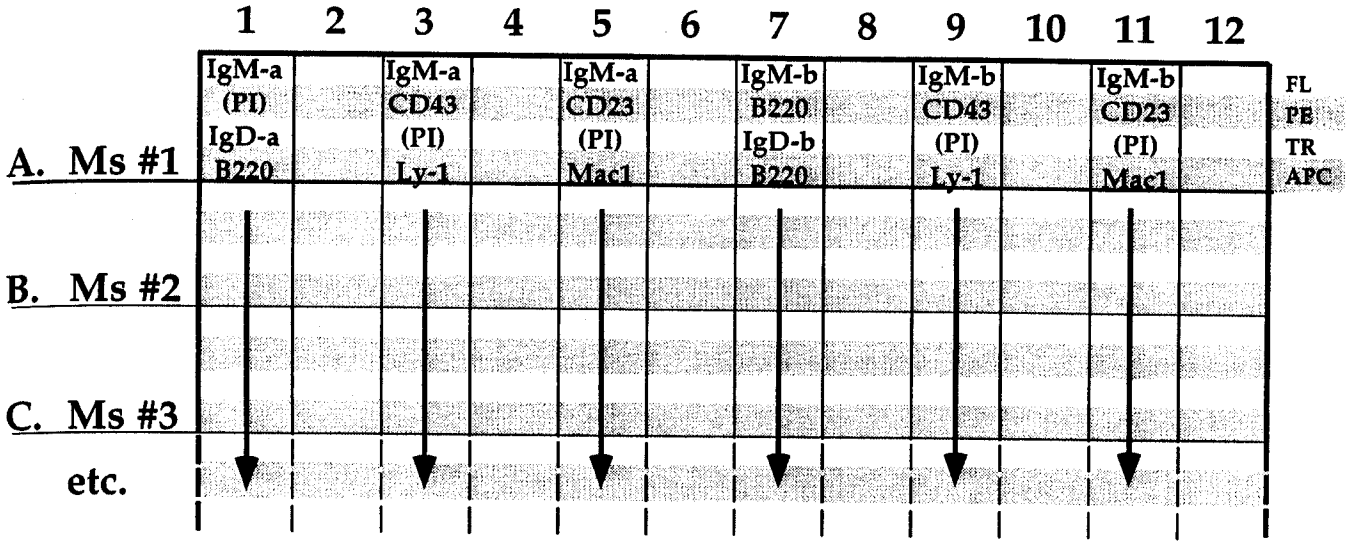


Fig. 49.1. *Sample layout for a staining experiment using a plate.* These stains would be used to evaluate peritoneal cavity B cells in reconstituted or immunoglobulin-heterozygous mice on a FACS dual-laser (488 and 600 nm excitation) instrument with four fluorescence channels Such mice contain both B cells which express a-allotype IgM and IgD and B cells which express b-allotype IgM and IgD. The target antigens are listed for each column with the appropriate fluorochome. FITC, PE, and APC reagents are direct conjugates. For TR, TR-AV is used to reveal biotinylated reagents. Each column contains one staining cocktail and is separated from the others by an empty column. Each row corresponds to peritoneal cavity cells from different experimental mouse (Ms). Eight animals can be accommodated on one plate.

sample-containing samples should also be separated by one empty well: this would mean that up to 24 wells per plate can be used). Only after all the first step reagents (typically about 50 μl) are added to the wells should the cells (typically, 0.5–2.0 $\times$ 10^6) be added. This ensures uniform staining and makes it easier to control the reagent incubation time.

When the stains are separated by one well in 96-well trays, cell transfer to tubes is particularly easy with a multichannel pipette. The test tube racks for the typically-used 12 $\times$ 75 mm tubes position these tubes at a distance exactly twice the distance between wells in a 96-well tray. Thus, a multichannel pipette with tips placed on every other position facilitates the transfer of cells to the "FACS tubes."

For some non-saturating reagents, it is important to control the incubation time during the staining procedure (see "Titration of antibody reagents" below). This is especially easy to do in plates: cells and reagents are simply aliquoted into adjacent columns. The staining is initiated by transferring an entire column of cells onto a column of stains using a multichannel pipette.

Washing steps are usually needed for quantitative enumeration of cell populations, although the amount of washing depends on the fluorochrome-reagents being used and how well the population of interest is separated from negative cells. For one-step stains, washing steps after the incubation with antibody reduces the non-specific background. This is particularly important when trying to discriminate dull from negative populations. For two-step stains, washing is also required to remove unbound reagent from the first step prior to adding the second step reagent.

Some reagents require more washing steps than others. We find that TR-avidin and Cy5-PE conjugates have the highest non-specific background and require the most washing, typically 3 spins per step when staining in microtiter plates. Two spins are often sufficient for one-step reagents. One or even no washes can be used when the positively stained cells are distinct from the negative cells. Note, however, that with some reagents used at high concentration, background fluorescence can be caused by reagent in solution. In general, when multiple cocktails of 3 or 4 antibody reagents each are used to evaluate numerous cell samples, we find it easiest to consistently wash 3 times after each reagent step, rather than evaluate the minimal number of washes for each reagent.

Note that "washing" steps are simply dilutions of reagent, and do not impart a physical force on the cells to remove nonspecifically-bound reagents form the cell surface. Thus, when staining is performed in tubes (for example, for sorting from large cell populations), a much larger dilution is obtained with each wash and fewer washing steps are required.

Titration of antibody reagents: choice of the optimal concentration of a conjugated antibody reagent

Consequences of nonsaturating stains

By definition, an antibody stain that is not in equilibrium does not saturate. The primary consequence of using antibody conjugates under nonsaturating conditions is that the final fluorescence achieved (per cell) will depend on three factors: the number of cells added, the concentration of antibody added, and the amount of time of the incubation. In many circumstances, only two of these three will affect the final stain intensity: antibody stains that do not saturate do so because either [1] there is not enough antibody present (i.e., antigen excess), or [2] the affinity constant is very low. In the latter case, there is usually still a significant excess of antibody over antigen–and therefore, the final fluorescence will be relatively insensitive to the antigen concentration. (i.e., 1 μg of antibody is enough to stain 10^8 cells with 10^4 antigens/cell: thus, at typical staining cell concentrations of 10^6 cells, there is still a 100-fold excess of antibody, and variations in cell number will not measurably affect the staining intensity). At

nonsaturating conditions when antibody is in excess, the fluorescence will also increase with time. Thus, for a 30 minute incubation, a three minute variation could lead to as large as a 10% variation in the final intensity (although the typical effect will be smaller, since the binding kinetics is nonlinear with respect to time). Of course, under these circumstances, the efficiency of the cell washes will also affect the final intensity.

Nonsaturating stains thus lead to time-dependent and volume-dependent staining intensities. This is undesirable in general, but when performing quantitative antigen density measurements, it becomes unacceptable. For some antibodies which do not saturate even at very high concentrations, the only way to perform quantitative measurements is to have a very strict protocol, in which the volumes of reagent and the incubation times are carefully controlled.

Theoretical considerations

The theoretical amount of antibody required for saturation staining can be calculated from standard equilibrium binding equations. For a "rule of thumb" calculation, we will ignore differences in monovalent versus bivalent binding and consider the binding problem to be a simple two-component macroscopic association. Standard binding equilibrium equation relates the concentrations of free antigen (Ag), free antibody (Ab), bound complex (C), to the binding constant (K):

$$Ab + Ag \Leftrightarrow C$$

These are related by the equation:

$$K = [C]/\{[Ab]\,[Ag]\} \quad (1)$$

rearranging gives

$$K\,[Ab] = [C]/[Ag] \quad (2)$$

Assume saturation is where more than 90% of the antigen is complexed with antibody, such that

$$[C]/([C] + [Ag]) \geq 0.90 \quad (3)$$

This means that the complex is at least 9 times as concentrated as the free antigen, that is,

$$[C]/[Ag] \geq 9 \quad (4)$$

The free antibody concentration is related to the total antibody concentration by:

$$[Ab] = [Ab_T] - [C] \quad (5)$$

where Ab_T is the total antibody. But, since almost all of the antigen is complexed, the concentration of the complex is approximately that of the total amount of antigen present:

$$[C] \approx [Ag_T] \quad (6)$$

Therefore, substituting equation (5) into (4), and (4) and (3) into (2), we get:

$$K\{[Ab_T] - [Ag_T]\} \geq 9 \quad (7)$$

Solving for the total antigen, we find that:

$$[Ag_T] \leq [Ab_T] - 9/K \quad (8)$$

Table 49-2. Cell number limitation for antibody staining

	[Ab]		
μg/100 μl	10	1	0.1
nM (approx.)	1000	100	10
K (Affinity, M-1)			
10^7	10^7	ns	ns
10^8	10^8	10^6	ns
10^9	10^8	10^7	10^5
10^{10}	10^8	10^7	10^6
10^{11}	10^8	10^7	10^6

Note: The number of cells that can be saturably stained was calculated from Equation 8, assuming 10^5 copies of antigen per cell, and a staining volume of 100 μl. ns = nonsaturating. In addition, the following conversions were used (in this example, the frequency of the positive cells is 100%, or 1.0):

$[Ag_T]$ = {(# cells) × (frequency of positive cells) × (10^5 copies/cell)}/{6.02 × 10^{23} molec/mol) × (10^{-4} L)}

$[Ab_T]$ = (μg IgG) × (1.5 × 10^{11} μg/mol) × (10^{-4} L)

Thus, the maximum amount of antigen that can be stained (at equilibrium) at saturation depends on two factors. The total amount of antibody must exceed the total amount of antigen and, at lower affinity constants, it must exceed it by progressively larger margins.

Practical consideration

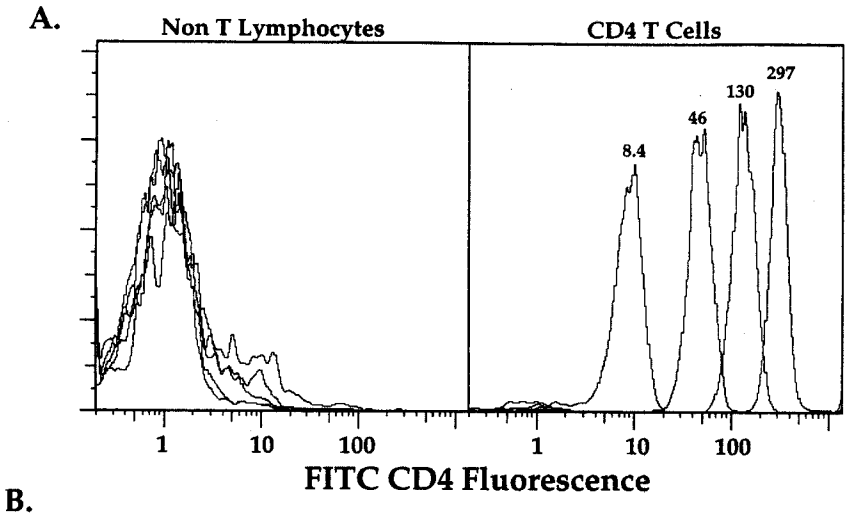
As an example, let us assume that we are staining an antigen on cells that is present at 100,000 copies per cell (an abundant antigen)–*and* that 100% of the cells in the population express this antigen. Typical antibody binding constants range from 10^7/M to 10^{11}/M. Table 49.2 gives the maximum number of cells that can be stained (in 100 μl) and still achieve saturation (>90% of antigen bound), given various affinity constants and total amounts of antibody used (in this example, an IgG).

At cell numbers *above* the maximum listed in Table 49.2, the final fluorescence will diminish approximately by the ratio of the cell number to the maximum listed. Thus, if using an antibody at 1 μg in 100 μl, with an affinity of 10^{10}/M, staining 10^8 cells would result in approximately 10% of the saturating fluorescence. Of course, this fluorescence may still be more than enough to achieve adequate resolution from unstained cells.

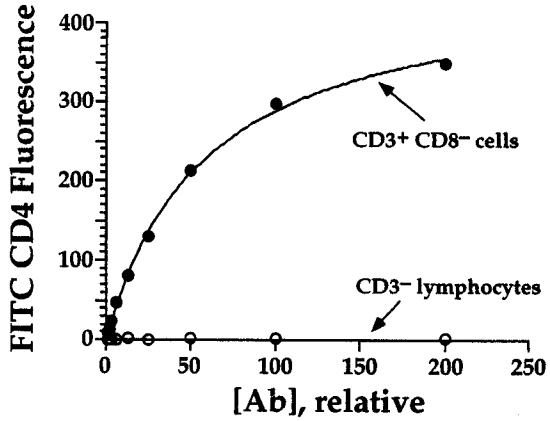
This brings about two important practical conclusions: the amount of antibody being used for analysis of 10^6 lymphocytes (for instance, during an exploratory phenotyping analysis) may not have to be increased at all when performing a preparative sort from 10^8 lymphocytes. Just as important, the amount of reagent recommended by a manufacturer for staining 10^6 lymphocytes *cannot* be decreased 10-fold when staining 10^5 lymphocytes.

Experimental titrations

Titrating reagents is relatively simple. Usually, a 2-fold serial dilution series is generated, starting at what is presumed to be a saturating concentration of reagent (usually, 2 μg of reagent for a 100 μl test is appropriate; however, if this amount turns out to be nonsaturating, then the titration must be repeated starting at higher concentrations). Stains are adjusted to have equivalent final volumes. It is generally best to include a second color which will positively identify the cells which also stain for the titrated reagent. (For instance, when titrating CD3, the counter-stain could be anti-TCR). This is so that the median fluorescence of the



B.



C.

[Ab]	CD4	nonT
200	350	1.5
100	297	1.5
50	212	1.3
25	130	1.1
12.5	80	1.2
6.25	46	1.1
3.12	24.1	1.0
1.56	13.8	0.93
0.78	8.4	0.9

Fig. 49.2. (**A**) *Histograms of FITC CD4 fluorescence, as a function of antibody concentration, for CD3⁻ lymphocytes (left), and CD3⁺CD8⁻ T cells (right).* The numbers over the peaks indicate the median fluorescence of the population. The shoulder of fluorescence in the left panel is due primarily to monocytes (which are $CD4^+$) that enter the lymphocyte scatter gate. (**B**) The fluorescences of these two populations are plotted as a function of the CD4 antibody concentration. The background fluorescence does not significantly increase over the range at which the CD4 reagent becomes saturating. The appropriate concentration to use would be more than 200 (relative units); in this case, the titration should be redone starting at higher concentrations of reagent. (**C**) Median fluorescences of the positive and negative population at each concentration of reagent.

positive and negative populations can be accurately determined–even if the antibody stain is so dilute as to generate overlapping distributions for the positive and negative populations. The second color should not be diluted: add it after generating the dilution series. Finally, add the number of cells to the stain that will typically be used under experimental staining conditions (such as 1 million). All incubations and washes should be performed under standard staining conditions.

After analysis, the median fluorescence of the positive and negative populations is computed and then plotted against the antibody concentration. A typical titration of FITC CD4 is shown in Figure 49.2. If possible, a nonlinear least-squares fit of equation (9) can be used to estimate the "goodness" of the titration.

$$F = B + F_{max}/(1 + k/Ab_T) \qquad (9)$$

where F is the cellular fluorescence; B is the cell-specific background fluorescence (autofluorescence); F_{max} is the fluorescence that would be obtained at 100% saturation; k is the antibody concentration giving half-maximal fluorescence; and Ab_T is the concentration of antibody. Since the value for B is part of the fit, no background subtraction need be applied. Also, the value obtained for B may be different for different cell types; that is, it is valid only for the cell type for which the titration was performed. Programs like JMP (SAS Institute) and Kaleidagraph (Synergy Software) on the Apple Macintosh® can fit such an equation. In general, the concentration of reagent to use should be at least 2-fold above a concentration which gives at least 90% of the saturating fluorescence.

In some cases, reagents do not saturate until extremely high concentrations (such as Fig. 49.3A). In these cases, it may be necessary to use nonsaturating concentrations of reagent, with the caveats outlined above.

Figures 49.3B and 49.3C illustrate the dependence on time and cell number of fluorescence. In Figure 49.3B, a reagent is titrated against three significantly different cell numbers. In this case, even a 20-fold increase in cell number (over the 1 million per test) only results in a 3-fold decrease in fluorescence at subsaturating concentrations of antibody. This indicates that even at this point,

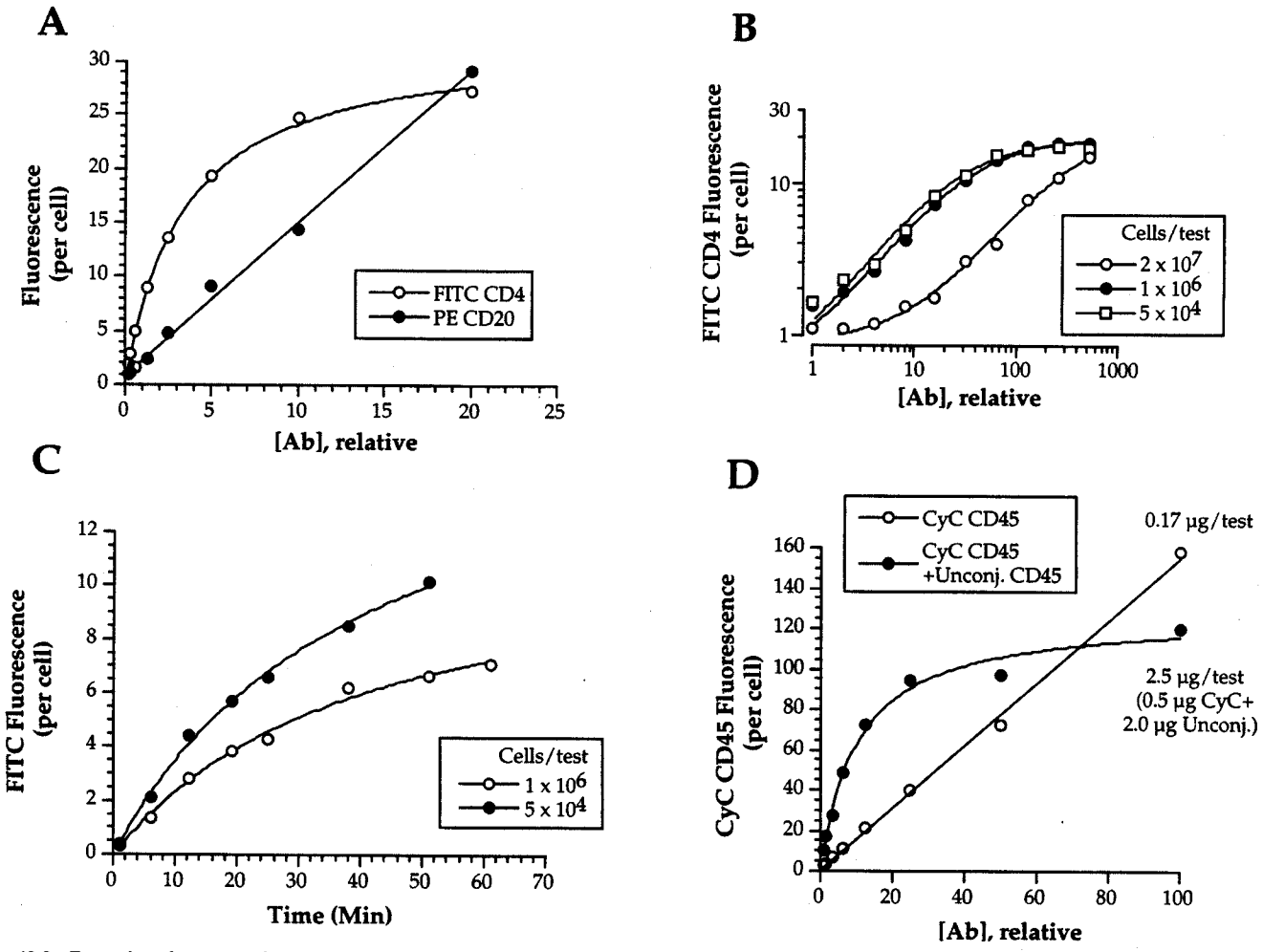


Fig. 49.3. *Examples of titrations for saturating and nonsaturating antibody reagents.* (**A**) Titration of two different antibodies. A standard binding curve is fitted to the data using a nonlinear least-squares algorithm. The FITC CD4 (Becton Dickinson, Leu3a) reagent saturates; that is, increasing the amount of antibody per stain above 20 µl would not result in significant increased fluorescence per cell. On the other hand, the PE CD20 (Becton Dickinson, Leu16) reagent does not saturate. In fact, the linearity of the data suggests that the saturating concentration is considerably above the amount of antibody contained in 20 µl of reagent (probably at least 10-fold more antibody is required). (**B**) The number of cells per test does not affect the staining concentration until the amount of antigen approaches the total antibody concentration. A FITC CD4 reagent was titrated against 3 different concentrations of cells. Cell numbers below 1 million per test do not affect the final fluorescence; the stain is sufficiently in antibody excess at this concentration of antigen. However, at 20-fold higher cell numbers, the cellular fluorescence at any particular concentration of antibody is about 3-fold decreased, indicating that the antigen concentration is beginning to approach the antibody concentration. (**C**) Kinetics of antibody binding at nonsaturating concentrations. When the antibody reagent is nonsaturating (i.e., dilute), then the incubation time significantly affects final cellular fluorescence. As the reaction approaches equilibrium, the effect of time diminishes significantly. (**D**) The CyChrome CD45 reagent does not saturate until approximately 2 µg per test. However, the cellular fluorescence achieved at this concentration of reagent would be well off-scale on most flow cytometers. (In this case, at 0.17 µg/test, the cells are already in the last decade of a 4-decade log amplifier). Therefore, the conjugated reagent was "doped" with unconjugated reagent. Shown is a 1:4 doping mixture. This mixed reagent is not only saturating, but on-scale in fluorescence.

the reagent is in excess over the antigen. Note that at the saturating concentration, even 20 × 10^6 cells can be stained with little loss in cellular fluorescence. *However, this does not mean that the reagent can be diluted 20-fold when staining only 1 × 10^6 cells!* Such a dilution would result in the loss of fluorescence because the binding reaction would not have reached equilibrium. Figure 49.3C illustrates that when the reagent is at subsaturating concentrations, binding kinetics can take much longer than a standard incubation time.

Finally, with the advent of extremely bright fluorophores, especially the phycobiliproteins, many reagents are so bright at saturation that they are "off-scale," even on a 4-decade logarithmic amplifier. In such cases, virtually all manufacturers have chosen to dilute the reagent so that it remains on-scale. This applies to many phycoerythrin (PE) reagents, and virtually all Cy5-PE tandem reagents. Therefore, many such reagents are sold at nonsaturating concentrations! Caution must be used when using such reagents for quantitative analysis.

The problem of too-bright reagents can be solved by inclusion of "cold" competitor, in the form of unconjugated reagent. The competitor must be the same antibody as is used for the conjugation, so that the same epitope is seen by both. Figure 49.3D illustrates the use of unconjugated antibody to "dope" a Cy5-PE tandem of CD45. CD45 is an antigen found at very high density on

lymphocytes; together with a bright fluorophore, the reagent is off-scale at approximately 20% of the concentration required to achieve saturation. However, a 1:4 mixture of the conjugate and the unconjugated antibody results in a saturating stain that remains on-scale for analysis.

Staining controls

Autofluorescence

All cells have an intrinsic fluorescence due to normal components such as flavins and cytochromes. This autofluorescence, which can limit the sensitivity of the measurement, is a function of cell size, cell type, and the excitation and emission wavelengths being used. For example, resting lymphocytes have relatively little autofluorescence, activated lymphocytes typically have more and granulocytes, and monocytes have the most. Tissue culture cells vary in autofluorescence and tend to have considerably more than similar cells prepared directly from the animal.

For any flow cytometry experiment, it is important to determine the autofluorescense level of every cell population being considered in each channel being measured. Usually this is accomplished by recording unstained cells. However, often it is useful to define particular leukocyte populations with counter stains in other channels. The intrinsic cell background should be used as the ideal background for negative cells when evaluating the titration of new reagents. Internal cell fluorescence is greatest toward the blue end of the spectrum; consequently, the fluorescein and PE channels have greater autofluorescence than TR and allophycocyanin (APC). Autofluorescence is even greater in the near UV. The deleterious consequence of autofluorescence (reduced sensitivity) can be alleviated by using bright reagents in the channels with the most autofluorescence, saving the dullest reagents for channels with the least autofluorescence. Enumeration of discrete cell populations which are well distinguished from the negative cells can be done with high autofluorescence. Even UV excitable dyes such as AMCA can be used in this situation. However, the enumeration of populations in which the antigen expression is a continuum from negative to positive, where "dull" cells need to be distinguished from negative cells, requires low autofluorescence. Consequently, we prefer to use APC conjugates for these antigens (for example, in enumerating the Ly-1 (CD5) surface antigen on B cells as described below).

Finally, it is possible to correct for the contribution of autofluorescence in one measurement channel (such as FITC) through the use of compensation from another measurement channel for which no fluorophore is present (such as the PE channel, when PE is not included in any stain). This procedure treats autofluorescence simply as another fluorochrome that is present on all cells. Thus, to correct for autofluorescence, one FACS channel is devoted to determining its magnitude in each cell; this channel is then used to estimate the amount of autofluorescence present in the other channels even when other stains are present (for a more detailed discussion of compensation, see Chapter 50).

Autofluorescence correction can be done either in real-time using standard compensation circuitry [3], or during post-hoc analysis using software compensation [4]. The principle underlying this correction is that the amount of autofluorescence in any two channels is proportionately related (i.e., a cell which has twice as much autofluorescence in the FITC channel will have twice as much autofluorescence in the PE channel). By determining the constant of proportionality relating the measured autofluorescences in the two channels, the relative amount of autofluorescence in one channel can then be estimated by measuring the amount in a second channel. (This is simply a normal compensation technique, as described in Chapter 50.) It is important to realize that this process is not a background subtraction (i.e., a constant subtracted from every cell measurement), but rather a means for quantitating the fluorochrome-specific fluorescence in the presence of autofluorescence. The only disadvantage of autofluorescence compensation is that it requires a dedicated parameter (i.e., the number of measured parameters is one more than the number of other fluorochromes being used). Autofluorescence compensation can significantly improve signal-to-background, allowing quantitation of dim fluorescence on even highly autofluorescent cells (such as [4]).

Reagent controls

The antibody reagents themselves can also contribute to background staining. It is common for many reagents to show some degree of staining independent of the target antigen. This nonspecific staining can be grouped into two classes: Fc-mediated (for immunoglobulin reagents only), and general "stickiness." It is relatively straightforward to evaluate Fc-mediated background staining. Different cell types have Fc receptors specific for immunoglobulin of particular isotypes. Monocytes in particular bind immunoglobulin this way. This effect can be evaluated using antibodies of the same isotype as the reagent, but whose specificity is irrelevant (i.e., not on the cells of interest). This class of non-specific staining can often be blocked by pre-incubating cells with serum Ig, or with anti-Fc receptor antibodies which block the Fc-combining region, before staining. Fc receptors often have affinity for immunoglobulins from other species; that is, human cells may bind murine antibody reagents.

Isotype control reagents are a necessary but not sufficient measure of nonspecific staining. Every time a monoclonal antibody is conjugated to a fluorochrome (either small organic dyes or large proteins), a new complex is created which can change the charge, hydrophobicity, and aggregation state of the antibody. Over-conjugation can even alter the ability of the antibody to bind its target antigen. Since each conjugation is unique, isotype controls, even when coupled to the same fluorochrome, do not necessarily control for this stickiness. Some of this potential variability can be reduced by controlling the level of conjugation and by eliminating aggregates by either centrifugation or gel filtration before use. Even with these precautions, it is still helpful to evaluate background staining on a population known not to express the target antigen, provided that such a population can be identified. For example, IgD is dull to bright on various murine B cell populations, but negative on T cells. By counter-staining with a T cell marker (such as CD4) and a B cell marker (such as B220/RA3 to 6B2), the background staining of anti-IgD can be effectively evaluated. Ideally, the fluorescence profile on the CD4 T cells should be the same in the presence and absence of the anti-IgD reagent. If this is the case, the background can be attributed completely to the cell autofluorescence. One caution: sometimes a surface marker associated with a cell population is shown to be present at low levels on another population. Our favorite example is Ly-1 (CD5), which was originally known as a pan-T cell marker. However, it is also present, albeit at lower levels, on a distinct subpopulation of B cells.

"Dump channel"

Rare cell populations (<1%) can be best identified using a cocktail of reagents. Ideally the cells should be positive for at least two markers and negative for another. The use of a negative channel eliminates cells that non-specifically bind all reagents, as well as eliminating particularly autofluorescent cells; in addition, it eliminates two-cell events ("doublets") containing different cells individually expressing the different positive markers. To this end, it is particularly useful to make a cocktail of antibodies specific for unwanted cells. For example, we often use a mixture of T Cell, macrophage, and granulocyte markers to make up such a "dump channel" for our enumeration of antigen-specific B cells.

The PE channel is well suited for the dump, since we include propidium iodide (detectable in the PE channel) to discriminate live (PI-negative) from dead (PI-positive) cells [5]. PI is a DNA intercalating dye which is excluded by intact cell membranes. However, when cells die, membrane integrity fails, and PI can enter and stain the nucleus. PI is very important for small cell populations because it gives a more complete exclusion of dead cells than scatter alone. Dead cells often stain non-specifically and can be mistakenly identified as a distinct population.

When properly executed, the "dump" channel will contain positive signals for all unwanted events. This leaves the remaining channels for positive discrimination. In more routine use, with larger populations, this exclusion parameter may consist of only PI.

Compensation

Proper setting of compensation values to correct for the spectral overlap of the various fluorochromes is important for any multiparameter FACS analysis. Errors in the compensation set-up are common in the immunology literature, so we will present some detail on how to recognize and avoid these issues. The emphasis in this section is on reagent choice, compatibility, and evaluation. Instrumental considerations and methods for determining the correct values are presented in Chapter 50. Here we discuss some of the consequences of incorrect settings and the difficulty in catching these errors post hoc. One preliminary warning: MAb reagents prepared with different fluorochrome preparations may have different spectral properties. This is especially common for the phycobiliprotein and tandem reagents and less of a problem for the small organic dyes such as FITC. Thus, it is wise to check the compensation values for each set of reagents prior to any FACS analysis.

While compensation has the extremely beneficial effect of making the measurements independent of each other (i.e., removing the contribution of a fluorophore from all but one channel), it has two potential disadvantages. First, incorrect compensation can lead to significantly wrong biological conclusions (see below). Second, compensation inherently broadens the distribution of a fluorescence marker. Compensation involves the subtraction of one or more signals from a particular signal. Therefore, the electronic errors of the individual measurements of each channel involved in compensation are propagated through to the final output. This can result in an apparent increase of the coefficient of variation (CV) of a fluorescence measurement. However, for most applications this increased CV does not affect analysis.

Intralaser compensation

An analysis of splenic T cells with different compensation values is presented in Figure 49.4. The cells were stained with three MAb reagents: 1) FITC labeled anti-TCR, which identifies essentially all splenic T cells, 2) biotin-labeled anti-CD8, which is revealed with Texas Red-Avidin (TR-AV) and identifies about half of the T cells and 3) allophycocyanin (APC)-labeled CD4, which identifies the other half of splenic T cells. There are essentially no T cells in the spleen which are double-positive for CD4 and CD8. TR and APC are both excited with a dye laser (excitation 600 nm) and have significant overlap of their emission spectra.

Two populations of T cells can be readily identified even without compensation (Fig. 49.4, bottom left). However, the false impression is given that the "CD4 cells" express some CD8 and the "CD8 cells" express a substantial amount of CD4. As indicated by the diagonal orientation of the contours, the signal in the two channels is highly correlated: this is expected because (in reality) only one reagent contributes to each population and the "spillover" fluorescence (in the "wrong" channel) is proportional to the true fluorescence (in the "right" channel). Note that the TR-reagent makes a larger contribution to the APC channel than the APC-reagent makes to the TR channel (24.9 vs. 8.2%).

Proper compensation is when the median signal of a singly stained population (such as APC only) equals the median signal of an unstained population in the overlap channel (TR). This setup yields an accurate picture of the surface antigen distribution. T cells appear positive for either CD4 or CD8, but not both (Fig. 49.4, top left). Modest errors in setting the compensation values can affect the interpretation of the data. In the middle panel, both the APC and TR signals are undercompensated by 20% of their proper values (19.9 and 6.6% respectively). This gives the false impression that the CD4 T cells are dull for CD8 and that the CD8 T cells are dull for CD4. Most importantly, this undercompensation cannot be deciphered from this single FACS plot. The compensation is just sufficient so that the contours do not show the diagonal characteristic of the uncompensated plots. Note that the situation becomes especially difficult to unravel when novel populations are being investigated and when the two markers appear on the *same* cells; for example, CD4 and CD8 on cells in the thymus. (Similar problems can arise when compensation is set too high.)

Problems with compensation are often not apparent when only one of the compensation channels is viewed. In the middle and right columns of Figure 49.4, FITC-labeled anti-TCR, which is not involved in the TR/APC compensation, is presented on the abscissa. In the uncompensated profile, there appear to be two populations of T cells, one bright, and one intermediate, for CD4. This duller population does not stain with CD4 at all, but is simply the CD8 T cells. Note that if the TR reagent had been a little brighter, the contour plots would show a single CD4 T cell population. When the TR reagents are eliminated (Fig. 49.4, bottom right), the CD8 positive T cells are readily apparent as the TCR^+ and $CD4^-$ population. With proper compensation, the correct FACS profiles are obtained in the presence or absence of the TR reagent. With undercompensation, once again it appears that there is a population of T cells which are dull for CD4.

Including stains with fewer reagents is an extremely effective way to build a multi-parameter FACS analysis protocol and evaluate artifacts. As seen in Figure 49.4, the TCR-CD4 plots are

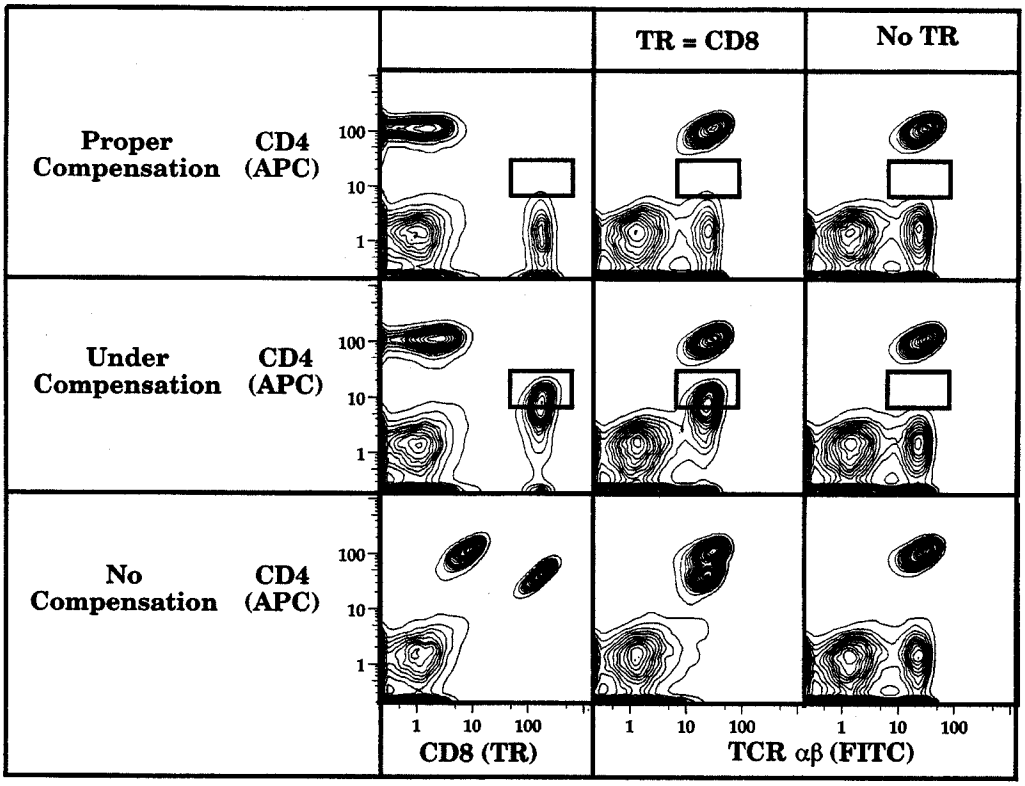


Fig. 49.4. *Consequences of improper compensation.* Spleen cells from a Balb/c mouse were stained with APC-anti CD4, FITC-anti-TCR$\alpha\beta$. For the profiles in the left and center column, biotin-anti-CD8 was also included and revealed with TR-Avidin. Proper compensation values for the spectral overlap were determined with Shiva software (M. Bigos, Stanford University) using cells stained with only one reagent. The proper values are 24.9% for the TR signal in the APC channel and 8.2% for the APC reagent. For the under-compensated profiles, both compensation values were reduced by 20%, to 19.9% and 6.6%, respectively. All plots are 5% probability contours.

all correct in the absence of the CD8 stain in the TR channel, regardless of the compensation value used (since compensating from a channel with no fluorescence has no effect). Compare and contrast the CD4 versus TCRab profile. The CD8 reagent is absent in the right column. With proper compensation, both stains give the correct result. With under-compensation, or even no compensation, the profile without the TR stain still gives the correct result. There is simply no TR signal to be detected in the APC channel. This example suggests an important point: it is best to build 3 or 4 parameter analysis protocols by adding one component at a time.

Interlaser compensation

In all of the cases described above, spectral overlap occurs only in channels which detect fluorescences excited by the same laser. However, compensation becomes more complicated from a practical standpoint when the dyes are excited by different lasers. For example, in the dual laser set-up described in chapter FACS-1 (488 and 600 nm excitation) a PE reagent can be excited by the dye laser and emit in the TR channel (in addition to the excitation by 488 nm, with orange emission). This effect is most visible when bright PE reagents are used. Current sorters do not allow for real-time interlaser compensation. Consequently, this crosstalk can limit the ability to accurately phenotype cells which are bright for a PE reagent and dull for a TR reagent. The problem can be reduced in two ways. First, the dye laser wavelength can be increased to about 606 nm to decrease the PE excitation, and thus decrease the PE signal in the TR channel. The optimal wavelength will depend on the specific PE preparation. Note, however, that no adjustment will help if there is residual phycocyanin in the preparation. The drawback to the wavelength change is that the TR signal itself is also decreased, although to a lesser extent. A second approach is to detect the lower density antigen with the PE reagent, and the higher density reagent with the TR reagent.

Experiment design

Selecting, preparing, and using fluorescent reagents

Designing a successful FACS experiment is only rarely accomplished without trial and error. Many important and complicated considerations have been discussed above. Here we summarize the important steps and provide some specific examples. Designing and completing a FACS experiment typically requires several smaller experiments. The process is an iterative one, which can even span several publications, in which initial experiments will often reveal the need for additional antibody-reagents, additional controls, altered staining combinations, and different protocols. It is always best to approach a novel FACS experiment by performing a small subset of the stains first and building upon the results, layer by layer, until the degree of desired complexity is attained.

1. Determine the samples to be analyzed. List all tissue types (peripheral blood, spleen, bone marrow, thymus, etc.) and all of

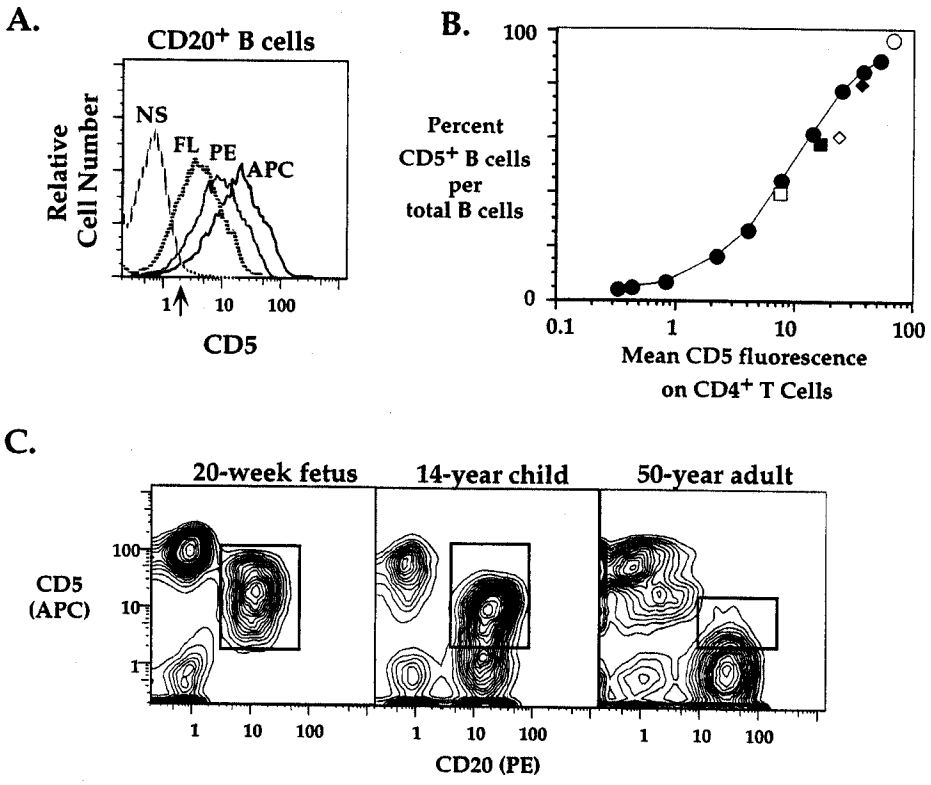


Fig. 49.5. *Better resolution of CD5 expression on B cells with an optimal APC-anti-CD5 reagent.* (**A**) Data are from multiple stainings of the same 20-week fetal spleen. Histograms of CD5 expression are shown after gating for B cells by CD20. The arrow defines the cutoff for $CD5^+$ B cells. The measured value of B cells which are $CD5^+$ 90% with APC-anti-CD5, 80% with PE-anti-CD5, and 62% with FL-anti-CD5. Gates are used such that <2% of the B cells are positive with CD20 alone in the FL channel (NS), or in other channels. Isotype matched controls labeled with APC or FL give the same background levels (<2%). (**B**) The calculated level of $CD5^+$ B cells depends on the anti-CD5 reagent. Data are from analysis of a single 21 weeks fetal spleen. Anti-CD20 (APC or FL) is used to identify all B cells; anti-CD4 (FL or PE) is used to identify a subset of T cells with the highest and most uniform CD5 expression (CD8 and γδ T cells are duller for CD5, and the ratio of CD4 to CD8 T cells varies among individuals). The anti-CD5 reagents are: (circles), two different APC conjugates; (diamonds), two different PE conjugates; (squares), two different fluorescein conjugates. The reagents used for the open symbols were also used in panel A. Points for the solid curve were generated by titrating unlabeled anti-CD5 ("cold competitor") in the presence of a constant, optimal amount of APC-anti-CD5. (**C**) In the fetal spleen nearly all of the B cells are $CD5^+$, whereas in the adult they are mostly $CD5^-$. At adolescence, there are approximately equal numbers of $CD5^+$ and $CD5^+$ B cells and two distinct populations can be observed. These plots have 5% probability contours.

the individual animals or patients that need to be analyzed, including any biological controls. The number of tubes in the experiment will be the product of the number of individuals, the number of tissue types, and the number of stains per tissue. It is important to have enough cells for the control stains (see #5, below). Consider grouping samples by tissue type, in order to maintain uniform sample handling over time.

2. Select the reagents to use. List all of the cell populations that need to be identified in each tissue and the antigens that are needed to reveal them. In particular, group together antigens that must be simultaneously measured. This will tell you the minimum number of colors that you will need to perform your experiment. Consider bringing all stains up to this number of colors (for instance, if one of your stains requires 4 colors, and two require 2 colors, then consider combining the latter two stains into one 4-color stain). It is likely that some reagents, such as pan-T Cell or pan-B cell markers, will be used in multiple stains. You may need to redesign the stain combinations depending on the availability of the reagents.

3. Determine the optimal antibody-fluorochrome combinations. For each combination of stains, assign each antibody to a fluorochrome that is being used in your experiment. During this process, you should consider two factors: antigen abundance and compensation requirements. In general, put brighter fluorochromes on the less abundant antigens. Fluorochrome brightness (which takes into account several factors including autofluorescence) can be loosely listed as follows (decreasing brightness) APC > Cy5-PE > PE ≫ TR > FITC ≫ Coumarins (for example, see Fig. 49.5). Indirect reagents are likely to be brighter than their direct counterparts (i.e., since TR is typically used as an avidin conjugate for biotinylated reagents, the TR reagents are often much brighter than FITC). To minimize potential problems with compensation, avoid combinations of a bright PE reagent and a dim TR reagent (see "Interlaser compensation" above). Also consider the use of a dump channel with PI to exclude dead cells and a cocktail of antibody reagents to excluded specific cells which are not of interest in a particular stain (See "Dump channel" above). Again, steps 2 and 3 will have to be repeated depending on reagent availability.

4. Prepare reagents. Buy, borrow, steal, or make the appropriate reagents. It is often useful to have the same antibody conjugated to more than one fluorochrome. As outlined in the section above

("Titration of antibody reagents"), *titrate each reagent* to determine its binding properties. This should be done for both "home-made" and commercial reagents. When possible, use reagents at saturating concentrations to ensure uniform staining intensities. The titration process will also allow you to determine the relative brightness of each reagent for your experiment. It is also good to confirm the specificity of the reagent and to evaluate the general level of non-specific binding. With some effort, it is possible to establish a highly effective repertoire of good reagents which can be used for many experiments.

5. Determine what controls are needed for each experiment. At a minimum, compensation controls must be included. These consist of samples stained with only one reagent at a time. Ideally half the cells should be positive and half should be negative. Stains for splenic or peripheral blood T or B cells are usually good. The brightest possible reagent for each fluorochrome should be selected for compensation. The reagent need not be one used in your study; however, it should be at least as bright as any in your study. A bright population can also by generated by combing two reagents which are specific for the same cells. For phycobiliprotein reagents (PE, APC, Cy5-PE), and especially for tandem dyes (Cy5-PE) you should have a conjugate made from the same lot of fluorophore as your reagent set. Note that some of these reagents may have different compensation values than others, so you should at some point test all your reagents for spectral overlap.

Other controls may be necessary for the staining protocol. These can include isotype controls (to estimate nonspecific binding), second-step controls (in multi-step stainings), etc. In general, these controls should be done for every reagent and every cell type; however, they do not necessarily have to be done in every experiment. Finally, control samples which are unstained can be useful. This can help identify problems with the compensation and reveal the background staining on a particular subset of cells.

6. Stain and analyze the samples. Try to use a uniform staining protocol to ensure reproducible staining. Use appropriate staining medium (such as deficient RPMI as described above); if possible, include azide and keep cells cold to prevent antibody sloughing or capping. When immunofluorescence staining is combined with other staining (for instance, for intracellular glutathione, calcium, or DNA (Chapters 53 and 54), or for apoptosis (Chapter 55)), you will need to determine whether the antibody staining should precede or follow the other procedural steps.

Collect the appropriate number of cells for the populations being analyzed, as described in Chapter 50. Typically 30,000 cell collections are suitable for three of four color analysis of lymphocyte populations; however, more cells should be collected for rare populations.

Analyze the data carefully *before* doing the next round of staining. This will tell you which aspects of items 1 to 5 above need to be changed. For example, Are the reagent combinations and controls appropriate? Do some populations need to be further phenotyped? Decide which new antibody reagents, antibody-reagent combinations, and controls need to be included in the analysis and proceed with another round.

Choosing reagent combinations

The choice of reagent combinations can effect the biological conclusions that are reached. Reagent choices are generally flexible when discrete, mutually exclusive, populations are being evaluated; however, they are crucial when different populations with a continuous expression of antigen, are being enumerated. For example, we found this to be the case in our the evaluation of the number of B-1 cells (CD5 B cells) present in human tissue during ontogeny [6].

Table 49-3. Potential stain combinations to quantitate B-1a cells

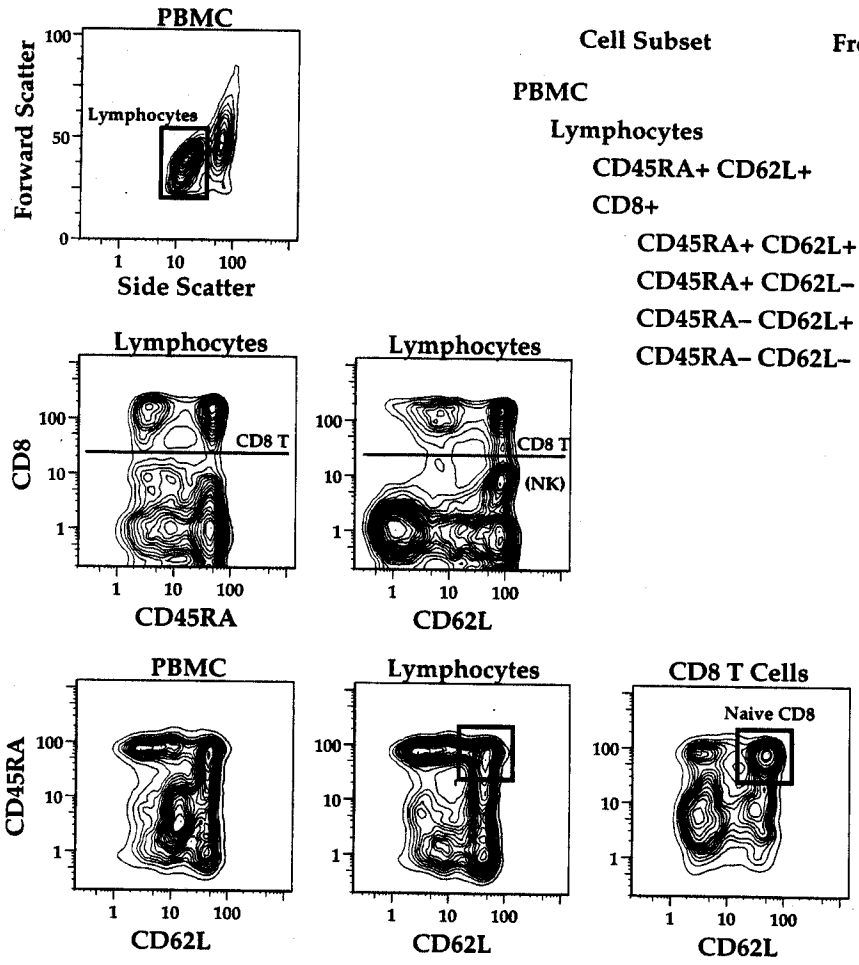
			Stain 2		
Channel	Conjugate	Stain 1	Tube 1	Tube 2	Stain 3
FITC	Direct	IgM	IgM	IgM	IgM
PE	Direct	PI	PI	PI	IgD
TR	Bi (+TR-Av)	IgD	PI	IgD	PI
APC	Direct	Ly-1	Ly-1	PI	Ly-1

Note: Propidium iodide fluorescence can be quantitated in the PE, TR, and APC channels. Since PI-positive cells are excluded from further analysis based on the channel that contains no reagents, the presence of PI fluorescence in channels used for antibody reagents is not a problem.

Example 1: Bright reagents reveal dull populations. There are marked differences in the perceived level of B cells ($CD20^+$) which are $CD5^+$ when different reagents are used (Fig. 49.5A). Our brightest reagent, an APC-anti-CD5, indicates that $\geq 90\%$ of the B cells in this fetal spleen are $CD5^+$. In comparison, fluorescein reagents (one of ours and one obtained from Becton Dickinson) tested on the same fetal spleen sample yields only 62% $CD5^+$ B cells. PE reagents give intermediate results. This increase is not due to non-specific staining. The entire B cell population in fetal spleen shifts as an ensemble to higher levels of CD5 with increasingly bright anti-CD5 reagents while the background staining on non-specific cells (non T, non B cells) does not increase, suggesting one $CD5^+$ B cell population in the fetal tissue. In contrast, the majority of B cells in adult spleen does not shift above the autofluorescence level with the same increasingly bright anti-CD5 reagents. During adolescence, when the number of $CD5^+$ and $CD5^-$ B cells is about equal, two relatively distinct populations can be observed with the probability contour plots (Fig. 49.5C).

The dependence of the calculated percent of $CD5^+$ B cells on reagent brightness is further emphasized (Fig. 49.5B). CD4 T cells, which are bright and uniform for CD5 expression, provide an excellent internal measure for the anti-CD5 reagent brightness. A well-defined curve is generated by titrating unlabeled anti-CD5 in the presence of a standard, optimal amount of APC-anti-CD5. APC-isotype and unstained controls are quite clean in the APC channel, with $<2\%$ of the $CD20^+$ cells appearing to be $CD5^+$. Other anti-CD5 reagents, including those labeled with PE and FL, also fall on this curve. In our hands, APC-anti-CD5 reagents are generally better than PE reagents, which in turn are better than FL-conjugated reagents. Imperfect stains deviate from the curve. For example, the data point designated with an open circle was from a slightly sticky APC-anti-CD5 reagent which also binds nonspecifically to non-T and non-B cells. Similar deviations are seen if the reagent is used in great excess, or if aggregates are not removed from the reagent prior to staining.

Example 2: Not all reagent combinations are equal. In addition to brightness, difficulties with compensation can often give misleading results when a single cell population of interest is being revealed by a pair of reagents with overlapping spectra. This is particularly problematic when populations with a continuous expression of antigen from negative to positive are being enumerated. Even with proper compensation, there is a broadening of the



Cell Subset	Frequency (%)
PBMC	100.0
Lymphocytes	65.2
CD45RA+ CD62L+	15.3
CD8+	12.3
CD45RA+ CD62L+	4.1
CD45RA+ CD62L−	2.6
CD45RA− CD62L+	4.5
CD45RA− CD62L−	1.3

Fig. 49.6. *Multiparameter FACS is required to uniquely identify some cell subsets.* Human PBMC were stained to resolve T cell subsets, with the goal of uniquely enumerating CD8 naive T cells. We have shown that this particular population of cells disappears progressively in HIV-infected adults and children [8, 9] In this example, a three-color combination consisting of fluorescein-CD62L (L-selectin), PE-CD45RA, and Cy5-PE-CD8. For each plot, the gating used to display the events in that plot is indicated above the plot. Cell subset frequencies are given in the table on the top right. (**Top**) Forward and side scatter signals for all events (all PBMC). The lower-scatter population consists primarily of lymphocytes; the higher side-scatter population is primarily monocytes. (**Middle**) CD8 T cells are the brightly CD8-staining population. NK cells express CD8 at lower levels, and can be clearly distinguished on the right plot as CD62L+. Both CD62L and CD45RA divide CD8 cells into two subsets each. However, it is impossible using only these two colors to know whether the CD45RA− cells are also CD62L−, if they are CD62L+, or if they are split by CD62L as well. In other words, using two two-color stains as shown here, we can identify that there are at least 2 and up to 4 different CD8 subsets that can be identified with these markers. (**Bottom**) On the right, we can see that in fact there are actually at least four subsets of CD8 T cells identified by the CD45RA and CD62L markers. Naive T cells can be uniquely identified by the co-expression of CD45RA and CD62L; all other CD8 subsets are memory. The same gate identifying naive T cells is shown on the middle plot (all lymphocytes) for comparison. (**Table**) Frequencies of subsets within PBMC are shown for a selection of phenotypes. Note that the (typical) frequency of naive CD8 T cells in healthy adults is approximately 4 to 10% of PBMC (4.1% in this example). These plots have 5% probability contours.

signal in the opposite channel because the difference of two measurements, each with their own CV, is used to obtain the final value.

Sometimes it is possible to choose reagents combinations which avoid potential problems. As an example, consider three potential stains for enumerating murine Ly-1 B cells (B-1a cells) in the peritoneal cavity, using a two laser (488 + 600 nm), 4 fluorescence channel flow cytometer (see Table 49.3, PI is propidium iodide, which discriminates live from dead cells). Two stains require one tube each; stain #2 requires two tubes.

Three surface antigens (IgM, IgD, and Ly-1/CD5) are expressed at different levels on different B cell populations [7]. Ly-1 is bright on T cells, dull on B-1a cells, and negative on B-2 (conventional B) cells; IgD is bright on B-2 cells and dull on B-1a cells. The discrimination of the Ly-1-dull B-1a cells from other B cells requires some care. As with the example for human CD5 B cells above, APC is the fluorochome of choice for CD5 because it is very bright and there is low autofluorescence in the channel used to measure it. With respect to compensation, Stain 1 is the most difficult to execute correctly, because it uses a reagent (TR-Av/Bi-IgD) which is bright on the cells that are not of interest (B-2 cells). Therefore, either slightly incorrect TR-APC compensation, or the mere broadening of the compensated signal as discussed above, will lead to incorrect quantitation of dully-positive cells (B-1a) in the other channel (APC-Ly-1). Consequently, for this stain it is paramount to set the compensation correctly: preferably with the reagents being used for the analysis,

and with computer software to determine the exact settings. Under-compensation of the TR-Av/Bi-IgD stain will over-count B-1a cells, and over-compensation will under-count these cells.

These potential problems can be avoided or at least evaluated by omitting the TR IgD reagent (Stain 2, tube 1). This will yield an accurate IgM versus Ly-1 profile, but does not give any information on IgD levels. A separate stain could be used for IgM-IgD (Stain 2, tube 2), but this does not allow for the simultaneous analysis of the three surface antigens. The best solution is to change the IgD reagent from bi/TR-AV to PE (Stain 3), since full phenotype information is still available, and the resulting B1-a/B2 quantitation is not sensitive to the TR/APC compensation.

Example 3: The power of multiparameter analysis. Recently, we identified a population of CD8 T cells that progressively declines during the progression of HIV disease [8, 9]. The disappearance of these naive CD8 T cells may play a central role in immunodeficiency. Figure 49.6 demonstrates how these cells can be phenotyped, showing not only the power but also the necessity of multiparameter analysis.

Indeed, the loss of the naive CD8 T cells had been missed by several researchers. This was primarily because only two-color combinations of markers were used to try to quantitate these cells. As shown in Figure 49.6, neither CD45RA nor CD62L alone can uniquely identify these cells. Also, using CD45RA and CD62L together is not sufficient, since many non-CD8 T cells coexpress these markers.

In general, the information content of a multiparameter experiment will increase geometrically with the number of independent parameters. In fact, as the example in Figure 49.6 shows, it may be impossible to quantitate certain cell subpopulations unless at least three immunofluorescence colors (together with scatter measurements for size) are made simultaneously. Examples of requirements of four colors are already in the literature [10, 11] and undoubtedly even more colors will be required in the future.

However, there are other advantages to using as many simultaneous markers as possible. It reduces the number of samples that need be analyzed. There is no information lost by using different fluorochromes on each of three antibodies in one stain, and analyzing each color as if it were a one-color experiment, compared to using the same fluorochrome on each antibody in three different tubes. This reduction in sample number leads to several benefits: less sample preparation time, less reagent expenditure (if counter-stains are being used), less instrument time, and less sample requirement. The latter can be especially important for precious samples, such as blood from infants; the others translate into economic benefit. Using simultaneous markers also gives the ability to explore the possibility that antigen expression is not independent; that is, that there are subsets of cells that express unique combinations of these markers.

Disadvantages of using increased color combinations must also be considered. First of all is the increased complexity in instrument set-up. Compensation must be appropriately set, and calibration is somewhat more complex when multiple lasers are involved. Indeed, inappropriate compensation can lead to fallacious conclusions, as demonstrated in Figure 49.4.

It should be noted, however, that data analysis is *not* necessarily more complex with multiple colors. At the simplest level, each color would be treated as an independent stain, thus, the other stains can be ignored and the analysis is no more complex than a single sample with that stain. Of course, the data analysis is necessarily much more complex when dependent stains are selected (as in Fig. 49.6), but impossible without the additional colors.

Acknowledgments

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and with computer software to determine the exact settings. Under-compensation of the TR-Av/Bi-IgD stain will over-count B-1a cells, and over-compensation will under-count these cells.

These potential problems can be avoided or at least evaluated by omitting the TR IgD reagent (Stain 2, tube 1). This will yield an accurate IgM versus Ly-1 profile, but does not give any information on IgD levels. A separate stain could be used for IgM-IgD (Stain 2, tube 2), but this does not allow for the simultaneous analysis of the three surface antigens. The best solution is to change the IgD reagent from bi/TR-AV to PE (Stain 3), since full phenotype information is still available, and the resulting B1-a/B2 quantitation is not sensitive to the TR/APC compensation.

Example 3: The power of multiparameter analysis. Recently, we identified a population of CD8 T cells that progressively declines during the progression of HIV disease [8, 9]. The disappearance of these naive CD8 T cells may play a central role in immunodeficiency. Figure 49.6 demonstrates how these cells can be phenotyped, showing not only the power but also the necessity of multiparameter analysis.

Indeed, the loss of the naive CD8 T cells had been missed by several researchers. This was primarily because only two-color combinations of markers were used to try to quantitate these cells. As shown in Figure 49.6, neither CD45RA nor CD62L alone can uniquely identify these cells. Also, using CD45RA and CD62L together is not sufficient, since many non-CD8 T cells coexpress these markers.

In general, the information content of a multiparameter experiment will increase geometrically with the number of independent parameters. In fact, as the example in Figure 49.6 shows, it may be impossible to quantitate certain cell subpopulations unless at least three immunofluorescence colors (together with scatter measurements for size) are made simultaneously. Examples of requirements of four colors are already in the literature [10, 11] and undoubtedly even more colors will be required in the future.

However, there are other advantages to using as many simultaneous markers as possible. It reduces the number of samples that need be analyzed. There is no information lost by using different fluorochromes on each of three antibodies in one stain, and analyzing each color as if it were a one-color experiment, compared to using the same fluorochrome on each antibody in three different tubes. This reduction in sample number leads to several benefits: less sample preparation time, less reagent expenditure (if counter-stains are being used), less instrument time, and less sample requirement. The latter can be especially important for precious samples, such as blood from infants; the others translate into economic benefit. Using simultaneous markers also gives the ability to explore the possibility that antigen expression is not independent; that is, that there are subsets of cells that express unique combinations of these markers.

Disadvantages of using increased color combinations must also be considered. First of all is the increased complexity in instrument set-up. Compensation must be appropriately set, and calibration is somewhat more complex when multiple lasers are involved. Indeed, inappropriate compensation can lead to fallacious conclusions, as demonstrated in Figure 49.4.

It should be noted, however, that data analysis is *not* necessarily more complex with multiple colors. At the simplest level, each color would be treated as an independent stain, thus, the other stains can be ignored and the analysis is no more complex than a single sample with that stain. Of course, the data analysis is necessarily much more complex when dependent stains are selected (as in Fig. 49.6), but impossible without the additional colors.

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