Immunophenotyping

There are four basic methods for staining cells with antibodies for immunophenotyping by flow cytometry. The first method (see Basic Protocol 1) is an indirect one that employs a primary unconjugated monoclonal antibody followed by a secondary, fluorochrome-conjugated polyclonal antibody. Because the target antibody is not conjugated with a fluorochrome in this method, a second fluorochrome-conjugated polyclonal antibody—derived from a different animal species and directed against the IgG from the species that generated the first antibody—is used. An intact polyclonal antibody preparation should never be used for immunophenotyping; the fluorochrome-conjugated $F(ab')_2$ fragment should always be used (see Critical Parameters).

The second method (see Alternate Protocol 1) is also an indirect method, in this case employing a hapten-conjugated primary antibody followed by a fluorochrome-conjugated polyclonal antibody against the hapten. Examples of haptens are digoxigenin, di- or trinitrophenol, and sometimes biotin. A variation on this method included in Alternate Protocol 1 employs a biotinylated antibody followed by a fluorochrome-conjugated streptavidin. The fourth method (see Alternate Protocol 2), which is the method of choice, employs a directly conjugated monoclonal antibody against the desired antigen.

Combinations of the above methods are also described for two-color staining (see Basic Protocol 2 and Alternate Protocols 3 and 4) as well as three- and four-color staining (see Alternate Protocols 5 to 9). The ethidium monoazide (EMA) procedure for detecting nonviable cells in a cell population is also included (see Support Protocol 1). Finally, analysis of the data acquired from flow cytometry using cells stained by the above procedures is detailed (see Support Protocols 2, 3, and 4).

BASIC INDIRECT STAINING

BASIC PROTOCOL 1

This is the most traditional method of staining. It uses unconjugated primary antibody followed by fluorochrome-conjugated secondary antibody. The most commonly used fluorochromes are fluorocein (FITC), phycoerythrin (PE), PE-Cy5 tandem complex, and PerCP. The PE—Texas red tandem complex is utilized to a lesser extent, mainly because of the lack of availability of directly conjugated reagents. Cy5 and allophycocyanin are likely to become important fluorochromes with the availability of dual laser excitation. Antibodies are available from several suppliers—e.g., Becton Dickinson Immunocytometry, Caltag Labs, Coulter, Gen Trak, Pharmingen, Sigma, and R & D Systems (see *SUPPLIERS APPENDIX*).

Materials

3 mg/ml normal goat IgG
$5–10 \times 10^6$ cell/ml target cell suspension in PBS (*APPENDIX 2A*)
Unconjugated monoclonal antibody against cell-surface antigen of interest, appropriately titered (*UNIT 4.1*)
Isotype control: myeloma protein of appropriate isotype
Erythrocyte-lysing solution (see recipe), 4°C
Fluorochrome-conjugated polyclonal goat anti–mouse IgG $F(ab')_2$, appropriately titered
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
2% formaldehyde (see recipe)

Centrifuge and rotor capable of $2000 \times g$, refrigerated

Contributed by C.C. Stewart and S.J. Stewart
Current Protocols in Cytometry (1997) 6.2.1-6.2.18

1. Add 67 µl of 3 mg/ml goat IgG per ml of target cell suspension (200 µg/ml IgG final). Incubate 10 min in an ice bath.

   See Critical Parameters for additional discussion of incubation temperature.

   The IgG is added to block all Fc receptors and prevent nonspecific binding; see Critical Parameters.

2. Add the appropriate amount of unconjugated monoclonal antibody to a fresh tube, then add 50 µl of blocked cell suspension from step 1. Prepare an isotype control tube and a blank tube with no added reagents (just cells) in the same manner.
3. Incubate 15 min in an ice bath, then add 3 ml of 4°C erythrocyte-lysing solution.
4. Centrifuge 3 min at 1500 × *g*, 4°C. Decant the supernatant and resuspend cells in the residual solution.
5. Add the appropriate amount of fluorochrome-conjugated goat anti–mouse IgG $F(ab')_2$. Incubate cells 15 min in an ice bath, then add 3 ml PBS.
6. Centrifuge 3 min at 1500 × *g*, 4°C. Decant the supernatant and resuspend cells in the residual solution
7. Add 200 µl of 2% formaldehyde and incubate ≥1 hr at room temperature before acquiring data.

   See Critical Parameters for additional discussion of fixation time.

ALTERNATE PROTOCOL 1

INDIRECT STAINING USING BIOTINYLATED OR HAPTEN-CONJUGATED ANTIBODY

This technique should be used when only biotinylated or hapten-conjugated antibodies are available.

Proceed as in the basic indirect staining procedure (see Basic Protocol 1) with the following modifications at steps 1, 2, and 5.

Additional Materials *(also see Basic Protocol 1)*

3 mg/ml normal mouse IgG
Biotinylated or hapten-conjugated monoclonal antibody against cell-surface antigen of interest, appropriately titered (*UNIT 4.1*)
Isotype control: biotinylated or hapten-conjugated myeloma protein
Fluorochrome-conjugated goat anti-hapten $F(ab')_2$ *or* fluorochrome-conjugated streptavidin, appropriately titered

1a. Use 3 mg/ml normal mouse IgG in place of the normal goat IgG.

2a. Use biotinylated or hapten-conjugated monoclonal antibody in place of the unconjugated monoclonal antibody. Use biotinylated or hapten-conjugated monoclonal antibody against irrelevant antigen for isotype control.

5a. Use fluorochrome-conjugated goat anti-hapten $F(ab')_2$ or fluorochrome-conjugated streptavidin in place of the fluorochrome-conjugated goat anti–mouse IgG $F(ab')_2$.

ALTERNATE PROTOCOL 2

DIRECT STAINING PROCEDURE

Directly conjugated antibodies offer faster staining and are the most convenient tools in clinical immunology, as the mixtures of antibodies can be subjected to a high level of quality control. These directly conjugated antibodies are available with all the fluorochromes from the suppliers named in Basic Protocol 1.

Proceed as in the basic indirect staining procedure (see Basic Protocol 1) with the following modifications at steps 1, 2, and 5.

Additional Materials *(also see Basic Protocol 1)*

3 mg/ml normal mouse IgG
Fluorochrome-conjugated monoclonal antibody against cell-surface antigen of interest, appropriately titered (*UNIT 4.1*)
Isotype control: fluorochrome-conjugated myeloma protein

1a. Use 3 mg/ml normal mouse IgG in place of the normal goat IgG.

2a. Use fluorochrome-conjugated monoclonal antibody in place of the unconjugated monoclonal antibody. Use fluorochrome-conjugated myeloma protein for isotype control.

5a. Omit step 5 of Basic Protocol 1 (addition of secondary antibody); also omit subsequent centrifugation (step 6). Proceed directly with formaldehyde fixation (step 7) using cells suspended in the residual erythrocyte-lysing solution (see step 4).

As a monoclonal antibody directly conjugated to fluorochrome is used, no labeled secondary antibody is needed.

TWO-COLOR IMMUNOPHENOTYPING USING UNCONJUGATED PRIMARY ANTIBODY/FLUOROCHROME-CONJUGATED SECONDARY ANTIBODY IN COMBINATION WITH BIOTINYLATED ANTIBODY

BASIC PROTOCOL 2

The four basic procedures for staining cells with antibody (i.e., indirect using unconjugated primary antibody and fluorochrome-conjugated secondary antibody, indirect using biotinylated antibody, indirect using hapten-conjugated antibody, and direct using fluorochrome-conjugated monoclonal antibody) can be combined in various ways to produce simultaneous evaluation of multiple antigens. This protocol combines the first two of these indirect staining procedures. There are two general rules that must be adhered to for obtaining valid results with this protocol. First, the unconjugated primary antibody/fluorochrome-conjugated secondary antibody-labeling step must be performed separately and before labeling with the next procedure. Second, when the first labeling reaction is complete but before addition of subsequent antibodies, blocking (see Critical Parameters) must be performed with IgG from the species from which the unconjugated antibody was derived (e.g., mouse IgG for mouse antibodies).

Materials

3 mg/ml normal goat IgG
$5–10 \times 10^6$ cell/ml target cell suspension
Unconjugated monoclonal antibody against cell-surface antigen of interest, appropriately titered (*UNIT 4.1*)
Isotype control: unconjugated myeloma protein
Erythrocyte-lysing solution (see recipe)
Fluorochrome-conjugated polyclonal goat anti–mouse IgG $F(ab')_2$, appropriately titered
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
3 mg/ml normal mouse IgG
Biotinylated monoclonal antibody against a second cell-surface antigen of interest, appropriately titered (*UNIT 4.1*)
Isotype control: biotinylated myeloma protein
Fluorochrome-conjugated streptavidin, of concentration such that appropriate quantity is contained in 10 µl
2% formaldehyde (see recipe)

Centrifuge and rotor capable of $2000 \times g$, refrigerated

Label with unconjugated primary antibody and fluorochrome-conjugated secondary antibody

1. Add 67 µl of 3 mg/ml goat IgG per ml of target cell suspension (200 µg/ml IgG final). Incubate 10 min in an ice bath.

   See Critical Parameters for additional discussion of incubation temperature.

   The IgG is added to block all Fc receptors and prevent nonspecific binding; see Critical Parameters.

2. Add the appropriate amount of unconjugated monoclonal antibody to a fresh tube, then add 50 µl of blocked cell suspension (from step 1). Prepare an isotype control tube and a blank tube with no added reagents (just cells) in the same manner.

3. Incubate 15 min in an ice bath, then add 3 ml erythrocyte-lysing solution.

4. Centrifuge 3 min at 1500 × *g*, 4°C. Decant the supernatant and resuspend cells in the residual solution.

5. Add the appropriate amount of fluorochrome-conjugated goat anti–mouse IgG $F(ab')_2$, Incubate 15 min in an ice bath, then add 3 ml PBS.

6. Centrifuge 3 min at 1500 × *g*, 4°C. Decant the supernatant and resuspend cells in the residual solution.

Label with biotinylated antibody and fluorochrome-conjugated streptavidin

7. Add 10 µl of 3 mg/ml mouse IgG to each tube. Incubate 10 min in an ice bath.

8. Add the appropriate amount of biotinylated monoclonal antibody (or biotinylated isotype control). Incubate 15 min in an ice bath, then add 3 ml erythrocyte-lysing solution.

9. Centrifuge 3 min at 1500 × *g*, 4°C. Decant the supernatant and resuspend cells in the residual solution.

10. Add 10 µl fluorochrome-conjugated streptavidin. Incubate 15 min in an ice bath, then add 3 ml PBS.

11. Centrifuge 3 min at 1500 × *g*, 4°C. Decant the supernatant and resuspend the cells in the residual solution.

12. Add 200 µl of 2% formaldehyde and incubate at least ≥1 hr at room temperature before acquiring data.

    See Critical Parameters for additional discussion of fixation time.

ALTERNATE PROTOCOL 3

TWO-COLOR IMMUNOPHENOTYPING USING BIOTINYLATED ANTIBODY IN COMBINATION WITH HAPTEN-CONJUGATED ANTIBODY

This modification of Basic Protocol 2 provides a method for staining cells with a biotinylated and hapten-conjugated antibodies. One fluorochrome is conjugated to the biotin while the second antibody to the hapten(s) is conjugated with the other fluorochrome(s). While hapten-conjugated antibodies have been used in research, they have not had general use in clinical immunophenotyping. Because many different haptens could be used, each with their specific fluorochrome-conjugated second antibody, they present a potential strategy for creating complex multicolor combinations of primary antibodies, such as FITC, PE, PE–Texas red, and PE-Cy5 or PerCP. For two-laser excitation, the PE–Texas red is replaced by using either APC or Cy5.

Additional Materials *(also see Basic Protocol 2)*

3 mg/ml normal mouse IgG
Biotinylated monoclonal antibody against one cell-surface antigen of interest and hapten-conjugated monoclonal antibody against a second antigen, appropriately titered (*UNIT 4.1*)
Isotype control: biotinylated and hapten-conjugated myeloma protein
Fluorochrome-conjugated streptavidin, of concentration such that appropriate quantity is contained in 10 µl
Fluorochrome-conjugated monoclonal antibody, of concentration such that appropriate quantity is contained in 10 µl

Proceed as in the basic protocol for two-color immunophenotyping (see Basic Protocol 2) with the following modifications at steps 1, 2, and 5.

1a. Use 3 mg/ml normal mouse IgG in place of the normal goat IgG.

2a. In place of the unconjugated monoclonal antibody, use a combination of appropriate quantities of a biotinylated monoclonal antibody against one antigen of interest and a hapten-conjugated antibody against a second antigen. Prepare an isotype control tube and a blank tube with no reagents (just cells) in the same manner.

5a. Use 20 µl of a combination of fluorochrome-conjugated streptavidin and fluorochrome-conjugated anti-hapten monoclonal antibody in place of the fluorochrome-conjugated goat anti–mouse IgG $F(ab')_2$. Continue with subsequent centrifugation and resuspension (step 6), but omit additional treatment with biotinylated antibody and streptavidin (steps 7 to 11). Proceed directly with formaldehyde fixation (step 12) using cells suspended in residual PBS from step 6.

TWO-COLOR IMMUNOPHENOTYPING USING BIOTINYLATED ANTIBODY IN COMBINATION WITH DIRECTLY CONJUGATED ANTIBODY

ALTERNATE PROTOCOL 4

Sometimes the desired fluorochrome-conjugated antibody is not available but a biotinylated one is. This situation is especially apparent when a PE–Texas red tandem is desired. By combining the biotinylated antibody with the directly conjugated ones, complex multicolor cocktails can be produced.

Additional Materials *(also see Basic Protocol 2)*

3 mg/ml normal mouse IgG
Biotinylated monoclonal antibody against one cell-surface antigen of interest and fluorochrome-conjugated monoclonal antibody against a second antigen, appropriately titered (*UNIT 4.1*)
Isotype control: biotinylated and directly conjugated myeloma proteins
Fluorochrome-conjugated streptavidin, of concentration such that appropriate quantity is contained in 10 µl

Proceed as in the basic protocol for two-color immunophenotyping (see Basic Protocol 2) with the following modifications at steps 1, 2, and 5.

1a. Use 3 mg/ml normal mouse IgG in place of the normal goat IgG.

2a. In place of the unconjugated monoclonal antibody, use a combination of appropriate quantities of a biotinylated monoclonal antibody against one antigen of interest and a fluorochrome-conjugated monoclonal antibody against a second antigen. Prepare an isotype control tube and a blank tube with no reagents (just cells) in the same manner.

5a. Use 10 µl of appropriately titered fluorochrome-conjugated streptavidin in place of the fluorochrome-conjugated goat anti–mouse IgG $F(ab')_2$. Continue with sub-

sequent centrifugation and resuspension (step 6), but omit additional treatment with biotinylated antibody and streptavidin (steps 7 to 11). Proceed directly with formaldehyde fixation (step 12) using cells suspended in residual PBS from step 6.

ALTERNATE PROTOCOL 5

THREE-OR FOUR-COLOR IMMUNOPHENOTYPING USING UNCONJUGATED ANTIBODY, BIOTINYLATED ANTIBODY, AND DIRECTLY CONJUGATED ANTIBODY

Staining with three, four, or more colors can be accomplished by putting the antibody and second reagent steps together appropriately. To do this, one must always stain cells first with an unconjugated primary antibody and second fluorochrome-conjugated antibody. This is the only step in which a primary antibody cannot be combined with the others.

The two most extensively used three-color combinations are FITC, PE, and PE-Cy5 tandem complexes or PerCP. The fourth color can be a PE–Texas red complex for single-laser excitation. The APC or Cy5 fluorochrome is used for the fourth color when a second laser operating at 635 nm is used. For example, PE-Cy5 second antibody could be used with the unconjugated primary antibody, a PE–Texas red–streptavidin could be used with the biotinylated antibody, and primary antibodies directly conjugated with FITC and PE could be used to complete the four-color staining paradigm.

Additional Materials *(also see Basic Protocol 2)*

One or two fluorochrome-conjugated antibodies against cell-surface antigens of interest, appropriately titered (*UNIT 4.1*)

Proceed as in the basic protocol for two-color immunophenotyping (see Basic Protocol 2) with the following modification at step 8.

8a. Add appropriate amount of one or two fluorochrome-conjugated monoclonal antibodies along with the biotinylated monoclonal antibody.

ALTERNATE PROTOCOL 6

THREE- OR FOUR-COLOR IMMUNOPHENOTYPING USING BIOTINYLATED, HAPTEN-CONJUGATED, AND DIRECTLY CONJUGATED ANTIBODIES

Whenever possible, it is better not to use an unconjugated primary antibody with a second fluorochrome-conjugated antibody. This is because two extra steps are required, and if blocking is not properly done, the conjugated antibodies added subsequently can bind to the second fluorochrome-conjugated antibody leading to artifactual data. Biotinylated, hapten-conjugated, and directly conjugated primary antibodies can be combined into cocktails as described in *UNIT 4.1*. The fluorochrome-conjugated streptavidin and anti-hapten second antibody can also be combined into a cocktail to produce a more efficient and flexible staining procedure.

Additional Materials *(also see Basic Protocol 2)*

3 mg/ml normal mouse IgG

Hapten-conjugated antibody against cell-surface antigen of interest, appropriately titered (*UNIT 4.1*)

One or two fluorochrome-conjugated monoclonal antibodies against cell-surface antigens of interest, appropriately titered (*UNIT 4.1*)

Isotype control: biotinylated, hapten-conjugated, and directly conjugated myeloma proteins

Fluorochrome-conjugated streptavidin, of concentration such that appropriate quantity is contained in 10 µl

Fluorochrome-conjugated anti-hapten monoclonal antibody, of concentration such that appropriate quantity is contained in 10 µl

Proceed as in the basic protocol for two-color immunophenotyping (see Basic Protocol 2) with the following modifications at steps 1, 2 and 5.

1a. Use 3 mg/ml normal mouse IgG in place of the normal goat IgG.

2a. In place of the unconjugated monoclonal antibody, use a combination of appropriate quantities of biotinylated, hapten-conjugated, and one or two directly conjugated monoclonal antibodies. Prepare an isotype control tube and a blank tube with no reagents (just cells) in the same manner.

5a. Use 20 µl of a combination of fluorochrome-conjugated streptavidin and fluorochrome-conjugated anti-hapten monoclonal antibody in place of the fluorochrome-conjugated goat anti-mouse IgG $F(ab')_2$. Continue with subsequent centrifugation and resuspension (step 6), but omit additional treatment with biotinylated antibody and streptavidin (steps 7 to 11). Proceed directly with formaldehyde fixation (step 12) using cells suspended in residual PBS from step 6.

THREE- OR FOUR-COLOR IMMUNOPHENOTYPING USING BIOTINYLATED ANTIBODY AND DIRECTLY CONJUGATED ANTIBODIES

ALTERNATE PROTOCOL 7

Another strategy for multicolor immunophenotyping is to use a biotinylated primary antibody in combination with two or three directly conjugated antibodies. This procedure is most relevant because of greater availability for biotinylated reagents. All primary antibodies can be combined into a cocktail (see *UNIT 4.1*).

Additional Materials *(also see Basic Protocol 2)*

3 mg/ml normal mouse IgG
Biotinylated monoclonal antibody against cell-surface antigen of interest, appropriately titered (*UNIT 4.1*)
Isotype control: biotinylated and fluorochrome-conjugated myeloma proteins
Two or three fluorochrome-conjugated monoclonal antibodies against cell-surface antigens of interest, appropriately titered (*UNIT 4.1*)

Proceed as in the basic protocol for two-color immunophenotyping (see Basic Protocol 2) with the following modifications at steps 1, 2 and 5.

1a. Use 3 mg/ml normal mouse IgG in place of the normal goat IgG.

2a. In place of the unconjugated monoclonal antibody, use a combination of appropriate quantities of biotinylated antibodies and two or three directly conjugated monoclonal antibodies. Prepare an isotype control tube and a blank tube with no reagents (just cells) in the same manner.

5a. Use 10 µl of fluorochrome-conjugated streptavidin in place of the fluorochrome-conjugated goat anti-mouse IgG $F(ab')_2$. Continue with subsequent centrifugation and resuspension (step 6), but omit additional treatment with biotinylated antibody and streptavidin (steps 7 to 11). Proceed directly with formaldehyde fixation (step 12) using cells suspended in residual PBS from step 6.

ALTERNATE PROTOCOL 8

THREE- OR FOUR-COLOR IMMUNOPHENOTYPING USING UNCONJUGATED PRIMARY ANTIBODY AND BIOTINYLATED SECONDARY ANTIBODY IN COMBINATION WITH DIRECTLY CONJUGATED ANTIBODIES

Another strategy is to use a primary antibody followed by a biotinylated second antibody that is then colored with a fluorochrome-conjugated streptavidin. When applying this strategy, stain cells with the unconjugated primary antibody first, and follow this with the biotinylated second antibody. After blocking with normal mouse IgG, a cocktail containing all the other directly conjugated antibodies and the fluorochrome-conjugated streptavidin can then be added to finish the staining.

Additional Materials *(also see Basic Protocol 2)*

Biotinylated polyclonal goat anti–mouse IgG $F(ab')_2$ (Caltag Labs)
Isotype control: unconjugated and fluorochrome-conjugated myeloma proteins
Fluorochrome-conjugated streptavidin, of concentration such that appropriate quantity is contained in 10 µl
Two or three fluorochrome-conjugated monoclonal antibodies against cell-surface antigens of interest, appropriately titered (*UNIT 4.1*)

Proceed as in the basic protocol for two-color immunophenotyping (see Basic Protocol 2) with the following modifications at steps 5, 8, and 10.

5a. Use biotinylated goat anti–mouse IgG $F(ab')_2$ in place of the fluorochrome-conjugated goat anti–mouse IgG $F(ab')_2$. Prepare an isotype control tube and a blank tube with no reagents (just cells) in the same manner.

8a. Add 10 µl fluorochrome-conjugated streptavidin along with a combination of two or three fluorochrome-conjugated monoclonal antibodies in place of the biotinylated monoclonal antibody.

9a. Omit step 10 of Basic Protocol 2 and subsequent centrifugation and resuspension (step 11). Proceed directly with formaldehyde fixation (step 12) using cells suspended in residual PBS (step 9).

ALTERNATE PROTOCOL 9

THREE- OR FOUR-COLOR IMMUNOPHENOTYPING USING A COMBINATION OF DIRECTLY CONJUGATED ANTIBODIES

The easiest, most efficient, and least troublesome procedure is to use all directly conjugated antibodies. Except for the PE–Texas red reagents, just about everything is available from one supplier or another. In addition, if a reagent is not available with the desired fluorochrome, it can be obtained as a custom conjugate from Becton Dickinson Immunocytometry, Caltag Labs, or Molecular Probes. In some instances, it may be necessary to supply them with the purified antibody.

Additional Materials *(also see Basic Protocol 1)*

3 mg/ml normal mouse IgG
Fluorochrome-conjugated monoclonal antibodies against cell-surface antigens of interest, appropriately titered (*UNIT 4.1*)
Isotype control: fluorochrome-conjugated myeloma proteins

Proceed as in the basic indirect staining procedure (see Basic Protocol 1) with the following modifications to steps 1, 2, and 5.

1a. Use 3 mg/ml normal mouse IgG in place of the normal goat IgG.

2a. Use a combination of fluorochrome-conjugated monoclonal antibodies in place of the unconjugated monoclonal antibody. Prepare an isotype control tube and a blank tube with no reagents (just cells) in the same manner.

5a. Omit step 5 of Basic Protocol 1 (addition of secondary antibody); also omit subsequent centrifugation (step 6). Proceed directly with formaldehyde fixation (step 7) using cells suspended in the residual erythrocyte-lysing solution (see step 4).

As monoclonal antibodies directly conjugated to fluorochromes are used, no labeled secondary reagents are needed.

EMA PROCEDURE FOR DETECTING NONVIABLE CELLS IN A CELL POPULATION TO BE FIXED

SUPPORT PROTOCOL 1

Dead cells bind antibodies nonspecifically. Each antibody, however, binds dead cells to a different extent—which could lead to misinterpretation. It is important, therefore, to minimize dead cells in the analysis window. Ethidium monoazide (EMA) stains the dead cells, making them much brighter than the viable cells.

EMA staining is done on a separate aliquot of the same cell suspension used in any of the staining procedures in this unit. The EMA tubes are analyzed on the flow cytometer along with the other immunophenotyping panels as described in Support Protocol 4 (also see Critical Parameters).

For additional discussion of the purpose of EMA staining and the difference between EMA and other stains for nonviable cells (e.g., propidium iodide), see Background Information.

Materials

Cell suspension for analysis
Erythrocyte-lysing solution (optional; see recipe)
EMA working solution (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
2% formaldehyde (see recipe)

12 × 75–mm test tubes
Centrifuge and rotor capable of 2000 × *g*
Fluorescent desk lamp
White pan

1. Add 50 µl of cell suspension to a 12 × 75 mm test tube. If necessary, add 3.5 ml of erythrocyte-lysing solution to lyse erythrocytes.
2. Centrifuge 3 min at 1500 × *g*, 4° to 25°C. Decant the supernatant and resuspend cells in the residual solution.
3. Add 5 µl EMA working solution.

   Determine optimal concentration for working solution for each batch of EMA.

4. Lay tube(s) flat in a white pan 20 cm beneath a fluorescent desk lamp at room temperature and irradiate for 10 min with fluorescent light. At end of irradiation, add 3.5 ml PBS.
5. Centrifuge 3 min at 1500 × *g*, 4° to 25°C. Decant the supernatant and resuspend cells in the residual solution.
6. Add 0.5 ml of 2% formaldehyde and incubate at least 1 hr before acquiring data.

DATA ANALYSIS FOR FLOW CYTOMETRIC IMMUNOPHENOTYPING

Two basic ways to analyze flow cytometry data are the marker approach (described in Support Protocol 2), in which a marker is placed on a histogram to designate positive and negative cells, and the template approach (described in the Commentary), in which a region is drawn circumscribing the geometric pattern created by the cell populations. The marker approach (see Support Protocol 2) is well suited for the measurement of one or two colors of fluorescence, but for three and four colors, the template approach (see Background Information) may be more meaningful.

SUPPORT PROTOCOL 2

Marker Approach Using Population Gate

This approach is illustrated using five-parameter data. There are two ways to address analysis with the marker approach. The first, described in this protocol, is to establish a "population" gate using FS versus SS. The second (see Support Protocol 3) is to establish a "cell" gate using SS versus cell-specific antibody. For both these approaches, the region R9 is used for this purpose. The procedure here is illustrated with human leukocytes. This protocol requires listmode files with the acquired data.

1. Display bivariate plots of all parameters:

   3 parameters = FS vs. SS and green fluorescence univariate histogram

   4 parameters = FS vs. SS and green fluorescence vs. orange fluorescence (see Fig. 6.2.1)

   5 parameters = FS vs. SS, green fluorescence vs. orange fluorescence, red fluorescence vs. orange fluorescence, and red fluorescence vs. green fluorescence (see Fig. 6.2.2 and Fig. 6.2.3)

2. To establish the desired population—e.g., lymphocytes—that express CD45 brightly, load the listmode file of cells stained with CD45 and CD14.

3. Refer to Figure 6.2.1A and draw a region (R9) to circumscribe the lymphocyte cluster and another region (R2) to circumscribe the granulocytes completely in the FS versus SS plot.

   This latter region is drawn in the FS versus SC display so that some granulocytes that overlap B cells that are less CD45-bright than T cells and NK cells can be excluded.

4. Draw a region R3 (Fig. 6.2.1B) around the CD45-bright–CD14-negative cells in that bivariate plot. Create the Boolean logical gate "R3 and NOT R2" and gate the FS versus SS display (Fig. 6.2.1C).

   The $CD45^+$ cells (i.e., all lymphocytes) and the $CD14^-$ cells (i.e., NOT monocytes) then exclude the granulocytes (R2) by scatter.

5. Gate the CD45 versus CD14 display using R9 (Fig. 6.2.1D). Create results and determine the percentage of lymphocytes (CD45-bright) inside the region R9 and their purity from the cells inside R3.

   The purpose of this step is to determine the purity and the percentage (or yield) of lymphocytes that are in R9.

6. Load the file containing the unstained cells (or an isotype control file; Fig. 6.2.2), gated on region R9 and adjust quadrant markers in the green fluorescence versus orange fluorescence and red fluorescence versus green fluorescence display so that <2% of the events are beyond the marker.

   The purpose of this step is to determine markers for positive and negative cells.

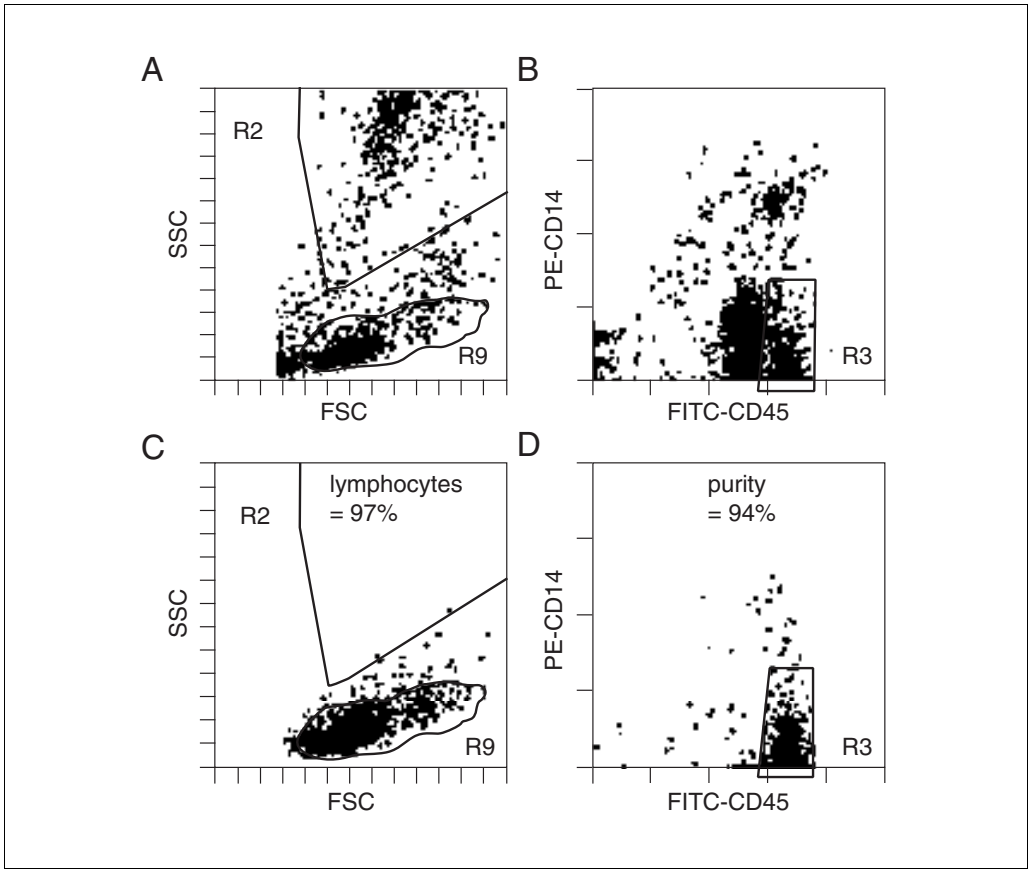

Figure 6.2.1 Bivariate plots for establishing a lymphocyte gate.

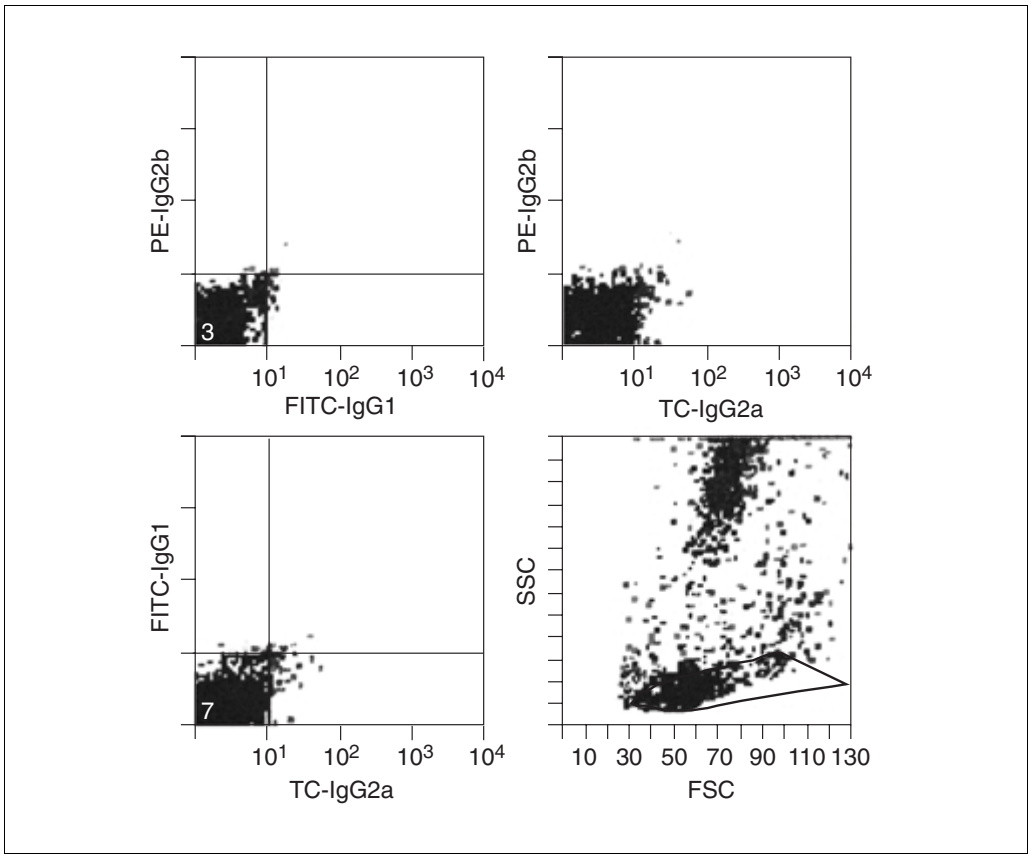

Figure 6.2.2 Isotype control bivariate displays. **See color figure.**

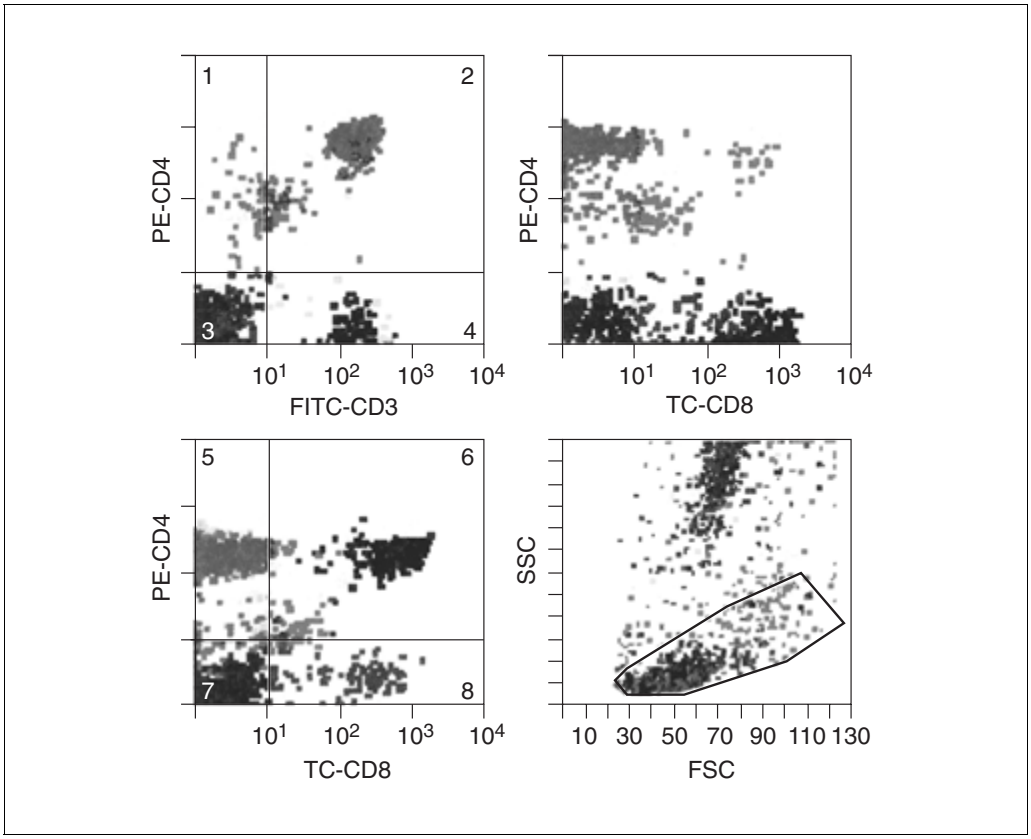

Figure 6.2.3 Bivariate displays of stained cells. **See color figure.**

7. Create the Boolean logical gates for all the combinations as shown in Table 6.2.1 and assign a color to each.

   The purpose of this step is to create the four (two-color) or eight (three-color) possible binary populations of positive and negative cells and to identify them by their color.

8. Load each file containing stained cells and record the results as shown in Figure 6.2.3 and Table 6.2.1.

   The purpose of this step is to analyze the acquired data.

SUPPORT PROTOCOL 3

Marker Approach Using Cell Gate

Instead of a population gate in FS versus SS, a cell gate can be used around cells stained with a specific antibody. This requires that the gating antibody be present in every tube. The gating antibody could be a lineage-specific one like CD3 for T cells, CD19 for B cells, or CD34 for hematopoietic progenitor cells. It might also be a combination like CD56/NOT CD3 for NK cells, or CD45 for all leukocytes.

1. Display bivariate plots of SS versus the gating antibody, FS versus SS, and antibody 1 versus antibody 2.

   For four-color data, the SS versus gating antibody, antibody 1 versus antibody 2, antibody 3 versus antibody 2, and antibody 1 versus antibody 3 are displayed as shown in Figure 6.2.4.

2. In the SS versus gating antibody view, draw region R9 and gate all other histograms on R9.

   This step provides the gate for the cells of interest.

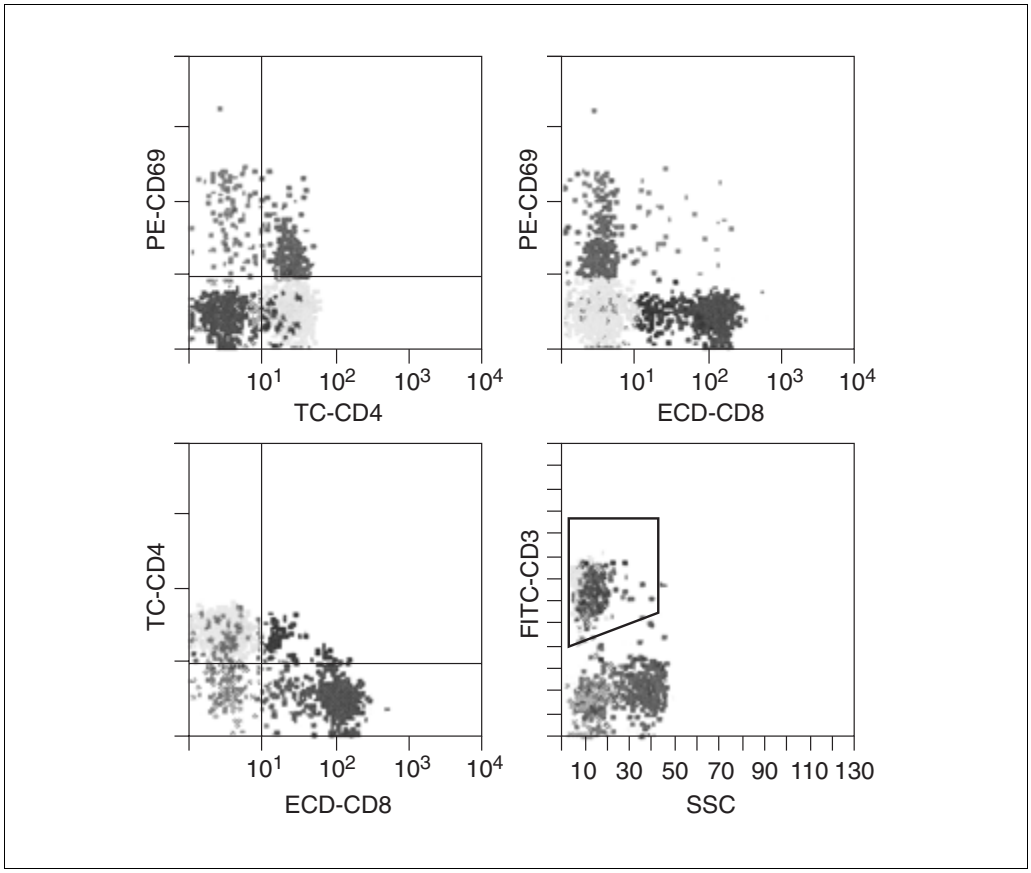

Figure 6.2.4 Four-color bivariate displays. **See color figure.**

3. Set quadrant markers using the file from cells stained with only the gating antibody, with or without an isotype control, so that <2% of events are beyond the marker.

   This step provides the markers to distinguish positive and negative cells,

4. Create the Boolean logical gates for all the combinations and assign a color to each, as shown in Table 6.2.1.

5. Load each file containing stained cells and record the results.

Table 6.2.1 Analysis of Data Using Quadrant Markers

No. of colors	Boolean expression	Cluster color	% positive cells in Figure 6.2.3
One color			
– – –	R3 and not R8	Black	1
+ – –	R4 and R5	Yellow	3
Two colors			
– + –	R1 and not R6	Cyan	2
+ + –	R2 and R5	Green	49
– – +	R8 and R3	Brown	6
Three colors			
+ – +	R6 and R4	Blue	17
– + +	R1 and R8	Violet	1
+ + +	R2 and R6	Red	4

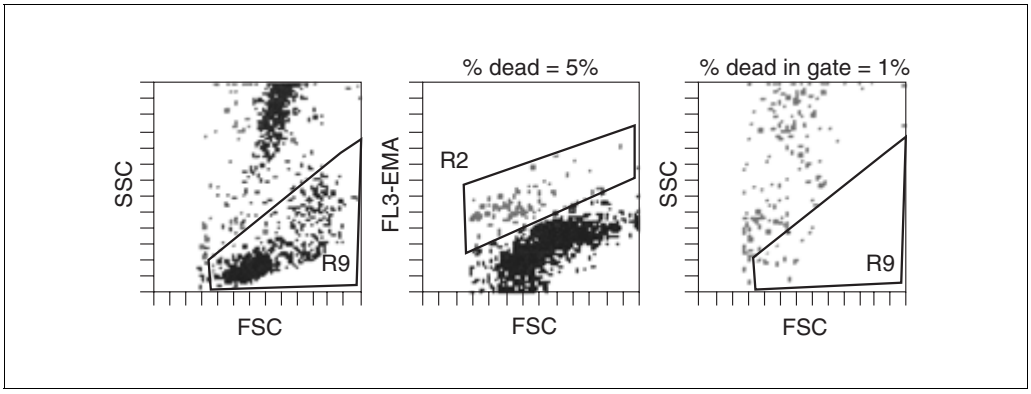

Figure 6.2.5 Determination of dead cells using EMA. **See color figure.**

SUPPORT PROTOCOL 4

Determining Viable Cells

Because EMA staining (Support Protocol 1) is done on a separate aliquot of cells (see Critical Parameters), the location of dead cells is determined in the FS versus SS plot so they can be gated out of the analysis. Refer to Figure 6.2.5.

1. Display FS versus SS and FS versus EMA fluorescence. Draw a region R2 around the dead cells.
2. Gate FS versus SS on R2. Draw a gating region R9 so that >95% of cells are viable.

   By gating the FS versus red fluorescence display on R9, the percent of dead cells in R9 can be determined.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A;*** *for suppliers, see* ***SUPPLIERS APPENDIX.***

EMA solution

Prepare a stock solution of 5 mg/ml ethidium monoazide (EMA; Molecular Probes) in PBS (*APPENDIX 2A*) and store up to 2 years at −20°C; wrap aluminum foil around the container to keep in total darkness. Prepare working dilution of 5 µg/ml biannually, divide into 50-µl aliquots in 0.5-ml microcentrifuge tubes, and store at 4°C (wrapped in aluminum foil). Open tubes one at a time as necessary.

CAUTION: *EMA is a DNA dye and should be handled using protective clothing and gloves. Never pipet by mouth.*

Erythrocyte-lysing solution

4.13 g ammonium chloride (154 mM final)
0.5 g potassium bicarbonate (10 mM final)
0.0185 g tetrasodium EDTA (0.082 mM final)
500 ml double-distilled H_2O

Formaldehyde, 2%

200 ml 10% formaldehyde (ultrapure, EM Grade; Polysciences)
800 ml Dulbecco's PBS (Life Technologies)

COMMENTARY

Background Information

Antibodies

The antibodies used as second antibodies or antibodies to haptens in these protocols are almost always polyclonal antibodies. These are derived from the serum of mammals that have been immunized with the immunoglobulin fraction of serum obtained from the animal species from which the first antibody was derived or with a hapten that has been conjugated to a large protein such as poly-L-lysine. Serum-derived polyclonal antibodies consist of IgM, all subclasses of IgG, and IgA. All of these antibodies will have specificities for the immunogen. Because pentameric IgM and quadrameric IgA are present, the $F(ab')_2$ fragments should always be used, as all antibodies will then be dimeric. This produces a uniform preparation with the lowest amount of undesired cell binding. Polyclonal antibodies are usually purified by passing the $F(ab')_2$ preparations over a column composed of protein A or protein G or anti-IgG directed against the animal's purified IgG light chains or heavy chains. The latter method is best because only those immunoglobulin molecules specific for the immunogen are recovered.

Detection of dead cells

Propidium iodide (PI) stains DNA and is commonly used to detect dead cells in a fixed preparation because it is excluded by viable cells with intact membranes. In flow cytometry, use of viable cells presents both a health hazard and the inconvenience of requiring immediate evaluation; therefore cells are frequently fixed. Unfortunately, when cells are fixed all cells stain with PI because they are no longer viable. PI stains reversibly, and will leak out of cells during fixation and spread through the whole preparation. This process also occurs with other dyes, including 7-aminoactinomycin D (7-AAD). Ethidium monoazide (EMA) provides a solution to this problem (Riedy et al., 1991). Like PI and 7-AAD, it stains the DNA of dead cells, but not viable ones. When stained cells are exposed to light, an azide group on the dye is photoactivated and covalently bonds to DNA and histones. After exposure, dye that has not been bound can be washed away and the dead cells are permanently stained. Fixation will not change the situation.

Data analysis

Software for the analysis of immunophenotyping data has evolved over the years as the number of antibodies combined together has increased. The fundamental approach is to set a marker to resolve antibody-positive cells from negative ones. The most primitive analysis strategy is to create a gate in the FS versus SS view and apply it to all files. Next, the antibody-fluorescence histogram is displayed. For a single antibody a univariate histogram is used. For antibodies in combination, bivariate histograms are displayed and quadrant markers are inserted. The position of markers is set using unstained cells or an isotype control. There is no rule for the percentage of events allowed above the marker, but usually it is <1% to 2%.

The number of bivariate histograms (BH) increases with the number of antibodies (n) according to the formula: $BH = (n-1) + (n-2) + \ldots + (n-n)$. The number of bivariate histograms that require quadrant markers is $(n-1)$. The number of binary populations that can be resolved by the quadrant markers expressed in terms of Boolean algebra is 2^n. Thus, 3 antibodies provide 8 populations, 4 provide 16, 5 provide 32, etc. Clearly the ability to visualize all of these populations becomes increasingly difficult (see *UNIT 10.4*).

As interest in measuring more parameters increases, new approaches to data analysis are required. One such approach is cell gating, where one antibody is used to resolve cells of interest, such as CD45 for lymphocytes, CD3 for T cells, CD19 for B cells, CD14 for monocytes, and CD34 for hematopoietic progenitor cells. Other antibody combinations—e.g., CD4/CD8 for T cells or anti-kappa/anti-lambda for B cells, are used to resolve the population subsets.

Another approach is to use a template composed of regions that define all the populations resolved by the antibody combination. This approach assumes that distinct clusters occur as well-resolved geometric shapes in the various bivariate views of multidimensional space. Thus, in one view a cluster may appear homogeneous, but a second view reveals two or more clusters. Regions linked by Boolean algebra are used to define all distinct clusters until all are homogeneous. Homogeneity does not imply any particular geometric shape, only that no further separation occurs and all kinds can be found.

The template approach can be automated using clustering algorithms. Although several

attempts have been made to apply these mathematical approaches, the results have not been promising, mainly because each file represents an entirely new experience for the algorithm. Applying neural networks or classification and regression trees provides the added dimension of experience for analysis of high-dimensional data.

Critical Parameters

Washing

Recently, several suppliers have introduced procedures with fluorochrome-conjugated antibodies that require only lysing erythrocytes but not washing the specimen. This may seem to be a good idea, even though it is really a bad one for several reasons. First, the supposition is fewer cells are lost because they are not washed away. In reality, positive cells are lost to detection because the nonspecific binding increases markedly for most antibodies. Although the stained cells do not change in brightness, the increased nonspecific binding and fluorescence in the cell stream causes a decreased signal-to-noise discrimination. When cells are washed, some may be discarded along with the supernatant fraction, but the solution to this problem is faster centrifugation ($1500 \times g$, rather than $300 \times g$) as recommended in this protocol. The higher speed does not increase cellular aggregation and no intact cells are found in the discarded wash supernatant.

Proponents of the lyse/no wash system also believe it saves time by eliminating washes. This is a false belief as well. Using the bare minimum of 1 ml lysing reagent results in a final volume >3× that of the washed cells (after resuspension in 2% formaldehyde)—data acquisition using the flow cytometer will also take >3× as long for the same number of cells. In addition, erythrocyte lysis is likely to be incomplete, leaving intact erythrocytes whose presence in gating regions will affect the calculations for percent positive cells.

Washing all samples is strongly recommended. There are several important reasons for this recommendation. First, it is desirable to remove unreacted antibody. Second, higher backgrounds will be evident without washing, which may result in dim antigen expression being missed.

Blocking

Intact antibodies, especially monoclonal antibodies, bind to any cell that has unoccupied Fc receptors. Even when the cells are derived from blood that contains all immunoglobulin isotypes, these receptors are not fully saturated because the immunoglobulin concentration in blood is nonsaturating. For this reason, serum should never be used for blocking in these protocols. It is necessary to add a higher concentration of IgG to block all receptors so that the antibody binds only to its epitope and not to Fc receptors as well. All hematopoietic cells have Fc receptors except T cells and erythrocytes.

Sequence of reactions

When a second conjugated polyclonal antibody is used, that staining reaction must always be performed first and the IgG block must be from the same animal species as the polyclonal antibody. It cannot be assumed that all Fab sites on the second antibody have bound epitopes on the primary. To the extent these are free binding sites, addition of another primary antibody can bind to these free sites rather than to its epitope. Thus, any cell that is positive for the unconjugated primary antibody can also be positive for all others, unless these free binding sites are blocked. This is done by adding purified IgG from the same species as the one from which the primary unconjugated antibody was derived. Failure to perform this procedure in the correct order will result in flawed data.

Temperature

The temperature of staining has not been indicated. Many protocols recommend room temperature (~22°C). The authors recommend placing the rack of tubes in an ice bath (4°C). Many cell types—e.g., myeloid cells—still function at room temperature, albeit more slowly, and can internalize bound antibodies.

Fixation

A minimum fixation time of 1 hr has been indicated in the various protocols. This is because known hazardous virus will be inactivated, making the samples safer to handle while acquiring data. The FS versus SS distribution of aldehyde-fixed cells changes markedly during the first 8 hr because the continuous cross-linking of proteins results in changes of cell shape and granularity. Because of this, the FS versus SS profile for granulocytes (and to a lesser extent for monocytes) that is obtained after 1 or 2 hr will look different from that obtained after longer fixation times. Lymphocytes are not affected because of their low granularity. A 12-hr fixation time is recommended for complete stabilization. This time

period is most convenient because it corresponds to an overnight fixation. Fixed suspensions should be evaluated within 5 days because autofluorescence will increase with longer storage times.

Dead cells

Dead cells bind antibodies nonspecifically, and their presence can lead to misinterpretation. In most instances the use of EMA enables the establishment of a gate to exclude nearly all dead cells based on an FS versus SS gate. The authors use the approach of defining a region on the desired population to optimize exclusion of dead cells so that the detector otherwise allocated to EMA can be used for another fluorochrome. If the gated region on the desired cell population contains >5% dead cells (i.e., <95% viable cells in the region), one can accept that the results of analysis may be flawed to the extent the result is caused by nonspecific antibody binding to dead cells. One can decrease the gate size to reduce the percentage of dead cells, even if it means decreasing the percentage of desired cells evaluated, or one can terminate evaluation of the data—bad data is worse than no data. EMA (like PI or 7-AAD) can be added as a third color after staining cells with FITC- and PE-conjugated antibodies.

Data analysis

For single-color analysis, a simple univariate histogram suffices because cells are either negative or positive for the antibody. When two antibodies are combined, four populations (see Table 6.2.1 and Fig. 6.2.1) can be resolved. For three-antibody combinations there are eight populations; but because two bivariate views are needed to resolve their combination, Boolean expressions are required and a color must be assigned to them so each cell population can be visualized in all the bivariate views.

The two regions R9 and R2 are drawn in the FS versus SS plot for determining lymphocyte purity because region R3 must be extended somewhat into the space occupied by granulocytes (CD45-dim) if all B cells are to be included. Some CD45 monoclonal antibodies do not resolve B cells, which are dimmer than T cells and NK cells, from the brighter CD45-dim granulocytes. Because these granulocytes are excluded by forward scatter gating on R9, they also need to be excluded when backgating on R3. This is accomplished using the Boolean combination "R3 and NOT R2." In this way the percentage of lymphocytes in the gate R9 can be determined. The cells in R3, when gated on R9, contain the purity of the lymphocyte gate. In the example shown in Figure 6.2.1, 97% of lymphocytes—the cells in R3 that are inside R9—are counted, with a purity of 94% for cells in R9 that are also in R3. These percentages should be >90% for lymphocytes. Large lymphocytes, which are most important in disease, are frequently excluded from the analysis. Note that the large cells with low SS to the right of the dense cluster have been included in R9 because the lymphocyte gate (R9) circumscribes only the dense lymphocyte cluster. These cells are very important and should always be included even though they often reside under the monocyte cluster.

R9, rather than R1, is used as the gate region because most analysis software applications assign colors in a hierarchical fashion. If R1 were the gate, it would not be possible to assign colors to the various cell populations resolved by the Boolean logical gates shown in Table 6.2.1.

It is desirable to use multiparameter software that allows at least nine regions, as shown in Figures 6.2.1 and 6.2.2 and Table 6.2.1. Regions 1 to 8 are used for the quadrant markers and regions 9 and higher are used for gating.

Some software applications attach a color based on a Venn spectrum to the gate logic, in a hierarchical order instead of allowing user-selected colors for each population. In viewing multiparameter data, this software restriction is often undesirable because the color mixtures can duplicate the background color and the population will completely disappear from the view. The hierarchical nature associated with gating means the gate must always be last. Because of hierarchical color assignment, if the gate region is first, it will not be possible to assign colors to any other region. Thus, it must be last so that its color does not dominate. Color assignments for quadrant markers combined by Boolean equations must be assigned by the user and not the software.

Anticipated Results

The most important single potential problem with results stems from the quality of the antibody. Refer to *UNIT 4.1* for a more complete discussion of these problems. The objective of these protocols is to resolve epitope-positive cells from negative ones. To do so, there must be enough epitopes, and the antibody's fluorochrome must be bright enough for the investigator's instrument to measure. One may not be able to do anything about the number of epitopes but the fluorochrome and the instrument

are within the investigator's power. Often, stream-in-air detection is less sensitive than stream-in-liquid (flow-cell cuvette) detection. Thus, the instrument plays a role in sensitivity. Because PE-containing fluorochromes are five to seven times brighter than the others, a strategy to use them for conjugation to antibodies that will be used to detect the cells with the least number of epitopes can markedly improve results. Finally, Fc-receptor binding, dead cells, and nonspecific binding affect both the result and its interpretation. Fc-receptor binding can be eliminated by appropriate blocking; dead cells can often, but not always be removed from analysis by appropriate gating. Nonspecific binding problems can be reduced by washing.

When a cell population is poorly resolved by one antibody, it can often be well resolved by combining it with a second to pull the population away from other cells. Even if both antibodies are directed toward antigens in low abundance, their combined effect can be rewarding. This is one of the best reasons for performing multicolor flow cytometry.

Investigators have been taught to expect that immunophenotyping by flow cytometry will resolve positive and negative cells. Clearly, if one desires to have a CD4 count or a CD34 count, this is what to expect. But the pathologist does not expect this result when using immunohistochemistry to identify cells in a tissue. When cell populations are to be identified, it is possible to combine antibodies that resolve different cells and their pattern in bivariate plots can be used to identify them. In the case of pathology, their pattern is the expected result, and deviations from the known pattern may indicate abnormal processes such as malignancy.

Time Considerations

One should always set up the necessary tubes and reagents prior to getting cells. If 96 tubes (one full standard rack) are used as an example, this preparation should take ~30 min. The time to prepare cells depends on where they come from; nevertheless, they should be obtained just before they are to be stained, whenever possible. Counting, washing, and blocking takes at most 15 min. Adding cells to the antibody cocktails takes another 5 min. For each block of steps, 20 min is required. Thus—in a procedure where staining with an unconjugated antibody (20 min) and subsequent staining with secondary antibody (20 min) is combined with a block (10 min) prior to adding a biotinylated antibody combined with directly conjugated antibodies (20 min) and finally adding fluorochrome-conjugated antibody or antibodies (20 min)—up to 90 min may be necessary for staining. For acquiring data on the instrument, the authors usually require 1 min per tube. This will vary depending on cell concentration and the number of events acquired. It is difficult to evaluate analysis time, because this depends on how much prior knowledge of the samples is available. If it is a routine evaluation where the analysis protocol is known, it may require only 1 to 2 min per sample. Thus, 96 samples can take as little as 4 hr to process from start to finish.

Literature Cited

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Key References

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Provides additional information on instrument setup, data acquisition, and data analysis.

Contributed by C.C. Stewart and S.J. Stewart
Roswell Park Cancer Institute
Buffalo, New York