

Preparation of Cells and Reagents for Flow Cytometry

Flow cytometry is a widely used method for analyzing the expression of cell surface and intracellular molecules (on a per cell basis), characterizing and defining different cell types in heterogeneous populations, assessing the purity of isolated subpopulations, and analyzing cell size and volume. This technique is predominantly used to measure fluorescence intensity produced by fluorescent-labeled antibodies or ligands that bind to specific cell-associated molecules.

A procedure for direct and indirect staining of single-cell suspensions of lymphoid tissue or peripheral blood lymphocytes (PBL) to detect cell surface membrane antigens is presented (see Basic Protocol 1). In addition, four support protocols present methods for fluorescence labeling of purified antibodies (see Support Protocols 1 to 4).

A protocol for flow cytometric analysis of intracellular antigens in single-cell suspensions (e.g., mononuclear cells from human or murine peripheral blood or bone marrow, lymphoid cell suspensions, cells grown in suspension cultures, or dissociated tissues; see Basic Protocol 2) is also included. The procedure outlined in Basic Protocol 2 involves successive steps of fixation, membrane permeabilization, staining with directly labeled or unlabeled antibody, and washing to exchange solutions and remove excess unbound antibody. Alternate Protocol 1 describes intracellular staining of unfixed cells in the presence of a detergent. Finally, Alternate Protocol 2 describes staining nonviable cells to facilitate discrimination of dead cells in fixed or permeabilized cell preparations.

Accurate quantitative results on each staining experiment depend on the correct determination of the reactivity of the antibody with the intracellular antigen. This requires optimization of antibody staining by titration of the reagents and the use of appropriate staining controls. The Commentary presents a discussion of these topics (see Critical Parameters).

IMMUNOFLUORESCENCE STAINING OF SINGLE-CELL SUSPENSIONS FOR DETECTION OF SURFACE ANTIGENS

BASIC PROTOCOL 1

Immunofluorescence staining for flow cytometric analysis involves making a single-cell suspension from lymphoid tissues or peripheral blood, successive binding steps in which cells are incubated in tubes or microtiter plates with unlabeled or fluorescent-labeled antibodies or ligands, and wash steps using excess buffer to remove unbound antibodies. Support protocols describe the preparation of conjugated antibodies, while choice of label is discussed in Critical Parameters.

A successful staining procedure is dependent on optimization of experimental conditions through titering of reagents and use of appropriate controls. The Commentary presents a thorough discussion of these strategies (see Critical Parameters).

Materials

- Sample material: lymphoid tissue (*UNIT 3.1*) or single-cell suspension of human peripheral blood (*UNIT 7.1*)
- Staining buffer (see recipe), 4°C
- Labeled or unlabeled antibody (see Support Protocols 1 to 4) diluted to the appropriate concentration as determined by titering (see Critical Parameters)
- Propidium iodide (PI) solution (optional; see recipe)
- Fixation solution (optional; prepare immediately before use; see recipe)
- 12 × 15-mm round-bottom test tubes *or* 96-well round-bottom microtiter plates

Immuno- fluorescence and Cell Sorting

100- μ m nylon mesh (optional)
Sorvall RT-6000B with H-1000B rotor (or equivalent)

Additional reagents and equipment for trypan blue exclusion (*APPENDIX 3B*)

Prepare cells for antibody staining

1. Disrupt sample material in a small petri dish containing 5 ml staining buffer, 4°C.
Tissue may be disrupted by teasing with fine forceps, by mashing between two frosted microscope slides, or by mashing with a ground glass stopper.
2. Remove cell clumps and debris by allowing them to settle 2 to 3 min in a 12 \times 15-mm round-bottom test tube before transferring the supernatant to a second tube, or by passing the suspension through 100- μ m nylon mesh into a test tube.
3. Centrifuge cell suspension 8 min in a low-speed centrifuge at 300 \times g (e.g., 1000 rpm in a H-1000B rotor), 4°C, and discard supernatant. Resuspend cell pellet in 10 ml staining buffer, 4°C.
4. Determine viable cell count by trypan blue exclusion (*APPENDIX 3B*).

Stain cells with antibody

5. Centrifuge cell suspension (from step 3) 8 min at 300 \times g, 4°C, and discard supernatant. Resuspend cell pellet to 2 \times 10⁷ cells/ml in staining buffer, 4°C.
If using the biotin-avidin staining system, be certain that the staining medium contains no FBS (0.1% BSA is an appropriate substitute) or biotin.
6. Add 50 μ l cell suspension (10⁶ cells) to 12 \times 75-mm round-bottom test tubes or the wells of a 96-well round-bottom microtiter plate.
7. Add 10 μ l appropriately diluted, labeled antibody to each tube or well containing cells and mix gently. Incubate 20 min in an ice bath.

The same method is used for staining in microtiter plates as in test tubes, except as noted in step 10. There is less chance of spillover between samples when using test tubes. Different times or temperatures of incubation are sometimes required for antibodies that bind at low affinity.

Wash cells in preparation for flow cytometry

8. Wash cells by adding 2 ml staining buffer, 4°C.
9. Centrifuge cell suspension 6 min at 300 \times g, 4°C. Discard supernatant by aspiration or rapid inversion of the tubes.
10. Repeat wash steps 8 and 9 one time.

If microtiter plates are used for staining, wash cells three to five times with 100 μ l staining buffer, 4°C each time. Centrifuge plates 3 min at 500 \times g (1500 rpm in H-1000B), 4°C and discard supernatants as above.

*For detecting unlabeled primary antibodies with labeled second antibodies, or for use of the biotin-avidin system and/or multicolor staining, add each reagent separately and repeat the incubation and wash steps with the addition of each antibody. For multicolor analyses, and in cases where no antibody interactions are possible, two reagents (i.e., two primary antibodies) can be added simultaneously (see *Critical Parameters*).*

11. Resuspend stained cell pellets in 400 μ l of 4°C staining buffer. Keep cell suspension on ice until analyzed by flow cytometry.

*The cell suspension can also be treated to detect dead cells by adding 10 μ l propidium iodide solution to each tube prior to analysis. It is occasionally necessary to fix cells before analysis (see *Critical Parameters*). Cells can be fixed by resuspending pellet in fixation solution. Fixed cells can be stored for \leq 1 week at 4°C.*

IMMUNOFLUORESCENCE STAINING OF FIXED AND PERMEABILIZED SINGLE-CELL SUSPENSIONS FOR DETECTION OF INTRACELLULAR ANTIGENS

BASIC
PROTOCOL 2

This method of preparing cells for intracellular staining retains the scatter characteristics of cells on the flow cytometer, preserves surface immunofluorescence, and allows accurate measurement of cellular DNA content by yielding good coefficients of variation on DNA histograms. It is therefore particularly suited for multiparameter flow cytometric analysis. The technique can be performed with low cell numbers (i.e., as few as 2.5×10^5 cells) because cell loss is minimal.

In addition, as discussed in the Commentary (see Critical Parameters) the protocol can be easily modified to facilitate the detection of intracellular antigens which differ in their sensitivity to denaturation or in the retention of the antigen-antibody complex after permeabilization of the cell. The degree of fixation can be varied by increasing the concentration of paraformaldehyde in the fixation solution. Tween 20 can be replaced with an alternate detergent if the antigenic protein is more resistant to extraction from a permeabilized cell.

For simultaneous analysis of cell-surface and internal antigens, cells that have been stained for expression of cell-surface antigens can also be stained for expression of intracellular antigens using this protocol. Fluorochrome selection and appropriate antibody combinations are discussed in the Commentary (see Critical Parameters). Do not use peridinin chlorophyll protein (PerCP) –labeled antibodies for cell-surface staining, as PerCP fluorescence is lost after permeabilization.

Materials

Cell sample: mononuclear cells derived from human or murine peripheral blood, bone marrow, thymus or spleen; cells grown in suspension cultures; or dissociated tissues (UNIT 3.1)

PBS (APPENDIX 2A) without Ca^{2+} and Mg^{2+} , 4°C

Fixation solution (see recipe), 4°C

Permeabilization solution (see recipe)

Fluorochrome-labeled, biotin-labeled, or unlabeled antibody (see Support Protocols 1 to 4 or use commercial supplier) appropriately diluted in staining buffer (see recipe)

Washing buffer (see recipe)

Second-step fluorochrome-labeled antibody or avidin/streptavidin

PBS containing 1 mg/ml propidium iodide (PI) or 7-aminoactinomycin D (7-AAD; optional; see recipes for PI and 7-AAD stocks)

12 × 15–mm round-bottom test tubes

Sorvall H-1000B rotor or equivalent

62- μm nylon mesh (Small Parts; optional)

1. Place $\sim 10^6$ cells into a 12 × 15–mm test tube and add 1 to 2 ml PBS without Ca^{2+} or Mg^{2+} . Centrifuge 5 min at $300 \times g$ (e.g., 1000 rpm in a H-1000B rotor), 4°C.

The ability to discriminate between dead and live cells by their light-scatter patterns is often lost in fixed or permeabilized cell preparations; therefore, if considerable numbers of dead cells are present, they should be removed by Ficoll-Hypaque separation prior to fixation and permeabilization. Alternatively, dead cells can be identified by staining with a fluorescent dye and exclusion by flow cytometric analysis (see Alternate Protocol 2).

2. Remove supernatant by aspiration or rapid decanting and discard. Add 875 μl cold PBS to cell pellet and mix gently. Add 125 μl cold fixation solution and mix again. Incubate 1 hr at 4°C.

Immuno-
fluorescence and
Cell Sorting

5.3.3

A 1-hr incubation gives optimal light-scatter discrimination between different cell types (e.g., between lymphocytes and monocytes) on the flow cytometer. The incubation time may be shortened to 30 min for cell preparations that contain only one cell type. As discussed in the Commentary (see Critical Parameters and also see Troubleshooting), different paraformaldehyde fixative concentrations and incubation temperatures may be needed for optimal retention of a particular intracellular antigen.

3. Centrifuge cell suspension 5 min at $300 \times g$, 4°C . Remove supernatant by aspiration or rapid decanting and add 1 ml permeabilization solution to the cell pellet. Mix gently and incubate 15 min at 37°C .

4. Add 1 ml PBS. Centrifuge cells 5 min at $300 \times g$, 4°C . Remove supernatant by aspiration or rapid decanting.

A cell pellet may not be visible after the fixation step because fixed cells aggregate less well and therefore tend to spread out at the bottom of the tube.

5. *For unlabeled, fluorochrome-, or biotin-labeled antibodies:* Add 100 μl of the appropriate working dilution of antibody in staining buffer to cell pellet. Mix well. Incubate 30 min at 4°C . Add 1 ml washing buffer and centrifuge 5 min at $300 \times g$, 4°C . Remove supernatant and wash pellet again with 1 ml washing buffer. Centrifuge 5 min at $300 \times g$, 4°C .

If cells have been stained with a fluorochrome-labeled primary antibody, proceed directly to step 7a or 7b, as needed.

A blocking step involving preincubating cells 1 min with 50 μl heat-inactivated human AB serum or normal mouse serum before adding the antibody dilution may reduce nonspecific binding. Human AB serum cannot be used, however, if simultaneous analysis of cell-surface immunoglobulin chains is to be performed. Longer incubation times and higher staining temperatures (e.g., 22° to 25°C or 37°C) may be required for some antibodies, for instance for IgM antibodies that will cross cell membranes less effectively and will diffuse more slowly inside the cell compared to IgG antibodies. See Critical Parameters and see Troubleshooting for further discussion.

6. *To stain cells again after unlabeled or biotin-labeled first antibodies have been used:* Repeat step 5 using second-step fluorochrome-labeled polyclonal antibody or avidin/streptavidin.

Labeled avidin/streptavidin or $F(ab')_2$ fragments are the reagents of choice for intracellular second-step staining because they eliminate the possibility of Fc binding.

- 7a. *If simultaneous analysis of DNA content will not be performed:* Resuspend cells at a concentration of $1\text{--}2 \times 10^6$ cells/ml in staining buffer. Filter samples through 62- μm nylon mesh to remove clumps, if necessary. Analyze samples on flow cytometer within 2 hr of staining. Keep samples at 4°C , protected from light, until flow cytometric analysis.

Cells that have been fixed and permeabilized will have altered light-scatter properties compared to untreated cells—i.e., usually less forward scatter signal on the flow cytometer. For best results, analyze the cells on the flow cytometer as soon as possible. Samples can be held longer, depending on the cell type and the retention of the antigen-antibody complex. For extended storage (e.g., >16 hr) resuspend cells in 1% paraformaldehyde to prevent deterioration.

- 7b. *If simultaneous measurement of intracellular antibody staining and DNA content is to be performed:* Resuspend cells at a concentration of $1\text{--}2 \times 10^6$ cells/ml in 20 $\mu\text{g}/\text{ml}$ 7-AAD or 10 $\mu\text{g}/\text{ml}$ PI in PBS. Incubate at least 30 min at 4°C . Filter samples through nylon mesh to remove clumps, if necessary. Analyze samples on flow cytometer

within 2 hr of staining. Keep samples at 4°C, protected from light, until flow cytometric analysis.

PI is used in combination with fluorescein isothiocyanate (FITC)-labeled antibodies; 7-AAD can be used with FITC-labeled antibodies and must be used with phycoerythrin (PE)-labeled antibodies (see Critical Parameters).

LABELING ANTIBODY WITH FLUORESCHEIN ISOTHIOCYANATE (FITC)

SUPPORT PROTOCOL 1

Conjugation of fluorescein isothiocyanate (FITC) to purified antibody is an extremely valuable technique for identifying surface molecules using either fluorescence microscopy or flow cytometry. In the procedure that follows, the amino groups of the antibody molecule are coupled with fluorescein derivatives. Following removal of unbound FITC, the fluorochrome/antibody ratio is determined and the labeled antibody is used in the basic (see Basic Protocols 1 and 2) and alternate protocols (see Alternate Protocols 1 and 2).

Materials

- 1 to 2 mg/ml purified monoclonal antibody (*UNITS 2.4 & 2.5*)
- FITC labeling buffer, 4°C (see recipe; prepare ≤ 2 weeks before use)
- 5 mg/ml FITC in anhydrous dimethylsulfoxide (DMSO)
- Dialysis buffer, 4°C (see recipe)
- Sephadex G-25 column (Pharmacia Biotech PD-10; optional)
- Additional reagents and equipment for dialysis (*APPENDIX 3H*)

Conjugate FITC and antibody

1. Dialyze purified monoclonal antibody against 500 ml FITC labeling buffer at 4°C with two to three changes over 2 days (*APPENDIX 3H*).

This step removes free NH_4^+ ions and raises the pH to 9.2. Generally, up to 5 ml of 1 to 2 mg/ml antibody can be dialyzed against 500 ml buffer.

2. Determine antibody concentration based upon A_{280} .

Concentration of antibody (mg/ml) = $A_{280} \times 0.74 \times \text{dilution factor}$.

3. Add 20 μl of 5 mg/ml FITC in anhydrous DMSO for each milligram of antibody. Incubate 2 hr at room temperature.

Both the dye and organic solvent must be anhydrous; prepare FITC/DMSO solution immediately before use.

4. Remove unbound FITC by dialysis in 500 ml dialysis buffer at 4°C with two to three changes over 2 days. Alternatively, filter on a Sephadex G-25 column.

Determine FITC/antibody ratio

5. Dilute FITC-IgG complex with dialysis buffer so that A_{280} is < 2.0 .
6. Determine and record A_{280} and A_{492} .
7. Calculate protein concentration:

$$\text{protein (mg/ml)} = \frac{A_{280} - (A_{492} \times 0.35)}{1.4}$$

where 1.4 is the reciprocal of the FITC-conjugated antibody molar coefficient.

8. Calculate moles of protein:

$$\text{protein (moles)} = \frac{\text{protein (mg/ml)}}{1.5 \times 10^5}$$

$$\text{FITC (moles)} = \frac{A_{492}}{0.69 \times 10^5}$$

where $1.5 \times 10^5 =$ mol. wt. Ig and $0.69 \times 10^5 =$ mol. wt. FITC.

9. Determine fluorochrome/protein (F/P) ratio:

$$\text{F/P} = \frac{\text{moles of FITC}}{\text{moles of protein}}$$

An F/P ratio of 5 to 6:1 is usually optimal for flow cytometry.

SUPPORT PROTOCOL 2

LABELING ANTIBODY USING LONG-ARMED BIOTIN

Biotin is a naturally occurring vitamin with a molecular weight of 244 Da. It has an extremely strong affinity for avidin ($K_d = 10^{-15}$ M); thus, biotin-labeled antibodies can be detected using commercially available avidin coupled to fluorochromes. Because the binding of biotin or the subsequent binding of avidin may induce changes in protein structure, many companies now supply biotin containing a spacer between the protein-binding site and the avidin-binding site (sometimes known as long-armed or spacer biotin). Biotin can also be easily coupled to antibodies via a hydroxysuccinimide ester, usually without disturbing the biological properties of the antibody.

Follow the method for FITC conjugation (see Support Protocol 1), substituting the following reagents and steps as indicated.

Additional Materials (also see Support Protocol 1)

Succinimide ester labeling buffer (see recipe)

10 mg/ml long-armed biotin (Zymed) in anhydrous dimethylsulfoxide (DMSO)

1. Dialyze (*APPENDIX 3H*) 1 to 2 mg/ml purified antibody as for FITC labeling (see Support Protocol 1), using succinimide ester labeling buffer instead of FITC labeling buffer.
2. Determine protein concentration based upon A_{280} .

$$\text{Concentration of antibody (mg/ml)} = A_{280} \times 0.74 \times (\text{dilution factor}).$$

3. Add 10 μ l of 10 mg/ml long-armed biotin in anhydrous DMSO for each milligram of antibody. Incubate 1 hr at room temperature.

Both the dye and organic solvent must be anhydrous; prepare biotin/DMSO solution immediately before use.

4. Remove unbound biotin by dialysis at 4°C as for FITC labeling (see Support Protocol 1, step 4).

Biotin/protein ratio cannot be determined spectrophotometrically, but titration comparison of the same antibody labeled with FITC can indicate whether relabeling is necessary.

LABELING ANTIBODY WITH TEXAS RED

Texas Red, the sulfonylchloride derivative of sulforhodamine 101, has been used for many years in dual-laser multiparameter flow cytometry; however, directly labeling antibodies with this dye can be difficult, depending upon the class of the antibody and host species (Titus et al., 1982), as concentrations required to achieve adequate dye/protein ratios often precipitate the antibody-dye conjugates. The recent development of the modified Texas Red-X succinimidyl ester has greatly improved Texas Red labeling, allowing a greater range of antibodies to be labeled with substantially less precipitation of antibody-dye conjugates. The procedure is similar to the protocol for biotin labeling (see Support Protocol 2), with the modifications detailed below.

Materials

- 1 to 2 mg/ml purified monoclonal antibody
- Succinimide ester labeling buffer, 4°C (see recipe)
- 5 mg/ml Texas Red-X succinimidyl ester (Molecular Probes) in *N,N*-dimethylformamide (DMF)
- Dialysis buffer, 4°C (see recipe)
- Stabilizing buffer (see recipe)

- Dialysis tubing
- Sephadex G-25 column (Pharmacia Biotech; optional)

1. Dialyze purified monoclonal antibody against 500 ml succinimide ester labeling buffer at 4°C with two or three changes over 2 days (APPENDIX 3H). Allow ≥4 hr between buffer changes.

For discussion of dialysis and a detailed procedure, see UNIT 2.7.

2. Determine antibody concentration based upon A_{280} and adjust to 1 to 2 mg/ml.

Concentration of antibody (mg/ml) = $A_{280} \times 0.7 \times \text{dilution factor}$.

3. Add 5 μ l of 5 mg/ml Texas Red-X succinimidyl ester for each milligram of antibody. Incubate 1 hr at room temperature.

Both the dye and organic solvents must be anhydrous; prepare Texas Red-X/DMF solution immediately before use.

4. Remove unbound Texas Red-X by dialysis at 4°C as in step 1, but using dialysis buffer. Alternatively, filter on a Sephadex G-25 column.

5. Remove any precipitated antibody by centrifuging 3 min at $10,000 \times g$, 4°C.

6. Determine Texas Red/antibody ratio by measuring A_{596}/A_{280} .

A ratio of 0.5 to 0.7 usually gives the best results and probably represents two to three Texas Red molecules bound per antibody, based upon a molar extinction coefficient for antibody bound to Texas Red of $8.4 \times 10^4 M^{-1}$ at 596 nm (Titus et al., 1982).

7. Dilute Texas Red-Ig complex solution 1:1 with stabilizing buffer.

LABELING ANTIBODY WITH PHYCOBILIPROTEINS

Coupling phycobiliproteins such as phycoerythrin (PE) and allophycocyanin (APC) to antibodies is more difficult than labeling with FITC (see Support Protocol 1) or biotin (see Support Protocol 2). A sulfhydryl-maleimide linkage is used to couple the antibody to the phycobiliprotein. The unbound antibody and phycobiliprotein are then separated by size on a gel-filtration column.

The procedure described here is for PE-antibody coupling. The step for APC coupling is identical except where noted.

Additional Materials (also see Support Protocol 1)

- 10 to 25 mg/ml phycoerythrin (PE)
- Coupling buffer, pH 5.5 and 7.5 (see recipe)
- Sulfhydryl addition reagent: *N*-succinimidyl-*S*-acetylthioacetate (SATA; Calbiochem); store under nitrogen after opening
- Dimethylformamide (DMF)
- Heterobifunctional cross-linker: γ -maleimidobutyric acid *N*-hydroxysuccinimide ester (GMBS, Calbiochem; store under nitrogen after opening)
- Deacetylation buffer (see recipe)
- Tetrahydrofuran (THF)
- Cysteine
- Running buffer, degassed (see recipe)
- AcA 34 column (IBF Biotechnics)

Prepare the PE-SATA conjugate

1. Dialyze PE against 500 ml coupling buffer, pH 7.5 as for FITC labeling (see Support Protocol 1, step 1 and *APPENDIX 3H*). Use sufficient PE to give a PE/IgG (w/w) ratio of 3:1.

The precise concentration of PE must be determined by spectrophotometric measurements at A_{596} and the concentration adjusted with coupling buffer to within the indicated range.

2. Dilute *N*-succinimidyl-*S*-acetylthioacetate (SATA) to 1 mg/ml in DMF.
3. Add 10 μ l diluted SATA solution for each milligram of PE to be labeled. Incubate 30 min at room temperature.
4. Dialyze PE-SATA conjugate in 500 ml coupling buffer, pH 7.5 at 4°C with two to three changes to remove unreacted SATA. Store at 4°C for later use.

Label the antibody and isolate the conjugate

5. Dialyze purified antibody in 500 ml coupling buffer, pH 7.5 as described in step 1 of FITC-labeling protocol (see Support Protocol 1) to a final IgG concentration of ≥ 1 mg/ml.
6. Dilute γ -maleimidobutyric acid *N*-hydroxysuccinimide ester (GMBS) to 2 mg/ml (7.14 mM) in THF.
7. Deacetylate PE-SATA conjugate from step 4 by adding 100 μ l deacetylation buffer for each milliliter of PE-SATA. Incubate 1 hr at room temperature.
8. Add 10 μ l diluted GMBS solution for each milligram of antibody to be labeled. Incubate 30 min at room temperature.
9. Wash one Sephadex G-25 column for each 2.0 ml IgG-GMBS conjugate solution to be loaded by adding 10 ml coupling buffer, pH 5.5 (per column). Load 2.0 ml IgG-GMBS solution onto the washed column. Monitor eluate spectrophotometrically

using a 280-nm filter and collect the portion represented by the first peak. Proceed immediately to step 10.

The first peak is the GMBS-labeled antibody. The second peak is free GMBS and should be discarded.

Couple PE-SATA and IgG-GMBS conjugates

10. Mix deacetylated PE-SATA conjugate from step 7 with IgG-GMBS conjugate from step 9 immediately after isolating the latter. Incubate 2 hr at room temperature.

Use a 3:1 ratio of PE to IgG for optimum yield, but use a 2:1 ratio of APC/IgG.

11. Quench residual maleimide groups by adding cysteine to twice the antibody concentration.

For example, add 25 μ l of 0.1 mg/ml cysteine (569.5 μ M) per milligram of IgG.

12. Separate PE-IgG conjugate from unconjugated PE and free IgG using an AcA 34 column. Sample volume to be loaded onto column should be between 0.5% and 4% of total bed volume of column. Pour an appropriately sized column using degassed running buffer according to manufacturer's directions.

Due to slow packing and running rates, it generally requires one night to pack a column and an additional night to isolate the sample; therefore it is advisable to pack the column before labeling.

13. Load sample onto column and run column at manufacturer's suggested rates. Several peaks will appear on the column printouts. The first peaks are PE-IgG conjugates with more than one PE-per-IgG. The peak with one PE-per-IgG will appear immediately before the unconjugated PE peak. The last peak will be unconjugated IgG.

Confirm number of PE-per-IgG using flow cytometry techniques or spectrophotometrically using A_{596}/A_{280} ratios. Best results have come from using the one-PE-per-Ig conjugate.

IMMUNOFLUORESCENCE STAINING OF UNFIXED CELLS FOR DETECTION OF INTRACELLULAR ANTIGENS

ALTERNATE PROTOCOL 1

This method for preparing cells for intracellular staining is very rapid, preserves immunofluorescence, and can also be used for measuring DNA content. It is particularly useful for cell preparations where the preservation of light scatter is less critical—e.g., for cell lines and for the staining of intracellular antigens that are denatured by cross-linking fixatives, such as paraformaldehyde.

For simultaneous analysis of surface and internal antigens, cells that have been stained for expression of cell-surface antigens can also be stained for expression of intracellular antigens using this protocol. Fluorochrome selection and appropriate antibody combinations are discussed in the Commentary (see Critical Parameters). Do not use PerCP-labeled antibodies for cell-surface staining, as PerCP fluorescence is lost after permeabilization.

Additional Materials (also see *Basic Protocol 2*)

0.3% and 0.1% (w/v) saponin (Sigma) in PBS (*APPENDIX 2A*): store \leq 1 month in amber container at 4°C

0.3% saponin in PBS with 10 μ g/ml PI or 20 μ g/ml 7-AAD (optional): add PI or 7-AAD stock to appropriate concentration (see recipes); prepare fresh before use and protect solution from light

Immuno- fluorescence and Cell Sorting

5.3.9

1. Place $\sim 2 \times 10^6$ cells into a 12×15 -mm test tube and add 1 to 2 ml PBS. Centrifuge 5 min at $300 \times g$ (e.g., 1000 rpm in Sorvall H-1000B), 4°C . Discard supernatant and wash pellet with another 1 to 2 ml PBS. Centrifuge 5 min at $300 \times g$, 4°C .

The light-scatter profiles of the cells on the flow cytometer will change considerably after permeabilization. This will also lead to a loss of discrimination between live and dead cells; therefore, whenever considerable numbers of dead cells are present, they should be removed by Ficoll-Hypaque separation prior to permeabilization or be identified by fluorescent-dye staining and excluded by flow cytometric analysis (see Alternate Protocol 2 and also see Critical Parameters for further discussion).

2. Dilute fluorochrome-labeled antibody in 0.3% saponin/PBS to an appropriate working dilution.
3. Remove supernatant by aspiration or rapid decanting and add 100 μl fluorochrome-labeled antibody in saponin to cell pellet. Mix gently without vortexing. Incubate 30 min at 4°C .

Saponin needs to be present during each antibody incubation and during each washing step to maintain membrane permeability. Blocking with human AB serum or normal mouse serum, before the addition of the antibody dilution, can reduce nonspecific staining. Longer incubation times and higher staining temperatures, e.g., 22° to 25°C or 37°C , may be required for some antibodies. For further discussion see Critical Parameters and also see Troubleshooting.

4. Add 2 ml PBS and centrifuge 5 min at $300 \times g$, 4°C . Discard supernatant and wash pellet with 1 ml of 0.1% saponin/PBS. Centrifuge 5 min at $300 \times g$, 4°C .

If cells have been stained with a fluorochrome-labeled primary antibody, proceed directly to step 6a or 6b, as needed.

5. To stain cells again after unlabeled or biotin-labeled first antibodies have been used, repeat steps 3 and 4 using second-step fluorochrome-labeled polyclonal antibody or avidin/streptavidin.

Labeled avidin/streptavidin or $F(ab')_2$ fragments are the reagents of choice for intracellular second-step staining because they eliminate the possibility of Fc binding.

- 6a. *If simultaneous analysis of DNA content will not be performed:* Resuspend cells at a concentration of $1\text{--}2 \times 10^6/\text{ml}$ in staining buffer in PBS (do not use 0.3% saponin/PBS). Filter cells through nylon mesh to remove clumps if necessary. Keep samples at 4°C , protected from light, until flow cytometric analysis.

Because cells were not treated with a fixative, samples should be analyzed on the flow cytometer as soon as possible.

- 6b. *If a simultaneous analysis of DNA content will be performed:* Resuspend cells at a concentration of $1\text{--}2 \times 10^6/\text{ml}$ in 0.3% saponin with 10 $\mu\text{g}/\text{ml}$ PI or 20 $\mu\text{g}/\text{ml}$ 7-AAD. If antibody is labeled with PE (see Support Protocol 4), use 0.3% saponin with 20 $\mu\text{g}/\text{ml}$ 7-AAD. Incubate 10 min at 4°C . Keep samples at 4°C protected from light until flow cytometric analysis. Filter through nylon mesh to remove clumps if necessary. Because samples are not fixed, analyze on the flow cytometer as soon as possible.

STAINING OF NONVIABLE CELLS WITH 7-AMINOACTINOMYCIN D FOR DEAD-CELL DISCRIMINATION

ALTERNATE PROTOCOL 2

This protocol describes the use of 7-aminoactinomycin D (7-AAD) for the fluorescent detection of nonviable (i.e., dead) cells that have lost membrane integrity in fixed or permeabilized cell preparations. DNA binding of fluorescent 7-AAD, which can leak out of stained cells into unstained cells after fixation or permeabilization, is blocked by including nonfluorescent actinomycin D (AD) in the fixation, permeabilization, staining, washing, and resuspension solutions.

Additional Materials (also see Basic Protocol 2)

- 1 mg/ml 7-aminoactinomycin D (7-AAD) stock solution (see recipe)
- Fixation solution containing 80 $\mu\text{g/ml}$ actinomycin D (AD; see recipe), 4°C
- 0.2% and 0.1% (v/v) Tween 20 in PBS containing 10 $\mu\text{g/ml}$ AD
- Staining buffer (see recipe) containing 10 $\mu\text{g/ml}$ AD
- Fluorochrome-labeled antibody in 0.3% (w/v) saponin/PBS containing 10 $\mu\text{g/ml}$ AD
- 0.1% saponin in PBS containing 10 $\mu\text{g/ml}$ AD

1. Place $\sim 10^6$ to 2×10^6 cells in a 12 \times 15–mm test tube and add 1 to 2 ml PBS. Centrifuge 5 min at $300 \times g$ (e.g., 1000 rpm in Sorvall H-1000B), 4°C. Discard supernatant and resuspend cells in 1 ml staining buffer. Add 1 μl of 1 mg/ml 7-AAD stock solution for a final concentration of 1 $\mu\text{g/ml}$. Mix well and incubate 20 min in the dark.
2. Centrifuge 5 min at $300 \times g$, 4°C, and remove supernatant by aspiration or rapid decanting. Resuspend pellet in 3 ml PBS and centrifuge 5 min at $300 \times g$, 4°C.

To stain fixed and permeabilized single-cell suspension:

- 3a. Remove supernatant by aspiration or rapid decanting and add 875 μl cold PBS to cell pellet. Mix gently.
- 4a. Add 125 μl cold fixation solution containing 80 $\mu\text{g/ml}$ AD and mix gently. Incubate 1 hr at 4°C.

Final concentration of AD in the 0.25% paraformaldehyde solution will be 10 $\mu\text{g/ml}$.

Complete removal of unbound 7-AAD is critical because residual 7-AAD will bind to the DNA of unstained live cells immediately when cells are permeabilized.

- 5a. Treat cells with Tween 20 and stain for immunofluorescent detection of intracellular antigens in fixed and permeabilized single-cell suspensions as described (see Basic Protocol 2, steps 3 to 6), except use 0.2% and 0.1% Tween 20/PBS and staining solutions containing 10 $\mu\text{g/ml}$ AD.

To stain unfixed cells permeabilized with saponin:

- 3b. Remove supernatant by aspiration or rapid decanting. Add 100 μl of the appropriate working dilution of fluorochrome-labeled antibody in 0.3% saponin/PBS containing 10 $\mu\text{g/ml}$ AD. Mix gently without vortexing. Incubate 30 min at 4°C.
- 4b. Add 2 ml staining buffer containing 10 $\mu\text{g/ml}$ AD. Centrifuge 5 min at $300 \times g$, 4°C. Discard supernatant and repeat wash with 1 ml of 0.1% saponin/PBS/AD. Centrifuge 5 min at $300 \times g$, 4°C.

Complete removal of unbound 7-AAD is critical, particularly when permeabilizing cells with saponin, because residual 7-AAD will bind to the DNA of unstained live cells immediately when cells are permeabilized.

- 5b. Stain cells for immunofluorescent detection of intracellular antigens in unfixed cells (see Alternate Protocol 1, steps 3 to 6), except use saponin and staining solutions containing 10 $\mu\text{g/ml}$ AD.

Immuno- fluorescence and Cell Sorting

5.3.11

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 5.

Actinomycin D (AD) stock solution, 1 mg/ml

1 mg actinomycin D (actinomycin C₁; Boehringer Mannheim)
50 µl absolute ethanol, -20°C
Mix well
Add 950 µl cold PBS (APPENDIX 2A)
Sonicate solution 10 min at 4°C or let sit a minimum of 24 hr at 4°C before use.
Store ≤1 month at 4°C.

AD does not dissolve readily, but it is most soluble at low temperatures.

7-aminoactinomycin D (7-AAD) stock solution, 1 mg/ml

1 mg 7-AAD (Calbiochem or Sigma)
50 µl absolute methanol
Mix well
Add 950 µl PBS (APPENDIX 2A)
Mix again
Store protected from light ≤1 month at 4°C

Working solutions (prepared immediately before use) contain 20 µg/ml 7-AAD. They should also be protected from light.

Coupling buffer, pH 5.5 or 7.5

0.1 M Na₂PO₄·7H₂O
0.1 M NaCl (APPENDIX 2A)
1 mM EDTA (APPENDIX 2A)
Adjust pH to 5.5 or 7.5 with concentrated HCl
Store ≤1 week at room temperature

Deacetylation buffer

Dissolve 3.47 g hydroxylamine (mono HCl; 0.5 M final) and 0.73 g EDTA (anhydrous free acid; 0.025 M final) in ~50 ml H₂O and adjust to pH 7.5 with solid anhydrous disodium hydrogen phosphate. Add H₂O to 100 ml final volume.

Dialysis buffer

0.1 M Tris-Cl, pH 7.4 (APPENDIX 2A)
0.1% (w/v) NaN₃
0.2 M NaCl (APPENDIX 2A)
Adjust pH to 7.4 with concentrated NaOH
Store ≤1 week at room temperature

FITC labeling buffer

0.05 M boric acid
0.2 M NaCl (APPENDIX 2A)
Adjust pH to 9.2 with concentrated NaOH
Store ≤1 week at room temperature

Fixation solution

2 g paraformaldehyde (electron microscopy-grade, Polysciences)
100 ml PBS (APPENDIX 2A)
Adjust pH to 7.2 with 0.1 M NaOH or 0.1 M HCl
Store protected from light ≤1 month at 4°C
To dissolve paraformaldehyde, heat solution to 70°C in a fume hood for ~1 hr. Cool to room temperature before adjusting pH. Check pH periodically.

continued

Do not heat the solution above 70°C. For best results, use only very pure preparations of paraformaldehyde (i.e., electron microscopy–grade from Polysciences). For intracellular antigens that are very sensitive to acid denaturation, use only freshly prepared fixation solutions as discussed in Critical Parameters.

Permeabilization solution: 0.2% (v/v) Tween 20

Mix 200 µl Tween 20 (Sigma) with 100 ml PBS. Store ≤1 month in amber container at 4°C. Warm to room temperature before use.

Propidium iodide solution

50 µg/ml propidium iodide
0.1% (w/v) sodium citrate
Store protected from light ≤1 year at 4°C.

Propidium iodide (PI) stock solution, 1 mg/ml

1 mg/ml propidium iodide in PBS (*APPENDIX 2A*)
1 mg/ml ribonuclease A (Sigma)
Store protected from light ≤1 month at 4°C.

Working solutions (prepared immediately before use) contain 10 µg/ml PI. They should also be protected from light.

Running buffer

81.82 g NaCl
4 ml glycerol
Dissolve in 3.8 liters PBS (*APPENDIX 2A*)
Adjust pH to 7.5 with concentrated HCl
Add PBS (*APPENDIX 2A*) to 4 liters

To degas buffer, place room temperature buffer in an Erlenmeyer flask equipped with a one-hole stopper and tubing (alternatively, a sidearm vacuum flask and stopper may be used). Apply vacuum through the tubing (or sidearm) while stirring buffer vigorously. Sample is degassed when no more bubbles rise out of solution.

Store at room temperature ≤1 week.

Stabilizing buffer

Hanks' balanced salt solution (*APPENDIX 2A*) without phenol red, containing:
0.1% (w/v) NaN₃
5.0% (w/v) BSA, fraction V
Store ≤1 year at 4°C.

Staining buffer

Hanks balanced salt solution (*APPENDIX 2A*) without phenol red, containing:
0.1% (w/v) NaN₃
1.0% (w/v) bovine serum albumin (BSA; fraction V)
Store at 4°C ≤1 year.

Succinimide ester labeling buffer

0.1 M NaHCO₃
0.1 M NaCl
Adjust pH to 8.4 with concentrated HCl
Store ≤1 week at room temperature

Washing buffer: 0.1% (v/v) Tween 20

Mix 100 µl Tween 20 with 100 ml PBS. Store ≤1 month in amber container at 4°C.

COMMENTARY

Background Information

In direct immunofluorescence staining, cells are treated with an antibody that has been conjugated to a fluorochrome (e.g., FITC; see Basic Protocols 1 and 2 and Support Protocol 1). In indirect staining (sometimes referred to as a sandwich technique), the primary reagent is not labeled but is detected by a second fluorochrome-labeled reagent. This second reagent may be an antibody with specificity for the first antibody. Alternatively, the avidin-biotin system can be used (see Support Protocol 2), whereby an antibody is conjugated to biotin and is detected with fluorochrome-labeled avidin (an egg-white glycoprotein with a very high intrinsic affinity for biotin). Another method of indirect staining uses aminophenyl hapten-conjugated antibodies, such as those made with arsanilic acid (Mishell and Shiigi, 1980). Detection of these antibodies is accomplished with fluorochrome-labeled antibodies specific for the haptens.

Indirect immunofluorescence offers several advantages over direct immunofluorescence. For instance, biotinylated antibodies can be detected with avidin conjugated to any number of different fluorochromes that are inexpensive, of high quality, and widely available from a number of commercial sources. In immunofluorescence using two antibodies, the second specific for the first, detection of a first antibody that is unpurified and unlabeled can be achieved using a second purified, labeled antibody. In addition, indirect immunofluorescence often amplifies the fluorescence signal.

Coordinate expression of more than one antigen or surface molecule on a given cell can be analyzed using multicolor staining. Multicolor staining usually combines direct and indirect immunofluorescence in a single protocol. This maintains specificity within a multicolor labeling system without sacrificing its versatility.

Staining of intracellular antigens for flow cytometry depends on techniques that fix or permeabilize the membranes of cells in solution to allow access of antibodies to internal cellular components. Most of the methods that have been developed use formaldehyde in combination with a detergent or alcohol (ethanol or methanol) to prepare cells for intracellular staining (for reviews see Jacobberger, 1989, and Clevenger and Shankey, 1993). Some methods use detergents without prior fixation of cells (Schroff et al., 1984; Jacob et al., 1991).

Alcohols coagulate cellular proteins and solubilize lipids. Thus, they simultaneously fix and permeabilize the cells. Formaldehyde cross-links proteins (Puchtler and Meloan, 1985) and when used alone, it does not provide adequate permeabilization; therefore, detergents are needed to solubilize membranes after formaldehyde fixation (Clevenger et al., 1985).

Alcohols create cellular debris which leads to clumping and subsequent cell loss. They also dramatically change the light-scatter profile of cells on the flow cytometer, which can make the identification of the cell populations of interest difficult. In contrast, fixation with formaldehyde maintains the light-scatter properties of cells and preserves surface immunofluorescence (Lanier and Warner, 1981); therefore, although it requires an extra step for permeabilization, formaldehyde fixation is the preferred method of preparing cells for intracellular staining for flow cytometry.

Basic Protocol 2 describes fixation using a low concentration of paraformaldehyde in combination with a mild detergent (Tween 20). It is particularly suited for simultaneous measurement of intracellular and surface staining as well as DNA content (Schmid et al., 1991). Furthermore, the procedure may be modified to permit detection of a diverse array of intracellular proteins (Clevenger and Shankey, 1993). By changing the formaldehyde concentration or the type of detergent used, the procedure can be used to detect proteins that differ in their susceptibility to antigenic denaturation and their resistance to extraction from a permeabilized cell (Mann et al., 1987). After fixation and permeabilization, the cells are stained with antibodies using standard immunofluorescent techniques for flow cytometry. Problems related to intracellular staining are discussed below (see Critical Parameters).

Critical Parameters

Preparation and storage of reagents

Although the protocols for labeling antibodies with fluorochromes and biotin are simple, optimal results are highly dependent upon the reagents used. The organic solvents (DMF, DMSO, and acetonitrile) and the dye powders used must be anhydrous. For this reason it is recommended that dyes be purchased in small amounts and stored in a desiccator. Organic solvents can be purchased packed under nitrogen in syringe vials. Solutions of FITC, Texas

Red, and biotin should be made just prior to use. It is recommended that the FITC labeling buffer and coupling buffer be made not more than 1 week before use. Texas Red labeling is more difficult than labeling with FITC or biotin, as it is more dependent upon the class and species of antibody to be labeled (Titus et al., 1982).

Immunofluorescence staining is simple to perform, but several factors can influence the intensity levels achieved and specificity of binding. It is important to keep the staining vessels partially submerged in an ice bath and to include sodium azide in the staining buffer. Modulation and internalization of surface antigens can occur as a result of antibody cross-linking (Taylor et al., 1971; Loor et al., 1972), which can produce a loss of fluorescence intensity. Low temperature and the presence of sodium azide prevent this phenomenon by inhibiting metabolic activity. Cross-linking of surface antigens can also be prevented by using the monovalent form of antibodies prepared as Fab fragments (Porter, 1959). It is important that antibody preparations be free of aggregates because these complexes also bind through the Fc portions of the immunoglobulin molecule, promoting nonspecific binding to surface Fc receptors.

The preferred reagent for formaldehyde fixation of cells for intracellular staining is a buffered solution made from paraformaldehyde, the solid polymer of formaldehyde. Commercially available formalin solutions can contain formic acid, which may be generated from formaldehyde by exposure to excess heat and light or produced during the manufacturing processes. In addition, commercially available formalin that is not buffered contains methanol as a stabilizing agent. Both formic acid and methanol can have detrimental effects on structures of intracellular antigens. When kept at 4°C, and protected from light, the paraformaldehyde solution described in this protocol is stable for at least 1 month as long as a pH of ~7.2 is maintained. It is advisable to check the pH periodically and discard the solution if it has become acidic; however, for antigens that are very sensitive to denaturation, only freshly prepared fixation solution should be used. Alternatively, a 16% electron microscopy (EM)-grade formalin solution sealed under nitrogen gas is available (from Polysciences) for laboratories that do not have access to a fume hood.

The permeabilization solution, which contains either Tween 20 (see Basic Protocol 2) or saponin (see Alternate Protocol 1), is stable at

least 1 month when stored at 4°C and protected from light. Again, use only pure preparations because contaminating substances may affect the antigenic properties of intracellular proteins.

Intracellular staining

To stain intracellular proteins, antibodies have to cross cellular membranes. An antibody's coupling with an intracellular antigen depends on its affinity for the antigen and how easily it can access the appropriate epitopes inside the cell. Using polyclonal antisera can therefore improve the staining of intracellular antigens because they represent mixtures of antibodies of different affinities that are directed against a variety of epitopes. Furthermore, antibodies of different isotype (IgG versus IgM) have different molecular weights and vary in their diffusion constants. Consequently, increased staining temperature and longer incubation time can improve reactivity with the intracellular antigen; however, low temperature (i.e., 4°C) favors the stability of antibody-ligand complexes. For staining of cells that have been permeabilized by saponin, the presence of detergent in the staining solution is required for antibody penetration (Jacob et al., 1991; Sander et al., 1991).

Antibodies purchased from various commercial sources can differ dramatically in their reactivity with a given intracellular antigen. It is advisable to buy only reagents that have been developed and tested for flow cytometry, especially ones known to be useful for intracellular staining. Ideally, several antibodies directed against the intracellular antigen of interest should be tested to determine which one gives the best separation between specific staining and background. Antibodies used for detecting proteins on immunoblots may not give good results in flow cytometric assays because they may not be directed against the conformation-dependent epitopes present in intact cells. Also, antibodies that react well with cells fixed onto slides may not adequately stain intracellular antigens of cells in solution.

Nonspecific binding of antibodies is a considerable problem in intracellular staining because there are many more components to which an antibody can attach itself inside the cell than there are on the cell surface. For this reason, monoclonal antibodies often give clearer results for intracellular staining than polyclonal antisera, which tend to show high background staining. Also, directly labeled antibodies are preferable for intracellular stain-

ing, because with second-step reagents, the nonspecific binding of the first and the second antibody have to be considered. When indirect staining must be done, avidin/streptavidin or F(ab')₂ fragments of antisera are the reagents of choice because of reduced Fc binding. Addition of serum (e.g., 50% heat-inactivated human serum or 20% normal mouse serum) to the staining buffer can decrease the level of nonspecific staining. Multiple washing steps with buffers that contain low concentrations of detergent can further reduce nonspecific background staining.

Careful titration of all staining reagents is mandatory to find the optimal antibody concentration. The optimal concentration may differ from the amount recommended for cell-surface staining. When an indirect staining system is used, both the first- and second-step reagents have to be titrated. This is usually done with a staining matrix to determine the antibody concentrations that give the best separation of staining above background and autofluorescence, which are higher in fixed than in unfixed cells. For studies of antigen quantification, it is important to determine the saturating amount of antibody to ensure that the fluorescence signal from each individual cell will represent the actual antigen content. In addition, cells that have not been fixed and permeabilized, and cells that do not express the intracellular antigen of interest, should be used as negative experimental staining controls. Cells that express the intracellular antigen of interest should be used as positive staining controls.

Basic Protocol 2, first described in Schmid et al. (1991), is well suited for combining cell-surface staining with intracellular staining. Surface staining is performed according to standard protocols. The cells are then fixed and permeabilized and the intracellular antigen is stained. There will be some loss of fluorescence intensity of cell-surface antigen staining after the fixation and permeabilization step; however, with FITC, PE, and Tricolor staining, even dimly expressed antigens are preserved sufficiently to allow discrimination from background. In contrast, the fluorochrome peridinin chlorophyll protein (PerCP) shows a dramatic loss of staining intensity after the permeabilization step. This makes PerCP unsuitable for combined cell-surface antigen and intracellular staining by the procedures described (see Basic Protocol 2 and Alternate Protocol 1).

Antibodies labeled with fluorochromes possessing different emission spectra must be used for combined surface and intracellular staining.

Again, directly labeled antibodies are preferable, because a second-step reagent will also bind to the surface antibody, unless a biotinylated antibody or an antibody made in a different species is used intracellularly.

The current method is also applicable to combining surface and intracellular staining with DNA staining (Schmid et al., 1991; Storek et al., 1992). Formaldehyde fixation is the preferred method for preserving cell-surface immunofluorescence for flow cytometry (Lanier and Warner, 1981); however, formaldehyde also rapidly cross-links nuclear histones, which will impair access of the dye (e.g., PI or 7-AAD) to DNA. A low concentration of paraformaldehyde (0.25%) is therefore used in Basic Protocol 2 to reduce the effect of cross-linking on nuclear proteins and, thus, improve the coefficient of variation on the resulting DNA histograms. Depending on the fluorochrome label of the antibody used for surface or intracellular staining, either PI or 7-AAD can be used for DNA staining, as discussed below.

Fixation and permeabilization

Basic Protocol 2 is simple to perform and will produce cell preparations with little debris. Cell loss is minimal and the cell populations are distinctly visible on the flow cytometer; however, this protocol may not be suitable for all cells and all intracellular antigens. Because the exact effects of fixation and detergent treatment on cellular membranes and antigens are not yet completely understood, the most critical parameter is the selection of an appropriate fixation and permeabilization procedure for a previously uncharacterized intracellular protein. Thus, the optimal procedure for a given antigen has to be determined empirically. This may mean that different concentrations of formaldehyde or an alternate fixation time or temperature have to be tried (Mann et al., 1987). Use of the detergent saponin may improve the stainability and retention of intracellular antigens (Franek et al., 1994) or permit the detection of intracellular cytokines (Sander et al., 1991).

However, because of the inverse relationship between the permeability of cells for probes and the retention of intracellular structures, the optimal detergent and its concentration have to be matched to the fixative and the antigen. For instance, Tween 20, which is used in Basic Protocol 2 for permeabilization after fixation, is a very mild detergent that is able to preserve the discrimination between cell clusters even when cell preparations are fixed in low concen-

trations of paraformaldehyde. Altering the concentration of Tween 20 or replacing it with another detergent (e.g., Triton X-100) can alter the light-scatter properties of cells considerably and affect the discrimination of cell clusters on the flow cytometer or cause extraction of cellular components.

Finally, to detect antigens that are denatured by cross-linking fixatives like formaldehyde, the optimal technique may be to treat cells with various concentrations of ethanol or methanol (Bauer and Jacobberger, 1994) or to use a protocol in which unfixed cells are permeabilized by saponin (Jacob et al., 1991).

The best way to determine whether a fixation protocol is suitable for staining a given antigen is to use cells that express the intracellular antigen of interest as a positive experimental control. In addition, the success of an individual staining experiment for a new intracellular antigen should always be verified by fluorescence microscopy, because flow cytometry cannot offer information on the physical location of the fluorescence in the cell. Localization of staining—e.g., nuclear versus cytoplasmic—is an important confirming parameter for antigens with a known intracellular location and may be affected by the fixation and permeabilization procedure (Clevenger and Shankey, 1993).

Discrimination of nonviable cells

Nonviable (i.e., dead) cells can bind to antibodies nonspecifically and therefore have to be excluded from flow cytometric analysis. Frequently, nonviable cells can be discriminated from live cells on the basis of light scatter. This discrimination, however, is often lost in fixed or permeabilized cell preparations; therefore, whenever there are considerable numbers of dead cells present, it is advisable to remove dead cells and clumps from the cell preparation by Ficoll-Hypaque separation. However, this can lead to cell losses and in some cases to the selective loss of cells of interest due to alterations in cell density.

Alternatively and preferably, dead cells can be distinguished from live cells by their uptake of fluorescent DNA dyes due to loss of membrane integrity. PI, which is commonly used for dead-cell discrimination, cannot be used because it will leak out of the stained dead cells and into the unstained cells after fixation and permeabilization. 7-AAD has also been used for dead-cell discrimination, and is particularly useful for combined staining with PE because its far-red emission can be effectively separated

from the orange emission of PE (Schmid et al., 1992). Staining of live cells by 7-AAD, which leaks out of dead cells after permeabilization, can be prevented by adding nonfluorescent actinomycin D (AD) to the fixing or permeabilization solution (Fetterhoff et al., 1993). This technique is described in Alternate Protocol 2 and its application is discussed below (see Anticipated Results).

Another method involves covalent binding of ethidium monoazide by UV exposure to the DNA of nonviable cells (Riedy et al., 1991). Recently, the use of the dye Tricolor (Caltag) for dead-cell discrimination in fixed or permeabilized cell preparations has been described (Levelt and Eichmann, 1994).

Choice of label

Fluorescein-conjugated antibodies are most commonly used for single-color flow cytometry analysis. Other conjugation methods are used for second-, third-, and fourth-color analysis of multiple parameters. Choice of label is largely dependent upon the flow cytometer available and the corresponding laser and optical filter combination. The number of parameters and the density of molecules per cell also dictate the number and type of fluorochromes used. Additional factors to consider include the ability of specific antibodies to withstand the conjugation procedures and retain activity as well as the availability or accessibility of target amino acids for covalent linkage to the fluorochrome.

Fluorochrome selection for intracellular staining

Fluorochromes for intracellular staining experiments should have low molecular weights, as otherwise the fluorochrome will reduce antibody mobility. Fluorescein isothiocyanate (FITC) is a small molecule and is commercially available conjugated to first- and second-step reagents. FITC has therefore been used extensively for intracellular staining, although its negative charge leads to an increase in nonspecific binding and its green emission spectrum is in the same range as cellular autofluorescence. Novel dyes that do not have these disadvantages and are small include 7-amino-4-methylcumarin-3-acetic acid (AMCA, available from Polysciences and Jackson Immunoresearch) and Cascade blue (Molecular Probes); however, both dyes require excitation in the UV range, an option that is not available with most benchtop flow cytometers.

Phycobiliproteins, such as phycoerythrin (PE), have much higher molecular weights and

are probably best used for combining cell-surface PE staining with FITC intracellular staining. Nevertheless, their size is in the same range as that of intact IgG and they are able to cross the cell membrane. They may be applicable for staining experiments where precise quantification of the intracellular antigen is less critical. When cell-surface staining is done with PE-labeled antibodies, 7-AAD must be used for DNA staining because of the extensive emission overlap between PE and PI (Rabinovitch et al., 1986; Schmid et al., 1991). When FITC is used for surface or intracellular staining, PI is the dye of choice for measuring DNA content, as it offers lower coefficients of variation on DNA histograms than 7-AAD and its emission signal can be easily separated from the FITC emission on the flow cytometer.

Multicolor staining

For multicolor staining, the order of reagent addition is determined by the specificities of the reagents used. If direct and indirect stainings are performed in the same protocol, the indirect is executed first. If two indirect stains are to be employed in the same staining procedure, both first antibodies may be added in the first incubation and the two second antibodies in the second incubation, as long as each labeled second antibody (or ligand) is specific for only one of the first antibodies and there is no interaction between the two second antibodies (or ligands). Alternately, each reagent may be added separately at different incubation steps.

Often, indirect staining systems may be used when the first antibodies are raised in different species. In this case, the second labeled antibodies each are specific for only one of the species and there is no cross-reaction. Multicolor staining can be carried out even if one of the second reagents does bind both of the first reagents by using a “cold block.”

For example, a staining protocol involving dual-species antibodies (added in sequential steps, with incubation and wash steps; see Basic Protocol 1) might proceed as follows: (1) add unlabeled rat anti-mouse CD8 (specific for mouse surface antigen); (2) add FITC-conjugated goat anti-rat immunoglobulin; (3) add purified rat immunoglobulin, incubate 10 min, then add biotin-conjugated rat anti-mouse CD4; and (4) add phycoerythrin-conjugated avidin. The excess rat immunoglobulin added at the beginning of the third step occupies any free binding sites on the labeled goat anti-rat immunoglobulin used in the second step and prevents it from binding the second rat antibody

used in the third step (the “cold block”). It is obvious from this sample protocol that the specificity of one of the reagents dictates the order of addition of reagents. The FITC-labeled goat anti-rat immunoglobulin has to be used before the biotin-conjugated rat anti-mouse CD4 so that it binds only the desired antibody—i.e., the rat anti-mouse CD8—and not the biotin-conjugated antibody that is detected with labeled avidin.

Controls for cell-surface staining

Single-color controls. To test for inappropriate interactions, multicolor staining protocols should include single-color controls where cells are stained with each fluorochrome-labeled reagent separately, then compared with an aliquot of the same cell sample stained with both fluorochrome-labeled reagents in the same tube. These controls should be performed for all combinations of direct and indirect staining methods involving more than one fluorochrome. Single-color controls are also useful for setting compensation in flow cytometric analyses when two fluorochromes have overlapping emission spectra (UNIT 5.2).

Reagent and cell sample controls. To determine background from direct staining, protocols often include negative controls with labeled irrelevant antibodies (e.g., antibodies that should not bind the cells used). The labeled irrelevant antibody can only determine general nonspecific binding of any labeled reagent to the cell population under examination. Positive and negative cell controls (cell samples to which the reagent should or should not bind) are used to determine the binding specificity of the labeled reagent and are especially useful in determining specificity of allotype- and species-specific reagents. An additional control for specificity in direct staining is the use of excess unlabeled antibody to show that it can inhibit binding of the same labeled antibody.

For indirect staining, either an irrelevant first antibody (usually of the same isotype) or no first antibody is used to establish background fluorescence or nonspecific binding contributed by the labeled second antibody.

Autofluorescence controls. Unstained cells (i.e., cells without any fluorochrome-conjugated reagent) are used to establish levels of background autofluorescence.

Controls for intracellular staining

Appropriate controls are essential for successful intracellular staining experiments because reagents can often bind nonspecifically

to cellular components that have been altered by the fixation and permeabilization procedure. Cells that express the internal antigen in question can serve as positive experimental controls. Cells known to lack the antigen being studied can control for the specificity of the antibody; however, appropriate negative control cells may not always be available. In some cases, it may be necessary to verify the flow cytometric results independently by immunoblotting or Western blot analysis.

Autofluorescence controls. Cells that have been fixed and permeabilized but not stained should be included in every assay to establish the level of cellular background fluorescence. This level will be higher in cells that were fixed and permeabilized compared to untreated cells and will differ among fixation techniques.

Reagent and cell-sample controls. To determine the degree of background caused by nonspecific binding of the heavy chain of the antibody, cells should be stained with isotypic irrelevant antibodies. Because this background is highly dependent on the protein concentration, control and relevant antibodies have to be compared at the same staining concentration. Unfortunately, manufacturers of commercial antibodies do not always list the protein concentration on antibody data sheets, but all companies should be able to provide this information on request.

To control for second-step reagents, cells are usually stained with the second antibody alone; however, cells that were incubated with an isotype-matched, unlabeled antibody that is nonreactive, followed by the second antibody, provide a more accurate control for background staining. Fluorescence measured above background is then considered to be caused purely by reactivity of the relevant antibody.

Single-color controls. In multicolor experiments, single-color control tubes containing cells that are stained with each reagent separately are used to equilibrate the flow cytometer for the amount of electronic color compensation for dyes with overlapping fluorescence emission spectra. Then, cells that have been stained with multiple reagents are analyzed with identical instrument settings. Single-color control tubes also allow determination of unwanted reagent interactions that can occur whenever cells are incubated with more than one antibody or dye. This is especially important when one directly labeled reagent is combined with one unlabeled reagent that requires a second step. In this case, antibodies made in different species have to be used, blocking steps

are needed, and staining controls are essential to confirm that there is no cross-reactivity between the reagents. Although some companies (e.g., Tago and Caltag) now offer highly purified and specific second-step reagents, directly labeled antibodies are preferable whenever two reagents are to be combined.

Troubleshooting

Cell-surface staining

The goal in immunofluorescence staining is to attain a high specific signal with minimal background fluorescence. Background fluorescence caused by nonspecific and unreliable binding is most often due to the use of an incorrect concentration of reagent. Titration will quantitate the minimal amount of antibody needed to achieve saturation.

Another common problem is the lack of specificity of second antibodies. Often, these are not monoclonal antibodies but are affinity-purified from antisera. Isotype- and species-specific second antibodies from commercial sources are notoriously unreliable. These reagents must be tested to determine specificity using a panel of monoclonal antibodies of known isotype and species origin as first reagents. Cross-reactions are most often encountered in multicolor staining systems where two first antibodies produced in different species (or different isotypes in the same species) are detected with species-specific (or isotype-specific) second antibodies that were labeled with different fluorochromes. Therefore, each second-step reagent should be tested for reactivity on both first reagents individually to test for specificity. Adding individual reagents at separate incubation steps and using a “cold block” (see Critical Parameters) to prevent inappropriate binding can reduce cross-reactivity.

A high fluorochrome (or biotin)/protein ratio in the conjugation can improve signal-to-noise ratios. The use of newer fluorescence probes, such as phycobiliproteins, offer higher levels of fluorescence and better emission characteristics and are more hydrophilic (which reduces nonspecific binding), thereby improving sensitivity in fluorescence assays.

Methods for conjugation with phycobiliproteins using sulfhydryl-maleimide linkages are presented in Support Protocol 4 (Duncan et al., 1983; Tanimori et al., 1983), although other linkages can be used (Kitagawa et al., 1981; Hashida et al., 1984; Blattler et al., 1985).

High backgrounds can be caused by a number of conditions. Autofluorescence from the

cell population often contributes background fluorescence and cultured cells can be especially troublesome. Autofluorescence can be reduced by using cells in an exponential growth phase, appropriate optical filters, and reagents that have been conjugated with red fluorochromes. Treatment *in vivo* or *in vitro* with drugs (some of which may fluoresce) or antibodies may increase nonspecific background. Short incubations of 3 to 4 hr at 37°C may be helpful for removing *in vivo* bound antibodies. Cell suspensions should be treated to remove dead cells because the latter promote aggregation and nonspecific absorption of labeled reagents, especially when large numbers are present. Residual dead cells can be excluded from the flow cytometric analysis by adding the propidium iodide solution as indicated in the basic protocol.

IgG antibodies can bind to Fc receptors regardless of their antigen specificity. This problem can be minimized by ultracentrifugation of antibody preparations to remove aggregates (e.g., using a Beckman Airfuge) 15 min at 100,000 × *g* just prior to staining. Binding to Fc receptors can also be prevented if antibodies are prepared as Fab or F(ab')₂ fragments (Porter, 1959; Parham, 1983), which removes the Fc portion of the IgG molecule. Another approach to reduce nonspecific binding to Fc receptors is to block the binding sites with unlabeled antibodies specific for Fc receptors or with an excess of serum. In this approach, care must be taken to ensure that labeled second antibodies do not recognize the blocking antibodies.

In some cases, stained samples may be fixed with dilute solutions of paraformaldehyde (Lanier and Warner, 1981), but this process can

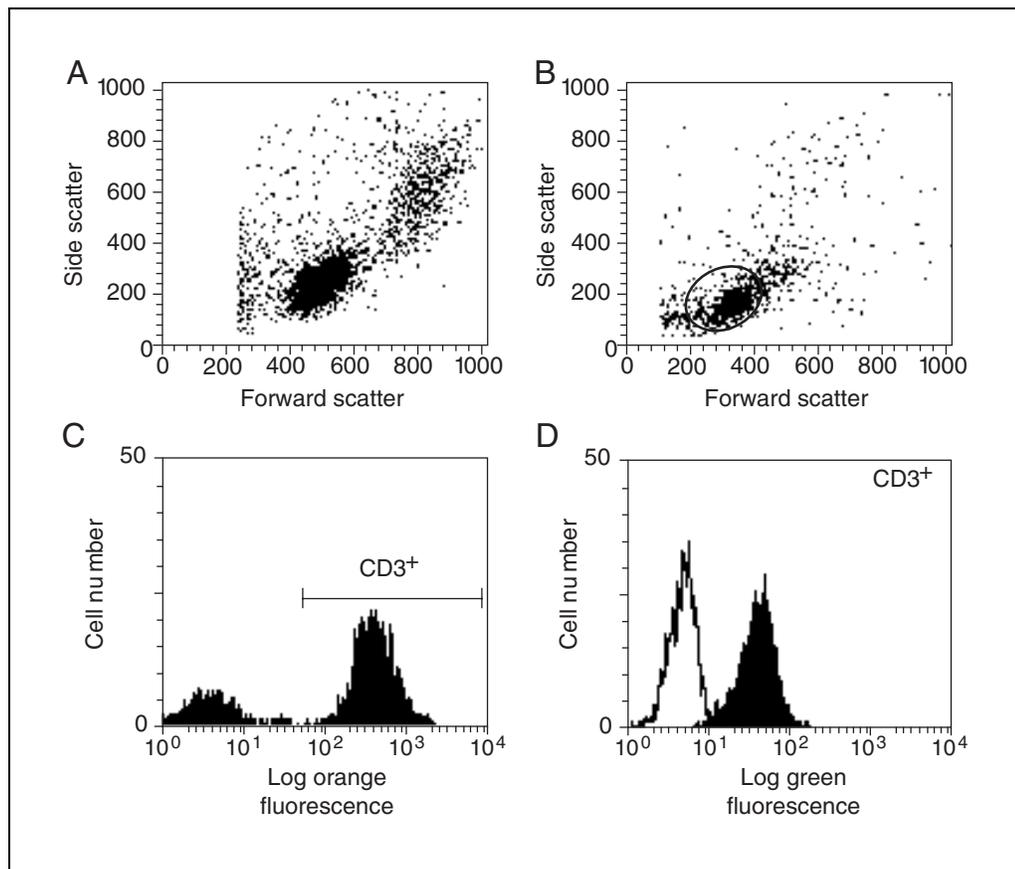


Figure 5.3.1 (A) Forward scatter (FSC) versus side scatter (SSC) dot plot of untreated peripheral blood mononuclear cells (PBMC). For panels B-D, PBMC were first stained for CD3 with a phycoerythrin (PE)-conjugated antibody. They were then fixed and permeabilized as described in Basic Protocol 2 and stained with either mouse IgG₁-FITC control or anti-Bcl-2-FITC monoclonal antibody (Dako). (B) FSC versus SSC dot plot with the gate set around lymphocytes. (C) Single-parameter histogram of CD3-PE fluorescence of cells within the scatter gate with a gate set on CD3⁺ cells. (D) Overlay of mouse IgG₁ fluorescence and Bcl-2 fluorescence of CD3⁺ cells within the scatter gate.

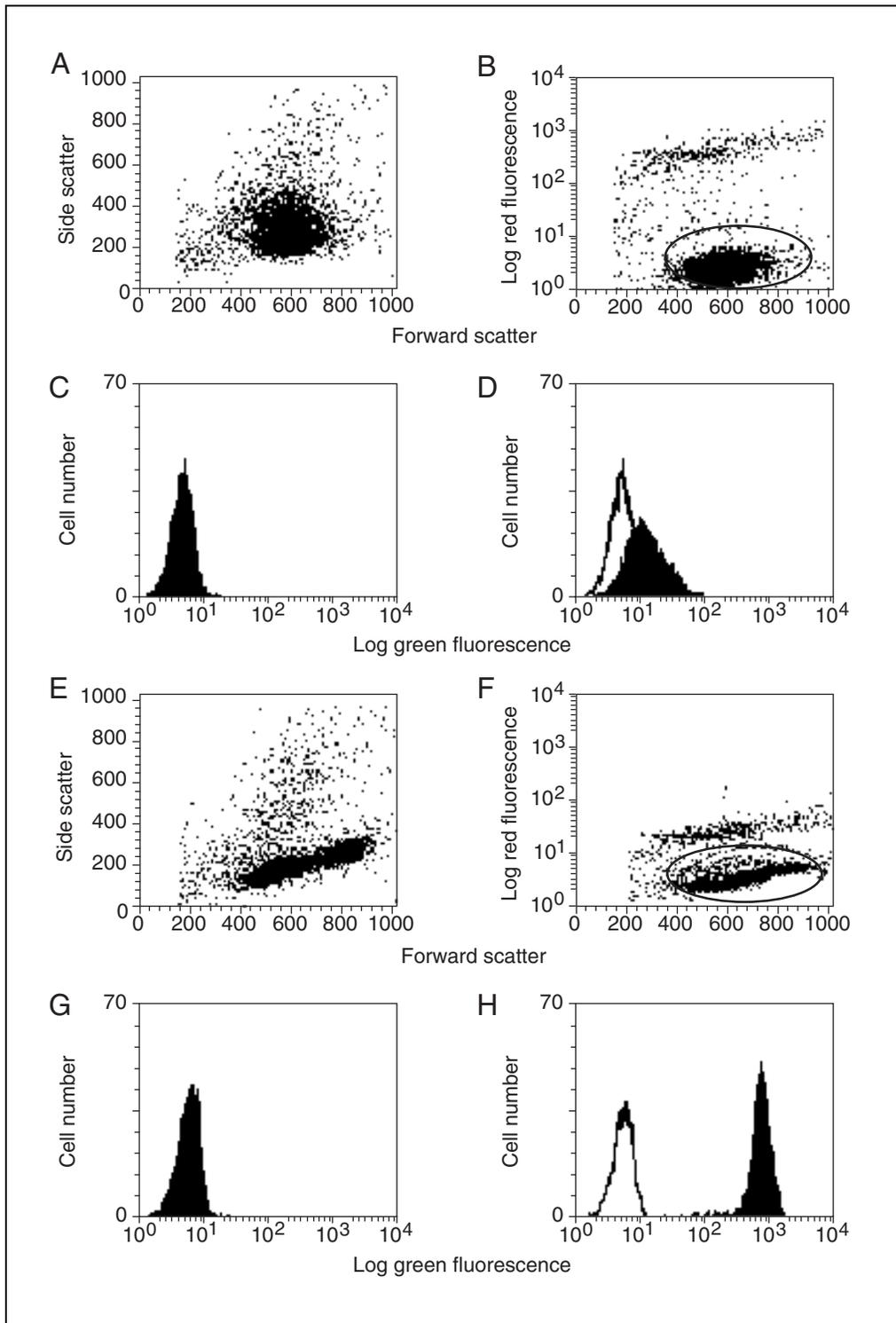


Figure 5.3.2 Human leukemic pre-T cells (SUP-T3) cell-surface stained with either mouse IgG₁-FITC control or CD3-FITC monoclonal antibody (panels A-D). 7-AAD was added for dead cell discrimination, where indicated, at a concentration of 1 $\mu\text{g}/\text{ml}$. (A) FSC versus SSC dot plot. (B) FSC versus 7-AAD fluorescence dot plot with the gate set around live cells. (C) Mouse IgG₁-FITC (background) fluorescence of cells within the gate. (D) Overlay of CD3-FITC fluorescence and background fluorescence of cells within the gate. Panels E-H: SUP-T3 cells treated with 7-AAD as described in Alternate Protocol 2 and fixed and permeabilized as described in Basic Protocol 2. Cells were stained with either mouse IgG₁-FITC control antibody or CD3-FITC. (E) FSC versus SSC dot plot. (F) FSC versus 7-AAD fluorescence dot plot with the gate set around live cells. (G) Mouse IgG₁-FITC (background) fluorescence of cells within the gate. (H) Overlay of CD3-FITC fluorescence and background fluorescence of cells within the gate.

increase the nonspecific background fluorescence. Aged solutions of paraformaldehyde can contribute to background fluorescence, and propidium iodide cannot be used to exclude dead cells once samples are fixed. It is preferable to analyze freshly stained live cells whenever possible. Obviously, this is not possible with biohazardous samples (UNIT 7.1).

Intracellular staining

When Basic Protocol 2 is performed with appropriate reagents and all the experimental and staining controls, the flow cytometric results should be reliable and quantitative. If intracellular staining is inadequate (i.e., no staining above background or only very dim staining in cells that are known to express the internal antigen) or if the localization of staining as verified by fluorescence microscopy is not consistent with the known location of the intracellular antigen, consider modifying the staining procedure (e.g., increasing the incubation time or temperature). If this is not successful, try changing the method of fixing and permeabilizing the cells. Increase the formaldehyde concentration to 1%, 2%, or 4%; use Triton X-100 or saponin as the detergent; use saponin without fixation; or use alcohol fixation. One of the many methods available should prove to be optimal for flow cytometric identification of cells expressing the intracellular antigen of interest (Clevenger and Shankey, 1993).

Anticipated Results

Data viewing and analysis will vary according to the exact set of parameters measured and the needs of the investigator. The results shown in Figures 5.3.1 and 5.3.2 can be considered fairly standard for detection of intracellular antigens. As for flow cytometric data analysis for surface immunophenotyping, cell samples are usually gated on forward versus side scatter to exclude debris and clumps and to focus on the cell type of interest. Alternatively, for exclusion of nonviable cells, a gate is set on forward scatter versus DNA fluorescence. Two examples are provided to illustrate analysis of intracellular antigen expression.

Figure 5.3.1 shows the measurement of expression of the mitochondrial protein Bcl-2 in T cells. Although the light scatter in the peripheral blood mononuclear cells was altered after fixation and permeabilization (Fig. 5.3.1B) compared to that of an untreated sample (Fig. 5.3.1A), it was possible to set a gate on lymphocytes. Then CD3⁺ T cells were identified

on a single-parameter histogram and their background and Bcl-2 fluorescence were displayed (Fig. 5.3.1D). More than 98% of the T cells expressed Bcl-2, a protooncogene which has been associated with cell protection from apoptosis. The presence of Bcl-2 in most normal T cells and B cells has been demonstrated previously (Aiello et al., 1992).

Figure 5.3.2 shows the results of an experiment in which a human leukemic pre-T cell line was first stained for surface expression of CD3. Cell viability was determined to be <80% by trypan blue staining. 7-AAD was added to exclude nonviable cells by flow cytometric analysis. Although discrimination of dead cells from live cells was not distinct on the FSC versus SSC dot plot (Fig. 5.3.2A), 7-AAD⁺ dead cells were clearly separated from 7-AAD⁻ live cells (Fig. 5.3.2B). These 7-AAD⁻ cells expressed only low levels of the CD3 antigen on the cell surface (Fig. 5.3.2D). In contrast, after fixation and permeabilization, a very high level of CD3 expression was detected in the pre-T cells (Fig. 5.3.2H), a finding consistent with previous reports of intracytoplasmic staining of CD3ε chains in T cells (Anderson et al., 1989). Again, a gate was set on 7-AAD⁻ cells, which were still clearly distinguishable from nonviable 7-AAD⁺ cells (Fig. 5.3.2F), despite loss of 7-AAD fluorescence in dead cells in the permeabilized cell preparation compared to unpermeabilized cells.

Time Considerations

Cell-surface staining. The time to perform Basic Protocol 1 should be ≤1 hr once a single-cell suspension is prepared. Multicolor staining procedures will be variable in length depending on the direct and indirect staining combinations (which determine the number of binding and wash steps).

Intracellular staining. After preparation of the single-cell suspension, Basic Protocol 2 can be completed in ~2 to 3 hr, depending on how long the cells are kept in the fixing solution (30 min to 1 hr) and whether a direct staining or indirect staining procedure was performed. The time needed for adequate fixation (at least 30 min) and permeabilization (15 min) will be advantageous for processing larger numbers of samples simultaneously and uniformly. Additional time is needed for surface staining (~30 min) prior to intracellular staining and for DNA staining (~30 min).

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Key References

- Clevenger and Shankey, 1993. See above.
- In-depth discussion of background information and excellent review of intracellular staining methods for flow cytometry and their application.*
- Jacob et al., 1991. See above.
- Describes Alternate Protocol 1 and shows examples of its application.*
- Schmid et al., 1991. See above.
- Describes Basic Protocol 2 and provides examples of its application.*

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