

Fluorochrome Production

Production of antibodies to specific antigens requires a complex process that starts with the immunization of an animal with an antigen. The animal will begin to produce antibodies to the foreign antigen and the spleen will fill with cells producing antibodies. After a certain period, the animal is sacrificed and the cells of the spleen are harvested.

The cells undergo a process called fusion where they are mixed with a cancer-derived immortalized cell line. A substance such as polyethylene glycol is added, which disrupts the cell membranes. This causes some of the cell line cells to fuse with some of the spleen cells. These cells are diluted in multiple-well plates and later screened to assess antibody production. The successfully fused cells now have the ability to produce antibody and are also immortalized so that they can continue to grow and produce antibody. Once a suitable cell line is chosen, the antibody is produced on a larger scale and may be purified before the final step, which is conjugation with a fluorochrome for use in flow cytometry.

Sample Preparation

The most important rule when preparing a sample for analysis by flow cytometry is to have as few clumps and as little debris as possi-

ble. Samples from cultured cells present some problems such as cell line cross-contamination, cell cultures that have degenerated too quickly, and *Mycoplasma* contamination. Cultured cells that are adherent should be removed from the flask or dish using enzymes that do not cause an alteration of surface antigens. Using a sample from tissue can be challenging because the cells must be removed using physical manipulation, by incubation with an enzyme, or by a combination of both.¹ If cells from a blood source are to be examined, red cells need to be lysed with ammonium chloride or other commercially available lysing reagent.

When labeling cell surface antigens, fresh, unfixed cells are incubated with the fluorescently labeled antibody. Sometimes the cells are fixed after the labeling of the cell surface, especially if an intracellular antigen is to be analyzed. For intracellular nuclear or cytoplasmic antigens, the cells must be permeabilized, which allows the stain to enter the cell. Several antigens are adversely affected by some fixatives, and for this reason the procedure must be optimized for each protein being studied. Several methods are used to permeabilize cells, including the use of fixatives, alcohols, or detergents. Resources for procedural recommendations are available from antibody or permeabilization buffer manufacturers.

Direct Cell Labeling

Cells can be stained or labeled with the use of a direct or an indirect method. In the direct method (by far the most common) the fluorochrome-labeled antibody is simply incubated with the cells. Typically, a wash step after the incubation period removes any unbound antibody. Samples containing red cells should be lysed with ammonium chloride or other commercially available lysing reagent.

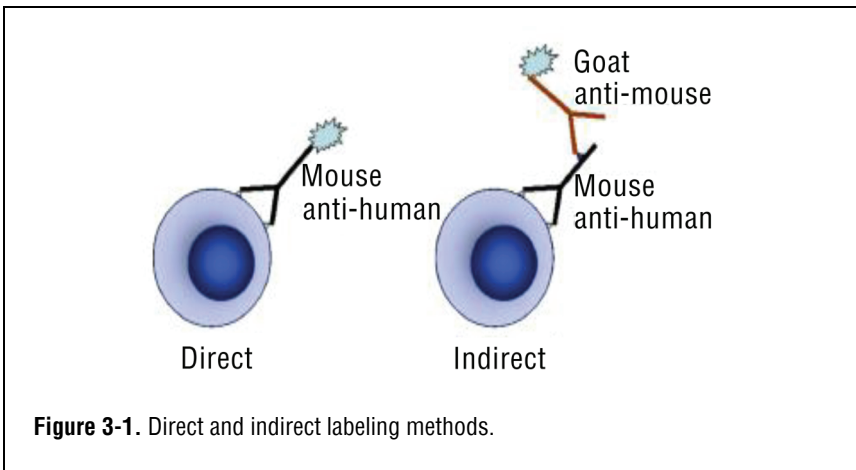
Indirect Cell Labeling

In indirect cell labeling, the cells are incubated with a primary antibody that does not have a fluorochrome and then washed. Next, they are incubated with a secondary antibody that does carry a fluorochrome and attaches to the primary antibody. Primary and secondary antibodies must be chosen carefully to ensure that they

are compatible. Figure 3-1 illustrates the difference between the direct and indirect staining methods.

Multiple antibody indirect labeling can be challenging because many primary/antigen-specific antibodies are derived from mouse monoclonals, and many secondary antibodies are directed against a species, for example goat anti-mouse. In this case, even if several different primary antibodies were used, they all would stain positive with the secondary antibody. Specific procedural recommendations for direct and indirect staining are available from fluorochrome manufacturers.

When performing surface and intracellular staining on the same sample, the surface staining is usually conducted before fixation and permeabilization. There are some antigens on the cell surface that can survive the permeabilization process, so they can be stained at the same time as the intracellular



antibody, keeping in mind that the labeling will no longer be specific to the antigens on the surface of the cell.

Selecting the Proper Concentration of an Antibody

Manufacturers often suggest the appropriate fluorochrome concentration for staining; however, it may not be optimal for every sample or procedure. The fluorochrome may be titrated to determine the optimal amount required for a given application. The titrating can be performed using serial dilutions and staining of cells known to be positive for the given antigen. The user can start with the recommended stain-

ing concentration from the manufacturer as "X" and then perform serial dilutions higher and lower than the recommendation (ie, $\frac{1}{4}X$, $\frac{1}{2}X$, X, 2X, 4X). Often the manufacturers' staining recommendations are stronger than necessary, so the titrating can also help conserve on the cost of these reagents. If an isotope control is needed, it is important to include it in the titrating optimization to define the negative and positive staining populations. Figure 3-2 shows a serial dilution vs fluorescence titrating curve. To quantify the number of antigenic sites on a cell, the use of an antibody close to the saturation point is essential. The saturation point means that every antigen site is labeled with the fluorescent antibody.

