Cells in the Microscope: Immunofluorescent labeling

Goal for the module: To visualize cellular components in the microscope using the technique of indirect immunofluorescence.

Introduction: The technique of immunofluorescence takes advantage of the remarkable specificity of antibodies to localize a particular molecule, the antigen, in cells and in tissues. In this method, the antigen, which can be almost any cellular component, is localized in its native location in a fixed specimen. First, you need to obtain antibodies that recognize the antigen. Antibodies are serum glycoproteins that are generated by cells of the immune system in response to a foreign substance, the antigen. Antibodies bind to antigens by their variable regions (see diagram). The most common class of antibodies is called IgG (immunoglobulin G). In many instances, antibodies can be obtained from a commercial source; in other cases the investigator produces the antibodies in the lab. To do this, a preparation of the purified antigen is injected into a suitable host animal, often a rabbit. Over time, the animal launches an immune response, and antibodies can be collected from the serum, and purified. The fixed cells are then incubated with the antibodies, rinsed and the antibodies localized. In direct antibody staining, the antibodies that recognize the antigen, called the primary antibody, are labeled with a fluorescent dye. However, antibodies are expensive, and often in short supply, and labeling them with a dye is an inefficient process. To overcome these limitations, an approach called Indirect Immunofluorescence is typically used. In this method, the primary antibody is unlabeled. It is localized in a second step, by using secondary antibodies that recognize the primary antibodies. Secondary antibodies are produced by immunizing a host animal with antibodies from a different species. For example, the primary antibody that you will use was made in a rat. To make secondary antibodies to use with this primary antibody, the rat IgGs are injected into a goat. The goat will generate antibodies to the rat IgGs, most of which will recognize the Fc portion of the primary antibody (see diagram). These secondary antibodies, called goat anti-rat antibodies, are purified and labeled with a fluorescent dye. An advantage of this method is that several secondary antibody molecules can bind to the primary antibody, so the signal is amplified. Another advantage is that
secondary antibodies labeled with different dyes are readily available, facilitating double labeling experiments.

**Figure 1: antibody structure**

![Antibody structure diagram](image)

**Figure 2: Indirect Immunofluorescence**

![Indirect Immunofluorescence diagram](image)

In this lab, you will perform immunofluorescence, and image the stained cells in the microscope.

**Reagents:**
- Coverslips of fixed cells
- Fine forceps
- Small beakers
- Humid chamber
- Glass slides
- Mounting media
- Ice bucket
- Staining reagents, pipettors
Procedure:

A. Indirect Immunofluorescence: Tubulin

1. Obtain a coverslip of fibroblasts that have been fixed for you. Take an aliquot of anti-tubulin primary antibody (already diluted and ready for use). These antibodies were raised in a rat.

2. Make a humid chamber, and pipette 50 ul of primary antibody onto the parafilm. Invert the coverslip onto the drop, and incubate for 60 minutes at 37 C.

3. When the incubation is nearly over, make another humid chamber, and place a 50 ul drops of secondary antibody (goat anti-rat FITC) on the parafilm. Cover with a lid that is foil covered (secondary antibodies are light sensitive).

4. When the first incubation is done, remove the coverslip from the primary antibody, rinse 3 X in PBS-Tw-Az by dunking 10 times in each beaker, and invert the coverslip on the drop of secondary antibody in the humid chamber. Incubate at 37 C for 30 minutes. Rinse again, and mount the coverslip on drop of Vectashield mounting media that contains DAPI, using a clean glass slide that has been labeled with your name, date and the experiment. This coverslip is now labeled for microtubules and DNA.

B. Indirect Immunofluorescence: Part II

During the incubation with primary antibody, perform indirect immunofluorescence with an antibody that recognizes a different antigen (your-favorite-antigen). You can choose to stain either: gamma tubulin, a protein of the Golgi apparatus, or cell-cell junctions. Use a coverslip of epithelial cells that have been fixed for you. Start this experiment while the first coverslip (from part A) is incubating. Each of these antibodies was raised in mice. The secondary antibody is goat-anti-mouse FITC. When the antibody incubations are done, mount the coverslip in Vectashield containing DAPI. This coverslip is now labeled for your-favorite-antigen and DNA.

C. Control Experiments

It is important to verify that the antibodies that you are using are specific for the antigen you wish to study.

1. No primary antibody. To demonstrate that the staining that you see results from a specific interaction of the primary antibody with the antigen, you can omit the primary antibody from the first incubation and use only the secondary antibody. One group can perform this control for the tubulin antibodies.

Note: because the antibodies that you will use have been well characterized, you need not perform these controls (2 & 3).

2. Preadsorption. To do this control, the antigen is incubated with the antibodies, allowed to incubate and spun to remove antibody-antigen complexes. The resulting supernatant, the preadsorbed serum, is then used in place of the primary antibody. What might you expect to observe? Why is this an important control experiment?

3. Dilution series. Antibodies are highly specific, but can bind to non-specific sites in the tissue or cells that you wish to study. To circumvent this problem you can (1) use the lowest dilution of the primary antibody that results in staining. This dilution is determined empirically for each batch of antibody. (2) you can pre-incubate the cells or tissue in a
solution of protein that will bind non-specifically to “sticky” sites in the cells. This solution is called the blocking solution or “block”. We diluted the primary antibodies into bovine serum albumin (BSA, 1mg/ml) this functions as the block.

D. Fixation

While your coverslips are incubating in primary antibody, you will learn how to fix cells for immunofluorescence. It will be easiest if one group at a time uses the fume hood downstairs.

   a. obtain a coverslip of live cells in a small Petri dish of growth media.

   b. remove the media: hold the dish at an angle and pipette the media from the corner of the dish.

   c. using the same method of holding the dish at an angle, rinse the coverslip by adding PBS (warm), and removing with a clean Pasteur pipette. Repeat twice. Be gentle: if you pipette directly on the coverslip of live cells, they can be dislodged from the coverslip.

   d. IN THE FUME HOOD, and WEARING GLOVES, remove the last rinse of PBS and add 1 ml of Extraction buffer, count to 10, and remove. Now add 1 ml of fixative (already prepared for you) to the dish containing the rinsed coverslip of cells. Swirl gently. Fix for 5 minutes.

   e. remove the coverslip with your fine forceps and dunk in 3 beakers of PBS-Tw-Az, in the hood. Place the coverslip of fixed cells in a clean 35mm Petri dish with PBS-Tw-Az. Dispose of the fixative in the proper container in the hood.

   f. the coverslip is now ready for staining. Given time constraints, we will have to save these coverslips to stain on another day. Put the coverslip in a clean 35mm Petri dish, wrap some parafilm around the edge to prevent evaporation, and store in the refrigerator.

Data collection and analysis:

1. Obtain paired images of the cells in phase and the microtubules in fluorescence at various magnifications. Include a marker bar in each image. What is the organization of microtubules in cells? Is the organization the same in all fibroblasts and epithelial cells (you observed microtubules in epithelial cells previously)? Document your findings. Is the staining similar in all areas of the coverslip? If not, what might be the reason?

2. Experiment with settings of the camera/software so as to obtain the best images of the microtubules.

3. Take preparations that are stained for a different antigen. Collect paired fluorescence and phase images. What is the location of this antigen? Collect sufficient data so that you can be sure that you can describe the organization.