**In-Person Immunocytochemistry Lab Manual Instructions**

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## Laboratory Exercise #8 Indirect Immunohistochemistry: Identifying Purkinje cells in the brain

**Learning goals:**

To learn about the most common applications for immunohistochemistry and immunocytochemistry

To perform immunohistochemical staining of tissue sections or neuronal cultures to reveal the Purkinje neurons in the developing rodent cerebellum

To perform chemical staining with Hoechst 33258 to reveal cell nuclei

**Background:**

**Immunohistochemistry** is the use of antibodies to stain histological sections of tissues. **Immunocytochemistry** is the use of antibodies to stain cultured cells. Antibodies in these types of experiments may be used to distinguish particular cell types from the other cells in the tissue or culture of interest, or to reveal specific subcellular structures such as the nuclear lamina, actin fibers, or organelles.

**Antibodies** are glycoproteins (proteins with sugars attached) that are made by vertebrates in response to exposure to a substance or chemical structure that is not normally found in that particular animal. The foreign substance that triggers production of the antibodies is called the **antigen**. Antibody molecules have a globular "Y" shape, with two sites that can bind specifically to the antigen, one at each tip of the "Y". The stem of the "Y" is called the constant region; it does not bind to the antigen, but is used by the animal to carry out several different functions, depending on its structure. For example, some constant regions target the bound antigen to be gobbled up by particular types of immune system cells; other constant regions target the bound antigen/pathogen to have holes poked in its membrane by still other components of the immune system. We can take advantage of the constant region as a "tag" in immunohistochemical procedures.

image removed due to copyright. Figure 1-16 from Janeway's Immunobiology (2005), Garland Science. Shows antibody structure.

Immunohistochemistry and immunocytochemistry are both designed to give **spatial** information: **Where** is this antigen within the cell/within the organ? And What happens to its localization after experimental manipulations or during development? Immunohistochemistry and immunocytochemistry are critical techniques in cell biology and developmental biology. Even for biochemistry and molecular biology, one of the first questions a scientist asks after cloning a new gene is, Where is its protein product expressed? Many disease states are the result of mis-localization of a mutant protein to an intracellular location where the protein function is inappropriate, or to a location where the protein cannot gain access to needed substrates or accessory molecules. In these cases, understanding the disease process hinges on understanding the intracellular localization of the protein, and immunocytochemistry is the method of choice.

When studying an intracellular antigen such as Calbindin-D28k, it is necessary to open some holes or pores in the cell membrane to allow antibodies to get in. This can be done by extracting the fixed tissue for 5 minutes in minus 20 degree C methanol, followed by 10-20 seconds in minus 20 degree acetone, followed by 15 minutes soaking on TBS to re-hydrate the tissue. Alternatively, no preliminary extraction is performed, but a dilute gentle detergent such as **Triton-X-100** can be included in the blocking and binding solutions to poke some holes in the membrane to allow antibody to enter the cells. This is the method of choice for staining calbindin in neurons.

In today's immunohistochemistry experiment, you will be given either thin sections of mouse brains or cultures of mouse brain cells on glass slides, and you will determine the distribution and morphology of the **Purkinje neurons** in the **cerebellum** at several different times during development. Purkinje neurons are the sole output neuron in the cerebellum, a brain structure that contributes to coordination of locomotion, balance, and the eye movements that you are making right now to read this Background information. **Calbindin-D28k** is a marker for Purkinje cells: within the cerebellum, only the Purkinje cells and their processes express this protein (although calbindin is also found in other parts of the brain). You will work with a primary polyclonal antibody raised in rabbits against calbindin, and with a goat anti-rabbit-fluorochrome-conjugated secondary antibody that will bind to the primary antibody. This combination of an invisible primary antibody and a secondary antibody that is bound to a detectable fluorochrome makes this an **indirect immunofluorescence assay**. (In direct immunofluorescence, the fluorochrome would be bound to the primary antibody, not to the secondary antibody, making the secondary antibody unnecessary). Antibody binding will be visualized via the fluorescence of the fluorochrome: in this case, Alexa 488, which fluoresces green. We will also take advantage of a fluorescent chemical counterstain, Hoechst 33258, which glows blue when bound to DNA. After completing the staining procedure, you will observe your slides through the microscope and note the locations and shapes of the Purkinje neurons.

A procedural note: It is customary in immunohistochemistry to include a no-primary-antibody control in the experimental design. It is also common practice to set up a range of primary antibody concentrations in order to optimize the specificity of the staining: too much antibody, and it will stick all over the sections, not necessarily only on its proper antigen. Too little antibody, and no staining will be detected. Usually a 3-4 log range of dilutions is used in an initial pilot experiment to determine the optimal conditions.

To allow visualization of cell nuclei, you will also stain the DNA with a chemical dye called Hoechst 33258. This dye will fluoresce blue upon DNA binding. This counterstain will permit you to observe the distribution of cell nuclei across the mouse cerebellar tissue sections.

**Procedure:**

**Summary of procedure:**

1. **Block nonspecific protein binding sites**
2. **Bind the primary antibody**
3. **Wash**
4. **Bind the secondary antibody**
5. **Wash**
6. **coverslip the slides and observe your results**

Before you begin the staining:

If you have thin sections of brain on a glass microscope slide, then using a PAP pen or other grease pen, draw a circle around the tissue sections to be stained. This circle will act as a corral to keep antibody solutions over the section and to prevent them from running off the slide.

If you have cultures of brain cells in multiwell slides, the silicone gaskets between the wells will serve to corral your antibody solutions, so no PAP pen or grease pen will be necessary.

You will be given slides in mid-staining. They will already have been blocked and have bound primary antibody overnight, and will be in one of the washes in step 3. Proceed from that step:

1. Block: tok-PBS (phosphate-buffered saline with potassium chloride) with
   10% normal goat serum, and
   If you have thin sections of brain: 0.2% triton-X-100, but
   If you have cultures of brain cells: 0.05% triton-X-100
   Incubate at least 1 hour at room temperature, humidified chamber

2. Primary Antibody: tok-PBS with
   1% normal goat serum, and
   If you have thin sections of brain: 0.2% triton-X-100, but
   If you have cultures of brain cells: 0.05% triton-X-100

   1:3000 final dilution of rabbit anti-calbindin antibody

   Incubate overnight at 4°C, humidified chamber

3. Wash: 3 times, with tok PBS in a large staining dish or Coplin jar
   If you have thin sections of brain: 20 minutes per wash
   If you have cultures of brain cells: 5-10 minutes per wash

4. Secondary Antibody: tok-PBS with
   1% normal goat serum, and
   1:5000 final dilution of Hoechst 33258 (you will be provided with a 1:100 preliminary dilution)
   If you have thin sections of brain: 0.2% triton-X-100, but
   If you have cultures of brain cells: 0.05% triton-X-100

   If performing non-fluorescent detection, add:
   1:1000 final dilution of Jackson Immunoresearch goat anti-

rabbit IgG-peroxidase conjugate
but if performing fluorescent detection, add:
1:3000 final dilution of goat anti-rabbit IgG Alexa 488 conjugate

Incubate room temperature 1 hour, humidified chamber

5. Note: For the rest of the procedure, protect your samples from the fluorescent room lights as much as possible, using aluminum foil tents and minimal room lighting.
Exposure to fluorescent light will fade your Hoechst 33258 and Alexa 488!

Wash: 3 times, with tok PBS, in a large staining dish
If you have thin sections of brain: 20 minutes per wash
If you have cultures of brain cells: 5-10 minutes per wash

6. For non-fluorescent detection methods only (if you're doing fluorescent detection, skip to step 8!):
Add substrate solution: For each 1 ml of substrate, mix together:

| | |
|---|---|
| 1 ml | TBS |
| 10 ul | diaminobenzidine 50 mg/ml stock |
| 6 ul | 3% hydrogen peroxide |

Incubate room temperature 3-5 minutes; monitor with microscope if available

NOTE: DAB is a carcinogen and should be handled ONLY WITH GLOVES! SAVE YOUR DAB WASTE FOR PROPER DISPOSAL.

7. When the color reaction is satisfactory, stop the reaction by removing the DAB solution back into its mixing tube, and flood the section with TBS. Discard this TBS rinse into the DAB waste container, and flood the section again with TBS.

8. For cultured cells on Lab-Tek wells: very carefully remove the silicon gasket using a razor and forceps.
For tissue sections with PAP pen "corrals": Rinse the section in distilled water. For all slides: Coverslip using Aquamount (your instructor will demonstrate how this is done). Allow the mounted slides to dry overnight, and seal the edges with nail polish, before observing on the microscope.

**Review Questions:**

1. What is the function of the blocking step?

2. What is the function of the washes?

3. What is the function of the triton-X-100? Hint: it is a detergent.

4. What is the function of the goat anti-rabbit Alexa 488-conjugated secondary antibody?

5. What does the Hoechst 33258 bind to/what intracellular structures does it reveal?

6. Choose 3 different timepoints during Purkinje cell development and describe how their morphology changes between each timepoint. Use images from the microscope to create figures for your report. Use arrows on those images to help guide the reader to the features you discuss in your text.

**References:**

Harlow E, Lane D (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Janeway CA, Travers P, Walport M, Shlomchik MJ (2004) Immunobiology: The immune system in health and disease (6th edition). New York: Garland Science.

# Laboratory Exercise #9. Fluorescence Microscopy

**Learning goals:**

To understand the principles behind fluorescence microscopy.

To use a research-grade fluorescent microscope to collect images of histological sections or cultured neurons with fluorescently-labeled antibodies, or cell nuclei stained with Hoechst 33258.

Note: This lab instruction set will be greatly enhanced if you can print it out in color!

**Background:**

## A. Principles of Light Microscopy and Fluorescence Microscopy

Fluorescence microscopy is one of the most useful—and beautiful!—microscopy techniques available to the life scientist. It can be used to visualize single or multiple different types of molecules within biological specimens. Adapted for fluorescence resonance energy transfer (FRET), it can be used to document the most intimate interactions between individual molecules. It is a powerful and inspiring tool in the immunologist's repertoire.

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**Figure 1. Sample fluorescence microscopy images.**
A, A dividing cell labeled with three different fluorochromes. (Alberts et al. 2008 p. 587).
B, HeLa cells stained with anti-vimentin and a flourescein-conjugated secondary antibody. From Andrew J. Willouer, Lycoming College Cell Biology student.

## A1. A sense of scale

Fluorescence microscopy is a special application of light microscopy, because this type of microscope is designed to use and detect light waves. Because of this, the technique can only be used to visualize objects similar in size to the light waves. Figure 2 shows the resolving power of different types of microscopy. Objects as small as protein polymers within cells (like microtubules, vimentin filaments, and common cytoplasmic proteins) are easily visualized using fluorescence microscopy.

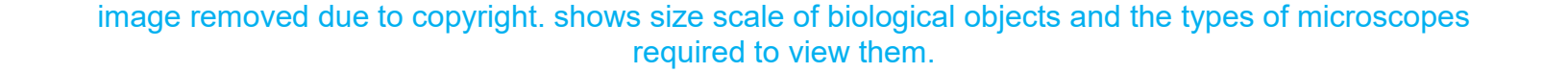


**Figure 2. Resolving power of different types of microscopy.**
Fluorescence microscopy is a subtype of light microscopy, and therefore can be used to visualize objects in the same size range as regular light microscopy. (Alberts et al. 2008, p. 581).
um (micrometer) – $10^{-6}$ m; nm (nanometer) – $10^{-9}$ m; A (Angstrom unit) – $10^{-10}$ m

## A2. Follow the light path

Figure 3A shows a typical research-grade light microscope in use. The light path used in this type of microscopy is diagrammed in Figure 3B. Notice that the light comes from a lamp beneath the stage and is focused onto the specimen by the lenses in the condenser. Then light is focused onto the eye of the observer by a combination of lenses in the objective and eyepieces. The light source

in this case is a modified, higher-intensity version of the common light bulb. Note that in this type of microscopy, the light is passing through the objectives in one direction only.

Figure 4 shows the light path in a typical fluorescence microscope. The fluorescent light comes from a special bulb containing an inert gas, such as mercury. An arc lamp is used to excite the mercury, which emits fluorescence across a variety of wavelengths. The fluorescent light passes through a barrier filter which can be chosen to select only certain wavelengths. A beam-splitting or **dichroic mirror** is used to deflect the fluorescent light through the objective and down onto the specimen. The light hits the specimen, and excites any fluorochrome that is sensitive to its particular wavelength. The fluorochrome emits light of a slightly longer (lower-energy) wavelength than the excitation light, which then bounces back up through the objective lens, the dichroic mirror, and a second barrier filter to eliminate undesired wavelengths. The lenses in the eyepiece focus the emitted fluorescent light up onto the eye of the observer.

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**Figure 3. Light microscope.**
A, A research-grade light microscope. B, Diagram of the light path in a compound microscope. (Alberts et al. 2008, p. 581).


**Figure 4. Diagram of the light path in a fluorescence microscope.**
Note the filter set: 2 barrier filters (labeled 1 and 3) and a dichroic (beam-splitting) mirror (labeled 2). This diagram illustrates the wavelengths of filters needed to visualize the fluorochrome fluorescein. (Alberts et al. 2008, p. 586).

**B. Visualizing molecules in biological specimens**

There are a few fluorescent molecules that specifically bind to particular partner molecules in a cell. DAPI is one of these molecules—it binds to DNA and fluoresces blue when DNA is bound. Likewise, Hoechst 33258 binds to DNA and fluoresces blue.

For most molecules biologists want to study, there is no fluorochrome that can be used to label them directly. In these cases, the biologist would usually use an **antibody**—a protein made by the immune system that specifically recognizes a target, called its **antigen**. Antibodies are made by injecting an animal with the desired antigen, then waiting for the animal to mount an immune response. After this time, the animal's blood can be drawn and tested to see if it contains antibodies of the desired specificity. These kinds of antibodies are called **polyclonal** antibodies, because the animals' blood would typically contain a wide variety (many clones = polyclonal) of antibodies against many different antigens, depending on the infections or substances to which the animal had been exposed.

In some cases, it is possible to remove the spleen from an immunized animal and fuse its cells with an immortalized myeloma cell line. This creates a **hybridoma**—a hybrid cell that has the antibody-secreting properties of the immunized animal's B cell and also the immortality of the myeloma parent cell. A panel of hybridoma lines can then be screened to identify those cells that secrete antibody against one particular antigen. These are called **monoclonal** antibodies, because they only react against one antigen.

Either polyclonal or monoclonal antibodies can be used for immunocytochemistry. Figure 5 shows how immunocytochemistry works. The antigen, usually in a cultured cell or tissue section mounted on a glass microscope slide, binds the **primary antibody**. Unbound primary antibody is washed off. Then a **secondary antibody** that recognizes the primary antibody is added. The secondary antibody can be bound or **conjugated** to a fluorochrome or to an enzyme to aid in visualization. Unbound secondary antibody is washed off, and the slide is coverslipped in preparation for imaging on the microscope.

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**Figure 5. Indirect immunocytochemistry.**
This is a very sensitive detection method, because multiple molecules of secondary antibody with the fluorochrome attached can bind to each primary antibody, amplifying the fluorescent signal. (Alberts et al. 2008, p. 589).

Figure 6A shows the excitation and emission wavelengths for the most commonly used fluorochromes. **You need to know the excitation and emission wavelengths that are appropriate for the fluorochrome you are using in order to set up the microscope filter sets properly to see the fluorescence.** Note that red light has a longer wavelength than violet light. Also note that the excitation light always has a shorter wavelength (higher energy) than tha emission light (lower energy) for any given fluorochrome. Figure 6B shows a beautiful example of multiple fluorescence imaging.

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**Figure 6. Fluorescent probes.**
A, Excitation and emission wavelengths for commonly used fluorescent probes are shown. DAPI or Hoechst 33258 are commonly used to stain DNA blue. FITC is fluorescein isothiocyanate, a green fluorochrome. Cy3 is a commonly used red flourochrome.
B, multiple fluorescent probe microscopy of a dividing cell. green = spindle microtubules visualized with FITC, red = centromeres visualized with Cy3, blue = DNA stained with DAPI.
(Alberts et al. 2008, p. 587).

**Procedure**

You will go to the Nikon TE2000 inverted fluorescence microscope room and/or work on a student-grade Nikon E200 fluorescent microscope for today's lab work. Dr. Morrison will help you to identify each of the parts of the microscope, referring to Figure 4 above.

You will use the Nikon microscopes to examine your slides of cerebellar sections or cultured cerebellar cells stained with fluorochrome-conjugated antibodies and/or Hoechst 33258.

If you are having difficulty obtaining an image, remember to trace the light path and make sure the correct filter sets and shutter positions are selected along the way.

Remember to save your images in a format that will allow you to insert them into a Microsoft Word document.

**Review Questions:**

1. What reagents and controls are needed for an indirect immunofluorescence assay?

2. What do the washing steps accomplish?

3. What does the Hoechst 33258 counterstain accomplish/make visible?

4. What does the green color indicate for this assay?

5. Why do you need different light filter sets to view the Hoechst 33258 stain and the anti-calbindin/Alexa 488 stains in this assay?

6. Compare and contrast the light paths for a regular light microscope and for a fluorescent microscope.

7. What is the purpose of the barrier filters and dichroic mirror in the fluorescent microscope?

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**References:**

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2008. Molecular Biology of the Cell, fifth ed. Garland Science Publishers, New York, p.581-589.

Harlow, E., Lane, D., 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Janeway, C.A., Travers, P., Walport, M., Shlomchik, M.J., 2004. Immunobiology: The immune system in health and disease, sixth ed. Garland Science, New York.