Chapter 5

Immunofluorescence of Cytoskeletal Proteins

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Mordacq, J. C., and R. W. Ellington. 1996. Immunofluorescence of cytoskeletal proteins. Pages 85-97, *in* Tested studies for laboratory teaching, Volume 17 (J. C. Glase, Editor). Proceedings of the 17th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 255 pages.

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Introduction

This laboratory exercise is used at Northwestern for the introductory biology sequence during the quarter that covers the topics of Cell Biology and Physiology. This is the last quarter of the introductory biology sequence. The majority of the students take this course during their sophomore year and most are either biology majors or students taking the pre-medicine curriculum. The students are taught in groups of 24 and work in pairs. This laboratory teaches the students the technique of immunofluorescence. This is a powerful technique commonly used in research and this exercise emphasizes the significance of being able to localize a molecule within a cell.

We have used a modified version of this laboratory that incorporates a second experimental specimen: bovine sperm cells can be used in addition to mouse 3T3 fibroblast cells. This modification can easily be made using the laboratory exercise written by Beth Retallack which is found in the proceedings from the 1994 ABLE meeting at Emory University (Retallack, 1995). This allows the students to see the cytoskeletal matrix in two different cell types and provides diversity within the laboratory so that most students are utilizing different conditions. This is a relatively inexpensive laboratory to run provided that you have a very expensive piece of equipment (fluorescent microscope). The students can easily see all of the different conditions with one microscope and a high resolution video camera.

Student Outline

Introduction

In this exercise, you will become familiar with a powerful technique commonly used in cell biology. This technique, called immunofluorescence, is used to detect and localize specific proteins or molecules within a cell. It is made possible by using antibody molecules that selectively recognize and bind to specific molecules (antigens). In addition to being useful for this technique, antibody-antigen interactions are utilized in several other common procedures, such as affinity chromatography and enzyme-linked immunoassays. Affinity chromatography is accomplished by chemically linking an antibody to a plastic resin which is then used to create a column much like the ones you used in B10-2. A protein solution is applied to the column, and only the protein to which the antibody is made (antigen) binds to the column. A solution of lower pH is then added to the column and the pure antigen elutes. In an enzyme-linked immunoassay, an antibody coated solid is incubated with the antigen solution to be assayed. An antibody linked to a enzyme marker is added to the mixture followed by the addition of a colored substrate for the marker. The result is a color producing reaction in which the amount of color is proportional to the amount of antigen present.

This technique is frequently used in a clinical medicine setting for the detection of proteins associated with disease such as infection with the HIV virus.

Fluorescence

The two most commonly used fluorescent dyes are fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (rhodamine). These molecules can be excited to a higher energy state by a wavelength of light. When the excited molecule drops back to its ground or normal energy state, excess energy is released in the form of light. It is this emission of light that results in fluorescence. Fluorescent molecules have characteristic excitation and emission wavelengths. FITC has an excitation maximum of approximately 495 nanometers (nm) and an emission maximum of 517 nm (green light). Rhodamine has an excitation maximum of 550 nm and an emission maximum of 580 nm (red light).

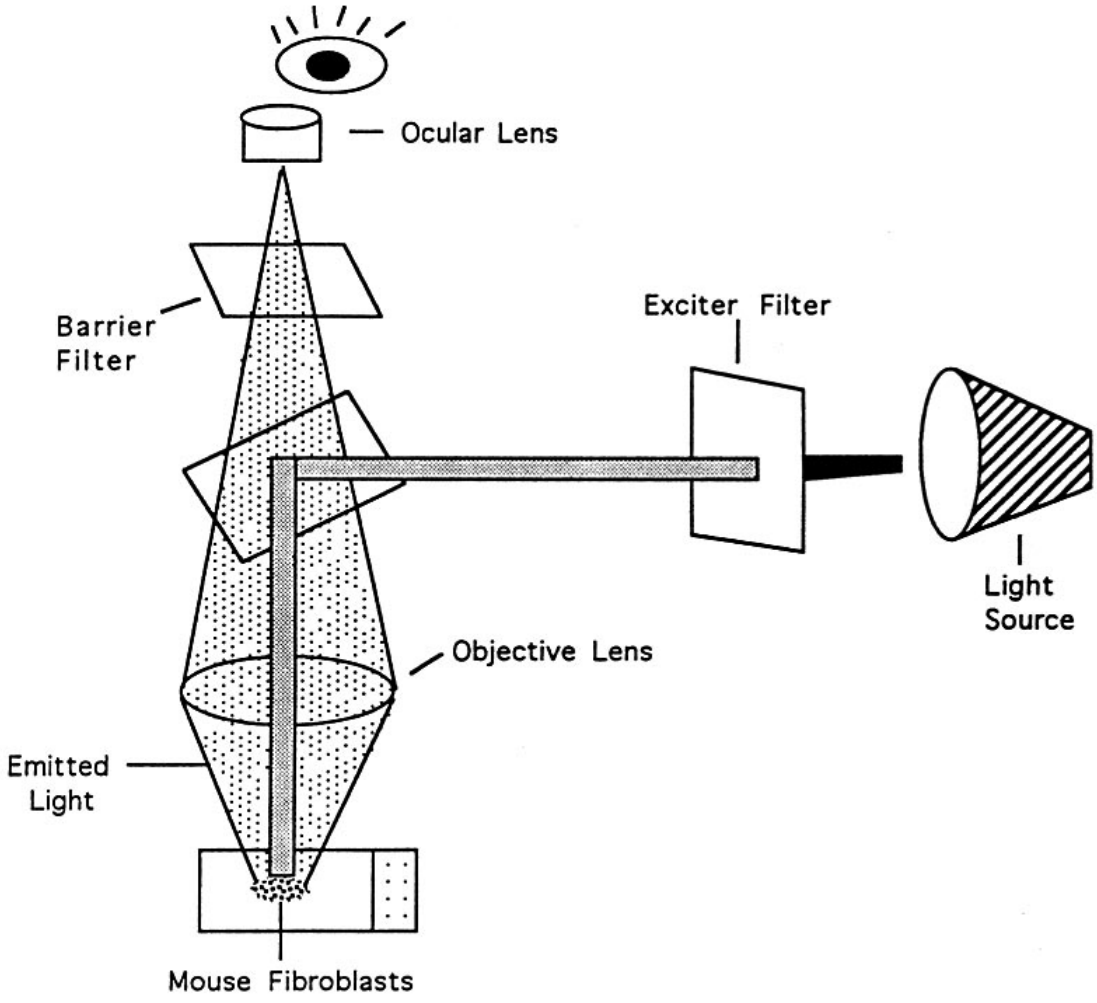

Figure 5.1. The fluorescent microscope

In order to excite the fluorescent dye and detect the resulting light it is necessary to use a special microscope. In many aspects, these microscopes are similar to the binocular microscopes that you used in B10-1; a set of objective lenses is used to magnify the specimen on the microscope stage. The difference lies in the way that the specimen is illuminated (the normal light source is not used). In its place, light emitted from a mercury arc or quartz lamp is passed through a series of exciter filters that are located between the lamp and the microscope stage (Figure 5.1). The exciter filters restrict the wavelength of transmitted light to a desired excitation wavelength (495 nm for FITC). The excitation beam of light is focused on the specimen located on the microscope stage which in turn causes the dye to fluoresce. The emitted light must then pass through a barrier filter on its way from the stage to the ocular lens. This filter allows only light of the desired emitted wavelength (517 nm for FITC) to pass. Thus, only the fluorescent light emitted by the sample is used to form a colored image.

Antibody-Antigen Interaction

Vertebrates have evolved a complex system to fight disease. This system, called the immune system, continually screens the blood for the presence of foreign substances. The majority of diseases are caused by bacteria, viruses, fungi, or protists. The immune system protects the body by attacking and destroying these invading microbes. The first clues of how this system works were provided by a country doctor named Edward Jenner in 1796. Jenner observed that individuals who had been infected previously with the often deadly disease smallpox were immune from future infections. He also observed that milkmaids who had caught a milder form of the disease (cowpox) almost never caught smallpox. Jenner hypothesized that cowpox somehow conferred protection against smallpox and tested this theory by infecting people with cowpox. Many of the experimental subjects became immune to smallpox.

It took centuries of experimentation to determine what it was in Jenner's cowpox experiment that was actually eliciting an immune response. Every cell, or in the case of smallpox, a virus, has on its surface a combination of proteins, carbohydrates, and lipids. These molecules are different for every organism and those on the surface of the smallpox virus are very different from those found on the surface of human cells. The molecules that are recognized as not being from your own body (non-self) are called antigens. The presence of antigens causes the infected individual to produce proteins called antibodies. The antibodies in turn recognize the antigens and help the body to fight off the infection by destroying the foreign microbes and defending against further infections by the same microbe.

The mechanism of the immune response is complicated and beyond the scope of this laboratory exercise. Instead, we will concentrate on how an antibody recognizes an antigen. Antibodies can be very specific for the antigen they recognize. In some instances, they can distinguish as little as one amino acid difference in two otherwise identical proteins. Antibody molecules are also called immunoglobulins. Antibody molecules consist of four polypeptide chains held together by disulfide bonds (Figure 5.2). There are two identical light chains which vary in molecular weight from 25–35 kilo daltons (kD) and two identical heavy chains that can range from 45–55 kD. The antibody molecule can be divided into a constant and a variable region. It is the variable region that determines the specificity of the antibody. Within the variable region are three hypervariable regions that together form a cleft which serves as the antigen binding site. Both arms of the antibody molecule have the same cleft. The antigen fits into one of the clefts much like a lock and key or a hand and a glove. In some ways, this interaction is similar to that of an enzyme and substrate.

Immunofluorescence

Two procedures are commonly used when doing immunofluorescence. The first procedure is called direct immunofluorescence and is a one-step process in which a fluorescent dye such as fluorescein is linked to antibody molecules that were produced in response to a specific antigen. The specimen that contains the antigen is incubated with the labeled antibodies, followed by a washing step to remove any unbound antibody. Then the preparation is examined using a fluorescent microscope.

The second procedure, called indirect immunofluorescence, will be utilized in this laboratory exercise. This two-step process begins by incubating unlabeled antibodies (primary antibody) with the specimen that contains the antigen of interest (Figure 5.3). The specimen is washed and then incubated with fluorescein or rhodamine labeled antibodies (secondary antibody) directed against the first antibody. In this instance, the first antibody becomes the antigen for the second antibody. The indirect method results in greater fluorescence when compared with the direct method because several secondary antibodies can bind a single primary antibody. In this exercise, you will be using primary antibodies produced in a rabbit and directed against either mouse actin or tubulin. The secondary antibodies (anti-rabbit antibody) were produced by injecting a goat with the primary antibodies and creating an immune response.

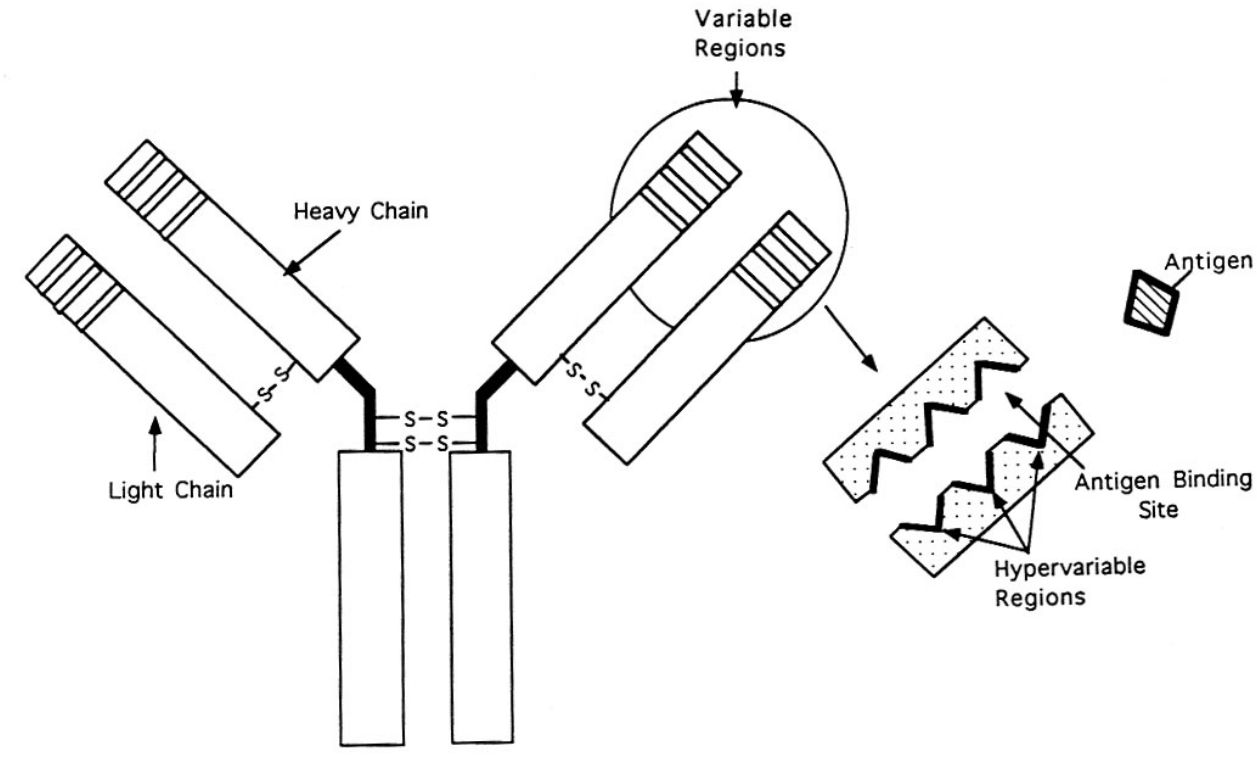

Figure 5.2. Antibody Structure.

Cytoskeletal Proteins

Eukaryotic cells contain three types of structures that comprise the cytoskeleton; microfilaments, intermediate filaments, and microtubules. These structures are found in the cytoplasm and are responsible for movement within the cell as well as giving the cell shape. Each type of structure has a different diameter and composition. The microfilaments consist mostly of the protein actin and are between 5 and 7 nanometers in diameter. These structures are thought to play a role in movement and locomotion. The intermediate filaments are made using one or more rod shaped proteins and are between 7 and 11 nanometers in diameter. There are several types of intermediate filaments each found only within certain cell types and all appear to be members of a single gene family. The microtubules are made using the protein tubulin and are between 20 and 25 nanometers in diameter. Microtubules are made up of α and β tubulin subunits each having a molecular weight of 55 kD. Besides being the components for structures like cilia and flagella, the microtubules form the centrioles and mitotic spindles that are necessary for the movement of chromosomes during cell division. A detailed discussion of these structures can be found in your text book.

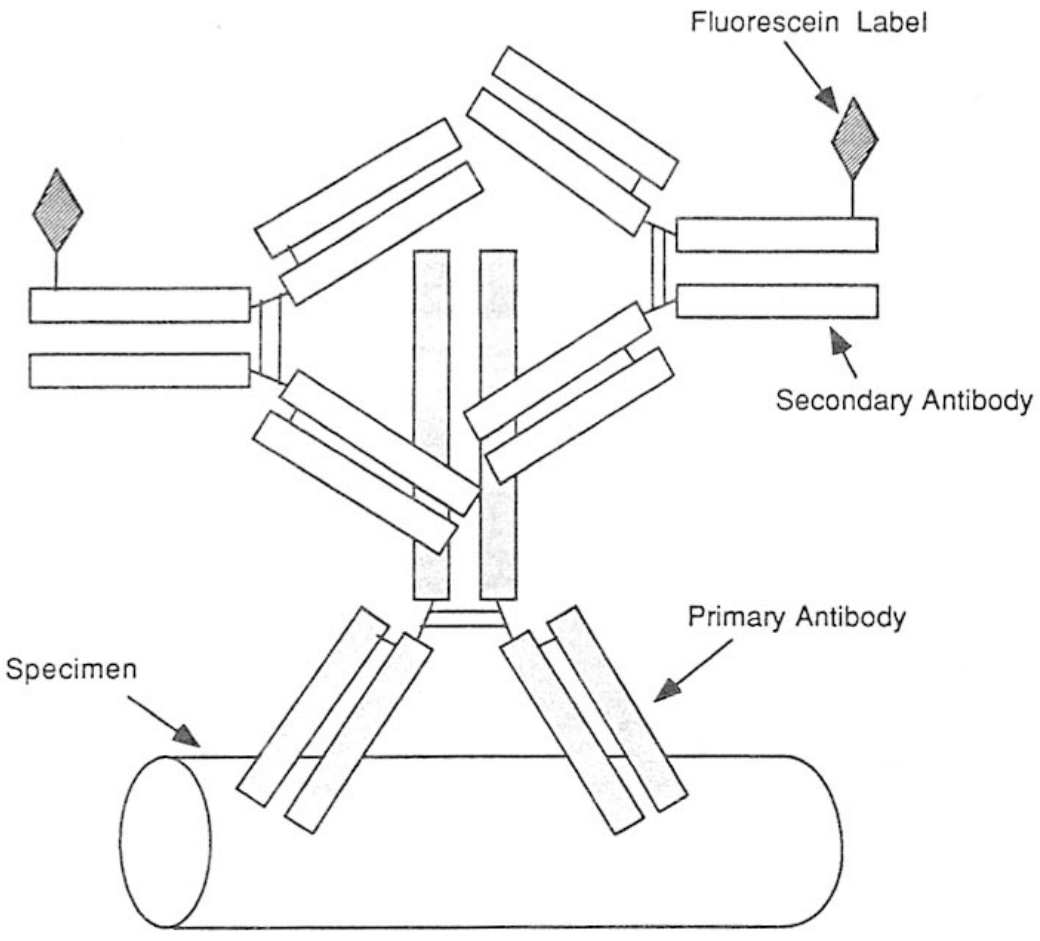

Figure 5.3. The indirect immunofluorescence method.

Laboratory Procedures

You will be working with a laboratory partner for this exercise. Your laboratory bench will be labeled directing you to use a specific set of antibodies. You will be using mouse fibroblast cells for this experimental procedure. The fibroblast cells grow in a medium supplemented with fetal bovine serum which is rich with growth factors the cells need to survive and multiply. Since these cells adhere to the growth surface, they are excellent specimens for this experiment. The one modification that has been made from the normal culturing procedure is that the cells will be grown on coverslips covering the surface of a petri dish. You will be working with three coverslips; one for the experiment, one for a control, and one for staining. You will also be using one of two different primary antibodies, anti-actin or anti-tubulin, and one of two different secondary antibodies, FITC- or rhodamine-conjugated antibody.

Part 1A: Washing and Fixing Fibroblast Cells

1. Fill a coverslip staining jar with Phosphate Buffered Saline (PBS). Take the jar and a pair of fine-tipped forceps to the TA bench. Gently lift a coverslip out of the petri dish and place the cell side (upper side) towards the taped side of the staining jar (Figure 5.4). This step is a crucial one for the rest of the experiment to work; the cells must be in the proper orientation so that they will be in contact with the antibody.

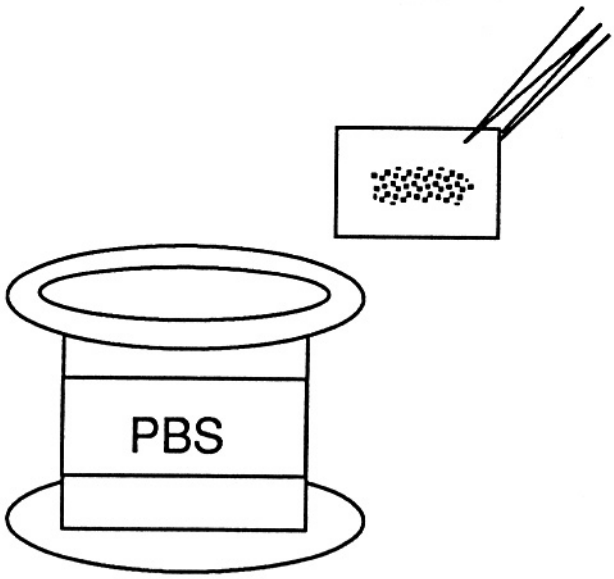

Figure 5.4. Cell Orientation.

2. Remove two more coverslips in the same manner.
3. Incubate the cells for two minutes.
4. Fill a second staining jar with PBS; transfer the coverslips to this jar and incubate for two minutes, again being careful of orientation. Change the PBS in the first staining jar and repeat the wash a third time.

5. One student from each pair should put on a pair of latex gloves and transfer the coverslips to a staining jar containing 2% paraformaldehyde. Paraformaldehyde is a fixative that cross links proteins to other proteins, thus protecting the structures within a cell from degradation. Gloves are necessary at this step because paraformaldehyde is toxic. Incubate at room temperature for 30 minutes. Carefully remove the gloves so that they can be used later.
6. While the cells are in paraformaldehyde, you will need to prepare a humidity chamber in which you will do further incubations. Wet a piece of paper towel with water and place it in the bottom of a large petri dish. Place two toothpicks on top of the paper towel followed by two toothpicks on top of the first two toothpicks at right angles. This will prevent the slides from coming in contact with the paper towel.
7. Following the 30 minute incubation, rinse the PBS staining jars with water and fill them with fresh PBS. Put on your gloves and transfer the coverslips from paraformaldehyde to PBS and incubate for two minutes.
8. Transfer the coverslips to the second jar containing PBS for two minutes. Change the PBS in the first staining jar and repeat the wash a third time.

Part 1B: Primary Antibody Incubation

1. In your kit you will find six microscope slides. Label one of the slides "control" and a second slide "antibody" on the frosted surface. Take both slides to the TA bench and add 50 μl of an antibody (either anti-tubulin or anti-actin) to the center of the antibody slide. Add of 50 μl PBS to the center of the control slide. Record the identity of the antibody you are using.
2. Using the forceps, remove a coverslip from the staining jar and touch the edge of it to a Kimwipe to remove excess PBS. Place the coverslip cell side down directly on the drop of antibody on the slide. Do the same for the second coverslip and the slide containing a drop of PBS.
3. Place both slides in the petri dish humidity chamber and place in the 37□C incubator for 30 minutes. During this time start Part 2 of this exercise.
4. During this incubation you will need to fill the staining jars with fresh PBS.
5. Following the incubation, remove the coverslips from the slides and place them in the staining jar containing PBS for two minutes being careful to keep the coverslip oriented so that you know which surface is covered with cells. Place the control coverslip in the front of the dish and the coverslip that was incubated with antibody in the back of the dish.
6. Transfer the coverslips to the second jar containing PBS for two minutes. These steps are required to remove any unattached primary antibody.
7. Change the PBS in the first staining jar and repeat the wash a third time.

Part 1C: Secondary Antibody Incubation

1. The following steps should be done quickly since the FITC- and rhodamine-conjugated antibodies break down when exposed to light.
2. Label the third slide from your kit "control" and the fourth slide "antibody" on the frosted surface. Take both slides to the TA bench and add 50 μl of FITC- or rhodamine-conjugated anti-IgG (secondary antibody) to the center of each slide.
3. Using the forceps, remove the coverslips from the staining jar and touch the edge to a Kimwipe to remove excess PBS. Place the coverslips cell side down directly on the drop of antibody on

each slide. Remember to place the control and antibody coverslips on the control or antibody slides.
4. Place both slides in the petri dish humidity chamber and place in the 37□C incubator for 30 minutes.
5. During this incubation you will need to fill the staining jars with fresh PBS.
6. Following the incubation, remove the coverslips from the slides and place in the staining jar containing PBS for two minutes being careful to keep the coverslip oriented so that you know which surface is covered with the cells. Place the control coverslip in the front of the dish and the coverslip that was incubated with antibody in the back of the dish.
7. Transfer the coverslips to the second jar containing PBS for two minutes. These steps are required to remove any unattached secondary antibody. Change the PBS in the first staining jar and repeat the wash a third time.
8. Label the fifth slide from your kit "control" and the sixth slide "antibody" on the frosted surface. Take both slides to the TA bench and add a drop of mounting medium to the center of each slide.
9. Using the forceps, remove the coverslips from the staining jar and touch the edge to a Kimwipe to remove excess PBS. Place the coverslips cell side down directly on the drop of mounting medium on each slide. Remember to place the control and antibody coverslips on the control or antibody slides.
10. Remove excess mounting medium by gently blotting with a Kimwipe. Place your slides in the petri dish humidity chamber and cover with foil until you can view them under the microscope. Place a piece of tape on the dish with both partners names.
11. View your slides and the slides of the other students under the fluorescent microscope. Mark down any observations in your lab manual that you might need to write in your report. When you are finished, put your slides in the petri dish and cover with the foil containing your name. Leave the slides on your bench so that your TA will be able to grade your work.

Part 2: Cell staining

1. If you were to look at the cells that were prepared in Part 1 using a light microscope, you would see nothing. When doing immunofluorescence, you often only see specific structures within a cell. In order to see the whole cell, it is necessary to use stain.
2. At the side of the room you will find several sets of staining jars. You will do the staining procedure at this setup and not at your bench. The stain will not come out of clothing, so be careful when staining your cells.
3. Remove the coverslip from PBS and place it in the staining jar containing Hematoxylin stain. Incubate for 4 minutes.
4. Transfer the cells to the staining jar containing water. Using the forceps, slide the coverslip up and down for one minute. This gentle agitation will rinse excess stain from the cells.
5. Transfer the cells to a second staining jar containing water and repeat step #4.
6. Transfer the cells to the staining jar containing acid alcohol (1% HCl, 99% ethanol). Using the forceps, slide the coverslip up and down ten times.
7. Transfer the cells to the staining jar containing water. Using the forceps, slide the coverslip or slide up and down for one minute.
8. Transfer the cells to a second staining jar containing water and repeat step #7.
9. Transfer the cells to the staining jar containing ammonia water. Using the forceps, slide the coverslip up and down six times.

10. Transfer the cells to the staining jar containing water. Leave the cells in water for 10 minutes.
11. Transfer the cells to the staining jar containing Eosin stain for 10 seconds.
12. Transfer the cells to the staining jar containing water. Leave the cells in water for 10 minutes.
13. Transfer the cells to the staining jar containing 95% ethanol. Leave the cells in ethanol for 2 minutes.
14. Transfer the cells to the staining jar containing 100% ethanol. Leave the cells in ethanol for 2 minutes.
15. Place the coverslip cell side up on a paper towel at your bench. When the cells are dry, add one drop of immersion oil to the cells. Put a slide on the coverslip, invert the slide, and wipe off any excess oil from the edges using a Kimwipe.
16. View your preparation using the light microscope that corresponds to your seat number. Mark down any observations in your lab manual that you might need to write in your report. When you are finished, leave the slides on your bench so your TA will be able to grade your work.
17. Put your microscope away following the steps that are posted on the door to the microscope cabinet.

Report

1 Describe what you saw on your slide and the slides of the other students. What differences did you see with the anti-tubulin vs. anti-actin antibodies? Is this consistent with what you know about the different types of structures that make up the cytoskeleton? What differences did you see with FITC- vs. rhodamine-conjugated antibodies?
2. What did you see on the control slide? Why were these conditions used as a control? Is this control really necessary and why?
3. Explain what you saw with the cells that you stained with hematoxylin and eosin.
4. Design an experiment using immunofluorescence, fibroblast cells grown in culture, and anti-tubulin or anti-actin antibodies.

Literature Cited

Retallack, Beth. 1995. Immunocytochemical analysis of sperm cytoskeleton. Pages 135–140, *in* Tested studies for laboratory teaching, Volume 16 (C. A. Goldman, Editor). Proceedings for the 16th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 273 pages.

APPENDIX
Preparator's Guide

Materials

Large tray with:
- 3 coverglass staining jars, taped and labeled: (per pair)
  - 2—"PBS"
  - 1—"Paraformaldehyde, Do Not Discard"
- 2 pair fine tipped forceps
- Large (6")glass petri dish with lid
- 4 toothpicks
- Paper towel
- 6 new microscope slides with frosted end
- Marker (Black VWR)
- Wash bottle with deionized water

On Lab Bench:
- Kimwipes
- Small trash cans
- Small tray with:
  - Lens paper
  - Immersion Oil
  - Cover slips
  - Clean used slides
- Roll of Aluminum Foil

On TA Desk:
- 7 Trays with:
  1. Ice bucket with anti-tubulin, 50 ul Eppendorf pipetman, yellow tips
  2. Ice bucket with anti-actin, 50 ul Eppendorf pipetman, yellow tips
  3. Ice bucket with FITC conjugated anti-IgG, 50 ul Eppendorf pipetman, yellow tips
  4. Ice bucket with rhodamine conjugated anti-IgG, 50 ul Eppendorf pipetman, yellow tips
  5. PBS in orange capped jar, 50 ul Eppendorf pipetman, yellow tips
  6. Ice bucket with Mounting Media

Several waste containers for used tips

Petri dishes of mouse fibroblast cells

On bench at side of room:
- 2 fluorescent microscopes
- 2 Oxford repipeters with PBS set to 9.5 ml
- Gloves: Small, Medium, Large, and Extra Large

Cover tops of bench with lab paper.

Line up staining jars on the lab paper.
1. Hematoxylin
2. Water
3. Water
4. Acid Alcohol
5. Water
6. Water
7. Ammonia Water
8. Water
9. Eosin

10. Water
11. 95% EtOH
12. 100% EtOH

Solutions

1. PBS

	1 liter
KCl	0.2 g
KH_2PO_4	0.2 g
NaCl	8.0 g
$Na_2HPO_4.7H_2O$	2.16 g

   Deionized water to 1 liter
   There is no need to pH this solution.
   Need approximately 60 ml/pair. Make approximately 10 liters.

2. 2% Paraformaldehyde (PFA) Fixative
   For 100 ml, heat 60 ml water to 60□C. Add stir bar.
   Wear gloves and weigh out 2 g paraformaldehyde.
   Add to water; cover with watch glass; stir.
   Transfer to fume hood; stir and heat on low maintaining 60^{o}C. Do not overheat.
   Add 1 drop of 2 N NaOH or 2 drops 1 N NaOH.
   The solution should become clear fairly rapidly, but there will be some small particles that will not dissolve. When clear, remove from heat and add 33.3 ml 3∞ PBS.
   Check pH and adjust to 7.2 with HCl
   Add Water to 100 ml.
   Check pH again.
   Filter with # 1 filter paper.
   Cool to room temperature.
   Make fresh daily.

3. Mounting Media (Sigma #1000-4)

Antibodies

1:40 dilution of anti-tubulin in sterile PBS (Sigma #T-3526)
1:100 dilution of anti-tubulin (monoclonal) in sterile PBS (Sigma #T-4026)
1:40 dilution of anti-actin in sterile PBS (Sigma #A-2668)
1:40 dilution of anti-actin (monoclonal) in sterile PBS (Sigma #A-5441)
1:80 dilution of FITC conjugated antibody in sterile PBS (Sigma #F-0511)
1:64 dilution of FITC conjugated antibody (for monoclonal) in sterile PBS (Sigma #F-4143)
1:64 dilution of rhodamine conjugated antibody in sterile PBS (Sigma #T-5268)
1:64 dilution of rhodamine conjugated antibody (for monoclonal) in sterile PBS (Sigma #T-7657)

Stains

Use labeled Coplin staining jars.

1. Harris hematoxylin (Sigma #HHS-32)
2. Tap water
3. Tap water
4. Acid Alcohol: 70% EtOH (99 ml), Conc. HCl (1 ml)
5. Tap water
6. Tap water
7. Ammonia Water: Tap water (100 ml), Ammonium hydroxide 28% (0.2 ml)
8. Tap water
9. Eosin (Sigma #HT110-1-32)
10. Tap Water
11. EtOH 95%
12. EtOH 100%

Tissue Culture

Mouse fibroblast cells are grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% calf serum.

Glass cover slips are baked to sterilize. Flamed forceps are used to place the cover slips (8 per) into a 100 ∞ 20 mm tissue culture dish.

Cells are grown to approximately 75% confluence and split 1:10 two days before lab.