Chapter 10

Immunocytochemical Analysis of Sperm Cytoskeleton

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Introduction

The cytoskeleton is a dynamic network of protein filaments, such as microfilaments, intermediate filaments, and microtubules, which form by the polymerization of different protein subunits found in the cytoplasmic pool. Microtubules are composed of tubulin, formed from globular protein subunits, and accessory proteins, the MAPs, which affect the stability and function of microtubules.

Antibodies are Y-shaped protein molecules formed from light and heavy polypeptide chains. Antigens are bound to specific sites on the antibodies. Other portions of the antibody differentiate the various kinds of antibodies. Immunoglobulin G (IgG), a common antibody formed during immune responses, differs according to which animal originally produced it.

In this lab, monoclonal antitubulin antibodies produced in mice will be used to label bull sperm microtubules and the location of these antibodies will be determined by use of a peroxidase conjugated secondary antibody. To visualize the tubulin, some sperm will be incubated with two substrates: hydrogen peroxide (H_2O_2) and an artificial reactant in its reduced form, diaminobenzidine ($DABH_2$) as:

$$H_2O_2 + DABH_2 \xrightarrow{\text{peroxidase}} 2H_2O + DAB$$
(colorless) (colored)

The peroxidase is conjugated to the secondary antibody. DAB then stains the tubulin-containing parts of the bull sperm.

This lab exercise was designed for second-year biology students and has been successfully completed by over 300 students in 12 lab sections. Recently it has been augmented by the addition of immunofluorescent analysis using FITC.

Materials

The following **solutions** should be prepared before the laboratory:

Sucrose (30%) in 30 mM Tris, pH 8.0 (Sigma #T1503, Trizma Base Reagent Grade, MW = 121.1)
Triton X-100 (1%) in 0.1 M KCl , 5 mM $MgSO_4$, 0.5 mM EDTA, 1 mM DTT (Sigma #D0632), 10 mM Tris, pH 7.0
Poly-L-lysine (Sigma # P-7890, Hydrobromide, MW = 17000) 100 µg per ml in H_2O
PBS (phosphate buffer solution). 10X stock solution as: 80 g NaCl, 2 g KCl, 21.6 g $Na_2HPO_4.7H_2O$ (11.5 g if anhydrous and 14.35 g if $.2H_2O$), and 2 g KH_2PO_4. Dissolve in 800 ml dH_2O, adjust pH to 7.4, and make up to 1 liter.
Methanol, very cold (ideally –20°C)

The following **antibodies** should be prepared as :

Primary: Mouse DMIA (in PBS) as a 1:25 dilution in PBS (Sigma #T9026)

Secondary: Antimouse IgG, peroxidase conjugated as a 1:25 dilution in PBS (Sigma #5906)
Frozen bull sperm (obtained from your the nearest Agriculture College)
DAB stain (Sigma #D4168). Carefully read the instructions that come with the DAB stain.

Notes for the Instructor

This lab is described here for a typical undergraduate lab of 24 students with six sit-down benches each with four students. Each student has a standard microscope equipped for bright-field and phase contrast microscopy, with an ocular micrometer (calibrated in an earlier lab by the student). To set-up for this lab the following will be required:

Slide Preparation

Frosted slides, new (4)
Weighboats (labelled: poly-L-lysine, distilled water, distilled water) (3)
Poly-L-lysine (30 ml bottle)
Forceps, blunt-end (2)
Squeeze bottle of distilled water
Styrofoam "meat" tray or other container for drying and temporary storage of the slides

Preparation of Bull Sperm

Microcentrifuge tube (1.5 ml) in single holder containing bull sperm (in fridge)
Sucrose (30%), on bench (100 ml bottle)
Pipet (10 ml), safety bulb, and rack
Centrifuge tubes (15 ml, round bottom) in a rack (2)
Pasteur pipet and bulb
Wax pencil (to put initials on centrifuge tubes)

On the Side Bench

Triton X solution (2 bottles)
Pipets (1 ml), safety bulbs, and rack (2)
Pasteur pipet and bulbs
PBS (2 bottles). Dilute 10X stock solution to 1X.
Pipets (1 ml), safety bulbs, and rack (2)
Microcentrifuge tubes

Preferably in a fume hood:
Distilled water in a bottle
Pipet (1 ml), safety bulb, and rack
Vortex mixer
DAB
Microfuge tubes (1.5 ml)

Staining

Poly-L-lysine-coated slides in drying container (4)
Moist chamber (suitable plastic container covered with wet paper towel held by clothespins)
Coplin jars (labeled: PBS #1, PBS #2, PBS #3, methanol) (4)
Ice bath (for methanol, use old margarine container) (1)

PBS (1 liter bottle)
Paper towels
Forceps, blunt-end (2)
Pasteur pipets and bulbs
Container for *used* pipets
Microcentrifuge tube in single holder (1)

Student Outline

A co-operative effort and teamwork, on the part of four students per lab bench, are required to do the experiment. Components of the procedure can be done by different students as:

Poly-L-Lysine-Coated Slide Preparation (Student #1)

Poly-L-lysine-coated slides will be provided for today's lab. Prepare four slides for the next lab session as follows: :

1. Obtain four new clean slides .
2. Place two slides in a shallow container and cover with poly-L-lysine for 10 minutes.
3. Using forceps, transfer the slides to another shallow container holding water and rinse. Transfer to a second shallow container holding water and rinse again.
4. Repeat this process for the other two slides.
5. Place all four slides in a drying container and cover loosely.
6. Put this container in the designated place so that the *coated slides* will dry at room temperature for the next lab group.
7. Recycle the poly-L-lysine solution from the shallow container by pouring it into the original bottle using the provided funnel.

Preparation of Bull Sperm (Student #2)

The bull sperm will be provided in small plastic capsules premeasured to 1.0 ml. This is enough to make 60 slides! Work with Student #2 from the next bench.

1. Retrieve the unprepared sperm from the freezer.
2. Put 10 ml of 30% sucrose in a centrifuge tube.
3. Layer the sperm *very carefully* on the 10 ml, 30% sucrose cushion by filling a disposable pipet with the sperm and then with the pipet tip against the side of the centrifuge tube slowly adding the sperm solution to the sucrose so as to form a layer on *top* of the sucrose.
4. Centrifuge for 10 minutes at 3500 rpm (setting # 6 on the bench top centrifuge). All six benches will co-ordinate for this step.
5. Discard the supernatant and resuspend the sperm in 1 ml of Triton solution. Leave for 5 minutes.

6. Layer the sperm solution on another 10 ml 30% sucrose cushion and repeat the centrifugation as before.
7. Resuspend the *prepared sperm* in 0.5 ml of PBS on the small table beside the fridge. Store at 4°C for the next lab group in the appropriately marked container.

Immunocytochemistry of Bull Sperm (Students #3 and 4)

1. Retrieve a drying container with four *coated slides* from the designated area and also your allotment of *prepared sperm* from the appropriate section of the fridge. Take a wax pencil and on each of the coated slides mark a circle in the area where you will be putting the sperm and the antibodies. This circle should be placed on the underside of the slide. You may wish to use a "tail" on each circle to help you identify the underside versus the topside of your slide! This "tail" works best if you always hold the frosted part of the slide in the same hand.
2. Spread *one drop* of the sperm solution on each of the four coated slides and allow the cells to settle for 30 minutes at room temperature in a moist chamber.
3. Fill a coplin jar 3/4 with PBS and another with very cold methanol (in an ice bath).
4. Rinse the four slides briefly in PBS and fix for 2 minutes in the cold methanol.
5. Replace the used PBS with fresh PBS, dumping the used PBS down the drain.
6. Rehydrate the cells in the fresh PBS for 5 minutes.
7. Drain off the excess PBS (but do not dry) and apply *one drop* (from a disposable pipet) of the primary antibody, DMIA, to two slides. Put all four slides in the moist chamber for 30 minutes.
8. Using three coplin jars, each filled 3/4 full with PBS, wash the four slides three times for 2 minutes per wash.
9. Drain off excess PBS, apply *one drop* of the secondary antibody, antimouse IgG peroxidase conjugated *from a disposable pipet* to all four slides. Leave in a moist chamber for 20 minutes.
10. Wash three times in PBS and drain.

DAB Reaction *(to be performed by two student volunteers)*

Prepare the DAB solution for the entire lab section as follows:

1. Each student will put on rubber gloves and open a DAB tablet package (silver foil) and a urea hydrogen peroxide (gold foil) and drop the tablets into a disposable container. *Do not touch the tablets with unprotected fingers!* Add 1 ml of distilled water. Vortex until dissolved.
2. When a bench is ready for the staining, using a disposable pipet, put a drop of the prepared DAB solution on each of the slides.
3. Stain the slides by allowing them to incubate at room temperature for 10 minutes.
4. Rinse briefly by quickly dipping the stained prepared slides in PBS twice. Drain using a paper towel. Put on a coverslip and examine using the 40X ocular.

Laboratory Report/Exercises

Phase Contrast

Set your microscope up for phase contrast. Using some *untreated* bull sperm (left over by the student #2), prepare a slide and observe at 40X.

1. Describe what you see.
2. Measure the length of five sperm tails (μm), seen with phase at high-dry and calculate their average length.

Immunocytochemistry

When you have the stained slides, switch your microscope to bright-field. Observe the prepared specimens using 40X.

3. Measure the length of five sperm tails (μm) in material treated with secondary antibody only and calculate their average length.
4. Measure the length of five sperm tails (μm) in material treated with both primary and secondary antibodies and calculate their average length.
5. What is the difference in average length between the tails of sperm treated with both antibodies and those treated with only secondary antibody?
6. Describe the appearance of the material treated only with the secondary antibody.
7. What structures are seen in the DAB-stained material treated with both primary and secondary antibodies that are not seen in the untreated specimens viewed with phase?
8. In the reaction,

$$H_2O_2 + DABH_2 \xrightarrow{\text{peroxidase}} 2H_2O + DAB$$

is the $DABH_2$ being reduced or oxidized? Explain.

9. Why is the peroxide necessary in the above reaction?
10. What is the source of the peroxide?
11. Why is the peroxidase necessary? How does the DAB stain the sperm tail?
12. What are the controls in the experiment? Why do you run controls? Is there a positive and a negative control in this experiment? If not, what would be done differently to add them?