

## Section 3. Classical Anatomy

### Overview

One cannot hope to understand the function of a system without understanding its physical layout. Neuroanatomy is a broad umbrella of methods that aim to provide this insight. You are probably already familiar with gross anatomy of the brain (sheep brain dissection). In this lab, we will expose you to some classical methods in neuroanatomy. This includes tissue sectioning, staining, and microscopy. These methods allow detailed examination of the microstructure of brain regions and are thus a key component of much systems research today.

During this lab, you will work to take some tissue through key immunohistochemistry steps, and ultimately visualize the pattern of primary antibody binding in the tissue. During several of these steps, you will need to wait for reactions to complete, so you will have time to learn the other key steps in anatomical methods. These include: tissue perfusion, brain extraction and blocking, cutting of frozen sections (microtome), mounting sections on glass slides, and basic microscopy

## Classical Anatomy

\*\*Each of the techniques that we use today could be a lab itself. Due to time constraints, we are doing the cooking show version of animal perfusion, tissue sectioning and immunocytochemistry.

### Immunocytochemistry of Brain Sections- Part 1, Teaching Staff

\*\*The teaching staff has performed this portion of the protocol prior to the start of the lab session

1. Put 500  $\mu$ l of blocking solution into a new vial.  
-Blocking solution contains 2% triton X plus 1% goat serum in phosphate buffered saline (PBS).
2. Add into it 2  $\mu$ l of primary antibody against NeuN.  
-This antibody is created in mice.
3. Hand mix solution and antibody.
4. Pipette the 500  $\mu$ l of blocking solution and antibody into a well of the 24-well plate.
5. With a brush, take the brain sections and transfer it into the well with blocking solution and antibodies.
6. We will keep the antibodies incubating with the brain section for 1 hour on the shaker.

### Immunocytochemistry of Brain Sections-Part 2

\*\*Students will begin with these steps when they arrive in the lab.

1. With the brush, take the sections from the antibody solution and transfer them into a new well with PBS. Every time you change the solution, you will put the solution into a new well and use the brush to transfer the tissue into it.
2. Wash the tissue in PBS on shaker for 3 minutes, and repeat this washing step twice.
3. While the tissue is in the last wash prepare 500  $\mu$ l of blocking solution, and add to it 4  $\mu$ l of biotinylated anti-mouse secondary antibody.
4. Hand mix solution and antibody.
5. Once you are done with the washings, put the blocking solution and secondary antibody into a well, and with the brush, put the brain sections there.
6. We will incubate this for 1 hour on shaker.
7. In 30 minutes from the start of this incubation, you will make the A+B solution (see below).

**\*\* Note:** While tissue incubates you will perform either the animal perfusion OR the tissue cutting. Whatever technique you do not do now, you will do while the tissue incubates with the A + B reaction.

### **Finishing the Immunocytochemistry- Visualization**

1. In a vial, add 50  $\mu$ l of solution A and 50  $\mu$ l of solution B to 2.5mL of PBS.
2. Incubate this mixture at your bench for 30 minutes prior to use.
  - The A and B solution must react with each other before they react with the tissue.
3. At the end of the secondary antibody incubation, wash off the secondary antibody by incubating the sections in fresh PBS (three times for 3 minutes as previously done).
4. When finished, add 500  $\mu$ l of the A and B reaction mixture to a new well and then add the tissue.
5. We will incubate this for 45 minutes on the shaker.

**\*\*Note:** Here you will perform the technique that you did not complete during the secondary antibody incubation (perfusion OR tissue cutting). If you finish either technique early, you may start to analyze the slides provided.

6. When the A and B reaction incubation is complete, wash off the tissue with fresh PBS (three times for 3 minutes as previously done).
7. When the washing is complete, fill a well with new PBS, and alert the teaching staff that you are ready to visualize your reaction.

**\*\*Note:** The following steps must be done under the supervision of the teaching staff.

8. Bring your well plate, brush and a timer to the fume hood.
9. In the fume hood, the teaching staff has prepared a visualization solution in a Petri dish.
  - The solution consists of
    - 2.5mL PBS
    - 50  $\mu$ l reaction buffer
    - 100  $\mu$ l DAB
    - 50  $\mu$ l H<sub>2</sub>O<sub>2</sub>
    - 50  $\mu$ l Nickel solution
10. Add your tissue to the Petri dish and start your timer.
11. We will monitor the reaction under a microscope while timing its duration.
  - Tissue should become darkened overall with darker patches where NeuN concentration localized.
  - Reaction time should be between 2-5 minutes.
12. When reaction is complete, immediately transfer tissue to the well with the new PBS.
13. Wash tissue with PBS two more times for 3 minutes.

## Slide Production- Tissue Mounting and Coverslipping

1. Fill your Petri dish with PBS (enough to fill bottom of dish).
2. Upon completion of PBS washes in the well plate, transfer tissue into the Petri dish with PBS.
3. Take a glass slide and place one side into PBS, avoiding tissue, while holding the other side.
4. Wet slide with brush.
5. While continually holding the slide, use the brush to transfer and flatten a tissue section onto the slide (Mounting).
  - This step may take some finagling; if you are having trouble, ask the teaching staff for assistance.
6. Continue with the rest of the tissue sections. Try to get as many sections on one slide as possible.
7. When finished with tissue mounting, let sections dry on the slide for 5 minutes
8. When the tissue is dry, place the slides into a coplin jar filled with 70% ethanol for 3 minutes.
  - This step begins the dehydration process that one must do prior to coverslipping.
9. When 3 minutes have elapsed, transfer the slides to the jar filled with 95% ethanol for 3 minutes. Repeat with process with 100% ethanol (2X) and with citrisolv (2X).
10. Upon completing the dehydration steps, add a few drops of permount on top of the tissue.
11. Angle the thin cover glass over one side of the slide, then apply the glass on top of the tissue.
  - An analogy to this step is creating a door hinge with the slide cover (door) and closing that door.
  - Be careful not to create bubbles over the sections.
12. Look at the section under the microscope. Write observations in your notebook on them.

## Mouse Perfusion

**\*\*Note:** This technique must be done entirely under the supervision of the teach staff!

**\*\*Safety goggles are required!**

1. The teaching staff will give you an anesthetized animal.
2. While waiting for the animal, prepare a 20 ml syringe with PBS, and a 20 ml syringe with 5% paraformaldehyde.
3. Get butterfly needle. Attach butterfly needle to PBS syringe (both are luer lock).
4. Flush or prime the butterfly needle with PBS so that you do not introduce air into circulatory system.
  - Air bubbles in line can compromise vessels and the entire procedure!
5. Do not begin until the animal is fully anesthetized (the animal does not respond when pinching the toes). The teaching staff must give permission before you begin.
6. Secure the animal arms into a perfusion tray with pins. Pins should push into tray.

7. With scissors, open the skin around the abdomen, push organs out of the way, and locate the lower wall of the chest cavity (diaphragm).
8. SUPERFICIALLY cut open the chest along the midline then make two lateral cuts at the top of the chest cavity cage to expose the heart.
  - The lungs collapse when you puncture the chest cavity, so you may not see them.
9. Pin down the rib cage flaps that you have created so that you have room to work.
10. Cut the animal's RIGHT atrium with the scissors (a small cut is sufficient).
  - You will know it is cut when you see blood flow from it.
11. While HOLDING ONTO THE BUTTERFLY, insert the butterfly needle into the animal's LEFT ventricle.
12. Start applying pressure to the syringe with PBS (slowly, at a rate of 2 ml per minute).
13. Push PBS through the circulatory system until all blood is drained from the body.
  - Indications that blood is gone: Liver and heart change from dark red to yellowed color.
14. When blood is drained, take the PBS syringe out of the butterfly luer lock and exchange it for a 20 ml syringe with paraformaldehyde.
15. Take needle out of heart, re-flush with new syringe for air bubbles (syringe exchange can induce air in the line), and reinsert the needle into the heart.
16. Apply pressure to the PFA syringe at a rate of 2 ml per min.
  - If done well, you may see the animal convulse as the PFA goes through system. (The animal is not conscious. PFA triggers the release of acetylcholine at the neuromuscular junction).
17. Once the PFA perfusion is complete, proceed to brain extraction.
18. Cut the head from the rest of the body.
19. Cut the skin off the skull.
20. Carefully, cut the periphery of the skull (without damaging the brain).
21. With a blunt tool, pull the brain out of the skull.
  - If you were cutting this tissue, then you would post-fix the brain with more PFA for 1 hour and then saturate it with 10-30% sucrose (takes about 2 days) prior to cutting.
22. The teaching staff will take care of carcass and PFA disposal.

### **Sectioning Tissue with a Freezing Microtome**

\*\* Note: The teaching staff will do steps #1-4 while you observe. We will set the microtome to cut 40 $\mu$ M slices.

1. Put dry ice in receptacle of the microtome stage.
2. When the stage is cold, place a small amount 10% sucrose on the stage of the microtome.
3. The blade should be above the stage. Position it above the sucrose mound, and carefully slide the blade over the mound to flatten it.
4. Once the sucrose is flat, place the brain on it, cover the edges with sucrose and cover the brain with dry ice to freeze.
  - We will cut coronal sections. How would you position the brain to get coronal sections?
5. While waiting, prepare a 24-well plate with PBS in each well. You should fill each well

one-quarter with PBS.

6. Once the brain is frozen, position the blade to be level with the top of the brain.
7. Very CAREFULLY and GENTLY, slide the blade forward to cut the brain.  
-If done properly, the brain section should be molten on top of the blade.
8. Pick up the tissue slice by sliding your brush down the blade over the tissue and put it into a well plate with PBS.
9. Wipe excess PBS off of the blade with a kim wipe (in the same motion that you used to remove the tissue from the blade).
10. Move the blade back to its original position and adjust stage up by 40 $\mu$ M (how you do will depend on microtome used; ask teaching staff at the time of cutting).
11. Repeat steps. Each student should cut at least 15 slices to get a feel for this procedure.
12. Place each tissue section into a new well. When you get to the end of your well plate, start at the first well and add more tissue to each one.

### **Observations to Make with your Nissl Stained Tissue**

For each species (human, primate, rat), you have a Nissl stained section that includes primary visual cortex (V1). Look at each slide under the scope at 40x and 100x magnification (objective lenses of 4x or 10x) and analyze the following:

1. **Neurons vs. glia.** Once you orient yourself to the cortex using the weakest objective lens (4x), zoom in by switching to higher objective lenses (be careful not to impact the slide!). If you look closely at the purple cells, you will see that some just have their nuclei stained (glia), while others have purple material in the cytoplasm (neurons). You might even be able to see the beginnings of dendritic and axonal processes extending from the neuron cell body.
2. **Blood vessels.** Can you identify blood vessels within your tissue? Are they easier to identify in human vs. animal tissue? Can you guess why or why not?
3. **Cortical measurements across species.** Use the computer-attached camera to get an image of the cortex so that you can compare and contrast V1 in the three species (see below for some help on using the camera and computer. Ask an instructor for help if you are stuck).
  - a) Measure the overall thickness of cortex (from top of layer 1 to bottom of layer 6). Compare your findings between the species.
  - b) Try to determine the boundaries of the individual cortical layers and measure the thickness of each cortical layer. To help you, below is a Nissl section of rhesus monkey cortical area V1. In looking at these, ask yourself: Which layer is the primary input layer to V1? Where do those inputs come from? Compare your findings between the species.
  - c) Measure the neuronal density of layer 4 (i.e. neurons per square mm). Compare your findings between species.

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