Lab 1. Microdissection

The goal of this "techniques" lab is to understand how to dissect and process tissues that have been incubated in bromodeoxyuridine (BrdU). BrdU is a synthetic thymidine analog that is incorporated into DNA during cell replication. It can then be located with immunocytochemistry using an anti-BrdU antibody. Cells that label with this antibody can be used as evidence that a cell has divided.

> *Juvenile crayfish will have already been incubated in BrdU labeling reagent at 5mg/mL for 24 hours before this lab.*

In this lab you will be dissecting the brain from a juvenile crayfish for use in immunocytochemical staining for ***serotonin*** *and* ***BrdU*** *in the next lab.*

The lobster atlas web site should help with crayfish anatomy :
http://www.wellesley.edu/Biology/Beltz_Lab/LobsterAtlas.html

I. Tips for Microdissection:

Micro dissections can take a long time so it is important that you understand how to optimize the use of the microscope and understand the tools that you will be using. Below are a few suggestions to make it easier for you:

- Get comfortable – make sure that your microscope is close to you on the bench and that your chair is the right height and in front of the microscope.

- Set the ocular distance (distance between your eyes) on the microscope. It is essential that you use can both eyes while dissecting and that you are not just looking out of one eye.

- Focus for each eye: to use a binocular microscope correctly it helps if you adjust the focus for each eye so that you can use both eyes equally and don't get tired squinting! The microscopes that you will be using have the ability to allow for variation in the accommodating abilities of each eye. To do this:

    - Focus on an object.
    - Close one eye (the better one, if you only have problems with one eye).
    - Get the object in very clear focus with this eye using the objective focus.
    - Now close this first eye, and look through the second eye. Focus on the object using the adjustment on the eyepiece.
    - The object should now be perfectly clear when viewed with both eyes.

- Adjust the lighting so that the object is illuminated as clearly as possible and with as few shadows as possible. Lighting may have to be readjusted several times during the dissection.

- "Find" your instruments under the microscope. One of the most difficult things to learn is to find the instruments that you are using under the microscope. A good place to start is to touch the instruments that you have in each hand under the microscope. Your brain can then accommodate to where your hands are.

- Be very careful with your instruments. Fine forceps and fine scissors are provided in the lab. These instruments are very expensive and quite delicate so it is important that you treat them carefully by following these few suggestions:

    - The instruments are provided in a Styrofoam tray – try to return them to the tray when you are not using them.
    - You will be provided with course and fine forceps and fine scissors. Fine forceps have very fine tips that connect when you squeeze the forceps. These tips are very easily broken or bent if they are not carefully handled so try not to drop them and avoid walking around the lab holding them when you are doing another task.
    - Fine scissors have equally fine and fragile tips so take care of them in a similar manner to the forceps.

- You are also provided coarse forceps with blue handles – these are sturdier than the extra fine forceps so use these for pinning or jobs that require more strength.
- Wash your own instruments by running them under distilled water and returning them to a paper towel on your instrument tray. Never dump them in the sink at the end of the lab or leave them in any kind of a pile where they could get damaged.

- Try to relax while you are dissecting. As with all new motor learning tasks, it is easy to get tense at the beginning when you are anxious about making a mistake. If you feel tense, take a break and stretch before you try again.

- Avoid doing anything that will make your hands shake; some people shake when they have coffee; others shake when they don't have caffeine! So try to avoid what makes your hands shake.

- Remember this is a motor learning task and you will get better as you do it more. We hope to have extra animals so that you can practice on one. Let your instructor know if you feel that you need extra practice.

II. Dissection of Juvenile Brains (review the video before and during as necessary)

1. Chill the crayfish for 5 mins in a Sylgard dish on ice.
2. Pin the head in a sylgard dish, ventral side up.
3. Gently remove all appendages projecting from the head, with fine forceps.
4. Remove mandibles and look for nerve cord.
5. Remove connective tissue above the cuticle plate.
6. Remove the cuticle plate with fine forceps – the brain is right below this.
7. Cut the lateral brain connections and cut the eyestalks. Cut the nerve cord as far posterior as possible so there is plenty of tissue to pin during fixation.
8. Put the brain into a small Sylgard dish with crayfish saline and pin the brain ventral side up (bumps up). Do not pin the brain but place the pins in the connectives.

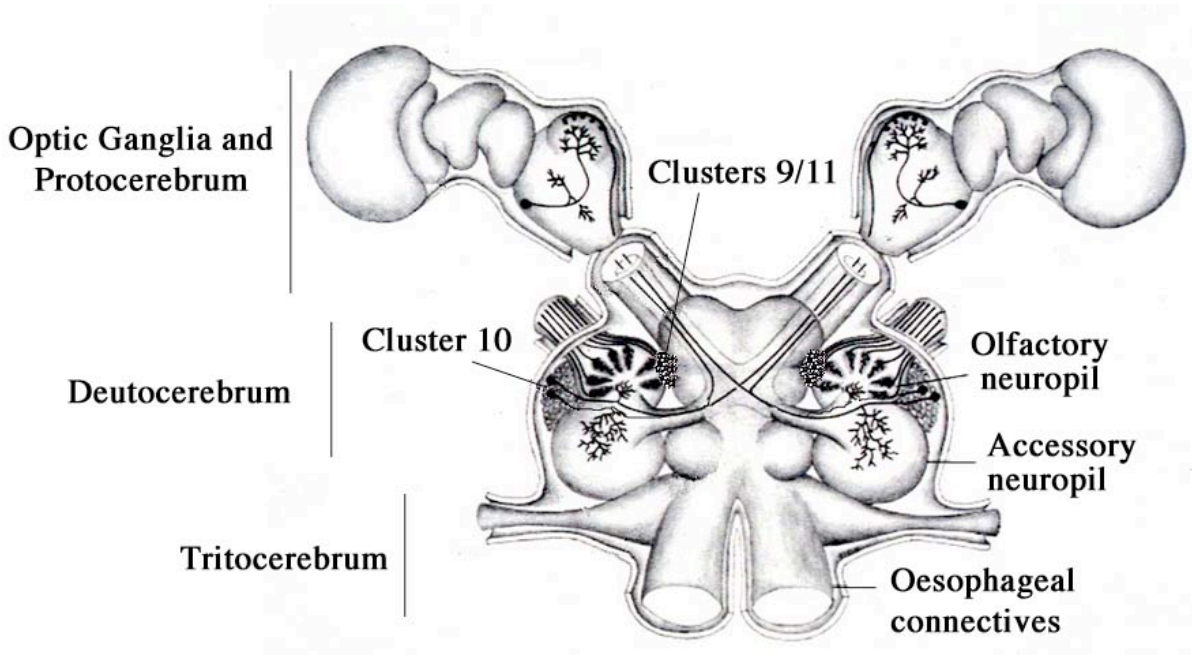

Figure 1 Drawing of the crayfish brain showing the three main divisions and the areas of life-long neurogenesis. Modified from drawing by David Sandeman; Mellon and Sandeman 1992.

III. Fixation and Rinsing

1. Remove the saline solution from the brain, and cover it with 4% paraformaldehyde fixative for 24-48 hours. Fixatives are chemicals that cross-link molecules, such as proteins, so that cellular structures remain intact in spite of tissue death. (Be careful: fixatives can also fix **human** tissues. Do not let fixatives come in contact with the skin and avoid breathing them).

TIP: *We will use a small refrigerator reserved for fixatives. Fixatives can linger or get spilled, and they may fix live materials that are subsequently put in the refrigerator.*

2. Rinse the brain six times in PB. Rinses should be at least 30 minutes apart (i.e., a maximum of six rinses in three hours) to rinse out the fixative so that the antibodies applied later will bind selectively and not be fixed to the tissue.

3. Place tissue in PB and store in the refrigerator until the next lab.

IV. Solutions (all solutions used for immunocytochemistry should be filtered).

1. **Crayfish saline**

Add the following to: 1L(1000ml) ddwater

NaCl	12.0g
KCl	0.4g
$CaCl_2$-$2H_2O$	1.5g
$MgCl_2$-$6H_2O$	0.5g
$NaHCO_3$	0.17g

2. **4% Paraformaldehye solution**

To make 200 ml:

50 ml dd H_20 at 60°C	8 g Paraformaldehyde
100 ml 0.2M Phosphate Buffer (pH 7.4)	50 ml ddH_20
1M NaOH	1M HCl

Add paraformaldehyde to the 50 ml of 60°C dd H_20 and stir solution for 10 mins. Clear solution with NaOH. Add remaining water and PB. Filter solution and adjust the pH to 7.4. Store the solution in an airtight container and in the fridge. Use within 1 week of making it. It works much better that way.

3. **O.1M PB**

Stock A: (pH 4.5) 26.7 $Na_2HPO_4.H_2O$/1000ml ddH_2O
Stock B: (pH 9) 28.4 Na_2HPO_4 /1000ml ddH_2O (53 g for heptahydrate)
Mix one part stock A, 4 parts stock B, for a) 0.2M solution; adjust pH to 7.3-7.4 with A or B. Mix 0.2M buffer with an equal volume of ddH_2O for a O.1M solution

4. **PBTx**

Add 400μl Triton x-100 for every 100 ml 0.1 M PB

Preparation for Week 2

For next week's lab you should:

1. Complete the rinses described in III. 2 above.
2. Read the manual (Lab II).
3. Do the sample dilution problems on page 15-17 of Lab II.
4. Prepare a flow sheet for the next 2 weeks, for the immunocytochemistry experiment.

Once you have read Lab 2, think about the following questions and be prepared to discuss them during the next lab:

1. In planning an experiment why is it important to pay attention to the hosts for primary and secondary antibodies ?

2. Is it possible to do a double-labeling experiments using antibodies raised in the same host?

3. What are the limitations of antibody-based methods?

4. Imagine that you use an antibody directed against antigen "A" to label tissues immunocytochemically, and you see lots of labeling. This labeling can be completely eliminated by preadsorbing the antibody with "A" prior to use. Therefore, is the molecule we are labeling identical to "A"? Why or why not?

5. In the previous example, how might you test the anti-A antibody for cross-reactivity with other molecules?

6. You have an antibody against the following peptide molecule:

   H-Met-Asn-Try-Leu-Ala-Phe-Pro-Arg-Met-NH_2

   You have characterized the antibody, and know that the epitope (recognition site) is in the Arg-Met-NH_2 region. Will this antibody also label the molecule:

   H-Try-Leu-Arg-Try-Phe-Phe-Pro-Leu-Arg-Met-NH_2

   How could you test this experimentally?

7. What is the essential difference between a monoclonal and a polyclonal antibody (see Lab II, Fig. 6)?

8. Why does anti-synapsin that is generated against *Drosophila* synapsin work in crayfish.

9. You have done an experiment where you see gorgeous staining in cortical neurons. However, your "no-primary control" has the same labeling pattern. Should you be concerned, or are you ready to publish your data? Why or why not?