BMD 415 Microscopic Anatomy Fall 2004
Lab Guide: Tissue Processing Procedures
UCOM 6138 & 6128

The class will be divided into lab groups. Each group will have access to the histology lab for about two weeks each. During your lab period you will be required to produce two finished microscope slides from each of two different tissues. Before starting to work in the lab, read through the lab manual to be familiar with the work involved, and watch the video demonstrations available on cd's that you may borrow.

The purpose of this section of the laboratory is to produce and analyze tissue samples microscopically. Students will dissect, embed, section, stain, and examine their specimens, and submit a written report of the results. Each student will choose two pathology specimens to examine. In addition to the written report, evaluations will be based on the quality of two slides from each tissue, lab notebooks, and lab performance. The schedule for the lab is a guideline; students will need to sign up to use equipment and make appointments with the lab assistant if help is needed.

This should be considered an experimental lab because no two tissues are ever alike in sectioning or staining properties. Even though the general procedure is described, you may need to experiment by trial and error in order to produce the best slides with your particular tissue block. You can vary tissue sectioning thickness, sectioning speed, temperature of the block, and staining times. Most of your grade will be based on the quality of your slides and this will depend on the carefulness of each step in the process. It is possible to have a perfect slide that is ruined at the very last coverslipping step. You will need to allow enough time for trial and error; don't plan on doing each step just one time.

Students **must** furnish and wear lab coats during lab. Many of the chemicals are toxic and should be handled with care; gloves will be provided and hands should be thoroughly washed after exposure. No food or drink is allowed in the lab. Pregnant students should notify the instructor of their condition.

Maintenance of the lab will be the responsibility of every student. All instruments, glassware, and counter tops must be left clean at the end of each lab period. Lab grades will take into account your cooperation in keeping the lab orderly.

The lab will be open from 8 AM – 5 PM, Monday-Friday. You must schedule your work so that you will be finished for the day, and the lab cleaned up, by 5 PM.

Each student will be required to maintain a notebook. The lab notebook should contain dates, times, and procedures followed with enough detail to explain to another student what you accomplished, and also to enable you to repeat the procedure at another time. Lab notes must be taken during each procedure, updated after each lab, and include observations and results. Attempts to write notes long after the actual lab almost always are inaccurate or incomplete. Accuracy is much more important than neatness, but notebooks must be legible. The lab notebooks should be available for inspection and evaluation throughout the quarter. Since they are in part a measure of each student's personal observations, they should not be copied or shared.

Any drawings, notes, etc., must be attached to notebook pages.

Steps in processing tissue for microscopy:
DISSECTION AND FIXATION

One requirement for microscopy is to stop the process of degeneration once the tissue is removed from a living organism. The process of fixation is designed to chemically "freeze" the tissue so that its structure is as near to the living state as possible. One of the most commonly used fixatives is buffered 10% formaldehyde. Fixatives usually crosslink proteins and prevent them from denaturing.

This step will involve dissecting two organs (~30 min.) and returning the next morning at approximately 10 a.m. to embed the tissue (~1 hr).

Choose a specimen from among the pathology bottles. The specimens are in 10% formaldehyde. Before opening the bottle, look up your specimen in an anatomy text book in the lab to familiarize yourself with the orientation of the specimen. Decide if you will want to cut a cross section, longitudinal section, include all layers of a structure, or if orientation will not matter.

Keep in mind that the bottles may not be correctly labeled and/or the specimen in the bottle may contain several tissue types in addition to the label. For example, a sample of intestine may have peritoneum attached to it. At this point is less important to know the identity of the organ than it will be at the end of this lab, when you will need to describe your tissue slides.

Your goal at this step is to hand-cut a sample of tissue large enough to have a representative slice, small enough to fit into the embedding mold, and thin enough for fluids to diffuse through (1-2 mm). In general this means the maximum dimensions will be 22 mm^2 x 1-2 mm thick. You must keep in mind that all tissues will be shared, so do not destroy, mutilate, or waste the specimen while dissecting your slice. Students after you will have to obtain their specimens from the same stockpile. Use a sharp scalpel and make three slices.

Do not let any of the specimens become dried out. Return the organ to the specimen bottle and place your tissue slices in a tissue cassette and label it (in pencil) with your name and the tissue (or a code, e.g. A1). Place your tissue cassette in a beaker of tap water and notify Lu Brown or Dr. Itaya that your tissue is ready to process. Clean up the dissection area.

DEHYDRATION, CLEARING, AND INFILTRATION

That evening your tissue will be placed in the automatic tissue processor for washing, dehydration, and clearing. (water wash until 11 p.m., 12-1 a.m. 80% alcohol, 1-2 a.m. 95% alcohol, 2-3 a.m. 95% alcohol, 3-4 a.m. 100% alcohol, 4-5 a.m. 100% alcohol, 5-6 a.m. 100% alcohol, 6-7 a.m. xylene, 7-8a.m. xylene, 8- a.m. xylene. The purpose of washing is to remove all excess fixative. The increasing concentrations of alcohol baths gradually remove all water and replace it with alcohol (dehydration). The alcohol is then replaced by xylene, which is immiscible with water but mixes with paraffin. The tissue becomes translucent at this stage so it is called clearing.

Next the xylene is replaced by paraffin, the infiltration step. The cassettes are placed in

melted paraffin (~60°C) baths 1 from 8-9 a.m., and bath 2 from 9-10 a.m. Cassettes are then transferred to a bowl of melted paraffin at the embedding table.

EMBEDDING

At 10 a.m. you should be ready to embed. The tissue will need to stay immersed in melted paraffin until properly placed in an embedding mold. Place a plastic tissue ring into a reusable tissue mold and fill with paraffin from the paraffin pot. Quickly place your tissue from the bowl into the mold with forceps. Use a wooden applicator stick to orient your tissue at the bottom and center of the mold. Make sure your tissue is oriented the way you want to be able to section it later. Clean up the area and complete your lab notes. Your lab notes at this point will be very important for later steps. Describe in as much detail as possible (including drawings) the steps in your dissection and the orientation of the slice.

The rationale for paraffin embedding: in order for tissue to be examined with a microscope, it must be sliced into thin sections, e.g., 5-40 μm, for transillumination. In order to make thin slices, the tissue must be firmer than it is in the fresh condition. One of the most common ways to section tissue for microscopic examination is to embed it in paraffin, which provides uniform physical support for sections to be cut without distorting tissue structure. Since paraffin is not miscible with water, this requires removing all aqueous solutions from the tissue in a process of dehydration, immersing the tissue in a solution miscible with paraffin (clearing agent), soaking the tissue in melted paraffin to allow the wax to infiltrate into the cells, and then allowing the paraffin to cool and harden. If tissue is inadequately dehydrated, e.g., hurriedly run through the alcohols, what do you think might happen during infiltration?

At this point the blocks need to cool to allow the paraffin to harden. If you want to section right away, you can put the semi-hardened blocks in a bath of ice water to speed up the process and allow sectioning in a few minutes. Ice is available in the back room across the hallway. When hard, twist the tissue mold to release the block and save the mold for reuse.

SECTIONING (~2 hours/block)
[Sign up to use the microtome]

Procedure

1. Fill water bath with hot water and add a pinch of gelatin. Avoid bubbles. Stir any bubbles to the surface and wipe them away by dragging a tissue across the surface.
2. Trim the excess paraffin, leaving a trapezoid shape, with narrow top and wider bottom edges parallel.
3. Examine the microtome and note the clamps for the tissue block and knife, the thickness control (in back), the thickness indicator (front), the large rotary fine advance knob (right side), and the coarse advance/retract knob (left side).
4. Clamp tissue block on microtome.
5. Carefully and slowly move the microtome blade toward the tissue block, then clamp the knife tightly in place.
6. Rotate the coarse/fine rotary knob to advance the tissue close to, but not touching the blade.

7. Rotate the fine advance knob. The first sections will usually be paraffin only and also only part of the tissue block.
8. Continue sectioning until the tissue block is being fully cut.
9. Section tissue at 5-10 μm (thinner is better).
10. Save sections (ribbons); handle with brushes.
11. Float sections on 40°C water bath.
12. Mount sections on albumin coated slides.
13. Dry overnight in warm oven (<50° C), or at room temperature.

Do not change microtome blades on your own. If you suspect that the blade needs changing, consult with Lu or Dr. Itaya. Last year one lab group used up all the blades for the semester.

Cut enough sections for 1-4 per slide, and at least 4-6 slides; you should prepare extra slides to test your staining times, and to be able to submit your best samples for evaluation. Tip: cooling your block in ice water may improve sectioning. Use as little albumin as possible and wipe excess albumin off with your finger or it will pick up eosin during the staining step. Watch for knife marks and chatter, or uneven sectioning. Observe your sections and collect only those that cut through the entire tissue block and have a uniform, even appearance. Discard sections with flaws.

STAINING - Hematoxylin and eosin (~2 hrs/tissue)

Practice coverslipping before you begin staining. There will be old slides available for practice. Ask a lab assistant for help with this step. Tip: use as little Permount as possible, usually about one drop for a 22x22 cover glass. Excess Permount will make extra work cleaning your slide later.

Never allow slides to become dry after beginning. Choose 3-4 of your worst slides for trial staining. Run the trial slides through the rehydration solutions first and place all test slides in hematoxylin. Remove the slides one at a time at 5 minute intervals and soak in the water bath. You should end up with slides stained in hematoxylin for 5,10,15, and 20 minutes. These slides

can be placed in the sodium bicarbonate for about 30 seconds, then soaked in water. Quickly examine these slides with the microscope to determine the best staining times. Well stained slides will have prominent blue nuclei. If none have blue nuclei, you can place them back in hematoxylin. The eosin staining time should be half the hematoxylin time. Follow the rest of the procedures with your trial slides and examine them after coverslipping. If none of the tissue is stained satisfactorily, you should run more staining trials before using up your best slides. During processing, check sections to make sure they stay on the slides, and check solutions for contamination from previous use. After the test run you should stain your good sections using the best staining times as soon as possible. If you wait, the stain may change from other students' use, i.e., become weaker.

1. Clearing agent, 3-5 min	Bath 1
2. Clearing agent, 3-5 min	Bath 2
3. 100% alcohol, 3-5 min	Bath 3
4. 95% alcohol, 3-5 min	Bath 4
5. 70% alcohol, 3-5 min	Bath 5
6. Running water, 1 min	
7. Hematoxylin, 10-20 min (rinse and check periodically for intensity)	Bath 6
8. Running water, 30 sec (until water is clear)	
9. Sodium bicarbonate solution, 10 sec (check for blue nuclei)	Bath 7
10. Running water, 30 sec	
11. Eosin, approximately 0.5x hematoxylin time	Bath 8
12. Running water, 5-10 sec	
13. 70% alcohol, 20 sec	Bath 9
14. 95% alcohol, 20 sec	Bath 10
15. 100% alcohol, 30 sec	Bath 11
16. 100% alcohol, 30 sec	Bath 12
17. Clearing agent, 3 min	Bath 13
18. Clearing agent, > 3 min	Bath 14
19. Coverslip with Permount	
20. Let dry at least overnight, preferably a week	
21. Clean slides after dry	

Expected results: nuclei - blue, cytoplasmic structures - pink. Staining should make tissue and cell structures visible, but not so dark to cloud structures. Staining is always a trial and error process because the attraction of tissue for stain is dependent on all previous steps, e.g., the kind of tissue, quality and length of fixation, infiltration, section thickness, adherence to slide, etc. In addition, it is almost impossible to judge how a tissue has stained until after it is coverslipped. Plan on several trials to determine the best staining for your tissue sections.

Observe the xylene. It should be clear. If it is milky, it has become contaminated with water and must be changed. Wear gloves to avoid skin contact with xylene and avoid breathing the fumes by

using the chemical hood.

MICROSCOPY

Examine slides grossly and then with microscopic objectives from 4x to 40x (oil immersion is rarely needed). Compare your sections with the histology atlases; identify as many structures as possible.

For each tissue sample that you have prepared, describe your observations in your own words with labeled drawings or photos of your slides. Your report should include a description of the structure and organization of your tissue at low power, showing the entire tissue block; medium power, showing the organization of tissues and cells into structural and functional specializations; and high power, describing any cellular specializations you can see (such as cell shape, membrane specializations, etc.). The report should relate structural specializations with function. Your report must be limited to your observations of your own slides; do not copy/paraphrase text or atlas descriptions. Any descriptions of structures that are not observable on the slides, or any omission of structures that are on the slide will result in penalties.

The lab report must be typed, double spaced, and cannot exceed two pages, excluding figures.

Figures may be drawn by hand or photographed, using the video camera, frame grabber, and computer in the lab. Photography must be carried out by Lu or Dr. Itaya. You may print the image or save to a disk for labeling and manipulating on your own computer (you will need a Photoshop-type program that can handle .jpg format images).

GRADING/EVALUATION

At the end of the lab, turn in your lab notebook and two slides of each tissue. Slides must be clean and orderly; dirty slides will not be evaluated.

The tissue processing portion of the course is worth 150 points, and will be graded on: lab notes, lab report, lab performance, and quality of slides. Lab notes will be graded for legibility and completeness. The written report will be evaluated on overall quality, completeness, organization and clarity, and a demonstration of understanding of histology. Lab performance will be evaluated for cleanliness, timeliness, and cooperation. Slides will be evaluated for neatness, quality of sectioning, staining, and coverslipping.

Results may be presented to the class at the end of the semester for extra credit, if time allows. Details will be announced in class.