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Animal Histology Lab Manual

BIOL 312

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Qatar University

Table of Contents

General Information **1**
Lab Safety 3
Notebooks 4
Project: Slide Preparation 6

Lab 1: Introduction to Microscopy **7**
Microscopy Exercises 9
1. Adjusting binocular eyepieces for Interpupillary Distance (IPD) 9
2. Adjusting for vision differences between your eyes 9
3. Setting up K hler illumination. 10

Lab 2: Sample Processing & Fixation **12**
Slide Preparation and Cell Stress 13
Fixation 14
Tissue Processing Exercises 15
1. Dissection of Animal and Start of Tissue Processing 15
2. Preparation of a Mammalian Blood Smear 17

Important Note **18**

Lab 3: Dehydration, Clearing, and Embedding **19**
Dehydration and Clearing 19
Infiltration and Embedding 20
Tissue Processing Exercises 20
1. Dehydration (to be done before the lab) 20
2. Infiltration and Embedding of Tissue Samples 21

Lab 4: Sectioning **22**
Tissue Processing Exercises 23
1. Use of a Microtome to Create Sections 23

Lab 5: Staining **26**
Hematoxylin 26
Eosin 27
Tissue Processing Exercises 28
1. Slide Staining with H&E 28

General Information

Welcome to the Histology Laboratory.

The course you're about to take is a work in progress – I will probably be still writing up some of the lab materials from week to week. It is also very dependent on the resources that are available to us from week to week, and as such, it may deviate a bit from the schedule listed in your syllabus.

This lab has two main goals:

1. to give you practical experience with histological slide preparation
2. to allow you to actually see examples of the cells, tissues and organs you learn about in lecture and teach you to identify them

As such, your time, especially in the early labs, will be divided between learning the practical aspects of slide preparation and viewing prepared slides of the cells and tissues you discussed in lecture.

This document focuses on giving you the theory and procedures relevant to practical slide preparation. In addition to this, I will be posting separate handouts detailing the slides you are to view in that lab and the cells and structures you are to identify. Taken together, this document and any additional handouts that will be posted online for you, will form the laboratory manual for this course. Please bring this document and the handouts to every lab, as some of the information presented in earlier labs may be relevant a little later.

It is important that you read through each laboratory exercise and theory before coming to the lab. This will help you understand why you will be doing certain things and will make it more likely that you will successfully complete the exercises in the allotted time, it also helps to ensure that the labs are conducted safely. Thus your preparation will be frequently tested using quizzes (these are likely to occur near the start of the lab).

It is important to note that due to the nature of the project in this course, there will be times (especially later in the semester) when you will need to perform work in the laboratory outside of the scheduled lab time. It should also be kept in mind that the protocols listed in this manual are not ideal – I've had to shorten many of the incubations due to time constraints in the lab.

I hope that you enjoy the labs and I would appreciate any comments or suggestions that you have for future improvements.

I wish you much success in this semester,

R. Stefan Rusyniak

Lab Safety

Your concern for safety should begin even before starting your lab activity. Please make sure you always read your lab manual / handouts, and think about each laboratory assignment before starting. Doing so, will alert you to any chemicals and other potential hazards that you might encounter in the lab that day. It will also ensure that you've had a chance to think about how you will perform certain activities and why they must be performed in certain locations or in a certain order.

Always remember that you are not the only person working in a lab and your actions and knowledge can affect and/or ensure the safety of others. For this reason please familiarize yourself with the locations of safety equipment (safety shower, eye wash station, first-aid kit, fire extinguisher, and blanket) in the lab, as well as the location of the medical clinic office in case medical assistance is needed. Also, please make sure your books, bags and clothing are placed out of the way where they are unlikely to get in the way.

In order to protect yourself and others follow basic safety procedures in the labs. Some of our classes involve the use of chemicals so please know and follow the following rules:

1. You must wear a laboratory coat for all laboratory work. You will not be allowed to work in the lab without one (and will be marked as absent).
2. Eating, drinking, and chewing gum are prohibited in the laboratory at all times.
3. Bulky clothes, abaya, long scarf, dangling jewelry and short skirts can be a hazard in lab. Please do not wear them to the lab or make sure they are secure and unlikely to cause problems.
4. Confine long hair and scarf when in the laboratory so that it will not catch on fire or come into contact with chemicals.
5. Wear shoes rather than sandals at all times in the laboratory. Wearing open-toed shoes is hazardous in a lab environment (you will be asked to leave the lab and marked as absent).
6. Special care is needed if you wear contact lenses since chemicals splashed in the eye may get under the lens and therefore be difficult to rinse.
7. Mascara has harmful effect on ocular lens (eyepiece) of microscope, and therefore should be avoided.
8. Do not use flammable liquids near open flames. Most organic liquids are flammable.
9. Smell chemicals carefully and only when instructed to do so. Waft odors towards your nose rather than sniffing directly.
10. Wash hands thoroughly with soap and water immediately at the end of each lab secessions and before leaving the lab.
11. Broken glass should be removed from a work area and placed in the glass receptacle.
12. Biological waste should be placed in the appropriate waste container.
13. Notify your lab instructor immediately if you are injured in any way.

Notebooks

A laboratory notebook contains an accurate and detailed account of experimental procedures and results. It needs to be neat, well-organized and as complete as possible. The level of detail should allow another researcher to repeat your experiment and replicate your results.

It is important to keep an accurate and detailed laboratory notebook. Sometimes, a small detail may not seem important when you first see it, but it might be useful when you're trying to analyze the results – especially when they are not what you expected. Being able to go back through your procedures and observations in your notes can help you explain why your results are different from you would expect – it might turn out that you skipped a step in the protocol, or that you made a mistake somewhere along the way (sometimes mistakes are good things – they lead to new discoveries).

The lab notebook is the property of the lab and not of the researcher who is writing in it. In a research setting, a laboratory notebook never leaves the lab. This is important, because many researchers working in that lab may need to refer to that notebook in order to perform their work.

> For example, you might be a graduate student who is studying a particular gene. When you finish your project, you will need to be able to refer to your notebook to write your thesis. However, others may need to be able to use your notebook as well.

- Perhaps you made some modifications to a procedure that gave improved results – other people in your lab would benefit from having access to your notes on this procedure.
- Maybe one of your friends in the lab asked you to complete some part of an experiment for them, you would put your notes and observations in the notebook and your friend would later need to access them.
- Also, after you have completed your studies and left the lab, other graduate students might be asked to continue your research to find out more – these new grad students will benefit from having access to your notes (so they don't have to start from the beginning).

Advice on keeping a “proper laboratory notebook” varies depending on who you ask in a teaching lab, but there are a few things that are commonly mentioned by most scientists. Because some research can lead to financially rewarding discoveries and a laboratory notebook can be used as evidence of prior discovery in cases of disputes over intellectual property / patents, there are a few commonly agreed guidelines for keeping a notebook. Much of it has to do with ensuring that the contents of the book cannot be easily altered:

1. Notebooks should be permanently bound. This means that is should not be possible to remove (or add) pages from them without it being easily noticed.
2. All pages should be numbered. Again this will make it easier to notice if a page has been removed.
3. You should have a date on every page. This allows the tracking of when an experiment was first performed or when a discovery was made.
4. You should write in pen and never use white-out or corrective tape. If you need to make corrections then just neatly cross out the incorrect material. A notebook that shows evidence of erasing or removal of data (use of corrective tape, etc.) is not trusted.
5. A laboratory notebook should have your name in it, and a contact number in case the book is found by someone who wishes to return it.

Because a laboratory notebook tends to contain the details of many experiments, it is also a good lab practice to include a Table of Contents on the first 1-2 pages. In the Table of Contents you should list the important experiments and items, and indicate on which pages those items can be found.

For the purposes of this course, you will be maintaining a notebook detailing your work on your slide preparation. Please ensure that it contains a complete record of your work – if you notice that the staining on a slide needs improvement and you want to redo it, it would be helpful to know exactly what you did the first time so that you can make the appropriate changes on your second try.

Remember to make your notes as you do the procedure. Having an accurate but somewhat messy description of what you did is more important than having a neat but inaccurate account written based on your memory a day or two after the lab was completed.

Project: Slide Preparation

In the first few weeks of this course, you will be introduced to the basic techniques in tissue processing and preparation of slides for light microscopy. These first few labs will allow you to gain a little bit of practical experience with the procedures and tools that you will need to use in your project.

For your project, you will be producing well-sectioned and well-stained slides of a particular organ. It is hoped that you will produce slides of good quality that can subsequently be used in this course in the coming years, thus it is important that your slides are able to show relevant features clearly.

The project work will be completed over the course of the rest of the semester and will need to be mostly done outside of regular lab hours. Please keep in mind that **you will be responsible for the whole project** and no one will be there to remind you to get started, so manage your time wisely. Also, please note that you may potentially have to perform some of these procedures several times before you are able to produce slides of good quality, so please don't put this off to the end of the semester.

In the end, you will be submitting your two best slides for grading along with a 2 page (maximum) summary of the histology of the organ/structure/tissues (introduction), and a description of the histological features of the slides you are presenting along with any relevant slide coordinates.

Lab 1: Introduction to Microscopy

Much of what biologists wish to study is not observable by our unaided senses. For this reason, many fundamental questions about the nature of living things simply could not be answered by early scientists. As a result, many tools have been developed over time to extend our ability to observe nature. One such invention was the microscope.

Microscopes of various designs have been around for quite some time, but modern microscopy is generally traced back to the mid-17th century, when Anton von Leeowenhoek was the first to report the existence of microorganisms, and Robert Hooke coined the term "cell" to describe the small compartments he observed in cork with his microscope. Von Leeowenhoek went on to describe a variety of different cell types and made many improvements to the optics of his microscopes to enhance their resolution.

Increased resolution and magnification are two crucial properties of microscopes, with resolution being the more important of the two. Magnification is the process of making an object appear larger than it really is, while resolution refers to the amount of detail that is discernible in the resultant image (Figure 1).

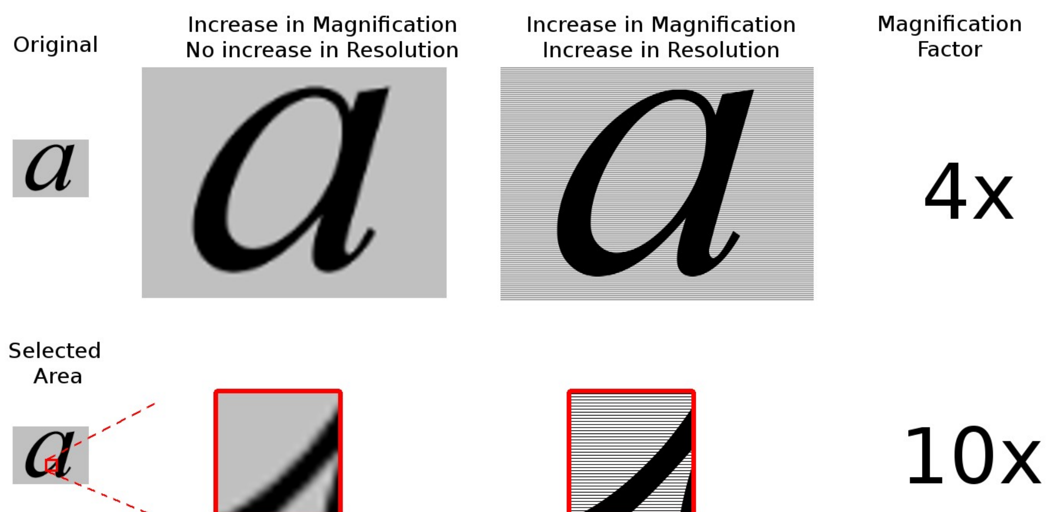

Figure 1. An illustration of the differences between magnification and resolution. The letter "a" was printed on a white background with very thin, black lines. Because the lines are very close together, they make the background appear gray. If the image is magnified without increasing resolution, no new detail is seen in the image (the background is gray). If resolution is increased along with magnification, then more detail is visible (thin lines in the background).

The lens system of a typical modern student microscope is designed to provide a resolution of about 0.5 micrometers. Careful matching of a light source and a precision lens system allows light microscopes in research labs to reach a resolution of 0.2 micrometers. This means that structures that are 0.2um apart can be seen as separate from one another.

For a compound light microscope, the specimen being examined is placed on a glass slide and viewed using light that is transmitted through the specimen. Light emitted by the light

source is focused onto the specimen and projected onto the objective lens by the condenser lens assembly. The objective lens then magnifies the image and conducts it to the ocular lens. The objective and ocular lenses are responsible magnifying the specimen, but it is the quality of the light and its focusing by the condenser assembly that determines the resolution and contrast of the image.

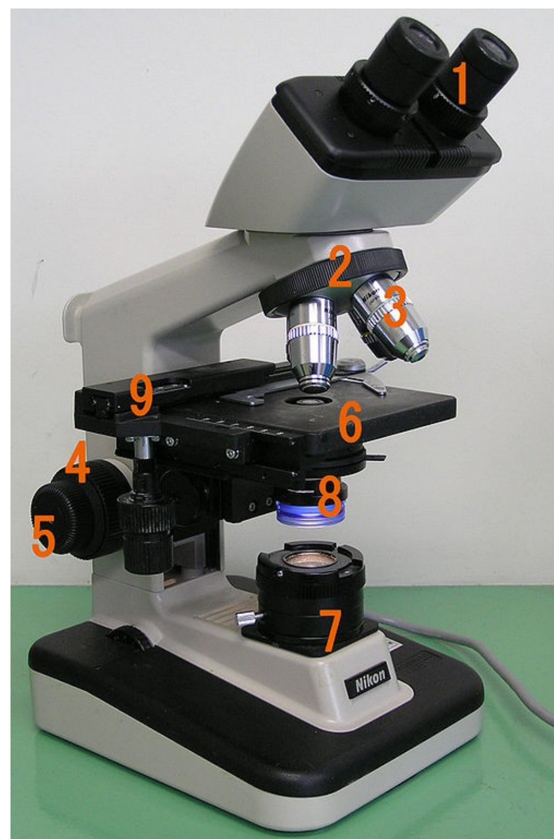

Figure 2: Parts of a compound microscope. (Image taken from Wikimedia Commons)

1. **Ocular lenses** (eyepieces). One of these usually has an adjustment ring for astigmatism, so please find it and use it to help you avoid eye strain. The binocular scopes can also be adjusted for interpupillary distance (the distance between the pupils of your eyes). Adjusting both of these features will help you customize the microscope for your comfort.

2. **Objective turret or nose piece.** Allows the changing of objective lenses to change the magnification. Be careful when swinging in the longer objective lenses – make sure they don't hit your specimen.

3. **Objective lens.** These lenses are often sold as a matched set and are parfocal. This means that once you get your specimen in focus with one of these lenses, you should only require the use of the fine focus knob to bring it back into focus after changing the lenses to a different magnification.

4. **Coarse focus adjustment knob.** Used bring things into partial focus, especially at lower magnifications. Avoid using this knob at high magnification.

5. **Fine focus adjustment knob.** Used to bring specimen into better focus. Should be the only adjustment needed at higher magnifications.

6. **Stage with slide-clip and location markings (9).** The clip is used to hold the slides securely in place as you move it around on the stage with the stage adjustment knobs. The location markings allow you to note down the specific locations of points of interest on your slides in case you need to find them again later.

7. **Light source (lamp housing) with a field-iris diaphragm.** The amount of light being emitted can usually be controlled through a separate control knob, and should never be set high (to protect your eyes). The top of the structure usually has an adjustment ring to control the opening of the field-iris diaphragm.

8. **Condenser lens assembly.** It usually includes the diaphragm lenses, condenser-iris diaphragm, and condenser centering controls. The position of the condenser lens assembly is controlled by a condenser focusing knob located near the fine and coarse-focusing knobs. You will be using the condenser lens assembly when calibrating the microscope. You are also likely to periodically adjust the condenser-iris diaphragm opening while viewing slides to help increase or decrease contrast of the image in your field of view.

In this part of the course, we will learn some of the ways in which microscopy is used in the study of cells. We will discuss some aspects of sample preparation for microscopy, you will prepare some slides and you will learn how to properly set up and use a compound light microscope. Later this term, you will also learn how to use it to do some viable cell counts on a cell culture, and you will learn about fluorescent microscopy. In today's lab, we will focus on the basics.

Microscopy Exercises

Materials

- Compound Light Microscope
- Prepared Slides

1. Adjusting binocular eyepieces for Interpupillary Distance (IPD).

The interpupillary distance is the distance between the centers of your two pupils and is unique o each person. During your first session with a new microscope, you should determine the correct setting for your eyes and set the microscope for that distance. During subsequent sessions, you should set the microscope to that setting.

If you look through the eyepieces and see two images, the interpupillary distance is not correct. To correct it, slide the eyepieces closer together or farther apart until the two fields merge to form a single circle of light. The interpupillary distance is now correct for you.

Procedure:

1. Examine the ocular eyepieces on your compound microscope and note how they move closer and farther apart.
2. Place a specimen on the stage, look down one ocular with one eye, and focus the specimen. Do not worry whether you are using one or both eyes or one or both oculars at this point.
3. Slowly move the two oculars closer together or farther apart until you can see the specimen with both eyes through both oculars. Instead of seeing two separate fields of view – one with each eye or overlapping fields of view, you should now see only one uniform field of view if you have adjusted the two eyepieces to the correct IPD for your eyes.
4. Record your IPD from the scale located near the ocular lenses of your microscope so you can quickly set up your microscope in future labs.

My Interpupillary Distance (IPD) is ____________________.

2. Adjusting for vision differences between your eyes.

Procedure:

1. With a slide on your microscope, locate the specimen under low power (4X or 10X) and then find a portion you wish to focus on.

2. Look at the specimen with only your right eye and get the slide into focus using the coarse and fine focus knobs. The left eyepiece is usually adjustable to compensate for refractive differences between your two eyes. Look for a diopter adjustment ring on the left eyepiece.

3. Close your right eye and look through the left ocular lens. Do not touch the focusing knobs. Adjust the focus for your left eye using only the diopter dial on the ocular lens.

4. Now you should be able to see the specimen in sharp focus for both eyes.

5. There is usually a scale under the diopter adjustment ring. Check to see if you can see any numbers on there and if they are positive or negative and record them for future reference.

My diopter setting is ____________________.

3. Setting up K hler illumination.

For a long time, the light source caused microscopists some challenges in obtaining good images. Lighting was generally uneven and the light filament of the light bulb (the source of the light) was often visible in the background of the images and did not allow for optimal resolution. This changed when August K hler developed a new method of illuminating microscopy samples in the late 19th century. Most modern microscopes now benefit from this innovation.   K hler illumination allows for very even lighting of the whole sample and optimal resolution when properly set up.

In this exercise, you will learn how to properly set up K hler illumination on your microscopes to allow you to properly align your lens system, leading to more optimal viewing of your samples and less eye strain and fatigue. This technique should be used every time you wish to view slides under a microscope and wish to obtain the best possible image.

Procedure:

1. Turn on your microscope, ensure that the objective lens is set to the lowest magnification, secure a slide on the stage, and focus on an area of the specimen. Which part doesn't really matter, we simply need to ensure that we have something in focus before we proceed.

2. Close the ***field-iris diaphragm***. This is located on the base of the microscope. Usually this is a plastic ring, built into the top of the lamp housing, that you can twist to open and close the diaphragm. Do this while looking through the ocular lens – this will allow you to see a bright spot of light appear in your field of view and get smaller. Once the ring-iris diaphragm is "closed" you will probably see a relatively small "fuzzy ball of light" on a slightly darker background. Your specimen will still be visible and in

focus. If your lens system is very "out of tune" the spot of light is likely to be near the edge or outside of your field of view.

3. Use the ***condenser focusing knob*** to adjust the height of the ***condenser assembly*** until the "ball of light" shows a well defined edge. The boundary between the light area and the darker area in your field of view will become clearly visible.

4. Once the bright spot is in focus, you will likely notice that it is not in the centre of your field of view. You will need to use the ***condenser centering controls*** (two long, metal knobs/screws) to adjust the location of the light region until it is centered. I suggest that you just get it close to the centre initially. Once it is close to being centered, open up the field-iris diaphragm until the edges of the light spot are very close to the edges of your field of view. Then, adjust the condenser centering controls again until it is centered.

5. Adjust the ***condenser-iris diaphragm*** opening to improve the contrast of the image

6. Open up the field-iris diaphragm to just beyond the field of view.

Your microscope should now be ready to use, but you should repeat the procedure when you change the objective lens to a different magnification to ensure that you're getting the best possible view of your specimen. This may seem like a lot of work for what seems like very minor improvements, but there will be times when it really makes a difference – the "small" improvement in resolution can be very important. Also, once you've done it a few times, the whole procedure takes no more than 30 sec.

Lab 2: Sample Processing & Fixation

There are several different ways to prepare samples for viewing under a microscope. There are “wet mounts”, where you can view a live specimen in water (you simply cover a drop of water with your specimen with a coverslip and view it), and “permanent slides” where you view a dead specimen. The advantage of the permanent slides is that they can be prepared in advance and viewed at any time, they can be prepared in a way that will enhance the contrast of the structures of the specimen to make it easier to see details, and they are permanent so you can view them again at another time and the structures you were viewing earlier are still in the same place. The disadvantage is that it takes longer to prepare a permanent slide.

Permanent slides can be prepared in different ways, this depends on the physical characteristics of the specimen to be viewed and the purpose of obtaining and viewing the sample.

If a sample is in liquid form, then it is prepared as a smear (sometimes labeled as “sm” on the slide or the slide inventory in the slide box). The procedure for preparing a smear is generally very quick and simple. A small amount of the sample is applied to the slide directly and pulled/drawn across the surface of the slide to generate a thin layer of the sample. It is then stained for a relatively short period of time (a fixative is often incorporated into the stain to preserve the sample), dried and coverslipped, and can be viewed under the light microscope. Thus a smear can be prepared and analyzed fairly quickly after obtaining the sample.

A quick way to prepare more solid samples is to perform a squash preparation (sometimes labeled “sq”). This is exactly what it sounds like – you simply squash your sample (if it's small and soft enough) down under a coverslip to produce a thin layer of cells on the slide (the sample is usually fixed before the squashing). The cover slip is then removed and the sample undergoes a quick staining procedure before being mounted and viewed under the microscope. This type of preparation is generally used for diagnostic purposes when the microscopist is looking for the presence of very specific features (ie. stage of mitosis or meiosis) and is not interested in the general appearance and organization of the tissue on the slide. In this situation it would be impractical and unnecessary to prepare the sample as a section. In fact, using a squash procedure might provide more examples of the required feature because more of the cells of interest would be spread across the surface of a single slide.

A spread is prepared in a similar way, but there is no force applied to the tissue. This type of preparation is used for very thin and delicate tissues, where the tissue is gently removed from it's initial location and is simply gently stretched across the surface of a slide. The thinness of the sample permits enough light to pass through it to allow the microscopist to see the structures and cells present in this tissue easily without having to section the tissue.

A tissue section is the most common type of slide that you will see in this course. This type of preparation is meant to give the microscopist the best possible view of how the tissue is organized and the appearance of the cells in the living state. For this reason, much effort goes into ensuring that the sample is well preserved as soon as it is taken, and that the processing of the tissue results in minimal unintended physical changes. In this type of preparation, the target tissue is fixed (preserved), embedded in a rigid supporting material, sectioned into thin slices and stained to increase the contrast between the different tissue components.

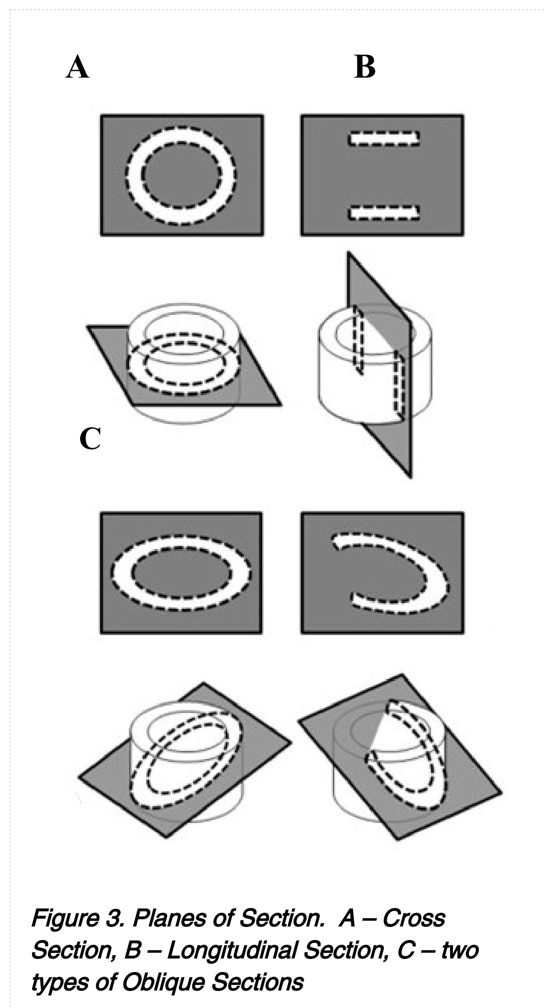

Figure 3. Planes of Section. A – Cross Section, B – Longitudinal Section, C – two types of Oblique Sections

Because different structures and organs are composed of cells organized in very specific patterns and layers, it is often useful to pay attention to the orientation of the sample as it is being sectioned. Sections are frequently made in cross-section (“cs” or “xs” on labels) or in longitudinal-section (“ls”), but sometimes it is difficult to specifically orient a structure if it is part of a larger sample and then it may appear to be in an “oblique” section. Oblique sections of different structures and tissues on a slide are quite frequent, so it is important to be aware of them and to be able to recognize them so that the slide can be interpreted accordingly. The importance of this will become apparent when we start looking at epithelia and cell types where shape is an important feature.

In today's lab, you will obtain a tissue sample for your project work and will also prepare a smear of a blood sample for viewing next week. We will spend the rest of the course viewing and producing sections.

Slide Preparation and Cell Stress

Cells maintain very tight control over their internal environment; this is called **homeostasis.** Anything that negatively impacts the cells' external environment (low levels of nutrients, low levels of oxygen, presence of toxins or free radicals, etc.) has the potential to affect their ability to maintain homeostasis and can thus cause the cells to become stressed. Such conditions tend to elicit a very specific stress response in cells and can affect their appearance under the microscope.

The reason that we start the discussion of tissue preparation for microscopy with the stress response, is that slide preparation involves the removal of cells from their normal environment and exposing them to stressors like the lack of oxygen and nutrients, and a lack of normal signaling molecules.

Prolonged exposure to these stress-inducing conditions can lead to physical changes in the cell as it struggles to maintain homeostasis, and potentially turns off a variety of non-essential pathways (and turns on stress-response pathways). As the cell shuts down any

non-essential processes in order to focus on simple survival, it tends to lose any specialized proteins and organelles/structures used to perform its specific functions. This is of concern to microscopists because it is these specialized proteins / organelles / structures that give the different cell types the characteristic appearance that we use to identify them.

This stress response is also of concern to pathologists. Pathologists need to be able to look at a slide of a biopsy and differentiate between normal/healthy cells and damaged/diseased cells. Disease in a cell is caused by different stressors (viruses, toxins, disregulation of homeostasis due to genetic mutation, etc.) and it is identified and located by comparing the appearance of those cells to normal cells. Differentiating between these diseased and normal cells would be very difficult if all the cells on the slide were responding to stress caused by their removal from the body.

The purpose of all the steps in slide preparation is to preserve the cells and tissues and show them in as close to their natural state as possible while making their details easily distinguishable under the microscope.

In preparation for microscopy, the tissues are first placed into a **fixative**. This preserves the tissue in its "natural state" and prevents the activation of processes that might affect its appearance. This tissue will later have to be sliced very thinly (**sectioning**) in order to allow light to easily pass through the tissue. Very thin sectioning also ensures that there is usually only one or two layers of cells on the slide making it easier to view and interpret.

Because sectioning generates such thin slices of tissue, there is very little material between the light source and the eye of the microscopist, this makes it very difficult to actually see any detail on the slide; structures need to block/absorb some light to appear darker than the background. **Staining** the section with combinations of dyes increases the contrast between the different components on the slide and allows us to see the tissue more clearly.

Unfortunately, the process is not quite that simple. Slide preparation is fairly prolonged process involving many steps.

The order of the main steps is as follows:

1. Fixation
2. Dehydration
3. Clearing
4. Embedding
5. Sectioning
6. Staining

Fixation

Fixation is the initial and most crucial step of the slide preparation process. As mentioned earlier, fixation is the process of preserving the cells and tissues in as close to their natural state as possible. In order to do this, the tissue sample must be placed into the fixative as soon as possible after removal from its natural environment (or as soon as possible after the death of the donor organism).

One of the main functions of the immediate use of a fixative is to terminate any cellular function in the specimen. This ensures that some of the degradative processes associated with cell stress and cell death will not have enough time to become activated or are stopped before damage to the cells can occur. The fixative will also do this to any microorganisms that may have been introduced onto the tissue sample during the dissection, and might otherwise have caused tissue damage. Thus the tissue sample will be preserved in the condition it was in at the time that it was extracted.

The fixative preserves the internal structure of the cells. It denatures and cross-links proteins to one another – acting like a glue – making them less soluble in water and thus less likely to be removed throughout the remaining steps of slide preparation. A similar effect is achieved in the extracellular matrix (ECM) which leads to tissue hardening and increased physical strength.

Additionally, the fixative tends to permeabilize the membranes (making them more porous to large molecules). This means that some molecules (like carbohydrates) may be lost from the cells in later stages of slide processing, but it also means that things like stains, antibodies (in the case of immunocytochemistry) and paraffin wax will be able to more easily enter the cell.

One of the drawbacks of chemical fixation (tissues can also be preserved by freezing) is that it can introduce some unintended physical changes like shrinkage – the cells and ECM can be caused to become smaller during this process (and thus become separated from surrounding ECM). The extent of the shrinkage is dependent on the specific chemical and procedure used.

There is a wide variety of fixatives used in tissue preparation, each with a slightly different set of properties (and disadvantages). They are selected based on which specific properties of each mixture would be most advantageous with that particular tissue and what it will be used for afterwards (ie. some fixatives allow protein cross-linking to be reversed and some allow for better penetration of antibodies).

In order to be effective:

- fixation should happen immediately after the acquisition of the tissue sample
- the tissue sample should be very small so that the fixative can efficiently penetrate the tissue
- the tissue should be placed into a relatively large (compared to the specimen) volume of fixative

Tissue Processing Exercises

1. Dissection of Animal and Start of Tissue Processing.

We generally receive fixed tissues from the animal research facility at QU, so you will not be doing this part in the lab. The following procedure would be typical of how your samples would be obtained, and is included here for completeness (in case you ever end up doing something like this in your research project).

You will notice that the procedure specifies that the organ needs to be cut into relatively small pieces – about 3-5mm per side. The smaller the piece of tissue, the more quickly the fixative can penetrate the sample. If you do take a larger sample, you should try to make sure that at least one of the sides is ~3mm or less in thickness – the thinner the side, the

more efficient the fixation will be. Also, you need to ensure that you work efficiently and get your tissues into fixative as soon as you can. This will help to ensure that what you see on your slides later is as close to a natural state as possible.

Sometimes a tissue sample needs to be washed in a buffer (PBS – phosphate buffered saline) before being placed in fixative; this is done to remove excess blood. In such cases, the buffer is kept cold. This helps preserve the tissue by slowing down all enzymatic processes, thus any degradative enzymes that might be produced before the tissue is placed in fixative will be significantly slowed down and their damage will be minimized.

Materials

- euthanized laboratory rat
- scalpel or scissors
- metal probes or pins (for moving organs and skin out of the way)
- forceps
- 10% formalin (10% formaldehyde, 1x PBS, pH 7.4)
- Jar or capped vial for fixative and tissue storage
- Benchkote paper

Procedure

1. Place the rat on its dorsal side (back) and secure it in place
2. From the throat of the rat, cut down the center of the animal until you reach the genitals.
3. Be careful not to cut too deeply, you only want to cut through the skin
4. At the end of your cut (by the genitals), cut laterally (to the sides)
5. Locate the bottom of the ribcage and make lateral cuts there as well
6. Open the flaps of skin to expose the abdominal cavity

The purpose of this lab is for each student to obtain some tissue samples that she will work on for the rest of the semester. Each student should try to obtain a different organ this way we can produce a variety of slides for use in this course. The liver is being used here simply as an example.

7. Locate the liver. It is a large, dark coloured organ just below the ribcage, composed of several lobes.
8. Cut off a portion of one of the lobes and place it on our work surface
9. Cut the liver fragment to an appropriate size for fixation. Remember to make at least one of the sides about 3mm in thickness.
10. Try to cut in such a way as to make the cut surfaces fairly smooth. Use a sharp scalpel and long smooth slicing motions.
11. Place the tissue fragment in some cold PBS for 2-3min.
12. Transfer the tissue into fixative. Do not just leave it while you work on the other tissue sample.

You should cut out a few samples of each organ. It's always better to have a few tissue samples available for your work – you will not get another chance to obtain more samples is something goes wrong. Also, please keep in mind that a living organism is being sacrificed for this, so please try to make sure that we get as much benefit from this sacrifice as possible.

You should especially try to take samples of the more “solid” organs like the heart (make sure you take samples that contain the whole thickness of a ventricle), kidneys, ovaries or testes, spleen, pancreas, tongue.

Also, try to take samples of some hollow organs like the aorta, the vena cava, esophagus, stomach (this might get smelly), small intestines (take sections from different parts along the length of the intestine), large intestine, appendix, gall bladder, urinary bladder, trachea and bronchi (the cartilage rings might make cutting a bit difficult here, so be careful). Try to cut out cross sections of these organs – the walls of the sections should be thin enough that you may not have to do much with these samples to allow for efficient fixation.

The fixation process generally takes place overnight at 0 – 4ºC (in most cases, it should not exceed 24hrs). Your technician or I will come in the next day to remove the fixative and place the samples into 70% ethanol. Please be sure to label your vials of fixative very clearly and put them in the place designated by the technician, so that we know which vials we need to work on tomorrow.

The 70% ethanol will keep the tissue preserved and start the dehydration process. When you come back next week, you will be able to continue the dehydration and the rest of the protocol.

2. Preparation of a Mammalian Blood Smear.

A blood smear (or blood film) is a frequently used diagnostic procedure in the health field. It allows medical doctors and pathologists to microscopically examine the various blood cells and quickly diagnose disorders that tend to show up in the blood. The blood smear is so common because it is quick and easy to prepare and can yield some important information about the health of a patient.

Your blood contains a mixture of cells. A majority of them are erythrocytes (red blood cells), while a small proportion (about 1-2%) are commonly known as “white blood cells” (leukocytes). The leukocytes are subdivided into several cell types, and tend to be found in the bloodstream in fairly consistent proportions. Noticing abnormal levels of one cell type or another can give doctors clues about the health problems that a patient might be presenting.

In this exercise you will be producing two blood smears for viewing under a microscope.

<u>Materials</u>

- Leishman's stain
- Glass slides
- Large coverslips (if available)
- Water, pH 6.8
- Running water (from a tap)
- Blood sample

Procedure

1. Place a small drop of blood in the middle of one end of a slide, near the labelled end (usually frosted).

2. Use the side of another slide as a spreader. Place the edge of this “spreader” against your slide and hold it at an angle (~30-40°) pointing away from the drop of blood.

3. Slide the “spreader” towards the drop of blood until it touches the edge of the droplet. The blood is likely to then spread sideways along the edge of the “spreader” by capillary action. You can slightly wiggle the “spreader” to help the blood migrate sideways.

4. Once the blood has spread from one edge of the slide to the other edge, pull the “spreader” down the length of the slide to “pull” the blood and spread it along the slide. You should do this in one smooth motion. Ideally, the smear should cover a majority of the slide and should end in a feathered/rounded edge.

Figure 4: Two peripheral blood smears suitable for characterization of cellular blood elements. Left smear is unstained, right smear is stained with Wright-Giemsa stain. (Image taken from Wikimedia Commons)

The Leishman's Stain

1. Allow the slides to air dry.

2. Put 4-8 drops of Leishman's stain on the slide (should be enough stain to cover the surface of the slide but not spill over the sides) and incubate at room temperature for 3 min. Leishman's stain includes methanol which will act as a fixative and preserve the cells.

3. Add an equal amount of buffered water, pH 6.8 (if you added 4 drops of stain, then add 4 drops of water). Add water slowly and mix it in with the stain by sucking the stain up and down with a plastic pipette.

4. Incubate the slides with the diluted stain for another 12 min. It is common, at this stage, to see some “filmy residue” on the slides – this is normal and can be ignored.

5. Wash off the stain under a slowly-running tap. Then put the slide down again, flood it with buffered water, pH 6.8 and incubate for one more minute.

6. Air dry the slide and mount with a coverslip if available.

Important Note

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Next week's lab involves several long incubations and needs to be started about 3hrs early in order for you to be able to complete it. Please be sure you arrange for someone to come in early before next week's lab in order to start the tissue processing for you.

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Lab 3: Dehydration, Clearing, and Embedding

Once the tissue is fixed, it will need to be infiltrated with wax (sometimes plastic) before it can be sectioned. The wax will make the tissue sample hard enough to cut into the very thin sections needed for microscopy (4-6µm). The problem with this is that wax needs to enter the cells and tissues, and fill the spaces that are occupied by water. Since water and wax do not mix, water needs to be completely removed from the tissues before wax can be applied. This is where the next two steps become important

Dehydration and Clearing

The replacement of water with something that is miscible with wax is a two stage process. First the water is replaced by ethanol, and then the ethanol is replaced by an organic solvent like xylene or toluene. The ethanol is used because it is miscible with both the water and the organic solvents.

The dehydration is done as a **graded alcohol series**. This means that the tissue sample is immersed in gradually increasing concentrations of ethanol until absolute ethanol (100% ethanol) is reached. The graded alcohol series is based on the simple concept of diffusion of a solute in a solvent and osmosis. Here is how this works in practice:

The tissue is initially placed in a low concentration ethanol solution (ie. 50%) for a period of time. During this time, the ethanol molecules move into the tissue and cells by diffusion while the water that is inside the cells and tissues leaves to dilute the ethanol in the solution (osmosis). The incubation times vary between protocols but generally range from ~10min to ~60min, and depend on the size and type of tissue being processed. At the end of that incubation, the concentrations of ethanol inside the tissue will have reached the same concentration as outside the tissue. Thus, 50% of the water inside the tissue has been replaced with ethanol.

The tissue is then placed into 70% ethanol and over time, the ethanol will be redistributed and replace more of the water inside the cells and tissues. At the end of this incubation, the concentration of ethanol inside the tissue will be 70%.

This is repeated with several more concentrations of ethanol until 100% ethanol is reached. This final incubation is usually repeated (with fresh 100% EtOH) to ensure that as much water as possible has been extracted from the tissue.

This process is gradual so that the tissue is not damaged or distorted by the process. A similar gradual procedure is sometimes followed in order to replace the ethanol with an organic solvent that is miscible with wax – this is called **clearing**. Gradual clearing however is not always done; sometimes the sample is simply placed in concentrated clearing reagent and the procedure simply involves relatively long incubations of the dehydrated tissue in several changes of the organic solvent. The number of changes and length of incubations again depends on the size and nature of the tissue sample. At the end of this process, the sample should be ready for infiltration by wax.

Infiltration and Embedding

The purpose of infiltration and embedding is to give the tissue rigidity before it is sectioned. Without this step, the tissue sample would simply be too soft to cut and it would be too difficult to control the orientation in which it is cut.

Infiltration is the process of replacing the clearing agent with an embedding material – often melted paraffin wax. The actual process is usually very similar to the graded series used in dehydration and in clearing (although some protocols deviate from this). Usually, the specimen is moved through several changes of melted paraffin to replace the clearing reagent with wax. The process can be sped up somewhat by performing it in a vacuum – the clearing reagent is usually volatile and tends to evaporate even more readily at the high temperatures (~60-65ºC) used to keep the paraffin in its liquid form.

Embedding involves carefully placing and orienting the infiltrated tissue into a mold containing solidifying paraffin wax. This mold will then be used to allow the wax around the specimen to set and form a solid support (known as a block) that can be easily held and manipulated by the apparatus used for sectioning (the microtome).

Tissue Processing Exercises

To make sure you have enough time to do this, **please complete Part 1 before the start of the lab** (you should try to start about 3hrs before the lab). If you can't come in for one of the changes, then please arrange with another student to do it for you.

1. Dehydration (to be done before the lab)

Before the tissue samples can be embedded in wax, the water must be gradually removed from the samples. This is done through a graded alcohol series – the alcohol replaces the water in the cells and tissues. The alcohol is then replaced by a clearing reagent like xylene or Histoclear, which is then gradually replaced by paraffin wax. The key here is the gradual replacement of one component by another (thus the use of graded series) until the tissue is infiltrated by a solvent that is compatible with the embedding medium (wax).

Materials

- Fixed tissue
- Graded Ethanol series
- Xylene
- Graded series of xylene mixed with paraffin
- Paraffin wax
- Tissue block molds
- Forceps for handling tissues in the solvents
- Forceps for handling infiltrated tissue

Procedure

Dehydrate the tissues by placing them in the following alcohol series.

1. 80% EtOH for 30min
2. 95% EtOH for 30min
3. 100% EtOH for 30min
4. 100% EtOH for 30min (fresh ethanol)
5. 100% EtOH for 30min (fresh ethanol)
6. Xylene for 15min
7. Xylene for 15min (fresh xylene)

2. Infiltration and Embedding of Tissue Samples

Processed samples will now undergo infiltration, where the clearing reagent will be replaced by paraffin wax. It is absolutely crucial that the tissue be well dehydrated and cleared before infiltration is attempted. Wax mixes very well with any left over clearing reagent and allows it to escape easily, but it will not mix or allow the removal of any remaining water or dehydrating reagent. If there is still any water or ethanol in the tissue, it will remain there and generate artefacts on the slides later. This is why the previous steps are so important.

The next part of the experiment will be done with warm solutions and on warming plates (to keep the wax in a liquid state until we're ready for it to solidify). The paraffin wax is usually kept about 2ºC above its melting point (~60ºC) until the final step where it is allowed to gradually solidify around your sample. Wax infiltration is sometimes done in a vacuum – this helps remove any left over xylene more efficiently and allows the wax enter into the cells more easily.

Infiltrate the tissues with paraffin as follows:

1. 50% paraffin / 50% xylene for 15min
2. 100% paraffin for 1hr
3. 100% paraffin for 1hr (fresh paraffin – should be free of any contaminating clearing reagent)
4. Pour a small amount of paraffin into a mold
5. Use **warm** forceps to transfer your sample into the mold
6. Place the mold on ice (or any cold surface) and gently press the tissue into the bottom. This will allow a thin layer of the paraffin to solidify around the tissue and hold it in place. Think about the orientation of your sample at this point – ie. do you want it to be in cross section?

When the tissue is properly oriented, allow the wax around the sample to set. It will start to become more opaque.

7. Add the plastic tissue cassette on top of the mold and add paraffin to the mold until it reaches the top of the plastic cassette. Make sure your cassette is labeled.
8. Allow the wax to cool to room temperature and let it set for about 30min before removing the mold.
9. Store your blocks at 4ºC overnight (or until the next lab)

Lab 4: Sectioning

Once your tissues are embedded in a solid resin or paraffin, it can be cut into thin sections for your slides. Generally, only a very small amount of the tissue will be sectioned and the rest can be stored for later use if warranted (ie. if you're doing research on something and will likely need to do experiments on that tissue again). The nice thing about embedded tissue is that at this point it is well preserved and can be stored for long periods of time.

The reason that “only a very small amount of tissue” is ever used is that the sections of the tissue are extremely thin – generally 4-10μm in thickness – so, even if you take 100 such sections, you will only have removed a very small amount of your total tissue sample.

Sections for light microscopy are usually cut using a precision tool called a **microtome** (tissues preserved by freezing are cut using a similar device called a cryostat). The microtome allows you to mount your block on a moving arm which will pass over a very sharp knife. It does so in a cyclic fashion as illustrated below:

1. the arm with your block starts above the blade and moves down
2. the arm moves to the knife and past it cutting off a thin section
3. the arm then starts to move upwards towards its original position
4. the arm moves forward by 4-10μm (depending on the setting)
5. the arm is ready to continue the cycle adding more sections to the “ribbon” that is generated as each section is attached to the previous one.

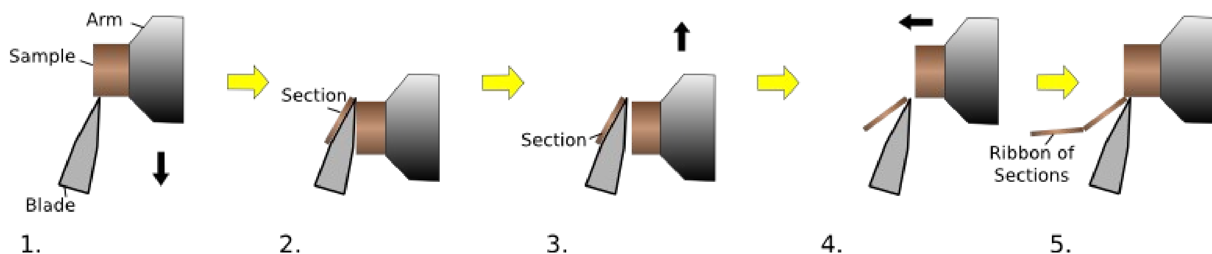


Figure 5. Microtome Sectioning Cycle. Sample is aligned just above the blade (1), the arm moves down (2) causing a thin section to be produced (3). The arm moves up, and forward by a few micrometers (4), and is aligned above the blade for another section (5).

This process can generate a fairly long ribbon as more sections are attached to the previous ones. These sections are very thin and tend to become wrinkled in the process of being sectioned. Gently warming the sections on the surface of water before placing them on slides allows the sectioned tissue to straighten out. This process is generally done in a water bath at ~50-55ºC – usually about 10ºC less than the melting point of the paraffin wax.

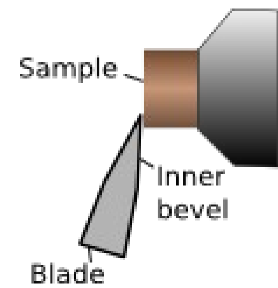
Sectioning is a bit of an art – it tends to require lots of practice before someone can consistently generate good sections of tissue. Here are a few tips to keep in mind:

– keep the tissue block cold (it makes the wax and tissue more rigid)

– the blade should be sharp and cold

– the angle of the blade needs to be adjusted optimally – this means that the inner bevel of the cutting edge should be about 0.5º away from being parallel to the cut surface of the block

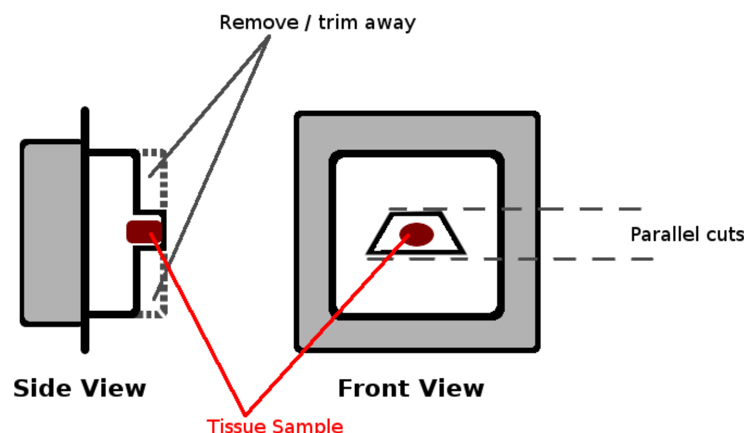
– sections should be cut fairly slowly



Tissue Processing Exercises

1. Use of a Microtome to Create Sections

Today, you will process the paraffin blocks you made last week. You will trim the block and section your tissues. When trimming the block, you should try to produce a "trapezoid" shape around the sample, with the cuts above and below the sample being parallel to each other (this will help obtain a straight "ribbon"). Try to remove enough paraffin from the block



to leave about 2-3mm of wax around the sample.

Once trimmed, you will mount the block in a microtome and generate some sections for staining next week.

Materials

- prepared paraffin blocks from the previous week
- sharp knife or razor blades
- microtome
- microtome blade
- fine brushes (for handling the sectioned tissue)
- water reservoir (for floating sections)
- glass slides (4-5 per group)

Caution

Microtome blades are extremely sharp. Always work very carefully when the blade is present in the microtome and **never** try to catch it if it falls. A natural reaction is usually to try to catch a falling object, so you really must be mindful of this.

Procedure

1. Use a sharp knife or razor blade to remove/trim excess wax from around your specimen. Be sure to leave straight edges around the sides of the remaining wax.
2. Mount the paraffin block in the microtome. Be sure that the bottom of the trapezoid you trimmed out is parallel to the blade.
3. Bring the arm down to the blade
4. Adjust the angle of the blade to ensure a good section
5. Start by making thicker sections (~10µm) until you've started cutting into your tissue sample.
6. Change the thickness to 5-8µm and continue cutting.
7. Start sectioning the tissue (adjust the angle of the knife if needed) until you've generated a short ribbon
8. Use your paintbrush to gently lift the section away from the blade so that more sections can be added onto the ribbon

There are a few common reasons why you might be having a hard time getting a ribbon:

- Your knife is dirty (has wax residue on its sides).
  - Use a brush dipped in xylene to clean off any wax residue from the sides of the blade. Be sure to brush upwards (towards the sharp edge) this way you don't damage your brush on the blade.
- Your knife is dull.
  - Try loosening the knife and moving the blade over a bit to a (hopefully) sharper region. Don't forget to tighten the clamps that hold the knife afterwards – a loose blade can also cause problems with sectioning.
- Your knife is at the wrong angle.
  - Adjust the angle of the blade
- Your knife is too warm
  - Remove the knife and place it in a freezer for a little while, or hold an ice cube against the knife while it is still mounted in the microtome.

9. Once you've generated a ribbon of 4-7 sections, use your paintbrushes to lift the ribbon off the blade and float them on the surface of warm water (you can also store your ribbons in a dry box if you want to mount them on a slide later)
10. Allow the sections to float on the water's surface for about 5-10min to flatten out
11. Gently separate the ribbon into smaller groups of sections – remember that you will need to cover them with a coverslip at the end, they will have to fit under one.
12. Gently insert the edge of a slide below the surface of the water next to the floated ribbon.

It might be a good idea to look at the condition of the slide before using it and to clean it with some ethanol and a Kimwipe before using it. This will remove any dust and particulate matter from the slide before you put your tissue sections on it. Be sure to use a Kimwipe and not just any tissue, Kimwipes are used because they are lint-free – they won't leave behind any paper fibers or dust on the slides.

13. Position the slide under the ribbon and slowly lift the slide out of the water, lifting the sectioned ribbon with it.
14. Stand the slide on its edge against a support and allow excess water to drain from the slide. Allow 15-20min for this.
15. Place the slide on a slide warmer (or oven if available) at ~45ºC overnight to allow the water to evaporate and help your sectioned tissue adhere to the slide. Make sure the slides are covered in some way (a shallow box is often used) to prevent dust from settling on your sections.

These slides can then be stored until needed or undergo the staining procedure.

Aside for a few pigment-producing cells, most cells are transparent. Additionally, the process of sectioning into very thin sections leaves very little on a slide that can absorb or deflect light. For this reason, tissue needs to be stained in order to make the different features more easily visible and the improve the contrast between different parts of a cell. There are some stains and techniques which can specifically bind to certain structures to easily help microscopists identify them, but most stains are relatively non-specific and are only used to make things easier to see in general.

Most stains interact with their targets through a charge-charge interaction. Such stains are frequently subdivided into acidic and basic stains. An acidic stain has a coloured component (**chromophore**) and a functional group that is anionic and can interact with positively charged cell components on a slide. Such components are often referred to as being **acidophilic**. A basic stain has a cationic functional group and can bind to acidic cellular components – these components are referred to as **basophilic**. Two such common stains in histology are Hematoxylin and Eosin.

Hematoxylin

Hematoxylin is a natural dye extracted from logwood (*Haematoxylon campechianum*). Hematoxylin is a basic (cationic) dye and interacts with acidic cell components. Acidic cell structures will tend to stain a blue or purple colour on the slides, whereas other components will remain unstained. The actual colour and the intensity of the staining depend on a few factors including the pH of the stain. The intensity of the colour is also dependent on the amount of that particular cell component in that part of the cell.

Cell or tissue components that stain with Hematoxylin are frequently referred to as being "basophilic" because they interact with a basic dye.

Can you think of any components of a cell that might be acids and thus pick up some Hematoxylin stain? Before you answer that question, please read the "Common Misconception" box to the right.

Stain Preparation

Hematoxylin is generally prepared in ethanol (it's more soluble in ethanol than in water) and oxidized to produce the active component of the stain – hematein.

Common Misconception

Students often think of H+ (the proton) as an acid. It is understandable, since it is the component we use to measure acidity of a solution (pH), but a proton is not an acid.

Careful reading of your 1st year Biology or Chemistry textbook will reveal that acids are molecules that have a tendency to lose protons in solution. So, the molecule that lost that proton, was the initial acid. Thus, it is cellular components such as these that can be stained by Hematoxylin.

The process of preparing this stain can be quite simple:

Dissolve hematoxylin in ethanol, plug the container with cotton to prevent contamination while allowing exposure to oxygen, leave exposed to light and air for several weeks to

several months. Over time, this produces a mixture that contains increasing amounts of the active component.

This protocol has been modified by many researchers to speed up the production of hematein by the addition of oxidizing agents – usually mercuric oxide or sodium iodate. Sodium iodate is more commonly used these days to avoid generating mercury waste.

The staining effectiveness/intensity of hematein is also frequently improved by the addition of a metal salt (a “mordant”) to the mixture during stain preparation. Aluminum or iron salts are commonly added (other metal salts can also be used), generating colours ranging from purple to dark blue. Aluminum salts are generally referred to as “alums”.

The addition of a weak acid to this mixture appears to further enhance the stain's ability to interact with certain cellular components. It is thus a part of many of these modified protocols.

Eosin

Eosin is a red/pink synthetic dye derived from fluorescein (a fluorescent organic compound with a variety of uses). Eosin is an acidic (anionic) dye and is often used to stain basic (cationic) cell and tissue components. It is usually used as a counterstain to Hematoxylin because structures that do not stain with Hematoxylin will frequently bind Eosin, and the resulting deep-blue and red colours provide a very good contrast to allow easy differentiation between different cellular and tissue structures.

Eosin is mostly used to stain proteins. It generally binds to basic amino acids like arginine and lysine which tend to be protonated at most pH ranges used in slide preparation and thus have a positive charge (are cationic). Structures that bind eosin tend to be described as “acidophilic” or “eosinophilic”.

The colour and the amount of staining in some of the regions of a particular tissue will also at times be determined by the relative amounts of cationic and anionic tissue components. Areas that have more cationic components will tend to bind more eosin and appear more red or pink, while areas containing more anionic components will bid more hematoxylin and appear more purple.

Practical Connection

Your protocol tells you to partially dehydrate your samples through an ethanol series (from 50% up to 90% EtOH) before staining with Eosin. The dehydration is then continued after the eosin staining (to 100% EtOH).

If you were to stain the slides with eosin before starting that whole dehydration procedure, you would likely see that much of the stain would be lost from your tissues because of eosin's increased solubility at the lower ethanol concentrations.

Stain Preparation

Eosin is soluble in water and ethanol, but it's solubility in ethanol low at higher concentrations (ie. 95% or 100% EtOH), thus treating your tissues with high ethanol concentrations after staining is less likely to remove any bound stain from your slides (see Practical Connection).

It comes in two varieties. Eosin Y produces a slightly more yellow-orange colour compared to Eosin B, which has a slight bluish tint to its colouration (an “imperial red”). The two varieties only differ in the amount of bromine attached to the fluorescein molecule. The addition of acetic acid to the eosin Y solution tends to deepen the colour and make it more red.

Tissue Processing Exercises

1. Slide Staining with H&E

Today, you will be using Hematoxylin and Eosin to stain the sections you have generated. Since most stains are water-soluble, they need an environment that is free of wax. This is why one of the first steps in staining is the removal of the embedding medium, and at least a partial rehydration of the tissue. Pass the slides in the various solutions provided in the following order to obtain a tissue section stained with H&E:

Materials

- Coplin jars
- Xylene
- 100% Ethanol
- 95% Ethanol
- 90% Ethanol
- 80% Ethanol
- 70% Ethanol
- 50% Ethanol
- Slides with animal tissue sections [4 slides per group]
- Forceps

Procedure

Move slides through the Coplin jars in the following sequence:

1. Xylene for 10min
2. 100% Alcohol for 2min
3. 95% Alcohol for 2min
4. 90% Alcohol for 2min
5. 80% Alcohol for 2min
6. 70% Alcohol for 2min
7. 50% Alcohol for 2min
8. Stain in Hematoxylin for 10-15min
9. Rinse in Distilled water for ~5-10sec (just dip your slide several times in the water and transfer it to the next jar)
10. Distilled water for 30sec - 1min
11. 50% Alcohol for 2min
12. 70% Alcohol for 2min
13. 80% Alcohol for 2min
14. 90% Alcohol for 2min
15. Stain in Eosin for 2-3min
16. Rinse in Distilled water for ~5-10sec (just dip your slide several times in the water and transfer it to the next jar)

Caution

Xylene is classified as a moderate hazard. It should be used in a well-ventilated area, and proper safety equipment including gloves should be worn.

To prepare a permanent slide, the stained tissue section is preserved by **mounting** the slide using a “mountant” like Canada balsam. This will make a permanent seal between the slide, the tissue and the cover slip. Resins like Canada Balsam are not water soluble and thus require that the tissue be completely dehydrated before they can be applied. For this reason, we did not incubate the slides in water for very long in the previous step – we do not want to rehydrate the tissue now that we're processing the slides through an increasing alcohol series.

17. 95% Alcohol for 2min
18. 100% Alcohol (fresh ethanol) for 2-5min
19. 100% Alcohol (fresh ethanol) for 2-5min (this is a second ethanol wash)
20. Xylene for 2min
21. Put a very small drop of Canada Balsam directly on top of your tissue.
22. Place the cover slip on the stained tissue section and place on a warming plate.