Neuroscience Methods

VII. Histology I: An Introduction

Materials
Fixed rat brain
Cryostat, microtome, or other means of sectioning
Subbed microscope slides
Materials for cresyl violet staining protocol (see methods section)
  - absolute ethyl alcohol
  - distilled water
  - cresyl violet acetate (e.g. sigma #C5042)
  - glacial acetic acid
  - clearing agent (e.g. histoclear - Baxter #C4200-1)
Staining dishes and slide racks
Mounting media (e.g. Permount)
Q-tips
Coverslips
Low power light microscope or a light box and magnifier
Stereotaxic atlas, such as Paxinos and Watson [1]

Purpose
To become familiar with basic histological technique. To use staining techniques in combination with a stereotaxic atlas to identify anatomical structures in sectioned brain tissue.

Background

Histology refers to the microscopic study of tissue. The visualization of the brain’s cytoarchitecture is an important complement to the studies of function and gross anatomy performed in other labs. It is used in a number of areas essential to neuroscience research, such as identifying nuclei affected by a lesion and verifying the placement of a subcortical electrode.

Histology also refers to the techniques used to prepare tissue for microscopic study. This includes not only staining tissue for light and electron microscopy, but also more advanced techniques such as tracing fiber tracts, identifying receptor types present in a given brain region, and mapping the distribution of the expression of a particular gene. These latter techniques are more properly described as histochemistry, which is the (art and) science of localizing the various chemical constituents of cells. This is an exciting field that makes use of methods from areas as diverse as biochemistry, molecular genetics and image processing.

5 Contributed by M. Jones, Spring 1996
In this lab, we will prepare sections of brain tissue for viewing using a light microscope. In order to be visualized, the tissue must first be fixed and stained. **Fixing** prevents enzymatic and other postmortem changes that degrade the tissue. Typically, the animal containing the brain of interest is sacrificed and **perfused**, i.e. the blood is drained from the body and a fixative solution is pumped into the vascular system. Removing the blood improves the results of staining, and the fixative preserves and usually hardens the brain so that it can be **sectioned** (cut into thin slices) without tearing. The sections, cut sufficiently thin to view using a light microscope, are then stained to bring out cellular details that would otherwise be difficult to observe.

There is a wide variety of staining techniques. In neuroscience, perhaps the most familiar is **Golgi staining**. For reasons still unknown, this technique stains only a few cell bodies in the tissue in their entirety, thereby allowing a detailed visualization of individual neurons (note a Golgi stain can take several months to complete!). Other techniques include **myelin stains** for visualizing fiber bundles, **degenerating-axon stain** which specifically stains dying axons, and several techniques for **cell body** (Nissl) staining.

In this lab we will be using **cresyl violet**, a Nissl stain that colors cell bodies a brilliant violet. Nissl is a term used by classical cytologists for the endoplasmic reticulum. Since all cells contain ER, cresyl violet will stain both neurons and glia. An outline of the procedure consists of three steps:

1. Sectioning the brain.
2. Placing the brain sections on slides and applying the stain.
3. Viewing the stained sections in conjunction with a brain atlas.

Routine staining for light microscopy typically requires sections of about 50µm in thickness. While crude sections may be prepared with a razor blade and a steady hand, sectioning is usually performed using a cryostat, microtome, or vibratome. Each of these provide some means of securely holding the tissue and passing a razor or special knife through it at a precise angle. With each pass of the blade the tissue is advanced a specified distance such that uniformly thick sections are obtained. Because even fixed tissue can be difficult to section cleanly, the tissue is usually frozen (cryostat) or embedded in paraffin (wax) when using a microtome. The vibratome uses a vibrating blade so freezing or embedding is usually not required.

The staining procedure consists of sequentially dipping the slides in about a dozen different solutions for specified amounts of time (see next page). Although the sequence of solutions may appear to be arbitrary, it is designed to accomplish the following:

1. An initial alcohol soak removes lipids (fats) and fixation chemicals from the tissue.
2. The sections are submerged in stain.
3. The tissue is dehydrated (water is driven out) by a series of alcohol baths.
4. The unstained parts of the tissue are made transparent by a clearing agent.

The series of decreasing alcohol baths between the initial soak and the stain are necessary because the stain is water-based (i.e. made up in water) and so the tissue would not stain well if placed directly in the stain following the initial alcohol soak because alcohol and
water are not miscible. Also note that an acidic bath is included in the dehydration sequence. This serves to remove excess stain from the tissue.

Collections of stained cell bodies form structures that can be identified with the aid of a stereotaxic atlas. An atlas is a collection of pictures of brain sections with accompanying diagrams of structures that can be identified in each section. One such figure is provided in Appendix A. This plate includes an indication of the section’s position, a view of the section itself, and a sketch of the major structures which can be identified. While this plate show only a coronal section, most atlases also include sections along sagittal and/or horizontal planes, and often include coordinate axes so that identified structures may be precisely located using a stereotaxic apparatus.
Method

1. Your TA will provide you with a slide containing 2-5 sections. Carefully blot away any excess moisture from around the tissue. Allow to dry sufficiently (5-15 min) so the sections do not fall off during staining.

2. Insert your slide into one of the carriers provided. Pass the carrier through the following sequence of baths, observing the times indicated:

   - 95% ETOH 15 min.
   - 70% ETOH 1 min.
   - 50% ETOH 1 min.
   - DH2O 2 min.
   - DH2O 1 min.
   - Cresyl Violet Stain 2 min.
   - DH2O 1 min.
   - 50% ETOH 1 min.
   - 70% acid ETOH 2 min.
   - 95% ETOH 2 min.
   - 95% ETOH a few dips
   - 100% ETOH 1 min
   - Histoclear 5 min

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6 ETOH = ethyl alcohol; DH2O = distilled water; all solutions 200 ml
7 add 1.25g cresyl violet acetate and 0.75 ml glacial acetic acid to 250 ml warm DH20, cool and filter
8 2 ml glacial acetic acid in 200 ml 70% ETOH
9 Some people find histoclear vapor to be irritating. You may want to wear a mask during this procedure.
After finishing this “gauntlet”, let the carrier drain on a paper towel for about 30 seconds, then remove the slides from the carrier. Wipe off excess clearing agent from around the tissue. Using the shaft of a Q-tip, immediately apply a small drop of mounting media to each section and slowly lower a coverslip onto the slide. Gently press out any air bubbles that may have formed. Note that mounting media tends to get everywhere except where you want it to - wear gloves and be as careful as possible.

**Viewing**

Using either a light box and a magnifier or a light microscope on low power, examine your stained sections. Cell bodies will appear as violet to dark blue. Fiber tracts will remain white or light colored. Under higher magnification, some of the subcellular organelles may be visible (usually the cell nucleus).

Compare your stained sections to the illustrations in the stereotaxic atlas. Note that the anatomy of the rat brain (especially the hippocampal structures) differs greatly from that of the human brain. Pick your best section and make a rough sketch. Identify at least 5 structures using the atlas, including at least one nucleus and one fiber tract. What is the approximate position along the rostral/caudal axis of your section? Using the atlas, order all the slides in your group starting with the most rostral and ending with the most caudal. Are there sections missing in your collection?
Suggestions for Further Study

Many of the histology books at Norlin library are somewhat dated. While the reference section is your best bet for standard textbooks, for hands-on information, try Palkovits and Brownstein [2], Davenport [3], Humason [4], Mesula [5] and Luna [6]. These contain excellent tips on technique and include scores of recipes for various protocols. For books focusing more on the chemistry of staining, look around in the QH320’s. Here you will also find books on light and electron microscopy, and techniques such as freeze-fracturing. For a fascinating look at “applied histology”, try the Center for Human Simulation web site (http://www.uchsc.edu/sm/chs). The people at CHS have fixed an entire human body, sectioned at regular intervals, then by combining the cross sections of organs in sequential sections, reconstructed the 3D geometry of several organ systems!

Study Questions

1. How does “histology” differ from “histochemistry”?

2. What does cresyl violet stain? Why does removing blood from the tissue improve the results of staining (be specific)?

3. The following are some problems that can occur during staining. Give a probable cause of each.
   a) One or more chemicals used in fixation reacted with the stain, ruining the section.
   b) Water remained in the section, resulting in a foggy slide.
   c) The finished section was too light (list two corrections).

4. What are some uses of stained sections?

5. Staining can be classified as progressive, in which the section is removed from the stain once sufficient stain has been absorbed, and regressive in which the section is over stained and the excess stain removed using a solvent. Which type of staining is the cresyl violet protocol?
References


Appendix A - Sample Plate from Palkovits and Brownstein [2]
ANSWERS

Question 1)

Histology refers to the microscopic study of tissue and the techniques used to prepare tissue for microscopic study.

Histochemistry, on the other hand, is the science of localizing the various chemical constituents of cells. This includes identifying receptor types present in a given brain region and mapping the distribution of the expression of a particular gene.

Question 2)

Cresyl violet is a Nissl stain, and Nissl is a term used by classical cytologists for the endoplasmic reticulum. Therefore, cresyl violet stains the cell’s endoplasmic reticulum.

Because blood is composed of blood cells and is responsible for the transport of myriad other cells, and because these cells all contain Nissl substance (endoplasmic reticulum) any remaining blood in the tissue will be deeply stained by cresyl violet, and the “important” features of the structure will be obscured by this artifactual staining.

Question 3)

a) One or more chemicals used in fixation reacted with the stain, ruining the section.
   The initial alcohol soak was not thorough enough to remove all the fixation chemicals.

b) Water remained in the section, resulting in a foggy slide.
   Water is removed from a sample prior to staining by dehydration: “running” the sample through a series of graded alcohol baths of increasing concentration. If water remained in a sample, resulting in a foggy slide, it would be highly likely that the sample was passed too quickly through the alcohol baths, or the samples were not passed through the highest concentration alcohol bath (100% alcohol). Stopping prior to the 100% alcohol bath would leave 5% water in the sample if the subsequent bath was 95% alcohol.

c) The finished section was too light (list two corrections).
   If a resulting section is too light, then either the staining time was too short, or too much stain was removed during the dehydration and clearing stages of the protocol.

Question 4)

Stained sections are used to verify the location of an electrode that had been lowered into the brain using stereotaxic coordinates. Stained section also reveal the cellular morphology of different areas of the brain. Stained sections are also used in the creation of stereotaxic atlases, and are used to identify the boundaries of different nuclei.
Question 5)

Cresyl violet is a *regressive stain*. If the samples were directly removed from the staining bath, dehydrated, and cover slipped, it would be impossible to discern any cellular morphology. The clearing agent is necessary to remove excess stain from non-nissl containing structures (i.e. fibers of passage). The clearing agent is responsible for the staining “regression.”