

ADDING TECHNIQUES TO THE NEUROBIOLOGY OF
AGING LABORATORY AT TCU

by

Ryan Gessouroun

Submitted in partial fulfillment of the requirements for Departmental Honors in the

Department of Neuroscience.

Texas Christian University

Fort, Worth, Texas

May 2, 2014

ADDING TECHNIQUES TO THE NEUROBIOLOGY OF
AGING LABORATORY AT TCU

Project Approved:

Supervising Professor: Gary Boehm, Ph.D

Department of Psychology

Brenton Cooper, Ph.D

Department of Psychology

Ronald Pitcock, Ph.D

John V. Roach Honors College

ABSTRACT

For my honors project I will meet frequently with the graduate students working in the laboratories of Dr. Boehm and Chumley, to help refine, fine-tune, and write up the biological and/or behavioral laboratory protocols that will then be combined with our previously existing laboratory manual. This manual, which includes highly specific recipes and laboratory protocols, is of critical importance to proper lab function and laboratory productivity, and will help the lab practice consistent methodology for years to come. In doing this project, not only will I learn the ins and outs of varied laboratory procedures/techniques, but I will benefit both undergraduate and graduate students alike who will utilize this resource as a reference.

TABLE OF CONTENTS

<u>INTRODUCTION</u>	1
<u>IMMUNOHISTOCHEMISTRY</u>	2
BRDU Protocol.....	3
BRDU Staining.....	5
Marielle’s Awesome BrdU Protocol.....	8
Tomato Lectin Staining.....	10
Iba-1 Staining.....	11
Rabbit Anti-Iba1 Staining Protocol: Free Floating Sections	13
LBC-1-IFU Protocol	15
<u>SOLUTION CHEMISTRY</u>	16
Making 30% Sucrose for Cryostat.....	17
Making DEPC Water	18
Making LPS	19
Making Scopolamine	21
Blocking Buffer	22
Making Sterile Saline.....	23
RIPA Lysis Buffer	24
Scopolamine.....	25
Making Cresyl Echt Violet	26
<u>HANDS-ON PROCEDURES AND LAB EQUIPMENT INSTRUCTIONS</u>	27
Measuring RNA Concentration Using NanoDrop.....	28
Perfusion and Heart Puncture Protocol.....	30

Perfusions.....	36
For Perfusions.....	37
Thin Sectioning.....	38
Vivarium Daily Check.....	39
Using the Confocal.....	41
Autoclave Protocol.....	46
EDTA in Tubes to Avoid Blood Clotting.....	48
Vibratome Protocol.....	49
<u>BEHAVIORAL TESTING AND TRAINING PROTOCOLS</u>	53
Passive Avoidance Conditioning.....	54
<u>BIOLOGICAL ASSAY PROTOCOL</u>	57
Protein Assay & ELISA Instructions.....	58
Total RNA Isolation from Punch Tissue using Rneasy Kit.....	59
DC Protein Assay.....	61
First Solo Western Blot.....	63
Protein Extraction.....	65
Staining Brain Sections.....	66
Strip & Reprobe Protocol (PVDF Membranes).....	67
Western Blot.....	68
Blood/Serum Collection Protocol.....	74
Sera Protocol.....	75
Cresyl Violet Stain Protocol.....	77

INTRODUCTION

A successful lab is always growing and changing, constantly adapting. This is the state of laboratories around the world as each discovers new information themselves and learn of the discoveries of others. For neurobiology laboratories, this could be in the discovery of a new gene that further predisposes an individual to Alzheimer's Disease. Very differently, the advancement could come from a new technique for behavioral assessment in mice. This is very exciting, but also poses a problem for a laboratory. Every lab has many moving parts: supervising researcher, perhaps graduate students and undergraduate students, administrative members and suppliers. For all of these entities to come together as one cohesive unit, a lab must rely on its protocols. Protocols are obtained both from within the lab and externally. But in order to establish a setting in which new discoveries *can* be made, an up-to-date protocol agenda is vital.

My project seeks to solve the problem outlined above. The current state of the neurobiology lab IS very disjointed. The laboratories of Dr. Gary Boehm and Dr. Mike Chumley work together with a great deal of overlap. Over the past few years, the lab has grown significantly both in size and in its research endeavors. However, it has done this with the status of its protocols lagging behind. A large number of protocols existed as handwritten notes or on scratch paper floating from person to person throughout the lab. Many of the protocols had notes written on an originally printed document that amend the protocol to the correct version. For the lab to continue to move forward, these needed to be compiled into one central location. This project creates a common methodology for the lab.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a widely used biological technique used to identify specific chemical substances with the use of antibodies. Antibodies, either obtained from an animal or synthesized artificially, are extremely specific to their substrate. A significant portion of experimentation involves the detection of a certain substance in response to a stimulus; in the case of our lab, that substance is usually proteins produced in learning and memory. In order for the antibodies to provide analyzable evidence, a detection method must be utilized. There are numerous techniques used for detection in immunohistochemistry. One detection method utilizes fluorescent molecules which can be bound to the antibody, making it visually detectable.

BRDU Protocol

TBS = PBS

Wash in PBS 3x5 min.

2-12 plates

Pre Inc. in H₂O₂

.6% H₂O₂

Hydrogen Peroxide

Fridge door 30%

V₁C₁ = V₂C₂

for 30min on slanter

HCl → 30 min in incubator with rotator.

500μL/section

50mL clonical in 37° water bath.

Take off HCl

Add Borate at room temp.

5min

Suck off

New 5 min

Suck off

New 5 min

Wash off borate w/ PBST

3x5min

Blocking

3% donkey + PBST

$$V_1C_1 = V_2C_2$$

$$100 \times X = 15 \times 3$$

$$450\mu\text{L} (\text{in } 45\text{mL})$$

$$(450\mu\text{L in } 15\text{mL})(1 \times 3)$$

BRDU Staining

Standard Method: ABC Method or LSAB Method

Enhanced Method: Polymeric Methods

Chromogen Substrate:

Reagent: DAB

Incubation Time/Temperature: 1-3 minutes/room temperature

Counterstain:

Reagent: Gill's Hematoxylin or Mayer's Hematoxylin

Staining Time: 30 seconds

Results:

Staining Pattern: Nuclear

Images: [Search image](#)

Additional Information:

Tissue Type: BrdU Incorporated tissues

Fixation: Formalin-fixed paraffin sections

Positive Control: BrdU incorporated tissues

Negative Control: Omit primary antibody, isotype control, absorption control

Blocking: 2-5% normal serum to reduce unspecific background staining; 0.5-3% H₂O₂ to block endogenous peroxidase activity; avidin/biotin to block endogenous biotin activity if necessary

Notes:

Denature DNA by incubating sections in 2N HCl for 30 minutes at 37°C, and neutralize the acid by immersing sections in 0.1M borate buffer for 2x5 min. (12.1M)

2N HCl:

10N HCl ----- 20 mL

Distilled Water -----80 mL

Mix well.

Alternate: 165 mL reagent grade 10N HCl + 835 mL H₂O = 1000mL 2N HCl

0.1M Borate Buffer, pH 8.5:

Sodium Borated (MW 381.4) ----- 3.8g x10= 38g

Distilled Water -----100 mL x 9.5= 950 mL

Bring up to 1L

Mix to dissolve and adjust pH to 8.5

Description: To measure DNA synthesis or cell proliferation, 5-bromo^{2'}-deoxy-uridine (BrdU) can be incorporated into DNA in place of thymidine. Cells, which have incorporated BrdU into DNA, can be quickly detected using a monoclonal antibody

against BrdU. The binding of the antibody is achieved by denaturation of the DNA. This is usually obtained by exposing the cells to acid, or heat.

Primary Antibody:

Name: BrdU Antibody

Clone: BU1/75, ICR1, Rat anti-BrdU

Supplier: Accurate Chemical & Scientific

Catalog Number: OBT0030

Dilution: 1:100 using IHC-Tek Antibody Diluent (Cat # IW-1000 or IW-1001) to reduce background and unspecific staining and serum blocking step is NOT needed.

Incubation Time/Temp: 60min/room temperature.

Antigen Retrieval:

Device: IHC-Tek Epitope Retrieval Steamer Set (Cat#IW-1102)

Buffer/pH value: IHC-Tek Epitope Retrieval Solution (Cat #IW-1100)

Heat/Cool Temperature: 95-100°C/room temperature

Heat/Cool Time: 20minutes/20minutes

Detection Methods:

Marielle's Awesome BrdU Protocol

1. Pick sections
2. Wash sections in **PBS** 3x for 5 min.
3. Pre-incubate section in 0.6% Hydrogen Peroxide (500 μ L/section) for 30 min on the slanted rotator. For 24 sections, 0.3mL of 30% H₂O₂ in 15 mL of milip coater.
4. Wash in PBS 3x for 5 min.
5. During the wash place 2N HCl (the amount needed) in a 50mL conical tube in 37°C water bath.
6. Put the 37°C 2N HCl on sections (500 μ L/section) and place in 37°C incubator (on rotator) for 30 min.
7. Aspirate or pipette off the HCl
8. Add 0.1M borate buffer (at room temp, 500 μ L/section) for 5 min.
9. Repeat step 8 for a total of 3 times
10. Wash off borate buffer with **PBST** 3x for 5 min.

Blocking:

1. Add 3% Donkey serum + PBST to sections, 500 μ L/section
2. Incubates room temp for 2 hours on rotator

Note: always make extra. 1350 μ L of Donkey into 45mL of PBST

Primary:

1. Rat anti-BrdU at 1:250, (2 μ L/of rub//section)(500 μ L/section) in 3% Donkey Serum + PBST
2. Place in cold room on rotator over night
3. Wash 3x15min in PBST

Note: 13mL of Donkey + PBST, 52 μ L of rat BrdU

Secondary:

1. Donkey anti Rat HRP 1:250 (500 μ L/section) in 3% Donkey + PBST
2. Incubate 4hrs at room temp on rotator
3. Wash with PBST 3x15min
4. Then wash one time with PBS for 10min.

DAB Stain:

1. One drop of Substrate Buffer 1 (#1) per 1mL of DI water (500 μ L/section)
2. MIX WELL
3. Add one drop of #2 and one drop of #3 per mL of water.
4. MIX WELL
5. Use within 30min of making, and keep in dark until ready to use (make sure it warms to room temp)
6. Add 500 μ L/section and place on rotator for 5min.
7. Wash 3x for 5min with MILIPORE WATER
8. Add enhancer if needed (see manufacturer protocol)
9. Mount the slices on slides
10. Add one drop of VectaMountAQ to each brain slice and coverslip
11. Allow to dry (approxiiimately 30min)

Tomato Lectin Staining

- Day 1
 - 1. Wash 5x15' PBST
 - 2. O/N 4° with PBST + BigTynylated tomato lectin
 - 3. Leave overnight
 - 1:250 or 4 μ L/mL of PBST
 - 500 μ L per section
 - "Slanter"
- Day 2
 - 1. Wash 5x15 PBST
 - 2. 4 hr at RT with PBST + Peroxidase conj SA
 - 1:500 or 2 μ L/mL PBST
 - 500 μ L per section
 - RT "Slanter"
 - 3. Wash 5x15' PBST
 - 4. Wash 1x10' PBS
 - 5. Do DAB staining- Mexy Dev. Color
 - Quickly so be prepared to wash sections
 - 6. Mount in Vectamount AQ and coverslip
 - 7. Dry O/N
 - 8. Seal with Noul Polish

Iba-1 Staining

1. Wash sections with water to remove trace.
 - a. 10 min.
 - b. 3 changes during 10 min.
2. Add 1mL Sodium Hydrobromide (0.5% in PBS) to each section
3. Incubate at room temperature on rocking for 30min
4. Remove Sodium Hydrobromide by pipettor to waste bottle.
5. Wash 3x10 min with PBS.
 - a. Methanol → 5mL of methanol.
 - b. 45mL DiH₂O
6. Add 0.3% H₂O₂ in 10% MeOH to each section
7. Incubate at room temperature for 30min rocking making 50mL. 0.5mL of 30% H₂O₂ (500μL)
8. Wash 3x10 min in PBST
9. Block overnight at 4° or 4hr at room temperature with 3% Donkey Serum in PBST (make enough to add 500μL to each well x2). Keep in fridge 3μL/100μL.
10. Remove block and add 500μL fresh block to each well
11. Titrate in 1° Ab as per plate diagram.
12. Overnight at 4° rocking (walk-in fridge)
13. Wash 1x5 min and 3x10 min with PBST.
14. Add 2° Ab in PBST as per plate diagram.
15. Incubate 4° overnight or 4 hours room temperature.

16. Repeat step 13.

17. _____

Rabbit Anti-Iba1 Staining Protocol: Free Floating Sections

Day 1

1. Wash sections in DI H₂O to remove trace PFA.
 - a. Wash for 10 min with 3 changes rocking.
2. Add 1mL sodium hydrobromide (.5% with PBS) to each section.
 - a. Dispose of in the sodium hydrobromide waste.
3. Incubate at room temperature rocking for 30 min.
4. Wash with PBS 3x10 min.
5. Add 500 μ L of 0.3% H₂O₂/10% MeOH in PBS to each section (fridge)
6. Incubate at room temperature for 30 min rocking.
7. Wash 3x10 min in PBST
8. Block overnight at 4°C or 4 hrs at room temperature rocking
 - a. Block: Vectastain
 - b. 3 drops (150 μ L) of Goat serum
 - c. 10mL of PBS
 - d. 500 μ L of block per well.

Day 2

9. Add 500 μ L of 1° Ab (Rab-anti-Iba1) at desired concentration diluted in Block (or PBS)
10. Incubate overnight at 4°C rocking.

Day 3

11. Wash 1x5 min and 3x10 min with PBST
12. Add 500 μ L of 2 $^{\circ}$ Ab in PBS to each well
 - a. Vectastain Kit:
 - b. 1 drop (50 μ L) of Ab
 - c. 10mL of PBS
13. Incubate overnight at 4 $^{\circ}$ C or 4 hrs at room temperature rocking.

Day 4

14. Wash 1x5 min and 3x10 min with PBST
 15. Add ABC in PBS, 500 μ L in each well.
 - a. Vectastain Kit:
 - b. 2 drops (100 μ L) of Reagent A
 - c. 10mL of PBS
 - d. 2 drops (100 μ L) of Reagent B
 - e. Mix immediately and allow to stand for 30 min.
 16. Incubate for 30 min at room temperature rocking.
 17. Wash 1x5 min and 3x10 min with PBS (not PBST!)
 18. Add 500 μ L of DAB stain to each well
 - a. To make DAB, follow manufacture protocol with kit
 19. Wash off excess DAB with several (3-4) washes of DI H₂O
 20. Mount sections onto slide and apply cover slip
- Note:* Keep slides refrigerated.

LBC-1-IFU Protocol

1. Deparaffinize sections if necessary and hydrate to distilled water.
2. Incubate slide in Luxol Fast Blue Solution for 24 hours at room temperature or 2 hours at 60°C.
3. Rinse thoroughly in distilled water.
4. Differentiate section by dipping in Lithium Carbonate Solution (0.05%) several times (up to 20 seconds).
5. Continue differentiation by repeatedly dipping in Alcohol, Reagent (70%) until grey-matter is colorless and white-matter remains blue.
6. Rinse slide in distilled water.
7. Incubate slide in Cresyl Echt Violet (0.1%) for 2-5 minutes.
 - a. Heat and spin for 2 minutes
 - b. 100µL
 - c. 400µL Di H₂O
 - d. 1.25mL acetic acid then filter
8. Rinse quickly in 1 change of distilled water.
9. Dehydrate quickly in 3 changes of absolute alcohol.
10. Clear as desired and mount in synthetic resin.

SOLUTION CHEMISTRY

Solutions are of widespread use throughout the lab. It is vital to have both accurate and precise solutions. These solutions vary greatly in chemical makeup, concentration, and purpose. Especially for undergraduates to participate in this process of the experimentation, it is necessary to have calculated and exact recipes for making the solutions used in the lab. It is equally important to have them easily accessible and their creation easy to understand, and this master list of protocols fulfills that need.

Making 30% Sucrose for Cryostat

To make 100mL

1. 30g of sucrose
2. Raise 1xPBS 6uel (sp????) to 100mL
3. Use 5 mL/brain
 - a. Wait until brain sinks, then imbed brain.

Making DEPC Water

Bake Bottle

- Cooper Lab
- 300° for 5 hrs (liquid setting)
- Add 1mL/L DEPC to distilled water.
- Add 5 tablets/L of phosphate buffer to DEPC
- Shake well to dissolve
- Store in Fridge

Making LPS

Materials:

- Saline
- LPS
- Syringe
- Serological Pipet
- 25 mL Serological Tips
- 50 mL conical tube
- 1 Medium tube
- LPS tubes
- Regular pipet with tips
- 1 mg LPS + 26.66 mL saline = 37.5 $\mu\text{g/mL}$

Procedure:

1. Draw up 26.66 mL of saline with the serological pipet and put it in the conical tube.
2. Using the syringe, draw up a good amount of saline and put it in the medium sized tube (the amount of liquid does not matter since this will be used to draw up the LPS and wash the inside of the vial).
3. Using the syringe, draw up a few mLs of saline from the medium sized tube and inject it into the LPS vial. Make sure to hit the center target on the lid of the vial.
4. Invert the syringe-tube together several times. Be careful not to shake the vial.

5. Draw the liquid back up into the syringe slowly and inject this into the conical tube.
6. Repeat steps 1-5 three times.
7. Pour any left over saline from the medium tube back into the conical tube.
8. Using scissors, cut the aluminum band around the vial.
9. Using forceps, remove the rubber stopper.
10. Pipet saline from the conical tube to the LPS vial back and forth to wash the inside of the bottle.
11. Rinse the pipet tip.
12. Set the pipet to 900 μL per LPS tube.
13. Fill 29 vials. If fewer or more, check your concentration.
14. Cap the tubes. Do not stick your hand inside so that they remain sterile.
15. Wipe everything down with ethanol and assume LPS is everywhere as you clean.
16. Label the box with red tape and write the following: 250 $\mu\text{kg}/\text{dose}$, 37.5 $\mu\text{g}/\text{mL}$ concentration, Lot # from bottle, Date, and your initials.
17. Put in freezer, including unused tubes.

Making Scopolamine

- SCOPOLAMINE + $1\mu\text{g}/\mu\text{L}$ in Saline. Same as $1\text{mg}/\text{mL}$
1. Weigh out Scopolamine: between 5-10mg onto weigh paper folded into 4 triangles making it easier to pour into the tube.
 2. Add sample volume saline as #mg of scopolamine.
 - a. Ex: $5.8\text{mg} + 5.8\text{mL}$
 3. Mix.
 4. Give to mouse at 3x weight.
 - a. $30\text{g mouse} = 90\mu\text{L I.P.}$

Blocking Buffer

- PBST, 5% milk, 0.02% Azide (in 10% stock)
- Make 500mL
 - Don't heat, but stir bar.
 - 500mL of PBST
 - 25g milk
- Primary.

Making Sterile Saline

- 100% ethanol spray down
- pH PBS to 7.4 do not overshoot or else start over.
- 2 micron filters, inside is steril (Nalgene). Don't touch inside.
- Use autoclaved bottle. Take off lid.
- Screw Nalgene into bottle.
- Attach hose to make side.
- Turn on pump with foot under hood.

-
- Same Nalgene.
 - Re-use until it does not filter anymore
 - Put it back in bag and leave in hood.
 - Don't touch the blue bottom.

RIPA Lysis Buffer

- 10 mL/40 fold. Make up different with H₂O (Millipore, 7.8mL)
- 2 50 μ L Tris- shelf
- 5000 μ M/150 = 33.3 fold dilution
- 300 μ L of NaCl- shelf
- 1000 μ L of NP-40 (10% stock) – fridge
- 500 μ L of DOC (deoxycolate) – benchtop
- 100 μ L SDS
- Add protease inhibitors (kept in box in freezer)
 - 100X stock \rightarrow 1X
 - 100 μ L \rightarrow 10mL of RIPA

Scopolamine

- $3\text{mg/kg} = 3\mu\text{g/g}$
 - $35\text{g Mouse} \times 3\mu\text{g/g} = 105\mu\text{g/mouse}$
 - $45\text{g mouse needs } 3\mu\text{g/g} = 135\mu\text{g} = 135\mu\text{L}$
 - $1\mu\text{g}/\mu\text{L}$
-

- $20\text{mg/kg} = 20\mu\text{g/g}$
- $30\text{g mouse} \times 20\mu\text{g/g} = 600\mu\text{g/mouse}$

Making Cresyl Echt Violet

- 400mL
 - 2g crystal violet
 - 320mL DI H₂O
 - 80mL 100% ethanol

Procedure:

1. Add cresyl mix
 2. Add ethanol mix
 3. Filter crystal sout
-
- 45mL Cresyl violet
 - 15 drops of acetic acid (mask) for when you dip slides.

HANDS-ON PROCEDURES AND LAB EQUIPMENT INSTRUCTIONS

In the laboratory, highly technical and sensitive machinery is used to obtain experimental results. These techniques, such as how to obtain the hippocampus from the brain of a mouse, and machines, such as the fear conditioning boxes, require a great deal of practice and expertise. In order for these items to be used by a number of individuals, a clear and easy to understand list of protocols is very important. The list compiled below is a comprehensive grouping of the procedural techniques used in the lab as well as the instructions for navigating laboratory technology.

Measuring RNA Concentration Using NanoDrop

- Now that we have isolated the RNA we need to measure the concentration so we can dilute all the samples to the same concentration for RT-PCR.

Procedure:

1. Start the NanoDrop program.
2. Load 2 μ L of RNase-free water to the pedestal and click “OK” in order to perform the first “**blank**” read. (P2)
3. On the right hand set the wavelength to read RNA rather than DNA.
4. **Clean the NanoDrop** optical reading field by spraying a small amount of RNA Zap and DEPC water on a kimwipe.
5. Carefully, pipet **2 μ L of RNase-free water** and do a **second “blank”** read by hitting the blank button.
6. Once again, **clean the NanoDrop** optical reading field by spraying a small amount of RNA Zap and Nuclease-free water on a kimwipe.
7. Make sure you type in the correct **Sample ID for each RNA sample**.
8. Carefully, **pipet 2 μ L of sample onto the optical reading field** and put the arm down and press the “play” button.
9. The sample should make a column of liquid through which light will be emitted to determine the optical density of RNA.
10. Once the reading appears, lift up the arm and **save the sample**. Pipet off as much of the sample that you can and return it to the sample tube.

11. **Clean the NanoDrop** optical reading field by spraying a small amount of RNA Zap and Nuclease-free water on a kimwipe.
12. Repeat for each sample and make sure the **save the readings** with an appropriate file name in the appropriate BL folder.
13. Samples must contain at least 40ng/ μ L of RNA to be used for RT-PCR. If less, try a re-read.
14. **Print the RNA report** containing all the RNA data for the day by clicking “report” button and then print the report.
15. **Put the samples in the freezer** in the appropriate box.

Perfusion and Heart Puncture Protocol

Materials:

- Perfusion/Heart Puncture Data Sheets
- 23g cc syringes
- 2-21g winged infusion canulae that have been “stretched”
- Ether cone lined thinly with cotton
- Glass anesthetizing box with layer of cotton & wire
- Anesthetic ether
- 10% Formalin
- 0.9% saline
- Perfusion Pump & Two Perfusion Set-Ups
- Forceps
- Scissors
- Rubber Gloves (many pairs)
- 10 x 75 mm glass culture tubes
- Jars for brains
- Microcentrifuge Vials
- Paper Towels

Set Up (days before):

1. Label brain jars, micro-centrifuge tubes and paper towels for each subject being perfused. Use permanent marking pen.
2. Cover the jar and vial; label with clear tape.
3. Store in respective holders in perfusion order.
4. Make sure you have enough saline, formalin and ether.
5. Make customized perfusion data sheets and fill in weight, S#, DOB, etc.
6. Set up your perfusion pump and set-ups under the hood, including an empty mouse tub lined with a plastic bag.

Heart Puncture Perfusion Procedure:

1. Pour some ether on cotton in anesthetizing box and ether cone.
2. Put ether cone in box and cover. Keep ether covered at all times.
3. Bring in cart load of mice and check to make sure order corresponds to that of perfusion sheets, brain bottles, towels and vials.
4. Place paper towels with correct S# for first two mice on perfusion boards (ALWAYS work left to right so the first mouse is on the left board).
5. Place an opened and evacuated syringe close to under hood where you will do the heart puncture.
6. Make sure both perfusion set-ups are set to run saline rinse first.
7. Place the first mouse in anesthetizing container. Put cone under rubber band on small, wooden perfusion board.

8. Watch the mouse carefully and remove it as soon as it is under (try not to let them go into convulsions, although with some strains, particularly DBA's, this is very difficult).
9. Pin mouse in spread eagle position on board with nose inside cone. Make sure body is fairly tight.
10. Adjust amount of anesthesia by moving cone towards or away from head. It is important to move quickly, but not frantically once the mouse is under. Efficiency and speed will improve with practice.
11. Evacuate the syringe again to make sure plunger action is smooth.

Note: The remainder of the heart-puncture procedure is for right-handers; lefties will have to adjust accordingly.

Heart Puncture:

1. Sit Down. Gently palpate the chest with left index finger and locate spot where heartbeat is strongest. If you divide the chest into four directional quadrants, this point is usually at/between the southwest and south southwest points.
2. Then, holding the syringe close to the needle/barrel joint, visualize this location in your mind as you remove index finger, put needle tip vertically over this point.
3. Tilt syringe 30° to the right. Insert needle slowly. If you are on target, blood should appear in the base of the syringe, but this is not always the case.

4. Slowly start to withdraw plunger. If no blood appears, gently move the needle up and down a little to try and get into the middle of the chamber. You can try restating, but with each additional poke, the animal goes further and further into shock and heartbeat gets more erratic.
5. Reminder: as you are doing this, keep an eye on the anesthetic state of the mouse.
6. Withdraw as much blood as you can, remove the from chest and take it off the syringe barrel. Expel the blood gently and slowly in the correctly labeled culture tube.
7. Replace needle, cover, and dispose in sharp container and mark down the amount of blood obtained and other pertinent information on perfusion data sheet.

Perfusion:

1. Pinch a good chunk of abdominal skin with large tissue forceps.
2. Cut an oval shaped area of skin off, running from genital area to bottom of rib cage and side to side.
3. Gently separate liver from diaphragm using forceps.
4. Grasp zyphoid process with forceps and lift rib cage up.
5. Cut diaphragm along interior of rib cage.
6. Cut an inverted “V” from the rib cage, starting laterally and ending medially near the top of the sternum. Be sure to keep scissor blades close to the under side of the rib cage to avoid cutting any organs.
7. Snip across the top of the sternum to remove this piece.

8. Grasp right atrium with small forceps and snip it off. You may have to remove some clotted blood from heart before this step.
9. Turn on flow to perfusion setup (set meter on pump to almost 4) and double check to see it is on saline rinse.
10. Hold heart with small forceps and insert cannula into lower left ventricle. Blood should begin flushing out the cut atrium.
11. Remove ether cone and put in anesthetizing jar. Don't let the cotton get all wet and icky.
12. When rinsing fluid runs clear, switch pump to formalin. Run formalin until the mouse is fixed; usually 2-5 minutes.
13. To test for "fixedness", check the rigidity of the neck by pressing nose towards chest. If there is resistance, then the subject is fixed. Sometimes, no matter how long you run fixative, an animal will not fix. In these cases, make sure to note "poor fix" on the perfusion sheet and reason, if you know one.
14. When animal is rigid, unpin from small board and place on paper towel with correct S#, which should be at the top of the set up board. Use paper towel to soak up fluids on small board.
15. Decapitate the carcass and cut a slit along the top of the skull.
16. Place head in properly labeled jar and fill jar $\frac{3}{4}$ full with 10% formalin from squeeze bottle and secure lid with tape.
17. Dispose of carcass in plastic lined mouse tub.

Note: Once you become familiar and comfortable with the heart puncture and perfusing procedure, you can operate two perfusion set-ups simultaneously, heart puncturing one while the other is rinsing and perfusing.

Clean Up:

- Empty all liquid blood and pickling wastes into appropriate container.
- Wash screens, basins, boards and instruments and get set to begin the next day.
- Rinse tubing with distilled water, discard cannula in sharps container and wipe down hood area.
- Let tubing hang and dry before storing in box in mouse perfusion supply drawer.
- Dry and put away surgical instruments.
- Let etherized carcasses sit under the hood for a few hours in open container before sealing plastic bag and discarding in freezer near loading dock.

Perfusions

pH 7.4 → 1xPBS, 4% PFA

To make 1xPBS:

- 1000 mL
- 100 mL 10xPBS
- 900 mL water

To make 4% PFA:

- 500 mL
- 8% PFA
- Add 500 mL 2xPBS
- When placing 8% PFA into 2xPBS, heat until in solution. Then test pH.

2xPBS:

- 100 mL of 10xPBS
- 400 mL of water

For Perfusions

- 1xPBS- pH 7.4
- 4% PFA- pH 7.4

To make 1XPBS

- 1000mL
 - 100mL 10XPBS
 - 900mL water

To make 4% PFA

- 500mL of 8% PFA
- Add 500mL 2XPBS

To make 2XPBS

- 100mL of 10XPBS
- 400mL of water

Thin Sectioning

- 6% agarose with 20 μ m

Vivarium Daily Check

Each of the following items must be completed daily in each of our three rooms, provided animals are present, in order to maintain a happy, healthy, and long-term animal colony.

It is your responsibility to check each item on this list after you have done it and return the page to the Daily Check Log. Failure to do so will result in reprimand and eventual expulsion from our lab. IT IS THAT IMPORTANT!

- Empty all water drain-off bins and clean any spills.
- Check each cage for food and flooding and replace as needed.
- Check water nozzle where cages flood and tighten as needed.
- Check for new pups in ALL breeding cages.
- Make sure there are at least 10 clean cages, food bins and card holders
- Clean work spaces with Quatricide (spray bottle)
- Sweep floor as needed.
- Enter temperature, humidity, and number of new litters of pups on the door.

These items should be performed for each room currently housing our animals. Please initial that you checked each of the following rooms:

Room 312: _____

Room 328: _____

Room 337: _____

Using the Confocal

- Stage Control
 - Control of y and x axis of slide stage
 - Focus Wheel
 - Control microscope and objectives 10X-63X
 - Illumination Pathway (light or laser)

 - Turn ocular System Online
 - Choose Filter Dappy, GFP or DS red.
-
- Find Area sections
 - Click offline (towards top)
 - Acquisition Tab
 - Design own configuration or set per objective
 - Folder Load: Chumley 20X
 - Imaging set up 00> when laser being used and wavelength of emission is picking up (400-700nm)
 - Light path
 - Emission Spectra

- You can adjust how much you want the spectra to be (i.e. accept more/less light) but you want to get that peak.
 - Don't want spectra to overlap
 - You can either have all 3 lasers fire at same time or you can use the laser one at a time.
 - Find 1 setting of light and keep it all through the exp. so you'll compare slides.
- Smart Setup
 - Choose dyes
 - Recent dyes
 - ly2
 - ly3
 - ly5
 - Dvil Chain List
 - Fast/speed/compromise with adjustments apply
 - Fluorochrome needs to be excited at 488, not 458.
 - Bump up intensities and gain on detectors until start seeing images
- Continuous to see signal
 - To put in new slide, switch ocular to online
 - Offline acquisition
- 488 → Florese green (more power) → external
 - 1. Main Power

- 2. Then turn key (fan starts)
 - When turning off, turn key and wait for fan to stop then turn off power
- 3. Turn on bright light source (epifluorescence).
 - Sensitive to time (anode/cathode melt inside into a plasma state)
 - If you leave it on for 15-20min, then you get a steady arch → keeps bright light lamp in better shape for longer.
- 4. Component Power
 - Power (switch near screen)
- 5. System plc (switch near screen)

Note: wait until screen is started up (says ready)

- 6. Turn on computer for the screen
- 2 users → LSM user (laser scanning microscope)
 - Software = ZEN (Zeiss efficient navigation)
 - Acquire data → new slides
 - Image processing
 - Play w/ images already taken
- Click (Start System)

- Work space windows
- Tabs/pull down menus
 - Occular → want to look through micros (filter wheels w/ dichroics. Filters incoming lights)
 - Use to find each you can't

- Turn lasos little black to 488.
- Laser \hat{e} (turn on right before ready to imagine, do not photobleach your image)
 - (Zeiss \rightarrow back it off after light comes off)
- Acquisition \rightarrow acquire data through box (spectral detector)
 - Prism moves light path and diffracts light into wavelengths \rightarrow detectors.
 - (3)
 - (cytochrome 2,3,5)
- Move stage default directions with software or can't turn ocular back on.

To take an image:

- Just a single plane—(snap) to take single picture, or take images of a stack to make a 3D image (maximum intensity image)
 - Stack selected.
 - Multi 0 analysis, set first and last image
 - Focus to where you want to start collecting, focus out to where to end.
 - Move to first/move to last. Collection 1 at 2.75 μ m intervals.
 - Want clean images.
 - Scan 4 times and take average.
 - Do 8 for really clear. Can alter time if needed.
 - Galley view \rightarrow 2-D player or 3D (max intensity image) all 7 images.

- Saving Images
 - File → export → save
 - Also save .lsm files for everything.
- Shut down computer.
- Systems off, components off
- Black box Idle, white box idle.
- Key off (wait for fan)
- Hydrolysis off.

Autoclave Protocol

- Click SELECT to run pre-set cycle
- Use Liquids 3 for any modifications to pre-set liquid cycles

Gravity Cycle

- Click SELECT to run pre-set cycle
- Use Gravity 3 for any modifications to pre-set liquid cycles

Vacuum Cycle

- Click SELECT to run pre-set cycle
- Use Vacuum 3 for any modifications to pre-set liquid cycles

Autoclave Usage:

- All material (liquids, glass, pipettes) MUST be sterilized in containers.
- Waste material in redbags should be no more than half full and fit in container.
- Please remove materials immediately after the cycle is completed.
- Do NOT leave any materials in the autoclave overnight.
- Orange gloves should be worn when taking materials out of sterilizer.
- Spills in the chamber should be immediately cleaned. Sterilizer is to be turned off to cool before cleaning.
- Record sterilizer usage in notebook.

- The sterilizer powers on at 7:00am and off at 10:00pm.
- First users must turn on Jacket and Generator prior to use.
 - Jacket Power On
 - Generator Power On
- Sterilizer takes about 15-30min to be ready for use.

Sterilizer Cycles

- Liquids- for any liquids or agar based materials
- Gravity- for sterilizing glassware
- Vacuum- for sterilizing pipettes/tips with need for drying.

Liquid Cycle

EDTA in Tubes to Avoid Blood Clotting

- 150mg of EDTA in 10mL H₂O
 - 50μL of EDTA/tube
- Rock, keep at room temperature.
 - Spin (cold room) 815g for 10min.
- Supernatant → -80°C

Vibratome Protocol

1. Inside the Tupperware container labeled, “Vibratome” are all of the supplies you will need.
2. Put on gloves and retrieve brain sample from the refrigerator.
3. Dump the formaldehyde (the liquid in the sample tube) into the aldehyde waste jar (found under the sink).
 - a. Use the cap as a barrier to ensure you do not lose the brain.
4. Remove a petri dish from the container and place the brain in it. Clean the formaldehyde off of the brain using PBS and tweezers.
5. Place the brain on a paper towel.
6. Keep the original tube the brain came in- they are reused, just place in sink and wash after you have finished slicing the brain.
7. Remove a single edge razor from the container and cut off the prefrontal cortex.
 - a. Approximately 1cm back from the front of the brain (where the Olfactory Bulbs are)
 - b. Make sure to make a straight cut, if it is cut at an angle the rest of your slicing can be skewed.
8. Place the brain back into the PBS
9. Retrieve the 3% agarose solution from the shelf in the back left of Chumley’s lab.
 - a. Fill a beaker 1/3 way full with water.

- b. Loosen the cap of the agarose 50mL tube and place the tube into water and put into microwave. Check on it every 15 seconds to ensure it doesn't boil over.
10. Get a 24 well plate and label it (using a permanent marker) with the Animal #
11. Get a plastic mold out of the Tupperware container and label it with the animal number, date, your initial and anything else on the brain tube.
12. Pour the melted agarose into the mold up to the small dashed line.
 - a. If there is any agarose left in the tube, leave it uncapped until it cools and place it back on the shelf.
 - b. If the tube is empty, place the used tube back on the shelf with the rest of the agarose. Make sure it is in the back.
13. Place the brain into the agarose: the sliced off section down, make sure it is perfectly vertical using the tongs.
 - a. Place the mold into the refrigerator until it hardens: about 25 minutes.
 - b. Clean the extra agarose off of the tongs and place them back into the container.
14. Remove the PBS+Azide (.03% solution) from the fridge and fill each well halfway
 - a. Use a serialological pipette (5mL)
15. Remove one package of blue blades from the "sharp" box in the container. Snap them in half to create 2 blades- cut off the "wings" of the blade at an angle to make fit into blade holder.
16. Tighten the platform onto the box (using the screw and red tool) and then tighten the box onto the machine with the lever on the front side.

17. Put the blade into the blade holder.
 - a. Make sure it is centered and level, tighten with red tool
18. Take the mold out of the refrigerator
19. Cut the corners of the formation with the razor blade from the container and remove the mold.
 - a. Make any necessary adjustments to ensure that the brain is level.
20. Use a dime size amount of superglue to attach the mold onto the plate.
 - a. Make sure the top of the brain is facing the machine with the skinny side of the mold up.
 - b. Let the glue dry.
21. Fill the box with PBS until it covers the blade.
22. Install the blade onto the machine.
 - a. Make sure the blade is set as far as it will go and the platform is all the way down.
23. Turn on the machine and light source.
24. Bring the blade up to where the mold starts and then press the button (with the arrows), then manually pull the blade forward until where the agarose ends and press the button again.
25. Set the machine to 50UM
26. Press “continuous” and then start.
27. Keep slicing at 50UM until you see the “old man” face, then switch to 30UM and allow the machine to recalibrate.
28. Once slices are back to normal, start collecting the samples.

29. Gently pull the agarose away from the sample while still in the PBS using two sets of tweezers.
 - a. Place the sample in the first well (A1)
 - b. Continue to place the samples into the wells horizontally. Once all wells are filled, start again back at A1 until the brain falls apart and no more samples can be taken.
30. Cover the samples and place them in the refrigerator.
31. Clean up all of the materials.
 - a. Place the blue blade into the sharps bin.
 - b. Use the blade from the container to scrape off the superglue from the platform.
 - c. Rinse all pieces in the sink and then put everything back onto the machine.
 - d. Then get as much of the agarose out of the drain as possible using tweezers.
32. Turn the machine off
33. Turn off the light source.
34. Cover the vibratome with its cover.
35. The end.

BEHAVIORAL TESTING AND TRAINING PROTOCOLS

Behavior is defined as an animal's response to stimuli. This is an extremely complex phenomenon with many factors at play. However, our lab relies on the assumption that observable behavior is both observable and an indication of underlying biological processes. Therefore, in order for the lab to draw meaningful conclusions in the realm of neurobiology, it is imperative to have precise biological testing and training protocols to reduce the effects of extrinsic factors that can arise from human error. This set of protocols will ensure that the present and future use of behavioral testing and training protocols will be both consistent and accurate.

Passive Avoidance Conditioning

Preliminary Set-Up:

- Make sure the boxes are configured for passive avoidance
 - The two beams proximal to the door on the left/dark side are disabled (done via micro-switches inside the back of the unit) and the left black door placed screwed on over the clear plastic door; the right hand side remains clear.
 - You also may want to use the small passive avoidance insert boxes which diminishes exploration time and helps decrease latency on Day 1- make sure they are not blocking the beam or blocking the gate from going up and down.
 - Make sure lights are off when testing. Be very careful when cleaning and working with the units, as they are fragile and expensive.
 - Do not dangle animals by the tail.

Procedure:

1. Read “Do’s and Don’ts notice” on the door.
2. Turn on switches on black boxes to the sides of each unit.
 - a. For all 6 boxes. If you are not going to use a box, you still must put a paper towel in it.
3. Find “Shortcut to Gemini” on the desktop (username: psy_work2; password: boehmlab4) and double-click it: make sure there are no mice in the boxes yet.
4. Using the box at the left, selective Passive avoidance.

5. Instead of loading all the parameters yourself, go to “Load Session” box at the top/right of the screen and double-click it, from which you can access a default file we use.
6. To select/load our default file, select in the little box the desktop, and find the file in the passive avoidance folder and Open. This will pull up a file for 60 mice (and our usual parameters), though you may not test that many mice for the given file. Note that you will need a paper test sheet that acts as your key for what the real subject numbers are. Do not lose this, so put it in the manila folder for the study.
7. Using your paper test sheet, decide how many you are going to run in each testing batch. Note that you must have paper towels (or something to block the beams) in the unused boxes. There will always therefore be testing groups of 6, even if you only run 3 or 4.
8. Click the Start Session box now, and give your file an appropriate file name, and select where you want to save it (desktop is recommended). After you do this, all 6 boxes will show up, with each showing you the green prompt for “ready”.
9. Once the lights are off and the white noise generator is on, your mice are ready for testing. Insert mice (and paper towels as needed) into the right hand/clear side of the unit and click the square “start” button on the front of each unit, when ready to start the 60 second acclimation countdown. After 60 sec, the light goes on and the door goes up. When the animal follows its natural tendency and crosses to the darkened side, the door goes down and the animal receives a 2 sec 0.4mA foot-shock. If it dashes across to the safe side, make a note of it on your test sheet, and you very well may need to throw out that animal (depending on whether or not you can tell if it got

shocked). Be sure to write down any useful notes on the test sheet about things that happened (e.g., mouse jumped out of box, crossed back to safe side on either trial, mouse looked ill, injection went poorly etc.). There is a 300 second maximum latency to respond. Once all are done in the group, take out animals (gently) and clean boxes carefully between groups.

10. When finished with your subjects, hit “Abort” button and file will be saved.
11. Back up your data on a keychain drive and to BL file folders and clean/sweep room, including cleaning out sink and emptying trash.

BIOLOGICAL ASSAY PROTOCOL

Biological Assays are essential to understand how an internal or external stimuli affects neurobiology at a cellular level. The behaviorally observed results of tests on learning and memory have a biological basis. Most commonly, that is quantified in proteins produced that are associated with learning and memory. This, of course, is not the only way. Biological Assay protocols are crucial to the lab because without them, there would be no observable biological basis for conclusions. The lab must have an up-to-date list of protocols for biological assays and a way to update that list as new techniques and modifications incur. This list of protocols fulfills that need.

Protein Assay & ELISA Instructions

Materials:

- 450 μ L of lysate/ 2 brains
- Protein Assay Dilution- 1:4
 - 5 μ L of sample
 - 15 μ L of buffer
- ELISA 2:1 dilution
 - 300 μ L of sample
 - 300 μ L of work. incub. buffer.

Procedure:

1. Put 200 μ L of sample in each well (instead of SD) before incubating overnight

Total RNA Isolation from Punch Tissue using Rneasy Kit

Things to do before starting:

- For all these steps use RNase-free filter tips (found only under the hood)
- Use RNase free tips
- RNase free tubes from box, pink tubes, Xtra bottom tubes.
- Ensure you have enough 70% ethanol. If not, make more.
- Add β -Mercaptoethanol (β -ME) to Buffer RLT before use. Add 10 μ L β -ME per 1mL of Buffer RLT. Make sure to do this under a fume hood. Can be stored up to 1 month.
 - To do 2 runs of 8 samples: 6.3mL Buffer RLT and 63 μ L β -ME
 - To do 2 runs of 16 samples: 12.6mL Buffer RLT and 126 μ L β -ME

Procedure:

1. Label all tubes. Set out all necessary pipets, tips, reagents in necessary locations. Fill one microcentrifuge tube with buffer RLT and one with ethanol to clean homogenizer.
2. **Pipette off the RNAlater** used to store the samples and **add 350 μ L of Buffer RLT**.
3. Homogenize the tissue at level 6 in the buffer RLT until no visible tissue pieces remain. **Clean the homogenizer** with ethanol first, then buffer before each sample.
4. Centrifuge at **full speed for 3 minutes**. Carefully, pipet off the supernatant without disturbing the pellet and deposit into a new labeled tube.

5. Add 350 μ L of 70% ethanol (out of the centr. into ice, 13,000 RPM pull foam first. Turn pipet to 500 μ L to control the amount) to the collected supernatant and mix by pipetting. **Do not centrifuge.**
6. **Transfer the entire sample (700 μ L)** into an Rneasy spin column.
7. Close the lid, and centrifuge for **26 seconds at 10.8k RPM**. Turn to hold because only does min. Put in ice.
8. Save the collection tube but discard the flow-through into the waste beaker. Put collection tube back in bottom that was dumped.
9. Add **700 μ L Buffer RW1** to the Rneasy spin column.
10. Close the lid, and centrifuge for **26 seconds at 10.8k RPM**.
11. Discard the flow-through into the waste beaker.
12. Add **500 μ L Buffer RPE** to the Rneasy spin column.
13. Close the lid, and centrifuge for **26 seconds at 10.8k RPM**.
14. Discard the flow-through into the waste beaker.
15. Add **500 μ L Buffer RPE** to the Rneasy spin column.
16. Close the lid, and centrifuge for **2 minutes at 10.8k RPM**.
17. Place the Rneasy spin column into new 2mL collection tube (provided in the kit).
18. Close the lid, and centrifuge at **full speed for 1 minute**.
19. Place Rneasy spin column in a new 1.5mL collection tube and **add 25 μ L of Rnase-free water** to the spin column membrane.
20. Centrifuge for **1 minute at 10.8k RPM** to elute the RNA. **Repeat this spin cycle.**
21. Finally, **discard the pink filter tops**. Your sample should be in the collection tube.
22. Now measure RNA concentration using **Nanodrop**.

DC Protein Assay

- Flat bottom assay plates.
- To make A': 20 μ L of s/1mL of A
 - 5 μ L of sample in each well
 - 25 μ L of A' in each well
 - 200 μ L of B into each well
- 1.52 mg/ μ L of gamma globulin
 - Add 47.36 μ L of gamma globulin to 52.63 μ L of RIPA = 100 μ L
 - 0.8 μ g/mL
 - 1:2 Senal dilution
 - 50 μ L of gamma globulin to SO buffer = 0.4
 - 50 μ L of .4 to SO buffer = 0.2
- Make duplicates on plate.
- When output is up, look at standard corrected #'s.
 - Linear to get data.
- Place those #'s in spl_hipp_cort4x.xls spreadsheet.

Protein #	Dilution Factor	Stock pro concentration	Amt of Sample	(Blue stuff) Amt Buffer	Amt lysis buffer

B	C (1,2,4)	D	E	F	
		$B \times C = D$	$100/D = E$	50 μ L	

For 100 μ L of protein in buffer to boil

All tubes want to be 1 μ g/ μ L of protein

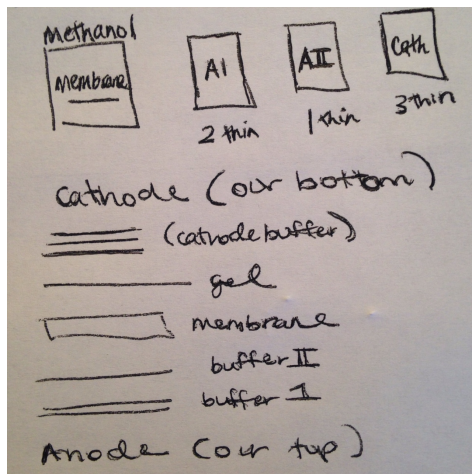
First Solo Western Blot

- RIPA MP40 & Blue Stuff
- Take stock tissue & dilute w/ RIPA and blue stuff
 - Put in boil tubes (tube heater to 100). 2 minutes

	Amount Tissue	RIPA	Blue Stuff
Spleen	46.6 μ L	3.4 μ L	50 μ L
Hippocampus	19.7 μ L	30.3 μ L	50 μ L
Cortex	24.7 μ L	25.3 μ L	50 μ L

- Put in beads to cool and mix by flicking.
- Load Gel → put gel in blorad holder and runner WISDS running buffer.
 - Pull combo out before pouring SDS
- SDS Running Buffer
 - Ladder- 10 μ L
- LDR
 - S10, H10, C10, S20, H20, C20, S30, H30, C30
- Constant Voltage
 - At 200V
 - 45:00 time- hit running man.
 - Build cathode and anode.
- To Pour Gel
 - Need big glass place (spacer plates) and small glass plate (Shoa Plates)
 - Plate holders w/ rubber stopper

- See previous page for upper & lower stacking gels.
- To Run western
 - Drop membrane into methanol
 - Rinse w/ DI Water
- In Preparation Place



- .15 amps for 45 min.
- Membrane into poncou for 10min (reddie)
 - Make sure there is protein
- Pour Poncou back into canister,
- Rinse with ddH₂O and let dry.

Protein Extraction

1. Remove tissue and wash in PBS
2. Chop into pieces and add cold RIPA (labeled tubes, set #1). Buffer. Make fresh.
3. Homogenize
4. Incubate on ice for 1 hour (pour your gels in this time)
5. Centrifuge at $>10,000$ RPM in cold room (30mins)
6. Remove lysate (called whole cell lysate, labeled tubes set #2) to new labeled at ice cold tube.
7. Perform protein assay.

Note: at this point, WCL's can be stored in freezer.

- Aliquot into 2 - multiple freeze/thaws not recommended
- Use excel format to determine amounts of protein

Staining Brain Sections

- **Day 1**
 - Rinse section in PBS (3x10 min)
 - Block in 5% PBST + donkey
 - Store overnight in cold room slanter.
- **Day 2**
 - IBA-1 Antibody
 - PBST + 5% Donkey + 1:500 μ L (brain section)
 - Store overnight in cold room.
- **Day 3**
 - 15 min wash with PBST (x5)
 - 2nd - Dx goat Hrp 1:1000
 - In pBST + 5% Donkey
 - 10 μ L in 10mL
 - 4 hrs – room temperature
 - 15 min wash with PBST (x5)
 - 15 min wash with PBS (x1)
 - DAB Staining
 - Clean off slides
 - Sliding → Vectomount AQ
 - Roll out. Drop on each section and cover slip
 - Leave over night. Around all edges dap and dry.

Strip & Reprobe Protocol (PVDF Membranes)

1. Reactivate PVDF in MeOH, wet.
2. Stripping Buffer: 62.5 mM Tris pH 6.8, 2% SDS, 100mM BME

20% SDS (x) = 75mL (2%)	→ 7.5mL
2M Tris (x) = 75mL (62.5mM)	→ 2.343 mL
380 μ L BME/50mL	→ 570 μ L
	→ 64.487mL H ₂ O

75mL

3. Incubate 60°C x 20min
4. Wash 2x10 min in PBST.
5. Rinse in PBST.
6. Block in 5% milk/PBST begin Western Blotting protocol from the blocking step.

Western Blot

Note: We do not have the luxury of performing this experiment over 2 days. So, we will use three separate lab sessions and your instructor will do some of the additional work.

Outline:

Day 1: On this first day, we will pour the Polyacrylamide gel that you will use on the following lab day. This procedure takes practice. So, we will take our time and concentrate on following the directions so that we have a useable gel for the following session.

Day 2: The second Western Blotting day will require that you have completely read the procedure before you arrive. This day will be a long one. We will prepare and load our protein samples, run the gel, and then immediately transfer the gel to our membrane. Your instructor will make sure that the gel is blocked before your third session.

Day 3: Again, this day will take time. You will arrive and add a primary antibody to your blot and incubate for one hour. Then after a 15 min wash, you will add secondary:HRP antibody and again incubate for an hour. Finally, following a 15 min wash, you will add substrate until you get a color change. After a wash in water to remove the substrate, we will analyze the results of the blot.

A more detailed description of **Day 1** follows below. **Day 2** and **Day 3** will utilize kit components similar to the ELISA lab and you will receive the .pdf document associated with this kit on eCollege before your class.

Gel Preparation (Professor will do as a demonstration):

1. **Resolving gel:** Combine the reagents below according to the percentage of gel recommended by your instructor. Do NOT bubble the mixture or you will have bubbles in your gel.

Lower Gel	7.5% x 2	10% x 2	10% x 4	12.5% x 2	12.5% x 4	15% x 2	10% x 5
30% Acrylamide/Bis	4mL	5.3mL	10.6mL	6.7mL	13.4mL	8mL	13.25mL
Lower Gel Buffer	4mL	4mL	8mL	4mL	8mL	4mL	10mL
Water	8mL	6.7mL	13.4mL	5.3mL	10.6mL	4mL	16.75mL
10% APS	60μL	60μL	120μL	60μL	120μL	60μL	150μL
TEMED	8μL	8μL	16μL	8μL	16μL	8μL	20μL

2. **Gently** top the acrylamide mixture with a layer of 95% ethanol. This will prevent air from touching the acrylamide, a step necessary to speed-up polymerization. Also, **very gently**, top the remaining acrylamide mix in your tube. This will give you a control sample in which to test whether polymerization has occurred.
3. After polymerization has occurred, pour off the alcohol and allow the top of the resolving gel to dry.

4. **Stacking gel:** Combine the reagents below according to the number of stacking gels needed.

Upper Gel	2 Gels	4 Gels	5 Gels	10 Gels
30% Acrylamide/Bis	0.75mL	1.5mL	1.825mL	3.75mL
Upper Gel Buffer	1.25mL	2.5mL	3.125mL	6.25mL
Water	3mL	6mL	7.5mL	15mL
10% APS	20 μ L	40 μ L	50 μ L	100 μ L
TEMED	10 μ L	20 μ L	25 μ L	50 μ L

5. Make sure the comb is inserted completely and with no bubbles associated with the lanes.
6. Once the gel has polymerized, remove it from the gel casting apparatus and cover with paper towel damped with a small amount of upper gel buffer. Wrap the gel: paper towel sandwich in saran wrap and store until the next lab session in a Ziploc Bag.

For Transfer:

- **Anode I:**
 - .3M Tris-base pH 10.4 \rightarrow 2L = 72.66g Tris base.
 - 10% methanol (add when using). 1L = 36.33g

- 1L = 900 mL of ddH₂O + 36.33g Tris base + 100 mL methanol.
- **Anode II:**
 - 25mM Tris base.
 - 1L = 3.0275g Tris base.
 - 2L = 6.055g Tris base.
 - pH= 10.4
 - 10% methanol (add when using)
- **Cathode:**
 - 25mM Tris base.
 - 1L = 3.0275g Tris base, 3g Glycine
 - 2L = 6.955g Tris base, 6g Glycine
 - 40mM Glycine
 - pH= 9.4

For Western Blotting Exercise:

- **4x Upper Gel Buffer- 500mL**
 - 400mL ddH₂O
 - 30.3g Tris-Base
 - pH to 6.8 with concentrated HCl (≈20mL)
 - 20mL of 10% SDS
 - Bring to 500mL with ddH₂O
- **4x Lower Gel Buffer- 500mL**

- 400mL ddH₂O
- 90.9g Tris-Base
- pH to 8.8 with concentrated HCl
- 20mL of 10% SDS
- Bring to 500mL with ddH₂O
- **10X Running Buffer- 1L**
 - 800mL ddH₂O
 - 14.4g Glycine
 - 1.0g SDS
 - 3.025 g Tris-Base
 - Bring Volume to 1L with ddH₂O
- **10X Running Buffer- 2L**
 - 1600mL ddH₂O
 - 28.8 g Glycine
 - 2g SDS → cast
 - 6.050g Tris-Base
 - Bring Volume to 2L with ddH₂O
- **1X Towbin Transfer Buffer- 1L**
 - 600mL ddH₂O
 - 3g Tris-Base
 - 14.4g Glycine
 - 200mL Methanol
 - pH to 8.3

- Bring Volume to 1L with ddH₂O

Blood/Serum Collection Protocol

1. Collect blood in 1.75mL tube and place on ice.
2. Allow blood to clot (30min-1hr)
3. Warm tube to 37°C and dislodge clot from side of tube.
4. Spin 1200RPM (500g) for 30min.
5. Remove serum and place in new labeled tube.
6. Freeze at -80°C

Sera Protocol

1. Following heart puncture with a 23g needle, remove the needle from the syringe barrel and gently expel the blood into a marked, 10x75mm glass culture tube.
2. Record the amount of blood obtained and any other pertinent information on data sheet. Dispose of entire syringe in appropriate sharps receptacle.
3. Following all perfusions for the day, place all samples in refrigerator. They may remain there for 4-24 hours to clot.
4. Set up a weight-balanced list of samples (two columns) and keep with perfusion sheets. This will be used to place the samples in the centrifuge. Note which samples will need “dummy” tubes with water for counterbalance. Label laminin microcentrifuge tubes for all samples .6cc and above. Label = S#Lam.
5. Turn on centrifuge shortly before spinning to allow it to come to temperature (8°C).
6. Place samples in rotor(s) according to your balanced sample list. Use dummy tubes filled with appropriate amount of water where necessary. It is very important that the samples be balanced since an off balance load will result in poor Sera samples.
7. Spin at 3500rpm for 15 minutes.
8. Decant Sera into labeled microcentrifuge tubes (cover S# on tube with tape to protect) using micro pipette set at 60mL. If you have a good sample, it will take several withdrawals to get all the sera. Decant the last 60mL into the laminin tube. Use one tip for each S#. Be extremely careful not to put samples in the wrong tube. To help prevent this from happening, place emptied blood tubes back in rack, in the order in which you decant them. Do the same with the ser and laminin samples, using a

- second tube holder. This way, if you do use an incorrect sera tube, you can retrace your steps and determine where the error occurred. Make sure you relabel any tubes if you do make a mistake.
9. Put sera in super cold freezer immediately following directions on freezer. Samples are basically stored in containers by study, except laminin samples.
 10. Rinse blood clots out of glass tubes and dispose in lab glass bucket. Turn off centrifuge, toss tips, clean up and put everything away.

Cresyl Violet Stain Protocol

Solutions Needed:

- Cresyl Violet Stain (0.1% Cresyl Violet Acetate)
- 70% Ethanol
- 95% Ethanol
- 100% Ethanol
- Xylene
- 95% Ethanol + 10% Glacial Acetic Acid (4-5 drops)

Recipe for Cresyl Violet Stain (500mL)

- 500mL H₂O
- 0.5g Cresyl Violet Acetate (1g Cresyl Violet Acetate/1L H₂O)
- 1.25mL Glacial Acetic Acid (2.5mL 100% Glacial Acetic Acid/1L H₂O)

Stir on heat (60°C) until majority of crystals are dissolved*. Let the solution cool and store in dark bottle. Reheat to 60°C and filter before every use.

*If possible, stir solution for a couple of days with heat, and then filter again. If you still get specks of solid on sections, use a Bottle Top Filter (rather than a fluted paper) for better filtration.

Procedure

1. Parafia Slides

2. Run slides through jars again, but backwards starting from Xylene and ending in diH₂O (rehydrating). Step 1.
3. Dip and agitate gently in Cresyl Violet Stain for about 3-4 minutes or until you feel it has stained.
4. Rinse off all excess stain in H₂O.
5. Place in 70% ethanol with 6-7 dips.
6. Alternate between the 95% ethanol and 95% EtOH + Glacial Acetic Acid (this will help differentiate the stain) until you get the differentiation you want.
7. Finish out by running down the rest of the jars from 95% to 100% to Xylene.
8. Coverslip with Permount and let it dry overnight.