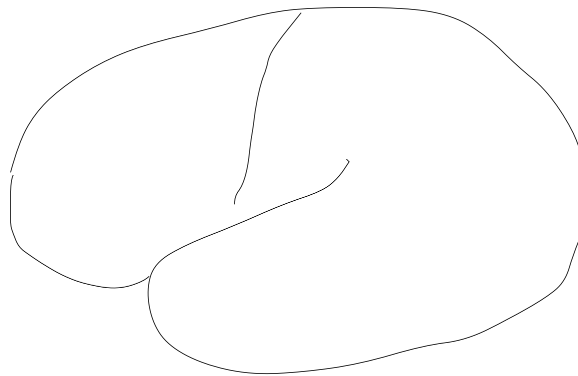
THE NEUROANATOMICAL DYSLEXIA RESEARCH LABORATORY MANUAL



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BUFFERS

PBS (Phosphate Buffered Saline), 0.05 M

For 1 liter:

13.4 g $Na_2HPO_4 \cdot 7H_2O$, Dibasic heptahydrate
8.0 g NaCl

Dissolve in 1 liter dH_2O
pH = 7.40, Store at 4°C

TBS (Tris Buffered Saline), 0.05 M

For 1 liter:

6.05 g THAM, Tris (Hydroxymethyl) Aminomethane
8.0 g NaCl

Dissolve in 1 liter dH_2O
pH = 7.60, Store at 4°C

NaPb (Sodium Phosphate Buffer)

0.2 M

For 1 liter:

5.52 g NaH_2PO_4 (Monobasic) in 200 ml dH_2O
22.72 g Na_2HPO_4 (Dibasic Anhydrous) in 800 ml dH_2O

Combine or from the start dissolve reagents in 1 L dH_2O
pH = 7.40, Store at 4°C

0.1 M

For 1 liter:

2.76 g NaH_2PO_4 (Monobasic, in 200 ml dH_2O)
11.36 g Na_2HPO_4 (Dibasic Anhydrous, in 800ml dH_2O)

Combine or from the start dissolve in 1 L dH_2O
pH = 7.40, Store at 4°C

Tris

0.05 M

For 1 liter:

6.05 g THAM
Dissolve in 1 L dH_2O
pH = 7.60, Store at 4°C

0.1 M

For 1 liter:

12.10 g THAM in 1L dH_2O
pH = 7.60, Store at 4°C

0.1 M Acetate Buffer

For 1 liter:

13.6 g sodium acetate · $3H_2O$ in 1 L dH_2O

pH = 5.00, Store at 4°C

Citrate Buffer

For 25 ml:

5.1 g Citric Acid
4.7 g Sodium Citrate

Dissolve in 25 ml dH_2O.

pH = 3.60, Store at 4°C

10 N NaOH

40 g NaOH pellets in 100 ml dH_2O
Do not adjust pH

1 N NaOH

4 g NaOH pellets in 100 ml dH_2O
Do not adjust pH

30% Buffered Sucrose

For 100 ml:

30g sucrose in 100ml 0.1M NaPb

pH = 7.40, Store at 4°C

10% Buffered Sucrose

For 100 ml:

10 g sucrose in 100 ml O.1M NaPb

FIXATIVES

We have been using a recipe wherein temperatures never exceed 60°C. Apparently, when the temperature exceeds 60°C it destroys the fixative although some sources suggest heating the fixative to 70°C. However, temperatures well below 60°C will cause an incomplete dissolution of the fixative.

All of the following procedures, except pH adjustment, are performed under the hood with gloves .

2% Paraformaldehyde/0.05% Glutaraldehyde

Make 10% buffered sucrose prior to the perfusion. This will usually be needed in the perfusion so it is better to make it beforehand.

For 1 liter:

Heat approximately 200 ml dH_2O to 60°C. Add 20 g Fisher paraformaldehyde to the heated water. Stir for 5 minutes. Add 1N NaOH slowly until solution clears completely. Cool solution to 40°C. Add 2 ml 25% glutaraldehyde. Bring volume up to 500 ml with dH_2O. Add 500 ml 0.2 M NaPb. Filter.

pH = 7.40, use the same day the fixative is made.

4% Paraformaldehyde

Make 10% buffered sucrose prior to the perfusion. This will usually be needed during the perfusion so it's better to make it beforehand.

For 1 liter:

Heat approximately 300 ml dH_2O to 60°C. Add 40 g Fisher paraformaldehyde to the heated water. Stir for 5 minutes. Add 1 N NaOH slowly until the solution clears completely. Cool solution to 40°C. Bring volume up to 500 ml with dH_2O. Add 500 ml 0.2M NaPb. Filter.

pH = 7.40; use the same day the fixatve is made.

NCAM

For 1 liter:

Heat approximately 200 ml dH_2O to 60°C. Add 2.5 g Fisher paraformaldehyde to the heated water. Stir for 5 minutes. Add 1 N NaOH slowly until solution clears. Cool to room temperature. Filter.

pH = 7.40

Then add:

16.2 g glucose (Dextrose)
400 μl $CaCl_2$
20 ml 25% glutaraldehyde
400 ml 0.2 M NaH_2PO_4 (11.04 g/400 ml dH_2O)
100 ml 0.2 M NA_2HPO_4 (2.84 g/100 ml dH_2O)
10 ml dimethyl sulfoxide

Bring up to volume with dH_2O.
pH = 7.40

Modified Zamboni's

- 4% Paraformaldehyde, 0.08% glutaraldehyde, 15% picric acid

For 1 liter:

Heat approximately 300 ml dH_2O to 60°C. Add 40 g Fisher paraformaldehyde to the heated water. Stir for 5 minutes. Add 1 N NaOH slowly until solution clears. Cool solution to 40°C. Add 3.2 ml 25% glutaraldehyde. Add 500 ml 0.1 M NaPb. Add 150 ml acqueous picric acid. Bring up to volume with dH_2O. **Do not adjust pH**.

Low-High Paraformaldhyde

Rapid flush with 4% para (1.3 -1.4 of total fix volume) at pH = 6.00 for good penetration throughout tissues, but weak fixation. Then flush with the remaining 4% para at pH = 9-11 for weak penetration and strong fixation.

PLP Periodate-Lysine-Paraformaldehyde

For 400 ml:

Solution A: Dissolve 3.65 g L-lysine in 100 ml dH_2O.
Add 20 ml 0.1 M Na_2HPO_4 (1.42 g/100 ml dH_2O).
Bring up to volume with 0.1 M NaPb. Filter.

pH = 7.40; Store at 4°C. This solution is stable for one month.

Solution B: Heat 150 ml dH_2O to 60°C.
Add paraformaldehyde to the heated water.
Stir for 5 minutes.
Add 1N NaOH slowly until the solution clears completely
Filter.

pH = 7.40; store at 4°C. Solution is stable for 1 day only.

Combine solutions A and B and add 256 mg sodium-m-periodate. After perfusion immerse in fixative for 30 minutes.

Bouin's

For 250 ml:

187.5 ml picric acid, saturated solution
62.5 ml 37% v/v formalin
12.5 ml glacial acetic acid

Do not adjust pH.

PERFUSION

The wash, usually 0.9% saline, is run through the aenesthetized animal at a high rate in order to remove all of the blood from the animal. **This step is critical**. If all the red blood cells are not removed, they will block capillaries preventing parts of the brain from receiving adequate fixative. The resulting brain will be difficult to cut and the staining may be dull with a high background. For the adult mouse only a small amount of restriction should be placed on the flow of the wash. For the adult rat, let the wash flow as fast as possible.

1. Aenesthetize the animal and pin down to styrofoam block, making sure that the pins do not press down too hard on the animal. The best placement is through loose skin under the arms and legs.
2. Remove skin over the abdominal cavity and cut through muscle layer. Cut through the diaphragm and remove the sternum carefully to expose the heart. Do not cut lung tissue. Cut pericardium.
3. Place needle in left ventrical in a longitudinal orientation. The heart *should not* be beating fast.
4. Cut the right atrium and turn on the wash.

5. When the liver is clear, after approximately 1 minute, switch to fixative, which should also be flowing at a rapid rate. The perfusion is good if the animal “dances” upon receiving the fixative. After a few minutes the neck and tail should be stiff.

HOW TO USE THE PH METER

Place the beaker of solution you will pH on the stir plate. Add a magnetic stir bar, to expedite the whole process.

Calibrate the pH meter. The slope should be set at 84% and the temperature must be set for 22°C. The pH button should be pressed inward.

1. Wash the probe twice by spraying with dH_2O and carefully dabbing the probe dry. Do not touch the glass membrane inside the plastic.
2. Immerse the pH probe in a beaker filled with fresh Buffer Solution, pH=7.00. Press the standby button and release. This will give you the reading. Move the calibration knob until it reads 7.00. Depress the standby button so it stays depressed. Wash the probe twice with dH_2O.
3. Immerse the pH probe into the solution. Depress the standby button to obtain a reading.
4. To create the desired pH one must add either acid (HCl) or base (NaOH) in weakly (1N) or heavily (10N) concentrated solutions. If you want the pH to be smaller (lower in number) you must add acid, and if you want the pH to be larger (higher in number) add base. Always try to add as concentrated solution of acid or base as possible to minimize the change the overall volume of the mixture. For example, a liter solution that needs to drop in pH by 2 full points should use the concentrated NCl, not the 1N HCl.
5. After the desired pH has been obtained, remove the probe and wash twice with dH_2O. Depress the standby to the inward position. Immerse in the storage solution. Write down on the label of your mixture the pH you obtained.

FROZEN TISSUE

Cutting

1. Animal tissue must be placed in 10% buffered sucrose until the brain sinks and then 30% buffered sucrose until the brain sinks. The sucrose acts as a cryoprotectant.
2. Place the platform on the sliding microtome. In the well pour a small amount of 100% EtOH and add dry ice in the well and on the platform.
3. After ten minutes, when the platform is very cold, build a small base of ice on which you will mount the tissue. Squeeze water from a pasteur pipette onto the platform.
4. Remove the tissue from the 30% buffered sucrose and, for the brain, cut the cerebellum in half in order to make a stable base.

   Place the tissue in a cup of dry ice and let freeze for several minutes. Cover the tissue with dry ice.

5. Remove the frozen tissue from the dry ice and place on the flat surface of the ice mount on the platform.

6. Build up the mount with more water so that the cerebellum is completely covered in ice.
7. Make a small nick on the right ventral side of the brain. This will aid in orientation during mounting and later examination.
8. With the wooden blocks, cover the entire brain with dry ice. Then adjust the platform so that the brain is level.
9. The brain is ready to cut. During the cutting it is necessary to periodically build up the dry ice both on the platform and in the well. Do not let the brain thaw. Cut slowly and evenly and remove the sections with a brush. Wipe away the water on the blade after each cut or several cuts. Store the tissue in a buffer in a section box at 4°C.

Mounting

1. Transfer sections from the compartments to a dish filled with mounting solution.

Mounting solution:

For 1 liter:

"subbing solution"	500 ml
0.05 M Tris	500 ml

2. Place the first few sections to be mounted in another dish with mounting solution. Mount the sections on a subbed slide, in order going posteriorly with the sections all having the nicks on the same side. After a few minutes adjust and remove creases from sections that are drying.
3. When slide is done, place upright to air dry. After completely air dry, place on slide warmer at 56°C overnight.

"Subbing Solution"

For 1 liter:

1 L dH_2O
0.5 g chromium potassium sulfate
5.0 g gelatin (60 bloom).

Subbing Slides:

For 1 liter:

dH_2O	1000 ml
Warm on hot plate	
Chromium potassium sulfate	0.5 g
Gelatin (60 bloom)	5.0 g

Making "subbed" slides:

1. Dip in rack of slides in warm dH_2O
2. Dip rack in hot "subbing" solution
3. Place rack in oven at 34°C overnight.

STAINS

Hematoxylin and Eosin - frozen, cryostat, paraffin on slides

Protocol

Step		Time
1.	Dip in 100% EtOH to fix	
2.	Dip in 70% EtOH	
3.	dH_2O	2 min.
4.	Filter hemotoxylin, stain	5 min.
5.	dH_2O	10 sec.
6.	Acetic acid - H_2O	1 min. or until background is clear
7.	dH_2O	5 min.
8.	Blueing reagent	30 sec. - 1 min. (under hood)
9.	dH_2O	5 min.
10.	Eosin	3 min.
11.	95% EtOH	10 min.
12.	95%	2 min.
13.	100% EtOH	2 min.
14.	Xylenes	3 min.

Recipes

Acetic acid - H_2O

dH_2O	339.5 ml
Glacial acetic acid	10.5 ml

Results

Nuclei - blue
Cytoplasm - pink
blood vessels - red

Hemotoxylin and Eosin - Celloidin, floating

Protocol

1. Filter hematoxylin, stain 5 min.
2. dH_2O wash 30 sec.
3. Differentiate in 1% acid alcohol, until light
4. dH_2O wash 30 sec.
5. Dip for a few seconds into dH_2O with a few drops of ammonium hydroxide in it. This is a blueing agent.
6. dH_2O wash 30 sec.
7. Eosin 1min.
8. dH_2O wash, settles stain 5 min.
9. 70% EtOH 1 min.
10. 80% EtOH 1 min.
11. 95% EtOH 1 min.
12. Store in alpha terpineol until ready to coverslip

Recipes

1% Acid Aclohol

70% EtOH	99ml
1 N HCl	1 ml

Results

Nuclei	blue
Cytoplasm	pink
Blood vessels	red

Masson's Trichrome, free-floating, frozen sections

Protocol

1.	dH_2O	1 min.
2.	Weigert's Iron Hematoxylin	30-45 sec
3.	dH_2O	1 min.
4.	Masson's fuschin OG	5 min.
5.	1% acetic acid	3 min.
6.	5% phosphotungstic acid	5 min.
7.	1% acetic acid · H_2O	3 min.
8.	2% light green	5 min.
9.	1% acetic acid	1 min.
10.	dH_2O	1 min.
11.	Mount sections in mounting solution	
12.	80% EtOH	1 min.
13.	95% EtOH	1 min.
14	Xylene	1 min.

Recipes

Weigert's Iron Hemotoxylin

Solution A

Hematoxylin	1g
95% EtOH	100ml

Solution B

Ferric chloride	2.5 g
Ferrous sulfate	4.5 g
Concentrated HCl	2 ml
dH_2O	298 ml

Filter both solutions A and B before mixing. Mix either in equal portions or 1-3 ratio. You'll have to see which works best.

2% Light Green Solution

light green SF yellowish	2.0 g
dH_2O	98 ml
Glacial acetic acid	0.5 ml

1% Acetic acid H_2O

dH_2O	99 ml
Glacial acetic acid	1 ml

Masson's Fuchsin - Ponceau Orange G

Stock solution:

Ponceau 2R	2g
Acid Fuschin	1g
Orange G	2g
0.2% Glacial acetic acid - H_2O	300 ml

Working:

Stock Masson's Fuchsin OG	10 ml
0.2% Acetic Acid H_2O	90 ml

Results

Nuclei - black
Collagen - blue
Blood vessels - red

Loyez stain

for frozen sections stored in formalin, free-floating

A stain for myelin

Protocol

1. dH_2O 30 sec.
2. Incubate 6 hours in 2% Ferric ammonium sulfate
3. dH_2O 30 sec
4. Incubate with freshly made stain overnight at room temperature, using just enough stain to cover sections
5. Wash twice in dH_2O 30 sec. each time
6. Differentiate in 2% Ferric ammonium sulfate until the grey matter appears - look under microscope
7. Wash 3 times in dH_2O 30 sec. each time
8. Differentiate in Weigert's solution, can go back and forth between water and Weigert's to obtain desired background
9. Wash 3 times in dH_2O, but for the second time add two drops of ammonium hydroxide
10. Mount in mounting solution
11. Wash twice with 100% ethanol
12. Xylene 1 min.

Recipes

Stain:

Hematoxylin	1g
100% EtOH	10 g
Dissolve hematoxylin in gentle heat.	
Add	dH_2O 100 ml
	sat'd lithium carbonate 2 ml

Weigert's Solution

Potassium ferricyanide	2.5 g
Sodium borate	2.0 g
dH_2O	100 ml

PTAH

A stain to visualize astrocyte gliosis

Protocol

Step		Time
1.	dH_2O	30 sec.
2.	Incubate in saturated $HgCl_2$	3 hours
3.	Lugol's Iodine	5 min.
4.	dH_2O	30 sec.
5.	95% EtOH	5 min.
6.	dH_2O	30 sec.
7.	Potassium permaganate 0.25%	5 min
8.	Oxalic acid, 5%	5 min
9.	Wash 5 times with dH_2O	30 sec. each time
10.	Stain with PTAH	24 hours
11.	100% EtOH, 2 washes	10 sec each
12.	Xylenes	1 min.

Recipes

<u>Zenker's Saturated $HgCl_2$</u>

dH_2O	950 ml
Mercuric Chloride	50 g
Potassium Dichromate	25 g
Sodium sulfate	10 g

Make under hood with mask and gloves, to avoid inhaling the compound. Add 50 ml glacial acetic acid before use; must make fresh each time.

<u>Lugol's Iodine</u>

dH_2O	400 ml
Potassium Iodide	8 g
Iodine	4 g

<u>PTAH Stain</u>

dH_2O	1000 ml
Phosphotungstic Acid	20 g
Hematoxylin	1 g

Dissolve hematoxylin and phosphotungstic acid in separate portions of dH_2O. Dissolve hematoxylin with gentle heat, mix in flask. The mixture must mature. This can be done by keeping in sunlight for 3 weeks or spontaneously by adding 0.2g potassium permaganate.

Cresyl Violet

Stains only cell bodies
For celloidin embedded tissue

Protocol

1.	dH_2O	1 min.
2.	CV	3 min.
3.	dH_2O	1 min.
4.	80% EtOH	5 min.
5.	80% EtOH	5 min.
6.	95% EtOH with dropper of collophonium	20 min.
7.	95% EtOH	2 min.
8.	95% EtOH	3 min.

Store in alpha-terpineol
Before mounting and coverslipping, transfer sections to Xylene

Recipes

Working solution CV, 0.5%

0.5 g in 100 ml dH_2O
Collophonium
35 g white rosin in 100 ml 100% EtOH

Congo Red

a stain for amyloid, a material that appears in tissue as a result of disease

Protocol

1.	100% EtOH	2 min.
2.	95% EtOH	2 min.
3.	50% EtOH	2 min.
4.	Congo Red	5 min.
5.	Differentiate in Potassium Hydroxide	3 min.
6.	dH_2O	30 sec.
7.	Hematoxylin, filter	3 min.
8.	Tap water	5 min.
9.	95% EtOH	2 min.
10.	100% EtOH	1 min.
11.	Xylenes	1 min.

Recipes

Congo Red

Congo Red	0.5 g
50% EtOH	100 ml

Potassium Hydroxide

80% EtOH	100 ml
Potassium Hydroxide	0.2 g

Results

Amyloid - red

Nuclei - blue

Oil Red O for Lipids

For formalin-fixed tissue

Protocol

1.	dH_2O	30 sec.
2.	Oil Red O	10 Min.
3.	dH_2O	30 sec.
4.	Hematoxylin	3 min.
5.	Tap water	3 min.
6.	Scott's solution	3 min.
7.	dH_2O	5 min.
8.	Mount with glycerol jelly	

Recipes

Oil Red O

Stock Solution:

100% EtOH	100 ml
Oil Red O	0.5 g

Working solution:

Stock solution	6 ml
distilled H_2O	4 ml

Scott's Solution

dH_2O	500 ml
Magnesium Sulfate	10 g
Sodium Bicarbonate	1.0 g

Results

Areas which are brilliant red contain lipids
Nuclei - blue

Thionin -

Stains cell bodies

	Cryostat		**Frozen**	
1.	100% EtOH	5 sec.	dH_2O	3 min.
2.	70% EtOH	5 sec.	Thionin	5 min.
3.	dH_2O	1 min.	dH_2O	30 sec.
4.	dH_2O	1 min.	95% EtOH	10 sec.
5.	Thionin	5 min.	100% EtOH	2 min.
6.	dH_2O	10 sec.	100% EtOH	2 min.
7.	95% EtOH	10 sec.	100% EtOH	2 min.
8.	100% EtOH	2 min.	Xylenes	1 min.
9.	100% EtOH	2 min.	Coverslip	
10.	100% EtOH	2 min.		
11.	Xylenes	1 min.		
12.	Coverslip			

Recipes

Thionin

dH_2O	930 ml
Sodium acetate	37 g
Thionin	0.5 g
Glacial acetic acid	30 ml

Bring up to volume with dH_2O.

pH = 4.2-4.4

Filter, Store at room temperature.

PIPETTES

Using the pipette

For any volume under 3 ml that needs measuring one can attain high accuracy with the eppendorf digital pipette.

The pipettes have three stops when depressing the plunger. The first stop is used when drawing the liquid into the pipette. The measurement on the pipette is accurate at this stop.

When expelling the liquid from the pipette first depress the plunger to the first stop, going back and forth a few times to remove all of the liquid. You may want to depress the plunger to its second stop to aid in expelling the liquid. To be consistent, it is important to depress the plunger the same number of times for each sample. Also touching the tip to the sides of the beaker or test tube will aid in removing more fluid.

Once you've finished using the pipette, go to a trash can and depress the plunger to the third stop. This last stop will eject the pipette tip. The yellow tips go on the pipettes with the yellow band, and the blue tips go on the pipettes with the blue band.

Also, remember that pipettes are always more accurate towards the middle of the range than at their extreme. Try not to touch the pipette tip ends with your fingers.

IMMUNOHISTOCHEMISTRY

In all washes and as a base for the antibody diluent, we use PBS or TBS. The addition of the salt to the phosphate or tris buffers inhibits low-affinity binding of non-specific serum proteins thereby reducing low-affinity background staining.

The Antibody Diluent

The antibody diluent is used to dilute the antisera. The diluent base is PBS or TBS (depending on the antibody involved, the spec. sheet should make some suggestion) to which is added Triton X-100. The Triton X-100 is a detergent that aids in antibody penetration of the section.

The antibody diluent for a given stain contains normal serum from the same host species providing the bridge (2°) antibody.

For example:

1° antibody: Rabbit Anti-GFAP
2° antibody: Biotinylated anti-rabbit IgG (made in goat)

The anti-rabbit antiserum we use is made in goat. Therefore, we use a goat serum. We use both 3% and 5% serum solutions. The primary, secondary, and any tertiary antibodies of a given stain will all use the same antibody diluent.

Antibody Diluent base of 1L

1L PBS
3 ml Triton X-100
pH = 7.40,
for TBS **pH = 7.60**
Store at 4°C

For the stain, make up 50ml of 3% normal serum in Antibody Diluent:

1.5 Normal serum in 50 ml Antibody Diluent base.
Stable for 3 days after the addition of the serum.
Normal serum is added to portions of this base.

Examples:

1° antibody	2° antibody	Serum Species
Mouse anti-NF	Rabbit anti-mouse IgG	Rabbit normal serum
Rabbit anti-VIP	Goat anti-rabbit IgG	Goat
Mouse anti-glutamate	Horse anti-mouse IgG	Horse
Rabbit Anti-GABA	Goat Anti-Rabbit IgG	Goat
Rabbit Anti-GFAP	Goat Anti-Rabbit IgG	Goat
Mouse Anti-RGF (radial glial fiber)	Rabbit Anti-Mouse IgG	Rabbit

DAB

3,3′-Diaminobenzidine is a useful colored end product because it is insoluble in alcohol. The brown color of DAB can be enhanced by treatment with osmium tetroxide, nickel chloride, silver nitrate, or other metallic salts such as cupric sulfate. Since DAB is a benzene derivative, it is considered a possible carcinogen and great care should be exercised to avoid inhalation of the powder and contact with the skin.

10% DAB Stock Solution

Since DAB, 3,3′-diaminobenzidine, is carcinogenic, it is convenient to store it in frozen aliquots, thereby minimizing exposure. 1 ml aliquots of 10% DAB are stored at -20°C in microcentrifuge tubes. Wear a mask, goggles, and gloves when weighing the DAB and clean all areas where DAB *may* have been spilled. Bleach will colorize the DAB, but will not "deactivate" it. Add to 0.1M Tris solution under the hood. Discard or store all contaminated material.

10% DAB: 2 g DAB in 20 ml 0.1M Tris buffer

DAB Working Solution

Add 250 μl 10% DAB and 15 μl H_2O_2 to 50 ml 0.05M Tris. After development discard this solution in an appropriately labeled waste bottle.

0.5% Cupric Sulfate/0.9% Saline

This solution, like many metallic salts, osmium tetroxide, nickel chloride, silver nitrate, enhances the brown color of DAB.

1 L dH_2O
5 g cupric sulfate
9 g NaCl
Store at 4°C

Thionin

37 g Sodium acetate in 940 ml dH_2O
0.5 g Thionin
30 ml Glacial acetic acid

pH = 4.2-4.4
Filter; store at room temperature

When a new antibody arrives:

1. The first step is to find, on the specification sheet, at what temperature the antibody needs to be stored. Sometimes an unreconstituted antibody will need to be stored at -20°C while its reconstituted form will need +4°C.
2. Then determine from the spec. sheet whether reconstitution is necessary, or how much double-distilled H_2O is needed. Then label microcentrifuge tubes for small aliquots of the antibody (usually under 100μl). Pipette the antibody in the labeled tubes and store.

To begin a new stain:

1. To determine the companion sera needed for a stain, we need to know the donor species of the antibody.

   1° antibody:______________ Anti-________________
   Donor Species Antigen

   For example:
   68kD NF, Boehringer Mannheim

   1° antibody: Mouse Anti- Neurofilament
   Donor Species Antigen

2. The 2° antibody must be directed against the donor species of the 1° antibody.

   2° antibody:______________ Anti-________________
   Donor Species Antigen

   The donor species of this antibody is determined by what is available.

   For our example:
   1° antibody: Rabbit Anti- Mouse
   Donor Species Antigen

3. Now, we have a secondary antibody. The normal serum used to dilute antisera will be from the same species as the secondary antibody.

   For our example:

   1° antibody: Mouse Anti-Neurofilament
   2° antibody: Rabbit Anti-Mouse IgGs

   Normal serum in antibody diluent: Rabbit

4. The tertiary antibody or compound we will use will depend on the characteristics of the secondary.
   If we have a biotinylated secondary antibody, we will use the ABC (avidin-biotin complex) kit. If we *do not* have a biotinylated secondary antibody, then we will need a tertiary antibody that will bind to the secondary in our stains. In some cases, the secondary binds to the primary antibody *and* the tertiary antibody.

For our example:

2° antibody: Rabbit Anti-Mouse IgG
3° antibody: Mouse Peroxidase Anti-Peroxidase

The Rabbit Anti-Mouse will bind to the Mouse Anti-Neurofilament and the Mouse Peroxidase Anti-Peroxidase.

The tertiary antibody or compound will contain a peroxidase molecule (or other enzyme) that will develop the chromogen.

Antibody Dilutions

The proper dilution method is to make both constituents add up to the whole. For example, for a 1:10 dilution of antibody to serum, we would use one part antibody and nine parts serum for a total of 10 parts. However, when extremely high dilutions are needed, for example 1:100 or higher, it is satisfactory to use one part antibody to 100 parts serum instead of the proper one part antibody to 99 parts serum. The slight inaccuracy will not affect your results.

When pipetting small amounts of antibody, minor errors can be significant. Therefore, for all pipetting of an antibody use the adjustable pipettes. An adjustable pipette has greater accuracy near its mid- range than at its extremes. So, if we wanted to pipette 200 μl, we would use an adjustable pipette with a range 100μl to 1000μl instead of one with a range of 10 to 100 twice.

Immuno Counterstain

Methyl Green/Alcian Blue

Protocol

1.	dH_2O	5 min.
2.	0.5% cupric sulfate/saline	5 min.
3.	dH_2O	30 sec.
4.	methyl green/alcian blue	5 min.
5.	dH_2O, two changes	30 sec. each
6.	95% EtOH	1 min.
7.	100% EtOH, two changes	1 min. each
8.	Xylenes	1 min.

Recipe

For 400 ml:

A 2 g alcian blue in 200 ml 0.1 M acetate buffer

B 4 g methyl green in 200 ml 0.1 M acetate buffer.

Let mixture stir overnight. Extract 8 times with separation funnel and chloroform. To extract, add 50 ml of chloroform to the separation funnel and shake vigorously although let the cap sit loosely to release pressure. Let the mixture sit for a few minutes to allow it to separate into two phases. Use about 50 ml chloroform each time. Do this step under the hood. Discard the bottom phase in a chloroform waste bottle.

When all methyl green is extracted, mix A and B. Filter. Store at room temperature.

GABA

Incstar Cat. #20094

Fixation:

After perfusion remove brain from skull and immerse in 10% buffered sucrose overnight at 4°C. The following day immerse in 30% buffered sucrose at 4°C until the brain sinks. The tissue will keep without sodium azide for approximately two weeks.

Sectioning:

Cut the brain on a sliding microtome (frozen) at 30μm. Sections are stored in 0.1 M NaPB at 4°C. The sections will keep without sodium azide for approximately two weeks.

Immunohistochemistry:

All steps are carried out on the shaker table and at room temperature unless otherwise specified.

1. Transfer tissue to netted carriers in beakers of 5ml PBS. This protocol is calculated for the 5ml volume.
2. Wash twice in 5ml PBS for 5 min. each wash.
3. Incubate with 0.6% H_2O_2 solution for 20 min. **For 5 ml volume**: 100μm 30% H_2O_2 in 5ml PBS.
4. Wash twice in 5ml PBS for 5 min. each wash.
5. Add 1° antibody diluted 1/325 - 1/500 and incubate overnight at 4°C. **For 5ml volume**: 15μl anti-GABA antibody (pre-diluted) in 5 ml antibody diluent.
6. Wash twice in 5ml PBS for 5 min. each wash.
7. Incubate with Biotinylated Anti-Rabbit IgG (BARI) diluted 1/60 for 2 hours at room temp. **For 5 ml**: 83.4 μl BARI in 5ml antibody diluent.
8. Wash twice in 5ml PBS for 5 min. each wash.
9. Incubate in ABC complex for 2 hours at room temp. **This solution must be made 30 min. in advance. For 5ml**: 2 drops A, 2 drops B, in 5ml PBS.
10. Wash twice in 5ml PBS for 5 min. each wash.
11. Wash twice in 5ml 0.05M Tris for 5 min. each wash.
12. Develop with 0.05% DAB, 0.01% H_2O_2 in 0.05 M Tris until desired staining is obtained. **This step to be conducted under the hood with gloves. For 5ml**: 250μl DAB, 15 μl H_2O_2 in 50ml 0.05M Tris.
13. Place sections in petri dish with a few ml of 0.05M Tris and swirl around to remove DAB.
14. Place sections in mounting solution and mount onto subbed slides. Dispose of all DAB-contaminated solutions in waste bottle.
15. Place slides on slide warmer and dry at 56°C for 45 min.

GFAP

Incstar Cat. #22522

Fixation:

After perfusion remove brain from skull and immerse in 10% buffered sucrose overnight at 4°C. The following day immerse in 30% buffered sucrose at 4°C until the brain sinks. The tissue will keep without sodium azide for approximately two weeks.

Sectioning:

Cut the brain on a sliding microtome (frozen) at 30μm. Sections are stored in 0.1 M NaPB at 4°C. The sections will keep without sodium azide for approximately two weeks.

Immunohistochemistry:

All steps are carried out on the shaker table and at room temperature unless otherwise specified.

1. Transfer tissue to netted carriers in beakers of 5ml PBS. This protocol is calculated for the 5ml volume.
2. Wash twice in 5ml PBS for 5 min. each wash.
3. Incubate with 0.6% H_2O_2 solution for 20 min. **For 5 ml volume**: 100μm 30% H_2O_2 in 5ml PBS.
4. Wash twice in 5ml PBS for 5 min. each wash.
5. Add 1° antibody diluted 1/25 and incubate overnight at 4°C. **For 5ml volume**: 200μl Anti-GFAP antibody in 5 ml antibody diluent.
6. Wash twice in 5ml PBS for 5 min. each wash.
7. Incubate with Biotinylated Anti-Rabbit IgG (BARI) diluted 1/60 for 2 hours at room temp. **For 5 ml**: 83.4 μl BARI in 5ml antibody diluent.
8. Wash twice in 5ml PBS for 5 min. each wash.
9. Incubate in ABC complex for 2 hours at room temp. **This solution must be made 30 min. in advance. For 5ml**: 2 drops A, 2 drops B, in 5ml PBS.
10. Wash twice in 5ml PBS for 5 min. each wash.
11. Wash twice in 5ml 0.05M Tris for 5 min. each wash.
12. Develop with 0.05% DAB, 0.01% H_2O_2 in 0.05 M Tris until desired staining is obtained. **This step to be conducted under the hood with gloves. For 5ml**: 250μl DAB, 15 μl H_2O_2 in 50ml 0.05M Tris.
13. Place sections in petri dish with a few ml of 0.05M Tris and swirl around to remove DAB.
14. Place sections in mounting solution and mount onto subbed slides. Dispose of all DAB-contaminated solutions in waste bottle.
15. Place slides on slide warmer and dry at 56°C for 45 min.

Glutamate

Incstar cat. #22523

Fixation:

After perfusion remove brain from skull and immerse in 10% buffered sucrose overnight at 4°C. The following day immerse in 30% buffered sucrose at 4°C until the brain sinks. The tissue will keep without sodium azide for approximately two weeks.

Sectioning:

Cut the brain on a sliding microtome (frozen) at 30μm. Sections are stored in 0.1 M NaPB at 4°C. The sections will keep without sodium azide for approximately two weeks.

Immunohistochemistry:

All steps are carried out on the shaker table and at room temperature unless otherwise specified.

1. Transfer tissue to netted carriers in beakers of 5ml PBS. This protocol is calculated for the 5ml volume.
2. Wash twice in 5ml PBS for 5 min. each wash.
3. Incubate with 0.6% H_2O_2 solution for 20 min. **For 5 ml volume**: 100μm 30% H_2O_2 in 5ml PBS.
4. Wash twice in 5ml PBS for 5 min. each wash.
5. Add 1° antibody diluted 1/200 and incubate overnight at 4°C. **For 5ml volume**: 25μl Anti-Glutamate antibody (pre-diluted) in 5 ml antibody diluent.
6. Wash twice in 5ml PBS for 5 min. each wash.
7. Incubate with Biotinylated Anti-Mouse IgG (BAMI) diluted 1/60 for 2 hours at room temp. **For 5 ml**: 83.4 μl BAMI in 5ml antibody diluent.
8. Wash twice in 5ml PBS for 5 min. each wash.
9. Incubate in ABC complex for 2 hours at room temp. **This solution must be made 30 min. in advance. For 5ml**: 2 drops A, 2 drops B, in 5ml PBS.
10. Wash twice in 5ml PBS for 5 min. each wash.
11. Wash twice in 5ml 0.05M Tris for 5 min. each wash.
12. Develop with 0.05% DAB, 0.01% H_2O_2 in 0.05 M Tris until desired staining is obtained. **This step to be conducted under the hood with gloves. For 5ml**: 250μl DAB, 15 μl H_2O_2 in 50ml 0.05M Tris.
13. Place sections in petri dish with a few ml of 0.05M Tris and swirl around to remove DAB.
14. Place sections in mounting solution and mount onto subbed slides. Dispose of all DAB-contaminated solutions in waste bottle.
15. Place slides on slide warmer and dry at 56°C for 45 min.

Neurofilament

68, 160, 200kD fragments: Boehringer Mannheim Cat #814326, 814334, 814342
SMI 31, 32: Sternberger-Meyer Cat #AMI 31,32

Fixation:

After perfusion remove brain from skull and immerse in 10% buffered sucrose overnight at 4°C. The following day immerse in 30% buffered sucrose at 4°C until the brain sinks. The tissue will keep without sodium azide for approximately two weeks.

Sectioning:

Cut the brain on a sliding microtome (frozen) at 30μm. Sections are stored in 0.1 M NaPB at 4°C. The sections will keep without sodium azide for approximately two weeks.

Immunohistochemistry:

All steps are carried out on the shaker table and at room temperature unless otherwise specified.

1. Transfer tissue to netted carriers in beakers of 5ml PBS. This protocol is calculated for the 5ml volume.
2. Wash twice in 5ml PBS for 5 min. each wash.
3. Incubate with 0.6% H_2O_2 solution for 20 min. **For 5 ml volume**: 100μm 30% H_2O_2 in 5ml PBS.
4. Wash twice in 5ml PBS for 5 min. each wash.
5. Add 1° antibody diluted 1/33 for 68-200 kD, 1/200 for SMI 31,32 and incubate overnight at 4°C. **For 5ml volume**: 150μl for 68-200 kD Anti-Neurofilament antibody (pre-diluted) or 25μl SMI 31,32 Anti-Neurofilament antibody in 5 ml antibody diluent.
6. Wash twice in 5ml PBS for 5 min. each wash.
7. Incubate with Rabbit Anti-Mouse IgG diluted 1/20 for 2 hours at room temp. **For 5 ml**: 250 μl Rabbit Anti-Mouse IgG in 5ml antibody diluent.
8. Wash twice in 5ml PBS for 5 min. each wash.
9. Incubate in with mouse PAP diluted 1/250 for 2 hours at room temp. **For 5ml**: 20 μl mouse PAP in 5 ml antibody diluent.
10. Wash twice in 5ml PBS for 5 min. each wash.
11. Wash twice in 5ml 0.05M Tris for 5 min. each wash.
12. Develop with 0.05% DAB, 0.01% H_2O_2 in 0.05 M Tris until desired staining is obtained. **This step to be conducted under the hood with gloves. For 5ml**: 250μl DAB, 15 μl H_2O_2 in 50ml 0.05M Tris.
13. Place sections in petri dish with a few ml of 0.05M Tris and swirl around to remove DAB.
14. Place sections in mounting solution and mount onto subbed slides. Dispose of all DAB-contaminated solutions in waste bottle.
15. Place slides on slide warmer and dry at 56°C for 45 min.

Neuropeptide Y

Cambridge Research Biochemicals Cat. #CA 295

Fixation:

After perfusion remove brain from skull and immerse in 10% buffered sucrose overnight at 4°C. The following day immerse in 30% buffered sucrose at 4°C until the brain sinks. The tissue will keep without sodium azide for approximately two weeks.

Sectioning:

Cut the brain on a sliding microtome (frozen) at 30μm. Sections are stored in 0.1 M NaPB at 4°C. The sections will keep without sodium azide for approximately two weeks.

Immunohistochemistry:

All steps are carried out on the shaker table and at room temperature unless otherwise specified.

1. Transfer tissue to netted carriers in beakers of 5ml PBS. This protocol is calculated for the 5ml volume.
2. Wash twice in 5ml PBS for 5 min. each wash.
3. Incubate with 0.6% H_2O_2 solution for 20 min. **For 5 ml volume**: 100μm 30% H_2O_2 in 5ml PBS.
4. Wash twice in 5ml PBS for 5 min. each wash.
5. Add 1° antibody diluted 1/1000 and incubate overnight at 4°C. **For 5ml volume**: 5μl Anti-Neuropeptide-Y antibody in 5 ml antibody diluent.
6. Wash twice in 5ml PBS for 5 min. each wash.
7. Incubate with Biotinylated Anti-Rabbit IgG (BARI) diluted 1/60 for 2 hours at room temp. **For 5 ml**: 83.4 μl BARI in 5ml antibody diluent.
8. Wash twice in 5ml PBS for 5 min. each wash.
9. Incubate in ABC complex for 2 hours at room temp. **This solution must be made 30 min. in advance. For 5ml**: 2 drops A, 2 drops B, in 5ml PBS.
10. Wash twice in 5ml PBS for 5 min. each wash.
11. Wash twice in 5ml 0.05M Tris for 5 min. each wash.
12. Develop with 0.05% DAB, 0.01% H_2O_2 in 0.05 M Tris until desired staining is obtained. **This step to be conducted under the hood with gloves. For 5ml**: 250μl DAB, 15 μl H_2O_2 in 50ml 0.05M Tris.
13. Place sections in petri dish with a few ml of 0.05M Tris and swirl around to remove DAB.
14. Place sections in mounting solution and mount onto subbed slides. Dispose of all DAB-contaminated solutions in waste bottle.
15. Place slides on slide warmer and dry at 56°C for 45 min.

RAT-401

Radial Glial Fibers

Sue Hockfield - Yale University

Fixation:

After perfusion remove brain from skull and immerse in 10% buffered sucrose overnight at 4°C. The following day immerse in 30% buffered sucrose at 4°C until the brain sinks. The tissue will keep without sodium azide for approximately two weeks.

Sectioning:

Cut the brain on a sliding microtome (frozen) at 30μm. Sections are stored in 0.1 M NaPB at 4°C. The sections will keep without sodium azide for approximately two weeks.

Immunohistochemistry:

All steps are carried out on the shaker table and at room temperature unless otherwise specified.

1. Transfer tissue to netted carriers in beakers of 5ml PBS. This protocol is calculated for the 5ml volume.
2. Wash twice in 5ml PBS for 5 min. each wash.
3. Incubate with 0.6% H_2O_2 solution for 20 min. **For 5 ml volume**: 100μm 30% H_2O_2 in 5ml PBS.
4. Wash twice in 5ml PBS for 5 min. each wash.
5. Add 1° antibody diluted 1:4 and incubate overnight at 4°C. **For 4ml volume**: 1 ml Anti-Radial Glial Fiber antibody (Rat-401;pre-diluted) in 3 ml antibody diluent.
6. Wash twice in 5ml PBS for 5 min. each wash.
7. Incubate with Rabbit Anti-Mouse IgG diluted 1/20 for 2 hours at room temp. **For 5 ml**: 250 μl Rabbit Anti-Mouse IgG in 5ml antibody diluent.
8. Wash twice in 5ml PBS for 5 min. each wash.
9. Incubate in with mouse PAP diluted 1/250 for 2 hours at room temp. **For 5ml**: 20 μl mouse PAP in 5 ml antibody diluent.
10. Wash twice in 5ml PBS for 5 min. each wash.
11. Wash twice in 5ml 0.05M Tris for 5 min. each wash.
12. Develop with 0.05% DAB, 0.01% H_2O_2 in 0.05 M Tris until desired staining is obtained. **This step to be conducted under the hood with gloves. For 5ml**: 250μl DAB, 15 μl H_2O_2 in 50ml 0.05M Tris.
13. Place sections in petri dish with a few ml of 0.05M Tris and swirl around to remove DAB.
14. Place sections in mounting solution and mount onto subbed slides. Dispose of all DAB-contaminated solutions in waste bottle.
15. Place slides on slide warmer and dry at 56°C for 45 min.

Somatostatin

Reichlin-N.E. Medical/Tufts

Fixation:

After perfusion remove brain from skull and immerse in 10% buffered sucrose overnight at 4°C. The following day immerse in 30% buffered sucrose at 4°C until the brain sinks. The tissue will keep without sodium azide for approximately two weeks.

Sectioning:

Cut the brain on a sliding microtome (frozen) at 30μm. Sections are stored in 0.1 M NaPB at 4°C. The sections will keep without sodium azide for approximately two weeks.

Immunohistochemistry:

All steps are carried out on the shaker table and at room temperature unless otherwise specified.

1. Transfer tissue to netted carriers in beakers of 5ml PBS. This protocol is calculated for the 5ml volume.
2. Wash twice in 5ml PBS for 5 min. each wash.
3. Incubate with 0.6% H_2O_2 solution for 20 min. **For 5 ml volume**: 100μm 30% H_2O_2 in 5ml PBS.
4. Wash twice in 5ml PBS for 5 min. each wash.
5. Add 1° antibody diluted 1/100 and incubate overnight at 4°C. **For 5ml volume**: 50μl Anti-Somatostatin antibody in 5 ml antibody diluent.
6. Wash twice in 5ml PBS for 5 min. each wash.
7. Incubate with Biotinylated Anti-Rabbit IgG (BARI) diluted 1/60 for 2 hours at room temp. **For 5 ml**: 83.4 μl BARI in 5ml antibody diluent.
8. Wash twice in 5ml PBS for 5 min. each wash.
9. Incubate in ABC complex for 2 hours at room temp. **This solution must be made 30 min. in advance. For 5ml**: 2 drops A, 2 drops B, in 5ml PBS.
10. Wash twice in 5ml PBS for 5 min. each wash.
11. Wash twice in 5ml 0.05M Tris for 5 min. each wash.
12. Develop with 0.05% DAB, 0.01% H_2O_2 in 0.05 M Tris until desired staining is obtained. **This step to be conducted under the hood with gloves. For 5ml**: 250μl DAB, 15 μl H_2O_2 in 50ml 0.05M Tris.
13. Place sections in petri dish with a few ml of 0.05M Tris and swirl around to remove DAB.
14. Place sections in mounting solution and mount onto subbed slides. Dispose of all DAB-contaminated solutions in waste bottle.
15. Place slides on slide warmer and dry at 56°C for 45 min.

VIP

Incstar Cat. #20077

Fixation:

After perfusion remove brain from skull and immerse in 10% buffered sucrose overnight at 4°C. The following day immerse in 30% buffered sucrose at 4°C until the brain sinks. The tissue will keep without sodium azide for approximately two weeks.

Sectioning:

Cut the brain on a sliding microtome (frozen) at 30μm. Sections are stored in 0.1 M NaPB at 4°C. The sections will keep without sodium azide for approximately two weeks.

Immunohistochemistry:

All steps are carried out on the shaker table and at room temperature unless otherwise specified.

1. Transfer tissue to netted carriers in beakers of 5ml PBS. This protocol is calculated for the 5ml volume.
2. Wash twice in 5ml PBS for 5 min. each wash.
3. Incubate with 0.6% H_2O_2 solution for 20 min. **For 5 ml volume**: 100μm 30% H_2O_2 in 5ml PBS.
4. Wash twice in 5ml PBS for 5 min. each wash.
5. Add 1° antibody diluted 1/333 and incubate overnight at 4°C. **For 5ml volume**: 15μl anti-VIP antibody (pre-diluted) in 5 ml antibody diluent.
6. Wash twice in 5ml PBS for 5 min. each wash.
7. Incubate with Biotinylated Anti-Rabbit IgG (BARI) diluted 1/60 for 2 hours at room temp. **For 5 ml**: 83.4 μl BARI in 5ml antibody diluent.
8. Wash twice in 5ml PBS for 5 min. each wash.
9. Incubate in ABC complex for 2 hours at room temp. **This solution must be made 30 min. in advance. For 5ml**: 2 drops A, 2 drops B, in 5ml PBS.
10. Wash twice in 5ml PBS for 5 min. each wash.
11. Wash twice in 5ml 0.05M Tris for 5 min. each wash.
12. Develop with 0.05% DAB, 0.01% H_2O_2 in 0.05 M Tris until desired staining is obtained. **This step to be conducted under the hood with gloves. For 5ml**: 250μl DAB, 15 μl H_2O_2 in 50ml 0.05M Tris.
13. Place sections in petri dish with a few ml of 0.05M Tris and swirl around to remove DAB.
14. Place sections in mounting solution and mount onto subbed slides. Dispose of all DAB-contaminated solutions in waste bottle.
15. Place slides on slide warmer and dry at 56°C for 45 min.

BDA

Molecular Probes Cat # dilution 10%

Fixation:

After perfusion remove brain from skull and immerse in 10% buffered sucrose overnight at 4°C. The following day immerse in 30% buffered sucrose at 4°C until the brain sinks. The tissue will keep without sodium azide for approximately two weeks.

Sectioning:

Cut the brain on a sliding microtome (frozen) at 40μm. Sections are stored in 0.1 M NaPB at 4°C. The sections will keep without sodium azide for approximately two weeks.

Histochemistry:

All steps are carried out on the shaker table and at room temperature unless otherwise specified.

1. Need 2 small 50 ml beakers per compartment being reacted.
2. In each beaker, put 5 ml of 0.3% Triton X100 and 3.5 μl Avidin.
3. Divide tissue from one staining compartment into two beakers.
4. Incubate for 1 – 1.5 hours.
5. Wash twice in 5ml PBS for 5 min. each wash.
6. Develop with metal enhanced DAB (18 ml buffer, 2 ml diluted DAB) until desired staining is obtained. **This step to be conducted under the hood with gloves**.
7. Place sections in petri dish with a few ml of PBS and swirl around to remove DAB.
8. Place sections in mounting solution and mount onto subbed slides. Dispose of all DAB-contaminated solutions in waste bottle.

CRYOSTAT

Quick Freeze

1. Cut head off and remove brain if old enough
2. Get slurry ready so when brain is out you can proceed without delay.

   Slurry - in a small plastic cup put methyl butane in another cup of liquid nitrogen
   - add small pieces of dry ice
   - when there is a frost on outside of cup, it is ready
   - two should be made so one is always ready
   - when brain is in slurry, there should be no bubbles rising from slurry, as this can crack brain
3. When brain is removed, dip a slide into slurry
4. Put a spot of oct embedding medium on a slide and quickly dip into slurry.
5. Set brain onto a spot of oct, and immerse for 1 minute into slurry.
6. Put brain that's now embedded in oct into a scintillation vial full of slurry, and put into freezer until it is ready to be cut on cryostat.

Cutting

1. Turn on first thing in the morning. Takes 30-45 min. to get to -18°C.
2. Embedding - embed on chuck; be careful not to let brain thaw.
   a. lay on an even base of oct on chuck and let it freeze

b. push in timer freezer on left of cryostat, this makes platform much colder so embedding goes faster, lasts for 5 min. after you set it.
c. transfer brain from scintillaton vial to the cap of the vial, which should be filled with *methyl butane* - important to keep brain cold.
d. take brain from cap when base is ready and trim the bottom flat so it will sit evenly - use razor blade.
e. put a spot of oct onto frozen base, and using tweezers position the brain olfactory bulbs up onto the base and into the spot of unfrozen oct.
f. using Histofreeze (be sure the nozzle is set on low) carefully spray the oct holding the brain till frozen. Hold bottle upright and use tube to direct spray downward. Don't spray oct hard or you will push the oct around and get bubbles or uncovered spots of the brain.
g. slowly and carefully cover the whole brain with oct. The better you do this step, the easier the brain will cut. Use Histofreeze after adding each layer, and be sure it is frozen before adding another. Freeze the oct quickly after you put it on the brain so the brain doesn't thaw.
h. after brain is completely covered, carefully trim around the sides.

Note: The brain should be oriented on the chuck so when it is cut the blade comes from underneath, or proceeds ventrally to dorsally (a). This way the molecular layer on the top of the brain may be preserved from cutting artifacts, or from the sections rolling. This is oriented by facing the hole in the arm of the chuck out towards you when it is in the platform. This hole is where the screw goes when the chuck is attached to the mount on the cryostat (b). This hole should face you, and the brain should be placed ont he chuck with the olfactory bulbs up and the dorsal (upper) side facing you (c). When the brain is mounted in the cryostat, the ventral (bottom) side will be cut first and the dorsal last.

3. Mount brain in cryostat, using screw to tightly secure it. The holder the chuck fits into should be positioned straightly before proceeding, so if you are on the 2nd or 3rd brain remember to realign it.
4. The top should be trimmed, and the brain positioned correctly to the blade.
    a. to move the brain back and forth manually, the handcrank advancement mechanism must be disengaged. By the black wheel in the back of the cryostat there is a switch that disengages it. If you turn the wheel and there is a screeching noise, the handcrank is not disengaged. When disengaged *the handcrank will move the mount up and down*, but not forward the amount it is set (for us, 8μm).
    b. use the wheel to move the brain foward manually, and the handcrank to move the brain up and down, and in this way the top of the oct can be trimmed off to a flat surface. The flat surface should be trimmed so that it is perpendicular to the blade, you should reposition the mount in the up and down direction to achieve this.
    c. once the olfactory bulbs (in deskulled brains) or a recognizable part of the brain (in brains with skull) is found the brain can be oriented right to left. The brain can be repositioned and trimmed so the right and left hemispheres are equal in size. For this trimming, the handcrank should be

re-engaged so the brain only advances 8μm, in order to preserve as much of tissue as possible.

d. before proceeding to taking series, the oct around the brain should be trimmed into a square, as it cuts cleaner. The mount should be locked into a position above the blade so it doesn't move while you trim it with a razor blade. This is done with a knob on the handcrank. When the knob is on the bottom of the circle, it will push into a hole and lock the mount into position.

5. Cutting - use subbed slides
    a. take 5 sections onto slides and then cut 5 discarded sections. The sections are transferred from the blade to the slide by pressing the slide to the blade in a smooth motion.
    b. the slides should be marked with strain, litter, sex, age, position in uterus, and slide number and letter. The slides start as 1a, 2a, 3a, 4a, 5a, the second set is 1b, 2b, 3b, etc.
    c. when taking sections start with all the slides to the left on the metal platform on top of the cryostat. As you put a section onto the section, place it to the right. After 5 sections are taken all the slides should be to the right. Then move them all over to the left and start over. This removes any confusion as to which slide you are on, or which ones have sections and which don't.
    d. while you are cutting, you may want to clean the blade. Use 100% alcohol, and make sure it has evaporated before you touch the roll bar to the blade. Never get any solution on the roll bar. It may be wiped with a tissue or your fingers, and it can be sprayed with Histofreeze.
6. After cutting you *dip the slides in acetone* for a few seconds, and then set them out to dry. Then put them into a slide box with a desiccator packet and put in freezer or stain immediately.

Troubleshooting

Rolling:

If sections are rolling up, the roll bar may be positioned wrong. It is repositioned by loosening the screw on top, and using the side screw to move it back and forth. The roll bar should be even with the top of the blade, and can be gauged by moving a finger back and forth across the roll bar and the blade.

Mushing:

1)Always keep track of the orange light to the right of the indicator panel. When it is on, the temperature in the chamber is above what it is set for. It is OK if it's on for a little while, but if the sections start to crinkle or mush the chamber may be too warm, so close the lid and let the chamber cool.

2.) If sections continue to mush you can spray the brain or the blade with Histofreeze. Check for bubbles or holes in the oct as well. If there is a bubble in the oct it can be filled by placing some oct on your finger and pressing it up to the hole. Let the oct of the mount melt a little so it will hold the fresh oct better, and then freeze. Also the blade may need to be cleaned. You may just need to trim the bottom of the oct, as a ragged leading edge can affect cutting.

Tearing:

If there is tearing it may be something mentioned above, or a nick in the blade. The blade can be loosened and moved.

Cracking:

If the sections crack or roll up from the bottom, the mount is too cold. You can warm up the mount by gently placing a thumb or finger up against it for a second. DO NOT RUB! Just let the warmth from your digit gently melt it a little. Don't let it get too warm though, or it will start to mush. If the sections continue to roll you may need to trim the bottom of the mount. You should trim periodically while you cut, as the smallest square around the mount is usually the easiest to cut. This rolling is a good reason to orient the top of the brain away from the blade.

Sticking:

If the sections stick to the roll bar, try to wipe it off with a tissue, or with your finger. You could also give it a blast with the histofreeze. The mount may also be too warm.

<u>Notes</u>: Basically you need to keep the right temperature and so you either warm things up or cool things down. You can also try to vary your cutting speed or rhythm but this you will learn how to do with practice.

IMMUNOGLOBULINS AND C3-EMBRYOS

Sacrifice and fast-freeze

To preserve mother and fetus, follow 1-4 only; to preserve only fetuses, follow only 1,3 and 5.

1. Anesthetize pregnant mother and make abdominal incision, revealing pups.
2. Insert butterfly needle in left ventricle, cut right atrium, initiate saline perfusion
3. Remove pups, decapitate them, and immerse their heads immediately in Methyl Butane which, after having dry ice added, has come to a slow “boil”. Allow heads to fix in cold Methyl Butane for 1minute. Carefully remove heads from the dry ice slurry and store them in a portion of the slurry in a scintillation vial at -20°C (in freezer or cryostat).
4. Initiate fixative perfusion in mother
5. Sacrifice mother by either cutting diaphragm or overdose of ether.
   •immerse her head in Bouin’s for paraffin
   •extra pups also in Bouin’s

Sectioning

Mount brain on cold chuck using OCT medium. Be certain not to allow any thawing of the tissue; use Histofreeze generously to keep OCT medium and brain tissue constantly frozen.

Mount 6-8μm sections on subbed slides and allow them to air-dry until all sections are mounted and opaque. Quick-fix in room temperature acetone for 5 seconds and air-dry again. Initiate immunohistochemistry unless slide must be stored. In this case, keep slides in airtight boxes with Drierite packets at -20°C overnight or -70°C for longer periods.

Immunostaining

Note: Each rinse consists of two 5-minute washes of the slides in whichever buffer. Each antibody incubation and rinse is performed on the agitator.

Transfer slides from either -20°C or -70°C to ice cold acetone and postfix for 10 minutes. (Material coming directly from sectioning may bypass the acetone postfix.)

1. Rinse slides in PBS.
2. Soak slides in 1% H2O2 in methanol to block endogenous peroxidase activity for 20 minutes. (9 ml 30% H2O2 in 300 ml methOH).
3. Rinse slides in PBS.
4. Soak slides in 5% normal rabbit serum in PBS for 10 minutes to block nonspecific binding of antisera to tissue. (15 ml NRS in 300 ml PBS).

5. Soak slides in 1° antiserum for 2 hours at room temperature.
   - C3 (1/b2500)-200μl Ab in 100ml diluent - Ig fraction Goat Anti-Mouse C3 #224085
   - Igs (1/500)-200 μl Ab in 100 ml diluent - IgG fraction Goat Anti-Mouse
   - Immunoglobulins (IgA, IgG, IgM) #0211-0231
   - IgG (1/250)-400 μl Ab in 100 ml Ab diluent - Goat anti-Mouse IgG #0611-0081
6. Rinse slides in PBS
7. Soak slides in 2° serum for 2 hours at room temperature.
   - Rabbit immunoglobins to goat immunoglobins #035 7228
   - The dilution used is 1/50 (6ml Ab in 300 ml Ab diluent).
8. Rinse slides in PBS.
9. Soak slides in 3° antiserum for 2 hours at room temperature.
   - Peroxidase anti-peroxidase goat PAP #0100-1221
   - The dilution used is 1/500 (600 μl Ab in 300 ml Ab diluent).
10. Rinse slides in PBS.
11. Rinse slides in Tris Buffer (0.05M, pH 7.60)
12. CAUTION!: Use hood and gloves for development!

Develop slides in .05% DAB and .005% H2O2 in Tris until desired staining is achieved. (2ml 10% DAB and 60μl 30% H2O2 dissolved in 400 ml Tris buffer). To stop development, transfer slides to fresh Tris buffer.

Mounting

1. Rinse slides in Tris buffer
2. Soak slides for 3 minutes in 0.5% cupric sulfate/saline
3. Rinse slides in tap water for 3 minutes
4. Counterstain- Immerse slides in Methyl Green/Alcian Blue for 3 minutes
5. Rinse slides with tap water 2x 2 minutes each
6. Rinse slides with 95% ethanol for 3 minutes
7. Rinse slides with 100% ethanol 2x 3 minutes each
8. Immerse slides in fresh xylene for 5 minutes
9. Coverslip with Permount

REMOVING BRAINS FROM SKULLS

1. Remove lower jaw by breaking mandibles with big scissors.
2. Remove loose tissue from back of neck and the top of the cerebellum.
3. Remove the tissue on underside up to hard palate. Don't take too much from sides or you will go into cortex. Tissue in a ring around the beginning of the spinal cord should come off underneath and follow up around to the top, exposing plate over cerebellum.
4. Chip away plate over cerebellum with small bites using the Rongeur's. Note that the dark horizontal line on skull is where cortex begins.
5. When you have removed plate up to where the cortex begins (dark line) carefully work the Rongeur's under the bone and chip off a small piece. Do this to the right side, in the middle you may take off cortex. Using this opening work up under the bone again and remove the rest of the skull on the right side, except the small bits at the top that don't pull away.
6. Remove the skull to the left by sliding Rongeur's under bone from the side, and pulling up.
7. Chip off the small pieces of bone over prefrontal cortex, and carefully remove skull from the sides.
8. Insert Rongeur's into eye sockets and squeeze out olfactory bulbs. Then you should be able to pull the brain away from the remains of the head.

CELLOIDIN

Embedding

5 day dehydration procedure - Start on Monday

Mon:	dH_2O
Tues:	80% alcohol
Wed:	95% alcohol
Thurs:	100% alcohol
Fri:	solution of 50% ether/50% alcohol 100%
Fri evening:	3% celloidin; stays for 1 week

After 1 week, embed in 12% in a plastic boat (1-2 days). When hard let sit in 80% for at least a few hours, then cut from blocks and mount.

Recipes

Make solution of one half ether and one half 100% alcohol for a total of 1500 mls. Dissolve celloiden in this, turning often to mix. Soften celloiden first in the 100%.

3% -45 g	smaller 3%	22.5g in 375s
6% - 90g	smaller 12%	30 g in 125s
12% - 180g		

PARAFFIN PROCESSING

Embedding

Take brains through the following series:

H_2O	4 hours
50% ethanol	4 hours
70% ethanol	overnight
95% ethanol	1 hour
95% ethanol	2 hours
100% ethanol	2 hours
100% ethanol	3 hours
Cedarwood oil #1	2 days, or until sunken - can be stored in cedarwood oil
Cedarwood oil #2	2 days
Cedarwood oil #3	2 days
Toluene	1 hour - should not stay overnight
Toluene	1 hour
Paraffin #1	2 hours - oven 54-56°
Paraffin #2	2 hours
Paraffin #3	2 hours

Embed in fresh paraffin

Let block harden at room temperature. Store in fridge for long periods.

Sectioning

Paraffin sections are cut on a rotating microtome at the deisred thickness and transferred using a brush to a 45°C water bath containing a teaspoon of gelatin. Sections are then mounted onto subbed slides, air dryed, and stored or stained.

Hydration of Paraffin Sections/Staining

Take slides through the following series:

Before xylene bath, place sides in warmer and heat to 60°C for 30 min.

Paraffinized xylene	5 min.
Fresh xylene	5 min.
Fresh xylene	5 min.
100% ethanol	3 min.
100% ethanol	3 min.
95% ethanol	3 min.
95% ethanol	3 min.
H_2O	3 min.
H_2O	3 min.

Stain as desired

ANTIBODY INFORMATION

Cooper Biomedical - Cappel Worthington

Pooled Ig's:

Goat Anti-Mouse Immunoglobins (IgG, IgA, IgM)
Cat #0211-0231
AB Protein - 5.0mg/1ml
Comes as a liquid. Aliquot into 50μl and store at -20°C

C_3:

Goat Anti-Mouse C_3
Cat #0211-0601
AB protein - 4.0mg/1ml
Comes as a liquid. Aliquot into 50μl and store at -20°C

IgA:

Goat Anti-Mouse IgA (affinity purified)
Cat #0611-3141
AB Protein - 1.0mg/1ml
Comes as a liquid. Aliquot into 50μl and store at -20°C

IgG:

Goat Anti-Mouse IgG
Cat #0611-0081
AB Protein - 1.0mg/1ml
Comes as a liquid. Aliquot into 50μl and store at -20°C

Peroxidase Anti-Peroxidase:

Goat Anti-PAPCat #0100-1221
Restore with 1ml ddH_2O
Store in 20μl aliquots at -20°C

Address

Cooper Biomedical, Inc. (Cappel Worthington)
Scientific Division
One Technology Court
Malvern, PA 19355

1-800-523-7620

Dako Corporation (Dakopatts)

Rabbit Anti-Goat:

Cat #7228
Restore with 2ml ddH_2O
Store in 200μl aliquots at -20°C

Rabbit Anti-Mouse:

Code #Z259
Do not freeze.

Mouse PAP

Code #B650
Restore with 1.0ml ddH_2O
Do not freeze.

Address

Dako Corporation
22 North Milpas St.
Sant Barbara, CA 93103

1-800-235-5743, 5763

Inc & Incstar (Immunonuclear Corporation)

Rabbit Anti-Rat IgG

Cat #014
AB Code PF785
Restore with 200 μl ddH_2O
Store at -20°C.

Rabbit Anti-Mouse VIP

Cat #20077
Restore with 100μl ddH_2O
Store at +4°C for 30 days

Rabbit Anti-GFAP:

Cat #22522
Do not freeze
Comes as a liquid — use at 100%.

Address

INC
1951 Northwestern Ave.
PO Box 285
Stillwater, MN 55082

1-800-328-1482 Mary Frick - immuno specialist

ICN Biomedical

F.I.T.C. Conjugated Anti-:

prepared in goat

Rabbit IgG

Cat #65-173
Comes as aliquid
Store at -20°C.

Address

ICN
330 Highland Ave.or PO Box 1200
Costa Mesa, CA 92626
Lisle, IL 60532

714-545-0113

Vector Laboratories

Biotinylated Anti-Rabbit IgG:

goat origin, H + L, affinity purified
Cat #BA 1000
Protein (Biotin IgG) = 1.5mg/1ml
Restore with 1ml ddH_2O
Store at +4°C

Address

Vector Laboratories, Inc.
30 Ingold Road
Burlingame, CA 94010

415-697-3600

Sternberger-Meyer Clono Pap

Rat Peroxidase Anti-Peroxidase:

Cat #RC 15
rat origin monoclonal
Store at -20°C
Aliquot

Address

Sternberger-Meyer Immunocytochemicals, Inc.
3739 Jarrettsville Pike
Jarrettsville, MD 21084

301-557-7582

Boehringer Mannheim

68, 160, 200 kD Neurofilament:

Restore 40 μg in 2ml ddH_2O
Cat #81432b
Do not freeze
Store a +4°C.

Address

Boehringer Mannheim Biochemicals
PO Box 50816
Indianapolis, IN 46250

Order: 800-262-1640
Technical Service: 800-428-5433

Pelfreeze Biologicals

MAS 050 Rat IgG Anti-:

Cat #MAS 050C

Mouse T-lymph

Monoclonal a Thy-1
Ig Content - 2mg/ml
Comes as a liquid. Store at -20°C in 20 μl aliquots

Rabbit Anti-Rat IgG:

Ca #11690-2
5 ml

Address

Pelfreeze Biologicals
PO Box 68
Rogers, Arkansas 72756

1-800-643-3426

Reichlin — N.E. Medical/Tufts

Somatostatin:

Restore to make 50μl
Dissolve in NaPb. Dilute 1/100.
Store at -20°C. 180μl aliquots can be thawed and refrozen a few times.
Remove from freezer and dilute 1/60 in antibody diluent.

Cambridge Research Biochemicals

Neuropeptide Y:

Cat #295
Rabbit origin — neuropeptide tyrosine
Reconstitute with 100 μl ddH_2O
Store unopened vial at -20°C
Store frozen aliquots at -20°C
Avoid thawing, refreezing

VIP:

Cat #CA-340
Rabbit origin
Restore with 100μl ddH_2O
Store unopened vial at -20°C; Store frozen aliquots at -20°C
Avoid thawing and refreezing

Address

Cambridge Research Biochemicals, Ltd.
Button End, Harston
Cambridge CB2 5NX ENGLAND

0223-871674

Gibco Labs

3175 Staley Rd.
Grand Island, NY 14072
1-800-828-6686

Janssen Life Sciences

1) orders, shipments
40 Kingsbridge Rd.
2) tech assist, prices, misc.
Piscataway, NY 08854
3) billing, credit
1-800-624-0137

Miles Scientific (of Miles Labs)

30 West 475
North Aurora Rd.
Naperville, IL 60566
1-800-348-7465

American Histology
7746 Lorraine Ave., Bldg. 208
Stockton, CA 95210
209-477-5109

Immunogold Info:

Constance diFiglia with Kay Fields
Department of Neurology
Bldg. Forch, Rm. 147
Albert Einstein College of Medicine
Bronx, NY 10461
212-430-3163

Dr. Kuljis on computer [KULJIS@YALEMED]

CHEMICAL SOURCES

Sigma	Fisher	Other
Alcian Blue	Ammonium Hydroxide	Acid Fuschin — MCB
Calcium Chloride	Buffer Solution, pH=7.00	Blueing Reagent - Lerner Labs
Citric Acid	Chromium Potassium Sulfate	Cedarwood Oil — MCB
Congo Red	Collophonium	Cresyl Violet — Chromo-Gesellschaft-Roboz Surgical
Cupric Sulfate	Ferric Ammonium Sulfate	Eosin Solution — Richard Allen
DAB	Formalin	Glutaraldehyde — TAAB
Dimethyl Chloride	Histofreeze	Liquid Nitrogen — 7th floor radiology 4th floor dish room
Ferric Chloride	Hydrochloric Acid, Conc.	Normal Serum — Vector Laboratories
Ferrous Sulfate	Hydrochloric Acid, 1N	OCT — Tissue-Tek
Gelatin (60 bloom)	Mercuric Chloride	Paraffin — Tissue Prep
Glacial Acetic Acid	Methanol	Potassium Chloride, Sat'd — Corning
Glycerol	Methyl Butane	Sodium Phosphate, Monobasic — Electron Microscopy Sciences
D-(+)-Glucose	Paraformaldehyde	
Glycerol Jelly	Parlodion Strips	
Hematoxylin Solution	Permount	
Iodine	Picric Acid, Saturated	
L-lysine	Potassium Dichromate	
Light Green SF Yellowish	Potassium Ferricyanide	
Lithium Carbonate	Sodium Acetate	
Magnesium Chloride	Sodium Borate	
Magnesium Sulfate	Sodium Hydroxide pellets	
Methyl Green	Sodium Hydroxide, 1N solution	
Oil Red O	Sodium Phosphate, Dibasic Heptahydrate	
Orange G	Sucrose	
Oxalic Acid	THAM	
Phosphotungstic Acid	Thionin	
Ponceau 2R	Xylenes	
Potassium Hydroxide		
Potassium Iodide		
Potassium Permaganate		
Sodium Chloride		
Sodium Citrate		
Sodum m-periodate		
Sodium Phosphate, Dibasic Anhydrous		
Sodium Sulfate		
Triton X-100		

GLOSSARY

Antibody - a serum protein that is formed in response to exposure to an antigen, and reacts with high specificity with that antigen to form immune complexes in vivo and in vitro.

Antigen - a substance that appears foreign to the host which stimulates formation of a specific antibody and which will react with the antibody created.

Avidin - an egg-white glycoprotein that will bind non-immunogenically with four molecules of biotin. Avidin binds biotin with very high affinity allowing this method to have greater specificity than other direct or indirect methods.

B-Cell - the lymphocytes which manufacture antibodies after exposure to an antigenic compound.

Conjugated Antibody - when a marker is chemically linked to an antibody molecule. This can be a flourescent label such as flourescein and rhodamine, or an enzyme like horseradish peroxidase, or a linking molecule like biotin.

Direct Method - In immunohistochemistry, a specific or primary antibody is conjugated to a molecule such as an enzyme. A chromagen that reacts with the enzyme is added to create the colored end-product.

Donor Species - the species in which the antibody is raised.

Endogenous Peroxidase - the peroxidase enzyme that is present mostly in red and white blood cells. It is necessary to block this endogenous peroxidase with a dilute solution of H_2O_2, or it will react with the chromagen and impart a false signal.

Epitope - a portion of a molecule that can induce specific antibody production. Otherwise known as antigenic determinants. A single antigenic molecule can contain several epitopes.

Indirect Method - an unconjugated primary antibody binds to the antigen in the section. Then, to visualize this complex, a conjugated or bridge secondary antibody that will bind to the primary antibody is incubated with the section. A chromagen can be added after this step or after the binding of a tertiary antibody. The secondary or tertiary antibody will contain an enzyme that will react with the chromagen to create a colored end-product. Both types of these indirect methods enable the initial signal to be augmented.

Monoclonal Antibody - A monoclonal or single cell antibody is a fraction of antibody produced by a single B-cell line (known as a clone) so all of the antibodies are generated to a single epitope.

Negative Control - Omit the primary antibody and in place of it use an identical dilution of non-immune (not exposed to the antigen in question) serum from the same animal species as the primary antibody.

Non-Specific Staining - when a colored-end product results from any reaction other than the antigen-antibody-enzyme complex, otherwise known as background staining.[1]

Peroxidase Anti-Peroxidase (PAP) Complex - an antibody that will bind to peroxidase molecules and a peroxidase enzyme conjugated antibody which are bound together.

Polyclonal Antibody - Each B-cell can form antibodies to only one epitope. A group of B-cells can produce antibodies against several different epitopes. The resulting antibody fraction is called a polyclonal (many cells) antibody.

Positive Control - In order to ensure that the staining is the result of the antibody-antigen complex we desire, we need to evaluate to what antigen the antibody is binding. A positive control would involve incubating the antibody with a section and a quantity of the known antigen. The known antigen should bind to all of the antibody so that no antibody s free to bind the antigen in [2]the section. The section would then be blank after development with a chromagen.

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[1] This staining can be of three types:

1. Background - when in some way the chromogen is bound to something other than the antigen in question.
2. Cross reactivity - an antibody reacts with another epitope.
3. Endogenous Peroxidase.

[2]