CHRONOAMPEROMETRY

Ascorbic Acid (FW 176.1)

STOCK	20 mM		0.18 g	in 50 ml ddH_2O
DILUTION	250 µM	from 20 mM	500 µL	in 40 mL PBSlite

NOREPINEPHRINE (FW 169.2)

STOCK	200 µM		.0017 g	in 50 ml ddH_2O
	500 µM		.0042 g	in 50 ml ddH_2O
	2 mM		.0169 g	in 50 ml ddH_2O
DILUTION	2 µM	from 200 µM	400 µL	in 40 mL PBSlite
	2 µM	from 500 µM	160 µL	in 40 mL PBSlite
	2 µM	from 2 mM	40 µL	in 40 mL PBSlite

DOPAMINE (FW 189.64)

STOCK	200 µM		.0019 g	in 50 ml ddH_2O
	500 µM		.0047 g	in 50 ml ddH_2O
	2 mM		.0190 g	in 50 ml ddH_2O
DILUTION	2 µM	from 200 µM	400 µL	in 40 mL PBSlite
	2 µM	from 500 µM	160 µL	in 40 mL PBSlite
	2 µM	from 2 mM	40 µL	in 40 mL PBSlite

NAFION-COATING PROCEDURES
Spin the Wheel of Nafion Roulette

1) Freund RK, Gerhardt GA, Marshall KE, & Palmer (2003) Alcohol 30:9-18

Each microelectrode was coated with Nfrion it enhance sensitivity and selectivity to norepinephrine:

1) Fibre-length 150-200 μm
2) Before coating, high-speed, 5-Hz chonoamperometric recording were carried out in 0.1 M PBS for 10 min to test the patency of the glue interfaces and to determine the general recording characteristics for each microelectrode.
   - typical background oxidation currents are -1.5 to -6 x 10^{-9}A
3) Rinse microelectrode tip in distilled water and dry at **200°C for 5 min**
4) Immerse in Nafion (5%)
   - give 2-3 coats and dry for 5 min at **200°C** after each immersion

"The improved high-temperature (**200°C**) drying procedure for the Nafion coating has been shown to increase the temporal performance and selectivity of the microelectrodes for measures of norepinephrine."

2) Yavich L, Jakala P, & Tanila H (2005) J Neurochem 95:641-650

1) Fibre-length 250-300 μm
2) Dip electrode in Nafion (5%) solution 3x
3) After each dip allow the electrode to dry at 20 for 1 minute

PBS 0.05 M
2L; sodium phosphate mono, 2.8g; sodium phosphate Dib, 11.34; NaCl, 11.68

AA
0.18g/50ml = 20 mM

L-Glu
0.169g/50ml = 20mM
0.084g/100 ml = 5mM

KCl 70mM in a 100ml
0.52g; KCl, 0.46g; NaCl, 0.037g $CaCl_2$

KCl 120mM in a 100ml
0.894g; KCl, 0.17g; NaCl, 0.037g $CaCl_2$

DA (200uM) for CNS ejection
10 ml final volume saline
add
100 ul of AA 20 mM
1 ml of DA 2mM

pH solution to 7.2-7.4

Calibration Solutions

A) Ascorbic Acid

Chemical Location: On shelves near balances.

Recipe: Chemical/ conc.	MW(g/mole)	Grams	Solvent/ volume
20 mM L-Ascorbic Acid	176.1	**0.18**	**50 mL** ddH_2O

Container: Store solution in 100 ml glass bottle.

Storage: Make fresh daily

Special instructions: Solution turns yellow when it oxidizes.

B) Dopamine (3-Hydroxytyramine)

Chemical Location: DA is on the shelf. Perchloric acid ($HClO_4$) is in Corrosives cabinet.

Recipe: Chemical/ conc.	MW(g/mole)	Grams	Solvent/ volume
2 mM Dopamine•HCl	189.6	**0.038**	**99 mL** ddH_2O **1 mL** $HClO_4$

Container: Store solution in 100 ml glass bottle.

Storage: At low pH this solution lasts months.

Special instructions: Make solution in 100 ml volumetric. If it turns color, dispose of it

Dopamine:

200uM Dopamine in Saline w/ Ascorbate: for 10 ml final volume, first add about 5 ml of filtered physiological saline. Then add 50 micro liters of 40 mM Ascorbic Acid. Add 1.0 ml of 2.0 mM Dopamine, and the saline to volume. Then add 0.1 M NaOH, a few micro liters at a time, until a pH of 7.2-7.4 is attained. Do not go over pH 8.0; if you do, Dopamine will auto-oxidize, and you will have to start over. You can use the ascorbic acid to bring the pH down if you go past 7.4

500uM Dopamine in Saline w/ Ascorbate: for 10 ml final volume, first add about 3ml of filtered physiological saline. Then add 50 micro liters of 40 m Ascorbic Acid. Add 2.5 ml of 2.0 mM Dopamine, and the saline to volume. Then add 0.1 M NaOH, a few micro liters at a time, until a pH of 7.2-7.4 is attained.

2mM Dopamine Stock in 0.01M Perchloric Acid

DA F.W. = 189.6 g/mole → aka "3-Hydroxytyramine" on bottle 1/30/07 KW

To make 100ml, weigh out 0.0379g DA, and add to 50ml DIUF H2O in a volumetric flask. Add 10 ml of 0.1 M perchloric Acid, then fill to the volumetric mark with DIUF H2O. Mix with a magnetic stir bar, and transfer to an amber 100ml I-Chem bottle.

- make this ~~in~~ completely in .1M perchloric Acid not just 10ml 6/22/06 FCT

PBS Lite

0.05 M Phosphate Buffered Saline

Recipe for 2 and 4 liter prep

1. Transfer ~ 1800ml of deionized, filtered H20 to a 2 liter Nalgene container. This is for 2 liter prep.
2. For a 4 liter prep just double the deionized H20 to ~3600ml into a 4 liter Nalgene container.
3. Add, while mixing with a stir bar:

Chemical	F.W.	Amount, Grams 2liter / 4liter
SODIUM PHOSPHATE (NaH2PO4) (MONOBASIC, MONOHYDRATE)	137.9	**2.8 / 5.6**
SODIUM PHOSPHATE (Na2HPO4) (DIBASIC, ANHYDROUS)	141.9	**11.34 / 22.68**
SODIUM CHLORIDE (NaCl)	58.44	**11.68 / 23.36**

4. Stir until all chemicals are dissolved, Transfer to a 2- liter or 4 –liter volumetric flask, and add dH20 to the volume mark.
5. Filter through a 0.2 micron nylon filter.
6. Test the pH; it should be 7.4

NOTE: Filtered PBS has a long shelf life.
Totals: 0.10 moles phosphate/2liters=0.05M
0.20 moles NaCl/ 2 liters = 0.10M
Molarity = 0.15M = 150mM (~300 mOsm)

Plating Bath

The plating bath is 1 M HCl, saturated with NaCl. The recipe below is for making 500 ml of 1 M HCl. To make the plating bath add about 60 ml HCl to a 100 ml beaker. Continue to add NaCl (stirring) until a 1/4" to 1/2" thick layer forms on the bottom.

Chemical Location: NaCl is on the shelf near balances. HCl is in the Corrosive cabinet.

Recipe:

Chemical/ conc.	Volume	Solvent/ volume
1M HCl	**45 ml** HCl	**455 ml** ddH_2O

Container: Store HCl solution in 1 L glass bottle (on shelf near plating baths).

Storage: Long shelf life.

Special instructions: See above. Also, stir the solution prior to plating any reference electrode. This redistributes the NaCl in solution. If the solution evaporates, add more acid and salt.

Intracranial Drug Application

A) 70 mM Potassium (with NaCl and $CaCl_2$)

Chemical Location: In Cabinets above balances.

Recipe: Chemical/Conc.	MW(g/mole)	Grams	Solvent/Volume
			100 ml ddH_2O
70 mM Potassium Chloride (KCl)	74.5	0.52	
79 mM Sodium Chloride (NaCl)	58.4	0.46	
2.5 mM Calcium Chloride-Dihydrate ($CaCl_2 \cdot 2H_2O$)	147	0.037	

Container: Store solution in 100 ml glass bottle in the refrigerator.
Storage: Solution lasts days to weeks (replace when cloudy or particles are visible).
Special Instructions: Make solution in 100 ml volumetric. Before use, check the pH of this drug at room temperature, ad adjust accordingly. Use a syringe filter when filing the pipette.

B) 120 mM Potassium (with NaCl and $CaCl_2$)

Chemical Location: In cabinet above balances.

Recipe: Chemical/Conc.	MW(g/mole)	Grams	Solvent/Volume
			100 ml ddH_2O
120 mM Potassium Chloride (KCl)	74.5	0.894	
29mM Sodium Chloride (NaCl)	58.4	0.170	
2.5 mM Calcium Chloride-Dihydrate ($CaCl_2 \cdot 2H_2O$)	147	0.037	

Container: Store solution in 100ml-glass bottle in the refrigerator.
Storage: Solution lasts days to weeks (replace when cloudy or particles are visible).
Special Instructions: Make solution in 100 ml volumetric. Before use, check the pH of this drug at room temperature, ad adjust accordingly. Use a syringe filter when filing the pipette.

C) 200 μM Dopamine (with 100 μM Ascorbate)

Chemical Location: Use 2 mM DA calibration solution and 20 mM AA calibration solution and dilute with physiological saline.

Recipe: Chemical/Conc.	Volume
0.9%NaCl (saline)	9 ml
200 μM DA	1 ml (of 2 mM DA)
100 μM AA	50 μl (of 40 mM AA)

Container: Store Solution in scintillation vial.
Storage: Solution lasts only one day. Light Sensitive.
Special instructions: Make and use the solution the same day. *Adjust the pH Carefully*. If you go above pH 8, DA and AA will oxidize and you need to start over. Use syringe filter when filling the pipette.

SECTION 1

Electrochemical Worksheets

The following page shows a sample worksheet used for *in vivo* electrochemical recording in the rat brain. It is useful to keep a written record of the responses as you obtain them, including the filename and TTL# for each signal. After a TTL marked signal comes back to baseline, press the **END** key. A small window will appear, listing some of the parameters (amplitude, reduction/oxidation, ratio, rise time, *etc.*) for that particular signal. Other columns on the worksheet are provided to indicate the brain coordinates (Position) and the pressure ejection parameters (PSI * Sec and Volume). Following an experiment data can be graphed and further analyzed using the Analysis module of the FAST-16 program.

Electrochemistry Work Sheet

Experiment#:__________ WE Type: Multi Single Dagger GEC
Date: / /
Drugs:______________ WE Cal. Factor: E1________________ E2________________
Frequency:_________ Puffer tip diameter: P1________________µm P2________________µm
Applied Pot.__________V WE-Puffer tip dist. WP1________________µm WP2________________µm
Ref. VS Ag/AgCl:______

Rat/Monkey Strain:_____ Sex:_______ Age:______ Wt:______ **Bregma Coord:**
Anesthetic dose: MAX ______________cc MIN________________cc AP ______ ML______
______ ______
______ ______

Position	PSI*Sec	Filename	TTL#	Amp.	Ratio	Volume	$T_r/T_{1/2}$	Comments
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SECTION 2

Reference Electrodes

In order to maintain a stable reference potential, the chemical reaction at the reference electrode must be reversible. The most common reference electrode is a silver wire plated with chloride. The reversible reaction is:

$$Ag + Cl^- \text{ <<----->> } AgCl + e^-$$

For best results, the reference electrode should be re-plated before every experiment. After plating, the reference electrode should be compared to a stable reference obtained from a commercial source. The voltage difference between the silver/silver chloride wire and the stable reference should be less than 15 mV measured in a 3 M NaCl solution.

Plating Procedure:

Plating bath:	1 M HCl solution (prepared in distilled water) *saturated* with NaCl.
Power supply:	0.5-2.0 ampere 9-12 volt DC power supply
Wire:	Two Teflon coated silver wires (0.008" bare)

1) Start by stripping off 4-5 cm of Teflon from one wire and about 1 cm from the other wire. These wires should have the same amphenol connectors that are used on the carbon fiber working electrodes.
2) Connect the wire to be plated (with 1 cm bare silver) to the Anode (+) of the 12 volt power supply.
3) Connect the wire with the longer (4-5 cm) bare end to the Cathode (-).
4) Place both wires in the HCl bath and turn the power on. Watch for bubbles to roll off the counter electrode.
5) Allow the plating process to continue for 15-30 minutes (until wire to be plated turns "silver" in color).
6) Put the newly plated reference electrode in a solution of 3 M NaCl and compare against a stable reference with a multimeter (acceptable range ± mV).
7) Leave the reference in 3 M NaCl until you are ready to position it in your preparation (keep in solution in the dark or they can also be stored dry in the dark).

SECTION 3

Electrode Supplies

The following supplies are used in the manufacture of electrodes. We also list addresses for companies where you can obtain reference electrodes, Nafion, pipette glass, and sticky wax for attaching electrodes to pipettes or other holders.

A. Graphite-epoxy paste
PX grade GRAPHPOXY
Dylon Industries Inc.
7700 Clinton Road, Cleveland, OH 44144
800-237-8246

B. Epoxy
Epoxylite #6001-M
The Epoxylite Corporation
1066 Arundel Ave. ,Westerville, OH 43081

C. Sticky Wax (fusing electrodes & pipettes)
Kerr Brand "STICKY WAX"
SYBRON
Emeryville, CA 94608
For local distributor try dental suppliers

D. Wire
RadioShack
26 gauge; product # 910-4212

Newark Electronics
28 gauge lacquer-coated copper wire
Product #38F388
(consult phone book for local number)

E. Carbon
33 µm fibers carbon monofilament
(1g spool containing 1,320 linear ft for $1,122)
Specialty Materials, Inc.
Lowell, MA 01851
978-934-7599/978-322-1900
www.specmaterials.com

5 µm fibers
Amoco (consult phone book for local number)
Greenville, SC 29601

F. Electrodes and Electrode Accessories
Quanteon Limited Liability Company
105 Parker Lane, Nicholasville, KY 40356
ASTeCC Bldg., Rm. A364, Univ. of Kentucky
Lexington, KY 40536
859-296-9286 or 859-257-2300 x 270
http://cenet/

G. Gold pin connectors
Sager Electronics (distributor)
www.sager.com
Mill-Max Mfg., Corp. (manufacturer)
Product #: 3603-0-07-15-00-00-08-0
1-800-724-3870

H. Nafion - 5% w/v solution
25 or 100 ml quantity #27, 470-4
Aldrich Chemical Co.
Milwaukee, WI 53233

I. Reference Electrodes
Model #RE-5B
Bioanalytical Systems
2701 Kent Ave., West Lafayette, IN 47906
1-800-845-4246

Flexible Reference Model #MF-2079
Microelectrodes Inc
Londonberry, NH 03053
603-668-0692

J. Electrode Glass
Sodalime (4mm O.D.; 0.7mm wall thickness)
(for working electrodes)
Schott Rohrglas @26005
Glass Warehouse
800 Orange/P.O. Box 1039
Millville, NJ 08332

3-Barrel Glass
World Precision Instruments
175 Sarasota Ctr. Blvd.
Sarasota, FL 34240-9258
941-371-1003

Microfilament (1 mm O.D.; 0.58 mm I.D.)
For single barrel pipettes, #6015
A-M Systems, Inc.
11627-A Airport Rd., Everett, WA 98204
206-353-1123 or 800 426-1306

SECTION 4

Troubleshooting

A. Basic Recording Problems

Problem:
I cannot get any waveforms during calibration or electrode verification.

Possible Solutions:
- Power to the system interface is off.
- Electrodes are not connected to the headstage nor have cold solder junctions.
- Gains are not set high enough for an electrode.
- The Analog-to-Digital cable has been jarred loose or the board is not connected.
- Tip of working electrode is not in solution.
- Potential ground loop in the system.

Problem:
The baseline in Acquisition mode, or Calibration mode in chronoamperometric or fast cyclic waveforms continues to rise.

Possible Solution:
- Bad reference electrode.
- Working electrode is wet at the wire/electrode interface.
- Fiber electrode is cracked allowing solution to enter electrode shank; switch to a new electrode.

Problem:
The baseline in Acquisition or the waveforms in Calibration continues to fall.

Possible Solution:
- Bad reference electrode.
- Working electrode is over coated with Nafion.
- The working electrode may need a few more minutes to stabilize.

Problem:
The signal is noisy.

Possible Solution:
- A/C interference from heating pads or lights.
- Poor grounding of computer.
- Bad reference electrode.
- Damaged or bad working electrode.

B. Pitfalls

Pitfall:
Electrode/wire interface gets wet.

Possible Solutions:
- This will increase the background current and contribute to noise.
- Dry the electrode in an oven for 5 minutes at 85°C.

Pitfall:
Cracks in the raising interface will allow solution to contact fibers inside the shank, increasing noise and raising background signals.

Possible Solutions:
- Replace electrode.

Pitfall:
Waveform of chronoampere signal does not have a uniform exponential decay.

Possible Solutions:
- Glue covering carbon fiber surface, particle of glass on tips or broken fiber.
- In all cases, cut tip of fibers to expose new surface.

Pitfall:
Prolonged exposure to brain can cause alterations of electrode surface.

Possible Solutions:
- We do not recommend recording with one electrode for several experiments. Use new electrodes daily if possible.

Pitfall:
Calibrations conducted using medium to high micromolar concentrations of the monoamines can result in adsorption of substances to the surface of electrodes.

Possible Solutions:
- Perform all calibrations of electrodes using solutions ranging from 0.05 to 10 µM. For 5-HT studies, calibrate using 0.05 to 3 µM solutions. After calibrating with 5-HT, remove the electrode from the beaker promptly.

Pitfall:
Non-linear calibration, roll-off of calibration curve, or data sets show a flatline response at the peak of release.

Possible Solutions:
- Calibration gain settings were too high for this type of signal. Electrodes should be recalibrated over a higher range of concentrations and lower gain settings.

Pitfall:
Electrodes are left in the air after insertion into brain tissue, which causes adsorption of blood, and related tissue to electrode. This can cause substantial loss in the apparent sensitivity of the electrode.

Possible Solutions:
- Rinse electrodes with distilled/ deionized water after removing from tissue and store in PBS or artificial CSF.

Pitfall:
Reference electrode is not properly coated with AgCl and will not hold a stable potential.

Possible Solutions:
- Make fresh reference electrodes every day (See Section 3).

Pitfall:
System interrupts will interfere with data acquisition.

Possible Solutions:
- IVEC disables the mouse so mouse movement won't interfere. However the keyboard is still active, so avoid laying anything on the keyboard or pressing keys during calibration and data acquisition.

Pitfall:
Electrode response times are slow.

Possible Solutions:
- Nafion coating is too thick on electrode surface. Replace electrode; Nafion cannot be removed from most electrode surfaces.

SECTION 5

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FIXATIONS AND PERFUSIONS

Stock Solution - 20% paraformaldehyde (PAF)

Do all steps in a fume hood; the PAF powder and fumes from the solution are dangerous to breath.

Weigh out 200g of PAF and add distilled water to a total volume of 800ml. Stir and heat until 65°C and add 1 to 2ml or 1 to 2 Pasteur pipets full of 5N NaOH to clear solution. Then filter solution and Q.S. to 1 liter. Keep refrigerated in an airtight container, stays good for 2 to 3 weeks.

200g - 1L
100g - 500mL
50g - 250mL

"Superfix" – SF

This is a low pH – high pH fix. Two fixatives need to be made and both kept in ice baths.

Prefix: 2% PAF IN 0.1M Na Acetate pH 6.5
For perfusions of 1 to 2 rats 500ml is enough, for more, one liter.

500ml	1 liter
250ml distilled H_2O	500ml distilled H_2O
4.1g Na Acetate	8.2g Na Acetate
50ml 20% PAF	100ml 20% PAF
Add H_2O to 475ml	add H_2O to 975ml
pH to 6.5	pH to 6.5
Q.S. to 500ml	Q.S. to 1 liter

"Superfix": 2% PAF + 0.1% glutaraldehyde in 0.1M Na Borate pH 8.5
For perfusions of one rat.

500ml H_2O
38.14g Na Borate – low heat until dissolved then cool to room temp.
100ml 20% PAF
Add distilled H_2O to 975ml
pH to 8.5
Q.S. to 1 liter

Just before you are ready to perfuse add 4.0ml of 25% glutaraldehyde or 2.0ml of 50% glutaraldehyde to fixative and mix thoroughly.

To perfuse: Anesthetize rat with urethane until sedate. Be sure all fixes are ice cold.

Prefix – 3 min.
"Superfix" – 30 min.
Postfix *in situ* overnight or longer if need be at 4°C.

4% PAF in 0.1M Phosphate Buffer pH 7.4

500ml solution

250ml distilled H_2O
11.12g $Na_4P2O7 \cdot 10H_2O$ pyrophosphate tetrasodium
3.5g $NaH_2PO_4 \cdot H_2O$ sodium phosphate monobasic, monohydrate
100ml 20% PAF
add H_2O to 475ml
pH to 7.4
Q.S. to 500ml

1 liter solution

500ml distilled H_2O
22.24g $Na_4P_2O_7 \cdot 10H_2O$ pyrophosphate tetrasodium
7g $NaH_2PO_4 \cdot H_2O$ sodium phosphate monobasic, monohydrate
200ml 20% PAF
add H_2O to 975ml
pH to 7.4
Q.S. to 1 liter

To perfuse: anesthetize rat with urethane 1.25g/kg until sedate
Saline flush – 3 min.
4% PAF in 0.1M phosphate buffer – 10 min.
Postfix *in situ* overnight at 4°C.

→ use for perfusion, fresh tissue, etc.

Sodium sulfide perfusions

This fixation step is used predominately for Timm stain.

Sulfide "stinky – 0.37% sodium sulfide solution pH 7.2
Make in fume hood

900ml distilled H_2O
11.7g $Na_2S \cdot 9H_2O$ sodium sulfide nonahydrate
11.9g $NaH_2PO_4 \cdot H_2O$ sodium phosphate monobasic, monohydrate
pH to 7.2
Q.S. to 1 liter

Make up 4% PAF or "Superfix" as described previously.

To perfuse: Anesthetize rat until sedate

4% PAF fixation		"Superfix"	
Saline flush	- 3 min.	Saline flush	- 3 min.
"Stinky"	- 5 min.	"Stinky"	- 5 min.
Saline	- 2 min.	Saline	- 2 min.
4% PAF fix	- 10 min.	Prefix	- 3 min.
		"Superfix"	- 30 min.

Postfix *in situ* 1 day or longer at 4°C.

EM Fix - 1.0% PAF + 1.5% glutaraldehyde in 0.1M phosphate buffer pH 7.4

500ml solution

250ml H_2O
11.12g $Na_4P_2O_7 \cdot 10H_2O$ pyrophosphate tetrasodium
3.5g $NaH_2PO_4 \cdot H_2O$ sodium phosphate monobasic, monohydrate
25ml 20% PAF
30ml 25% glutaraldehyde
Add H_2O to about 475ml
pH to 7.4
Q.S. to 500ml

1 liter solution

500ml H_2O
22.24g $Na_4P_2O_7 \cdot 10H_2O$ pyrophosphate tetrasodium
7g $NaH_2PO_4 \cdot H_2O$ sodium phosphate monobasic, monohydrate
50ml 20% PAF
60ml 25% glutaraldehyde
Add H_2O to 975ml
pH to 7.4
Q.S. to 1 liter

To perfuse: Anesthetize rat until sedate
Saline flush - 3 min.
EM Fix - 10 min.
Postfix *in situ* overnight at 4°C.

Immersion Fix For Human Tissue

2% PAF and 2% Acrolein in 0.1M Phosphate Buffer

250ml distilled H_2O
11.0g pyrophosphate tetrasodium
3.5g sodium phosphate monobasic, monohydrate
40ml 20% PAF
pH to 7.4
Q.S. to 490ml
Add 10ml acrolein before immersion fixation

When the tissue has been removed and is ready for immersion fix drop the tissue into the solution and gently rotate the tissue for about 1 to 2 days.

PVP - cryoprotectant/storage for fixed tissue.

250mL PBS
150 g sucrose (30%)
5 g polyvinylpyrrolidone (PVP; 1%)
150 mL ethylene glycol (30%/vol)
Bring to 500 mL with dH_2O

PVP cryoprotectant

PVP cryoprotectant for fixed tissue

250 ml 0.2M PBS (adj to final volume of **500 ml** with $d.H_2O$ yielding 0.1M PBS)

30%	sucrose	150 g / 500 ml of PBS
1%	polyvinyl pyrollidone (PVP)	5g / 500 ml of PBS
30%	ethylene glycol	150 ml /500 ml of PBS

adjust to 500 ml with $d.H_2O$.

DeOLMOS CYROPROTECTION SOLUTION

This solution is used for long-term storage of tissue if there is a possibility of future use of sections. The sections generally look very good but never as good as a fresh immuno run, so that it would be better to do immunocytochemistry asap.

30% sucrose, 1% polyvinyl-pyrrolidone, 30% ethylene glycol in 0.06M sodium phosphate buffer

0.06M phosphate buffer

9.50g sodium phosphate dibasic $Na_2HPO_4 \cdot 2H_2O$ or
5.96g sodium phosphate dibasic anhydrous

2.12g sodium phosphate monobasic $NaH_2HPO_4 \cdot H_2O$

Dissolve into 700ml distilled H_2O

METHOD

300g sucrose
10g polyvinylpyrrolidone
300ml ethylene glycol
700ml 0.06M sodium phosphate buffer

Dissolve sucrose in the phosphate buffer, warming gently with continuous stirring. When cool add polyvinylpyrrolidone and ethylene glycol.
Store at 4°C for up to 12 months.

Buffers

0.2M Sodium Phosphate Buffer

Solution A

Sodium phosphate monobasic $NaH_2PO_4 \cdot H_2O$
27.80g to 1 liter distilled H_2O

Solution B

Sodium phosphate dibasic $Na_2HPO_4 \cdot 7H_2O$
53.65g to 1 liter distilled H_2O

Volume of 0.2M buffer to make pH specific 0.1M phosphate buffer (total volume 200ml)

pH	Volume (ml) 0.2M monobasic	Volume (ml) 0.2M dibasic
5.7	93.5	6.5
5.8	92.0	8.0
5.9	90.0	10.0
6.0	87.7	12.3
6.1	85.0	15.0
6.2	81.5	18.5
6.3	77.5	22.5
6.4	73.5	26.5
6.5	68.5	31.5
6.6	62.5	37.5
6.7	56.5	43.5
6.8	51.0	49.0
6.9	45.0	55.0
7.0	39.0	61.0
7.1	33.0	67.0
7.2	28.0	72.0
7.3	23.0	77.0
7.4	19.0	81.0
7.5	16.0	84.0
7.6	13.0	87.0
7.7	10.5	89.5
7.8	8.5	91.5
7.9	7.0	93.0
8.0	5.3	94.7

G L O S S A R Y

ABC	Avidin-biotin complex
CaBP	Calcium binding protein
CalR	Cal Retin
CC	Cholecystokinin
CHROMO	Chromogratin
DYN	Dynorphin
GABA	Gamma - aminobutyric acid
GAD	glutamate decarboxylase
GFAP	glia fibrillary acid protein
HsAMs	Horse anti-mouse
HSP	heat shock protein
5-H-T	serotonin
m	Mouse monoclonal
NHsS	Normal horse serum
NPY	neuro-peptide
NP	neurophysin
NRbs	Normal rabbit serum
PV	parvalbumin
RbASh	Rabbit anti-sheep
RG	reactive glia
SS	somatostatin
TH	tyrosine hydroxylase
TR	Texas red
VIP	vasoactive intestinal peptide

Antibody Dilutions

REAGENTS FOR IMMUNOCYTOCHEMISTRY

NHsS 10% and NRbS 10%		1ml → 9ml
HsAMs	1:400	5µl → 2ml
Prot A	1:400	10µl → 4ml
RbASh	1:400	5µl → 1 ml
TR Anti-mouse	1:200 1:400	5µl → 1ml 5µl → 2ml
TR Anti-Rabbit	1:200 1:400	5µl → 1ml 5µl → 2ml
ABC	1:1000	1µl A + 1µl B → 1 ml
ABC Elite	1:1000	1µl A + 1µl B → 1 ml

Tris D

ANTIBODIES

GluR2/3 chemicon polyclonal rabbit

Company and Cat. #	Antibody	Made From	Dilutions
Regeneron	BDNF 1:1 – brain derived nerve growth factor	Rabbit polyclonal	1:10,000 1:20,000
Chemicon – BM	BrDU 1:1 - bromodeoxyuridine	Mouse monoclonal	1:1000
Sigma	CaBP 1:1 calcium binding protein or calbindin	Mouse monoclonal	1:100,000
Chemicon Cat. # AB5054	CALR 1:1 calrentinin bound and unbound	Rabbit polyclonal	1:30,000 1:10,000 immunofluorescence
Calbiochem Cat. # PC38-100UL	CFOS (Ab-5) 1:1	Rabbit polyclonal	1:40,000
Chemicon Cat. # AB131	GABA 1:10	Rabbit polyclonal	1:10,000
Boehringer Mannheim (BM)	GAD 65 1:1	Mouse monoclonal	1:5000
Chemicon (Chem)	GAD 67 1:1	Rabbit polyclonal	1:3000
~~BM Cat. # 814369.~~ Chemicon MAB3402 →	GFAP 1:1 – glia fibrillary acidic protein	Mouse monoclonal	1:1000
Chemicon Cat. # MAB377	NeuN 1:1	Mouse monoclonal	1:5000
Peninsula Labs Cat. # IHC7172	NPY 1:2 neuropeptide Y	Rabbit polyclonal	1:30,000
Sigma Cat. # P3171	PV 1:1 parvalbumin	Mouse monoclonal	1:100,000
Privately supplied	PROX 1:1	Rabbit polyclonal	1:30,000
Peninsula Labs Cat. # IHC8004	SS-28 1:1 somatostatin	Rabbit polyclonal	1:8000
Günter's lab Private supply	SS14W1 1:1 somatostatin	Rabbit polyclonal	1:5000
Arc BD Biosci	Arc	Mouse mono	

→ α GFAP Chemicon Mab 3402

16a

Antibody Dilutions

NOTE: *(All antibodies are rabbit except those indicated)*

⟶ = into

PRIMARY ANTIBODY	DILUTIONS	
CCK 4/86 (1:2)	1:5000	1µl ⟶ 2.5ml
	1:6000	1µl ⟶ 3ml
	1:8000	1µl ⟶ 4ml
CCK-8 CRB (1:2)	1:2000	1µl ⟶ 1ml
mCaBP (1:1) (Sigma)	1:10,000	1µL ⟶ 10ml
	1:100,000	1ml (1:10,000) +9ml (TRIS B) 10ml (1:100,000)
	1:500,000	1ml (1:100,000) +4ml (TRIS B) 5ml (1:500,000)
	1:1,000,000	1ml (1:100,000) +9ml (TRIS B) 10ml (1:1,000,000)
CaBP-Baimbridge (1:1)	1:1000	1µl ⟶ 1ml
	1:2000	1µl ⟶ 2ml
CaBP Pike (1:5)	1:5000	1µl ⟶ 2ml
CAL R (1:10)	1:5000	2µl ⟶ 1ml
mcfos (1:1)	1:10,000	1µl ⟶ 10ml
	1:50,000	1ml (1:10,000) +4ml (TRIS B) 5ml (1:50,000)
	1:100,000	1ml (1:10,000) +9ml (TRIS B) 10ml (1:100,000)
mCHROMO (1:1)	1:10,000	1µl ⟶ 10ml
	1:50,000	1ml (1:10,000) +4ml (TRIS B) 5ml (1:50,000)
DYN(1:2)	1:1000	2µl ⟶ 1ml
	1:2000	1µl ⟶ 1ml
	1:4000	1µl ⟶ 2ml

Antibody Dilutions

GABA (Chem) (1:10)	1:5000	2µl → 1ml
	1:10,000	1µl → 1ml
GABA (ETI) Rat (1:1)	1:1000	1µl → 1ml
GABA (ETI) Guinea Pig (1:1)	1:1000	1µl → 1ml
GABA (Sigma) (1:1)	1:5000	1µl → 5ml
GAD 1440 Sheep (1:20)	1:10,000	2µl → 1ml
	1:20,000	1µl → 1ml
	1:25,000	2µl → 2.5ml
	1:50,000	1µl → 2.5ml
GAD (Chem) (1:1)	1:200	5µl → 1ml
GFAP (1:1)	1:5000	1µl → 5ml
mHSP (1:10)	1:20,000	1µl → 2ml
	1:50,000	1µl → 5ml
5-HT (serotonin) (1:2)	1:1000	2µl → 1ml
NPY (1:2)	1:1000	2µl → 1ml
	1:2000	1µl → 1ml
	1:4000	1µl → 2ml
NP E.Z. (1:1)	1:1000	1µl → 1ml
NP-II (1:2)	1:1000	2µl → 1ml
PV301 (1:1)	1:1000	1µl → 1ml
	1:2000	1µl → 2ml
	1:5000	1µl → 5ml
PV302 (1:1)	1:1000	1µl → 1ml
	1:5000	1µl → 5ml
mPV (1:2) (Sigma)	1:10,000	1µl → 10ml
	1:100,000	1ml (1:10,000) +9ml (TRIS B) 10ml (1:100,000)
	1:500,000	1ml (1:100,000) +4ml (TRIS B) 5ml (1:500,000)
	1:1,000,000	1ml (1:100,000) +9ml (TRIS B) 10ml (1:1,000,000)
mRG mouse (1:1)	1:1000	1µl → 1ml

Antibody Dilutions

SS20 (1:2)	1:1000	2µl → 1ml
	1:2000	1µl → 1ml
SS73 (1:2)	1:1000	2µl → 1ml
	1:2000	1µl → 1ml
S-SS309 (1:2)	1:5000	2µl → 5ml
S-SS320 (1:2)	1:5000	2µl → 5ml
mSS-AB mouse (1:1)	1:1000	1µl → 1ml
	1:5000	1µl → 5ml
mSS-8 mouse (1:1)	1:5000	1µl → 5ml
	1:10,000	1µl → 10ml
	1:20,000	1µl → 20ml
mSS-10 mouse (1:1)	1:5000	1µl → 5ml
mTH-AB mouse (1:2)	1:1000	2µl → 1ml
	1:5000	2µl → 5ml
VIP12/86 (1:2)	1:2000	1µl → 1ml

SOLUTIONS

10% Triton X (store in refrigerator)
10ml Triton X
90ml distilled water

BSA - Bovine Serum Albumin 5mg/ml
500mg BSA (Sigma VI) dissolved in 75ml of distilled water
Q.S. to 100ml with distilled water. Freeze 10ml aliquots.

Tris Buffer - 0.1M, pH 7.6
96.96g Tris HCl
22.24g Tris Base
Q.S. to 8 liters with distilled water and check the pH
Store the buffer in an 8-liter carboy at room temperature.

24.24 g Tris HCl for 2L
5.56 g Tris Base for 2L

Tris A - 0.1%Triton X in Tris Buffer
10ml 10% Triton X and 990ml Tris buffer.
Refrigerate.

Tris B - 0.1% Triton X and 0.005% BSA in Tris Buffer
10ml 10% Triton X
10ml BSA (5mg/ml) bovine serum albumin
980ml Tris buffer
Refrigerate.

Tris C - 0.005% BSA in Tris Buffer
10ml BSA (5mg/ml)
990ml Tris buffer
Refrigerate.

Tris D - 0.1% Triton X and .005% BSA in 0.5M Tris Buffer
60.60g Tris HCl
13.9 g Tris Base
10ml BSA (5mg/ml)
10ml 10%Triton X
Q.S. to 1 liter. Check pH 7.6. Refrigerate.

Tris E - 0.005% BSA in 0.5M Tris Buffer
30.30g Tris HCl
6.85g Tris Base
5ml BSA
Q.S. to 500ml
Check pH

7% Agar - For Embedding Tissue

7g agar into 100ml Distilled water
Heat until agar has melted and has a thick consistency
Pour agar into molds and orient tissue with the area to cut facing down

POLYCLONAL RABBIT

IMMUNOCYTOCHEMISTRY PROTOCAL FOR VIBRATOME SECTIONS

The entire process is carried out in plastic compartment boxes. The sections are agitated on a rotator during the entire process.

1. To cut sections on a Vibratome you will need razor blades (either single edge injector-type blades or, for more difficult tissue, the double edge razor blades platinum chrome), a mounting block, an adhesive such as Loctite industrial adhesive or Crazy Glue, a paint brush, forceps and dental wax or a tissue mold. Take the tissue and cut with an industrial single edge razor blade to the desired area making sure that there is a flat surface area to glue the tissue. Blot the tissue dry and add a small drop of adhesive to the mounting block and place the tissue on the adhesive. Rotate the tissue to get an even layer of adhesive and press lightly on the tissue for about a minute. Put the mounting block into the stage of the Vibratome and tighten the chuck. Wash the injector-type razor blade with 95% alcohol to rid of any residue oil and then clamp it to the blade holder of the Vibratome. Add Tris buffer to the bath. To cut the tissue, set the speed to setting 5 and the amplitude to setting 7 depending on your Vibratome. If the tissue is soft and doesn't cut properly, slow down the speed and increase the amplitude. If that doesn't work than embed the tissue in 7% agar. Begin sectioning by first advancing to the tissue and than trimming it until you get a full section. Cut 50 microns section for immunocytochemistry and 25-30μ for light microscopy (LM) or Timms stain. Pick up the sections with a brush just as they're coming off the blade and put them in Tris buffer in the box on bin. Depending on the size of the bins put 4-8 sections in each bin. For LM pick up sections with a brush and mount on treated slides.

2. Wash the sections with Tris buffer for 3 x 5 min. to wash out any remaining fixative. Transfer the sections with a brush to a rinsed box with fresh Tris buffer. During the entire process the sections should be transferred to a clean or washed box when going to the next solution.

3. Wash the sections with 1% hydrogen peroxide (1ml of 30% hydrogen peroxide to 29ml of Tris buffer) for 30 min. Any endogenous peroxidase in the tissue will result in the sections bubbling. Normally there is peroxidase in the tissue, which results in high background staining if not treated with hydrogen peroxide. The hydrogen peroxide binds to the peroxidase resulting in a release of oxygen and the peroxide is then washed out.

4. Wash in Tris for 5 min.

5. Wash in Tris A for 10 min.

6. Wash in Tris B for 10 min.

7. Incubate sections in 10% Normal Goat Serum in Tris B for 1 hour. This is to bind non-

specific antigens.

8. Wash in Tris A for 10 min.

9. Wash in Tris B for 10 min.

10. Incubate sections in primary antibody (made up in Tris B) at the appropriate dilution overnight on a rotator in the cold room. Inadequate movement of the sections results in poor antibody penetration. Make sure there is enough volume of primary antibody to cover the sections because too little will result in light staining. The rule of thumb is 1ml for every section, such as a rat brain coronal section.

Next:

Note: sections can stay in primary antibody for longer than a day but not more than a week.

11. Wash in Tris A for 10 min.

12. Wash in Tris B for 10 min.

13. Incubate sections in biotinylated Goat Anti-Rabbit IgG (1:1000 dilution in Tris B, i.e., 1µl/ml Tris B) for 45 min. at room temperature on a rotator.

14. Wash in Tris A for 10 min.

15. Wash in Tris D for 10 min.

16. Incubate sections in ABC Elite (avidin-biotin horseradish peroxidase complex from Vector Labs) at 1:1000 dilution (1µl A + 1µl B to 1ml of Tris D) for 1 – 2 hours. Allow the complex to form for 30 min. before using.

17. Wash in Tris buffer for 3 x 5 min.

18. The sections are incubated in DAB (diaminobenzidine tetrahydrochloride) solution for an average of 15-30 min. depending on the optimal staining of each antibody and its background. DAB is carcinogenic so avoid breathing the powder; wear gloves and a facemask. Work in a fume hood.

To make DAB add 100ml of Tris buffer to 50mg DAB (individual bottles of preweighed DAB are available from Polysciences). Filter. The DAB from Polysciences does not need to be filtered normally. Add to the filtrate the following:

a) 0.1ml aliquot of glucose oxidase (Sigma: 30mg/10ml distilled water and freeze

aliquots);

b) 0.2ml of aliquot of ammonium chloride (2.0g ammonium chloride/10ml distilled water and freeze aliquots;

c) 0.8ml aliquot of D (+) glucose (2.5 glucose/10ml distilled water and freeze aliquots). Shake the contents together. Before adding sections, allow DAB solution to sit for 5 min. to generate reducing equivalents enzymatically.

19. Remove sections from DAB solution when staining is judged to be optimal and wash in Tris buffer for 3 x 5 min. To dispose of the DAB mix it with a generous amount of bleach to break down the DAB structure. When the mixture is a clear yellow pour it down the drain and run plenty of water afterwards.

20. Mount the sections on gelatin-and- alum treated slides and air dry overnight. Dehydrate in ethanol and xylene and coverslip with Permount. The protocol for dehydration is:

70% ethanol 2-3 min.
95% ethanol 2-3 min.
100% ethanol 2-3 min. 2 changes
Xylene 2-3 min. 2 changes

IMMUNOCYTOCHEMISTRY PROTOCOL FOR MOUSE MONOCLONAL ANTIBODIES ON VIBRATOME SECTIONS

1. Vibratome sections as described previously in main protocol.
2. Wash sections with Tris buffer for 3 x 5 min.
3. Treat sections with 1% hydrogen peroxide (1ml of 30% hydrogen peroxide to 29ml of Tris buffer) for 30 min.
4. Wash in Tris for 5 min.
5. Wash in Tris A for 10 min.
6. Wash in Tris B for 10 min.
7. Incubate sections in 10% normal horse serum made in Tris B for 1 hour. This is to bind nonspecific antigens.
8. Wash in Tris A for 10 min.
9. Wash in Tris B for 10 min.
10. Incubate sections in primary mouse antibody (made up in Tris B) at the appropriate dilution and volume overnight, or up to one week, on a rotator in the cold room.

Next Day:

11. Wash in Tris A for 10 min.
12. Wash in Tris B for 10 min.
13. Incubate sections in biotinylated horse anti-mouse IgG 1:400 (5µl + 2ml Tris B) for ~~45~~ 1 hr. min. at room temperature on the rotator. The kit is from Vector Labs.
14. Wash in Tris A for 10 min.
15. Wash in Tris D for 10 min.
16. Incubate sections in ABC Elite 1:1000 (5µl + 5µl) to 5ml Tris D for 1 – 2 hours. MUST BE AT LEAST 30 Min before use.
17. Wash in Tris buffer for 3 x 5 min.

IMMUNOCYTOCHEMISTRY FOR VIBRATOME SECTIONS WITHOUT TRITON

This protocol is used for GAD antibodies that do not use Triton.

1. Follow steps to hydrogen peroxide.
2. Wash in Tris 2 x 5 min. (or one 5 min. wash and one 15 min. wash with Tris if you are running other sections that are being treated with Tris A).
3. Wash in Tris C for 15 min.
4. Incubate in 10% normal serum for 1 hour of whatever the secondary antibody is made of; i.e., 10% normal horse serum if the secondary antibody is biotinylated HsAMs.
5. Wash in Tris buffer for 5 min. (or 10 min. if running other sections in Tris A).
6. Wash in Tris C for 10 min.
7. Add primary antibody to Tris C at the appropriate dilution and incubate overnight or longer.

Next day:

8. Wash in Tris buffer for 5 min. (or 10 min.).
9. Wash in Tris C for 10 min.
10. Make up secondary antibody either biotinylated Goat Anti-Rabbit 1:1000 or biotinylated horse anti-mouse 1:400 as previously described.
11. Wash in Tris buffer for 5 or 10 min.
12. Wash in Tris C for 10 min.
13. Make up ABC Elite in Tris E at 1:1000 dilution and incubate for 1 – 2 hours.
14. Wash 3 x 5 min. Tris buffer.
15. Follow DAB steps as previously described.

BRIDGING (PAP) AND Avidin-Biotin Complex AMPLIFICATION METHOD

Suggestion from Cynara Y.Ko, Ph.D at Jackson ImmunoResearch Labs (West Grove, Pennsylvania)

1) All steps up to secondary incubation performed as in normal protocol.

2) Incubate in secondary IgG at RT for 1 hour
NOTE: They used goat α-rabbit IgG (1:50)

3) Wash

4) PAP complex incubation at RT for 1 hour (they used rabbit PAP; 1:500)

5) Wash

6) Incubate 1-2 hours in ABC Elite (Vector)

7) Wash

8) Visualize as per normal method (DAB)

DOUBLE BRIDGING WITH
Peroxidase-Anti-Peroxidase (PAP)

Technique modified from Milner and Bacon (1989) J Comp Neurol 281:479-495 for α-tyrosine hydroxylase and rabbit PAP

1) All steps up to secondary incubation performed as in normal protocol.

2) Incubate in secondary IgG at RT for 1 hour
NOTE: They used goat α-rabbit IgG (1:50)

3) PAP complex incubation at RT for 1 hour (they used rabbit PAP; 1:500)

4) Repeat incubation in secondary IgG (1 hr; RT)

5) Repeat incubation in PAP complex (1 hr; RT)

6) Visualize as per normal method (DAB)

BDNF IMMUNOCYTOCHEMISTRY PROTOCAL

DAY 1

1. Rinse 3 x 15 minutes in KPBS.
2. Incubate 0.5% H_2O_2 in KPBS for 30 minutes.
3. Rinse for 5 minutes KPBS.
4. Blocking step – Incubate for 20 minutes in 4.0% normal goat serum in KPBS B.
5. Incubate with primary antibody Rb A BDNF 1:10,000 in 4.0% NGtS and KPBS B overnight. That is 1µl + 10ml 4.0% NGtS in KPBS B.

DAY 2

6. Rinse 10 x 10 minutes in KPBS E.
7. Incubate with biotinylated goat anti-rabbit at 1:1000 in KPBS D for 60 minutes.
8. Rinse 4 x 15 minutes in KPBS C.
9. Incubate in ABC Elite 1:500 final dilution in KPBS A for 60 minutes. Before use mix 20µl A and 20µl B to 1ml in KPBS A and let stand for 30-60 minutes. Immediately before use dilute with 9ml KPBS A for the final dilution of 10ml of ABC Elite complex.
10. Rinse 2 x 10 minutes in KPBS.
11. Rinse 2 x 5 minutes in Tris buffer.
12. Make up 100ml of DAB in Tris buffer and put in DAB substrates (aliquots of glucose oxidase, ammonium chloride and D (+) glucose). Next take out 20ml of the DAB solution and add 50mM NiCl (nickel chloride hexahydrate 237.7 MW) or 237mg to solution. Filter the DAB-NiCl solution and use immediately. Incubate until optimal staining has been achieved.
13. Rinse 3 x 5 minutes in Tris buffer.
14. Mount sections in Tris buffer on treated slides and air dry.
15. Dehydrate in alcohol and xylene and use Permount to coverslip slides.

Stock solutions

BDNF STAINING

1. Stock KBPS

0.5M Potassium Phosphate Dibasic (K_2HPO_4) 174.18 F.W.

87.09g K_2HPO_4
1000ml distilled H_2O

0.5M Potassium Phosphate Monobasic (KH_2PO_4)

34.02g KH_2PO_4
500 ml distilled H_2O

1000ml Potassium Phosphate Buffer Saline (**KPBS**) – 50mM or 0.05M
pH 7.2 – 7.4

80ml 0.5M dibasic buffer
20ml 0.5M monobasic basic
8.77g NaCl
Q.S. 1 liter

2. Make enough KPBS to make all solutions.

KPBS A – KPBS + 1% BSA - also make enough to make all other solutions

10g BSA/ 1 liter KPBS

KPBS B – KPBS + 1% BSA + 0.4% Triton-X (TX)

100ml KPBS A + 4ml 10% TX

KPBS C – KPBS + 0.25% BSA

25ml KPBS A + 75ml dH_2O = 100ml KPBS C

KPBS D – KPBS + 1% BSA + 0.02% TX

100ml KPBS A + 0.2ml 10% TX

KPBS E – KPBS + 0.25% BSA + 0.02% TX

100 ml KPBS C + 0.2ml 10% TX

BROMODEOXYURIDINE (BrDU) PROTOCOL AND DOUBLE LABELING

1. Wash sections in 2x ssc (2x standard saline citrate) solution 2 x 5 min.
2. Incubate sections at 65°C in a water bath, preferably one with a shaker, for 2 hrs in 50% formamide in 2x ssc. Do this in fume hood or keep the water bath covered. If there is no shaker, manually shake sections every 15 min.
3. Wash in 2x ssc for 5 min.
4. Incubate in 2N HCL at 45°C water bath for 30 min. on a shaker or manually shake the sections every 15 min.
5. Wash in 0.1M Boric acid pH 8.5 for 10 min. on shaker. Now all steps should be done on a shaker without the water bath.
6. Wash 4 x 5 min. in Tris buffer.
7. Treat sections in 1% H_2O_2 for 30 min.
8. Wash sections in Tris buffer for 5 min.
9. Wash in Tris A and Tris B for 15 min. each.
10. Treat sections in 10% normal horse serum in Tris B for 1 hr.
11. Wash in Tris A and Tris B for 15 min.
12. Incubate sections in BM (Chem)-MBrDU 1:1000 in Tris B at 4°C on a shaker for 1 to 2 days.
13. Wash in Tris A and Tris B for 15 min. each.
14. Treat sections in Vector Labs biotinylated HsAMs 1:400 in Tris B for 45 min.
15. Wash in Tris A and Tris D for 15 min. each.
16. Treat sections in Vector Labs ABC Elite 1:1000 in Tris D for 1 to 2 hrs.
17. Wash in Tris buffer 3 x 5 min.

18. Make up 100ml of DAB in Tris buffer and put in DAB substrates (aliquots of glucose oxidase, ammonium chloride and D(+)glucose). Next take out 20ml of the DAB solution and add 25mM NiCl (nickel chloride hexahydrate 237.7 MW) or 119mg to solution. Filter the DAB-NiCl solution and use immediately. Incubate until optimal staining has been achieved. You should get black nuclear staining.

19. Wash in Tris buffer 3 x 5 min.

20. Wash in Tris A and Tris B for 15 min. each.

21. Treat sections with 10% normal horse serum in Tris B with an Avidin D blocking agent from Vector labs. Use 4 drops of Avidin D per ml of 10% normal serum. You may need to Q.S. to your final volume when making this solution. Incubate for 1 hr.

22. Wash in Tris A and Tris B for 15 min. each.

23. Incubate sections into second primary antibody with the Biotin blocking agent from Vector Labs. Use 4 drops of Biotin blocking agent per ml of final dilution of antibody. You may need to Q.S. to your final volume when making your antibody dilution. Incubate for 1 to 2 days at 4°C. For Sigma-MCaBP 1:100,000 make up a 1:10,000 initial dilution and then make up from that the final dilution.

Sigma-MCaBP 1:100,000
1µl → 10ml Tris B 1:10,000
1ml (1:10,000) + 40 drops Biotin blocking agent
Q.S. to 10ml in Tris B 1:100,000

24. Wash in Tris A and Tris B for 15 min. each.

25. Treat sections in biotinylated HsAMs 1:400 from Vector Labs for 45 min.

26. Wash in Tris A and Tris D for 15 min. each.

27. Treat sections in ABC Elite from Vector Labs at 1:1000 in Tris D for 1 to 2 hrs.

28. Wash in Tris buffer 2 x 5 min.

29. Wash in distilled water 2 x 5 min.

30. Do the *Novared* stain. *Novared* is from Vector Labs. Prepare the substrate solution as follows:

    To 5ml of distilled water add 3 drops of Reagent 1 and mix well.
    Add 2 drops of Reagent 2 and mix well.
    Add 2 drops of Reagent 3 and mix well.
    Add 2 drops of the Hydrogen Peroxide solution and mix well.

    Incubate sections with the substrate at room temperature until suitable staining occurs. This may take 5-15 minutes; anything longer may increase background staining. The stain should appear brick red. The BrDU/CaBP double labeling should appear as a red neuron stain for CaBP with a black nuclear stain for the BrDU showing that there is co-localization.

31. Wash in distilled water 2 x 5 min.

32. Wash in Tris buffer 2 x 5 min.

33. Mount sections in Tris buffer on glass slides and air dry.

34. Dehydrate in alcohol and xylene and use Permount to coverslip slides. The *Novared* should not fade.

BrDU PROTOCOL AND DOUBLE LABELING FOR MOUSE TISSUE

1. Wash sections in 2x ssc (2x standard saline citrate) solution 2 x 5 min.
2. Incubate sections at 65°C in a water bath, preferably one with a shaker, for 2 hrs in 50% formamide in 2x ssc. Do this in fume hood or keep the water bath covered. If there is no shaker, manually shake sections every 15 min.
3. Wash in 2x ssc for 5 min.
4. Incubate in 2N HCL at 45°C water bath for 30 min. on a shaker or manually shake the sections every 15 min.
5. Wash in 0.1M Boric acid pH 8.5 for 10 min. on shaker. Now all steps should be done on a shaker without the water bath.
6. Wash 4 x 5 min. in Tris buffer.
7. Treat sections in 1% H_2O_2 for 30 min.
8. Wash sections in Tris buffer for 5 min.
9. Wash in Tris A and Tris B for 15 min. each.
10. Treat sections in 10% normal horse serum in Tris B for 1 hr.
11. Wash in Tris A and Tris B for 15 min.
12. Incubate tissue with Vector Labs M.O.M. mouse Ig blocking reagent for 1 hr. (2 drops to 2.5ml Tris B)
13. Wash in Tris A and Tris B 10 min. each.
14. Incubate in M.O.M. diluent for 5 min. (600µl to 7.5ml Tris B).
15. Incubate sections in BM (Chem)-MBrDU 1:1000 in M.O.M. diluent in Tris B at 4°C on a shaker for 1 to 2 days.
16. Wash in Tris A and Tris B for 15 min. each.
17. Treat sections in Vector Labs biotinylated HsAMs 1:400 in Tris B for 45 min.
18. Wash in Tris A and Tris D for 15 min. each.
19. Treat sections in Vector Labs ABC Elite 1:1000 in Tris D for 1 to 2 hrs.

20. Wash in Tris buffer 3 x 5 min.

21. Make up 100ml of DAB in Tris buffer and put in DAB substrates (aliquots of glucose oxidase, ammonium chloride and D (+)glucose). Next take out 20ml of the DAB solution and add 25mM NiCl (nickel chloride hexahydrate 237.7 MW) or 119mg to solution. Filter the DAB-NiCl solution and use immediately. Incubate until optimal staining has been achieved. You should get black nuclear staining.

22. Wash in Tris buffer 3 x 5 min.

23. Wash in Tris A and Tris B for 15 min. each.

24. Treat sections with 10% normal horse serum in Tris B with an Avidin D blocking agent from Vector labs. Use 4 drops of Avidin D per ml of 10% normal serum. You may need to Q.S. to your final volume when making this solution. Incubate for 1 hr.

25. Wash in Tris A and Tris B for 15 min. each.

26. Incubate sections into second primary antibody with the Biotin blocking agent from Vector Labs. Use 4 drops of Biotin blocking agent per ml of final dilution of antibody. You may need to Q.S. to your final volume when making your antibody dilution. Incubate for 1 to 2 days at 4°C. For Sigma-MCaBP 1:100,000 make up a 1:10,000 initial dilution and then make up from that the final dilution.

    Sigma-MCaBP 1:100,000
    1µl → 10ml Tris B 1:10,000
    1ml (1:10,000) + 40 drops Biotin blocking agent
    Q.S. to 10ml in Tris B 1:100,000

27. Wash in Tris A and Tris B for 15 min. each.

28. Treat sections in biotinylated HsAMs 1:400 from Vector Labs for 45 min.

29. Wash in Tris A and Tris D for 15 min. each.

30. Treat sections in ABC Elite from Vector Labs at 1:1000 in Tris D for 1 to 2 hrs.

31. Wash in Tris buffer 2 x 5 min.

32. Wash in distilled water 2 x 5 min.

33. Do the *Novared* stain. *Novared* is from Vector Labs. Prepare the substrate solution as follows:

    To 5ml of distilled water add 3 drops of Reagent 1 and mix well.
    Add 2 drops of Reagent 2 and mix well.
    Add 2 drops of Reagent 3 and mix well.
    Add 2 drops of the Hydrogen Peroxide solution and mix well.

    Incubate sections with the substrate at room temperature until suitable staining occurs. This may take 5-15 minutes; anything longer may increase background staining. The stain should appear brick red. The BrDU/CaBP double labeling should appear as a red neuron stain for CaBP with a black nuclear stain for the BrDU showing that there is co-localization.

34. Wash in distilled water 2 x 5 min.

35. Wash in Tris buffer 2 x 5 min.

36. Mount sections in Tris buffer on glass slides and air dry.

37. Dehydrate in alcohol and xylene and use Permount to coverslip slides. The *Novared* should not fade.

SOLUTIONS FOR BrDU PROTOCOL

2X SSC – STANDARD SALINE CITRATE – 0.3M NaCL AND 0.3M Na CITRATE

8.77g NaCL
4.41g Na Citrate (trisodium salt Sigma C-7254)
Q.S. to 500ml H_2O

2N HYDROCHLORIC ACID

4.98ml concentrated HCL into 30ml H_2O
83ml conc. HCL into H_2O
417 ml

0.1M BORIC ACID Ph 8.5

6.18g Boric acid into 1 liter H_2O
pH with 5N NaOH to 8.5

HISTOLOGICAL STAINS AND PROCEDURES

Treated Slides

Before picking up any sections all microscope slides must be coated with gelatin and chromium potassium sulfate solution.

In approximately 1 liter of distilled H_2O add 5g of gelatin and 0.5g of chromium potassium sulfate 12-hydrate ($CrK(SO_4)_2 \cdot 12H_2O$). Heat solution until all has dissolved. Pour solution into a large staining dish and dip slides several times and allow to dry either in a drying oven or air dry.

* leave overnight to cool

1% Cresyl Violet Solution

5g cresyl violet into 500ml distilled H_2O
Stir for 2 hrs
Filter solution
Can be stored at room temperature for 1 year.

0.1% Cresyl Violet Solution – counterstaining solution

Mix 10ml 1% cresyl violet solution into 90ml distilled H_2O.

<u>To Coverslip</u> – dip sections several times for each step

1.	70% ethyl alcohol	2 – 5 min.
2.	95% ethyl alcohol	2 – 5 min.
3.	100% ethyl alcohol	2 – 5 min.
4.	100% ethyl alcohol	2 – 5 min.
5.	Xylene	2 – 5 min.
6.	Xylene	2 – 5 min.
7.	Coverslip with Permount. If the Permount is too thick add some xylene to loosen it.	

Cresyl Violet – Nissl Stain

1. 70% ethyl alcohol 2 – 5 min.
2. 95% ethyl alcohol 2 – 5 min.
3. 100% ethyl alcohol 2 – 5 min.
4. 100% ethyl alcohol 2 – 5 min.
5. Xylene 5 min. for 30-50µl sections or longer for thicker sections.
6. 100% ethyl alcohol 2 – 5 min.
7. 100% ethyl alcohol 2 – 5 min.
8. 95% ethyl alcohol 2 – 5 min.
9. 70% ethyl alcohol 2 – 5 min.
10. Distilled water wash 3 changes.
11. 1% cresyl violet 30 sec.
12. Distilled water wash 3 changes.
13. Acetic acid sol.: 1 – 2 ml of acetic acid in approx. 250ml of distilled water. Dip the sections to destain until there is an appropriate Nissl stain.
14. Distilled water wash 3 changes.
15. 70% ethyl alcohol 2 – 5 min.
16. 95% ethyl alcohol 2 – 5 min.
17. 100% ethyl alcohol 2 – 5 min.
18. 100% ethyl alcohol 2 – 5 min.
19. Xylene 2 – 5 min.
20. Xylene 2 – 5 min.
21. Coverslip with Permount.

Counterstain – Cresyl Violet

1. 70% ethyl alcohol 2 – 5 min.
2. 95% ethyl alcohol 2 – 5 min.
3. 100% ethyl alcohol 2 – 5 min.
4. 100% ethyl alcohol 2 – 5 min.
5. Xylene 5 min.
6. 100% ethyl alcohol 2 – 5 min.
7. 95% ethyl alcohol 2 – 5 min.
8. 70% ethyl alcohol 2 – 5 min.
9. Distilled water wash 3 changes.
10. 0.1% cresyl violet counterstain sol. 2 min.
11. Acetic acid sol. – 1-2 ml of acetic acid in approx. 250ml distilled water. Dip sections to destain and leave a very light background stain.
12. Distilled water wash 3 changes.
13. 70% ethyl alcohol 2 – 5 min.
14. 95% ethyl alcohol 2 – 5 min.
15. 100% ethyl alcohol 2 – 5 min.
16. 100% ethyl alcohol 2 – 5 min.
17. Xylene 2 – 5 min.
18. Xylene 2 – 5 min.
19. Coverslip with Permount.

AChE-Metachromatic Nissl Stain

Solutions

- **50 mM Sodium Acetate Buffer pH 5.0**
  - 1 litre distilled H_2O
  - 6.8 g Sodiium Acetate → 2.0508 g Na^+ acetate (ANHYD) in 500 ml
  - 1.0 g Copper Sulphate ($CuSO_4.5H_2O$)
  - 1.2 g Glycine
- **Esterase Incubation Solution**
  - **prepare this solution fresh and discard after use.**
  - 200 ml Sodium Acetate Buffer
  - 232 mg S-acetylthiocholine iodide
  - 6 mg ethoprpazine
- **Acid-Acetone**
  - 100 ml Acetone
  - 100 ml Glacial Acetic Acid
- **0.1% Cresyl Violet**
  - 1000 ml distilled H_2O
  - 1 g Cresyl Violet
  - Stir with spin bar several hours and filter
- **5% Acetic Acid**
  - 190 ml distilled H_2O
  - 10 ml Glacial Acetic Acid
- **10% Potassium Ferricyanide**
  - 20 g Potassium Ferricyanide
  - 200 ml distilled H_2O

Procedure

1. Esterase Incubation Solution . . . . 2 Hrs -Overnight (2-4 days for fixed)
2. Acid-Acetone. . . . . 5 min
3. 0.1% Cresyl Violet. . . . . 5 min
4. 5% Acetic Acid. . . . 1 min (or until differentiated)
5. 10% Ferricyanide. . . . . 2 min
6. distilled H_2O. . . . . 1 min
7. acetone. . . . . 10 sec
8. acetone. . . . . 30 sec
9. Xylene. . . . . 1 min
10. Xylene. . . . . 1 min
11. coverslip

Metachromatic Nissl

A — **ACID ACETONE**

100 ml	Glacial Acetic Acid
100 ml	Acetone

B — **CRESYL VIOLET**

1.0 L	Distilled H_2O
1.0 g	Cresyl Violet

This solution should be mixed as long as possible and filtered before use.

C — **10% ACETIC ACID**

20 ml	Acetic Acid
180 ml	Distilled H_2O

STAINING PROCEDURE:

1.) 5 minutes in acid/acetone.
2.) 1-2 minutes in distilled H_2O.
3.) 30 sec - 1 minute in 10% acetic acid.
4.) Rinse briefly (5-10 seconds) in first acetone
5.) Rinse 30 seconds in second acetone.
6.) 1 minute each in 2 changes of xylene.

BDHC Stain

Perfuse animal with 0.01-0.1% glutaraldehyde such as "Superfix", anything higher will not work as well and without glutaraldehyde the stain will wash out.

Make these solutions in advance.

Phosphate buffer sol.

For the stain to work the pH of this buffer can be no higher than pH of 6.8. A good range of pH is between 6.0 to 6.8 where the low pH gives a very dark blue green stain to a very light blue at the higher pH. At a pH higher than 6.8 the staining looks browner than blue and for double staining you may not be able to tell BDHC (Benzidine Dihydrochloride) stain from DAB. So the best thing would be to try various range of pH to get the most optimal staining.

Sol. A: 0.2M sodium phosphate monobasic
$NaH_2PO_4 \cdot H_2O$ 13.8g/500ml d. H_2O

Sol. B: 0.2M sodium phosphate dibasic
$Na_2HPO_4 \cdot 7H_2O$ 26.85g/500ml d. H_2O

Using pH meter, start with Sol. A (low pH) and add Sol. B (high pH) to obtain desired pH. Store at room temperature.

For washing sections – 0.01M Na Phosphate

5ml 0.2M Na phosphate + 95ml d. H_2O = 100ml 0.01M Na phosphate

BDHC Stain Chemicals

Weigh out -10mg BDHC (Benzidine Dihydrochloride)
- 25mg Na nitroferricyanide (prusside)

into 1.5ml tubes and store at –20°C. This may not be necessary to do it is just for convenience.

BDHC Solution

A. Make up 10mg BDHC in 95ml distilled water and stir for 30 min. If BDHC is in a tube wash out well BDHC tends to stick to the walls of the tube. While BDHC is stirring wash the sections in phosphate buffer.

B. Add to BDHC solution 25mg Na nitroferricyanide and 5ml of 0.2M Na phosphate after the last 5 minutes of wash of sections.

C. For staining add to the BDHC solution 2µl of 30% H_2O_2 in 12ml of the BDHC solution made in Step B. Add sections immediately. This solution deteriorates within 30 min. Staining time is 5 – 10 min.

Note: The most crucial part are steps B and C. Step A can stay longer, but once you add the other substances time is of the essence.

Procedure

1. After ABC incubation wash in Tris buffer 2 x 5 min.
2. Wash in 0.01M Na phosphate buffer 2 x 5 min.
3. Incubate sections in solution made in Step B for 10 min.
4. Incubate sections in solution made in Step C for 5-10 min.
5. Wash sections in 0.01M Na phosphate buffer for 2 x 5 min.
6. Mount sections in 0.01M Na phosphate buffer.
7. Dry sections, and then dehydrate in graded alcohols and xylene and coverslip.

local cubicle

CYTOCHROME OXIDASE

DAY 1 (Before the staining)

- Prepare PB▮ (0.1M and pH 7.6) (Stored at 4°C)
- Prepare Sucrose Buffer (1 L PB▮ + 100 gr Sucrose) (Stored at 4°C)
- TRIS Buffer (1 L): - cupboard 25C unit

90.75 o 363 mL dH2O
967.5 o 387 mL HCl 0.1N (the original dilution is 10N, so you must dilute 100 times the original dilution. To obtain 1L: 990mL dH2O + 10mL HCl 10N)
1.25 o 5 mL DMSO
62.5 o 250 mL main solution (250mL dH2O + 6 gr TRIZMA BASE)
25 o 100 gr Sucrose
68.75 o 275 mg CoCl2 - 4°C cabinet

Note: this buffer must be prepared one or two days before the staining, and stored at 4°C. Its colour must turn gray-green.

DAY 2 (Staining day)

- Prepare Staining Solution: (heating-shaking device) (dark room)

200 o 800 mL PB▮ (0.1M and pH 7.6)
15 o 60 mg Cytochrome-C - freezer in chemical room
4 o 16 mg Catalase (Sigma) - freezer in chemical room
10 o 40 gr Sucrose
0.5 o 2 mL DMSO
100 o 400 mg DAB - Freezer in immunoroom (Harley)

Note: when the solution reaches 37°C, put it into the stove.

- Prepare Glutaraldehide (800mL) ->

196 o 784 mL Sucrose Buffer
4 o 16 mL Glutaraldehide 25%

- Prepare Formaldehide: (3.7% final) (-> 4°C)

180 o 720 mL Sucrose Buffer
20 o 80 mL Formaldehide 37% (4°C)

- STAINING PROTOCOL:

1. Glutaraldehide + Sucrose Buffer	5 min
2. Sucrose Buffer	5 min
3. Sucrose Buffer	5 min
4. Sucrose Buffer	5 min
5. TRIS Buffer	10 min
6. Rinse in PB▮	
7. Incubate in Staining Solution (37°C & shaking)	1 hour
8. Formaldehide + Sucrose Buffer	30 min
9. Ethanol 30%	5 min
10. Ethanol 50%	5 min
11. Ethanol 70%	5 min

Degeneration Stain

This is a silver stain that shows degenerating neurons. Animals are to be perfused with 4% PAF in 0.1M phosphate buffer pH 7.4.

Stock Solutions

Protect sol. C from light. Store all solutions at room temperature, stable for several months.

Sol. A. 9% w/v (weight to volume) sodium hydroxide (NaOH)
9g into 100ml d. H_2O or 90g into 1 liter

Sol. B. 16% w/v ammonium nitrate (NH_4NO_3)
16g into 100ml d. H_2O or 80g into 500ml

Sol. C. 50% w/v silver nitrate $AgNO_3$
1g into 2ml d. H_2O or 3g into 6 ml

Sol. D. 1.2% w/v ammonium nitrate
1.2g into 100ml d. H_2O or 6g into 500ml

Sol. E. 300ml 95% ethyl alcohol
600ml d. H_2O
5g anhydrous sodium carbonate
Q.S. to 1 liter

Sol. F. 700ml d. H_2O
100ml 95% ethyl alcohol
15ml 37% formalin
0.5g anhydrous citric acid or 0.547g citric acid monohydrate
Adjust pH with Sol. A to 5.8-6.1
Q.S. to 1 liter

Sol. G. 0.5% v/v (volume to volume) acetic acid
5ml into 1 liter d. H_2O

Working Solutions

Prepare no more than 1 hour before beginning the staining procedure.

Pretreating Solution

Mix equal volumes of Sol. A and Sol. D

100ml A + 100ml D

Impregnating Sol.

Add 1.5 volumes of Sol. A to each volume of Sol. B. Then add 0.6ml of Sol. C for each 100ml of total volume.

60ml Sol. A + 40ml Sol. B + 0.6ml Sol. C (Stir sol.)

Washing Sol.

Mix 1ml of Sol. D to each 100ml of Sol. E

300ml E + 3ml D

Developing Sol.

Mix 1ml of Sol. D to each 100ml of Sol. F

100ml F + 1ml D

(2)

Degenerating Stain Procedure

Make sure no metal contaminates any of these solutions. Use a plastic compartment box with holes drilled in at the bottom and a nylon mesh glued around the base of the box to transfer the sections from one solution to the next. Use cotton tip applicators to push down sections if they happen to float on top.

1. Wash sections in distilled water 3 x 5 min. Rotate.
2. Pretreating Sol 2 x 5 min. Rotate.
3. Impregnating Sol. 10 min. Rotate.
4. Washing Sol. 3 changes within 5 min. Rotate.
5. Developing Sol. 1 min. Can be left in longer. Do not rotate.
6. Mount sections in 0.1M Tris buffer.
7. Dry sections.
8. Wash in Sol. G 3 x 10 min. Rotate.
9. Wash in distilled water dipping several times in several changes of water.
10. Dehydrate and coverslip with Permount. Protect sections from light. Store slides in a microslide box when not in use.

GLYCOGEN

Dimedone-PAS Method

(Kong et al. JNeurosci 22:5581-5587 and McManus, 1946)

1) If not already perfused (4% PFA-.1M PB)/cryoprotected (30% sucrose in PB or 4% PFA until sunk) fix fresh frozen tissue on undipped slides in 4% PFA in .1M PB for 20 minutes

2) Wash 3 x 5 minutes (PBS?)

3) Oxidize with 0.5% periodic acid (1 x 10 min at RT)

   - 0.5 g periodic acid up to 100 ml dH_2O

4) Wash dH_2O (?)

5) Saturated dimedone solution in dH_2O (20 minutes at 60 °C).
   - blocks aldehyde groups on non-glycogen substance

For 100mL .401g @ 19°C or .416g @ 25°C 1.15g @ 50°C

6) Rinse in dH_2O

7) Schiff's reagent (Sigma- in fridge) 15 minutes (RT)

   - Schiff's should be brought to RT before use
   - discard if pink (should be straw coloured)

8) React in running water

9) Air dry

10) Dehydrate and coverslip as per usual
   - 2-3 minutes 70% EtOH
   - 2-3 minutes 95% EtOH
   - 2-3 minutes absolute EtOH
   - 2-3 minutes absolute EtOH
   - 2 x 5 minutes xylene

Coverslip in neutral mounting medium

IMPORTANT NOTE: All steps (less 60°C incubation) should be performed in fumehood with appropriate protective gear for carcinogens and contact burns.

GLYCOGEN PHOSPHORYLASE HISTOCHEMISTRY

INCUBATION MEDIUM	50 mL	100 mL	200 mL
Na-ACETATE BUFFER	10 mL	20 mL	40 mL
DISTILLED H_2O	35 mL	70 mL	140 mL
EDTA	0.1 g	0.2 g	0.4 g
NaF	0.08 g	0.16 g	0.32 g
DEXTRAN FW 40,000	2.0 g	4.0 g	8.0 g
α-D GLUCOSE-1 PHOSPHATE	0.4 g	0.8 g	1.6 g
TOTAL GPase			
Adenosine Monophoshate (AMP)	0.04 g	0.08 g	0.16 g

pH to 6.0 wih NaOH

GLYCOGEN PHOSPHORYLASE
Staining Procedure

1.) Incubate slides in medium for 30 minutes at 37°C

2.) Dry for 30 minutes.

3.) Fix in 40% ethanol for 4 minutes.

4.) Dry for 15-20 minutes,

5.) Stain in Lugol's Iodine for approximately 3 minutes.

6.) Rinse briefly in 0.9% saline.

7.) Let dry for 30 minutes- overnight and coverslip.

Neurobiotin/Biocytin

Making Neurobiotin/biocytin

4% Neurobiotin in 1 M filtered potassium acetate =
4 mg Neurobiotin in 100 ul 1 M filtered potassium acetate
use 0.2 um filter for pot. acetate

1.5% biocytin =
1.5 mg biocytin in 100 ul 1 M filtered potassium acetate

Embedding slices
Remove slice from net with flat spatula, lifting underneath slice, transfer it to TRIS or ACSF. Make sure all side are immersed. Then transfer with a broken pasteur pipitte to a petri dish filled 1/2 way with fix. Lay filter paper on top of slice so it becomes immersed. Refrigerate for 1 day- 1 week. Then transfer to TRIS or phosphate buffer.

Make 4% agar by stirring 4g agar into steaming 100 ml dH20.
Do not boil.

Place drop of hot agar, i.e. before it's boiled but after its dissolved, onto the slice. Dry the slice so that it lies flat on the petri dish bottom before placing agar on top. Don't dry the slice too much so it is drying out; don't dry to little (you should see no moisture around the edges but the slice itself should be moist; adjust with kimwipes to drain excess moisture)

Let cool, then lift agar and slice off petri dish. Place in 2% paraformaldehyde and refrigerate overnight. Refrigerate leftover agar in the beaker it was made it also.

Resectioning
Remove agar from slice by gently peeling it off.
Make a square out of the extra agar. Glue to the Vibratome tray and make the top surface flat by cutting it like it was a brain. Use 50-100 um steps or else it will not work. Once the edge is smooth, reverse the blade 600 um above the surface and keep it there.

Dry the top of the block. Put a small amount of superglue on the surface near the cutting edge. Spread it out so there is a thin layer. Place the slice on top. CA1 should be cut before the dentate, so place CA1 at the cutting edge. Or place the EC near the cutting edge with CA3 the furthest away. These two options work best.

Slowly lower the blade in 50 um steps until a tiny piece of slice is cut. Depending on how much is cut, make the next section up to 75 um thick.

OVER

Neurobiotin/biocytin Processing

1. Incubate sections in 0.5% Triton in TRIS overnight.
or incubate in 0.5% Triton for 1 hr
0.5% = 5 ml 10% Triton in 95 ml TRIS

2. Wash sections 3x10 in TRIS A

3. Incubate 30 min in 10% methanol in (3% H_2O_2 in TRIS A)
This must be made up immediately before the incubation; it can not be made before hand. Monitor sections for bubbling; if there is a lot, stop and remake the solution because it was made wrong and the slices could disintegrate if left any longer!
(3% H_2O_2 in TRIS A)= 5 ml 30% in 45 ml TRIS A
10% methanol in (3% H_2O_2 in TRIS A) = 5 ml methanol in 45 ml (3% H_2O_2 in TRIS A)

4. Wash in TRIS A 3 x 10 min

optional: Wash in TRIS B 10 min

5. Incubate in ABC standard kit for at least 2 hr
5 drops A and 5 drops B in 30 ml TRIS is what you use for this step
Note phosphate buffer doesn't work

6. Preincubate in DAB
50 mg DAB in 100 ml TRIS and
- Use 50 ml of this and add 20 mg $NiNH_3SO_4$; incubate for 20 min; the $NiNH_3SO_4$ needs to be crushed manually to dissolve. You can do this most easily by taking a flat spatula and pressing it against each granule on the side of a tripour beaker.

7. Incubate in DAB
Use the other 50 ml of DAB-TRIS
Add 12.5ul of 30% H_2O_2
Transfer sections from the DAB-TRIS-Ni directly to the DAB-TRIS-H_2O_2 solution

8. Put sections into TRIS to stop the reaction.

9. Let dry after mounting. Let dry at least 8 hrs.

10. Dehydrate in 70% EtOH, 90, 95, 100, then place in xylene and coverslip in Permount.
Or
Wash in glycerol, mount in glycerol
Or
darken further by dipping in 1% osmium

Neutral Red Stain (Mesulam, 1978)

Solutions

1) Acetate Buffer (pH 4.8)

500 ml	0.1N acetic acid
750 ml	0.1N sodium acetate

2) Neutral Red Solution (1 L)

40 ml	acetate buffer
960 ml	1% (filtered) neutral red

Staining Procedure

1) Immerse sections in 'neutral red solution' (3 minutes)
2) Dehydrate using 15 sec in each of:
   - dH2O
   - 70% EtOH
   - 95% EtOH
   - 100% EtOH
   - 100% EtOH
3) Xylene (1 minute), xylene (1-30 minutes)
4) Coverslip

Neutral Red Stain (Johnson, 1978)

1) dH2O (a couple of quick dips to wash PBS)
2) Immerse sections in buffered neutral red solution for 3.5 min
3) dH2O- 10 sec
4) [optional] Chrome alum-copper sulfate (5 sec)
5) [optional] 50% EtOH 10-15 sec
6) 75% EtOH 10-15 sec
7) 95% EtOH 10-60 sec (until differentiated)
8) 100% EtOH 30 sec
9) 50% xylene + 50% EtOH 30 sec
10) Xylene (2 min), xylene (2-20 min)
11) Coverslip

Nitroblue Tetrazolium Method for Alkaline Phosphotase

SOLUTIONS:

Buffer Solution

0.2 M Tris-HCl, pH 9.5, containing 10 mM $MgCl_2$

Solution a

5 mg	5-bromo-4-chloro-3-indolyl phosphate (BCIP) is dissolved in:
0.1 ml	dimethyl formamide (DMF) then in:
1.0 ml	Buffer solution

Solution b

5 mg	Nitroblue tetrazolium (NBT) dissolved in:
0.1 ml	DMF

Solutions a and b are added, with continuous stirring, to 30 ml of the above buffer and filtered. Once filtered, incubate immediately for 20 min- 12 Hours. The intense blue-black reaction product at the site of alkaline phosphotase activity is soluble in alcohol and xylene, hence aqueous mounting is recommended.

Timm Stain

In order for this stain to work the animal must be perfused with sodium sulfide and then a fixative either 4% PAF or "Superfix". The sulfide binds to any zinc in the brain and becomes insoluble. Then the silver forms a metal-sulfide complex that becomes visible during the staining procedure.

Gum Arabic

It's easier to use pre-weighed Gum Arabic of 500g instead of weighing it. In a 2-liter beaker with 1 liter of distilled water, pour slowly the Gum Arabic using a T-Line laboratory stirrer. Once all the Gum Arabic is in continue stirring vigorously for 2 hours. Then cover and allow it to sit at room temperature overnight to let the air bubbles rise. Remove the white crusty top as carefully as possible and decant a very syrupy liquid in containers. The Gum Arabic can be stored in the refrigerator for several months.

To Prepare Sections for Timm Stain

Dip sections vigorously several times before going into the next alcohol change.

1. 70% ethyl alcohol
2. 95% ethyl alcohol
3. 100% ethyl alcohol
4. 100% ethyl alcohol – 5 min.
5. 95% ethyl alcohol
6. 70% ethyl alcohol
7. Distilled water wash 6-7 changes to make sure all alcohol is washed out.

OVER

Timm Stain Preparation

1 run

1. 120ml Gum Arabic

2. 20ml citrate sol.
   - 4.7g sodium citrate
   - 5.1g citric acid monohydrate
   - heat to dissolve
   - Q.S. to 20ml d.H_2O

3. 60ml hydroquinone
   - 3.4g hydroquinone
   - heat to dissolve
   - Q.S. to 60ml d. H_2O

4. 1ml silver nitrate
   - 425mg silver nitrate into 2.5ml d. H_2O

2 runs

1. 240ml Gum Arabic

2. 40ml citrate sol.
   - 9.4g sodium citrate
   - 10.2g citric acid monohydrate
   - heat to dissolve
   - Q.S. to 40ml d. H_2O

3. 120ml hydroquinone
   - 6.8g hydroquinone
   - heat to dissolve
   - Q.S. to 120ml d. H_2O

4. 2ml silver nitrate
   - 425mg silver nitrate into 2.5ml d. H_2O

Make steps 1-4 ready. Add step 1-3 together in a large Erlenmeyer flask and thoroughly mix. When sections are ready add step 4 to the Gum Arabic solution, and mix, and then pour solution in a staining dish with the sections. Place the staining dish in a water bath at 26°C and keep in the dark. The stain starts appearing within 10-15 min. After this time take out sections intermittently, rinsed with distilled water, to view the process of staining and put back in the solution if not ready. Total duration of development is 25-50 min. depending on how lightly or darkly you want the stain to go. Once the sections are ready rinse thoroughly with distilled water and coverslip.

Artificial Cerebral Spinal Fluid
Aston-Jones (Brain Res Bullet, 27:5-12)

CHEMICAL	CONCENTRATION	F.W.	g/L
NaCl	122 mM	58.4400	7.1297
KCl	3.1 mM	74.5600	0.2311
CaCl	1.3 mM	111.0000	0.1443
MgSO4	1.2 mM	120.3700	0.1444
NaH2PO4	0.4 mM	120.0000	0.0480
NaHCO3	25 mM	84.0100	2.1003

For final concentrations of GLUTAMATE or ISOPROTERENOL Q.S with above aCSF

GLUTAMATE	F.W	g/50 mL	g/100 mL
0.25 M	169.1000	2.1138	4.2275
0.5 M		4.2275	8.4550

ISOPROTERENOL	F.W	g/25 mL	g/50 mL
100 uM	247.7000	0.6193	1.2385
10 uM		0.0619	0.1239
1 uM		0.0062	0.0124

1.5M Phosphate Buffer pH 7.4

	100mL	1.00 L
$NaH_2PO_4 \cdot H_2O$	0.3962g	3.9620g
$Na_2HPO_4 \cdot 7H_2O$	3.2592g	32.5924g

For aCSF, make in 1.5M Phosphate Buffer, Q.S. with Phosphate Buffer

Artificial Cerebral Spinal Fluid (Harley)

		100mL	500mL	1.00 L
NaCl	147mM	0.8591g	4.2955g	8.5910g
KCl	3mM	0.0224g	0.1120g	0.2238g
$MgCl_2$	1 mM	0.0203g	0.1017g	0.2033g
$CaCl_2$*	1.3mM (add last)	0.0144g	0.0720g	0.1443g

ANESTHETICS & STOCK SOLUTIONS

Artificial CSF (Harley et al., 1996)

147 mM	NaCl
3 mM	KCl
1.3 mM	$CaCl_2$
1 mM	$MgCl_2$
1.5 mM	Sodium Phosphate Buffer, pH 7.4

AVERTIN

5 g	Tribromoethanol (TBE)
20 ml	Ethanol (not absolute)
5 ml	Tetra-amyl alcohol
250 ml	Saline (0.9%)

1.) Dissolve the TBE in 20 ml (near absolute) ethanol. Gentle heat may be necessary.
2.) Add the tetra-amyl alcohol
3.) Add saline (up to 250 ml)
4.) Store in refrigerator

INITIAL DOSE: 1.5 ml/100 g
SUPPLEMENTAL: 0.25 ml/100 g

Chloral Hydrate ~~(80 mg/ml)~~

4.0 g	Chloral Hydrate
~~50~~ 100 ml	~~Distilled water~~ saline

INITIAL DOSE: ~~0.5~~ 1 ml/100 g

Chloral hydrate
Higher concent
4.0g chl. Hy.
50mL (Q.S)
↳saline
Dose .5mL/100g

for acute only you can use higher dose. (intestinal impairments occur if you ~~do~~ use for survival surgeries)

Dibasic Stock (1 litre of 0.4 M)

56.78 g Sodium phosphate dibasic ($NaHPO_4$)
1000 ml Distilled water

Set the distilled water stirring and slowly add dibasic. Dibasic is difficult to dissolve and will go rock hard upon contact with water, so the solution needs to be in motion.

Hydrochloride (HCl) 2 M

0.7292 g HCl
10 ml Distilled H_2O

Krebs Bicarbonate-phosphate Ringer

6.9 g Sodium Chloride (NaCl)
0.4 g Potassium Chloride (KCl)
0.3 g Calcium Chloride ($CaCl_2$)
0.2 g Potassium Phosphate, monobasic (KH_2PO_4)
0.3 g Magnesium sulphate ($MgSO_4.7H_2O$)
Water: Make up to 1000 ml

Locke's Solution

0.9 g Sodium Chloride (NaCl)
0.024 g Calcium Chloride (CaCl)
0.042 g Potassium Chloride (KCl)
0.01-0.03g Sodium Bicarbonate
0.01-0.25g D-glucose
100 ml Distilled water

Lugol's Iodine

FROM CONCENTRATE

33 g Sucrose
20 ml Concentrated Lugol's Iodine
Distilled Water: make up to 300 ml

READY-TO-USE LUGOL'S

2.0 g Potassium Iodide (KI)
300 ml Distilled water
1.0 g Iodine (I_2)

Stir well- Store away from light.

Mammalian Ringer-Locke

9.0 g Sodium Chloride (NaCl)
0.25 g Potassium Chloride (KCl)
0.30 g Calcium Chloride ($CaCl_2$)
0.5 g Sodium bicarbonate ($NaHCO_3$)
1.0 g Glucose
Water: make up to 1000 ml

MonoBasic Stock

137.99 g Monobasic
1000 ml Distilled water

Na-Acetate Buffer

2.97 g Sodium Acetate (anhydrous)
400 ml Distilled H_2O
0.22 ml Glacial Acetic Acid

For glycogen phosphorylase histochemistry- pH to 5.6 with NaOH

NaOH (2 M)

0.8 g	Sodium Hydroxide (NaOH)
10 ml	Distilled water

Paraformaldehyde (1 Litre of 10%)

1000 ml	Phosphate buffer
100 g	Paraformaldehyde

Heat 500 ml of phosphate buffer to 50-55°C. Add the para to the stirring solution. It will take 30-60 minutes to dissolve. Add 500 ml of room temperature phosphate buffer. Allow tocool before handling.

NOTE: Do this in a fume hood. It stinks and is very bad for you.

Penicillin: Scheinpharm Penicillin G Sodium

5,000,000 IU Penicillin
30,000 IU x 2 per animal

Volume 41.7 ml saline
0.25 ml injection (i.m.)

Phosphate Buffer

pH	Monobasic Stock ml .1 M	Dibasic Stock ml .1 M
5.3	192	8
5.5	188	12
5.7	184	16
5.8	180	20
5.9	174	26
6.0	168	32
6.1	162	38
6.2	154	46
6.3	146	54
6.4	136	64
6.5	128	72
6.6	112	88
6.7	104	96
6.8	96	104
6.9	82	118
7.0	68	132
7.1	56	144
7.2	48	152
7.3	40	160
7.4	34	166
7.5	28	172
7.6	23	177
7.7	17	183
7.8	12	188
7.9	8	192

Perfusion Medium for Horseradish Peroxidase Stain

50 ml	10% Paraformaldehyde
50 ml	25% Glutaraldehyde
250 ml	0.2 M Phosphate buffer
150 ml	Distilled water

Procedure:

1.) 250 ml phosphate buffered saline (heparinized)
2.) 500 ml perfusion medium
3.) Decapitate brain
4.) Store in 30% sucrose in phosphate buffer
5.) Section ASAP

Perfusion Medium for Biocytin (also pCREB)

	500 ml	1000 ml
10 % Paraformaldehyde	200 ml	400 ml
25% Glutaraldehyde	10 ml	20 ml
0.1 M Phosphate Buffer	290 ml	580 ml

Phosphate-Buffered Saline (PBS)

1000 ml	0.1 M phosphate buffer, pH 7.4
9 g	Sodium Chloride

Keep at 4°C or room temperature. Discard if there are signs of infection.

Ringer's Solution

0.7 g	Sodium Chloride (NaCl)
0.0026g	Calcium Chloride (CaCl)
0.035 g	Potassium Chloride (KCl)
100 ml	Distilled water

SALINE- Physiological (0.9%)

NaCL	Distilled Water
0.9 g	100 ml
4.5 g	500 ml
7.2 g	800 ml
9.0 g	1000 ml

Tyrode's Solution

0.8 g	Sodium Chloride (NaCl)
0.02 g	Calcium Chloride (CaCl)
0.02 g	Potassium Chloride (KCl)
0.1 g	Sodium Bicarbonate
0.1 g	D-Glucose
0.1 g	Magnesium Chloride (MgCl)
0.005 g	Monosodium Phosphate
100 ml	Distilled water

Urethane

30.0 g Urethane
200 ml Make up to 200 ml with distilled water

INITIAL DOSE: 1.0 mL/100 g

4% PAF in 0.1M Tris Buffer

250ml distilled H_2O
6.06g Tris HCL
1.39g Tris Base
100ml 20% PAF
add H_2O to 475ml
pH to 7.4
Q.S. to 500ml

500ml distilled H_2O
12.12g Tris HCl (Trizma)
2.78g Tris Base
200ml 20%PAF
add H_2O to 975ml
pH to 7.4
Q.S. to 1 liter

To perfuse: Anesthetize rat with urethane 1.25g/kg until completely sedate.
Perfuse transcardiacally with the following:
Saline flush – 3 min.
4% PAF in 0.1M Tris buffer – 10 min.
Postfix brain *in situ* overnight or longer if need be at 4°C.