Basic Neuroanatomical Methods

This unit covers some of the basic procedures that are common to a wide range of neuroanatomical protocols. Among the neuroanatomical protocols covered in subsequent units are those for:

- labeling specific neuronal cell types on the basis of their neurochemical phenotype using immunohistochemical localization of antigenic sites on proteins or peptides (UNIT 1.2) or in situ hybridization histochemical localization of the messenger RNAs that encode such proteins or peptides (UNIT 1.3);
- localizing neurotransmitter receptor-binding sites (UNIT 1.4);
- combinations of the above methods that allow the connections between phenotypically characterized neurons to be visualized.

While each of these procedures requires certain specific histologic treatments, they all share some common methodologies. This unit provides some of the basic common protocols, as well as some standard staining protocols used as an adjunct to more specific neuroanatomical protocols.

Most neuroanatomical procedures involve the following steps: (1) brain preparation, (2) sectioning the brain, (3) labeling and staining the brain sections, (4) application of the sections to microscope slides, and (5) microscopic analysis. The sequence of the steps for labeling and staining the brain sections and applying sections to microscope slides will vary depending on the neuroanatomical method used. Procedures are provided for the preparation of unfixed, fresh brain tissue (see Basic Protocol 1) as well as for perfusion fixation of animals resulting in fixed neural tissue (see Basic Protocol 2). A variety of methods for sectioning brains are described, including frozen sectioning in a cryostat (see Basic Protocol 3), frozen sectioning with a microtome (see Basic Protocol 4), and sectioning with a vibratome (see Basic Protocol 5). The choice of sectioning method depends on how the brain has been prepared and what histochemical method is to be used. Methods for paraffin sectioning (see CPMB UNIT 14.1 and APPENDIX IA in this manual) or ultramicrotome sectioning (Bolam, 1992) are not covered in this unit. Three post-sectioning procedures are provided: defatting of slide-mounted sections (see Basic Protocol 6), thionin staining of the sections (see Basic Protocol 7), and coating of slides with photographic emulsion for autoradiography (see Basic Protocol 8). Finally, a procedure is described for subbing slides with gelatin, which is necessary in some protocols in order for the sections to adhere to the slides (see Support Protocol).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for care and use of laboratory animals.

PREPARATION OF UNFIXED FRESH-FROZEN BRAIN TISSUE

How the brain should be prepared for histologic processing depends on the specific neuroanatomical protocol to be used. Some protocols require the brain to be removed from the skull fresh—i.e., immediately following the killing of the animal—whereas other protocols require the brain to be perfused in situ with a fixative. The method of brain preparation used depends in part on the neurochemical that is to be localized; how a brain is prepared determines, in turn, how the brain should subsequently be sectioned.

Certain protocols require that the brain be removed from the skull fresh, without fixation. For example, autoradiographic localization of neurotransmitter receptor-binding sites is...
often dependent on such sites being maintained intact for ligand binding to occur. In other procedures, such as the localization of mRNA with in situ hybridization histochemical procedures, the brain is removed, sectioned in the fresh, frozen state, and sections are processed with fixative following their application to microscope slides. Alternatively, in some in situ hybridization protocols, prefixation may be performed prior to sectioning.

**Materials**

- Isopentane
- Dry ice
- Rat or mouse for study
- Anesthetic
- Dissection instruments:
  - Scissors
  - Spatula
  - Forceps
- Plexiglas or metal sieve-like basket and metal container large enough to hold it

1. Place ~300 to 500 ml isopentane in a metal container large enough to hold a corresponding sieve-like basket. Place the metal container with the isopentane in dry ice for 15 to 30 min, until the temperature of the isopentane reaches −70°C.

   *Isopentane may be reused, so it can be rebottled after the procedure is completed.*

2. Kill animal(s) with an overdose of anesthesia.

3. Remove the brain from the skull.

4. Place the brain on the mesh bottom of the sieve-like basket in a manner that preserves the normal shape of the brain.

5. Immerse the brain in the cooled isopentane for 20 to 30 sec.

   *The time of immersion is absolutely critical; it must be long enough to result in complete freezing of the brain, but not so long that the brain cracks. It may be necessary to test various times to determine the one that is optimal to meet these criteria.*

6. Rapidly remove the frozen brain from the isopentane, detach it from the mesh, and place it briefly on absorbent paper to remove excess isopentane.

7. Wrap the dried, frozen brain in foil and store at −20°C to −70°C until sectioning is performed.

   *Fresh-frozen brains can stored at −70°C for months (and even years) prior to being sectioned.*

**PERFUSION FIXATION**

Some protocols require that the brain be perfused with fixative via the circulatory system to achieve penetration of the fixative into the brain. Immunohistochemical procedures are among those that generally require perfusion fixation. The rationale behind this process is that fixation is necessary to stabilize the proteins or peptides in the brain in order to allow for subsequent antibody binding to the antigenic site on those proteins or peptides. The type of fixative required for optimal localization is a variable that is dependent on the antibody to be used and the antigenic target site. There are a wide range of perfusion-fixation methods that may be used; this protocol is only the most basic.
**Materials**

- Saline (0.9% w/v NaCl), 4°C
- Fixative solution for perfusion (see recipe), room temperature
- Rat or mouse for study
- Anesthetic
- Sucrose-infiltration solution, 4°C (see recipe)
- Peristaltic perfusion pump (e.g., Masterflex with variable-speed standard drives from Cole-Parmer)
- Masterflex Tygon tubing (0.25-in.)
- Blunt 13-G and 15-G hypodermic needles
- Surgical instruments (Roboz Surgical or Fine Science Tools) including:
  - Scalpel
  - Scissors
  - Clamps
  - Hegenbarth clip-applying forceps
  - Hemostats
  - Bone rongeur

**Prepare perfusion system and animal**

1. Place cold saline (0.9% NaCl) and room temperature fixative solution in separate flasks and set up the peristaltic pump, Tygon tubing, and perfusion instruments according to the manufacturer’s instructions in such a manner that the saline is first drawn through the pump into tubing that is to be connected to the animal. Use a valve system or other device to allow the fixative solution to be drawn through the tubing at a later point. Fill the system with saline.

   *Whichever method is used, it is important that air not be introduced during the switch between the two fluids.*

   *The Masterflex variable-speed pump (Cole-Parmer) provides a smooth flow rate from 0.06 to 3400 ml/min, depending on pump rotation speed and tubing diameter.*

2. Attach tubing primed with saline to a blunt 15-G hypodermic needle that will be used for perfusion.

3. Prepare rat or mouse for infusion by administering a lethal dose of anesthesia. Monitor it until the point when the animal fails to respond to pinching of the foot.

**Place perfusion needle into ascending aorta**

4. Make an incision through the abdomen just below the rib cage to expose the diaphragm. Make an incision in the diaphragm to expose the heart.

   *This procedure is most successful if the heart is still beating at the outset of perfusion.*

5. Open the thoracic cavity with two horizontal cuts through the rib cage on either side of the heart. Clamp the sternum with a hemostat and fold the cut rib flap headward to expose the heart.

   *The descending aorta may be clamped when the brain and head (but no other organs) are to be perfused.*

6. Make a small incision at the bottom apex of the left ventricle. Quickly insert a blunt 13-G hypodermic needle upward through the ventricle past the aortic valve so that it may be visualized ~5-mm inside the ascending aorta. Clamp the needle in place with a Hegenbarth clip-applying forceps or hemostat across the ventricle.

   *Viewing the ventrally exposed chest cavity, the left ventricle is to the right.*
**Initiate perfusion**

7. Begin perfusion of saline very slowly (i.e., 20 to 40 ml/min). Immediately after the peristaltic pump begins pumping the saline, cut the right atrium to allow an escape route for the blood and perfusion fluid.

   *Cutting the atrium may be delayed until it has become expanded by the introduction of perfused saline.*

   *It is recommended that the perfused saline be kept on ice, which helps reduce the possibility of coagulation of blood during the initial perfusion.*

8. Perfuse saline at a moderate to rapid rate (∼40 ml/min) and continue until the effluent runs clear, which may require 200 to 500 ml of solution.

9. After the effluent runs clear, stop the pump and introduce fixative into the peristaltic pump line running into the animal. Perfuse fixative at a moderate to slow rate (∼20 ml/min) such that ∼500 ml of fixative is perfused over 10 to 20 min.

   *When the fixative begins to enter the animal there may be some twitching of muscles, which provides an indication that the perfusion is proceeding properly. After several minutes there should be some indication of stiffening of the forelimbs and head, a further indication that the fixative is being properly perfused. Fixative should be kept at room temperature.*

10. Following perfusion of ∼500 ml of fixative, remove the brain from the skull using a bone rongeur.

   *Once this has been done, the brain may be post-fixed for a variable period of time in the same fixative as that used for perfusion. This initial post-fixation should optimally be done at room temperature. If this procedure is performed, it should be performed before sucrose infiltration.*

**Infiltrate brain with sucrose**

11. Transfer the brain to a vessel containing 4°C sucrose-infiltration solution. Incubate 24 to 48 hr at 4°C, until the brain sinks into the sucrose solution, indicating that sucrose has infiltrated the brain.

   *The purpose of sucrose infiltration is to prevent freezing artifacts in the tissue during sectioning.*

12. Section the brain (see Basic Protocols 3, 4, and 5).

   *Once the brain has been infiltrated with sucrose it is ready to be sectioned.*

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**BASIC PROTOCOL 3**

**CRYOSTAT SECTIONING OF FROZEN BRAIN TISSUE**

A cryostat is a microtome housed in a freezing chamber that allows the sectioning process to be performed at a temperature of −20° to −30°C. A cryostat is required for sectioning fresh-frozen brains, as the unfixed brain sections must be maintained in a frozen state until they are affixed to a microscope slide. Cryostat sectioning may also be used for perfusion-fixed brains, but is not mandatory because such brain sections retain morphologic integrity after being sectioned; they may thus be transferred to solutions directly from the knife prior to being mounted on subbed microscope slides. Certain fixatives—e.g., Histochoice—may require poly-L-lysine coated slides (see APPENDIX 2A).

**Materials**

- Dry ice (powdered or pellets)
- Embedding matrix (M-1 from Shandon/Lipshaw or OCT compound from Miles Labs)
- Brain tissue: fresh-frozen (see Basic Protocol 1) or perfusion-fixed (see Basic Protocol 2) and frozen on dry ice just prior to sectioning
Cryostat microtome
Specimen holder (cryostat chuck; metal platform for supporting specimen during sectioning)
Gelatin-subbed microscope slides (see Support Protocol)
Clean, soft paint brush (optional)
40°C warming plate (optional)
Small zip-lock bags

**Prepare brains for sectioning by mounting on a cryostat chuck**

1. Place specimen holder/cryostat chuck on dry ice and place embedding matrix or water on the surface of the specimen holder/chuck. As the embedding matrix or water begins to freeze, place the frozen brain, base-down, into it so that the brain adheres to the specimen holder/chuck.

2. Pour embedding matrix over the frozen brain to provide a thin coat that aids in maintaining the integrity of the brain sections during cutting.

   Alternatively, the tissue specimens may be frozen on coins with the embedding matrix. The specimens are then easily removed and stored indefinitely at −80°C in an air-tight container with some wet ice to prevent dehydration. This approach conserves specimen holders. When the specimen is to be sectioned, it is simply frozen to a holder with some more embedding matrix. If the specimen is to be saved after cutting some sections, it can be removed from the holder by placing the undersurface of the specimen holder in contact with warm water until the specimen detaches.

3. Place the brain, mounted on the specimen holder/cryostat chuck, in the cryostat microtome. Section the brain.

   The temperature used for cutting may need optimization for different tissue types.

   The use of talc-less gloves is recommended to avoid getting talc particles on the slides and tissue sections, which can absorb radiolabeled probe and produce spots on the film and nuclear emulsions.

**Cut the brain into sections and mount onto gelatin-subbed microscope slides**

4a. **Method 1:** Transfer sections from the specimen holder/chuck to a warm (40°C) gelatin-subbed slide.

   With this method the section adheres to the site that it touches; hence care must be taken to align the slide where one wants the section to adhere.

   IMPORTANT NOTE: The sections of fresh-frozen brains are fragile and must be handled with some care prior to affixing them to microscope slides.

4b. **Method 2:** Prechill slide and keep it cold in the cryostat. Position the cut brain section on the cold gelatin-subbed slide with a clean, soft brush. After the brain section is in position, warm slide by running a warm (40°C) steel bar on the back side of the slide, or place the slide on a 40°C warming plate, to allow the section to adhere.

5. Dry slides for 1 min on a 40°C warming plate. Place slides in storage bags (e.g., small zip-lock bags) and store at −20° to −80°C pending further processing.
SLIDING-MICROTOME SECTIONING OF FIXED BRAIN TISSUE

A sliding microtome is an instrument that may be used for sectioning fixed brains. For this procedure, the fixed brains must be cryoprotected by infiltration of sucrose to prevent freezing artifacts during the sectioning process (see Basic Protocol 2). Sucrose-infiltrated fixed brains are frozen and maintained frozen during sectioning. Sections are collected off the microtome knife and placed in a buffered saline (or cryoprotectant) solution and stored for subsequent histochemical processing.

**Materials**

- Sucrose-infiltrated fixed brains (see Basic Protocol 2)
- Dry ice
- Potassium phosphate–buffered saline (KPBS; see recipe)
- Sliding microtome with knife (Leica or American Optical) and sliding microtome stage
- Small brush
- Container for collecting brain tissue sections (e.g., 24-well Costar tissue culture plate)
- Gelatin-subbed microscope slides (see Support Protocol)

**Section brain**

1. Freeze sucrose-infiltrated fixed brain in dry ice.

2. Cool the microtome stage according to the manufacturer’s instructions.

   *The sliding microtome stage provides the platform on which the brain is held for sectioning. There are several types of stages. The simplest type has a trough into which dry ice is placed, which serves to maintain the brain frozen during sectioning. Another type of stage is connected by tubing to a CO₂ cylinder, which is used to generate dry ice around the brain. Some microtomes have a cooling apparatus through which coolant flows in order to maintain the stage and brain in a frozen state.*

3. Attach the brain to the stage by placing water on the prechilled stage and placing the brain on the water.

   *As the water freezes, the brain will freeze in place.*

4. Section the brain by sliding the microtome knife across the surface of the frozen brain. Carefully transfer the single cut sections from the knife surface with a brush to a dish containing a suitable buffer solution (e.g., KPBS).

   *The thickness of the sections is determined by the histochemical procedure to be performed, but sections between 10 and 50 µm are typically used.*

**Mount sections onto gelatin-subbed slides**

5. Float sections in a dish of KPBS.

6. Place a gelatin-subbed slide in the dish underneath the sections and position the section of interest onto the slide with a brush. Slowly raise the slide out of the dish and allow the section to dry onto the slide.

   *Once a section is dried onto the slide, the slide may be reimmersed in the dish and other sections affixed to it by repeating the process.*

7. If desired, stain dried, slide-mounted sections with thionin (see Basic Protocol 7).
A Vibratome (TPI; see SUPPLIERS APPENDIX) is an instrument that provides a vibrating knife to cut tissue. The advantage of this device is that the vibrating knife makes it possible to section brain tissue without the need for freezing and the consequent ultrastructural damage which is only partially averted via sucrose infiltration. While the Vibratome method is essential for electron microscopy, it also affords superior morphologic integrity of immunohistochemical labeling at the light-microscopic level.

**POST-SECTIONING PROCEDURES I: DEFATTING**

Brain sections cut fresh on a cryostat (see Basic Protocol 3) may be stored frozen until ready to use. For some procedures, such as in situ hybridization histochemistry, the brain sections may be fixed and defatted prior to histochemical processing.

**Materials**

- Fresh-frozen (unfixed) slide-mounted brain sections (see Basic Protocol 3)
- 4% formaldehyde in saline (see recipe)
- Acetic anhydride
- Triethanolamine/saline solution (see recipe)
- 70%, 95%, and 100% ethanol
- Chloroform
- Metal 30-slide rack (optional for small numbers of slides)
- 500-ml (or appropriate-sized) staining dishes

1. Thaw slides with sections at room temperature in the storage bags.
2. Fix mounted sections by immersing slides for 10 min in a staining dish with 4% formaldehyde/saline.
3. Add 1.25 ml acetic anhydride to 500 ml triethanolamine/saline solution in a beaker and stir rapidly for 20 sec. Immediately transfer solution to a staining dish containing the slides and immerse slides for 10 min.
4. Dehydrate sections by immersing slides successively for 1 min each in staining dish containing 70% and 95% ethanol, then twice for 1 min in 100% ethanol.
5. Defat sections by immersing in slides twice for 5 min in chloroform.
6. Remove chloroform by immersing slides twice for 1 min in 100% ethanol, then once for 1 min in 95% ethanol.
7. Allow slides to air dry and store in boxes at −20°C.

**POST-SECTIONING PROCEDURES II: THIONIN STAINING**

There are a number of basic histologic stains that are of considerable utility for neuroanatomical methods. These include cresyl violet, neutral red, hematoxylin/eosin, and thionin. In most cells of the brain, these stains label the cell body. Consequently, such staining is useful for visualizing numerous macroscopic neuroanatomical features related to brain organization, such as the cytoarchitecture of laminar differences in cortical areas that delineate different functional cortical areas, as well as the subdivision of areas such as the thalamus and hypothalamus into subnuclei. Moreover, thionin staining may be effectively used to determine the extent of neurotoxic lesions—e.g., 6-hydroxydopamine lesions of the dopamine neurons in the midbrain, substantia nigra, pars compacta, or excitotoxic lesions of the cortex and striatum.
Thionin is one of the most often used of the class of so-called Nissl stains. Most of its staining appears to be of RNA, which is particularly concentrated in the cell body and nucleus.

**Materials**

- Brain sections mounted on slides, preferably fixed
- Thionin solution (see recipe)
- 50%, 70%, 95%, and 100% ethanol
- Xylene
- 95% ethanol/1% acetic acid (optional)
- Coverslips
- Permount histological mounting fluid (e.g., Fisher)

1. Place slides with brain sections in staining racks. Dip successively in the following solutions for the indicated periods of time:
   - 1 min thionin solution
   - 2 min distilled, deionized H$_2$O
   - 1 to 2 min 50% ethanol
   - 1 to 2 min 70% ethanol
   - 1 to 2 min 95% ethanol
   - 1 to 2 min 100% ethanol
   - 1 to 2 min 100% ethanol
   - 2 min xylene
   - 2 min xylene.

   _The slide-mounted sections may be stored indefinitely in xylene until coverslipped._

2. Allow to dry, then apply coverslip over section using Permount. Examine by bright-field microscopy.

   _Staining should appear blue-purple with white matter relatively unstained. If white matter does not clear, additional destaining may be obtained with 95% ethanol/1% acetic acid._

3. If white matter has not destained sufficiently, pry off the coverlip (if already applied) and dip slide in the following solutions for the indicated periods of time:
   - 2 min xylene
   - 2 min xylene
   - 2 min 100% ethanol
   - 2 min 100% ethanol
   - 1 to 2 min 95% ethanol/1% acetic acid
   - 2 min 100% ethanol
   - 2 min 100% ethanol
   - 2 min xylene
   - 2 min xylene

   then apply new coverslip as in step 2.
PROCEDURES IN WHICH RADIOACTIVE PROBES ARE USED FOR LABELING MAY INVOLVE DIPPING SLIDES IN PHOTOGRAPHIC EMULSION TO OBTAIN CELLULAR RESOLUTION OF LABELING.

MATERIALS
- Emulsion: Kodak NTB-3 or Amersham LM-1 (thaw 30 min prior to use)
- 0.1% (w/v) Dreft detergent in H₂O
- Slide-mounted radioactively labeled tissue sections
- Dektol developer (Kodak)
- Stop bath: H₂O or 1.5% acetic acid in H₂O
- Rapid Fix (Kodak): prepare according to manufacturer’s instructions without hardener
- Darkroom with amber/red sodium photographic safelight and humidifier
- Slide mailer (2-slide or 5-slide, Shandon/Lipshaw or Thomas Scientific)
- 40° to 42°C water bath
- Blank microscope slides
- Light-tight slide boxes
- Staining racks and dishes
- Glass staining dishes (Thomas Scientific)
- Metal slide racks (Thomas Scientific)

COAT SLIDES WITH EMULSION
1. In darkroom under safelight conditions, melt emulsion completely in a 40° to 42°C water bath in a slide mailer or other suitable vessel in which slides may be immersed. Melt some emulsion separately in a beaker, which will be used to replenish the emulsion in the slide mailer as slides are dipped.
   - For Kodak NTB-3 emulsion: dilute gel form of emulsion 1:1 with 0.1% Dreft detergent.
   - For Amersham LM-1 emulsion: proceed to step 2 without dilution.
   - The vessel used for melting the emulsion should be shaped so as to minimize the volume of emulsion required to coat the slides.
2. Remove bubbles from emulsion (in both slide mailer and beaker) by dipping blank slide(s) in it up to 100 times. Let emulsion sit 15 min to settle.
3. Dip each slide into emulsion, wipe back of slide with a paper towel to remove emulsion, then stand slides upright on absorbent paper towels to allow excess emulsion to flow off slide.
4. Allow emulsion to dry for 1 to 3 hr in upright position.
5. Store slides in light-tight box at −20°C until time to be developed.
   - The exposure time required to obtain an adequate visible signal is determined empirically and varies depending on the isotope used and the amount of label in the tissue.

DEVELOP SLIDES
6. In darkroom under safelight conditions, dilute 1 part Dektol developer with 2 parts water in a staining dish. Cool developer solution on ice and maintain at 17°C during developing.
7. If necessary, thaw slides for 30 min prior to developing.
8. Place stop bath and Kodak Rapid Fix solutions in staining dishes.
9. Process slides in staining racks by passing them through the following solutions:

<table>
<thead>
<tr>
<th>Time</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min</td>
<td>Dektol developer, 17°C</td>
</tr>
<tr>
<td>1 min</td>
<td>stop bath</td>
</tr>
<tr>
<td>2 to 3 min</td>
<td>Kodak Rapid Fix.</td>
</tr>
</tbody>
</table>

10. Rinse slides in cold running tap water for 15 to 30 min. Air dry.

*The emulsion will come off the slide if rinse water is too warm.*

Following developing and thorough rinsing, slides may be processed further according to whatever specific labeling is required. A few examples include (1) staining slides with a counterstain such as thionin (see Basic Protocol 7) or (2) coverslipping slides without counterstaining, which involves processing them according to the procedure used for thionin staining with the omission of immersion in the stain.

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**PREPARATION OF GELATIN-SUBBED MICROSCOPE SLIDES**

Microscope slides generally need to be subbed in a solution that provides some adherence for the tissue sections, so that they will remain affixed during subsequent histologic processing. Slides are usually subbed in a solution of gelatin. For many histologic procedures, a single subbing process is sufficient; however, for other procedures slides need to be subbed twice to assure sufficient adherence. When there is any doubt, double subbing is preferred.

**Materials**

- Gelatin-subbing solution (see recipe)
- Glass slides
- Slide racks
- 40°C glassware-drying oven

1. Place slides in slide racks and dip for 1 min in gelatin-subbing solution.

2. Remove racks containing slides from subbing solution and shake to facilitate removal of excess subbing solution.
   
   *For twice-subbed slides, repeat steps 1 and 2.*

3. Dry slides in a 40°C oven overnight.

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**REAGENTS AND SOLUTIONS**

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.*

**Fixative solution for perfusion**

- 500 ml 8% formaldehyde stock solution (see recipe)
- 500 ml H₂O
- 3.2 g NaH₂PO₄
- 10.9 g Na₂HPO₄
- 9 g NaCl
- pH should be 7.4
- Store indefinitely at 4°C

**Formaldehyde stock solution, 8%**

- Heat 800 ml H₂O to 65°C, then add 80 g paraformaldehyde granules (EM grade, Electron Microscopy Sciences) in a fume hood. Stir for 1 min, then slowly add 2 to 4 ml 5 N NaOH (until solution is clear). Let solution cool, then filter through Whatman no. 1 filter paper. Finally, add H₂O to 1 liter. Store up to 2 weeks at 4°C.

*continued*
Formaldehyde in liquid form (formalin; 37%) contains impurities such as methanol (6% to 15%) and formic acid (<0.05%), which make it unsuitable for many histochemical applications. Formaldehyde solution for these purposes is best prepared from paraformaldehyde, which is heated at >65°C to break the polymer into formaldehyde. Formaldehyde prepared from paraformaldehyde may either be used soon after preparation or stored as a stock solution for preparation of relatively fresh fixative solutions.

CAUTION: Formaldehyde is toxic, and the preparation process involving heating results in considerable vaporization, which increases the hazard. It is essential to use appropriate safety procedures, such as working in a fume hood.

**Formaldehyde in saline, 4%**

500 ml 8% formaldehyde stock solution (see recipe)
500 ml H₂O
9 g NaCl
Store indefinitely at 4°C

**Gelatin-subbing solution**

Dissolve 3.0 g gelatin in 150 ml distilled water and heat to 50°C with stirring until gelatin is completely dissolved. Add 0.3 g chromium potassium sulfate dodecahydrate and 450 ml distilled water, then mix 15 sec and filter through Whatman no. 1 filter paper. Prepare fresh.

**Potassium phosphate-buffered saline (KPBS, 0.02 M, pH 7.4)**

0.45 g KH₂PO₄
3.81 g K₂HPO₄·3H₂O
9 g NaCl (sodium chloride)
H₂O to 1 liter
Stir to dissolve
pH should be ~7.4 at room temperature
Store up to several weeks at 4°C

*It is advisable to make up the KPBS fresh every month.*

**Sucrose-infiltration solution**

0.1 M sodium phosphate buffer, pH 7.4 (APPENDIX 2A)
0.9% (w/v) saline
20% to 30% (w/v) sucrose
Store indefinitely at 4°C

**Thionin solution**

9 ml glacial acetic acid
1.88 g thionin (Sigma)
1.08 g NaOH
H₂O to 750 ml
Store in a dark bottle indefinitely at room temperature and filter before each use

**Triethanolamine/saline solution**

Dissolve 74 g triethanolamine and 36 g NaCl in 3500 ml distilled water, then adjust pH to 8.0 with 5 N NaOH. Add water to 4 liters. Store indefinitely at room temperature.

*If solution is to be in phosphate-buffered saline add 0.122 g KH₂PO₄ and 0.815 g Na₂PO₄*

**LITERATURE CITED**


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