Appendix A: Nissl Staining Procedure

The following procedure has been developed by D.M. Simmons for staining frozen sections of tissue that have been fixed with aldehydes. The sections are mounted on subbed slides (see below) and dried thoroughly (several days at room temperature or overnight at 40-60°C). Leaving the sections in 70% ethanol overnight before staining is often helpful; the sections adhere more tightly to the slide, and the background is more uniform and free of nonspecific staining. If the original fixation was poor, adding 10% formalin to the alcohol may be helpful.

Staining Procedure

a) 95% ethanol: 2X3 min.
b) 100% ethanol: 2X3 min.
c) xylene: 2X15 min. (removes lipids)
d) 100% ethanol: 2X2 min.
e) 95% ethanol: 2 min.
f) 70% ethanol: 2 min.
g) 50% ethanol: 2 min.
h) distilled water: 2 min.
i) 0.25% thionin stain: 5-20 sec.
j) distilled water: 5-10 dips
k) 50% ethanol: 2 min.
l) 70% ethanol: 2 min.
m) 95% ethanol: 2X2 min. (use acetic alcohol if needed; see Notes)
n) 100% ethanol: 2X4 min. (second bath must be fresh)

o) clearing agent (e.g., xylene): 3X4 min.

p) coverslip from last change of clearing agent

Notes

1. If the staining remains too dark after 95% ethanol (step m), accelerate differentiation with 95% ethanol containing 1% acetic acid; time will vary from a few dips to a minute or so. If overdifferentiation occurs, go backward to step (i), and restain.
2. Slides can be left several hours to overnight in a fresh change of clearing agent.
3. Xylene and toluene are common organic solvent clearing agents with the high refractive index needed for microscopic preparations. However, they are quite toxic, and substitutes such as Hemo-D have been introduced in recent years. Hemo-D has virtually no tolerance for water contamination, and takes at least twice as long as xylene to clear adequately. The 100% ethanol preceding Hemo-D must be very fresh; if there is significant water contamination, a milky precipitate will be seen when transferring the slides into Hemo-D. The problem is obvious, and you must return them to an anhydrous alcohol before trying Hemo-D clearing again. Very slight water contamination presents a less obvious problem: the slides may pass through Hemo-D and look fine to the naked eye. However, under the microscope, they have a slightly "murky" appearance. Not enough time in Hemo-D may produce a similar effect. Thus, clear for at least 30 min. when using Hemo-D. The advantage of Hemo-D is its low toxicity, so that it is not essential to use a fume hood. A good compromise is two changes of xylene (in the hood) after 100% ethanol, followed by three changes of Hemo-D.

Formula for the Thionin Stain (step i)

1. Solution A: 5% NaOH (W/V): 5 g in 100 ml distilled water.

2. Solution B: 6% glacial acetic acid (V/V): 6 ml + 94 ml distilled water.
3. Buffer for dissolving thionin stain powder:
   a) 18 ml Solution A + 100 ml Solution B + 382 ml distilled water.
   b) adjust pH to 4.5 (with sodium hydroxide or acetic acid).
4. Heat the above buffer solution to steaming; add 1.25 g of thionin (C.I. 52000) and bring to a boil for 1 h. Let cool, and return volume to 500 ml with distilled water. Store in a dark bottle, and filter before use. Can be used for several months.

Preparation of Subbed Slides

1. Place new microslides in slide racks: use gloves. Soak for a minimum of 30 min. (overnight is better) in hot tap water with strong Alconox, or other laboratory glassware detergent.
2. Rinse 30 min. in hot running tap water, until all detergent foaming is gone.
3. Rinse 30 min. in several changes of distilled water.
4. In a dust-free area (very important), drain the slides briefly and immerse for 2 min. in subbing solution. Cleaned and dried slides can also be subbed (coated) if they have been kept free of dust.

   Subbing Solution (make fresh):

5 g	gelatin (USP)
500 ml	distilled water (stir and heat to dissolve; do not exceed 50°C)
0.5 g	chromium potassium sulfate (chroma alum); stir to dissolve

   Filter, and use warm. Avoid creating bubbles, as they will leave ridges and uneven rings of gelatin on the slides.
5. Drain in dust-free area and dry overnight at 30-57°C.
6. Let cool, and store in original slide boxes, free from dust. They remain good for at least two

months.