

Paraffin Processing of Tissue

Fixation of Tissues

1. Where the best possible morphology is required, animals should be anesthetized and subjected to cardiac perfusion with saline, followed by a 10% formalin flush. If biochemical studies need to be performed on the tissue, a 10% formalin flush should not be used as it may interfere with subsequent analysis.
2. For routine stains where perfusion is not required, tissue is sectioned and drop-fixed in a 10% formalin solution. Fixative volume should be 20 times that of tissue on a weight per volume; use 2 ml of formalin per 100 mg of tissue.
3. Due to the slow rate of diffusion of formalin (0.5 mm hr), tissue should be sectioned into 3 mm slices on cooled brain before transfer into formalin. This will ensure the best possible preservation of tissue and offers rapid uniform penetration and fixation of tissue within 3 hours.
4. Tissue should be fixed for a minimum 48 hours at room temperature.
5. After 48 hours of fixation, move tissue into 70% ethanol for long term storage.
6. Keep fixation conditions standard for a particular study in order to minimize variability. (Although set times are best, tissue may be fixed for substantially longer periods without apparent harm.

A few notes on fixation

The usual fixative for paraffin embedded tissues is neutral buffered formalin (NBF). This is equivalent to 4% paraformaldehyde in a buffered solution plus a preservative (methanol) which prevents the conversion of formaldehyde to formic acid. Because of the preservative, NBF has a shelf life of months, whereas 4% PF must be made fresh. Optimal histology requires adequate fixation, about 48 hrs at room temperature for thinly sliced tissues. Inadequately fixed tissues will become dehydrated during tissue processing, resulting in hard and brittle specimens. Alcohol based fixatives generally do not give good morphology but may be useful in special cases (such as BrdU staining). A particular challenge for the histopathology is immunostaining fixed specimens. In many cases formaldehyde fixation will prevent recognition of epitopes by the primary antibody. Occasionally, "antigen retrieval" procedures will improve results but usually frozen sections are a better bet. An alternative approach, suitable for thin or porous tissues, is to perform immunohistochemistry on fresh tissues and then post-fix and embed the tissues in paraffin.

Decalcification of bone (optional)

After fixation, bone must be decalcified, or else it won't cut on the microtome:

- Immerse tissue cassette in 11% formic acid with a stir bar overnight in a fume hood.
- Rinse in running water for 30- 60 minutes (the smell should be gone).

Storage in 70% Ethanol

After adequate fixation tissues are transferred to 70% ethanol and may be stored at 4°C.

Paraffin infiltration

In this procedure, tissue is dehydrated through a series of graded ethanol baths to displace the water, and then infiltrated with wax. The infiltrated tissues are then embedded into wax blocks. Once the tissue is embedded, it is stable for many years.

The most commonly used waxes for infiltration are the commercial *paraffin waxes*. A paraffin wax is usually a mixture of straight chain or n-alkanes with a carbon chain length of between 20 and 40; the wax is a solid at room temperature but melts at temperatures up to about 65°C or 70°C. Paraffin wax can be purchased with melting points at different temperatures, the most common for histological use being about 56°C–58°C. At its melting point it tends to be slightly viscous, but this decreases as the temperature is increased. The traditional advice with paraffin wax is to use this about 2°C above its melting point. To decrease viscosity and improve infiltration of the tissue, technologists often increase the temperature to above 60°C or 65°C in practice to decrease viscosity.

In the schedule below, it is presumed that the working day is from 8:00 a.m. to 5:00 p.m. If other than that, appropriate adjustments should be made.

Tissue preparation

Thickness	No more than 3 mm thick.
Area	20 mm × 30 mm.
Fixed tissue	Cut large organs into 3 mm slices and store in neutral buffered formalin for 48 hours. Select tissue from fixed areas, trim to size and refix until the evening. If the trimmed sample is visibly unfixed, refix for a further 24 hours.
Unfixed tissue	Slices of tissue should be <i>thoroughly</i> fixed before processing.
Times	All times in processing fluids for this schedule are for tissues 3 mm thick or less. Tissues thicker than that will require longer times.
Clearing agent	Xylene or another clearing agent that will clear tissues in similar times should be used.
Processing time	This schedule takes 12 hours, and processes overnight. On weekends tissues should be left in fixative until Sunday evening with a 48 hour delay.

Trim fixed tissues and keep in neutral buffered formalin (NBF) until ready to proceed. Put tissues in a labeled (usually with pencil, as solvents dissolve the ink) cassette.

Once fixed, tissue is processed as follows, using gentle agitation, usually on a tissue processor, as follows:

1. 70% ethanol for 1 hour.
2. 95% ethanol (95% ethanol/5% methanol) for 1 hour.
3. First absolute ethanol for 1 hour .
4. Second absolute ethanol 1½ hours .
5. Third absolute ethanol 1½ hours.
6. Fourth absolute ethanol 2 hour.
7. First clearing agent (Xylene or substitute) 1 hour.
8. Second First clearing agent (Xylene or substitute) 1 hour.
9. First wax (Paraplast X-tra) at 58°C for 1 hour.
10. Second wax (Paraplast X-tra) at 58°C 1 hour.

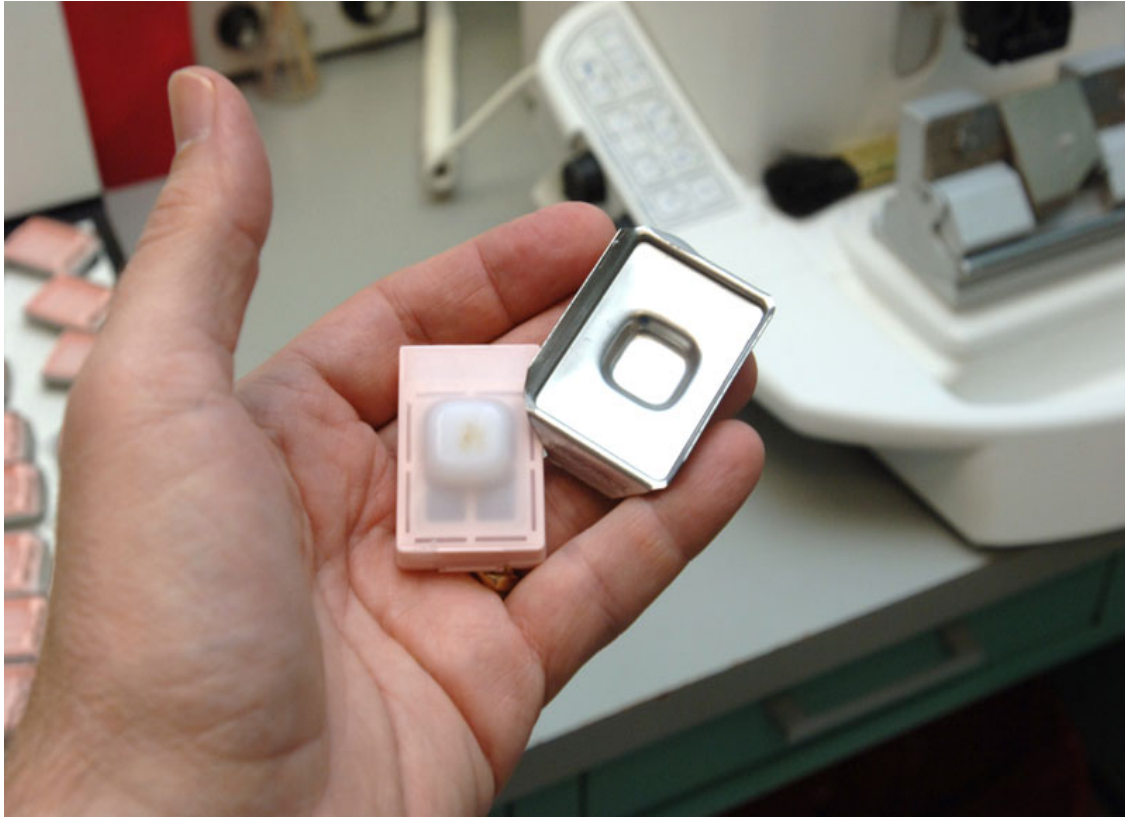
Due to the viscosity of molten paraffin wax, some form of gentle agitation is highly desirable. If the processor is to be run overnight it should be programmed to hold on the first ethanol bath and not finish until the next morning so the specimens do not sit in hot paraffin longer than the time indicated. If specimens are fresh they may incubate in formalin in the first stage on the machine. It is important to not keep the tissues in hot paraffin too long or else they become hard and brittle. Processed tissues can be stored in the cassettes at room temperature indefinitely.

Embedding tissues in paraffin blocks



Tissues processed into paraffin will have wax in the cassettes; in order to create smooth wax blocks, the wax first needs to be melted away placing the entire cassette in 58°C paraffin bath for 15 minutes. Turn the heat block on to melt the paraffin one hour before adding the tissue cassettes.

1. Open cassette to view tissue sample and choose a mold that best corresponds to the size of the tissue. A margin of at least 2 mm of paraffin surrounding all sides of the tissue gives best cutting support. Discard cassette lid.
2. Put small amount of molten paraffin in mold, dispensing from paraffin reservoir.
3. Using warm forceps, transfer tissue into mold, placing cut side down, as it was placed in the cassette.
4. Transfer mold to cold plate, and gently press tissue flat. Paraffin will solidify in a thin layer which holds the tissue in position.
5. When the tissue is in the desired orientation add the labeled tissue cassette on top of the mold as a backing. Press firmly.
6. Hot paraffin is added to the mold from the paraffin dispenser. Be sure there is enough paraffin to cover the face of the plastic cassette.
7. If necessary, fill cassette with paraffin while cooling, keeping the mold full until solid.
8. Paraffin should solidify in 30 minutes. When the wax is completely cooled and hardened (30 minutes) the paraffin block can be easily popped out of the mold; the wax blocks should not stick. If the wax cracks or the tissues are not aligned well, simply melt them again and start over.



The tissue and paraffin attached to the cassette has formed a block, which is ready for sectioning. Tissue blocks can be stored at room temperature for years.

Sectioning tissues

Tissues are sectioned using a microtome. Turn on the water bath and check that the temp is 35-37°C. Use fresh deionized water (DEPC treated water must be used if *in situ* hybridization will be performed on the sections). Blocks to be sectioned are placed face down on an ice block or heat sink for 10 minutes. Place a fresh blade on the microtome; blades may be used to section up to 10 blocks, but replace if sectioning becomes problematic. Insert the block into the microtome chuck so the wax block faces the blade and is aligned in the vertical plane.

Set the dial to cut 10 μM sections to order to plane the block; once it is cutting smoothly, set to 5 μM sections. The blade should be angled at 5°. Face the block by cutting it down to the desired tissue plane and discard the paraffin ribbon. If the block is ribboning well then cut another four sections and pick them up with forceps or a fine paint brush and float them on the surface of the 37°C water bath. Float the sections onto the surface of clean glass slides. If the block is not ribboning well then place it back on the ice block to cool off firm up the wax. If the specimens fragment when placed on the water bath then it may be too hot.

Place the slides with paraffin sections on the warming block in a 65°C oven for 20 minutes (so the wax just starts to melt) to bond the tissue to the glass. Slides can be stored overnight at room temperature.