Lesson 2

How to Prepare Histology Slides?

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It may be useful for medical students

to know a bit about

how to prepare histology slides as part of their general appreciation of laboratory biology and techniques used in medical research.

The *FIVE MAIN STAGES* in the preparation of histology slides are:

1. Fixing
2. Processing
3. Embedding
4. Sectioning
5. Staining

Paraffin Embedding Protocol

- Fixing in formaline (24 h.)
- Washing in running tap water (20 min.)
- 50% ethanol (20 min.)
- 70% ethanol (20 min.)
- 96% ethanol (2x 20 min.)
- 96% ethanol (for the night)
- Ethanol 100%: xylol (1:1; 20 min.)
- Xylene (3x 20 min.)- 3d shift t^o= 56 ^{o}C
- Xylene:Paraffin 1:1(20-25 min. t^o= 56 ^{o}C)
- Paraffin 1 (t^o =56 ^{o}C -1 h.)
- Paraffin 2 (t^o =56 ^{o}C - 1 h.)
- Paraffin 3 (cooling)

1.Fixing

- Samples of biological tissue are "fixed" to preserve the cells/tissue in as natural a state as possible and prevent postmortem decay (autolysis and putrefaction).
- Chemical fixatives are very carefully selected substances whose properties must meet many criteria.

Even the most careful fixation alters the sample to a certain extent and may potentially introduce artifacts that can interfere with interpretation of images of the fine detail of cells, incl. all their organelles, that can only be observed using an electron microscope (such fine detail of cells that can only be seen using electron microscopes is called "cellular ultrastructure").

Fixation of Tissues

- Where the best possible morphology is required, animals should be anesthesized 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.
- 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.
- 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.
- Tissue should be fixed for a minimum 48 hours at room temperature.
- After 48 hours of fixation, move tissue into 70% ethanol for long term storage.
- 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.

Fixing in Formalin

1. Formalin diffusion (1 mm/h)
2. Chemical Fixing

1.Fixing

- Because fixation is usually the first stage in a multistep process to prepare biological material for microscopy or other analysis, the choice of fixation method and specific fixative may depend on the subsequent processing steps appropriate in that particular case.

- **Chemical Fixation**
  In this case biological structures are preserved (both chemically and structurally) in a state as close to that of the living tissue as possible. This requires a chemical fixative that can stabilise the proteins, nucleic acids and mucosubstances of the tissue by making them insoluble.
- **Frozen Sections**
  Small pieces of tissue (typically 5mm x 5mm x 3 mm) are placed in a cryoprotective embedding medium then snap frozen in isopentane (an alkane) cooled by liquid nitrogen. Tissue is then sectioned in a freezing microtome or cryostat. Sections are then fixed by immersion in a specific fixative or series of fixatives for carefully controlled period of time.

Advantages - of fixation by frozen sections	Disadvantages - of fixation by frozen sections
•Give better preservation of antigenicity •Minimal exposure to fixative •Not exposed to the organic solvents	•Lack morphological detail •Possibility of biohazard

2.Processing

- Tissue processing is done to <u>remove water</u> from the biological tissues, replacing such water with a medium that solidifies, setting very hard and so allowing extremely thin sections to be sliced. This is important because biological tissue must be supported in an extremely hard solid matrix to enable sufficiently thin sections to be cut. Some typical values are:

5 µm thick for light microscopy

5 µm (i.e. 5 micrometres) = 0.005 mm = 0.000005 metre

80-100 nm thick for electron microscopy

80-100 nm (i.e. 80-100 nanometres) = 0.00008 mm to 0.0001 mm = 0.00000008 to 0.0000001 metre

3.Embedding

After tissues have been dehydrated and before they can be "sectioned" i.e. sliced very thinly they must be secured in a **very hard solid block.**

Different types of embedding techniques and materials are used depending on the sample being prepared and the other types of processing involved in preparing that particular sample.

In general, tissue samples are placed in molds together with liquid embedding material which is then hardened. **The result of this stage in the preparation of histology slides is hardened blocks containing the original biological samples together with other substances used so far in the preparation process**.

4.Sectioning

- Sectioning an embedded tissue sample is the step necessary to produce sufficiently thin slices of sample that the detail of the microstructure of the cells/tissue can be clearly observed using microscopy techniques (either light microscopy or electron microscopy).
- Possible orientations at which tissue samples may be sectioned include:

Vertical sectioning perpendicular (i.e. at right-angles) to the surface of the tissue. This is the most common method.

Horizontal sectioning is often done for the study of hair follicles and structures that include hairs, hair follicles, arrector pili muscles, and sebaceous glands in general. Such structures are sometimes called "pilosebaceous units".

Tangential to horizontal sectioning is done in chemosurgery, which is a form of microscopically controlled surgery used to treat certain types of skin cancer.

- The method used to actually cut sections from the hardened block of tissue depends on the type of microscopy that will be used to observe it and hence the thickness of sample required. In the case of samples to be studied using light microscopy, a steel knife mounted in a microtome may be used to cut 10µm tissue sections which are then mounted on a glass microscope slide. In the case of samples to be studied using transmission electron microscopy, a diamond knife mounted in an ultramicrotome may be used to cut 50 nm tissue sections which are then mounted on a 3-millimeter-diameter copper grid.

5.Staining

- Finally, the mounted sections are treated with an appropriate histology stain.
- Why are histology samples stained ?
- Biological tissue has very little variation in colours/shades when viewed using either an ordinary light (optical) microscope or an electron microscope. Staining biological tissues is done to both increase the contrast of the tissue and also highlight some specific features of interest - depending on the type of tissue and the stain used.

- **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.

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 max 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.

- 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.
- 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.
- Put small amount of molten paraffin in mold, dispensing from paraffin reservoir.
- Using warm forceps, transfer tissue into mold, placing cut side down, as it was placed in the cassette.
- Transfer mold to cold plate, and gently press tissue flat. Paraffin will solidify in a thin layer which holds the tissue in position.
- When the tissue is in the desired orientation add the labeled tissue cassette on top of the mold as a backing. Press firmly.
- 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.
- If necessary, fill cassette with paraffin while cooling, keeping the mold full until solid.
- 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.

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 *thoroughly* 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.