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Chapter 11

Paraffin block preparation and histological staining: Techniques for tissue structure analysis

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1. Introduction:

Fixation is vital for preserving tissue integrity for histological examination, aiming to maintain tissues in a lifelike state. Immediate fixation, post-surgery or autopsy, is crucial to prevent autolysis. Tissues are prepared for transformation into thin microscopic slices following fixation. One important method is to embed tissues in paraffin that matches their density. Using a microtome, paraffin-embedded tissues are cut into micrometer-thin slices, with paraffin serving as support. However, in order for water- and alcohol-soluble dyes to penetrate, paraffin must be eliminated during staining. The Hematoxylin and Eosin stain, commonly used in histology, gives distinct coloration to cellular components. Hematoxylin, which binds to nucleic acids, stains cell nuclei bluish-purple. Eosin, binding to cytoplasmic elements, imparts a pink hue to the background. Hematoxylin requires a mordant to enhance staining affinity. Mordants, high-molecular-weight metal salts, form strong bonds with tissues and dyes, ensuring effective staining.

2. Materials Required:

1. Aldehyde fixatives (e.g., formalin, 10% neutral buffered formalin, 4% paraformaldehyde, glutaraldehyde).
2. Large cover glass,
3. slides,
4. Glass vials,
5. Coplin jars.
6. Microtome, Blade,
7. Tissue,
8. Paraffin Wax

3. Procedure:

3.1. Tissue Fixation:

1. Using a sharp surgical blade, carefully remove any excess blood from the tissue of a recently euthanized animal and slice it thinly.
2. Make sure the fixative volume is 20 times the tissue weight before placing the tissue slices in a glass vial with a 10% formalin solution.
3. To accommodate the slow diffusion rate of formalin, slice tissue sections into 3 mm pieces before transferring them to formalin to ensure rapid and uniform fixation within 3 hours.
4. Fix the tissue for a minimum of 48 hours at room temperature.
5. Transfer the fixed tissue to 50% ethanol for 30 minutes, followed by 70% ethanol for long-term storage, maintaining consistent fixation conditions for each study.

3.2. Tissue Processing:

Gently transfer the fixed tissue through the following series of solutions:

1. Ethanol (70%) for 1 hour
2. Ethanol (95%) for 1 hour
3. First (100%) ethanol for 1 hour
4. Second (100%) ethanol for 1½ hours
5. Third (100%) ethanol for 1½ hours
6. Fourth (100%) ethanol for 2 hours
7. Immerse the sample in the first clearing agent (e.g., Xylene or its substitute) for 1 hour.
8. Transfer the sample to a second fresh clearing agent (e.g., Xylene or its substitute) for an additional 1 hour.
9. Place the sample in the first paraffin wax (e.g., Paraplast X-tra) at 58 °C for 1 hour in an incubator to initiate infiltration.
10. Move the sample to a second paraffin wax bath (e.g., Paraplast X-tra) at 58 °C for another 1 hour to ensure complete infiltration.

3.3. Setting up the Paraplast Block:

1. **Preparation:** Ensure the Paraplast block is thoroughly dry.
2. **Embedding:** Pour molten wax into the block without creating bubbles.
3. Using a hot needle to ensure appropriate orientation, carefully insert the tissue into the melted wax.
4. Before inserting the tissue into the wax block, make sure it hasn't cooled.
5. To set and solidify, place the block of Paraplast into the molten Paraplast and allow it to cool in cold water.
6. After pressing the Paraplast block into the molten Paraplast, let it cool in cold water to set and solidify.

7. **Storage:** Paraplast blocks allow for the permanent storage of paraffin-embedded tissues.

3.4. Sectioning of Paraffin-Embedded Tissue Using a Microtome:

3.4.1 Preparing the Paraplast Block:

1. Remove excess Paraplast from the stainless-steel mold using a razor blade, exposing the tissue surrounded by Paraplast.
2. Trim the face of the paraffin block carefully, ensuring that the tissue is encased within a 1 mm thick frame of paraffin on all sides.
3. Trim the block's upper and bottom borders to guarantee parallelism.
4. Trim the lateral surfaces to create a trapezoid-like face, facilitating proper orientation and sectioning.

3.4.2. Preparing the Microtome for Sectioning:

1. For ten minutes, place the Paraplast blocks face down on a heat sink or ice block.
2. Make sure the chuck mount is retracted before cleaning and lubricating the microtome.
3. Fit the microtome with a new blade.
4. For in-depth tissue analysis, set the section thickness to 10 microns, while thinner sections as thin as 5 microns are also a possibility.
5. Set the blade angle to 5º.
6. Note that blades can be used for up to 10 blocks before replacement if sections are not being properly made.
7. Secure the block into the microtome chuck with the wider end of the trapezoid aligned against the knife edge, ensuring that both the upper and lower surfaces remain parallel to the blade.
8. Maintain the blade angle without adjustment, moving the blade toward the block until it barely clears it, then lock it in place.

3.5. Sectioning Procedure:

1. Turn on the water bath and set the temperature between 37–40 °C.
2. Fill the bath with fresh deionized water. Begin by trimming the block to reach the desired tissue plane, discarding the initial paraffin ribbons.
3. As you continue sectioning the Paraplast block, complete trapezoid-shaped tissue sections will begin to appear.
4. Confirm that the sections form continuous ribbons during cutting for optimal handling and mounting.

3.6. Section Handling Procedure:

1. Once consistent ribboning is achieved, cut four additional sections. Use fine-tipped
2. forceps or a soft paintbrush to gently transfer the sections onto the surface of a 40 °C water bath.
3. Allow the sections to flatten and eliminate folds in the warm water. Then, carefully mount them onto clean glass slides. Limit each slide to no more than four sections to accommodate paraffin expansion, which can reach up to 20%.
4. Place the prepared slides on a warming block set to 55 °C—approximately 4–5 °C below the wax's melting point. Leave the slides until the wax surrounding the
5. tissue softens and turns translucent, ensuring the sections retain their original shape.
6. Once affixed, the tissue sections will appear translucent. Label each slide by either attaching pre-printed labels or marking directly on the slide using a diamond-tipped glass pen.
7. Store the slides at room temperature overnight to ensure proper adhesion before staining.

3.6. Staining Procedure:

1. Use Coplin jars for the staining process.
2. To deparaffinize the sections, immerse each slide in xylene (twice), ensuring the tissue remains firmly attached to the glass surface.
3. Rehydrate the tissue sections through a descending alcohol series:
   a) Xylene I – 5 minutes
   b) Xylene II – 2 minutes
   c) 100% ethanol – 3 minutes
   d) 95% ethanol – 3 minutes
   e) 70% ethanol – 3 minutes
   f) 50% ethanol – 3 minutes
   g) 35% ethanol – 3 minutes
   h) Distilled water – 3 minutes
4. Stain the tissue with Delafield’s hematoxylin for approximately 5 minutes.
5. Rinse the stained slides under running tap water. Examine briefly under a microscope: nuclei should appear dark blue, and cytoplasm should be light grey or pale blue.
6. Continue with:
   i) 35% ethanol – 2 minutes
   j) 50% ethanol – 2 minutes

7. If cytoplasmic overstaining occurs (appears blue), use acid alcohol (two drops of concentrated HCl in a Coplin jar of 70% ethanol) to gently decolorize until nuclei appear red-purple. Avoid excessive decolorization.
8. Immerse in 70% ethanol – 1 minute
9. Transfer to 70% ethanol saturated with lithium carbonate – 5 minutes or until nuclei appear blue. If under-stained, repeat the alcohol sequence and re-stain with hematoxylin.
10. Continue dehydration and counterstaining:
    a) 95% ethanol – 2 minutes
    b) Eosin Y – 2 to 5 minutes
    c) Rinse with 95% ethanol for 2–3 minutes to remove excess eosin. Extend time if the stain is too intense.
    d) 100% ethanol I – 2 minutes
    e) 100% ethanol II – 2 minutes
    f) Xylene I – 5 minutes
    g) Xylene II – 5 minutes
11. After the final xylene rinse, drain excess liquid.
12. Apply a small drop of mounting resin over the tissue sections.
13. Gently place a coverslip over the sections, ensuring no drying occurs during the process.
14. Allow the mounted slides to air-dry overnight at room temperature.

4. Precautions:

1. Fixation Time: Soft tissues (e.g., testis, lung) should remain in fixative for at least 15 minutes before sectioning. Proper fixation requires cutting after 30–45 minutes.
2. Avoid Wax Crystallization: To prevent wax crystals from forming due to sudden temperature changes between tissue and molten wax, embedding should be performed quickly and steadily.
3. Prevent Fogging: Rapid transitions between staining solutions help minimize humidity-induced fogging on slides.
4. Reagent Quality: Use only high-purity alcohol and reagents for optimal staining results.
5. Humidity Considerations: In humid climates or monsoon seasons, dehydration may slow down. Adjust immersion times or concentrations of 70%, 90%, and 100% ethanol and xylene accordingly.
6. Drain Excess Solution: During staining, gently tap the bottom of the slide on filter paper before moving it to the next solution. This helps remove residual liquid and ensures consistency.

5. References:

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