THE SCIENCE WORLD
A Monthly e Magazine
Popular Article
ISSN:2583-2212
December, 2022; 2(12), 2007-2011

An Overview: Histopathological Techniques

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https://doi.org/10.5281/zenodo.7411043

Introduction

Histological technique deals with the preparation of tissue for microscopic examination.The aim of good histological technique to preserve microscopic anatomy of tissue and makethem hard, so that very thin section (4 to 5 micron) can be made. After staining, the sectionshould represent the anatomy of the tissue as close to as possible to their structure in life. This is achieved by passing the total as selected part of the tissue through a series of process.

These processes are:

1. Fixation 2. Dehydration 3. Cleaning 4. Embedding 5. Cutting 6. Staining

Fixation:

This is the process by which the constituents of cells and tissue are fixed in a physical and partly also in a chemical state so that they will withstand subsequent treatment with variousreagents with minimum loss of architecture. This is achieved by exposing the tissue to chemical compounds, call fixatives.

Mechanism of action of fixatives:

Most fixatives act by denaturing or precipitating proteins which then form a sponge or meshwork, tending to hold the other constituents. Good fixative is most important factors inthe production of satisfactory results in histopathology.

Following factors are important:

 Fresh tissue Proper penetration of tissue by fixatives Correct choice of fixatives

No fixative will penetrate a piece of tissue thicker than 1 cm. For dealing with specimenthicker than this, following methods are recommended:

1. Solid organ: Cut slices as necessary as but not thicker than 5 mm.

2. Hollow organ: Either open or fill with fixative or pack lightly with wool soaked infixative.
3. Large specimen, which require dissection: Inject fixative along the vessels or bronchi asin case of lung so that it reaches all parts of the organs.

Properties of an Ideal Fixative:

1. Prevents autolysis and bacterial decomposition.
2. Preserves tissue in their natural state and fix all components.
3. Make the cellular components insoluble to reagent used in tissue processing.
4. Preserves tissue volume.
5. Avoid excessive hardness of tissue.
6. Allows enhanced staining of tissue.
7. Should be non-toxic and non-allergic for user.
8. Should not be very expensive.

Factor affecting fixation:

1. Size and thickness of piece of tissue.
2. Tissue covered by large amount of mucous fix slowly.
3. The same applies to tissue covered by blood or organ containing very large amount ofblood.
4. Fatty and lipomatous tissue fix slowly.
5. Fixation is accelerated by agitation.
6. Fixation is accelerated by maintaining temperature around $60^{o}c$.

Classification of Fixatives:

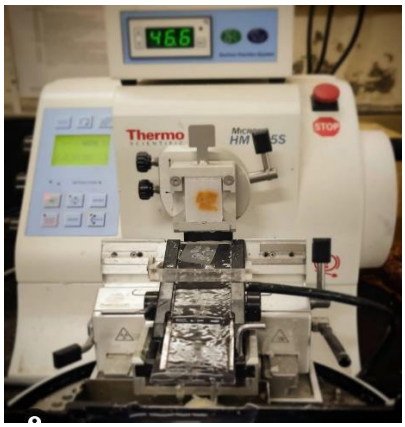
A. Tissue fixatives
 a. Buffered formalin b. Buffered glutaraldehyde c. Zenker's formal saline d. Bowen's fluid
B. Cytological fixatives
 a. Ethanol b. Methanol c. Ether
C. Histochemical fixatives
 a. Formal saline b. Cold acetone c. Absolute alcohol

Formalin is sold as 40% w/w solution of formaldehyde gas in water. It is used as 10% solution in water or normal saline. It does not precipitate protein but combine with NH_2 group to form an insoluble gel, preserve particularly all elements including fats. It keeps phospholipids in soluble in fat solvents. Tissue can remain in it for prolonged periods without distortion. It is compatible with most special stain. It is the cheapest and most popular fixative.

Tissue Processing:

In order to cut thin sections of the tissues, it should have suitable hardness and consistency when presented to the knife edge. These properties can be imparted by infiltrating and surrounding the tissue with paraffin wax, colloid in or low viscosity nitrocellulose, varioustypes of resins or by freezing. This process is called tissue processing. It is done in stages. It can be subdivided into dehydration, clearing, impregnating and embedding. It is importantthat all specimens are properly labeled before processing is started. For labeling, pen containing ordinary ink should not be used. Printed, graphite pencil written, type-written orIndia ink written labels are satisfactory. A system of transportation is required to carry the tissue through various steps in processing. The cut specimens are put in muslin cloth together with their labels

and are then transported from reagent to reagent in metal containersthat have perforated walls, so that the reagent enters into the tissues. Tissue processing is a long procedure and required 24 hours. Tissue processing can be done by manually or automatic tissue processing.



Sequence of manual tissue processing:

A. Dehydration:

Tissues are dehydrated by using increasing strength of alcohol; e.g. 70%, 90% and 100%. The duration for which tissues are kept in each strength of alcohol depends upon the size oftissue, fixative used and type of tissue; e.g. after fixation in aqueous fixative delicate tissueneed to be dehydrated slowly starting in 50% ethyl alcohol directly whereas most tissue specimens may be put into 70% alcohol. Delicate tissue will get high degree of shrinkage by two great concentrations of alcohol. The volume of alcohol should be 50-100 times thatof tissue

B. Clearing:

During dehydration water in tissue has been replaced by alcohol. The next step alcohol should be replaced by paraffin wax. As paraffin wax is not alcohol soluble, we replace alcohol with a substance in which wax is soluble. This step is call clearing.

Clearing of tissue is achieved by any of the following reagents:

- Xylene, Chloroform, Benzene, Carbon, Tetrachloride, Toluene

Xylene is commonly used. Small piece of tissue is cleaned in 0.5 – 1 hour; whereas larger (5 cm or more thick) are cleaned in 2-4 hours.

C. Impregnation with Wax:

This is allowed to occur at melting point temperature of paraffin wax, which is 54-60$_o$C. Volume of wax should be about 25-30 times the volume of tissues. The duration of impregnation depends on size and types of tissues and the clearing agents employed. Longerperiods are required for larger pieces and also for harder tissue like bones and skin as compared to liver kidney, spleen, lung etc. Xylene is easiest way to remove. Total durationof 4 hours is sufficient for routine impregnation.

Types of Wax employed for Impregnation:

1. Paraffin wax 2. Water soluble wax 3. Other material, like colloid in, gelatin, paraplast etc.Paraffin wax is used routinely. It has hard consistency, so section of 3–4-micron thickness can be cut.

Summary of Paraffin Wax Embedding:

Dehydration

70% alcohol 1 hour > 90% alcohol I 1 hour > 90% alcohol II 2 hours > 100% alcohol I 1 hour > 100% alcohol II 2 hours >100% alcohol III 2 hours

Clearing

Xylene I 2 hours > Xylene II 2 hours

Wax Impregnation

Paraffin wax 1 1 hour >Paraffin wax II 1 hour >Paraffin wax III 1 hour

D. Blocking:

Impregnated tissues are placed in a mould with their labels and then fresh melted wax is poured in it and allowed to settle and solidify. Once the block has cooled sufficiently to forma surface skin it should be immersed in cold water to cool it rapidly.

After the block has completely cooled it is cut into individual blocks and each is trimmed. Labels are made to adhere on the surface of the block by melting the wax with a metal stripsufficiently wormed.

Section Cutting:

- Before cutting section, block is trimmed and cooled
- Place tissue block in microtome and knife is adjusted at particular angle advance bladetoward block
- To remove crease on the section they are put in the water bath having temp. 1-2 °C belowthe melting point of paraffin
- To fix the section on the slide, the solution of albumin glycerin mixture is applied on theslide
- Slide is dried at room temp./automatic slide drier

Staining:

Staining is a process by which we give colour to a section. There are hundreds of stains available.

Procedure of staining:

Every stain is to be used according to a specified method. Staining can be done either manually or in an automatic stainer.

Manual Staining:

In a small laboratory when a few slides are stained daily, this is the method of choice. Although it is time consuming it is economical. Different reagent containers are placed in aspecial sequence and the slides are removed from one container to another manually.

***Automatic staining*:**

In a busy histopathology laboratory when hundreds of slides are stained daily, an automaticstainer

is required. This method has different containers of staining reagents. They are arranged according to the desired sequence. It has a timer, which controls the time for stay of slides in a given container. It has a mechanical device which shifts the slides from one container to next after the specified time. Advantages of automated stainer are:

a. It reduces the man power
b. It controls the timing of staining accurately
c. Large number of slides can be stained simultaneously
d. Less reagents are used

Slides stained either manually or by automatic stainer, pass through same sequences.

Haematoxylin and Eosin staining:

It is the most common used routine stain in histopathology laboratory.Reagents:

1. Harris's Haematoxylin 2. Acid alcohol 3. Ammonia water 4. Alcoholic eosin solution

Staining Procedure:

1. Put the sections fixed on slides in xylene for 3 minutes.
2. Then transfer to absolute alcohol for 3 minutes.
3. Transfer to 80% alcohol for 2 minutes.
4. Place in 50% alcohol for 2 minutes.
5. Wash the slide in running tape water for 1 minute and put in Harris's Haematoxylin for5-7 minutes.
6. Wash in running tape water for 30 seconds
7. Wash excess dye in 1% acid alcohol by continuous agitation for 15 second.
8. Wash in running tape water for 30 seconds
9. Give 2-3 dips in ammonia water solution until tissues attain a blue colour.
10. Wash in running tape water for 30 seconds
11. Counter stain with eosin for 3-5 minutes.
12. Wash in running tape water for 30 seconds
13. Dehydrate by keeping in increasing concentration of alcohol (2-3 minutes in 50%, 70%,95% and absolute alcohol).
14. Clear it in xylene and mount with DPX or Canada balsam.Result:

Nuclei - Bright blue
Muscle, keratin - Bright pink **Collagen and cytoplasm** - Pale pink**Erythrocytes** - Orange red

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