

Histopathology Techniques: Tissue Processing and Staining

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Method of Biopsy Taking:

1. Incisional biopsy
2. Excisional biopsy
3. Punch biopsy
4. Core needle biopsy
5. Curettage biopsy

Incisional biopsy:

In this method only a portion or wedge of tissue from a large lesion is taken and therefore, the procedure is strictly a diagnostic nature. It is performed when removal of entire lesion is impossible and often performed prior to major surgical procedure.

Excisional Biopsy:

In this technique, the entire lesion is removed, usually with a rim of normal tissue and therefore, the procedure serves the diagnostic and therapeutic function. Excising the entire lesion ensures sufficient tissue for histopathological examination, lessen the risk of tumour dissemination and eliminate sampling errors. It is performed when the lesion is smaller in size.

Punch Biopsy:

It is done by biopsy forceps. It is performed in the lesion of uterine cervix, oral cavity, esophagus, stomach, intestine and bronchus.

Core Needle Biopsy:

It is done with special type of wide bore biopsy needle. It permits a percutaneous approach to internal structures. Sampling errors are a significant problem in needle biopsy.

Curettage Biopsy:

Curetting are usually done for diagnosis of endometrial disease.

Choice of Biopsy Procedure:

The choice of appropriate procedure is dictated by anatomic consideration, biology of tumour type and by the request of pathologists.

Some General Rules for the biopsy Procedure:

1. The larger the lesion, the numerous the biopsies that should be taken from it because of the variability in the pattern that may exist and the fact that the diagnostic areas may be present only focally.
2. In ulcerated tumour, biopsies of the central ulcerated areas may show only necrosis and inflammation. Biopsies should be taken from the periphery that includes normal and diseased tissue.
3. The biopsies should be deep enough that the relationship between tumour and stroma can be properly assessed.
4. Deeply seated lesions are sometimes accompanied by a prominent peripheral tissue reaction which may be characterized by chronic inflammation, hyperemia, fibrosis, calcification and metastatic bone formation. If the biopsy is too peripheral, this may be the only tissue obtained.
5. When several fragments of tissue are obtained they should be sent to the pathology laboratory and all of them submitted for microscopic examination.
6. Crushing or squeezing of the tissue with forceps should be carefully avoided.
7. Once the biopsy is obtained, it should be placed immediately into container with adequate volume of fixative.

Handling of Specimen

Specimen should be transported in glass, plastic or metal container or in a plastic bag in 10% formalin. If formalin is not available at hand, place the specimen in refrigerator at 4°C to slow down autolysis. The container should have an opening larger enough so that the tissue can be removed easily after it has hardened by fixation. However, fresh material is needed for the following purpose:

1. Frozen section
2. Immunocytochemistry
3. Cytological examination
4. Microbiological sampling before histopathology
5. Chromosome analysis
6. Research purpose
7. Museum display

Requirement in Histopathology Dissection Room

1. A cutting board designed in such a fashion that all the fluids will flow directly into the sink.
2. Selves for specimen container.
3. Ready access to sink with hot and cold water.
4. Ready access to formalin.
5. Box of instruments including heavy and small scissors, different sized smooth and toothed forceps, a malleable probe, a scalpel handle, disposable blades, a long knife and pins for attaching specimen to a cork surface.
6. Labels.
7. A large formalin container.
8. Container with other fixative with instruction on how to mix them at the time of use.
9. Hard saw
10. Balance.

General Principle of Gross Examination:

1. Proper identification and orientation of the specimen.
2. Unlabelled specimen should never be processed.
3. A properly completed histopathology requisition form containing patient's name, age, sex, relevant clinical data, surgical findings, nature of operation and name of tissue submitted.
4. Careful search and examination of all the tissue submitted in order.
5. Surgeon should be instructed to submit all the material that they have removed, not the selected portion from it.
6. Place the specimen on cutting board in an anatomic position and record the following information:
 - a. Types of specimen
 - b. Structure included.
 - c. Dimensions
 - d. Weight
 - e. Shape
 - f. Colour
 - g. Consistency
 - h. Surgical margin, whether included or not involved by tumour.
7. Measurements are usually given in centimeter unless the specimen is very small in which mm can be used.
8. Endometrial and prostatic tissue should be measured by aggregate pieces in volume.
9. Endoscopic biopsies should be numbered.

Sampling for Histopathological Examination:

- Tissue submitted for histopathology must not be more than 3 mm thick and not larger than the diameter of slides used. Most specimens from solid tissues are cut in the form of pieces measuring 10 to 15 mm on the slides and 2 to 3 mm in thickness. Adipose tissue must be cut even thinner.
- Discrete areas of calcification or ossification should be taken out and should be decalcified in nitric acid.
- Small fragments of tissue must be wrapped in thin paper.
- If the fragments are very small, it should be stained by haematoxylin to facilitate their identification by the histopathologist.
- All tissue should be submitted in a diagnostic endometrial curettage. However, if the procedure was done for incomplete abortion and gross examination shows obvious product of conception, one representative section is more than adequate.
- Determination of surgical margin is helpful by painting them with India ink or a similar pigment before sectioning.

Histological Technique:

Histological technique deals with the preparation of tissue for microscopic examination. The aim of good histological technique to preserve microscopic anatomy of tissue and make them hard, so that very thin section (4 to 5 micron) can be made. After staining, the section should 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 various reagents 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 in the 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 specimen thicker 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 in fixative.
3. Large specimen, which require dissection: Inject fixative along the vessels or bronchi as in 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.

Temperature:

The fixation can be carried out at room temperature. Tissue should not be frozen once it has been placed in the fixative solution, for a peculiar ice crystals distortion will result.

Speed of fixation:

The speed of fixation of most fixative is almost 1 mm/hour. Therefore, a fixation time of several hours is needed for most specimens.

Amount of fixative fluid:

This should be approximately 10-20 times the volume of the specimen. Fixative should surround the specimen on all sides.

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 of blood.
4. Fatty and lipomatous tissue fix slowly.
5. Fixation is accelerated by agitation.
6. Fixation is accelerated by maintaining temperature around 60°C.

Classification of Fixatives:

- A. Tissue fixatives
 - a. Buffered formalin
 - b. Buffered gluteraldehyde
 - 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

Routine Formalin:

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 NH₂ group to form an insoluble gel, preserve particularly all elements including fats. It keeps phospholipids insoluble 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.

Ethyl Alcohol:

It is used in 90-100% strength. It precipitates albumin but not nucleoprotein. It causes shrinkages and hardening of tissues. It destroys mitochondria. It preserves glycogen and is useful for histochemistry of glycogen, uric acid, iron etc.

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, colloidin or low viscosity nitrocellulose, various types 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 important that 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 or India 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 containers that 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 mechanically.

Manual Tissue Processing:

In this process the tissue is changed from one container of reagent to another by hand.

Mechanical Tissue Processing:

Automatic tissue processors are available. In this processor, there are different jars containing reagents. These are arranged in a sequence. The tissue is moved from one jar to another by a mechanical device. Timings are controlled by a timer which can be adjusted in respect to hours and minutes. Temperature is maintained around 60°C.

The processing, whether manually or mechanically, involves the same steps.

*Sequence of manual tissue processing:**A. Dehydration:*

Tissues are dehydrated by using increasing strength of alcohol; e.g. 50%, 70%, 90% and 100%. The duration for which tissues are kept in each strength of alcohol depends upon the size of tissue, fixative used and type of tissue; e.g. after fixation in aqueous fixative delicate tissue need 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 concentration of alcohol.

The volume of alcohol should be 50-100 times that of 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 are cleaned in 0.5 – 1 hour; whereas larger (5 cm or more thick) are cleaned in 2-4 hours.

Impregnation with Wax:

This is allowed to occur at melting point temperature of paraffin wax, which is 54-60°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. Longer periods 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 duration of 4 hours is sufficient for routine impregnation.

Types of Wax employed for Impregnation:

1. Paraffin wax
2. Water soluble wax
3. Other material, like colloidin, gelatin, paraplast etc.

Paraffin wax is used routinely. It has hard consistency, so section of 3-4 micron thickness can be cut.

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 form a 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 strips sufficiently wormed.

*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 I	1 hour
Paraffin wax II	1 hour
Paraffin wax III	1 hour

Staining:

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

Classification of Stains:

Generally the stains are classified as:

- A. Acid stains
- B. Basic stains
- C. Neutral stains

All dyes are composed of acid and basic components. Dye is a compound which can colour fibres and tissue constituents.

Acid Dyes:

In an acid dye the basic component is coloured and the acid component is colourless. Acid dyes stain basic components e.g. eosin stains cytoplasm. The colour imparted is shade of red.

Basic Dyes:

In a basic dye the acid component is coloured and the basic component is colourless. Basic dyes stain acidic components e.g. basic fuchsin stains nucleus. The colour imparted is shade of blue.

Neutral Dyes:

When an acid dye is combined with a basic dye a neutral dye is formed. As it contains both coloured radicals, it gives different colours to cytoplasm and nucleus simultaneously. This is the basis of Leishman stain.

Special stains:

When a specific components of tissue e.g. fibrous tissue, elastic tissue, nuclear material is to be stained, certain special stains are used which specifically stain that component tissue.

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 a special 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 automatic stainer 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 tap water for 1 minute and put in Harris's Haematoxylin for 5-7 minutes.
6. Wash in running tap water for 30 seconds
7. Wash excess dye in 1% acid alcohol by continuous agitation for 15 second.
8. Wash in running tap water for 30 seconds

9. Give 2-3 dips in ammonia water solution until tissues attain a blue colour.
10. Wash in running tap water for 30 seconds
11. Counter stain with eosin for 3-5 minutes.
12. Wash in running tap 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

Special Stains:

1. **PAS** (Periodic Acid Schiff) stain: This stain demonstrates glycogen and neutral mucous substances, outlines basement membranes and reticulin and makes evident most types of fungi and parasites.
2. Stains for micro-organism:
 - a. **Gram-stain:** Gram stain allows the separation of bacteria those that retain the crystal-violet-iodine complex (gram-positive) and those that are decolorized by alcohol treatment and counterstained by eosin, safranin or fuchsin.
 - b. **Ziehl-Neelsen stain:** This stain detect acid fast bacilli.
 - c. **PAS stain:** It is used for fungi, amoeba and Tricomonas.
 - d. **Modified Giemsa** (2% Giemsa in water): Detects Helicobacter pylori.
3. **Congo-red:** It is used for identification of amyloid.
4. **Sudan-Black:** It is used for fat staining.
5. **Masson's Trichrome:** It is used for differentiation of connective tissue elements.
6. **Papanicolaou's stain:** It is used to stain cells in cervical and sputum smear for cytology.

Frozen Section:

The frozen section is a technique in which tissue is frozen rapidly to the temperature of -20°C and the sections are cut and stained. In this way tissue can be examined microscopically within 5-10 minutes of its removal from the body. It reduces the time of processing from 18 hours to 5 minutes. It has the disadvantage that only 8-16 micron thick section can be cut and finer details of tissue can not be examined. Frozen section is performed on a machine called cryostat. Following are the situations where frozen sections are helpful:

1. When rapid diagnosis regarding benign or malignant nature of lesion is required to decide the extent of surgery while the patient is still on the operation table.
2. When study of fat, proteins or antigenic markers is required, as they are destroyed by routine processing of tissue.

Precautions:

1. Frozen section is an emergency.
2. Laboratory workers should always be informed about frozen section before hand.

3. All preparations are completed before arrival of tissue.
4. Cryostat should preferably remain “ON” all the time to maintain its temperature at -20°C.
5. Deal with tissue urgently on arrival in the laboratory.

Procedure:

1. Gross examination of tissue by pathologist.
2. Tissue is placed in a metallic block is covered with approximate amount of isopentane. Isopentane has the property to freeze rapidly at -20°C.
3. Block-holder is placed over the freezing stage of cryostat close to glass door of cryostat to maintain its temperature.
4. Isopentane along with tissue is frozen within 1-2 minutes.
5. Open the door of cryostat. Transfer the block holder to its stage and fix it.
6. Spray the block and knife with isopentane to maintain temperature.
7. Trim the block with cutting mechanism adjusted at 25 micron thickness.
8. Before cutting the actual sections replace the “anti roll plate” . Make 8-10 micron thick sections.
9. Sections are transferred to slides, which are then rapidly taken to the staining rack.
10. Routinely frozen sections are stained with Haematoxylin and Eosin by following method. This method is called rapid haematoxylin and eosin staining.
 - a. Dip the slide in tap water once.
 - b. Dip in Harris haematoxylin for 1-2 minutes.
 - c. Rinse in tap water.
 - d. Differentiate in 1% acid alcohol, one dip only.
 - e. Bluing is done in ammonia, one or two dip only.
 - f. Rinse in running tap water.
 - g. Dip in eosin for 0.5 – 1 minute.
 - h. Rinse in water.
 - i. Dehydrate thorough 70%, 80%, 90% alcohols, one to two dips in each.
 - j. Keep in absolute alcohol for 1 minute.
 - k. Dip in xylene (Xylo) for 1-2 minutes.
 - l. Mount with DPX or Canada balsam.

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