**Dr. Iman**

**Histological Technique**

Histological technique deals with the prepration 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 (4-5 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 withstand subsequent treatment with various reagents with minimum loss of architecture. This is achieved by exposing the tissue to chemical compounds , called 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 apiece 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-Prevent autolysis and bacterial decomposition .

2-Preserves tissue in their natural state and fix all components.

3-Make the cellular component 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.

**Prupose of Fixation :**

1-To stabilize the protein in the tissue against (Autolysis) starts soon after the cell death. Enzyme causes the breakdown of protein and eventual liquefaction of the cell. So that autolysis is severe in tissue which are rich in enzymes ,such as the liver ,brain and kidney . By microscopy , autolyzed tissue presents a (washed –out) appearance with swelling of Cytoplasm eventually converting to a granular , homogeneous mass which fails to take up stains.

2-Stop putrefaction. If tissue is left without any preservation , then a bacterial attack will occuer, process is known as putrefaction.

3-Fixation also preserves cells and tissue constituents in as close a life –like state as possible allow them to undergo further preparative procedures without change .

4-Fixation cause hardening for the preserved tissue substances and protein, therefore ,it important subsequent sectioning of the tissue .

**Action of fixatives:**

Methods of Stabilizing Proteins:

1-**Heat** ( Physical method)- this method is becoming more used in the histology laboratories with the introduction of microwave fixation.

2-**Desiecation** (physical method )- Rarely used in histology. Geimsa stains are probably the most common used for this method .

3-**The** use of one or more chemical reagent (Chemical method ) – this is considered the primary method of fixation .

Classification of fixative :

A. Tissue fixatives

a. buffered formalin

b. buffered gluteraldehyde

c. Zenker 's formal saline

d. Bowen's fluid

B. Cytological fixative :

a. Ethanol

b. Methanol

c. Ether

C. Histochemical fixative :

a. Formal saline

b. Cold acetone

c. Absolute alcohol

**Chemical fixatives:**

 Chemical fixatives are used to preserve tissue from degeneration, and to maintain the structure and of sub-cellular components such as cell organelles (e.g. nucleus, endoplasmic reticulum , mitochondria ) .The most common fixative for light microscopy is 10% neutral buffered formalin (formaldehyde in phosphate buffered saline ).For electron microscopy , the most commonly used fixative is glutaraldehyde usually as 2.5% solution in phosphate buffered saline. Further fixative are often used for electron microscopy such as osmium tetroxide or uranyl acetate .

 Formalin fixative leads to degradation of mRNA , miRNA and DNA in tissue. However , extracted amplification and analysis of these nucleic acids from formalin – fixed , paraffin – embedded tissues possible using appropriate protocols (retrieving process ) .

 **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 tissue .It destroys mitochondria .

Other fixatives: 1-Buffered forma sline 2- 10% formalin 3-Suza, Bouin, Zinker Solution, Formaldehyde or Gluteraldehyde, Osimum tetroxide, Potassium permanganate.

**Frozen section fixation :**

Frozen section is a rapid way to fix and mount histological sections . It is done using a refrigeration device called a cryostat . The frozen tissue is sliced using a microtome and the frozen slices are mounted on a gelatinous coated slide and stained the same way as other methods. It is a necessary technique to fix tissue for certain stain such as antibody linked immunofluorescence staining. It can also used to determine if a tumor is malignant when it is found incidentally during surgery on a patient .

**Factors affecting fixation:**

**1-Tempracture**

 The factors influenced by temperature is the morphology of the tissue . Normally the fixation of specimens for standard histology is carried out at room temperature for convenience .For electron microscopy and some histochemical procedures , the temperature for fixation is usually 0-4 ºC.

 The use of chemical reactions at higher temperatures to penetrate tissue, including those involved in the fixation process will increase the rate of penetration of fixative to the specimen; however , an increase in temperature will also increase the rate of autolysis and diffusion of cellular components.

**2-Size**

 The penetration of fixatives into tissue is a relatively slow process and tissue blocks should either be small or thin in order to obtain satisfactory fixation. Large specimen should be opened and washed of contents or sliced thinly before placement in fixative. For routine processing the recommended thickness of the specimens should be no more than 3 mm thick .

 For shorter processing schedules the tissue be thinner sections , Ex.( Kidney, liver, heart , biopsies).

**3-Volume Ratio**

 The recommended ratio of the tissue volume to the fixative volume is at least 15-20 times greater than the tissue volume . If a specimen receives more than recommended volume the effect is none, we have plenty of fixative to tissue volume . However , if the volume of the fixative is less than the volume of tissue , we can see some problems such as under fixed tissue (poor fixation ).

**4-Time**

 It is common practice to fix 2 mm thick tissue blocks in buffered formalin for 4-8 hours .Large specimens and viscera are cut 5mm slices or viscera are emptied and pinned out on a board ,before fixing overnight in buffered formalin .This allows easier handling , examination and dissection .In the case of electron microscopy , diced tissue are fixed for 3 hour in glutaraldehyde before placing in a holding buffer such as sodium cacodylate. There is evidence that prolonged fixation in aldehyde can cause shrinkage and hardening of tissue and severe inhibition of enzyme activity .Prolonged fixation with oxidizing fixatives can degrade tissue by oxidative cleavage of protein and loss of peptide .

 The tissue should be placed in fixative immediately after surgery , as well as autopsies should be performed immediately after death .The more time it elapses between interruption of the blood supply and fixation the more post- mortem changes will be observed under the microscope.

**5-Penetration :** Fixatives penetrate the tissue at different rates .These rates can be affected by heat .The tissue is fixed starting at the periphery of the tissue and working inward toward the center of the tissue .

**7- Tissue Storage**

 Storing wet tissue is very important because often the tissue is needed for further studies . It the tissue has not been fixed and stored properly further studies are impossible . Tissue fixed in Neutral buffered Formalin are usually safe to use for further studies since they can remain in this fixative indefinitely , but this is not true for other fixatives . Non fix tissue may remain in 70 % methyl alcohol .

**8-PH**

The hydrogen ion concentration varies between fixatives, but in general , the pH should be kept in the physiological range , between pH 4-9 .When the formalin allowed to fall to a lower pH this can procedure formalin pigments.

 Even through in routine histology the pH is not usually critical , in electron microscopy it is very important .The pH for the ultrastructural preservation of great specimen the fixative should be buffered between 7.2 and 7.4 .

**8- Osmolality**

 The addition of a buffer to the fixative solution may alter the osmotic pressure exerted by the solution . Hypertonic solutions give rise to cell shrinkage whereas hypotonic fixatives result in cell swelling and poor fixation .With electron microscopy, the best results are obtained using slightly hypertonic solutions adjusted using sucrose.

**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 60ºC.

**Preparation of histological sections: -**

1. **Paraffin procedure:**
2. **Specimen** of tissue or organ is removed & cut into small pieces
3. **Fixation**: the small specimen them should be rinsed in suitable fixation such as 10% formation Bouin’s, neutral buffered formation etc. to preserve the normal morphology & facilities further processing.
4. **Washing**
5. **Dehydration**: after fixation, specimen should be dehydrated using ascending series of ethyl alcohols (70%, 80%, 90% & 100%).

 **5-Clearing**: a step in which the dehydrated specimen is placed in xylene or other alternatives. This step is essential before infiltrating the dehydrated tissue with paraffin because alcohol & paraffin do not mix. Clearing of tissue is achieved by any of the following reagents:

* Xylene
* Chloroform
* Benzene
* Carbon
* Toluene

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

**6-Infiltration**: a step in which the tissue infiltrated in paraffin. The melted completely replaces the xylene. This step can be conducted in an oven at a temperature just above the melting point of the paraffin.

Types of wax employed for impregnation:

1. Paraffin wax
2. Water soluble wax
3. Other material, like celloidin, gelatine, paraplast etc.

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

**7-Blocking**& **trimming:** when infiltration is complete, the specimen is transferred to an embedding mold (style) of fresh paraffin, left to be harden, then the access paraffin surrounding the specimen is trimmed away.

**8-Sectioning**: the block paraffin is secured to the microtome & desired thickness of section can be obtained. The serial sections resulted from the cutting or sectioning are called ribbon. One or more sections should carefully separate from the ribbon & transferred to the surface of warm water in a water path to soften & flattens the sections & eliminating the winkles if present. Flattened sections are transferred onto a slide which is then placed on a warming table for drying & subsequently to adhere to the surface of the slide. The prepared slide is ready to be stain with different stains.

**8-Staining**:

1. Dewax, a process in which paraffin is removed by xylene or the suitable solvent.
2. Rehydration in a descending series of ethyl alcohols.
3. Staining with the desired stain.
4. Dehydration with ascending series of ethyl alcohols.
5. Clearing with xylene to be transparent.
6. Mounting with the mounting media such Canada balsam or DPX and then is covered with the cover-slip.

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