Histological microtechniques

Lecture 1

Microtechniques (Microscopic preparations):

The microtechniques for tissue preparation consist of many steps to study the cellular structure which forming the body of the living human or animals, which can't see by the naked eyes, but can be seen by using ***Light microscope or Electronic microscope.***

Histology is the study of the microanatomy of cells, tissues, and organs as seen through a microscope.

Cytology (Cell biology) is a branch of biology that studies the structure and function of the cell, which is the basic unit of life.

Autopsy a small piece of nonliving tissue taken from dead organism (human or animals) for microscopical examination to know the reason of death.

Biopsy a small piece of living tissue taken from patient for microscopical examination to distinguish disease early.

Methods of taking biopsy:

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

Incisional biopsy:

*It is performed when removal of entire lesion is impossible.
*Often performed prior to major surgical procedure.
*Is strictly a diagnostic nature.

Excisional biopsy:

*In this technique, the entire lesion is removed, usually with a rim of normal tissue.

*It is performed when the lesion is smaller in size.

*The procedure serves the diagnostic and therapeutic function.

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.

Curettage biopsy:

*Curetting is usually done for diagnosis of endometrial disease.

Lecture 2

Some general rules in biopsy procedure

1. The larger the lesion, the numerous the biopsies that should be taken from it because of the fact that the diagnostic areas may be present only focally.

2. In ulcerated tumor, biopsies should be taken from the periphery that includes normal and diseased tissue.

3. Crushing or squeezing of the tissue with forceps should be carefully avoided.

4. Once the biopsy is obtained, it should be placed immediately into container with adequate volume of fixative.

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.

*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.

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.

*This is achieved by passing through a series of process.

These processes are:

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

Fixation:

In the fields of histology, pathology, and cell biology, fixation is the preservation of biological tissues from decay due to autolysis or putrefaction. It terminates any ongoing biochemical reactions and may also increase the treated tissues' mechanical strength or stability. Tissue fixation is a critical step in the preparation of histological sections, its broad objective being to preserve cells and tissue components and to do this in such a way as to allow for the preparation of thin, stained sections.

Process:

Fixation is usually the first stage in a multistep process to prepare a sample of biological material for microscopy or other analysis. Therefore, the choice of fixative and fixation protocol may depend on the additional processing steps

and final analyses that are planned. For example, immunohistochemistry uses antibodies that bind to a specific protein target. Prolonged fixation can chemically mask these targets and prevent antibody binding. In these cases, a 'quick fix' method using cold formalin for around 24 hours is typically used. Methanol (100%) can also be used for quick fixation, and that time can vary depending on the biological material. For example, MDA-MB 231 human breast cancer cells can be fixed for only 3 minutes with cold methanol (-20 °C). For enzyme localization studies, the tissues should either be pre-fixed lightly only, or post-fixed after the enzyme activity product has formed.

Types:

There are generally three types of fixation processes depending on the initial specimen:

Heat fixation: After a smear has dried at room temperature, the slide is gripped by tongs or a clothespin and passed through the flame of a Bunsen burner several times to heat-kill and adhere the organism to the slide. Routinely used with bacteria and archaea. Heat fixation generally preserves overall morphology but not internal structures. Heat denatures the proteolytic enzyme and prevents autolysis. Heat fixation cannot be used in the capsular stain method as heat fixation will shrink or destroy the capsule (glycocalyx) and cannot be seen in stains.

Immersion: The sample of tissue is immersed in fixative solution of volume at a minimum of 20 times greater than the volume of the tissue to be fixed. The fixative must diffuse through the tissue to fix, so tissue size and density, as well as type of fixative must be considered. This is a common technique for cellular applications. Using a larger sample means it takes longer for the fixative to reach the deeper tissue.

Perfusion: Fixation via blood flow. The fixative is injected into the heart with the injection volume matching cardiac output. The fixative spreads through the entire body, and the tissue doesn't die until it is fixed. This has the advantage of preserving perfect morphology, but the disadvantages are that

the subject dies and the cost of the volume of fixative needed for larger organisms is high.

Lecture 3

Chemical fixation:

In both immersion and perfusion fixation processes, chemical fixatives are used to preserve structures in a state (both chemically and structurally) as close to living tissue as possible. This requires a chemical fixative.

Crosslinking fixatives – aldehydes:

Crosslinking fixatives act by creating covalent chemical bonds between proteins in tissue. This anchors soluble proteins to the cytoskeleton, and lends additional rigidity to the tissue. Preservation of transient or fine cytoskeletal structure such as contractions during embryonic differentiation waves is best achieved by a pretreatment using microwaves before the addition of a cross linking fixative.

The most commonly used fixative in histology is formaldehyde. It is usually used as a 10% neutral buffered formalin (NBF), that is approx. 3.7%–4.0% formaldehyde in phosphate buffer, pH 7. Since formaldehyde is a gas at room temperature, formalin – formaldehyde gas dissolved in water (~37% w/v) – is used when making the former fixative. Formaldehyde fixes tissue by cross-linking the proteins, primarily the residues of the basic amino acid lysine. Its effects are reversible by excess water and it avoids formalin pigmentation. Paraformaldehyde is also commonly used and will depolymerize back to formalin when heated, also making it an effective fixative. Other benefits to paraformaldehyde include long term storage and good tissue penetration. It is particularly good for immunohistochemistry techniques. The formaldehyde vapor can also be used as a fixative for cell smears.

Another popular aldehyde for fixation is glutaraldehyde. It operates similarly to formaldehyde, causing the deformation of proteins' α-helices. However, glutaraldehyde is a larger molecule than formaldehyde, and so permeates membranes more slowly. Consequently, glutaraldehyde fixation on thicker tissue samples can be difficult; this can be troubleshot by reducing the size of the tissue sample. One of the advantages of glutaraldehyde fixation is that it

may offer a more rigid or tightly linked fixed product—its greater length and two aldehyde groups allow it to 'bridge' and link more distant pairs of protein molecules. It causes rapid and irreversible changes, is well suited for electron microscopy, works well at 4 °C, and gives the best overall cytoplasmic and nuclear detail. It is, however, not ideal for immunohistochemistry staining.

Some fixation protocols call for a combination of formaldehyde and glutaraldehyde so that their respective strengths complement one another.

These crosslinking fixatives, especially formaldehyde, tend to preserve the secondary structure of proteins and may also preserve most tertiary structure.

Precipitating fixatives – alcohols:

Precipitating (or *denaturing*) fixatives act by reducing the solubility of protein molecules and often by disrupting the hydrophobic interactions that give many proteins their tertiary structure. The precipitation and aggregation of proteins is a very different process from the crosslinking that occurs with aldehyde fixatives.

The most common precipitating fixatives are ethanol and methanol. They are commonly used to fix frozen sections and smears. Acetone is also used and has been shown to produce better histological preservation than frozen sections when employed in the Acetone Methylbenzoate Xylene (AMEX) technique.

Protein-denaturing methanol, ethanol and acetone are rarely used alone for fixing blocks unless studying nucleic acids.

Acetic acid is a denaturant that is sometimes used in combination with the other precipitating fixatives, such as Davidson's AFA. The alcohols, by themselves, are known to cause considerable shrinkage and hardening of tissue during fixation while acetic acid alone is associated with tissue swelling; combining the two may result in better preservation of tissue morphology.

Oxidizing agents:

The oxidizing fixatives can react with the side chains of proteins and other biomolecules, allowing the formation of crosslinks that stabilize tissue

structure. However, they cause extensive denaturation despite preserving fine cell structure and are used mainly as secondary fixatives.

Osmium tetroxide is often used as a secondary fixative when samples are prepared for electron microscopy (It is not used for light microscopy as it penetrates thick sections of tissue very poorly).

Potassium dichromate, chromic acid, and potassium permanganate all find use in certain specific histological preparations.

Mercurials:

Mercurials such as B-5 and Zenker's fixative have an unknown mechanism that increases staining brightness and give excellent nuclear detail. Despite being fast, mercurials penetrate poorly and produce tissue shrinkage. Their best application is for fixation of hematopoietic and reticuloendothelial tissues. Also note that since they contain mercury care must be taken with disposal.

Picrates:

Picrates penetrate tissue well to react with histones and basic proteins to form crystalline picrates with amino acids and precipitate all proteins. It is a good fixative for connective tissue, preserves glycogen well, and extracts lipids to give superior results to formaldehyde in immunostaining of biogenic and polypeptide hormones. However, it causes a loss of basophils unless the specimen is thoroughly washed following fixation.

HOPE fixative:

Hepes-glutamic acid buffer-mediated organic solvent protection effect (HOPE) gives formalin-like morphology, excellent preservation of protein antigens for immunohistochemistry and enzyme histochemistry, good RNA and DNA yields and absence of crosslinking proteins.

Lecture 4

2. Dehydration:

Before embedding the tissue sample, each tissue preparation must be dehydrated after fixation. Several methods for dehydration are used, but the most common, for both light microscopy and EM, is a series of alcohol solutions of increasing alcoholic concentration (e.g. 50%, 70%, 90% and 100%). Think of the dehydration process as similar to manual film development, where the picture-to-be is placed in a series of different solutions until finally the picture is exposed. Through the dehydration process, all the fixating solution and tissue fluid is removed. They are replaced with organic solvents such as xylol or toluol. Organic solvents are used because they are miscible with both the embedding media, paraffin, as well as the alcohol used to dehydrate the sample.

3. 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

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

Lecture 5

EMBEDDING

Embedding is the process in which the tissues or the specimens are enclosed in a mass of the embedding medium using a mould. Since the tissue blocks

are very thin in thickness, they need a supporting medium in which the tissue blocks are embedded. This supporting medium is called embedding medium. Various embedding substances are paraffin wax, celloidin, synthetic resins, gelatin, etc.

The choice of embedding media depends upon

- Type of microscope
- Type of microtome
- Type of tissue eg. hard tissue like bone or soft tissue like liver biopsy

Paraffin wax with a higher melting point (56 to 62^{o}C) is used for embedding. The molten wax is filtered inside the oven through a course filter paper into another container. This will protect the knife edge.

OTHER TYPES OF EMBEDDING MEDIA ARE:

Carbowax: It is a water-soluble wax. Therefore, tissues are directly transferred to water soluble wax after fixation and washing.

Methacrylate: It is easily miscible with alcohol and gives a clear and hard block when polymerized. Polymerization takes place in the presence of a catalyst. Any trace of water causes uneven polymerization and formation of bubbles in the block around the tissue.

Epoxy Resin (Araldite): Epoxy polymers of araldite is used in higher resolution work and to see greater details. Epoxy resins are used for electron microscopy. Epoxy polymers of araldite differ from methacrylate in that they are crosslinked causing the cured solid block of araldite to be insoluble in any solvent. Longer filtration is required because the viscosity of resin is greater than methacrylate. For electron microscopy araldite is obtained as casting resinCY212, a hardener DDSA and an amine accelerator, DMP (dimethylamino methyl phenol) Blocks are suitably cured before sectioning for 48 to 60 hours at 60oC.

Agar embedding: It is mainly used in double embedding. Multiple fragments and friable tissue may be impregnated in one block when sectioning on the cryostat. Another use of agar embedding is for FNAC specimens.

Celloidin media: Celloidin is a purified form of nitrocellulose. It is used for cutting hard tissues.

Gelatin: Its melting point is less than the melting point of agar. Gelatin may be used when frozen sections are required on friable and necrotic tissues.

TYPES OF MOULDS:

A variety of moulds are used for embedding. These may be LEUCKHARD embedding moulds (L mould) paper blocks, plastic moulds. Most of the laboratories use L moulds. L moulds are made up of metal, easy to procure, reusable and may be adjusted to make different size of blocks. One limb of the" L" is longer than the other. The two "Ls" are jointed to form a side of the rectangular box that act as a cast to make the mould.

L moulds

Plastic moulds: Most of the laboratories use plastic embedding rings now. These are relatively inexpensive, convenient and support the block during sectioning and are designed to fit it on the microtome. This eliminates the step of mounting or attaching the block on a holder (metal or wooden holder).

Advantages:

- Since the cassette is processed with the tissues and afterwards used for embedding, the writing has to be done once
- Cassettes are thin so less wax is required.
- The space required for filing the blocks is less.

Disadvantages:

- A special clamp has to be used in the microtome for this technique.
- The cassettes are shallow hence thin sections should be taken for processing.

Method of embedding:

1. Open the tissue cassette, check requisition form entry to ensure the correct number of tissue pieces is present.

2. Select the mould; there should be sufficient room for the tissue with allowance for at least a 2 mm surrounding margin of wax. Leuckhart mould method-This is the traditional embedding method. The “L moulds are adjusted according to the shape and size of the tissue. Glycerin may be applied to the L pieces and also to the metal or glass plate on which the moulds are placed for embedding. Simple glossed wall or floor tiles may also be used in place of glass plate.

3. Fill the mould with paraffin wax.

4. Using warm forceps select the tissue, taking care that it does not cool in the air; at the same time.

5. Place the tissue in the mould according to the side to be sectioned. This side should be facing down against the mould. A small amount of pressure may be used in order to have more even embedding.

6. Chill the mould on the cold plate, orienting the tissue and firming it into the wax with warmed forceps. This ensures that the correct orientation is maintained and the tissue surface to be sectioned is kept flat.

7. Insert the identifying label or place the labelled embedding ring or cassette base onto the mould.

8. Add more paraffin into the mould to fill the cassette and mould.

9. Cool the block on the cold plate.

10. Remove the block from the mould.

11. Cross check block, label and requisition form.

Lecture 6

Embedding and sectioning

Tissue that has been received in the laboratory needs to be prepared for sectioning. A variety of instruments are used to cut the sections and the protocol depends on the application. In most cases the tissue requires **embedding** in a medium, which allows thin sections to be cut cleanly; most tissues for routine histology are embedded in wax blocks. This requires that water is removed from the tissue and progressively replaced by wax, which can be solidified later to make a tissue block suitable for sectioning. The tissue is progressively dehydrated by immersing it in successively higher concentrations of alcohol (ethanol), before transfer to the organic solvent xylene and finally embedding in wax. Xylene is used at the final stage because wax is soluble in xylene, but not alcohol, so the wax can readily permeate the tissue. In a large pathology laboratory, much of this tissue processing is automated in order to save time and to produce consistent results.

A number of devices are available for cutting sections:

- **Microtome** cuts thin sections (1-50µm) from fixed, embedded tissue (1µm $=10^{-6}$ meters)
- **Vibratome** uses a vibrating blade to cut thicker sections from fresh or fixed tissue (up to 200µm).
- **Cryostat** cuts sections from deep-frozen blocks of unfixed tissue.

*Most sectioning in routine histopathology departments is done with a microtome producing sections of ~3µm thickness, from tissue that has been embedded in wax.

*The vibratome and cryostat are often used to cut unfixed sections, and these are often more suitable for antibody staining, but they are not the first choice for routine sectioning.

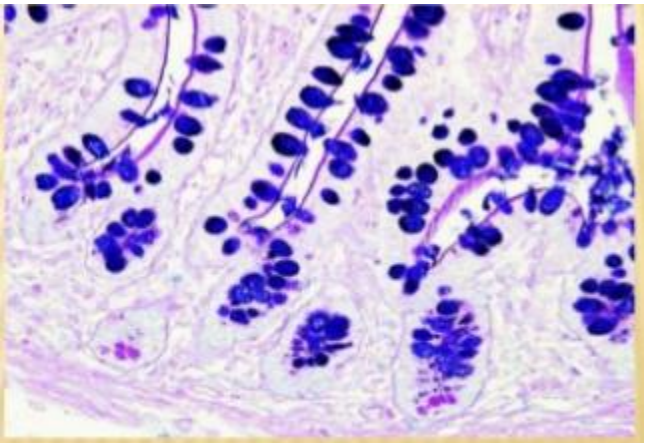
Lecture 7

Common Problems with Sectioning

- Cut too thin
- Cut too thick
- Dull Razorblade
- Sections cut in half

STAINING: is the process of applying dyes on the sections to see and study the architectural pattern of the tissue and physical characteristics of the cell.

On a chemical basis, certain parts of cells and tissues that are acidic in character (e.g nucleus) have greater affinity for basic dyes, while basic constituents (e.g cytoplasm) take more of the acid stains. In general, microscopic examination is facilitated if two contrasting stains are used; e.g hematoxylin which stains the nuclear detail, and eosin which brings out the cytoplasmic detail of the cell and the tissues architecture.

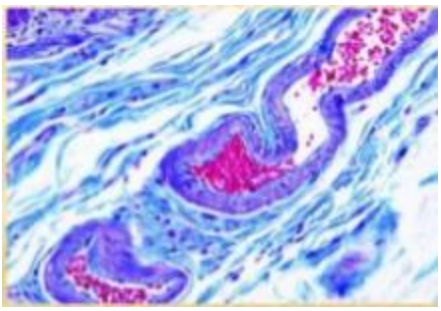


Alcian Blue and PAS (intestine). A stain that combines the properties of both Alcian Blue and Periodic Acid Schiff staining.

3 MAJOR GROUPS OF STAINING

1. **Histological Staining** - Process whereby the tissue constituents are demonstrated in sections by direct interaction with a dye or staining solution, producing coloration of the active tissue component.

   Micro anatomical or histologic staining is used to demonstrate the general relationship of tissues and cells with differentiation of nucleus and cytoplasm.



Gomori Trichrome Blue is used to stain and identify muscle fibers, collagen and nuclei

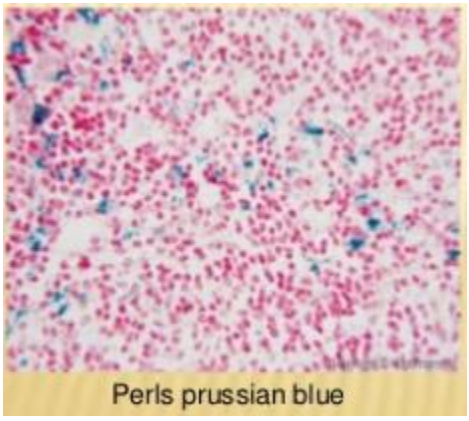
2. **Histochemical Staining (HISTOCHEMISTRY)**

- Various constituents of tissues are studied thru chemical reactions that will permit microscopic localization of a specific tissue substance.

Example:

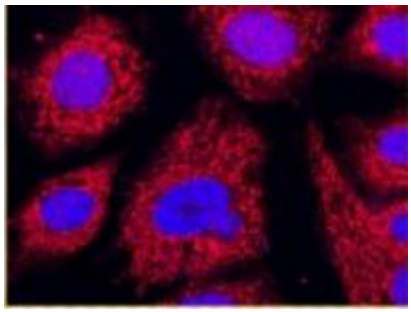
-Perls prussian blue reaction for hemoglobin

- Periodic Acid Schiff staining for Carbohydrates



3. **Immunohistochemical Staining**

- A combination of immunologic and histochemical techniques that allow phenotypic markers to be detected and demonstrated under the microscope, using a wide range of polyclonal or monoclonal fluorescent labeled or enzyme-labeled antibodies.



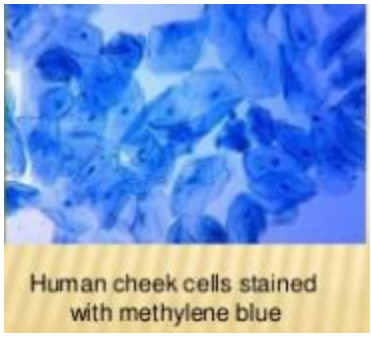
Confocal fluorescence micrograph of HeLa cells stained with monoclonal antibody against mitochondria enzyme and Cy3-conjugated anti-mouse antibody (red); rabbit polyclonal antibody to histones in DNA and Cy5-conjugated-rabbit antibody (blue).

METHODS OF STAINING:

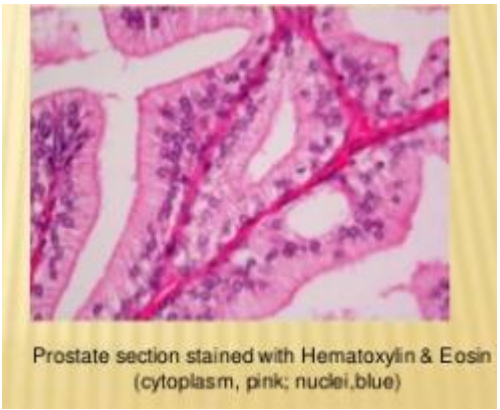
1. DIRECT STAINING

A process of giving color to the sections by using aqueous or alcoholic dye solution.

e.g methylene blue and eosin.



Human cheek cells stained with methylene blue



Prostate section stained with Hematoxylin & Eosin (cytoplasm, pink; nuclei,blue)

2. INDIRECT STAINING

A process whereby the action of the dye is intensified by adding another agent; either mordant or accentuator.

"MORDANT"

- serves as a link or bridge between the tissue and the dye, to make the staining reaction possible.

- e.g Potassium alum with hematoxylin in Ehrlich's hematoxylin, and iron in Weigert's hematoxylin.

"ACCENTUATOR"

- Does not participate in the staining reaction, but merely accelerates or hastens the speed of the staining reaction by increasing the staining power and selectivity of the dye.

- e.g Loeffler's methylene blue and phenol in carbol thionine and carbol fuchsin.

Lecture 8

Routine and Special Staining

Routine H&E staining and special stains play a critical role in tissue-based diagnosis or research. By colouring otherwise transparent tissue sections, these stains allow highly trained pathologists and researchers to view, under a microscope, tissue morphology (structure) or to look for the presence or prevalence of particular cell types, structures or even microorganisms such as bacteria.

In the histopathology laboratory, the term “routine staining” refers to the hematoxylin and eosin stain (H&E) that is used “routinely” with all tissue specimens to reveal the underlying tissue structures and conditions. The term “special stains” has long been used to refer to a large number of alternative staining techniques that are used when the H&E does not provide all the information the pathologist or researcher needs.

Dye origins

Hematoxylin is extracted from the logwood tree and purified. It is then oxidized and combined with a mordant (typically aluminium) to allow it to bind to the cell structures. Of the many hematoxylin preparations used in histology Gill's hematoxylin, Harris's hematoxylin and Mayer's hematoxylin are the most popular.

Eosin is formed by a reaction between bromine and fluorescein. There are two eosin variants typically used in histology: eosin Y which is slightly yellowish and eosin B which is slightly bluish. Eosin Y is most popular.

Some Common Special Stains

- Masson's Trichrome (skin). This stain is intended for use in histological observation of collagenous connective tissue fibers in tissue specimens. It is used to assist in differentiating collagen and smooth muscle in tumors and assists in the detection of diseases or changes in connective/muscle tissue.
- Periodic Acid Schiff (kidney). PAS staining is mainly used for staining structures containing a high proportion of carbohydrates such as glycogen, glycoproteins, proteoglycans typically found in connective tissues, mucus and basement membranes. Often used to stain kidney biopsies, liver biopsies, certain glycogen storage diseases in striated muscles and suspected fungal infections.
- Perls' Prussian Blue Iron (liver). This stain is used to detect and identify ferric (Fe^{3+}) iron in tissue preparations, blood smears, or bone marrow smears. Minute amounts of ferric iron (haemosiderin) are commonly found in bone marrow and in the spleen. Abnormal amounts of iron can indicate hemochromatosis and hemosiderosis.

Lecture 9

DECALCIFICATION

INTRODUCTION

The presence of calcium salts in tissues makes them hard. This causes damage to the knife, difficulty in cutting tissue. Calcium is normally present in bones and teeth. Calcium may also be present in normal tissues in pathological conditions like necrotic tissue in tuberculosis.

DECALCIFICATION

Aim – To remove calcium salts from the tissues and make them amenable for sectioning.

Preparation of tissues – The calcified hard tissues should be first cut into small pieces (2 to 6mm) with a thin blade, hacksaw or sharp knife in order to minimize the tearing of the surrounding tissues. This process is followed by fixation in buffered formalin or any other desired fixative. After fixation tissues must be thoroughly washed and excess fixative should be removed before the specimen is subjected to decalcification.

DIFFERENT METHODS OF DECALCIFICATION

1. Acid decalcification
2. Ion exchange resin
3. Electrical ionization
4. Chelating methods
5. Surface decalcification

Decalcification process should satisfy the following conditions

*Complete removal of calcium salts

*Minimal distortion of cell morphology

*No interference during staining

Decalcification is a straightforward process but to be successful it requires:

*A careful preliminary assessment of the specimen

*Thorough fixation

*Preparation of slices of reasonable thickness for fixation and processing

*The choice of a suitable decalcifier with adequate volume, changed regularly

*A careful determination of the endpoint

*Thorough processing using a suitable schedule

Lecture 10

Methods of Decalcification

Acid Decalcification – This is the most commonly used method. Various acid solutions may be used alone or in combination with a neutralizer. The neutralizer helps in preventing the swelling of the cells.

Following are the usually used decalcifying solutions:

1. **Aqueous Nitric Acid**

   Nitric acid - 5 ml

   Distilled water - 100 ml

   If tissue is left for long time in the solution, the tissue may be damaged. Yellow colour of nitric acid should be removed with urea. But this solution gives good nuclear staining and also rapid action.

2. **Nitric Acid Formaldehyde**

   Nitric acid - 10 ml

   Formalin - 5-10 ml

   Distilled water up to 100 ml

   Advantages:

   - Rapid action
   - Good nuclear staining
   - Washing with water is not required
   - Formalin protects the tissues from maceration

3. **Formic Acid Solution**

   Formic acid - 5 ml

   Distilled water - 90 ml

   Formalin - 5 ml

In this solution the decalcification is slow. If concentration of formic acid is increased the process is fast but tissue damage is more.

4. **Trichloroacetic Acid** - This is used for small biopsies. The process of decalcification is slow hence cannot be used for dense bone or big bony pieces.

Formal saline (10%) - 95 ml

Trichloroacetic acid - 5 gm

Lecture 11

Ion Exchange method: In these ammonium salts of sulfonated polystyrene resin is used. The salt is layered on the bottom of the container and formic acid containing fluid is filled. The decalcifying fluid should not contain mineral acid. X-rays can only determine complete decalcification. The advantages of this method are:

- Faster decalcification
- Well preserved tissue structures
- Longer use of resin

Electrolytic Method: Formic acid or HCl are used as electrolytic medium. The calcium ions move towards the cathode. Rapid decalcification is achieved but heat produced may damage the cytological details.

Chelating Agents: Organic chelating agents absorb metallic ions. EDTA can bind calcium forming a non-ionized soluble complex. It works best for cancerous bone. This is best method for decalcification of bone marrow biopsies as it preserves cytological details best. The glycogen of marrow is preserved.

EDTA Solution:

EDTA - 5.5 gm

Formalin - 100 ml

Distilled water - 900 ml

Surface Decalcification: The surface layer of paraffin blocks is inverted in 5% HCl for one hour. About top 30 micron is decalcified. It should be washed thoroughly before cutting.

Factors affecting rate of Decalcification

1. Concentration of decalcifying solution-Increased concentration of the decalcifying agent fastens the reaction.

2. Temperature-The rate of decalcification increases with rise of temperature.

3. Density of bone-Harder bone takes longer time to decalcify.

4. Thickness of the tissue-small tissue pieces decalcify earlier.

5. Agitation-Agitation increases the rate of decalcification.