Microtome Techniques

- The term **histochemistry** means study of chemical nature of the tissue components by histological methods.

- The cell is the single structural unit of all tissues. The study of cell is called **cytology**.

- A tissue is a group of cells specialized and differentiated to perform a specialized function. Collection of different type of cells forms an organ.

- **Histology** is the study of the cellular organization of body tissues and organs

- The structure of cells and tissues can be distinguished at two levels.

- The fine structure is that which can be distinguished at the level of light microscopy (a magnification of 1000 x or less). Electron microscopes are generally employed to study ultrastructure.

- The detailed structure of the cell cytoplasm, organelles and membranes that is not discernable with a light microscope.

- Cell structure is most commonly studied in slices of the tissue, called sections, that are thin enough to allow transmission of light or an electron beam.
- There are many methods of sectioning tissues, and sometimes particular tissues require special techniques.
- The method most widely employed is called the paraffin method.
- All histological procedures can be divided into a similar series of steps

- For the paraffin method these steps are as follows: (refer permanent slide preparation)

1. Tissue resection/collection of sample.
2. Fixation
3. washing
4. Dehydration
5. Clearing
6. Infiltration and embedding in paraffin
7. Sectioning with a microtome
8. Mounting on microscope slides
9. Clearing and Staining
10. Preparation of permanent mounts

I. Tissue resection

- Tissue resection is the surgical removal of all or part of an organ, tissue, or structure which will be used as the sample.

- These will be separated in to different anatomical regions depending on the nature study, it is then placed in fixative as the first stage of the histological procedure.

II. Fixation (refer fixation given in 1st part of this module)

- Fixation is the first step in any procedure in which tissue is to be preserved for histological study.

- Fixation is necessary to protect and harden the tissue against the deleterious effects of later procedures which otherwise would disrupt cellular structure beyond recognition.
- Furthermore, fixation minimizes autolysis (the degradation of cellular structure which results from the release of degradative enzymes from the excised tissue itself).

- The fixation process must be started as quickly as possible after removal of the sample.

- All fixatives distort tissue to a certain extent, but in general, proteins and cellular structure are preserved.

- Normally you choose a fixative containing several ingredients that balance out each other’s ill-effects. For eg, alcohol shrinks tissue and causes excessive hardening. You can counter these effects by adding an acid such as acetic acid, which swells tissue and prevents overhardening.

- One of the safest fixatives to use, which will not leave any toxic residues behind, is Carnoy’s fixative, a mixture of alcohol and acetic acid. It is not an ideal fixative. The addition of formaldehyde, for example, would give better preservation of cytological detail.

- The amount of time the specimen is left in fixative should depend on the size of the specimen and the type of fixative.

- Most specimens should be fixed 6–8 hours, or overnight. If the specimen is smaller than 5 mm on a side, then several hours will suffice.

- Tissues should be fixed and stored in the refrigerator at 4ºC.

- Unless a tissue is fixed soon after the removal from the body it will undergo degenerative changes due to autolysis and putrefaction so that the morphology of the individual cell will be lost.

III. Washing

- Following fixation, the tissue sample must be washed.
- This, as well as many of the operations that follow, can be done in the vial.
- To make a transfer of solution, you can either pour off the old solution into a finger bowl, or you can pipette off the old solution. The new solution is added directly to the vial. This avoids unnecessary handling that can damage the tissue.

- Washing is usually done in water. After the use of certain fixative it in urgent that the tissues be thoroughly washed in running water to remove the fixative entirely.

- For most fixatives, washing specimens in distilled water for 6–8 hours, or overnight, following fixation is sufficient. If you have chosen Carnoy’s as your fixative, then washing in 70% alcohol instead of water is both adequate and faster.

- Tissues treated with potassium dichromate, osimium tetraoxide and picric acid particularly need to be washed thoroughly with water prior to treatment with alcohol (for dehydration).

- Following washing, the specimen should be transferred to 70% alcohol and stored at 4ºC until it can be processed further. It can remain here for a number of weeks.

IV. Dehydration (refer permanent slides)

- To prepare the specimen for paraffin embedding, the specimen must be dehydrated through a series of alcohols up to absolute alcohol (ethanol is preferable to methanol, since it is less harsh on the tissues).This removes all the water, which is immiscible with paraffin.

- After washing specimens in water they should be placed in the lowest grade of the series, and then passed through solutions of higher grade.

Significance of graded alcohol series

- Alcohol should be allowed to diffuse into tissues gently.

- This is necessary to avoid violent diffusion currents which may shrink or distort delicate structures if they are suddenly transferred from an aqueous medium to strong alcohol.

- It is equally necessary to protect delicate and fragile tissues against the effects of local turbulence caused by rapid mixing of water and strong alcohol; leading to structural disruption.

•A higher concentration of alcohol initially is in inadvisable because this may cause very rapid removal of water may produce cell shrinkage.The usual procedure is to place the fixed tissues successively in alcohols of increasing strength for gradual dehydration.

•This is called as **graded series of alcohol**. The alcohol should be diluted with distilled water to prepare its grades.

•In most histological work, graded series may consist of 15%, 30%, 50%, 70%, 80%, 90%, and absolute alcohol.

V. Clearing

- Clearing as it describes a special property of the reagents that are used. They remove or clear the opacity from dehydrated tissues making them transparent. These fluids are called clearing agents.

- After the water has been removed, a clearing agent, such as xylene or toluene, which is miscible with both 100% alcohol and paraffin, makes a bridge between the alcohol and paraffin.

- The clearing effect is an added advantage but the essential process is dealcoholisation of the tissues, so that paraffin can be introduced inside the tissues.

- Clearing agent is required when the dehydrating agent is not miscible with the impregnating medium. It is essential for a clearing agent to be miscible both in dehydrating agent as well as embedding agent.

- Toluene is less harsh on tissue than xylene, causing less shrinkage and hardening, and so should be used instead of xylene if possible.

Commonly used clearing agents are as follows :

1. Xylene - It has a rapid action, Immersion time must not be prolonged otherwise the tissue become brittle.

2. Toluene and Benzene are similar in properties to xylene but are less damaging to the tissues on prolonged exposure.

3. Chloroform - It is slower in action but it causes less brittleness therefore tissue can be left in it overnight.

4. Carbon tetrachloride - It has similar properties to chloroform but is cheaper.

5. Cedar wood oil (Histological): It is good for treatment of delicate tissues as it has the least hardening effect.

• clearing agents are toxic. They should only be used in a ventilated hood (or outside, if necessary) and also must be put into special waste jars and disposed as a hazardous material

Techniques of clearing :

- If the tissue is being cleared in chloroform or carbon tetrachloride it may be left overnight.

- In Xylene, benzene or toluene one change after 30-60 minutes is satisfactory to give a clear translucent appearance to the tissue.

6. Paraffin Infiltration and Embedding

- After fixing, dehydration and clearing, the tissue become a bit hard. However the tissue still lack proper consistency for sectioning.

- They are not sufficiently rigid to be sliced into thin sections.

- The best method to cut sections of the material is to permeate it thoroughly with a medium that is fluid at one stage and solid when cooled or exposed to air.

- This medium should be soft enough to cut readily and hard enough to act as a support against the impact of knife and to hold the different parts in proper relation with each other after the sections have been cut.

- These functions are best performed if the corners, interstices, and cavities within the tissue are gradually filled with a substance which adheres firmly to the external and internal surface of the material.

•This process is called **infiltration** or **impregnation**. It is the complete removal of clearing reagents by substitution of paraffin or any such similar media.

•Prior to sectioning, the tissue block must be infiltrated with a material that acts as a support during the sectioning process. For the method described here, paraffin serves this purpose.

•During infiltration, the paraffin will equilibrate within the tissue block, eventually occupying all of the space in the tissue .

•After infiltration, the tissue is allowed to solidify in a mould, embedded within a small cube of paraffin. The supporting medium is called embedding medium or embedding mass.

•The paraffin you use will have a specified melting point. A melting point of 54ºC is typical and is suitable for sections that will be cut at 8 µm or thicker. Higher melting points indicate harder paraffin that can be cut into thinner sections.

•Impregnation with paraffin wax takes place in an oven heated to 56-60°C depending upon the melting point of the wax in use

a) Transfer to pure paraffin

- To transfer the tissues to molten paraffin, the clearing agent is poured off and is replaced with pure molten paraffin. The vial or the specimen tube is then kept back into the oven. Always throw the 1st changed wax, as it is meant only for making the tissue acquainted with its new medium. Make at least 2 more changes of fresh paraffin to get rid of all traces of xylene.

b) Length of time in each bath

•Vary with the material to be embedded. Generally 10 -20 minutes in each bath is sufficient for small pieces of tissues, 1 hr is sufficient in m,ost of the cases and 2 hrs in each is maximum.

•Insufficient infiltration in wax causes sections to wrinkle or join together to drop out from the wax. There is no basis, apart from experience, on which timing in each bath can be decided.

Time of impregnation

Depends on the following 3 factors

1. The size and type of tissue
2. The clearing agent employed
3. The use of vacuum embedding oven.

Embedding procedure:

- Embedding - It is the orientation of tissue in melted paraffin which when solidified provides a firm medium for keeping intact all parts of the tissue when sections are cut.

Techniques of casting

1. Molten paraffin wax which is heated at a temperature 2-3° above the melting point is poured into the mould to an adequate depth so as to cover the thickest tissue block. The paraffin blocks are generally prepared in a mould, using two L – shaped pieces of brass placed together to form 4 sides of a rectangle.

2. The wax touching the mould will quickly form a thin semi solid layers, Now introduce the tissue with a prewarmed forceps to prevent the wax to stick to it.

The tissue is pressed in this semisolid wax to orient it at the bottom of mould in a correct plane.

3. Fix the label in position by pressing one edge against solidifying wax usually sides of the mould are preferred.

4. As soon as a film of solid wax is formed on the surface, the whole block with mould are submerged in cold water at 20°C. If this is not done there will be crystallization of wax, using ice water to do initial cooling will also cause the block to crack.

5. When blocks are set hard they are removed from mould.

Trimming Paraffin Blocks

1. Rough trimming

- Remove the paraffin block from its mold, keeping track of the orientation of the specimen within it.
- Trim away excess paraffin using a safety razor blade.
- This should be done on a protective piece of wood or glass. Do not trim too close to the specimen. Then it has to be mounted on the microtome for sectioning.

7. Sectioning

- Sectioning is accomplished by using a cutting apparatus called a microtome, is a tool used to cut extremely thin slices of material, known as sections.

- The microtome will drive a knife across the surface of the paraffin cube and produce a series of thin sections of very precise thickness.

- The objective is to produce a continuous "ribbon" of sections adhering to one another by their leading & trailing edges.

•The thickness of the sections can be preset, and a thickness between 5 - 10 µm is optimal for viewing with a light microscope.

•The sections can then be mounted on individual microscope slides.

•Preparation and mounting of the embedded tissue block on the microtome is very important to successful sectioning. The paraffin surrounding the tissue block must be first trimmed, and then secured to a holder which is then mounted on the microtome.

Microtomes

•These are mechanical devices for cutting uniform sections of tissue of appropriate thickness.

• All microtomes other than those used for producing ultra thin sections for election microscopy depend upon the motion of a screw thread in order to advance the tissue block on knife at a regulated number of microns. Motion of screws can be direct or through system of gears or levers to magnify the movement. Microtomy is a method for the preparation of thin sections


Leica

8. Mounting of sections on microscope slides.

- In this procedure, the sections are permanently attached to microscope slides.

- The cutting process compresses the sections, and part of the mounting procedure is to expand the sections before they adhere to the slide.

- This can be done by floating the sections on warm water (5–10ºC below the melting point of the paraffin).

•Once a ribbon of sections begins to form, the end of the ribbon can be lifted with a moistened camel's-hair paintbrush.

•Some histologists float the sections in a water bath and then pick the expanded sections up on a slide.

• 1. Carefully transfer the sections to a solution held in a 45^{o}C water bath. Within a few seconds you should see the sections flatten and the wrinkles disappear.

2. Dip a clean microscope slide into the adhesive solution, and slowly pull it upward, out of the solution, allowing sections to adhere to the surface. Make sure that the slide is oriented with the label facing upward.

3. Dry the bottom of the slide and carefully blot excess adhesive from around the sections (be careful not to touch the sections themselves).

4. Allow the slides to dry overnight in the storage box.

•An adhesive is a substance which can be smeared on to the slides so that the sections stick well to the slides. Most of the tissue sections which are adequately thin and thoroughly dried without any air bubble trapped under them do not require an adhesive.

9. Clearing and staining.

- Before a section can be stained the paraffin must be removed, a process called clearing. After clearing, only the tissue remains adhering to the slide.

- Clearing is accomplished by passing the mounted sections through the solvent that dissolves the paraffin.

- using xylene or toluene, since the paraffin prevents staining of the tissues.

Staining

- The slides are then processed down to water, since most staining solutions are water-based.

- This requires a graded series of alcohols to prevent severe convection currents that would damage the tissues. Following staining, the slides are dehydrated again through a graded series of alcohol, cleared in xylene or toluene, and coverslips are applied using a plastic mounting medium that is miscible with the clearing agent.

- The sections, as they are prepared, are colourless and different components cannot be appreciated.

- Staining them by different coloured dyes, having affinities of specific components of tissues, makes identification and study of their morphology possible.

- Staining of histological sections allows observation of features otherwise not distinguishable.

- Certain terminologies used in the following account are given below.

1. Basic stains: basic dyes are alkaline and posses amino (NH2-) radicals and produce hydroxyl ions. These are cationic stains. These dyes posses strong affinity towards negatively charged structures such as nuclei. These components are called as basophilic.
Eg. Safranine, methylene blue, etc.

2. Acid stains: posses radicals that yield hydrogen ions such as sulphonic, carboxyl or hydroxyl groups. These stains are also called anionic and they have strong affinity for the basic components of cells such as cytoplasm. Eg; eosin, congo red

3. **Neutral stain:** those in which the acidic moiety as well as basic components of the dye are coloured. They stain both acidophilic and basophic structures. Eg., Romanovsky stain, which is the combination of methylene blue with eosin.

4. A **counterstain** is a stain with colour contrasting to the principal stain, making the stained structure more easily visible. example is eosin counterstain to haematoxylin in the H&E stain. Acid stain is usually employed as a counter stain after a nuclear stain.

5. Mordents: some stains cannot directly stain the tissue unless a mordent is present. The mordant binds to the chemical dye, helping to hold it so it remains stuck to the organism. A mordant is classically defined as an ion that binds a chemical dye and holds it down, such that the dye remains stuck on the tissue.

•Substance that causes certain staining reactions to take place by forming a link between the tissue and the stain. The link is referred as lake. Without it, dye is not capable of binding to and staining the tissue. e.g. Ammonium and Potassium alum for haematoxylin.

Eg., Gram’s Iodine is used a mordant (that fixes the crystal violet to the bacterial cell wall) in Gram satining procedure.

Gram staining is performed when the chemical dye crystal violet is mixed with the mordant iodine. Iodine and crystal violet form a large complex that precipitates out of solution. During the staining procedure, the bacteria are bathed in alcohol, which causes the cell walls to shrink. This shrinkage traps the iodine-crystal violet complex in the cell wall, which gives Gram-positive bacteria a purple color

6. Differential staining: I s a staining process which uses more than one chemical stain. Using multiple stains can better differentiate between different microorganisms or structures/cellular components of a single organism. One commonly recognizable use of differential staining is the Gram stain.

Gram staining uses two dyes: Crystal violet and Fuchsin or Safranin (the counterstain) to differentiate between Gram-positive bacteria (large Peptidoglycan layer on outer surface of cell) and Gram-negative bacteria.

7. Vital staining: Staining of structures in living cells, either in the body (in vivo) or in a laboratory preparation (in vitro). e.g. Janus green is taken up by living cells and stains the mitochondria. A **vital stain** is a **stain** that can be applied on living cells without killing them. Eg., janus green.

- The basic steps in staining and mounting paraffin sections are as follows:

1. Deparaffinization (clearing)
2. Hydration
3. Staining
4. Dehydration and clearing
5. Mounting

1. Deparaffinization

- Removal of wax is done with xylol.
- It is essential to remove the wax completely, otherwise subsequent stages will not be possible.
- At least 2 to 3 changes in xylol are given for suitable length of time.
- Sections of this stage should appear clear and transparent.
- Presence of any patches indicates the presence of wax and sections should be kept longer in the xylol

2. Hydration

- Most of the stains used are aqueous or dilute alcoholic solutions.
- Hence it is essential to bring the section to water before the stains are applied.
- The hydration is done with graded alcohols from higher concentration to lower concentration.
- Alcohol and acetone are miscible with xylol.
- First change is made to absolute alcohol or acetone followed by 90%, 70% alcohol and finally distilled water

4. Staining

- Various staining procedures are applied from this hydrated stage.
- The most common stain applied for histological study is Haemotoxylin and Eosin
- Washing and rinsing of tissue sections is a necessary part of most staining techniques.
- It eliminates carrying over of one dye solution to the next. Excess dye, mordants, or other reagents might react unfavourably or precipitate when placed in the fluid employed in the next step.

5. Dehydration and clearing

- Dehydration is done is graded alcohols or acetone from 70% to absolute alcohol or acetone.
- Dehydrating alcohol and acetones can remove some of the stains.
- Time has to be suitably modified to minimize fading of stains.

- Since alcohol and acetone are miscible in xylol, it is used for clearing the sections.
- Any sections from which water has not been completely removed would give a milky appearance after the first xylol. Such sections should be returned to absolute alcohol and the process repeated.
- Mounting is done after 2nd or 3rd xylol.

Staining schedule

Toluene (or xylene) I — ↓	20 min.
Toluene (or xylene) II — ↓	20 min.
100% ETOH — ↓	5 min.
95% ETOH — ↓	5 min.
70% ETOH — ↓	5 min.
Distilled water — I	5 min.
Alcian blue (filter before use)	10 min.
Distilled water — II	5 min.
Hematoxylin (filter before use)	2 min.
Tap water — several changes	1 min. each
70% ETOH — ↑	5 min.
Alcoholic eosin	5 min.

95% ETOH — ↑	1 min. dipping
100% ETOH I — ↑	1 min. dipping
100% ETOH II — ↑	1 min. dipping
Toluene (or xylene)* — ↑	2 min. and hold

6. Cover slipping and mounting

- The final step in this procedure is to permanently mount the sections under a coverslip.

- This is accomplished by covering the section in a medium that will harden and produce a clear binder between the slide and cover slip.

- The ideal mounting medium should not distort the stain color, or yellow and become brittle with age.

Mounting Coverslips

•Make quite sure that the sections are quite clear. Do not let the section go dry before mounting

1. Hold the slide between the thumb and the forefinger of one hand and wipe with a clean cloth both ends of the slides.

2.Place a drop of mountant on the slide over coverslip. Invert the slide over the coverslip and lower it so that it just adheres to the cover slip . Do not press or push the slide at all. It can damage the section.

3. After the mountant has spread to the edge of the coverslip wipe around it for neatness.

- If proper care has been taken there should be no air bubbles. If many are present, slide should be returned to the xylol to remove the coverslip.
- It will slip off and remounting is done. No attempt should be made to pull the coverslip.
- Slight warming of the slide from below will make the small air bubbles to escape from the slide of the coverslip.

- Mountants – canada balsom, D.P.X etc.

Criteria of acceptable mounting media

1. Refractive index should be as close as possible to that of glass i.e. 1.5.
2. 2. It should not cause stain to diffuse or fade.
3. 3. It should not crack or appear granular on setting.
4. 4. It should be dry to a nonsticky consistency and harden relatively quickly.
5. 5. It should not shrink back from edge of cover-glass.
6. 6. It should be free flowing and free from air bubbles.

•Staining of histological sections allows observation of features otherwise not distinguishable.

• For routine histological work, it is customary to use two dyes, one that stains certain components a bright color and the other, called the counterstain, that stains other cellular structures a contrasting color.

•While literally hundreds of staining techniques have been developed, the two stains most widely used for routine work are hematoxylin and eosin. Hematoxylin stains negatively charged structures, such as DNA, a blue color. Eosin imparts a red color to most of the other cell components.