S. S. College, Jehanabad

Department: Zoology

Class: M.Sc. Semester II

Subject: Zoology

Topic: Tissue processing

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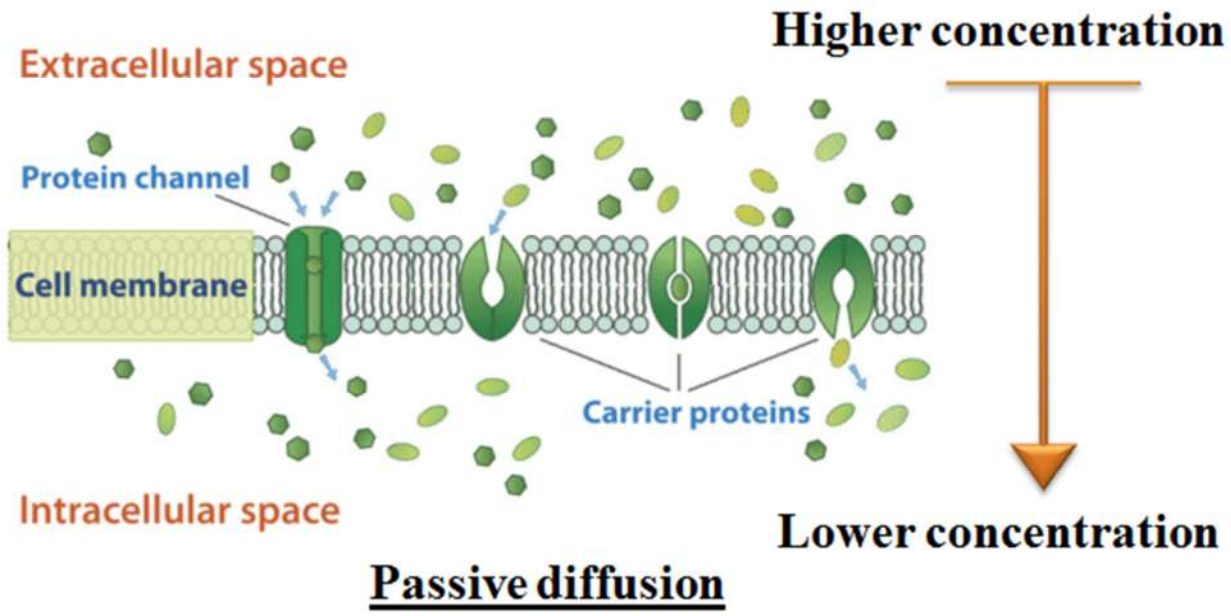
TISSUE PROCESSING

Tissue processing is a procedure of removing water from cells and replacing it with a medium which solidifies the specimen allowing thin sections to be cut for histological studies. Thus, it involves a to and fro movement of substances or reagent into and out of tissues through passive diffusion process. Since, it is a passive process; it requires no energy and involves movement of reagent from higher concentration to lower concentration as shown in figure below[1].



The rate of diffusion of solutes of reagent can be measured by Fick's law which states that rate of diffusion from higher concentration to lower concentration is proportional to area of surface on diffusion membrane and difference in concentration and inversely proportional to the thickness of surface over which diffusion takes place. Therefore, the equation can be written as;

$$\textbf{Rate of diffusion} \propto \frac{(\textbf{Area of diffusion surface}) - (\textbf{Difference in concentration})}{\textbf{Thickness of surface over which diffusion takes place}}$$

Where ∝ signifies 'proportional to'

Histological specimen preparation is carried through a series of stages from taking fixed tissue sample to the state where it is completely infiltrated with a suitable histological wax and embedded with suitable medium, normally paraffin wax, in readiness for cutting section on a microtome by a process known as **microtomy**. However, processing of a tissue specimen have significant number of variables that affect tissue processing and therefore needs to be considered during the process such as operating conditions which includes temperatures, the concentration of the reagents, and the properties of the tissue which includes size and thickness of the tissues. Mostly, these processes are done manually (hand processing); however for routine processes to handle large number of specimens, now generally (although, it is introduced in 1940), these processes are carried through automated machine known as automatic tissue processors which are of broadly two types – tissue transfer types and fluid transfer types.

[1] Adapted from "Tissue Sampling, Processing and Staining: https://tissue sampling.weebly.com/processing.html"

Tissue transfer processor (dip & dunk): The carousel or rotary is the most common model of automatic tissue processor which is characterized by the transfer of tissue, contained within a basket, through a series of stationary reagents arranged in-line or in a circular carousel or rotatory plan. It is provided with 9 – 10 reagent and 2 2- 3 wax positions, with a capacity of 30 – 110 cassettes depending upon the model. Agitation of fluid is achieved by vertical oscillation or rotary motion of the tissue basket which is occurring at 3 sec of intervals. This model of tissue processors allow maximum flexibility in the choice of reagents and schedule that can be run on them. In a comparative newer model, the tissue basket is enclosed within an integrated fume hood during agitation and transfer cycles to rule out the disadvantages of previous models like spilling, protection from electricity cut, etc.

Tissue transfer processor

Fluid transfer processor

Tissue transfer processor usually works in a following time schedule (however, it depends upon nature and size of the tissue, and urgency) as done manually:

Beaker I	**Fixative (formalin)**	**1 – 2 h**
Beaker II	Fixative	1 h
Beaker III	Fixative	30 – 45 min
Beaker IV	70% Ethanol	30 min
Beaker V	90% Ethanol	30 min
Beaker VI	Absolute ethanol	1 h
Beaker VII	Absolute ethanol	1 h
Beaker VIII	Methanol	30 min
Beaker IX	Xylene	1 – 2 h
Beaker X	Xylene	~1 h (45 – 60 min)
Wax bath I	At 45°C	2 h
Wax bath II	At 45°C	2 h

Fluid transfer processor (enclosed): In this type of processor, fluids are pumped to and from a retort in which the tissues remain stationary. There are 10 – 12 reagent stations with temperature

adjustable between 30° - 45°C, 3 – 4 paraffin wax stations with variable temperature settings between 48° - 68°C, and vacuum pressure options for each station. Agitation of fluid is achieved by tidal action[2]. Schedules of tissue processing is programmed and controlled by in-built computer with screen. Vacuum pressure cycles coupled with heated reagents allow effective reductions in processing times and improved infiltration of dense tissues. This type of processor has overcome the main drawbacks of the tissue-transfer processor and protects the tissue from drying up within the sealed retort.

Overview of the steps in tissue processing

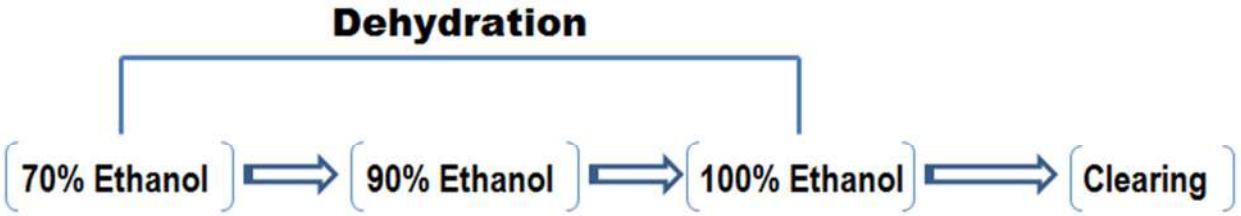
Tissue or organs taken as samples or specimens for histological studies or histochemical analysis need to be processed in the histology laboratory to produce glass microscopic slides that are viewed under the microscope. Different steps of tissue processing can be underlined as;

- Dehydration of tissue specimen
- Clearing of specimen
- Wax infiltration
- Embedding or blocking out

Dehydration of tissue specimen

The first stage in tissue processing is dehydration of tissue specimen that is a process by which water is removed from the tissue without any distortion. Though, some of the water which are either bond or free are removed also in the process of fixation, the proper dehydration of tissue specimen is necessary except where tissues are supported by an aqueous embedding medium such water soluble waxes. Dehydration is usually carried out using alcohol (largely ethanol).

In paraffin wax processing, dehydration of specimen fixed with aqueous fixatives such as formalin is usually initiated in 70% ethanol before progressing through 90% - 95% to absolute ethanol before processing the clearing stage. However, some specimens, which are properly fixed, is dehydrated by directly transfer to 95% ethanol. The time taken for dehydration of tissue is greatly dependent on tissue thickness, 1mm of tissue specimen needs up to 30 min of immersion in dehydrant solution.



Dehydrating agents: There are a number of chemical compound which are used in the dehydration process in tissue processing. Most commonly used dehydrating agent is ethyl alcohol. However, a number of dehydrating used in histological studies, which are as follows;

Acetone: It is a clear, colourless, flammable with a characteristic odour, low toxicity and is freely miscible with water. It is fast and effective as dehydrant and also act as a coagulant secondary fixative. It is ideal dehydrant for fatty tissue samples and the dehydrated samples can be directly transferred from acetone into paraffin wax.

[2] A force that is responsible for stretching a body towards and away from the center of mass of another body due to a gradient in gravitational field from the other body (as in tides due to moon's gravitational field)

Methanol: It is also a clear, volatile, flammable and poorly miscible in water. It tends to harden tissues more ethanol and is a poor lipid solvent.

Ethanol: It is also clear, colourless, flammable and hydrophilic liquids that are miscible with water. It also acts as secondary coagulant fixative in addition to its role as a dehydrating agent.

Isopropanol: It is less severe than ethanol but needs a tissue specimen to be transferred from 60% - 70% to absolute isopropanol to minimize shrinkage. It is completely miscible with water and most organic solvents and is fully miscible with molten paraffin wax. It shrinks and hardens the tissues and therefore, generally, used to dehydrate hard and dense tissues.

Butanol: Generally, N-butanol is used as a dehydrating agent. It is flammable with a distinct penetrating odour. It causes less hardening and shrinkage than alcohol but it is poorly miscible with water and paraffin wax, and therefore comparatively longer time is required for dehydration.

Phenol: It is clear hygroscopic crystals and is also available in a liquefied form. It is soluble in water, alcohol and most organic solvents. It develops a pink discolour on exposure to air and light.

Cellosolve: It is chemically ethylene glycol or 2-ethoxyethanol. It is colourless, almost odourless, flammable, and miscible with water and most other organic solvents. It is particularly used for preceding ester wax embedding. In order to avoid tissue shrinkage, they are transferred from aqueous fixative or 70% ethanol into full strength cellosolve.

Dioxane: It is also known as diethylene dioxide and is colourless, flammable and miscible with water and most organic solvents. It results in comparatively less shrinkage and hardening of tissue than ethanol. It is a rapid and has gentle action.

Dimethoxypropane (DMP) and diethoxypropane (DEP): These are flammable and miscible with wax. These are found suitable for rapid manual processing or machine processing and are comparable to conventional dehydration for tissue morphology and staining reactions.

Polyethelene glycol (PEG): It is clear, viscous liquids or solids of low toxicity. It is used in the dehydration of those tissue specimens which are affected by the solvents and heat of the paraffin wax method. At low temperature, it is liquid and dehydrate the tissue as it is passed through glycols of increasing molecular weight and viscosite, while at room temperature, high molecular weight PEG is solid and can be used for embedding.

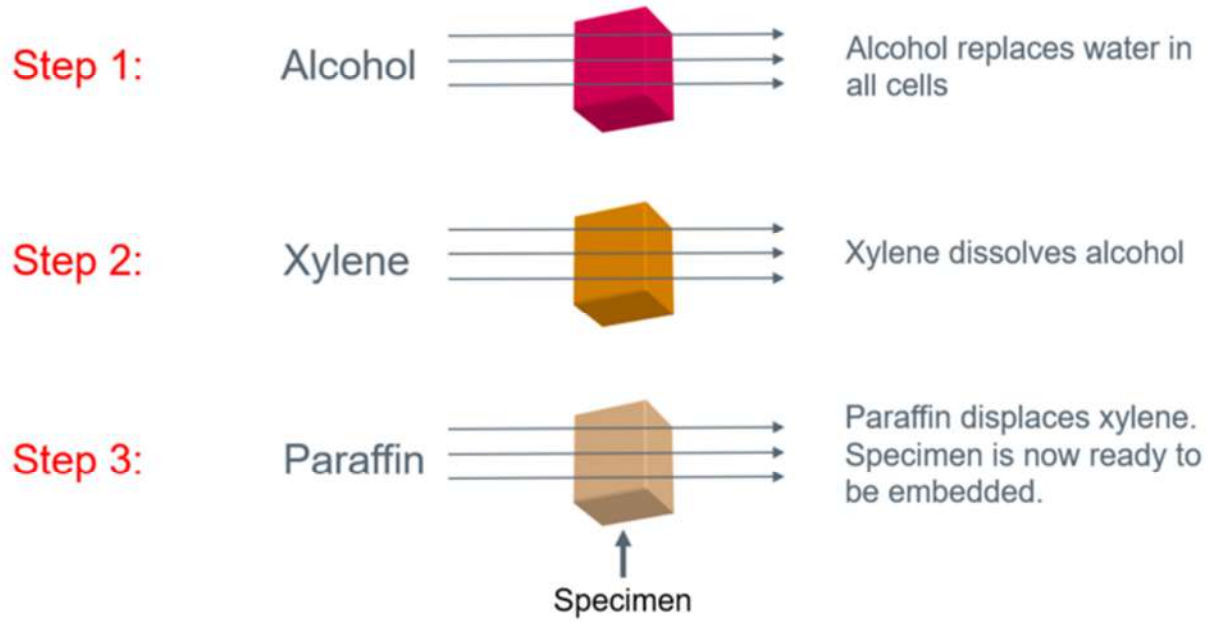
Tetrahydrofuran: It is also a colourless, highly volatile, flammable solvent with an offensive smell and completely miscible with water, most organic solvents, paraffin wax and mounting media. It dehydrates the specimen rapidly causing little shrinkage and hardening and is possibly the best of the universal solvents.

Clearing

Though, after dehydration process, the tissue specimens are water-free, infiltration with wax still can't be possible because ethanol is not readily miscible with paraffin wax. Therefore, an intermediate solvent that is miscible with both water and paraffin, is used. Due to this property, it displaces the ethanol in the tissue, and then this in turn displaced by molten paraffin wax. It is this process in tissue processing that is known as "clearing" and the reagent used is called a "clearing agent". Additionally, it can remove a substantial amount of fat from the tissue which otherwise presents a barrier to wax infiltration. When dehydrated tissues are placed into these

reagents, they are rendered transparent which also indicates the end point of the process. One negative aspect of using clearing agent is that it results in shrinkage of tissue due to dissolution of fats. Most commonly used clearing agent is xylene however; there are a number of clearing agents used in the clearing of dehydrated specimens which are as follows;

Amyl acetate, methyl benzoate and methyl salicylate: These are chiefly used as nitrocellulose solvents in double embedding techniques. These are ideal for manual processing as tissues may be left in them for extended periods without hardening. Since, these agents are difficult to remove from paraffin wax and therefore needs the use of toluene or similar solvent with one or two brief changes before passing through two or three changes of wax. These chemicals have low toxicity but strong penetrating odours and therefore needs good ventilation in the lab. However, they render the tissue specimen completely transparent and therefore used for clearing helminthes parasites for examination and whole mounting. Methyl salicylate clears tissues from 95% ethanol, hardens less, and causes minimal tissue shrinkage and hardening and tissue can remain in it indefinitely with being affected.



Benzene: It is more gentle and rapid than xylene and toluene and is probably the best transition solvent. However, due to its toxicity and carcinogenicity, it is not used in histochemical procedure.

Butyl acetate: It is generally used as a xylene substitute and nitrocellulose solvent.

Carbon tetrachloride: It is very toxic compound and therefore, it is discontinued in histological study.

Cedarwood oil: It is largely composed of cedrene and rapidly clears the tissues from 95% ethanol. It hardens the tissue least among all other transition solvents, but is difficult to eliminate from tissue during wax infiltration. It is particularly useful for processing dense tissues such as uterus or scirrhous (Greek; Skirrhos; Hard) carcinoma. Specimen can remain in cedarwood oil indefinitely without harm. Low viscosity refined oil could be used for cleaning. Formation of crystalline cedrol can be overcome by the addition of 1ml xylene or toluene to 80ml cedarwood oil. It is expensive.

Chloroform: It is expensive, volatile but slowly penetrating solvent. It causes less brittleness than xylene and is often used on dense tissues such as uterus. However, it dissolves some plastics and sealants so it is not generally used for the purpose.

Limonene (d-limonene): It is derived from citrus fruits and known to contain numerous chemicals that may act as transition solvents such as Histoclear and Citroclear. It is taken as xylene substitute.

Terpenes: These are isoprene polymers found in essential oils derived from some plants. These are oldest known transition solvents to be used in histology and include turpentine and oils of bergamot, cedarwood, clove, lemon, origanum and sandalwood. These are used soon after the tissue removed from 80% - 95% ethanol. They render tissue transparent and have a slow gentle non-hardening action. By diluting, tissue penetration and shrinkage can be minimized. They have low evaporation rates and therefore need one or more 30 min changes of toluene or similar solvent to remove before infiltration with wax.

Trichloroethane: It is commonly used as a substitute of xylene. It is a component of *Inhibisol* and *CNP30*. These solvents are stable to light but tend to slowly liberate hydrochloric acid on contact with water. Because of their high volatility, these compounds may achieve and exceed maximum allowable concentrations in poorly ventilated lab far more rapidly than xylene under the same conditions and therefore it is commonly not recommended.

Xylene and Toluene: These agents clear the specimen rapidly and rendered them transparent. Xylene hardens tissues fixed in non-protein coagulant fixatives and prolonged clearing in the solvent should be avoided. Industrial grade xylene may contain nearly 25% of other solvents such as ethyl benzene, with traces of benzene, odorous mercaptans and hydrogen sulphide. Only the sulphur and benzene-free solvent-grade xylene should be used for histological purposes.

Infiltration

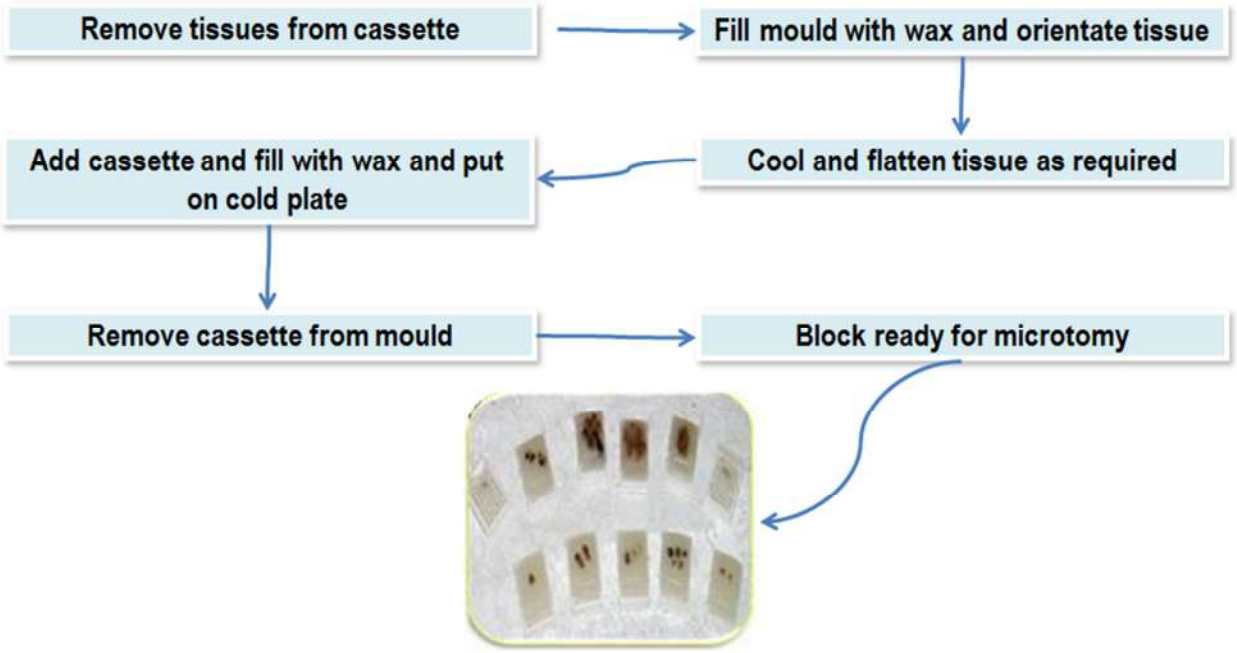
Infiltration is the saturation of tissue cavities and cells by a supporting substance which is generally the medium in which they are finally embedded. The properties of the medium should be similar to those of the tissue to be sectioned with regard to density and elasticity, and should also be molten at 30° to 60°C. The most common agent is paraffin wax which is molten when hot and solid when cold. However, a number of substances have been used for infiltration as well as embedding for eventual sectioning or microtomy.

Sometimes vacuum is used in infiltration to help complete impregnation of tissues with wax, which is usually carried out by using a molten wax or other medium under reduced pressure. Vacuum assistance helps to not only reduce the time tissues are subjected to heat but it also assists in the complete removal of any remaining solvent.

Embedding

Generally, paraffin embedding is employed to embed the tissue specimen and it is regarded as the standard method used in histology lab oratories practices. It is usually carried out using an embedding center and involves surrounding the tissues by an embedding medium such as paraffin which when cooled and solidified provides sufficient support for section cutting or microtomy. For microtomy, production of properly oriented and accurately labelled blocks is needed and therefore it is considered as one of the essential skills of trained histologists and

includes knowledge and understanding in areas such as tissue sampling, identification and anatomy.



Infiltration and embedding media: Though, most commonly used infiltration and embedding medium is paraffin. There are number of infiltration and embedding medium being used in histochemical procedure, which are as follows;

Celloidin (Low viscosity nitrocellulose): This medium is mainly used for preparing sections of soft tissues of mixed consistency such as an eyes and brain. It does not require heat, and the resultant block as rubbery consistency which gives good support to the tissues. However, it has some disadvantages such as inability to cut thin sections, storage of blocks in alcohol and speed of techniques that can take several weeks or months.

Ester wax: It is harder than paraffin wax and has a lower melting point i.e. 46° - 48°C. It is good for cutting hard tissues since it does not crumble.

Gelatin: It is reversible gel which can become irreversible by immersing in formalin. It is used as a support medium for frozen sections and sections of whole organs.

Paraffin wax: It is a polycrystalline mixture of solid hydrocarbons produced during the refining of coal and mineral oils. Its hardness or viscosity depends upon the molecular weight of the components and the ambient temperature. High molecular weight mixtures melt at higher temperature than waxes comprised of lower molecular weight fractions. It is marketed by its melting points which vary from 39°C to 68°C. Recently, some additives have been incorporated in paraffin wax that includes plastic polymers such as polyethylene wax which improves adhesion, hardness and plasticity, and dimethyl sulphoxide (DMSO) which reduce infiltration times and facilitates thin sectioning.

Resins: These are epoxy and acrylic compounds which are used mainly for the preparation of very thin sections and in the production of undecalcified sections of bone (In medical, it is required in the diagnosis of metabolic bone diseases such as osteoporosis and osteomalacia).

Water-soluble wax: Polyethylene glycols fall under this category. These are also used in dehydration and clearing. They are used for the demonstration of lipids and certain enzymes.

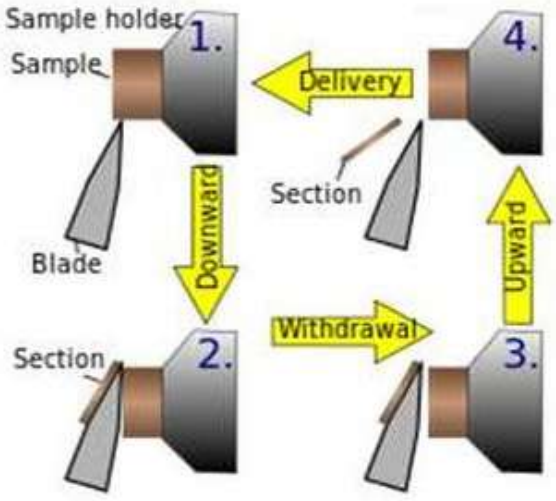
Microtomy

Microtomy or thin sectioning of tissue specimen for use in microscopy study is performed in specialized tool known as a **microtome**. A microtome is of commonly two type namely **rotary microtome** and **sledge microtome**. Microtomes use steel, glass, or diamond blades depending upon the specimen and thickness of the section required. Nowadays, disposable steel blades are generally used to prepare paraffin sections of tissues for light microscopy histology. The difference between these two is following;

In the rotatory microtome, the device operates with a staged rotary action and cutting is a part of the rotary motion. In this microtome, the knife is typically fixed in a horizontal position. Flywheel can be operated manually or they may be automatic or semiautomatic, and cut between 3 to 5 µm using paraffin wax block and thinner section if specimen is embedded in synthetic resin.

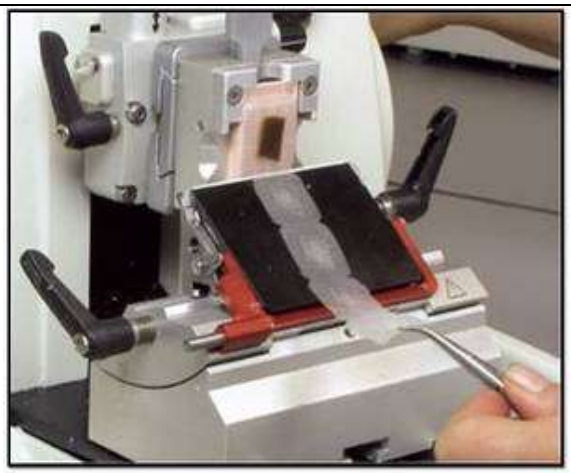
Rotary microtome

Sledge microtome

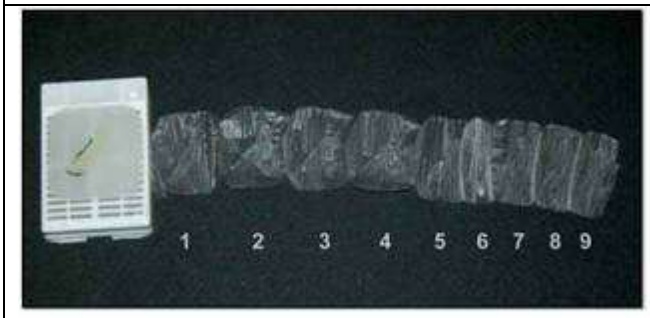


Principle of sectioning or cutting on a rotatory microtome

Paraffin block placed in holder and excess wax trimmed

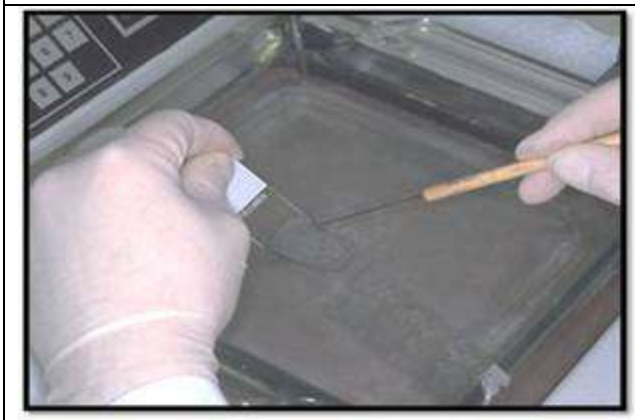


Paraffin sections cut at each turn of the flywheel



Ribbon of paraffin sections

Sections floated onto a waterbath



Section floated onto microscope slide

Unstained paraffin section on slide

These paraffin sections are then placed in a 60°C oven in order to melt the wax which helps to adhere the sections to the slide in readiness for the next stage of staining.

Frozen sectioning

It is a rapid way to fix and mount histology section using a refrigerator device called cryostat. A modified rotary microtome housed in a refrigerated cabinet. The temperature can be controlled with in -15^0c to -20^0c.The microtome is remotely operated from outside.

Freezing technique

1. Dry ice (Carbon di-oxide mixed with acetone with high pressure)
2. Liquid nitrogen (Iso-pentene cooled in liquid nitrogen)
3. Cold contact (Refrigerated test tube)
4. Commercial sprays.
5. Thermomodulate attachment.

Uses:

1. Urgent diagnosis: To detect whether a breast nodule is benign or malignant to decide lumpectomy or mastectomy.
2. To prevent dissolution or destruction of substance need to be examined.

a. Demonstration of fat in liposarcoma by Sudan black B.

b. Enzyme ATPase, NADPH in muscle biopsy for enzyme histochemistry.

3. For immunofluorescence studies like renal and skin biopsies to demonstrate deposit of antibodies, complements, fibrin and another antigen.
4. For silver and gold impregnation method in neurohistopathology.
5. To see excision margin whether it is free or invaded by tumor.
6. Diagnosis of hairspring disease or congenital mega colon in neonates and children.