H.S.C (VOCATIONAL)

MEDICAL LABORATORY TECHNICIAN

STD: XII (PAPER-2)

HISTOTECHNOLOGY

THEORY

INDEX

Topic 1: Introduction to Histotechnology

Objective: Students shall get an idea about the subject histotechnology. Student shall understand the various techniques involved in this & responsibility of technician.

1.1 Introduction-

Cells are the building blocks of all living things. Groups of these cells unite to perform a specific function. These groups of cells are called tissues. The tissues of the body consist of large numbers of cells and they are classified according to the shape, size and functions of the cells. Microscopic study of individual cell in a smear is called **cytology** and study of tissue is called **histology**.

Histology is the microscopic examination of normal tissue.

Histopathology means microscopic examination of diseased tissues to find out underlying pathology. Histopathological studies have proved to be one of the most effective means in diagnosing tissue abnormalities, benign and malignant tumors.

Histotechnology is the study of various techniques used to prepare tissue for microscopic examination.

The histopathological specimens are mostly collected by a surgeon in an operation theatre. The specimens are in the form of small pieces of tissues (biopsy) or it may be submitted as a whole organ or a mass.

Biopsy: It is the surgical procedure in which a piece of tissue is taken out of organ from a living person for histopathological examination.

Necropsy: When above procedure is done on dead body it is known as necropsy**.**

1.2 Role of Histotechnician-

Histotechnicians are neither involved in the collection of specimens nor in their laboratory evaluation.

Histotechnicians are responsible for handling & preparation of specimens to facilitate their gross & microscopic examination which is done only by histopathologist.

The final product turned out by a technician can favorably or adversely influence the diagnosis. Hence there should be perfection in technician's work.

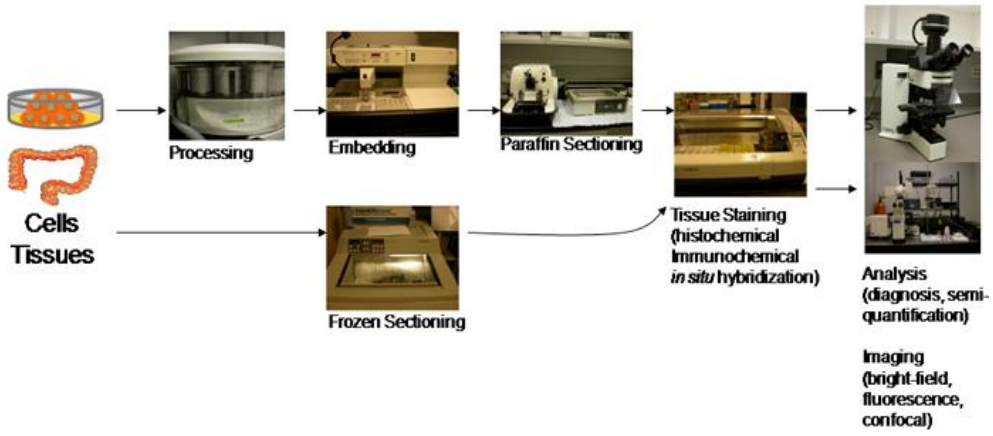

Figure 1.1 Tissue Sampling

1.4 List of important instruments used in histopathology laboratory

1. Microscope
2. Microtome usually Rotary
3. Knife sharpener (automatic knife sharpener or hone & strop)
4. Automatic tissue processor
5. Dissecting set for autopsy
6. Tissue flotation bath
7. Slide warmer
8. Cryostat
9. Freezing microtome

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Topic 2: Cell and Tissues

Objective: Students shall get knowledge of structure of cell, different types of tissues.

2.1. Structure of cell

The smallest, basic unit of the body is a cell, which is capable of doing all the functions independently.

The structure and function of a cell varies from organ to organ. For detail study of cellular structure, an Electron microscope is used. In unicellular organisms single cell carries out all functions while in multicellular different cells are for different functions, e.g. Respiration, excretion,digestion etc.

Cell consists of

1. Nucleus
2. Cytoplasm and cytoplasmic organelles

It contains **cell organelles** like Golgi apparatus, Endoplasmic reticulum, mitochondria, lysosomes, centriole & cell membrane.

In addition to this it contains **cell inclusions** like pigments, small particles of proteins, lipids & carbohydrates & thin filaments, secretary granules etc.

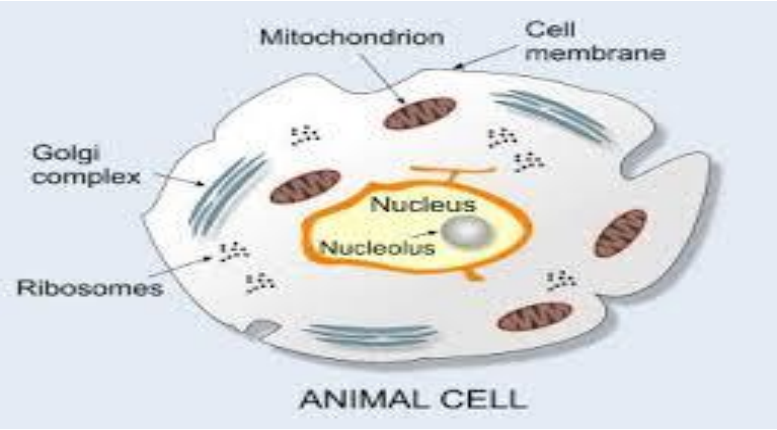


Figure 2.1.1

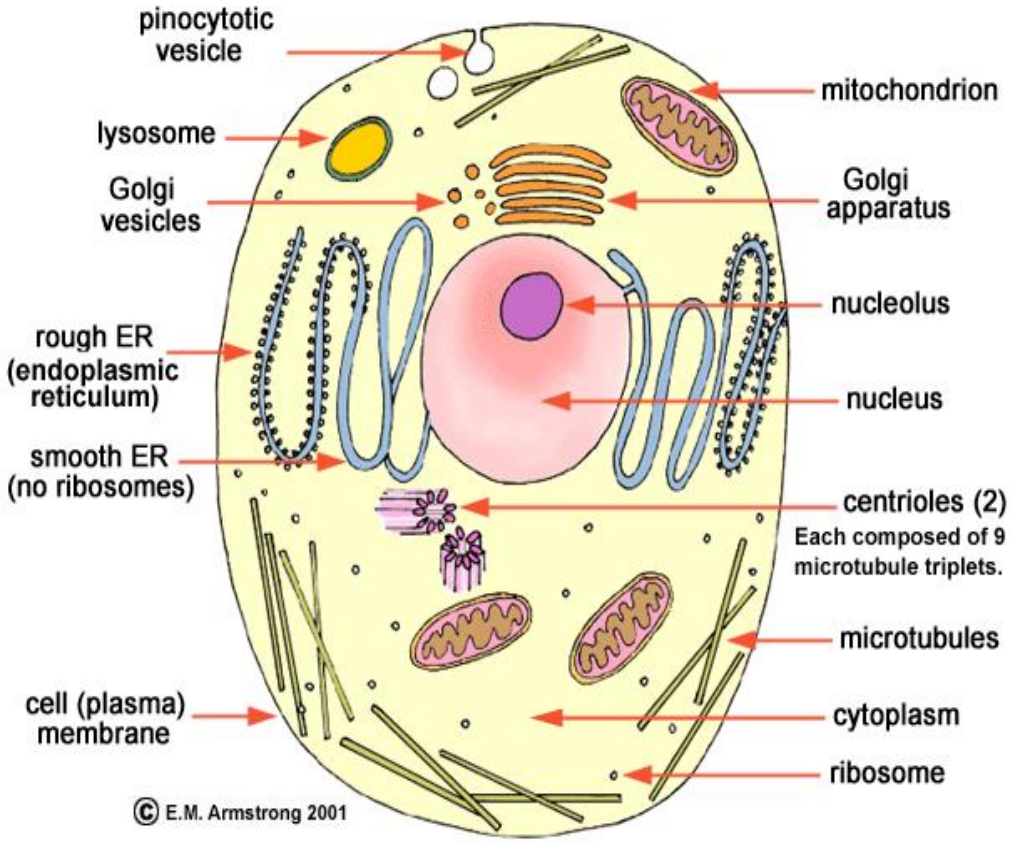


Figure 2.1.2 cell structure

Different parts of cell and its functions:

Sr. No.	Part of cell	Functions
1	Nucleus	Membrane bound structure that contains the cell's hereditary information
2	Plasma membrane	Maintains the integrity of the cell. It keeps the cell and its contents separate and distinct from the surrounding. It concern with

		function of protection, Secretion, absorption, excretion.
3	Mitochondria	Cell components that generate energy for the cell and are the sites of cellular respiration.
4	Endoplasmic reticulum (ER) Two types Smooth and Rough	Smooth ER- synthesizes lipids and steroid hormones, and is also associated with the detoxification of some drugs. Rough ER is studded with ribosomes. These are the site of synthesis of proteins
5	Golgi apparatus	The proteins move from the endoplasmic reticulum to the Golgi apparatus where they are 'packaged' into membrane-bound Vesicles called Secretory granules. The vesicles are stored and, when needed, move to the plasma membrane, through which the proteins are exported.
6	Ribosomes	They synthesize proteins from amino acids, using RNA as the template.
7	Lysosomes	Sacs of enzymes that digest cellular macromolecules such as nucleic

		acids.
8	Microfilaments.	Provide structural support and maintain the characteristic shape of the cell.
9	Microtubules.	These are contractile protein structures in the cytoplasm involved in the movement of the cell and of organelles within the cell.
10	Centrioles	Cylindrical structures that organize the assembly of microtubules during cell division.
11	Vacuoles	They collect waste products in a cell.

Types of cell (Depending upon ability to reproduce)

a. Permanent cells b. Labile cells

c. Stable cells

Permanent cells: These are incapable of multiplying under any situation.

E.g. Nerve cells

Labile cells: Multiply throughout life under physiological conditions to replace lost ones of their own kind.

E.g. Cells of epidermis & mucous membrane.

Stable cells: Do not divide normally but can multiply if the need arises.

E.g. Liver cells.

2.2 Tissues

Tissue :-Definition : An aggregate of same types of cells, having same general function, structure & united by varying amount of intercellular substance.

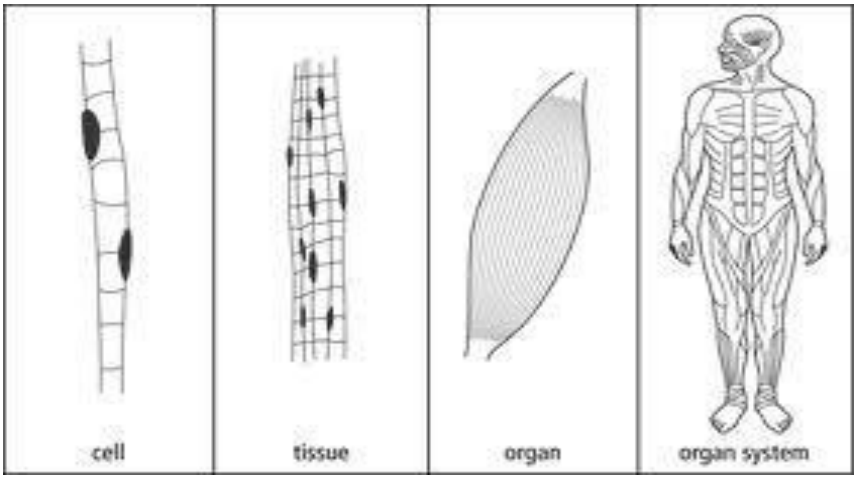
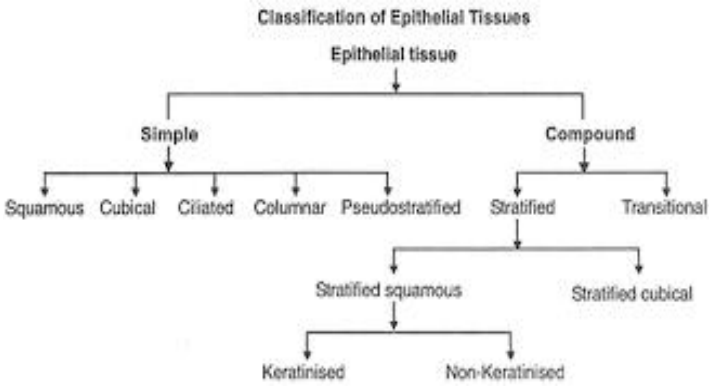


Figure 2.2.1

Tissue-Group of similar cells carrying out a specific function is known as tissue.

Basic tissues of body are

- Epithelial tissue
- Connective tissue
- Muscular tissue
- Nervous tissue



Epithelial tissue- It covers entire external surface of human body and inner surface of hollow organs. These cells lie on basement membrane.

Types of epithelial tissue:-

Sr. No	**Type**	**Description**	**Site where found**
1	Simple- Squamous Cuboidal Columnar- Ciliated Nonciliated	Single layer of cells lying on basement membrane. o Thin plate like cells o Cubical cells o Cubical but elongated cells with or without cilia.	 Pleural pericardial and abdominal cavities Endometrial glands Gastrointestinal tract (Nonciliated) Trachea and bronchial tree (Ciliated)
2	Compound (Stratified) o Squamous o Transitional	More than one layer of cells. Basal layer lies on basement membrane. o Plate like cells one above the other. o Basal squamous and then	 o Skin, Oral cavity, esophagus, Vagina. o Renal pelvis, ureter, urinary bladder,

	o Columnar	columnar cell. Look like pear shapes. o Basal columnar then stratification of cells	urethra o Pharynx, epiglottis, Fetal tissue

- **Simple squamous epithelium**

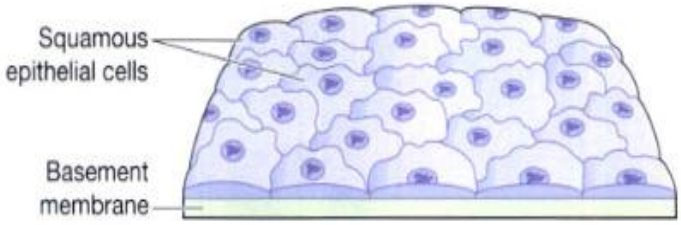


Figure -2.2.2

- **Simple Cuboidal Epithelium.**

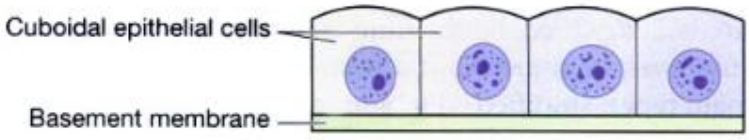


Figure 2.2.3

- **Simple Columnar Epithelium**

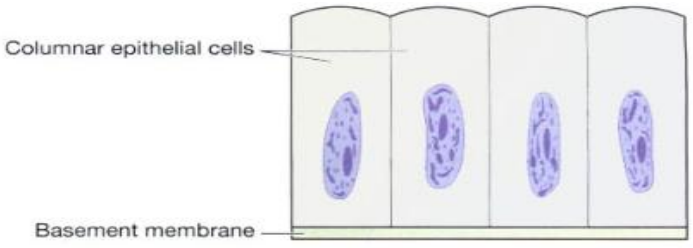


Figure 2.2.4

- **Ciliated Columnar Epithelium**

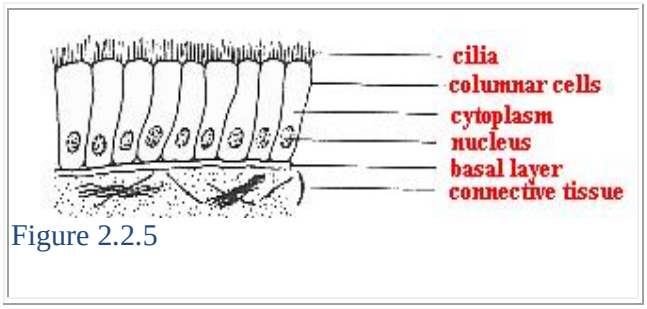


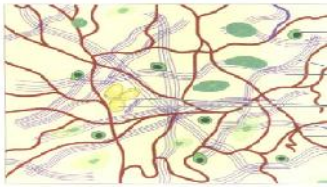
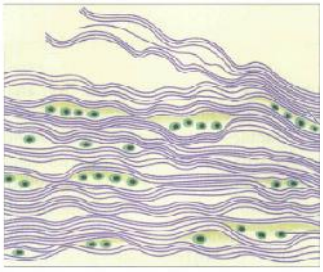
Figure 2.2.5

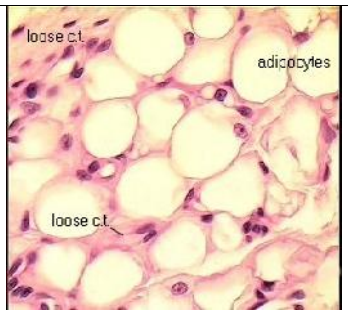
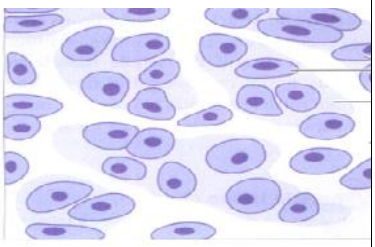
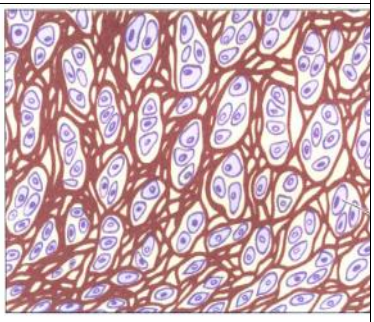
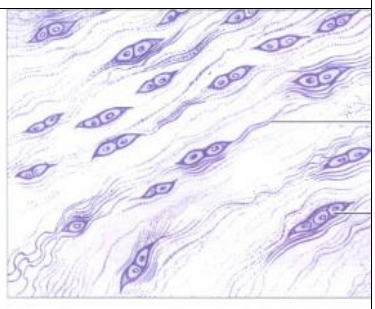
Connective tissue

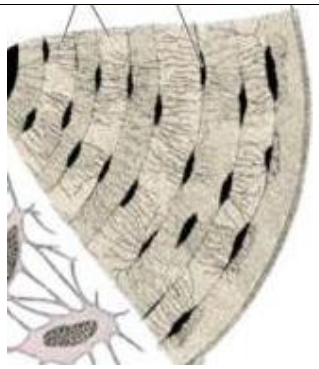
This is the most widespread and abundant type of tissue in the human body. Its function is primarily to support, anchor and connect various parts of the body. Although connective tissue exists in a number of forms, all types have three basic structural elements –

- Cells- Fibroblasts, Macrophages, Mast cells , Fat cells
- Fibres- collagen, Elastic and Reticulin fibres
- Intercellular substance (ground substance)

Types of connective tissue

Type	Site	Diagram
Loose areolar tissue	Immediately beneath the skin, in and around the blood vessel	 Figure 2.2.6
Dense fibrous tissue	Ligaments, tendons, periosteum, etc	 *Figure 2.2.7*

Adipose tissue	All subcutaneous tissue except eyelids, penis and inside the cranial cavity	 *Figure 2.2.8*
Cartilage • Hyaline	At joints and between bones • Covering ends of long bones, in costal cartilages, nose, larynx, trachea,etc	 *Figure 2.2.9*
• Elastic	➢ Lining of the ear, epiglottis and Eustachian tubes	 *Figure 2.2.10*
Fibrocartilage	Deepens cavities of bony sockets as in acetabulum, glenoid cavity, in semilunar cartilage.	 *Figure 2.2.11*

• Bone	Forms skeleton of body	 *Figure 2.2.12*

Muscular tissue.

Muscle is a tissue specialized for contraction and by means of which movements are carried out.

Muscular tissue is classified according to

A. Cross striations: - a. Striated muscles

b. Non striated or smooth muscles

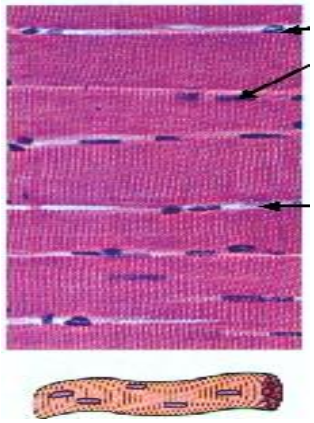
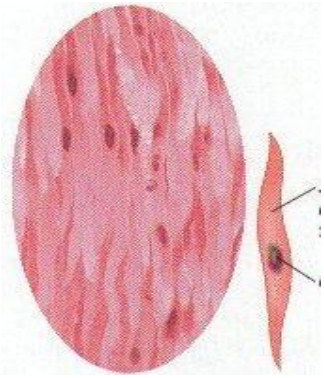
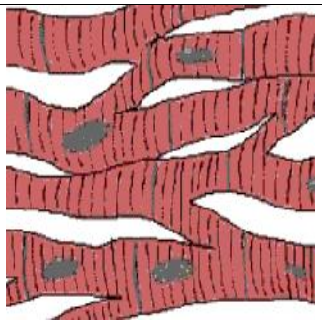
B. Nature of control: a. Involuntary b. Voluntary

C. Depending on distribution:-

a. Skeletal b. Cardiac c. Visceral (smooth)

There are three types of muscle tissue

1. Skeletal muscle
2. Smooth muscle
3. Cardiac muscle.

Type of muscle	Description	Location	Diagram
Skeletal	Voluntary. Individual muscle fibers are transversally striated by light and dark bands. No branching anastomosis.	Covers skeleton	 Figure 2.2.13
Smooth	Involuntary muscles. Activity is under the control of ANS. Spindle shape; do not show alternate light and dark bands.	Coats of blood vessels and lymphatics, walls of digestive tract, in hollow viscera, trachea ,bronchi, iris, ciliary muscles of eye	 Figure 2.2.14
Cardiac	Involuntary, striated but show branching anastomosis.	Heart	 Figure 2.2.15

Functions of muscular tissue:

- Locomotion of the body or bones.
- Development of muscular skills involved in our activity.
- Production of body heat.
- Support the organs within cavities.
- Changes in position of the body.
- Development of muscular skills involved in our activities and in our speech.
- Helping to protect blood vessels and assist in maintaining the circulation of the body by forceful contraction of the heart.
- Muscles help in respiration.

Nervous tissue

Neurons are the structural and functional unit of the nervous system. Nervous tissue contains two principal cell types.

- Neurons

  Neurons are nerve cells, sensitive to various stimuli. It converts stimuli to nerve impulse. It contains 3 basic portions. These are cell body, axons and dendrites.
- The neuroglia

  Neuroglias are cells that protect, nourish and support neurons. Clinically they are important because they are potential to replicate and produce cancerous growths.

  Nerve cell with its processes

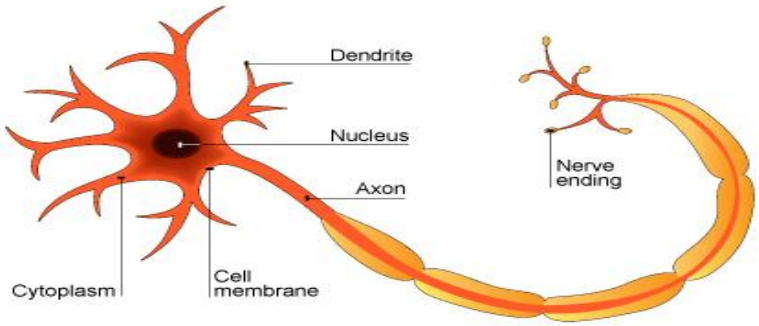
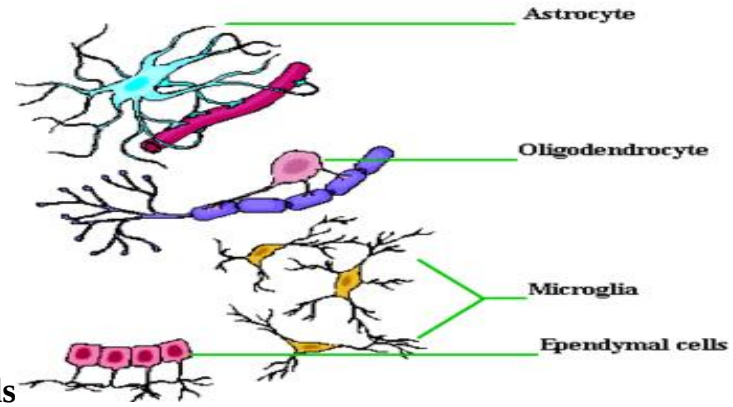


Figure 2.2.16



Neuroglial cells

Figure 2.2.17

Topic 3: Methods of examination of cells and tissues.

Objective- Students shall learn about various methods used preparation and examination of tissues.

3.1 Introduction

Different methods are used for examination of tissue with the aim of giving accurate diagnosis.

Details of investigations vary from laboratory to laboratory but basic principles are same. Methods of examination depends on number of factors like size and nature of tissue, structures to be studied, whether specimen is fresh or fixed, etc.

The different steps involved are as follows-

3.2 Collection & labeling of specimens

- The specimens processed in a histopathology laboratory are the tissues and organs removed from the living body and the organs obtained from autopsy after the death of the patient.
- Also biopsy specimens received for histopathology examination.
- Sometimes paraffin tissue blocks or slides for review are received.
- Immediately after removal from the body, minute tissue as well as large specimen, should be fixed in 10% formalin or other fixatives of choice which should be about 10-15 times the volume of the tissue. The specimen can be collected in plastic or glass containers.
- On arrival of specimen in HP lab it should be checked, confirmed and immediately stamped and dated. This should be accompanied by a requisition form bearing the essential details about the patient and the specimen. Accept it only if the information tallies. Once this is done, the specimen should given a number; for example in

B150/2014, B denotes the biopsy specimen, 150, the number of the specimen in serial order, and 2014, the year of the receipt of the specimen . A label containing name of patient, its identification number should be pasted on the containers. . This surgical or biopsy number along with the patient's name, age, sex, hospital number, ward and service should be entered in a separate register for reference.

3.3 Specimen rejection criteria: Specimen received in the laboratory should be rejected, if-

- Patient details on requisition & sample container are different.
- If details of specimen site is not documented.
- Specimen is not received in specified containers.
- The containers are improperly packed.
- Slides are broken or improperly processed.
- Insufficient contents are observed in paraffin blocks.

3.4 Gross examination of tissues

- Gross examination (grossing) is a process, by means of which specimen are inspected with the bare eyes to confirm identity of the specimen and to obtain useful diagnostic information, before being processed for further procedures and microscopic examination.
- Gross examination of surgical specimens is typically performed by a pathologist, or by a senior laboratory technician.
- All points regarding gross examination should be noted down.
- Pathologist selects area from which sections are to be prepared and pieces of tissues from those areas are cut and sent for further processing.
- Number of pieces which are cut are noted down.

Further processing depends whether specimen is fresh or fixed.

3.5 Examination of Fresh specimen- They are prepared as follows

- Squash preparation- The cells of the tissue are separated by squashing fresh minute tissue between two glass slides which are drawn apart. The cells are studied as wet preparation or they are stained with vital stain and examined microscopically. This helps to study cellular contents of the tissue.
- Teased preparation- Fresh specimen is immersed in saline and dissected with mounted needles. Pieces are taken on slides and mounted as wet preparation. Then they are examined by light microscope or by phase contrast microscope. Methylene blue can be used to enhance the recognition of cellular details.
- Impression method- Prepared by touching a freshly cut piece of tissue with the surface of slide. Cells from the tissue adhere to the slide and then they are examined with phase contrast microscope or by vital staining. It can be fixed and later on stained.
- Smears- Smearing a piece of fresh specimen evenly on slide is commonly used method in histopathology. It is examined after vital staining. It can be fixed immediately and stained by cytological stains.
- Frozen section- Fresh tissue is frozen to provide suitable consistency and then it is cut on freezing microtome and stained.

3.6 Examination of fixed specimen

The most effective means of studying normal and diseased tissue is to fix it, process it, embed it in paraffin wax, cut into thin sections, mount it on slides, stain it and examine it under microscope.

3.7 Report and Filing

After giving slides to pathologist he does histological diagnosis. Then reports are printed and dispatched.

Slides are preserved. Gross specimens are kept at least for 1 month before it is disposed off.

Note-

All surgical specimens sent for histopathology should be handled very carefully as it is not possible to repeat the sample

3.8 Types of biopsy

Incision biopsy: Removal of abnormal or diseased part of the tissue. Entire organ is not removed. This procedure is used for diagnostic procedure.

Excision biopsy: Removal of entire diseased part of the tissue with rim of normal tissue. This procedure serves both diagnostic and therapeutic purpose.

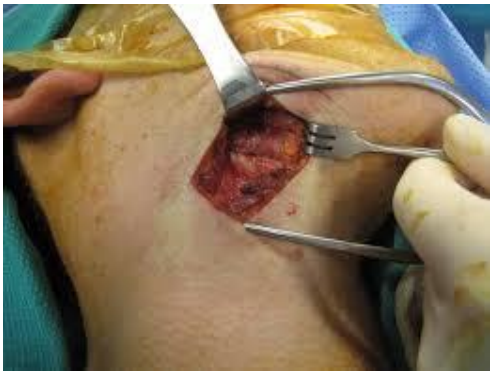


Fig.3.8.1 Excision of submandibular lymphnode

Punch biopsy: Removal of tissue bit from an ulcerative lesion.

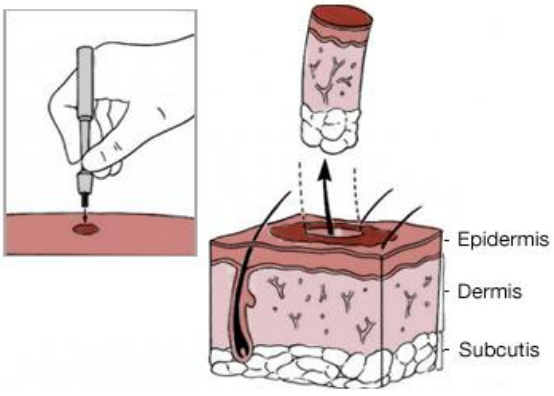


fig. 3.8.2

Cone surgery: It is related to uterine cervix only.

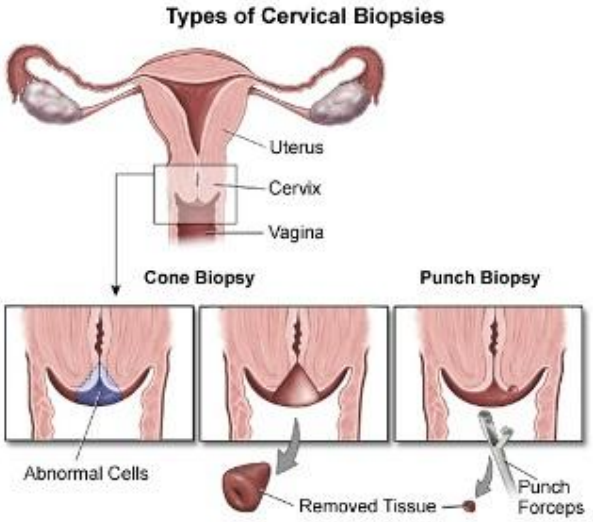


Fig. 3.8.3

Endoscopic biopsy: First organ is visualized for an abnormality and abnormal part is removed.

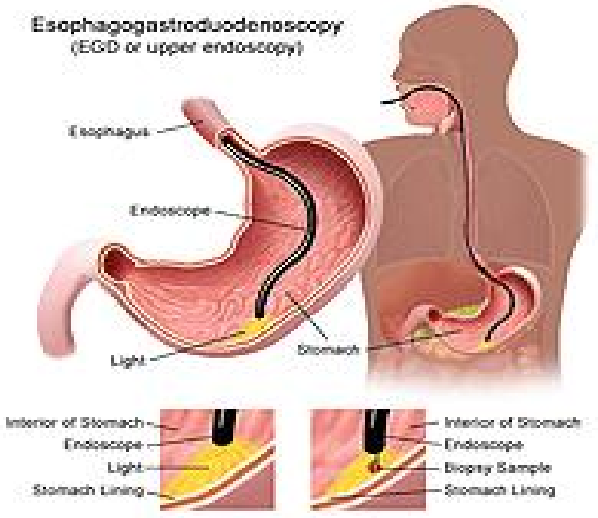


Fig. 3.8.4 Endoscopic biopsy

Needle biopsy: In this method, specimen is obtained with the help of special needle. This procedure is usually used for liver, kidney& bone marrow. Many times this is done under guidance of ultrasound.

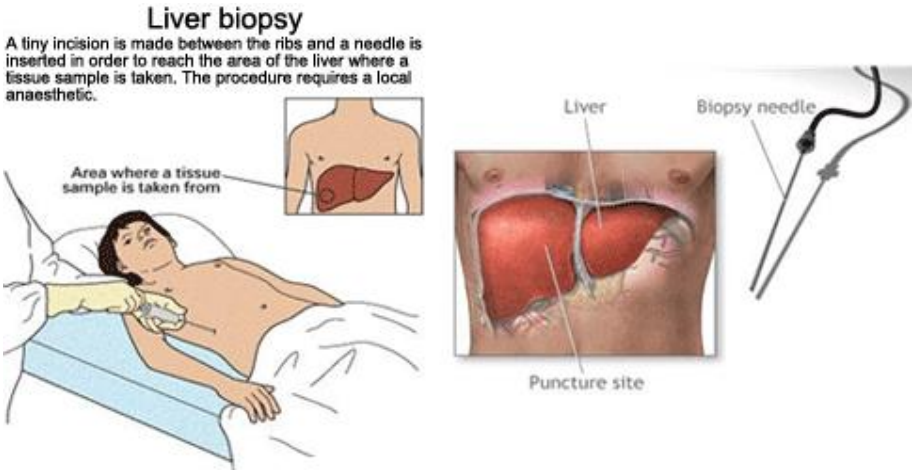


Fig. 3.8.5Liver biopsy

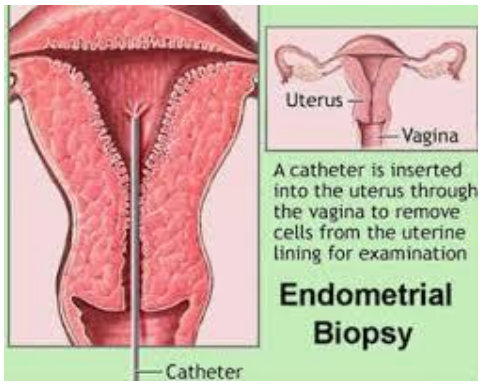


Fig. 3.8.6 Endometrial biopsy

Review Questions

- Name different steps of preparation of tissue.
- What are different methods of preparation of fresh specimen?
- How fixed tissue is prepared?
- What are different types of biopsies?

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Topic 4: Fixation of tissue

Objective- Students shall be able to classify fixatives and understand significance of fixatives.

4.1 Introduction

Various types of tissues are sent in Histopathology laboratory in order to identify underlying disease process which helps in diagnosis. The different organs/tissues should be properly preserved and processed so that pathologist can give correct histopathological diagnosis.

It tissue is not properly preserved it undergoes various changes like autolysis, putrefaction etc. Autolysis is the self digestion and destruction of tissue caused by action of intracellular enzymes whose normal behavior is altered causing breakdown of protein and liquefaction of cells. Putrefaction means the breakdown of tissue by bacterial action often with the formation of gases. This makes it difficult for pathologist to examine it under microscope and give diagnosis. To prevent this it is necessary to preserve and process tissue properly.

4.2 Definition and Objectives -

Fixation is the process of preservation of cells and tissue constituents in a condition identical to that existing during life.

The process of fixation is carried out twice.

1. At the operation theater as essential fixation or primary fixation.
2. Refixing is done in histopathology laboratory, after cutting the bits of tissue. The block of the tissue should be thick enough in order to penetrate the fixative in the reasonable short time..

Minute specimens should be wrapped in the cigarette paper or a lens paper and then placed in a special container like capsule.

Objectives-

- To prevent autolysis and bacterial decomposition and putrefaction.
- To coagulate the tissue so as to prevent loss of easily diffusible substances.
- To prepare the tissue against deleterious effects of various reagents used during preparation of tissue sections.
- To leave the tissue in a condition which facilitates differential staining with dyes and reagents.

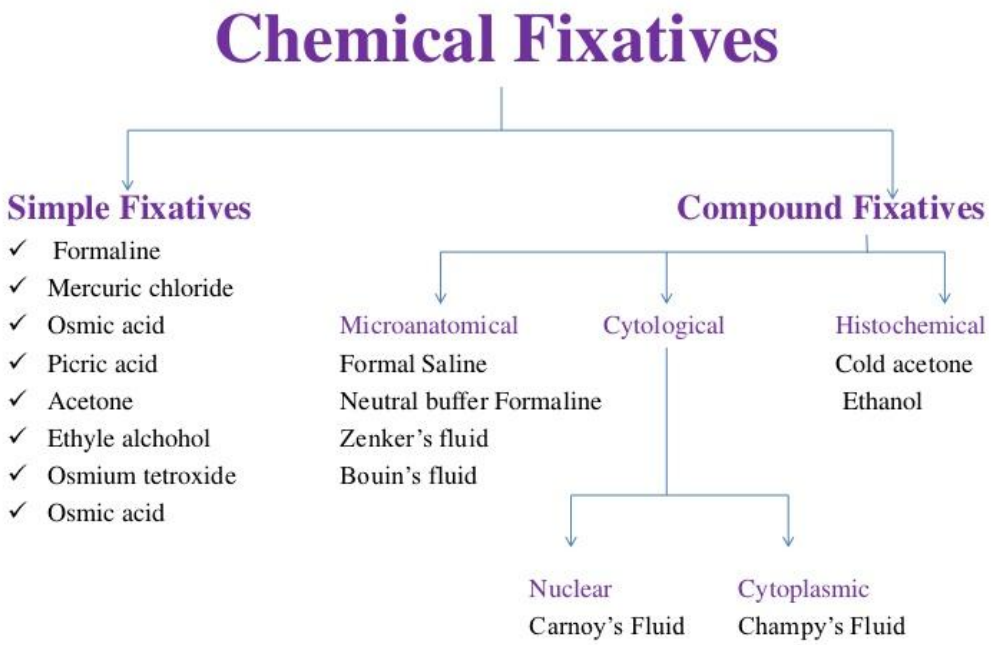
Adequate and complete fixation is the foundation of all good histological preparations. The length of time required for adequate fixation varies according to size and consistency of tissue and fixative used. Generally volume of the fixative should be 10times the size of tissue.

So, to fulfill aim there are certain criteria for an ideal fixative. They are

- Should prevent autolysis and bacterial decomposition.
- Should not shrink or swell the cell nor destroy the structure of the tissue.
- Should rapidly and evenly penetrate the tissue.
- Should give good optical differentiation.
- Should provide suitable consistency and render it amenable for easy manipulation.
- Should not interfere with staining.
- Should be non-toxic, non-corrosive and non-inflammable.
- Should be stable.
- Should be readily available and easy to prepare.
- Should allow long term storage of specimens without deterioration.

4.3 Classification of fixatives –

Fixatives are classified in to Simple and compound fixative.



4.3.1 Simple fixatives

Different simple fixatives are

- **Formaldehyde**-It is a gas which is soluble in water to approx. 40% weight. This saturated gaseous solution is available commercially as formalin. It preserves fats and proteins well.
- **Mercuric chloride ($HgCl_2$)-** It is a constituent of many fixatives. It is often used as saturated aqueous solution.
  Exposure of tissue to their action in excess will produce excessive hardness and make cutting difficult.
- **Picric acid**- This fixative is explosive when dry so it should be kept damp preferably under water. Generally it is used as saturated aqueous solution of 1% concentration.
- **Osmium tetroxide**- The solution is a strong oxidizing agent. It should be stored in dark. It fixes lipid permanently. It preserves cytoplasmic structures well so it is used for electron microscopy.

- **Acetic acid**- Generally bit is referred as glacial acetic acid because it solidifies at approx. 17^{o}C. It is not used alone as it causes swelling effect on tissue. It is used in compound fixative to counteract the shrinking effect of other agents.
- **Ethyl alcohol**- It is colorless, miscible with water and highly inflammable. Mainly it is incorporated into compound fixative. It is used as fixative for smears only.
- **Potassium dichromate**- Commonly simple fixative. At ph < 4.6 it precipitates proteins and fixes carbohydrates while at pH > 4.6 cytoplasm is well preserved and mitochondria fixed.
- **Chromic acid**- It is solution of chromium oxide in water. Stored as 2% stock solution. It is powerful oxidizing agent so should not be used with alcohol or formalin.
- **Acetone**- Mainly used for demonstration of enzymes. Glycogen is not well preserved.

4.3.2 Compound fixatives

These are products of two or more simple fixatives mixed together to obtain the combined effect of their properties. They are classified as

- Microanatomical
- Cytological
- Histochemical

4.3.2.1 Microanatomical Fixatives: -

- It is used to preserve various layers of tissue and cells in relation to one another so that a general structure may be studied.
- They are used routinely.
- They never over harden the tissue.

10% Formalin-

Commercial formalin	10ml
Distilled water	90ml

Advantages-

- It is ideal for surgical and postmortem specimens.
- It causes even fixation and produces very little shrinkage.
- Tissue can be kept infinitely in it.
- It forms the basis for museum technique.
- It is relatively inexpensive.
- It is readily available
- It is very easy to prepare.
- It is compatible with most of the stains.

Disadvantages-

- It is slow fixative.
- It forms black formalin pigment with blood.
- It has an unpleasant odor & its vapors irritate the eyes.
- Sometimes it causes allergic reaction on the skin.

So neutral formalin is used.

Neutral formalin

10% formalin	1000ml

Add excess amount of $Caco_3$

Fixation time- 5mm thick tissue is fixed in 6-12 hours at room temperature.

10% formal saline

Formaldehyde 40%	100ml
Sodium chloride	9gm
Distilled water	900ml

- It is ideal for fixing the brain.
- Fixation time for large tissue is 24 hours.

10% Buffered Formalin

Formaldehyde 40% (Formalin)	10ml
Sodium dihydrogen phosphate	0.4gm
Disodium hydrogen phosphate	0.65gm
Distilled water	90ml

- It prevents formation of acid formalin pigment.

Zenker's Fluid-

Stock solution-

Mercuric chloride (Hgcl2)	60gm
Potassium dichromate (K2cr2o7)	25gm
Sodium sulphate	10gm
Distilled water	1000ml

Working solution-

Add 5ml of glacial acetic acid to 95ml of Zenker's fluid just before use.

- It is commonly used as secondary fixative.
- Penetration power of the fixative is however poor and hence tissue should not exceed 5mm thickness.
- The zenker's fluid is recommended for small tissue like spleen, liver etc. RBCs are not preserved well.
- It is best for cytoplasmic and fiber stain.
- The tissue fixed in the zenker's fluid should be washed thoroughly under tap water before processing because the fixative contains mercuric chloride which forms blackening zone, this zone interferes with the stain while examining under microscope.

Helly's fluid (Zenker formal)

Stock solution

Sodium sulphate	1.0gm
Potassium dichromate	2.5gm
Mercuric chloride	5.0gm
Distilled water	100ml

Working solution

Prepared by adding 5ml of formalin to 95ml of stock solution.

Advantages

- Used as both micro anatomical and cytological fixative.
- Recommended for bone marrow and pituitary tissue.
- It gives excellent nuclear fixation.
- Staining of nuclei is more intense than after fixation with Zenker's solution and cytoplasmic granules are well preserved.

Disadvantages

- Fixation is slower than Zenker's fluid but gets completed in 24 hours.
- Tissues left in this fixative for more than required period form a brown scum over the surface.
- Removal of mercuric pigment with iodine is must.

Bouin's Fluid

Saturated aq.Picric acid	750ml
Formalin (37-40%)	250ml
Glacial acetic acid	50ml

Advantages-

- It is recommended for general purpose and for special study of hematology and lymphoid tissue.

- It fixes the tissue within 4-8 hours so it is a quick fixative.
- It gives brilliant staining with trichrome stain.
- It preserves glycogen well.
- Tissues can be transferred directly to 70% alcohol.

Disadvantages-

- Yellow color picric acid interferes in staining reaction.
- If the yellow color seen in the section then deparaffinize, wash and refix it.

Hiedenhains Susa-

Mercuric chloride	45gm
Nacl	5gm
Trichloro acetic acid	20gm
Glacial acetic acid	40ml
Formaldehyde 40%	200ml
Distilled water	800ml

Fixation time- For 7-8mm thick tissue 12-24 hours and for 3mm thick tissue 2-3 hours.

Advantages-

- It allows brilliant staining**,** e.g. silver impregnation method.
- It penetrates rapidly and fixes evenly.
- With the minimum shrinkage and hardening of tissue.

Disadvantages-

- Red blood cells are poorly preserved.
- The fixative contains mercuric chloride so it should be treated with iodine to remove mercury pigment.

4.3.2.2 Cytological fixatives -

Cytological fixatives are of two types

A. Nuclear fixative

B. Cytoplasmic fixative

A-Nuclear fixatives-

Carnoy's Fluid

Absolute alcohol	60ml
Chloroform	30ml
Glacial acetic acid	10ml

- It is a quick acting fixative.
- It is suitable for cytological and biopsy specimens.
- It is recommended for glycogen but readily removes the fat.
- It is used for study of chromosomes.

Advantages-

- This fixative produces good nuclear staining
- The tissue is transferred directly to the absolute alcohol.

Disadvantages

- Excessive shrinkage of tissue can occur..
- Red blood cells are haemolysed.

Clarke's fluid-

Absolute alcohol	75ml
Glacial acetic acid	25ml

- Fixation time-1-2 hours.
- It penetrates rapidly.
- It is good nuclear as well as cytoplasmic stain.
- It is ideal for smears.

B-Cytoplasmic fixatives-

Flemming's fluid-

1% chromic acid	15ml
2% osmium tetroxide	4ml
Acetic acid	1ml or less

- The period of fixation is 24-48 hours.

Advantages-

- Widely used for demonstration of chromosomes.
- This fixative is most commonly used for demonstration of myelin following primary fixation.
- It is an excellent fixative which is permanently fixes the fat.

Disadvantages-

- It is very costly.
- Deteriorates very rapidly and must be prepared before use.

Champy's fluid

3% Potassium dichromate	7ml
1% Chromic acid	7ml
2% Osmium tetroxide	4ml

- Fixation time 12hours for 2mm thick tissue
- It preserves mitochondria and lipids.
- It should be prepared fresh.
- It penetrates poorly and unevenly.
- Tissue must be washed overnight after fixation.

4.3.2.3 Histochemical fixatives –

These fixatives are used when it is necessary to demonstrate enzymes and other chemical substances of the cell.

They should not alter the normal chemistry.

They should not interfere with staining.

- For majority of histochemical methods cryostat cut sections of rapidly frozen tissue are preferred. These sections are used unfixed or fixed with a vapor fixative. Different vapor fixatives are
    - Formaldehyde
    - Acetaldehyde
    - Glutaraldehyde
    - Acrolein or chromyl sulphate
- Buffered formalin is most common fixative used for histochemical purposes.
- Absolute alcohol can be used as an alternative to buffered formalin.

Secondary fixation

This consists of fixing the tissue in formalin followed by Zenker's fluid or Susa fixative. It gives to firm texture to the tissue and improves staining qualities.

Freeze drying is an alternative to fixation. By this it is possible to preserve tissue with alteration in cell structure and chemical composition. This permits embedding in wax without dehydration by alcohol and clearing.

Review questions-

- Define fixation. What are aims of fixation?
- What are criteria for an ideal fixative?
- Classify fixatives. Give one example.
- Give composition of Zenker's fluid/Helly's fluid/Bouin's fluid/Flemming's fluid.
- What is secondary fixation?
- Name histochemical fixatives.

Topic 5: Decalcification

Objectives –Students can perform decalcification by different methods.

5.1 Definition-

Decalcification is the process by which calcium is removed from the fixed tissue.

Normally the calcium is present in the bones, teeth and cartilages but abnormally the calcium get deposited in different tissues in diseases like tuberculosis, bone tumor, etc. The presence of the calcium salts in the tissue renders it very hard to cut and also damages the microtome knife.

5.2 Methods of decalcification

- Acid method
- Chelating Method
- Ion exchange Method
- Electrophoretic Method

ACID METHOD –

It is most widely used method. The decalcifying fluid is the acid which removes calcium salt present in the tissue, thereby making the tissue soft enough for section cutting. The most common acid decalcifying fluids are

Formic acid –

10% formic acid	5ml
Distilled water	95ml

Formic acid is a good decalcifying agent widely used. It is very cheap, easy to prepare. It is slow in action but doesn't cause tissue damage

Decalcifying time is 2-7 days.

End point is detected by both X-ray and chemical method.

Nitric acid-

Conc. Nitric acid	5ml
Distilled water	95ml

5% Nitric acid is also a good decalcifying agent. It decalcifies the tissue very quickly. It is used for small pieces of bones. Decalcifying time is 4-14 hours. Over exposure impairs the nuclear staining.

End point is detected by both X-ray and chemical method.

Von Ebner's fluid-

Saturated solution of NaCl	50ml
Distilled water	42ml
Conc.Hcl	8ml

Rapid decalcifying agent time is 3-7days. It is ideal for teeth. No neutralization required. If end point exceeds impair staining.

End point is detected by both X-ray and chemical method.

Trichloro acetic acid (TCA)

It is specially used for the decalcification of the teeth and soft bone of the middle ear. 5% solution is used.

CHELATING METHOD-

This method is very slow. Time required is 3 weeks. Every three day interval fluid is changed and it is reduced to one day towards end of decalcification. It is generally used at pH 7.0.

EDTA is the best chelating agent.

EDTA	5.5gm
10% formalin	10ml
Distilled water	90 ml

<u>Advantages-</u>

- It causes very little or no damage to the tissue.

- The staining is very excellent.
- More appropriate for research applications where very high quality morphology is required or particular molecular elements must be preserved for techniques such as IHC, FISH or PCR.

Disadvantages-

- The process is very slow so not suitable for urgent specimens.

5.3Factors influencing the rate of decalcification

- Concentration

  The concentration of active agent will affect the rate at which calcium is removed. The concentration of active agent will be depleted as it combines with calcium and so it is necessary to use a large volume of decalcifier and renew it several times during the decalcification process.

- Temperature

  Increased temperature will speed up the decalcification rate but will also increase the rate of tissue damage so must be employed with great care.

- Agitation

  Gentle agitation may increase the rate slightly but as heat is produced it may cause tissue damage.

- Fluid access

  As with fixation fresh decalcifier should have ready access to all surfaces of the specimen. This will enhance diffusion and penetration into the specimen and facilitate solution, ionization and removal of calcium.

ION EXCHANGE METHOD

Principle- Calcium ions are removed from the solution are taken up by resins, thereby increasing the rate of solubility of calcium ions. Here formic acid is used.

Advantages-

- Penetrates very rapidly.
- Maintains cellular details.
- Staining is very good & brilliant.

Disadvantage-

- The end points can be detected by X-Ray only.

ELECTROLYTIC DECALCIFICATION

Principle- There is attraction of positively charged Ca ions to negatively charged electrodes. The tissue is placed in an electrolytic solution composed of 5% HCl and 5% Formic acid in equal parts.

Advantages-

- It is 3 times faster than any other method because acceleration is brought about by an electronic circuit.

Disadvantage –

- Heat produced during decalcification damages the tissue so not used in routine method.
- The end points can be detected by X-Ray only

5.4 Detection of end point of decalcification-

It is detected by three methods

1. Physical method

- Prick the needle in to the tissue.
- Press the tissue with the fore finger and thumb (Palpation).

2. By X-Ray technique

- This is an excellent method but expensive.
- Not always convenient and available.
- Not useful when radio-opaque metallic salt like $HgCl_2$ is used in fixative.

3. Chemical method-

   This is simple, reliable and inexpensive method.

   It is easy to perform.

   More suitable when acid decalcifying fluids are used.

Technique

- Take 5 ml of acid decalcification solution from under the specimen and place it in a test tube.
- Add a litmus paper. This will turn red due to strong acid of the decalcifying fluid.
- Add strong ammonia drop by drop till the medium becomes alkaline and litmus paper turns blue.
- If the solution is cloudy, indicates presence of calcium in large amount.
- Fluid should be changed and continue the decalcification.
- If there is no cloudiness add 0.5 ml of saturated aqueous Ammonium oxalate in the decalcifying fluid and keep it for 20-30 minutes.
- If turbidity appears continue decalcification.
- If there is no cloudiness then the decalcification is complete.
- Wash the tissue and transfer it to 70% alcohol.

Review questions-

- Define decalcification. Give its significance.
- Describe different methods of decalcification.
- How will you detect end point of decalcification?

Topic 6: Tissue Processing

Objective- Students shall carry out various steps included in tissue processing and prepare tissue for cutting on microtome.

6.1 Introduction

Tissue processing is the preparatory treatment of tissue before being sectioned which contains impregnation of specimen with an embedding medium to provide support and suitable consistency for microtomy.

A variety of materials and methods are available for embedding having special advantages and limitations. The different embedding media are as follows:

- The Paraffin wax
- The Ester wax
- The water soluble wax
- The cellulose nitrate
- Double embedding medium- It consists of paraffin wax and cellulose nitrate mixture.
- Freezing and freeze drying
- Gelatin and other substances

The paraffin wax embedding is most commonly used method.

6.2 Receiving tissues

Gross operated tissues with patient's details are received along with requisition form in histopathology laboratory for diagnosis.

Specimen of tissues is received in a container of fixative (volume 10times of specimen).

If tissues are not fixed should be sent immediately to the laboratory to avoid decomposition of tissue.

Note- All specimens in the laboratory should be handled very carefully, treat every specimen as infectious, so use disposable gloves and protective clothing.

6.3 Record –

After receiving tissue a brief description of the nature and its site of origin should be recorded along with patient's name, numbers date in a log (Register) book or in a working card.

In description of tissue, its number of pieces, size, color, appearance, consistency, and contour should be recorded and tissue requiring special attention should be marked.

6.4 Selection –

From gross specimen (received) of tissue only diseased tissue should be selected and cut or trimmed in small pieces. Always select multiple small pieces not greater than 4 mm in size for tissue processing.

Bone tissue or calcified tissues should be decalcified before tissue processing.

6.5 Labeling –

This is one of the important steps in tissue processing. After selection, tissues are labeled with the given number which should be written legibly and correctly & this label should pass along with tissue through all stages of tissue processing.

Most satisfactory labels are printed, graphite penciled, type written or India ink written, Tissue requiring special attention should be marked.

Very small biopsies like needle aspiration biopsies of kidney, liver etc may be wrapped in soaked filter paper.

And these tissues are put in the small **cassettes** before tissue processing.

The introduction of plastic embedding cassettes has greatly facilitated processing of tissue and reduced the risk of error.

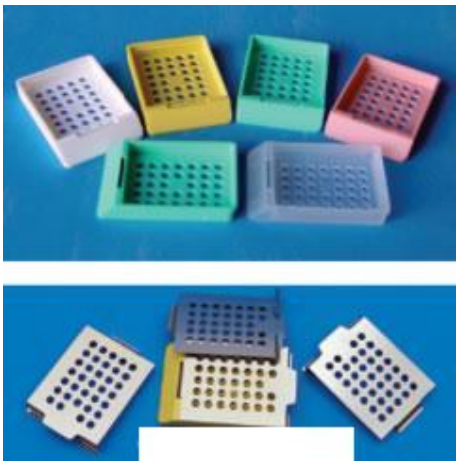


Fig.6.5.1 Tissue cassettes.

These cassettes are available in various sizes and colors with an integral lid and roughened sides which permit the necessary information to be recorded.

These cassettes with selected pieces of tissue inside eventually form part of the final paraffin wax block, during this procedure label should not be separated from the tissue.

After selection & preparation of cassettes for processing whatever of the remains of gross specimens of the tissue should be preserved until the final report of diagnosis of disease tissue comes & then this remains of tissue should be discarded.

6.6 Tissue processing using paraffin wax-

For embedding or making block of a tissue paraffin wax is the universally used. It is most satisfactory medium.

Paraffin wax is cheap, easily available and gives best results which are achieved most rapidly than other embedding medias like celloidin, carbowax etc.

It is carried out by two ways- Manual and Automatic tissue processing.

In both different steps involved are

- Dehydration
- Clearing

- Wax impregnation
- Embedding

6.6.1 Dehydration - Dehydration is the first step of tissue processing after fixation. Dehydration means removal of water from the tissue.

Necessity of dehydration

Paraffin wax is used for preparing tissue block; this paraffin wax is not miscible with water which is present in the tissue. This removal of water from the tissue or dehydration of tissue is necessary so that paraffin wax will enter into the tissue & preparation of tissue block becomes easy procedure.

Dehydration agents

For dehydration of tissue reagents commonly used are **ethyl alcohol**, **isopropyl alcohol. Acetone** & **dioxane** have also been used.

Dioxane - its fumes are toxic so it is rarely used.

Acetone - it causes shrinkage and make the tissue hard & dry, therefore it is used only for urgent biopsies.

Alcohol - It is clear, colorless, inflammable liquid with a pleasant odor and it is more suitable for dehydration of tissue, so it is routinely used as dehydrating agent. This consists of passing the tissue through a series of progressively more concentrated alcohol baths.

Procedure

- Tissues together with their identifying label in a cassette are transferred from one container of alcohol to another at the appropriate times, allowing them to drain for a few seconds between each change. The containers should be fitted with lids.
- The more delicate the tissue the lower is the grade of alcohol suitable for commencing dehydration and there should be smaller interval between each strength of the alcohol.

- The strength of the initial alcohol and the time required in each grade depends on the size and type of tissue and on the fixative which was used.
- For the final bath use pure or absolute alcohol which is free from water. The period necessary for dehydration may be reduced by processing at 37^0c instead of room temperature.
- Commonly used grades of alcohol are
    - 70%
    - 80%
    - 90%
    - 100%
- For delicate tissue gradation starts from 50%.

6.6.2 Clearing -

This is the next step after dehydration in tissue processing. Clearing also known as dealcoholization means removal of alcohol from the tissue by keeping it in antemedia or clearing agent like xylene.

Most of the antemedias raise the refractive index of the dealcoholised tissues there by imparting them a degree of transparency due to this transparency of tissue, this stage is called clearing and the medias are called clearing agents.

Necessity

The alcohol which was used for dehydration of tissue is also not miscible with paraffin wax therefore tissues are treated with such a reagent which will mix with both alcohol and paraffin wax and which in turn get removed from the tissue during impregnation.

Different clearing agents are used

Clearing agents

- **Xylene** - A rapid clearing agents suitable for urgent biopsies, It is highly flammable, tissues are rendered transparent by xylene. It clears 3mm thick tissue in half an hour.
  xylene fumes should not be inhaled so use protection.
  Other agents are
- **Toluene**- It is similar to xylene**.**
- **Chloroform-**It has high specific gravity so tissues float on the surface of it and takes longer time to clear. It is expensive.
- **Cedar wood oil**- It is slow in action and good clearing agent but it takes longer time to get removed from the tissue. So before impregnation tissues are kept in xylene for half an hour and then impregnated.

Procedure

- After dehydration tissues are put in clearing agent. Tissues not exceeding 3 mm in thickness are cleared in 15 - 30 minutes by immersing them in at least two sets of xylene jar for one hour each.
- Brain and blood containing tissues tends to become brittle if immersion is prolonged.

6.6.3 Infiltration and Impregnation

Infiltration - During this stage the clearing agent xylene is eliminated from the tissue by diffusion in the surrounding melted wax & the wax is then diffuses or infiltrate into the tissue to replace it.

Impregnation - Infiltrated melted wax get deposited into the tissue after completely removing clearing agent xylene. There should not be any trace of xylene in the tissue. This deposited wax in tissue is known as impregnated tissue.

Necessity –

Infiltration is required to make space for paraffin wax to enter inside the tissue.

Complete impregnation is necessary to provide suitable consistency to the tissue for microtomy.

Inadequate wax support will crack or crumble tissue section when its cutting is attempted on microtome and also it will lead to drying and shrinking of the embedded tissue blocks.

Procedure

- Tissues are directly transferred from clearing agent to a bath a fully melted paraffin wax kept in a oven for 50 – 60 minutes.
- Tissues are then transferred to second bath of melted paraffin wax for a similar length of time.
- Usually two changes of paraffin bath are sufficient for most of the tissue

The wax used should be of suitable melting point. This varies with the nature of the tissue. Hard tissue requires a higher melting point than soft tissues to give the necessary consistency and support as sections are cut. The wax commonly used has melting points in the range between 50^0 to 60^0.

If low melting point paraffin wax is used then hard tissue likes bone, fibromas require third change of paraffin wax.

This impregnated tissue with paraffin wax is then taken for preparing a tissue block or for embedding.

6.6.4 Embedding-

Embedding is the process of keeping impregnated tissue in molten paraffin wax and allowing it to solidify to provide support for section cutting.

Various moulds are used for it. They are

- L-shape metal moulds
- Plastic ice trays
- Paper boats
- Disposable plastic embedding rings

Most commonly L- shape metal moulds are used.

Technique –

- Two L- shape metal moulds are joined to form a square or rectangle depending on size of tissue.
- Apply glycerine on inside of mould to prevent sticking of wax.\
- Now pour molten paraffin wax in the mould.
- With the help of just warm forecep take out tissue from last paraffin bath and orient it in the mould in such a way that its cutting surface faces downwards towards the base of mould.
- Apply identification label to the side of the mould.
- Allow it to solidify at least for 15-30 minutes.
- After cooling take out mould from sides.
- Block is ready for cutting.

6.6.5 Manual tissue processing schedule

No	Procedure	Reagent	Time
1	Fixation	10% formalin	Overnight
2	Dehydration	80% alcohol 95% alcohol Absolute alcohol Absolute alcohol Absolute alcohol	9am—11am 11am—1pm 1pm—2.30pm 2.30pm—4pm Overnight

3	Clearing	Xylene Xylene	9am—11am 11am—1pm
4	Impregnation	Molten paraffin wax Molten paraffin wax	1pm—3pm 3pm—4pm
5	Solidity		Overnight
6	Warm and then vacuum.		9am—11am

6.7 Automatic tissue processor

Introduction-Automatic tissue processor is the valuable machine in the histopathology laboratory particularly where large numbers of tissues are processed daily.

These machines decrease both the time and labour necessary for processing tissue. The decrease in the processing time is due to the constant agitation, use of raised temperature which improves penetration of reagents in the tissue and produces more consistent result.

A variety of these machines are manufactured some act with tissue blocks in the basket being transferred from one container to another. Other machines design has a single central chamber into which processing fluids are transferred

The machines are equipped with electronic timer and processors which allow better flexibility of programming. There machines are usually equipped with a number of safety devices which warn for example overheating or underheating.

Containers –Special containers made of glass, stainless steel or plastic are provided with close fitting lids.

- The basket is of stainless steel with the die cut perforations.

- The cassettes are full perforated at the top, bottom and sides permitting free passage and draining of fluid.
- The machine has circular deck on which number a glass or steel containers are arranged specially for different reagents and tissues are shifted automatically from one container to another which is controlled by time device.
- Most machines incorporate at 12 stage cycle with the last two baths are thermostatically controlled for molten paraffin wax.

Note-

The care of automatic tissue processor is extremely important. Paraffin should be kept in the paraffin baths and removed from all other areas of instrument with soft clothe soaked in xylene. The receptacles and basket should be soaked in xylene and washed in very hot soapy water to remove all residual paraffin.

Procedure-

The processing schedule used with automatic tissue processor varies according to the type of tissue, the nature of the work, the clearing reagent and personal preference of pathologist.

Requirements

- Alcohol solutions of various concentrations e.g. 80 % 95 % absolute alcohol
- Xylene
- Paraffin wax
- Automatic tissue processor

Method

- The pathologist cut the pieces of tissue and places them in the tissue receptacles or basket with an indentifying number.

- Place the alcohol in ascending grade, xylene and paraffin wax in respective containers of the machine.
- The time level is set at zero and the machine is started at 4.30 pm.
- The basket with the cassettes automatically changes position and travel clockwise to take a bath in different reagents kept in the container in order to accomplish
  - Dehydration- Ascending grades of alcohol
  - Clearing- Xylene
  - Impregnation- Warm paraffin

6.7.2 Automatic tissue processing schedule

No	Timing	Reagent	Interval
1	Starting point	80% alcohol	0 hours
2	4.30-6.30pm	80% alcohol	2 hours
3	6.30-7.30pm	95% alcohol	1 hour
4	7.30-8.30pm	95% alcohol	1 hour
5	8.30-9.30pm	100% alcohol	1 hour
6	9.30-10.30pm	100% alcohol	1 hour
7	10.30-11.30pm	100% alcohol	1 hour
8	11.30pm-12.30am	Xylene	1 hour
9	12.30am-2.30am	Xylene	2 hours
10	2.30am-4.30am	Molten paraffin	2 hours
11	4.30am-6.30am	Molten paraffin	2 hours
12	6.30am-8.30am	Molten paraffin	2 hours

6.8 VACCUM – IMPREGNATION TECHNIQUES

The vacuum – impregnation technique depends on the production of negative pressure above the specimen in the impregnating wax. This pressure reduction hastens the extrusion of air bubbles and clearing agent from the tissue block facilitating rapid penetration by the wax. Automatic tissue processors normally have the facility to perform vacuum impregnation.

It is useful for

1. Urgent biopsies
2. Dense tissue
3. Lung tissue

Review Questions-

- Define tissue processing. Name different embedding media.
- Describe various steps involved in tissue processing?
- What are advantages of automatic tissue processor?
- Describe automatic tissue processor.
- Describe embedding technique.

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Topic 7: Section Cutting

Objective- Students must know different types of microtomes, microtome knives along with sharpening and technique of section cutting.

Introduction- Section cutting provides a thin section of tissue which makes it easy to examine the tissue under microscope, to detect if any pathology is present. It requires following things-

- Properly processed tissue
- A suitable microtome
- A sharp microtome knife
- A skilled microtomist

7.1- Microtomes

Microtome is an instrument used to take thin section of tissue for microscopic examination.

Microtomes were called “cutting machines” till 1839 and when Cheralier introduced the term “microtome”. Initially they were hand models. The first table model was introduced in 1840. The knife holder was introduced in mid-nineteenth century.

There are number of microtomes. The choice of microtome depends on type of specimen to be sectioned. The different types are

Cambridge rocking microtome-It is small, light weight microtome. Knife is fixed with edge uppermost and objects moves against knife in an arc of a circle. The cutting stroke is spring operated and somewhat less controllable than other types.

As it is light and jerky pulling action is required for sectioning, so it may move about on bench during sectioning. This is prevented by keeping microtome on nonslip material.

Figure 7.1.1 Cambridge Rocking Microtome

Advantages-

- It is used for paraffin wax embedded tissue mainly but can be used for frozen section.
- It is light weight and its mechanism is simple.
- The cost of knife and microtome is less.

Disadvantages-

- The sections are cut curved.
- No serial sections are possible.
- Not suitable for large blocks of hard material.
- The cutting angle of knife cannot be adjusted.
- It vibrates or may move on bench during cutting.

Base sledge microtome- It is large, heavy base microtome with two movable pillars holding the knife. The knife is fixed beneath which object mounted on in a heavy sliding base containing the feed mechanism. Base moves to and fro. This is done either manually or automatically.

Figure 7.1.2 Base Sledge Microtome

Advantages-

- Used for cutting large and hard tissue particularly bone and teeth.
- Also useful for tissues embedded in celloidin or LVN.
- Can be adapted for cutting frozen sections.

Disadvantages-

- Not suitable for serial sections.

Microtomes for frozen sections- It is of two types

a) **Freezing microtome**-It is attached to the edge of the bench. Here object is fixed and object moves. The object stage is connected to cylinder of compressed Carbon Dioxide for rapid cooling of specimen

Fig 7.1.3.A Freezing microtome

Advantages-

- Used for preparation of frozen sections of fixed or unfixed tissue.
- Used for urgent diagnosis.

Disadvantages-

- The knife and tissue blocks are exposed to atmospheric temperature and conditions after freezing.
- In-between cooling of specimen is required.
- Sections are thick.
- Ribbon of section is not possible.

b) **Cryostat-**It is a cold box containing microtome. Microtome is Rotary microtome. Temperature maintained is between -5 to -30^{0}c.

Figure 7.1.3.B Cryostat

Advantages-

- Fixed or unfixed tissue can be cut.
- Used for urgent diagnosis.
- Tissue blocks and the knife are maintained at same temperature.
- No in between cooling of specimen is required as temperature of chamber is between -5 to -30^{0}c.
- Artifacts are avoided.

Disadvantages-

- Sections are thick. So minor details cannot be studied.
- Serial sections are not possible.

Ultra microtome-This is highly specialized precision microtome. It is used for preparation of very thin sections for electron microscopy. It gives thickness range of 5-100nm (0.005-0.1µm). Specially constructed knives of plate glass are used which cut very small blocks of tissue embedded in synthetic resin.

Figure 7.1.4 Ultra microtome

Rotary microtome- This is most commonly used microtome. They are so called because the feed mechanism is actuated by turning a wheel at one side of a machine. Here knife is fixed and object moves against knife according to the thickness selected. One rotation of the wheel produces complete cycle of downward cutting stroke, an upward return stroke and activation of mechanism.

Figure 7.1.5 Rotary microtome

Parts of Rotary microtome-

- Block holder
- Knife clamp screws
- Knife clamps
- Block adjustment clamps
- Thickness gauge
- Angle of tilt adjustment
- Operating handle

Advantages

- The microtomes are heavy and stable.
- The knife angle is adjustable.
- The knife holder is movable.

- Excellent for cutting serial sections.
- Ideal for routine and research work.
- It is the only microtome used in teaching purposes.

Disadvantages

- The knives are expensive.

Care of microtome

- After use all paraffin and other residues on it should be removed by soft brush.
- All moving parts particularly the slides, the wheel, etc should be oiled with one or two drops of thin lubricant oil.
- The surface should be cleaned frequently.
- Clean the metal parts carefully with xylene.
- Put a cover on microtome when not in use.

7.2- Microtome Knives

Microtome knives are most important tool required for taking thin sections of wax embedded tissue and also tissue embedded in other media.

<u>Knife material</u>

- Microtome knives are usually made up of good quality steel.
- Other materials used are
    - Diamond knives used for cutting epoxy resin blocks in EM. They are very expensive.
    - Glass knives used for ultra microtome.
- Disposable knife- Nowadays these are commonly used. A lot of time for sharpening is saved and difficulty in sharpening of a knife is avoided. But relatively they are expensive and not as rigid as

other microtome knives. So there is tendency for some minor vibrations.

Types of knives- There are different types of knives. They are identified by their profile. They are divided in to four types.

- Profile A **Biconcave**- Length is 100-250mm. It has concavity on both sides. It is less rigid than others. Used with rocking microtome.
- Profile B **Plano concave**- One side is flat and other is concave with different degrees of concacavity. The plane surface is closest to tissue blocks. It is used for cellulose nitrate embedded tissue.
- Profile C **Wedge shape**- It is plane on both sides. Size varies from 100-350mm in length. This most commonly used knife in all types of microtomes and tissues embedded in paraffin wax, cellulose nitrate, synthetic resins and frozen section.
- Profile D **Tool edge-** It is plane on both sides with steep cutting edge. Stouter than conventional knives to give more rigidity. It is used to cut hard tissue such as undecalcified bone. The edge may be coated with tungsten carbide to increase life.

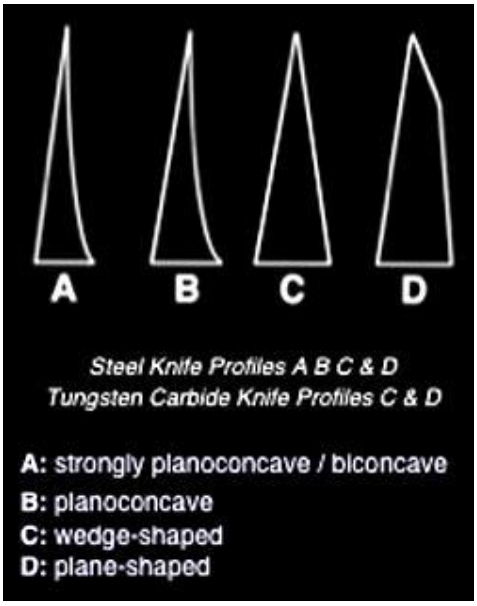

Figure 7.2.1 Microtome knives

Parts of a metal knife

- Heel- An angle formed by the cutting edge and the end of knife nearest the handle.
- Toe- It is the angle formed by cutting edge and the end of knife farther from the handle.
- Knife back- It is also known as honing guide because it gives necessary angle while honing and stropping. It maintains actual cutting facet during sharpening. Each knife has its own knife back. Biconcave knife do not require knife back while tool edge require back of special shape.
- Handle- Knives are provided with detachable handle which screws into one end of the blade.

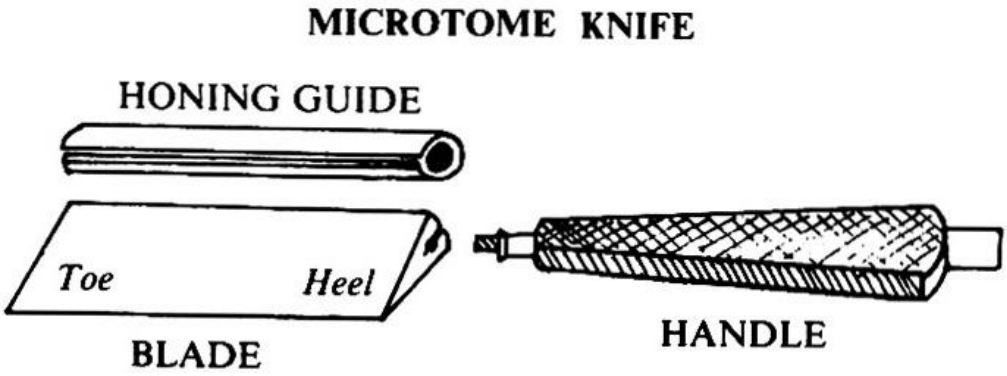

Figure 7.2.2 Microtome knife parts

Use and care of microtome knives

Good quality sections depend upon proper use and care of knives. If knife is used properly then it can give service for longer period of time. There are three main ways by which edge of knife get damaged-

a. Cutting hard objects
b. Careless storage
c. Improper handling during sharpening.

Care of microtome knife (Fig.7.2.3)

- Never keep the knife flat on the surface.
- Always keep the knife in its box when not in use.
- Clean the knife with xylene before and after use.
- When knife is to be stored for a long time smears it with light oil to prevent rusting.
- Always use back while sharpening.
- Always keep knife sharp.
- For cutting hard objects use separate knife.

Figure 7.2.3 Microtome knife with its box

Knife sharpening

A microtome knife should be sharp to obtain thin sections. A blunt or damaged knife edge may cause faults in cutting or cutting is not possible.

A knife edge should not show any indentations. It should be straight. But it doesn't mean that a perfectly straight knife edge is sharp.

Tests indicating condition of microtome knife edge-

- It should feel sharp.
- It should not show any macroscopic and microscopic nicks and teeth.
- It should easily cut detached human hair.

Knife sharpening is carried out by two methods

- Manual
- Automatic

Manual Method

This is carried out in two stages

1. Honing
2. Stropping

Honing

It is a process to remove all nicks and sharp pieces of metal and make the edge straight and sharp. The knife is moved on hard surface. It is known as hone (Sharpening stone). There are two grades of honing material available-

- Coarse
- Fine

Very badly nicked knives should be coarse honed first and then finished by fine honing.

There are different types of hones.

a. Carborundum- Artificial stone with coarse surface for badly nicked knives.
b. Belgium yellow (Belgium black vein) - A natural sandstone set on to a slate backing. Best for manual sharpening.
c. Arkansas- Hard pale yellow- white stone. It is natural stone.

d. Plate glass- used by applying an abrasive to the surface. For this aluminum oxide is used as abrasive material. Finest grade will be < 0.5µm.

   Both knife and plate should be thoroughly cleaned before going from one to another abrasive. Hones should be clean.

Honing should be done with knife back. It must fit the blade properly. Examine the knife under microscope after honing for even sharpness of cutting edge.

Technique of honing (Fig. 7.2.4)

- Clean the knife with soft cloth.
- Put the proper honing back.
- Keep hone on a bench of suitable height. Keep a damp cloth under the hone to prevent it from slipping.
- Clean the surface of hone and lubricate with oil or soapy water.
- Place the knife at one end of hone with the edge facing away from operator.
- Push the knife diagonally forward with cutting edge leading so that whole edge is equally ground by giving light but sufficient pressure. This is required to maintain edge of knife in contact with hone.
- Just before the edge reaches the end of hone, the knife is turned over on its back without lifting it. Then pull back the knife with cutting edge along the hone in a diagonal fashion.

  This is Heel to Toe movement.
- Examine the knife for its sharpness.

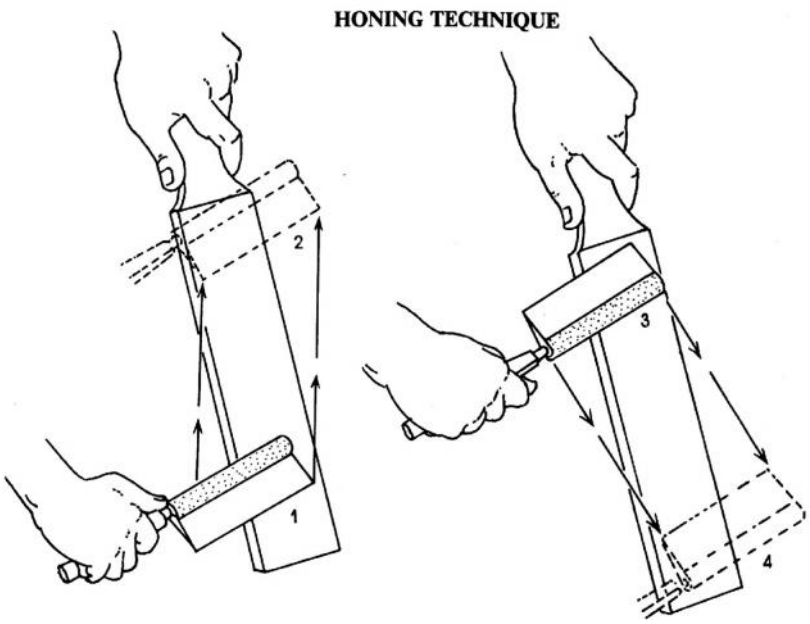

Figure 7.2.4

Stropping (Polishing)-

Stropping is the process of polishing the knife in order to remove buff formed during honing. It is done for further refinement to the knife edge. It is carried out on a softer surface such as leather with or without addition of fine abrasive.

Types of strop

Strops can be flexible or rigid. The rigid type (Paddle strop) is a leather strop stretched over a solid wooden block. This is more common and preferred to the flexible strop as it gives firm support during process. The movement of in knife in stropping is opposite to that used during honing. The strops may be hanging type. In hanging type they must be pulled as taut as possible.

Care of strops

- Strops should be kept in good condition.
- Strops must be clean, free of paraffin flecks, dust or any other abrasive material.

- Wipe the strop free of moisture with a clean, soft cloth and allow it to stand on its edge overnight.
- When not in use strops must be covered by polythene.

Technique of stropping (Fig. 7.2.5)

- Thoroughly clean the knife and dry it completely.
- Place the rigid strop on the bench or if hanging strop is there, it is pulled taut.
- Place the knife at one end of strop and polish it in a direction opposite to honing by giving sufficient pressure.
  This is Toe to Heel Movement.
- Examine the knife for its sharpness.

Note- Knives should be thoroughly cleaned after honing and stropping. This is to avoid contamination with traces of metal dust on knife which will get transferred to section during cutting and may give false positive Prussian blue reaction for ferric ions.

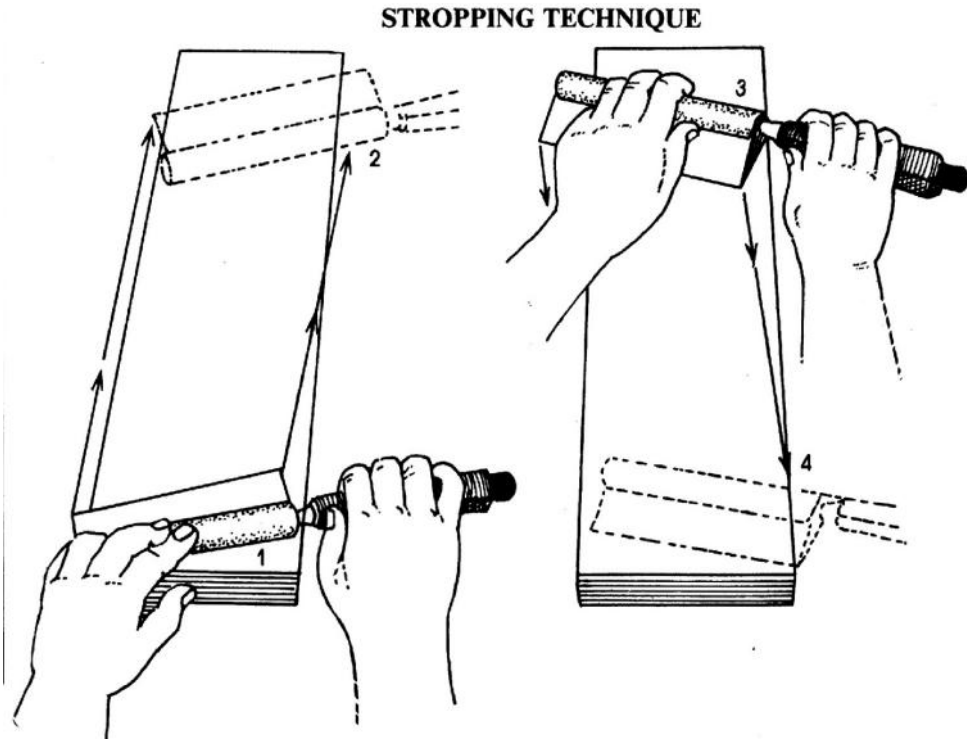

Figure 7.2.5

Automatic knife sharpener (Fig. 7.2.6 and 7.2.7)

These machines are fast becoming indispensible in histopathology laboratory.

Advantages

- Time saving.
- Easy to manipulate so that inexperienced technician can produce a well sharpened knife.

Disadvantages

- Expensive.
- Uncertainty of electric supply.
- Frequent repair problem can occur.

Machines may **semi-automatic** or **fully automatic.**

The semiautomatic machines require hand feeding of the knife against wheels made of glass or metals. But disadvantage of this is that manual

feeding of the knife across the revolving wheel causes uneven pressure and variation in the rate of honing leading to uneven knife edge.

In the fully automatic machines the knife is fitted in to a holder attached to the main spindle and allows the cutting edge to be in contact with the circular plate made of metal or glass. The abrasive is spread evenly on the surface of plate by oscillatory and rotator movement of device. The duration of sharpening time is set according to condition of knife. The knife automatically turns over from edge to edge at suitable interval. Abrasives are made of aluminum oxide, magnesium oxide or chromium oxide. Sizes varying from 0.05µ to 0.1µ.

Fig 7.2.6

Figure 7.2.7

7.3-Section cutting (Microtomy)

Requirements for section cutting are

- Properly processed tissue
- Suitable microtome in good condition
- A sharp knife
- Skill of microtomist

Different steps involved in section cutting are

1. **Attaching paraffin blocks to microtome-**

   Paraffin block should be attached properly to microtome head. It is attached by using object holder or carrier. The object holder may be a rectangular wooden block with grooved face or may be plane. It may be metal object carrier. Allow the paraffin wax to melt on the back of the block and then fix it to object carrier and then it is clamped to microtome head.

   First knife is fixed in knife clamp and tissue block is drawn across the knife edge. Positioning the knife edge helps in orienting the paraffin block.

2. **Orientation of block-**

   After attaching block, its proper orientation is necessary before it is cut. In rotary microtome screws are provided which help to change the block position as per the requirement. The orientation should be such that the top and bottom of the block are parallel and horizontal to the edge of the knife at the moment of impact. The block face should be as small as possible. There should be 3mm of paraffin around the tissue. Somewhat more paraffin should be available at the back of block.

3. **Trimming-**

   It is the removal of surplus wax present in front of tissue and exposure of complete surface area of specimen. After attaching and

orienting the block, set the gauze controlling the thickness of a section to 15µm. Use the extreme ends of knife or another knife to trim the block. Once the surface is exposed stop the trimming.

4. **Cutting the sections-**

   In properly trimmed block face, the top and bottom edges will be parallel; the block face touches the knife.

   Now replace the old knife with one which is reserved for section cutting. Adjust the thickness to 5µ. Raise the object just short of knife and begin the cutting. The centre of the blade is most desirable place to cut. Maintain a regular cutting rhythm. (The rate will vary with size of the block, the nature of tissue and type of microtome used.) With the act of cutting sections lie on knife. Thus a ribbon of section is produced. When a ribbon of 10-15 cm is formed, stop cutting and take the sections on slide.

5. **Attaching section to microscope slides-**

   After obtaining a ribbon of section, grasp the first section with a pair of fine forceps held in the left hand. A small camel hair paint brush held in the right hand can be used to brush away the last section from the knife. Lay them on a piece of black paper before mounting on a slide.

   Slides which are used are of variable sizes, commonly used is 76×26mm with thickness of 1-1.2mm. Immerse slide in 1% HCL in 70% alcohol and clean it with fluff free cloth.

   To allow section to remain attached to slide an adhesive mixture is used. Most commonly Mayer's glycerol egg albumin is used. It is prepared by mixing equal volume of egg white and glycerol and adding a small crystal of thymol to prevent bacterial growth. Apply this mixture thinly on a slide before taking section on a slide.

   Technique-

- Strip of individual sections are laid on surface of warm water taken in a beaker, the temperature of water should be 5-10^0 below melting point of wax. The sections will float and expand slightly and become flatten. Wrinkles may be removed by gentle stretching.
- Smear a clean slide with an adhesive.
- Dip that slide obliquely in a beaker.
- Draw the section towards slide with forcep or needle.
- As the selected edge of section touches slide, remove the slide along with section with upward and forward movement.
- Keep the slide in upright position to drain.
- Allow the slide to dry at room temperature but if quick drying is required it is done in an oven at 45-50^0C for 2 hours or more. Then slide is ready for staining.

Factors affecting cutting of section-

- Angle of tilt- It is the angle between surface of the block and a line bisecting the edge of the knife. Optimum tilt should be 20^0. Tilt $<20^0$ or $>20^0$ lead to undesirable sections.
- Rate of cutting- Each embedding medium has optimum cutting rate for given thickness of a tissue. Soft tissue embedded in soft medium requires slow smooth action. If it becomes forceful and fast, sections will compress.

  For cutting ribbon of sections from a paraffin block it requires more brisk and rhythmic rate of cutting.
- Temperature- Temperature has effect particularly on wax embedded tissue.

  In warm weather cooling of block is important to prevent softening of wax and to have thin sections. Tissue block is cooled by application of ice bags.

If surrounding temperature is low, then warming of atmosphere is necessary. This is done by Bunsen burner or electric lamp or by breathing on block before each section is cut.

Faults in section cutting-

Faults	**Cause**	**Correction**
Thick and thin sections	Insufficient tilt of knife	The angle between the block and the knife should be 5^0 to 8^0
Section split vertically	Damaged knife edge. Dirt in embedding media. Knife edge dirty.	Resharpen the knife. Filter the wax. Clean with xylol.
Sections have parallel lines across them (CHATTERS)	Hard tissue Tilt of the knife too much.	Soften the tissue by applying softener called mollifex on the block. Decrease the tilt of knife.
Width of section is less than that of block and the section is compressed.	Loss of bevel due to incorrect sharpening	Resharpen the knife using a back.
Sections roll up on cutting	Blunt knife Tilt is too much	Resharpen the knife. Adjust the tilt of the knife.
Sections crumble on cutting	Dehydration and clearing is not proper. Wax is too soft.	Reprocess the tissue Apply ice while

	Blunt knife.	cutting Sharpen the knife.
Ribbon fails to form	Paraffin too hard Tilt of knife too great Thick sections Dull knife	Use low melting point wax. Breathe on the knife and warm it slightly. Correct tilt. Cut thin sections Sharpen the knife.
Sections lifted off from the knife on up-stroke	Knife edge dirty. Knife tilt is less. Dull knife.	Clean with xylol. Increase tilt. Sharpen the knife.

Storage of sections-

- Unstained paraffin sections mounted on slides and properly dried can be stored indefinitely.
- Specially designed slide storage cabinets may be used.
- Cardboard boxes or trays are used for temporary storage of slides and for their movement within laboratory.

Review questions:

- Define microtome and microtomy.
- Enumerate different types of microtomes
- Describe rotary microtome.
- Classify microtome knives.
- What are different parts of microtome knife?
- How will you take care of microtome knife?
- Describe sharpening of knife.

- What is angle of tilt?
- How will you perform section cutting?
- Which different faults can occur in section cutting?

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Topic 8: Frozen Section

Objective- Students must know significance of frozen section and its advantages and disadvantages.

Introduction- Frozen section means tissues are embedded in ice and cut on freezing microtome.

8.1- Advantages and Disadvantages

Advantages-

- Helps in rapid diagnosis and intraoperative surgical consultation.
- Sections can be prepared in few minutes.
- For demonstration of lipids, amyloids, etc. Fats usually get removed during paraffin section which is stored by frozen section.
- In histochemistry especially with unfixed tissue where use of processing reagents would result in loss or alteration of the substance to be demonstrated which is preserved with frozen section.E.g. Enzymes
- For demonstration of components of CNS to avoid shrinkage and to allow better penetration of stains.
- For the preparation of sections of tissues like tendons which would be difficult to cut or flatten section after paraffin embedding.
- In fluoroscent antibody technique.

Disadvantages-

- Not possible to obtain ribbon of sections and serial sections.
- Sections are thick, so finer details cannot be studied.
- After cutting blocks should be stored in low temperature cabinet or in a jar of fixative at RT or processed to make paraffin blocks but artifacts may get produced.
- Staining is not satisfactory in the case of unfixed sections.

8.2- Preparation of material-

- A suitable block of fresh tissue is selected and it is trimmed with sharp scalpel so that its sides are parallel. The tissue block should be about 5mm thick.
- The block can be taken for cryostat sectioning or it can be fixed in 10% formalin or formal-alcohol (15ml of 40% formaldehyde mixed with 85ml of 95% alcohol.)
- As soon as tissue is received from OT it is transferred to a beaker and is boiled for 30-60 seconds along with fixative. Then wash in distilled water.

8.3- Freezing of tissue-

Freezing of tissue is carried out by adapting one of the following method.

- Using compressed Co_2
- Refrigerated microtome-Cryostat
- Thermoelectric cooling devices
- Dichlorofluoromethane aerosol spray.

8.4- Microtomy

After making tissue ice cool it is cut on

1. Co_2 freezing microtome
2. By using Cryostat.

8.4.1-Co_2 freezing microtome

It is a machine attached to the edge of the bench and connected to the Co_2 cylinder by specially strengthened narrow flexible metal tube to the object holder.

Object holder is hollow and there are holes which allow escape of Co_2 which will cool the specimen.

The valve of cylinder should be above the level of object stage to have proper flow of liq. Co_2 to enter in to stage. Cylinders should be in upright in position.

Freshly charged cylinders may contain some water. So it should be blown off by opening valve briefly before connecting to microtome.

Pressure of liq. Co_2 is 7000kPa. So blocking of connecting tube is possible. So length of tube should be as short as possible.

In this microtome object is fixed and knife moves.

Figure 8.4.1 Freezing microtome

Technique

- First lower the object holder. Place the specimen on the stage on a drop of water or on a piece of filter paper soaked in water.
- Insert the knife and tight it.
- Rack up the stage and bring the surface of the tissue to just below the knife edge.
- Adjust the desired setting 10-15µ.
- Lift the Co_2 control lever for 1-2 seconds with a pause of 3-4 seconds.
- Observe the tissue which will whiten as freezing starts from below upwards. Immediately before the block is completely white cease the freezing.
- Operate the microtome and cut the sections.

- Mount or take the section on slide. Sections are removed from the knife with finger or moistened brush and transfer it to a bowl of water. Then take them on slide. Method is **Leach** method.
    - Place clean slides in 2.5% gelatin in 1% phenol at 37^0C for few minutes.
    - Wipe one side of clean and allow to dry at room temperature.
    - Float section on to slide, drain off surplus fluid and allow drying at RT.
    - Place the slides for 5 minutes in closed jar containing 40% HCHO, the vapors of it act on gelatin and causes adhesion of section.
    - Sections which are not mounted should be stored in 5% formal saline.

8.4.2- Cryostat

Advantages of cryostat

- Tissues are kept cold even during act of cutting.
- Artifacts are avoided.
- Ideal for histochemistry and fluorescent antibody technique.
- Faster than freeze drying method.
- Few technical problems.

Construction

It is refrigerated microtome, temperature of which is between -5 to -30^0c. It consists of a microtome contained within a refrigerated cabinet designed to operate at -5 to -30^0c. Microtome in is Rotary type.

There is an anti-roll plate either of plastic material or in the form of glass plate. This is to have sections flat on the blade of knife for direct mounting on slide. The temperature of anti-roll plate should be equal to

the chamber temperature. It is aligned parallel to the knife edge. It should be firmly held in holder.

There is a window for keeping tissue inside.

Temperature varies according to the type of tissue and thickness of section required. Usually it is between -15 to -20^{0}c. For fatty material still lower temperature is required.

Figure 8.4.2 Cryostat

Technique-

- Blocks of fresh tissue up to 5mm thick are selected.
- Place a drop of water in the centre of the pre-cooled block holder.
- Place the tissue to be sectioned in the drop of water.
- Rapidly freeze the tissue- this is done by either standing the block holder in a bath of alcohol or acetone containing dry ice or by

placing the block holder in the special freezing attachment and exposing the tissue to Co_2 gas.

- When tissue is frozen position the holder in the microtome maintained at -15 to -20^{0}C. Close the window.
- Make the appropriate adjustment of knife edge so that it touches the block.
- Block is trimmed to its full face using one end of knife.
- Knife is moved to give fresh cutting edge.
- Position the anti-roll plate so that its edge is parallel and almost even with the knife edge.
- Set section thickness control usually at 5-10µm.
- Make sure that slides and stains are ready.
- Cut the sections with slow steady motion. The sections will move smoothly underneath the anti-roll plate.
- Open the window and anti-roll plate is flipped back.
- Section is picked up from the surface of knife on to a slide. The section should adhere to the slide which is slightly dried against back of hand. Then they are fixed in 5% acetic acid in absolute ethyl alcohol. Then perform staining.

8.5- Staining of frozen sections

Two most commonly used methods are

1. Haematoxylene and Eosin
2. Polychrome methylene blue

Haematoxylene-eosin staining

Reagents

- Harris's Haematoxylene
- Eosin

- 0.5% v/v hydrochloric acid
- Dilute ammonia water

Procedure

- Fix the air dried section in pure acetone for 15-20 seconds or in Formal-alcohol for 30-60 seconds.
- Place in water until cloudy appearance is washed away.
- Place in Harris's haematoxylene for 1-2 minutes.
- Wash in running tap water for 5-10 seconds.
- Dip in 0.5% w/v sodium borate or 2%v/v ammonia water until it shows blue appearance.
- Place in 705 ethanol for 5 seconds.
- Counter stain with 1% alcoholic eosin – 1-2 quick dips.
- Wash well in running water. Dehydrate through graded alcohol (80%-95%-100%).
- Clear in three changes of xylol.
- Mount in appropriate mounting medium.

Polychrome methylene blue staining

Reagents

1. Dichrome-eosin

Eosin	1gm
Potassium dichromate	1gm
Distilled water up to	100ml

2. Acetone-alcohol

Absolute alcohol	30ml
Acetone	25ml

3. Methylene blue stain

Methylene blue	1gm

Potassium carbonate	1gm
Distilled water up to	300ml

Procedure

- Stain the section in solution 1(dichrome-eosin) for 5-10 seconds.
- Wash in tap water.
- Differentiate in solution 2 (Acetone-alcohol).
- Wash in tap water.
- Stain in solution 3 (Methylene blue) for 10-30 seconds.
- Wash by using tap water.
- Dehydrate, clear and mount.

This method is recommended for rapid diagnosis with frozen section.

Review questions-

- What is frozen section? What are methods of freezing the tissue?
- What are advantages and disadvantages of frozen section?
- Describe procedure of section cutting on Co_2 freezing microtome.
- Describe cryostat.
- What are advantages of cryostat over Co_2 freezing microtome?
- Describe H and E staining method for frozen section.
- Describe Polychrome methylene blue staining for frozen section.

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Topic 9: Staining

Definition -

Staining is the process of coloring the tissue to facilitate their identification for histological diagnosis.

Significance -

- The purpose of staining is to optically differentiate the tissue components by variation in color.
- Unstained sections are of very little use for studying the internal structure of tissue as the cellular components cannot be clearly differentiated. Staining of the section enables us to study the physical characteristics and relationship of tissues and of their constituent cells.
- Cellular elements are transparent. To visualize them dyes are used. This is known as biological staining. These dyes or biological stains are natural or synthetic dyes.
- Stains may be used to define and examine bulk tissues like muscle fibers or connective tissue, cell populations like classifying different blood cells or organelles within individual cells.
- In biochemistry, it involves adding a class-specific (DNA, proteins, lipids, carbohydrates) dye to a substrate to qualify or quantify the presence of a specific compound.

Properties of dyes/stain-

- They are organic compounds.
- Most of them are water soluble.
- They color the tissue by diffusion.
- They color the tissue but color is not fast. It can be easily removed by washing in simple solution because stains are water soluble.

- Basic stains have affinity for acidic structure and acidic stains have affinity for basic structure.
- A successful staining method is both sensitive and specific.

Structure of Dyes-

There are two types of dyes- Natural and Synthetic

Natural dyes

Natural dye	**Source**	**Properties**
Haematoxylene	Heartwood of tree haematoxylon campechianum	In its natural form no staining capacity. It has to be oxidized into haematein and vused with metal to bind to tissue.
Carmine	Female cochineal bug	It is employed with salt of metal to stain.
Orcein	Lichen	It is one of the few vegetable dyes in histology. Stains elastic tissue.
Litmus	Lichen	Used as an indicator.
Saffron	Plant Crocus sativus	Incorporated in connective tissue stain.

Synthetic dyes-

They are large groups of organic compounds produced from coal. Now petroleum oils are used for their production. The primary products are hydrocarbons like benzene, toluene and naphthalene or phenols like phenol and cresol.

Their basic structure is quinoid compound where two oxygen atoms replace the hydrogen atoms of benzene ring. A quinoid compound is called chromophore. Compounds which contain chromophores are called chromogens.

Dyes are classified based on chromophoric group present are as follows

- Nitroso group
- Nitro group
- Azo group
- Thiazol group
- Anthraquinone group
- Xanthene group

Since the color of the chromogen does not bind to the tissue, auxochromes are needed to convert them into true dye. Auxochromes are acidic or basic compounds which confer the power of electrolyte dissociation and formation of salt. Some dyes contain an additional chemical group called modifiers which deepens the color of dye. They are either ethyl or methyl group.

Quinoid dyes are-	Basic and Acidic fuchsin
	Crystal violet
	Aniline blue
	Eosin
	Thionin
	Methylene blue
	Neutral red
	Haematein
Azo dyes are-	Orange G
	Congo red
	Trypan blue

Nitro dyes are- Picric acid

Acidic, Basic and Neutral Dyes

Acid stain/dye – This has negatively charged colored ions and positively charged colorless ions. It stains basic component of cell. Cytoplasm is basic in nature and so, it has affinity for acidic stains. Therefore it gets stained by acidic stains.

Examples of acidic stain -Eosin, orange -G-6 (OG-6), acid fuchsine.

Basic stain/dye - Basic stains have basic component colored and acidic component is colorless. This stains acidic component of cell. Nucleus is acidic structure and so, it has affinity for basic stain. Therefore it gets stained by basic stain.

Examples of basic stains- Haematoxylin, methylene blue, Basic fuchsine

Neutral dye – This contains both acidic and basic radicals which are colored and soluble in alcohol not in water. They stain nucleus as well as cytoplasm.

Examples – Romanowsky stains

Theory of staining

Staining occurs by physical and chemical methods.

Physical staining

Dyes can combine with tissues by adsorption which is a property of solid substances. Physical factors that are important for staining are **density** and **permeability** of tissues.

Dense tissues have large amount of protein chain per unit volume. They hold a large amount of dye and will remain colored after less dense tissues have been decolorized by differentiation.

Highly permeable tissue will be quickly stained and quickly differentiated while less permeable tissue require longer time to take up stain but resists differentiation.

Thus tissue must be denser and less permeable so that stain cannot be removed easily.

Chemical theory

A dye must ionize in solution to produce colored cations and anions. A basic dye has positively charged colored ions and negatively colorless ions. Like dye tissue also contains electrically charged positive and negative groups. Thus dye and tissue when combine with each other they ionize and electro-positive group of tissue will combine with electro-negative group of dye and vice-versa.

The intensity and fastness of stain depends on avidity of its ionized radicals for tissue components and from number and strength of these chemical bonds.

Thus dye staining is a combination of physical and chemical affinities between dye and tissue constituents.

Staining properties of dyes

The staining property of a dye may be microanatomical or cytological. Microanatomical stains are used to demonstrate the general relationship of tissues to each other while cytological stains demonstrate tiny structures in the nucleus and the cytoplasm and not the tissue types.

Direct staining-Some stains color the tissue perfectly when they are placed in simple aqueous or alcoholic solution of the dye. This is direct staining. E.g. Methylene blue, Eosin.

Indirect staining- Some stains require an additional intermediate substance called mordant which combines with tissue and then tissue-mordant complex combines with staining reagent which colors the tissue.

This is indirect staining.E.g. In Ehrlich Haematoxylene Potassium alum is used as mordant.

Accentuators – An accentuator is any chemical which do not combine with dye but increases the intensity of color. They are not essential for chemical union of dye and tissue. They are acids or alkalies added to acidic or basic dyes.E.g. KOH in Loeffler's methylene blue, Phenol in carbol fuchsin, etc.

Progressive staining- Stains which color the components in a specific order are called progressive stains.

Different elements of the tissue are colored in sequence and at the end of correct time in staining solution a good differential coloration of tissue are achieved.

Regressive staining- Tissues are first over stained and then differentiated by removing excess stain from unwanted parts of tissue.

Vital staining- When tissues are stained in a living condition it is called vital staining. It is of two types **supra vital and Intra vital**.

Supra vital-When living cells are stained after being removed from the body. Tissue is dissociated in staining fluid.

Intra vital- Staining of the cells while still part of the body. Dye is injected into living tissue.

With vital stain cytoplasm and cytoplasmic structures are stained. Nucleus is not stained. E.g. Mitochondria by Janus green.

Metachromatic staining- Some tissue components combine with dyes to produce a color different from color of original dye and different from color produced in rest of the tissue. This is known as metachromasia and dyes are called metachromatic dyes. g. Toludine blue, Thionine.

Metachromatic tissues are cartilage, connective tissue, Epithelial mucin, mast cell granules, etc.

Routine Staining Technique of Haematoxylin and Eosin

Routinely in histopathology **Haematoxylin and Eosin (H&E)** stain is used. Special stains are used if necessary.

Staining Equipments

- Staining dishes - Coplin jars with lid, which holds 5-10 slides or small jars which can hold single slide.
- Staining racks –Slotted racks with handles, capable of accommodating 20-30 slides are available with oblong aluminium trough.
- A Bunsen burner, 37^0C incubator, 60^0C oven
- Polythene wash bottles for distilled water and reagents.
- Cover slips of various sizes Commonly used are- 22×22mm, 26×22mm, 32×22mm, 42×22mm
- Staining machine – Automatic strainers are used now a days.

Reagents

- Haematoxylin

This is primary stain. It acts in conjunction with alum which acts as a mordant. It is basic stain and it stains the nuclei.

Preparation of Harris's Alum Haematoxylin –

Haematoxylin crystals	2.5 gm
Absolute alcohol	50 ml
Ammonium or potassium alum	50 gm
Distilled water	500 ml
Mercuric oxide	1.5 gm
Glacial acetic acid	20 ml

Dissolve haematoxylin in absolute alcohol and alum in water, mix, boil and add mercuric oxide and rapidly cool by plunging the flask into cold water. Add glacial acetic acid before use.

- Mercuric oxide ripens the stain immediately.
- The stain can be used immediately.
- Ammonium or potassium alum works as mordant.
- The stain deteriorates within 2-3 months. Precipitation is an indication of this.
- Prepare small quantity of the stain at a time.
- Glacial acetic acid gives precise and selective nuclear staining.

- Eosin

This is counter stain. It is acidic stain and it stains the tissue surrounding to the nucleus.

Stock solution –

Eosin Y	1 gm
Distilled water	20 ml
Dissolve over gentle heat	
Cool and add 95 % ethyl alcohol	80 ml

Working solution -

Stock solution	25 ml
80 % ethyl alcohol	75 ml

Filter before use.

15 ml of glacial acetic acid to 100 ml of stain gives deeper shades of red to the tissue.

Eosin aqueous solution

Eosin Y	5gm
Distilled water	100ml
Working solution	
Stock solution	1 part
Water	4 parts

- Dilute aq. HCl (0.5%)

Conc. hydrochloric acid-	2.5ml
Dist. Water	500ml

- Dil.Ammonical water

Strong Ammonia	1.5ml
Distilled water	500ml

- Xylene
- Different grades of alcohol.
- DPX mounting media

Procedure for staining paraffin sections-

Prestaining procedure

1. Drying- Slides should be completely dry.
2. Deparaffinisation- Paraffin wax is poorly permeable to stain, it is not miscible with water, the stain Haematoxylin is water based stain so it is necessary to remove it from the section. Paraffin wax is removed from section with the help of regent xylene (Two changes).
3. Removal of xylene with absolute alcohol- Xylene is not miscible with aqueous solutions and low grades of alcohol. So it is necessary to remove with abs. alcohol (Two changes).
4. Treatment with descending grades alcohol- Absolute alcohol is followed by 90%, 80%, 70% and for delicate tissue up to 50% alcohol. This is to avoid damage and detachment of section.
5. Water- Finally wash the section with water.

Steps 3, 4 and 5 constitute hydration of section.

Staining –

This involves treatment with single staining solution or use of more than one stain. Basic principle is either direct or indirect staining.

1. Haematoxylin – It is a primary stain used in H&E technique.This is basic stain and it colors nucleus. It is blue in color. It requires the help of substance alum or iron which is used as mordent. This is water soluble stain.
2. Decolourisation and Differentiation -After staining with haematoxylin next step is differentiation by acid alcohol. This step differentiates nuclei from cytoplasm. Stain is removed from the cytoplasm.
3. Treatment with ammonical water - Because of this, whole section becomes blue and nucleus becomes dark blue. (In this step we can use tap water instead of ammonical water because tap water is also alkaline in nature).
4. Counter stain - Eosin is used as counter stain. It is pink in color. It stains all tissue components except nucleus.

Post staining procedure

1. Dehydration – In most of cases paraffin sections are mounted in media miscible with xylene. So dehydration is done. It involves treatment with ascending grades of alcohol.
2. Clearing- To remove alcohol clearing is done with xylene (Two changes of xylene 1min each).
3. Mounting- Placing the cover slip on mounting media laid on stained tissue is called mounting.

   Functions of mounting

   - To flow between the slide and cover slip.
   - To fill the tissue and tissue cavities.
   - To release entrapped air bubbles.
   - To prevent damage to the section and for easy handling and storage.
   - It should have refractive index close to the glass (1.5).

- It should not be acidic and fade the color.

Commonly used mounting media are

Canada balsm

DPX (Distrene, plastisizer and xylene)

Technique of mounting

- After the slide is drained of xylol and wiped all around put a drop of xylol on the section (when DPX is used as it is thick).
- Take a cover slip and put a drop of DPX on it.
- The cover slip with DPX is gently lowered on the section.
- DPX spreads evenly. The cover slip is slightly pressed to remove the air bubbles.
- Excess DPX around the cover slip is wiped off with gauze soaked in xylene.
- Label the slide.
- Keep slide at RT for firm adhesion between section and cover slip.

Different steps of **H and E** staining

Name of step	**Reagent**	**Time**
Deparaffinisation	Xylene I Xylene II	5minutes 5minutes
Hydration	Absolute alcohol I Absolute alcohol II 90% alcohol 80% alcohol 70% alcohol Wash in tap water	1minute 1minute 1minute 1minute 1minute 1 minute
Staining-Primary stain	Haematoxylene	3-5 minutes

Differentiation	0.5% HCl	5-30 seconds
	Wash in water	
Bluing	Ammonia water	5 minutes
Rinse	95% alcohol	
Counter staining	Eosin	1 minute
Dehydration	Absolute alcohol I Absolute alcohol II	2 seconds 2 seconds
Clearing	Xylol I Xylol II	2 seconds 2 seconds
Mounting	DPX	

Special stains

Special stains and techniques are divided in to three groups-

I. For demonstration of particular parts of tissue usually components of connective tissue which are not seen by H & E stain.

Connective tissue stains

1. **Collagen and collagen fibres-**
   - Weigert's iron haematoxylene stain

     Collagen- Bright red

     Elastic- Dark (often black)

     Other tissue- Yellow
2. Reticular fibres-
   - Silver impregnation method

     Reticular fibres – black color
3. Elastic fibres
   - Verhoff's stain

     Elastic fibres- Black color

4. Masson's trichrome stain

   Nuclei- Black

   Collagen and mucus- Blue

   Muscle cytoplasm and keratin- Red

II. Stains for substances like iron, carbohydrates,etc. which do not take H & E.

1. Carbohydrates-

- Periodic acid schiff's stain(PAS)-

  Carbohydrates appear rose or purple red color.

2. **Amyloid**- It is a glycoprotein formed by combination of carbohydrates with proteins. Amyloidosis is a pathological condition in which amyloid (abnormal quantities of glycoprotein) gets deposited in various tissues and organs.

- Congo red stain-

  Amyloid appears deep pink or red color.

3. Pigments and minerals

Pigments

a. Melanin- It is granular black or yellowish brown pigment normally present in the skin, hair, eyes, etc. It is formed from tyrosine by the action of enzyme tyrosinase. It is insoluble in organic solvents but soluble in 1N NaOH. Solutions of ammonium silver nitrate are reduced to black metallic silver by melanin.

   In pathological conditions such as benign or malignant melanomas it is found in variable amount. it is identified by

   - Negative Prussian blue reaction
   - Positive argentaffin (silver) reaction- This depends on ability of tyrosine derivatives to reduce silver solutions to metallic silver.

b. Haemosiderin- Hb when released from RBCs is broken down in to iron free haematoidin and iron containing haemosiderin. It appears 2-3 days after release of Hb. It is present normally in spleen and bone marrow in small quantities. It is abundant in pernious anaemia and haemosiderosis.

It is granular yellowish brown pigment soluble in acids but not in alkalies.

It is detected by Prussian blue (Perl's reaction). It appears blue in color.

Minerals

A large variety of minerals are present but important are iron and calcium. It is detected by

a. Iron-

Prussian blue reaction- Bright blue color

b. Calcium-

Von kossa silver nitrate method- Black deposit

III. Stains for microorganisms-

1. Gram stain- Bacteria
2. Acid fast stain- Myco. Tuberculosis, M.leprae
3. Silver methanamine stain- Fungi
4. Giemsa stain- Bacteria and blood parasite.

Review questions

1. Define staining. What is sensitivity and specificity of a stain?
2. What is vital staining?
3. Classify dyes.What are natural and synthetic dyes?
4. Explain mechanism of staining.
5. Describe H & E stain.
6. Classify special stains. Give examples.

Topic10: Exfoliative cytology

Objective- Students shall understand the importance of exfoliative cytology and staining technique used to study different samples.

10.1- Introduction

Exfoliative Cytology is the study of the cells which are shed (exfoliated) spontaneously from the epithehal surfaces of the body or which may have been removed from such surfaces of membranes by physical means (e.g.scrapping)

Spontaneous exfoliation is a characteristic of normal epithelial surfaces. These surfaces as they grow continue to shed cells from the superficial layer and they become replaced by new cells. Cells of the malignant tumors exfoliate more readily than those from normal tissue. Exfoliated cells can be found in smears taken

- Directly from the epithelial membranes, or
- From certain accessible body cavities where they may accumulate. e.g. vagina, buccal cavity, etc.
- From a variety of body fluids and effusions including urine, pleural fluid and gastric juice.

10.2- Significance

It is recommended for

- Detection of malignant cells or precancerous lesions in the body.
- Detection of asymptomatic or precancerous cervical lesions in women.
- Assessment of female hormonal status in case of sterility and endocrine disorders.
- Determination of genetic (phenotypic) sex.

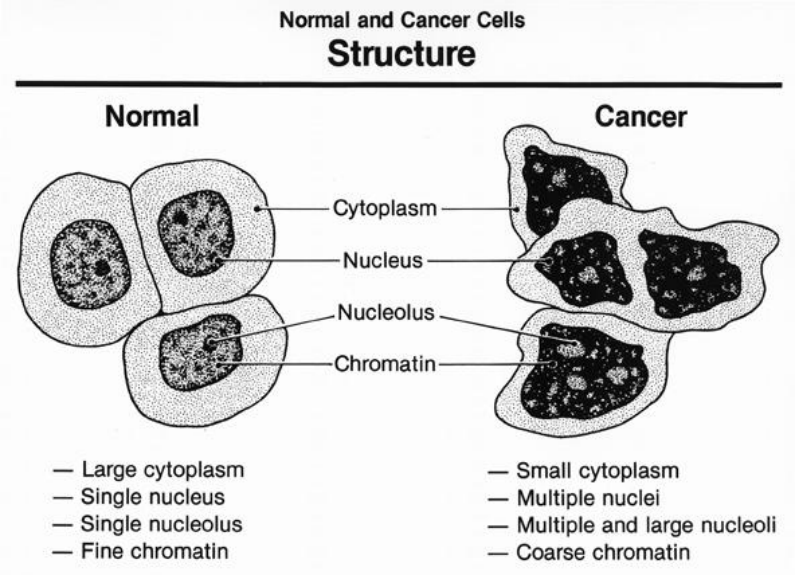
- Detection of the presence of infectious microorganisms.

Exfoliative cytology is very important in diagnosis of malignancy or cancer in various organs, especially those of urogenital tract & respiratory tract. This test is based on fact that cancer cells because of biochemical changes loose the stickiness and cohesiveness. Hence, they are readily shed from the epithelial surfaces, which are then studied for malignant changes.

10.3- Cytological changes in malignant cell

The cytological changes are based on the appearance of individual cell specially the nucleus which shows several abnormalities in a malignant cell,

- Enlargement of the nucleus without an increase in the overall size of the cell, giving a decreased cytoplasmic - nuclear ratio.
- Irregular nuclear outline and variation in size and shape.
- Hperchromasia: Due to high concentration of DNA in the nucleus of the malignant cells they stain more deeply with basic dyes.
- Multinucleation: More than one nucleus may be present due to abnormal cell division.
- Increase in the size of the chromatin particles which are distributed unevenly throughout the nucleoplasm.
- Increase in the size and the number of nucleoli.



10.4- Collection of material

The various specimens used for cytological examinations are

- Vaginal Smear
- Cervical smear
- Sputum Smear
- Urine Sediment
- Cerebro Spinal Fluid
- Nipple Discharge
- Gastric Washing
- Peritoneal, pericardial and pleural fluids

Some of the specimens are collected by the patients such as urine, sputum etc., but majorities of the specimens are collected by the attending physician during physical examination. These include vaginal and cervical smears, breast secretions, prostatic secretions, pleural fluid, etc.

A. Vaginal smears :

Here the material is obtained by aspiration from the posterior fornix of vagina by using a slightly curved glass pipette with s suction bulb. The aspirate is spread rapidly and evenly on a pre-labeled clean glass slide

which is immediately placed in a fixative before it dries up. Smears obtained by this method not only contain vaginal cells, but also the cells exfoliated from the other parts of female genital tract like cervix and endometrium.

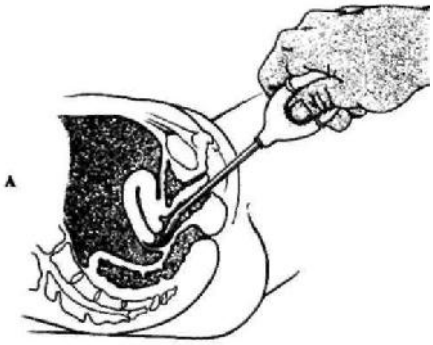


Figure 10.4.A.1

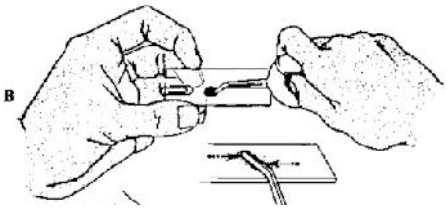
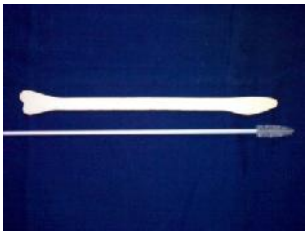


Figure 10.4.A.2

B. Cervical smears:

A speculum is inserted in to the vagina and the vagina and the uterine cervix is observed. A spatula is inserted into the cervical canal via speculum and rotated through360^{o} and the material is spread quickly over a clean slide and fixed immediately before drying.



Instead of spatula a cotton-wool tipped applicator can be used which is introduced into the cervix and the cervical surfaces are gently swabbed. The material from the swab is smeared on the slide and fixed.

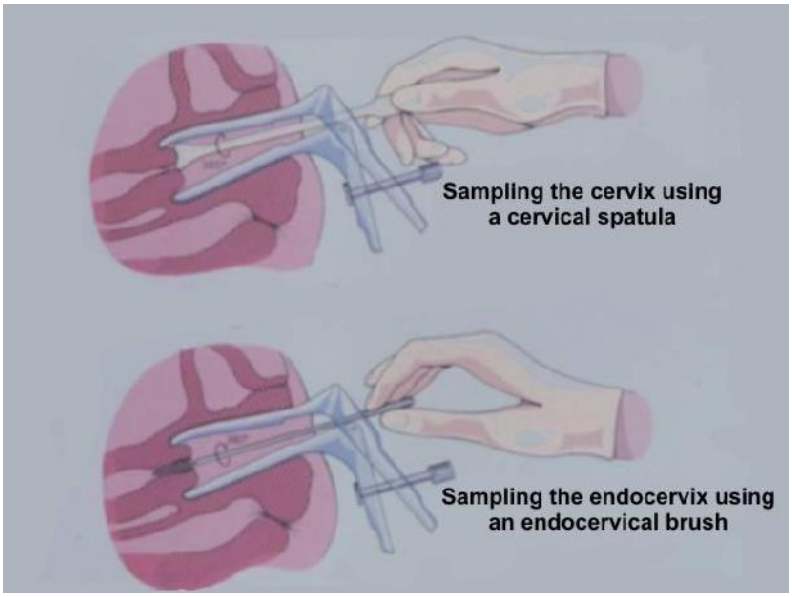


Figure 10.4.B

C. Sputum smears:

The sputum originates deep into the lungs. Hence, sputum specimen can only be obtained by deep coughing. It can also be obtained by Aerosol induced method wherein the patient is given inhalation of aerosol solution for 20 minutes to produce deep cough sample. It is collected in the early morning.It is advisableto take daily samples on 3

Figure 10.4.C

Consecutive days (3-9 days). The specimen should reach the laboratory within an hour after collection which is then smeared and fixed immediately. Sputum is loosened by a high speed blender or by proteolytic enzymes (e.g.papain. trypsin, etc.) or chemicals. The liquefied sputum is then taken for preparing the smear. The smear is fixed immediately.

D. Urine smears :

In males the early morning voided sample is taken. But, in female the urine specimen is obtained by catheterization to avoid contamination. In Deden's method, a large volume (400 to 500 ml) of urine is allowed to sediment in separating funnels. Collect the sediment, centrifuge and make the smear from the deposit on several albuminized slides. The smears are then fixed in ether-alcohol and stained.

In Sagi and Mackenzie's method, a large volume of urine (200 ml) is distributed in centrifuge tubes(50 ml each) carefully balanced and centrifuged at 2000 rpm for 20 minutes. The supernatant is discarded and the deposits from all the tubes are combined into one tube, a small amount of distilled water used as a vehicle. This tube is again centrifuged and the supernatant discarded. The sediment is spread evenly on to the slide with a wire loop and fixed immediately.

E. Cerebro-spinal Fluid

CSF is usually obtained through a lumbar puncture (spinal tap). During the procedure, a needle is inserted usually between the 3rd and 4th lumbar vertebrae and the CSF fluid is collected for testing.

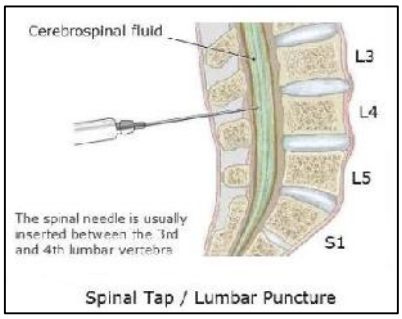
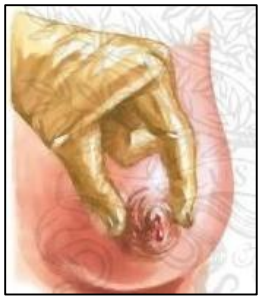


Figure 10.4.C

F. Nipple Discharge

Gently strip the sub-areolar area and nipple with the thumb and forefinger. Place the slide upon the nipple and draw it quickly across the nipple. If 1 drop is obtained, get another slide and do the pull-



apart technique. Immediately immerse the slide into a bottle of 95% isopropyl alcohol or use spray fixative.

G. Gastric Washing :

Gastric fluid undergo rapid degeneration and digestion at room temperature, so smears are prepared and fixed as early as possible after collection. The specimen is processed in the same way as for urine and smears are prepared from the sediment and fixed immediately.

H. Other Body Fluids

- Peritoneal fluid is withdrawn with a needle from the peritoneal cavity. The peritoneal cavity is the space between the two membranes lining the abdominal cavity.
- For collecting pericardial fluid, a small needle is inserted into the chest between the ribs into the pericardium (the thin sac that surrounds the heart) and a small amount of fluid is withdrawn.
- Pleural Fluid is collected by pleural tapping or thoracocentesis, a procedure in which a needle is inserted through the back of the chest wall into the pleural space (a space that exists between the two lungs and the interior chest wall) to remove pleural fluid.

10.5- Fixation

To prevent cellular distortion all the smear prepared for cytological studies should be fixed immediately before drying. Rapid fixation of smears is necessary to preserve cytological details of cells and tissues. Different fixatives used are

1. Ether alcohol mixture: This fixative was originally recommended byPapanicolaou. It consists of equal parts of ether and 95% ethyl

alcohol. It is an excellent fixative, but ether is not used in most of the laboratories because of its safety hazards, odor and hygroscopic nature.

2. 95% Ethyl Alcohol (Ethanol**):** The ideal fixative recommended in most of the laboratories for cytological specimen is 95% ethanol alone. It produces the characteristic effect desired on nucleus. It is a dehydrating agent and causes cell shrinkage as it replaces water,

3. 100% Methanol**:** 100% methanol is an acceptable substitute for 95% ethanol. Methanol produces less shrinkage than ethanol, but it is more expensive than ethanol.

4. Alternate fixing fluid is a mixture containing 3 volumes of tertiary butyl alcohol and one volume of 95% ethyl alcohol. It is equally effective and does not evaporate readily.

10.5- Staining Methods in Cytology

Georgios Nikolaou Papanikolaou

May 13, 1883 – February 19, 1962

Papanicolaou Staining Method

This is the most commonly used staining procedure in cytopathology laboratory. This technique is named after **Dr. George N. Papanicolaou,** the father of Exfoliative cytology and is devised for the optimal visualization of cells exfoliated from epithelial surfaces of the body. It is a popular method for the cytological examination of smears from female genital tract.

It gives a sharp nuclear staining, transparency of cytoplasm and good differential coloring of acidophilic and basophilic cells.

Steps of staining procedure-

1. Fixation

   The cytology smears are fixed in 95% ethyl alcohol or in other substitutes for a minimum of 15 minutes.

2. Nuclear staining

   It is done by using haematoxylin stain. Harris haematoxylin or its modified form is used. Papanicolaou staining is regressive method, in which first smear is over stained with haematoxylin and removes the excess stain by using a differentiating solution such as acid alcohol (0.05% HCl in 70% ethyl alcohol). As haematoxylin is used in an acid pH, a pink color will form and it is not stable. In order to make it stable, the compound is brought to alkaline pH (bluing) by treating with a weak alkaline solution. Running tap water which is slightly alkaline (pH 8) is used as bluing solution in small laboratories. Ammonium hydroxide solution (15 ml of ammonium hydroxide 28-30% w/v to 98.5 ml of 70% ethanol) can also be used.

3. Cytoplasm staining

   Cytoplasmic stains are OG-6 and EA-36. Both are synthetic stains and OG-6 is a monochrome stain while EA-36 is a polychrome stain.

4. Dehydration

Rinse the smears in absolute alcohol for two or three changes for the removal of water.

5. Clearing

During clearing, alcohol is being replaced with Xylene, which is also miscible in mounting medium.

The reagents used are:

Reagents

Harris's Alum Hematoxylin –

- Haematoxylin crystals 1gm
- Absolute alcohol 95% 10 ml
- Ammonium or potassium alum 20 gm
- Distilled water 200 ml
- Mercuric oxide 0.5 gm

Dissolve haematoxylin in 95% alcohol and alum in water. Mix haematoxylene solution with alum solution while alum solution is hot. Bring it to boil. Add mercuric oxide and cool rapidly in bath with running tap water or in ice cold water.

Orange G

- Orange G stock solution
  (0.5% solution in ethyl alcohol) 100ml
- Phosphotungstic acid 0.015gm

Polychrome stain EA 36

- Light green SF (yellowish)
  (0.1% Solution in 95% alcohol) 45ml

- Bismark brown
  (0.5% solution in 95% alcohol) 10ml
- Eosin yellowish
  (0.5% solution in 95% alcohol) 45ml
- Phosphotungstic acid 0.2gm
- Lithium carbonate
  (saturated aqueous solution) 1drop

Fig 10.5.1

Staining Technique

1. Remove slides from fixing jar.
2. Pass through descending grades of alcohol to water 6 dips each
   (Hydration of smear)
3. Rinse in distilled water.
4. Stain with Harris's haematoxylene 2-3 minutes
5. Rinse in tap water.
6. Differentiate in 0.25% aq. HCl
   (Until the cytoplasm is decolorized and nuclei retain the stain).
7. Rinse in tap water.
8. Blue in ammoniated water.
   (Until the desired staining density is reached).
9. Wash in running tap water 5 minutes

10. Pass through ascending grades of alcohol
    (Complete dehydration).
11. Stain in OG 6 — 2 minutes
12. Rinse in two changes of 95% alcohol.
13. Stain in EA 36 — 2-4 minutes
    (Until the desired intensity of color has been reached).
13. Rinse in two changes of 95% alcohol.
14. Pass through 100% alcohol.
15. Clear in xylol (3changes).
16. Mount and examine under microscope.

Results:

Nuclei	Blue
Acidophilic cells	Red to Orange
Basophilic cells	Green to blue green
Erythrocytes	Orange red.

10.6- Sex Chromatin

Sex chromatin, also called as “Barr body” was first described by Barr and Bertram in 1949 in the nerve cells of cats. It is a distinguishing characteristic of interphase nuclei of females in humans and certain other animals. In females the X-chromosome remains condensed during interphase and appears as a small mass of intensely staining chromatin against the inner surface of the nuclear envelope. This barr body is not found in nuclei of normal males. Sex chromatin can be seen in smears prepared from scrapping of the buccal mucosa.

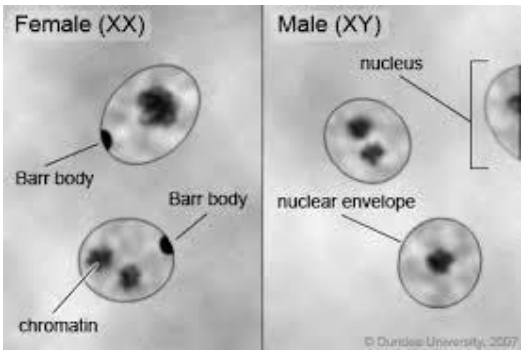


Figure 10.6.1

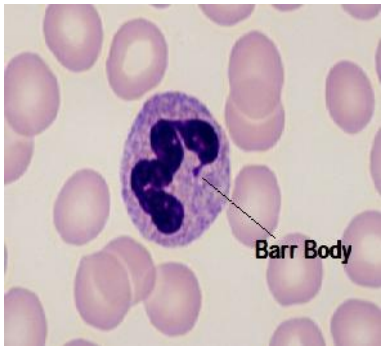


Figure 10.6.2

Similar phenomenon is seen as a “drumstick appearance “in the neutrophils in females. Here, the chromatin representing the condensed X-chromosome forms a minute separate lobule which is attached to the lobed nuclei of the neutrophils.

Sex chromatin staining

Preparation of smear:

The material is obtained from the buccal mucosa by scrapping the inside of the cheek with a spatula. The scrapping is smeared on to a clean glass slide which is immediately put in to equal parts of ether and ethyl alcohol and leave for at least 15 minutes.

Staining technique: (cresyl fast violet method)

This method described by Moore in 1962 is the best and most reliable method for staining sex chromatin.

Staining solution:

Cresyl fast violet acetate	0.5 gm
Distilled Water	100 ml.

Procedure:

- Fix the smear before drying in 95% alcohol for 30 minutes.
- Transfer to 50% alcohol for few seconds and then to dist. Water.
- Stain with Cresyl fast violet solution for 5 minutes.
- Rinse quickly in tap water.

- Dehydrate in 90% alcohol followed absolute alcohol.
- Clear in two changes of xylene, 3 minutes each.

Result:

Sex chromatin is seen as a deeply stained dot in the nucleus lying against the nuclear membrane.

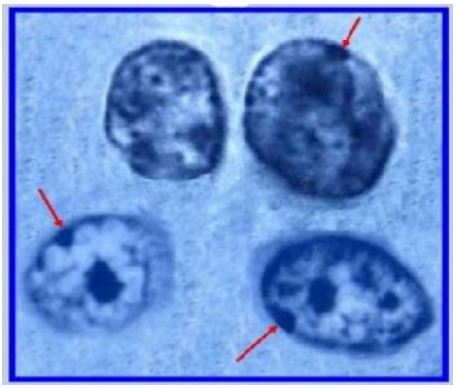


Figure 10.6.3

Review questions-

- Define exfoliative cytology. Give its significance.
- List various samples received in cytology laboratory.
- Which different fixatives are used in cytology?
- What is significance of fixing smear in wet condition?
- Describe pap staining method.
- What is sex chromatin? Give its significance.
- Describe sex chromatin staining.
- Describe morphology of sex chromatin.

Topic 11: Museum technique

Objectives:

1. To explain importance of museum in medical education.
2. To describe techniques of specimen preservation.

- **Definition & Significance:**

Museum is one of the important components of pathology department in teaching hospitals & Medical colleges. Museum provides good collection of specimens for Undergraduate & Postgraduate teaching. Museum serves important **functions** like

- Rare and important specimens become available for teaching and learning.
- Museum serves as permanent source of histological material which can be used by students as well as by researchers.
- Specimens are also useful for gross and microscopic study.

Museum technique means to collect process & display the common, unique pathological specimens. In medical museum intact specimens are mounted. All are not completely diagnosed on gross, histological diagnosis is done. The basic aim of museum technique is to keep specimen in such a way that the color of the specimen is restored. Commonly used color restoration technique is **Kaiserling method.** Various steps involved in preparation and storage of museum specimens are as follows.

1. **Reception of Specimens:**

- Specimens for museum are selected from specimens from operation theatre which are sent for histopathological examination and also from autopsy specimens.

- Any specimen received in the museum is registered in the museum register.
- Each specimen is given a number according to numbering system of museum. This number will be continued with specimen throughout its processing.
- The museum register should contain all necessary information about the specimen. (Clinical, gross & microscopic findings).

2. Preparation of the specimen:

- An ideal specimen is received fresh in unfixed state.
- However, it is mostly obtained from pathology laboratory after being examined, thus will already be formalin fixed.
- The specimen must never be allowed to dry.
- It must be kept away from water. Water causes hemolysis and discoloration.
- Specimen may be washed in normal saline.
- Dissection should be neat and with no irregular edges.
- Bisection should be done with long knife (30-35cm) so that an absolutely flat surface is obtained.
- If planning to use a specimen for museum, part of it can be kept without disturbing e.g. in kidney it can be bisected and one half is kept aside for museum.

3. **Fixation of the Specimen**:

- The objective of fixation is to preserve cells and tissue constituents in as close a life-like state as possible and to allow them to undergo further preparative procedures without change.

- Formalin fixation is the basis of museum preparation. For this Kaiserling technique and his modifications are used.
- Kaiserling recommended that the initial fixation be a neutral formalin (KI) solution and then transferred to a final preserving glycerin solution (KIII) for long term display. Color preservation is also maintained with these solutions.
- The specimen needs to be kept in a large enough container which can accommodate specimen along with 3-4 times volume of fixative.
- The fixative used is kaiserling I solution.

Kaiserling SolutionI:

- Formalin(40%) 400ml
- Potassium acetate 60 gm.
- Potassium nitrate 30gm.
- Distilled water 2000ml

Fix the specimen in this solution at least for two weeks. If specimens are large they take more time. If required organ should be injected with the solution.

4. **Restoration of color**:-

- During fixation, specimens lose their natural color; hence it is necessary to restore color of specimen.
- Remove the specimen from fixative, wash it in running water.
- Transfer it to Kaiserling solution II.

Kaiserling Solution II:-

- Industrial alcohol 80%

Watch the specimen during this stage and remove the specimen from alcohol when maximum color contrast is obtained. This requires

generally ½ to 1 hour. If correct time exceeds colors will begin to fade and cannot be brought back.

5. **Preservation of Specimen:**

- Wash the specimen in running tap water.
- Place it in Kaiserling Solution III

Kaiserling Solution III:

- Formalin (40%) 50ml
- Potassium acetate 1500gm
- Glycerine 3000ml
- Distilled water 9000ml

- The jar is filled with freshly filtered KS III.This solution Adjust the pH to **8** with NaOH. Specimens may be kept in this solution until they are ready for mounting in museum jar.
- Acts as permanent fixative.
- If the solution turns yellowish it should be replaced.

6. Presentation of Specimen (Mounting of specimen):-

- To display the specimens, the specimens are kept in Perspex containers of suitable size.
- Perspex is a high quality transparent plastic jar.
- If required centre plate is used for mounting.
- Nylon thread is used to type up the specimen on centre plate.
- The specimen is placed inside the glass jar of correct size supported by a Perspex sheet or glass rod to which it is stitched.
- The jar is filled with mounting fluid (KS III).
- Release the air bubbles.
- A hole is drilled into the top corner of the lid.

- A lid is placed on the jar and sealed on four sides by Araldite.
- A light weight is applied for two hours.
- After removing the air bubbles the hole is plugged by a Perspex plug and cemented.
- The specimens are arranged according to systems or pathology. Each and every specimen is accompanied with relevant in formation like an exhibit in the museum.
-

Figure 11.1

Review questions:-

1. What is museum technique? What is the importance of museum?
2. Describe fixation of museum specimens.
3. Enumerate various steps of museum techniques. How will you do color restoration?
4. Describe composition of kaiserling's mounting solution.

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Topic 12: Waste disposal

Objectives:

1. To impart knowledge regarding types of waste generated in histopathology section of laboratory.
2. To introduce various methods of disposal for waste

Introduction:

During the laboratory procedures in histopathological examination and cytological examination, significant amount of waste is generated. The waste can be in the form of human tissue waste, cotton, gauze waste or various body fluids & discharges received for cytological examination. All these waste carries risk of infection. Hence, proper knowledge about handling & disposal of waste generated in this section is necessary.

Types of waste:

According to the rules, waste generated during tissue processing, autopsy procedure and in cytological, examination will come under the category of "biomedical waste". Hence, all the rules & regulations about "biomedical waste" would be applicable to waste generated in this part of laboratory. Various types of wastes which are generated are specified as follows:

a) Human anatomical waste**:** This includes human tissues, organs, body parts etc.
b) Liquid waste**:** Waste generated from cytological section like used body fluids, discharge, swabs, endoscopic washings etc.

c) Solid Waste**:** This includes items contaminated with blood & body fluids. Used cotton swabs, gauze pieces will come under this category.

d) Waste Generated from disposable items**:** This can be in the form of waste sharps like needles, scalpels etc. & others as disposable gloves, disposable specimen containers, syringes etc.

Methods of Disposal:

Various methods which are commonly used & widely available are as follows:

I. **Human anatomical waste**: Human anatomical waste can be disposed off safely by two methods.

a) **Incineration:**

- Incineration means burning under high temperature. As a result organic, combustible waste is converted into inorganic incombustible matter (incineration ash). This significantly reduces waste volume and weight.
- For incineration to carry out special incinerator facilities are available in common in urban areas. The transportation of waste for incineration is carried in the manner mentioned herein below.
- The waste is collected in plastic bag of "Yellow" colour. The bag is sealed and sent for incineration. Incineration ash is disposed in municipal land fill.

b) **Deep burial**:

Deep burial can be used as method of disposal where incinerator facility is not available around, nearby places.

- **Advantages** of deep burial:

1. Transportation is not required
2. Sealing & packing in prescribed manner is also not necessary.

- **Limitations:**
  1. Isolated space is required in the surroundings of laboratory itself which is not feasible in crowded urban areas.
  2. Intermittent supervision is necessary for achieving the safety.

II. **Liquid Waste:** Liquid waste should be first decontaminated with chemical disinfectant or by autoclaving and then can be discarded into drains. This can be managed in the laboratory itself.

III. **Solid waste:** For disposal of solid waste “incineration” can be the method of choice. The waste is packed in “yellow” or “Red” plastic bags before sending for disposal.

IV. **Waste generated from disposable items:** Waste under this category is first decontaminated and then subjected for “shredding”. Shredding means breaking into small pieces so as to prevent unauthorized reuse. For disposal of such items the items are packed in “blue” or “white” translucent plastic bags which are puncture proof. Or it can be collected in “Red” colored plastic bag.

Segregation of Waste in color coded Bags

Review questions:

- What do you mean by “waste”?
- Enlist different types of wastes generated in this part of laboratory.
- Write a short note on ‘Incineration”
- Write about various methods of waste disposal.

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