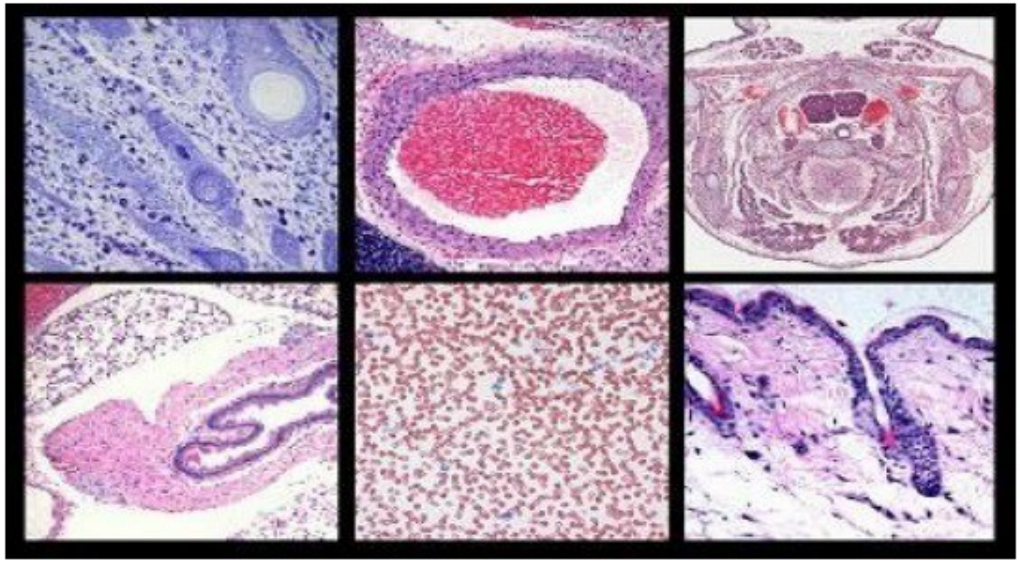
Lab 1: introduction of histopathology



Introduction

- Histopathology: is a branch of pathology which deals with the study of disease in a tissue section.
- Histology: is the study of tissues and their structures of plants and animals, it's commonly performed by examining cells of tissues under alight microscope or electron microscope.
- Pathology: is the study of diseases and of the changes that they causes changes in a person, an animal, or plant that are caused by diseases.
- The term **histochemistry** means study of chemical nature of the tissue components by histological methods.

•The cell is the single structural unit of all tissues.

• The study of cell is called cytology.

•A tissue is a group of cells specialized and differentiated to perform a specialized function.

• Collection of different type of cells forms an organ.

Types of material

1. **As biopsy:** A small piece of lesions or tumor which in sent for diagnosis before final removal of the lesion or the tumor **Incisional biopsy.**

◁ If the whole of the tumor or lesion is sent for examination and diagnosis by the pathologist, it is called **excisional biopsy.**

2. Tissues from the **autopsy** are sent for the study of disease and its course, for the advancement of medicine.

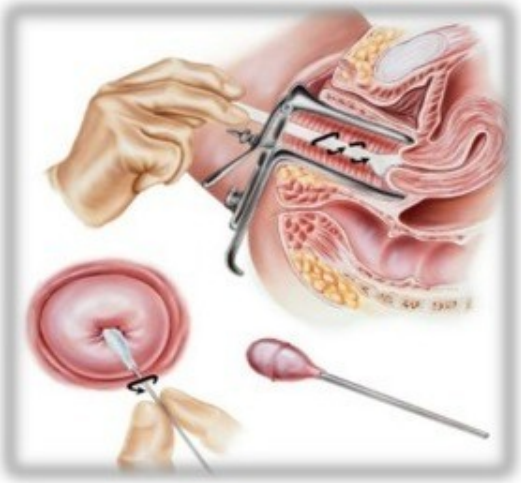
Types of Histopathological Preparations

1. Whole mounts: These are preparation entire animal eg. Fungus, parasite. These preparations should be **no** more than 0.2-0.5 mm in thickness.

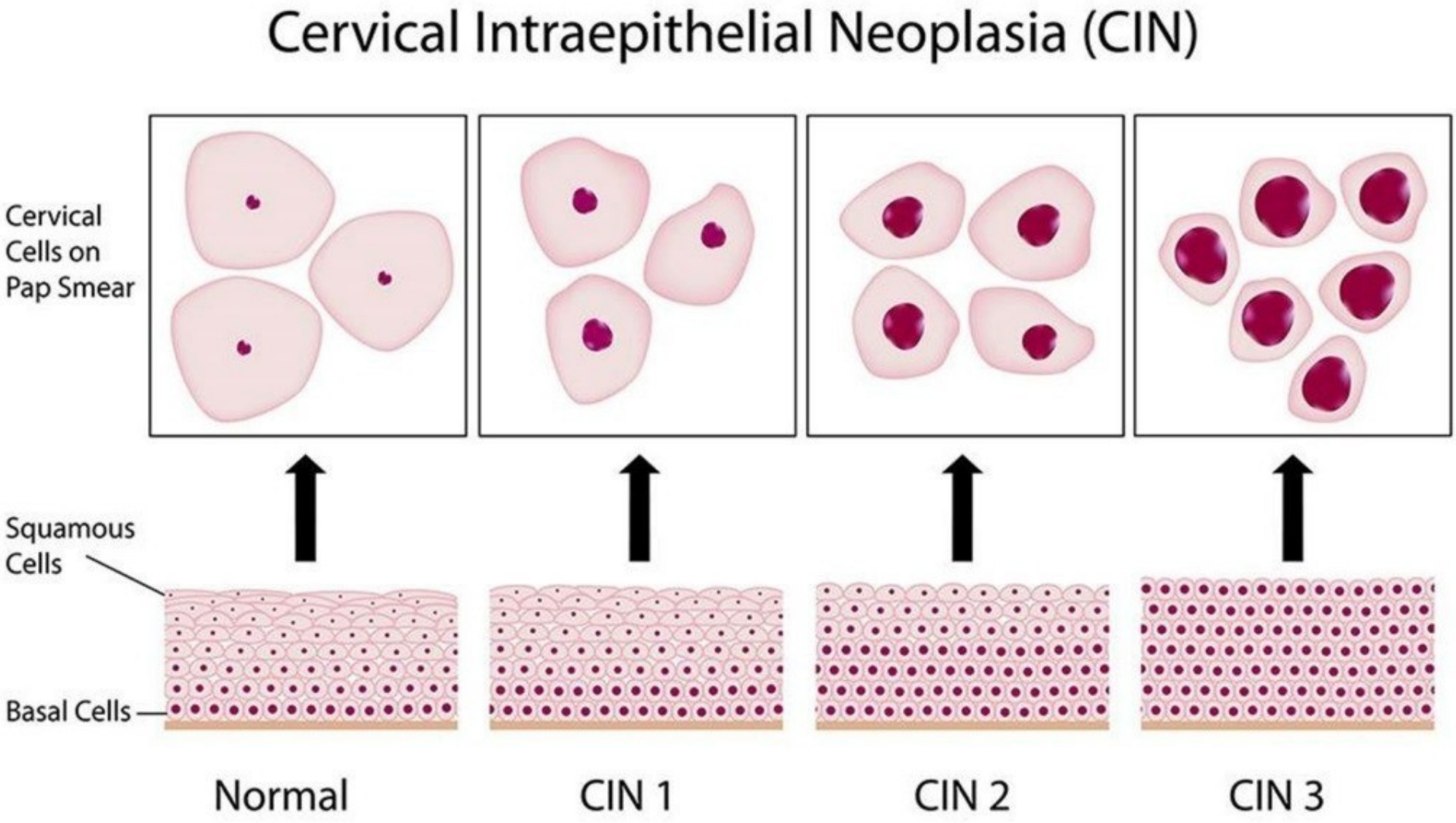
2. Sections: The majority of the preparations in histology are sections.

- The tissue is cut in about 3-5 mm thick pieces processed and 5 microns thick sections are cut on a microtome.
- There are 2 methods of hardening the tissues:
- One is by **freezing** them and the other is by **embedding** them in a hard material such at paraffin wax or gelatin.

3. Smears: Smears are made from blood, bone marrow or any fluid such as pleural or ascitic fluid, blood smear and pap smear.



Pap smear


Cervical Intraepithelial Neoplasia (CIN)
Cervical Cells on Pap Smear
Squamous Cells
Basal Cells
Normal
CIN 1
CIN 2
CIN 3

Responsibility of a technician

1. Specimen preservation.
2. Specimen labeling, logging and identification.
3. Preparation of the specimen to facilitate their gross and microscopy.
4. Record keeping.

To obtain these aims the following point need consideration

1. As soon as the specimen is received in the laboratory, check if the specimen is properly labeled with the name, age, Hospital Registration No. and the nature of tissue to be examined and the requisition form is also duly filled.

2. Also check if the specimen is in proper fixative.

◁ Fixative should be fifteen to twenty times the volume of the specimen.

◁ Add fixative if not present in sufficient amount.

3. Check if the financial matters have been taken care off.

4. Make the entries in biopsy register and give the specimen a pathology number called the accession number. Note this number carefully on the requisition form as well as the container. This number will accompany the specimen everywhere.

5. If the specimen is large inform the pathologist who will make cut in the specimen so that proper fixation is done. Container should be appropriate to hold the specimen without distorting it.

6. Blocks of tissues taken for processing should be left in 10% formalin at 60°C till processing. These would be fixed in 2 hours.

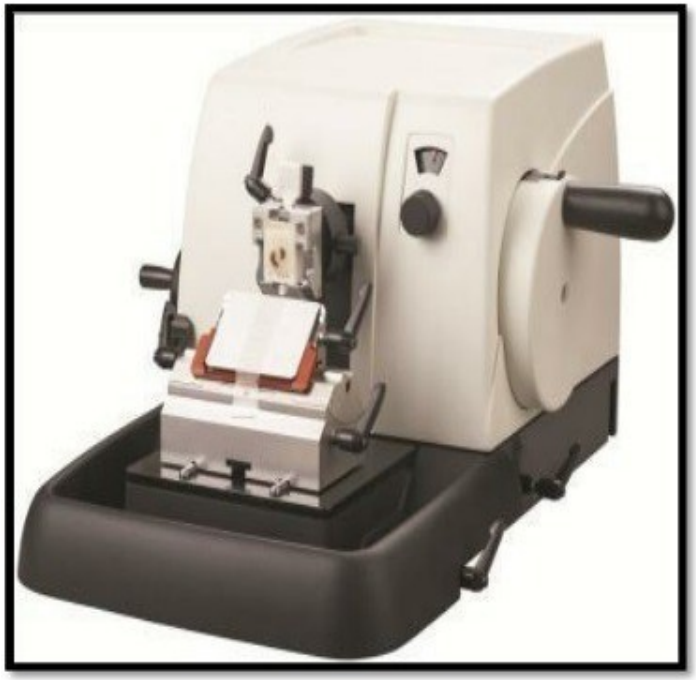
7. Slides should be released for recording after consultation with the pathologist.

8. Specimens should be kept in their marked container and discarded after checking with pathologist.

9- Block must be stored at their proper number the same day.

◁ **Note** the blocks have to be kept preserved for life long.

Equipment used during this course:

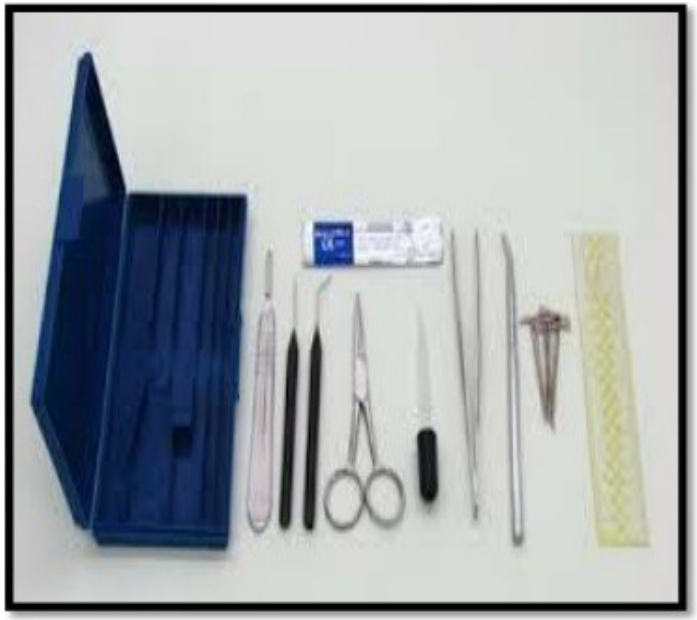


Microtome

Cryostat (frozen section)

Safety cabinet

Water bath



Dissecting tools

dispenser

Hot plate

Oven

Blocks

Staining machine

General safety rules

1. Never pipette by mouth.

2. Do not apply cosmetics and lip balm in the technical work areas.

3. Do not manipulate contact lenses in the technical work areas.

4. When diluting acids, always pour the acid into the water, never the reverse.

5. Wash hands frequently; hands should always be washed immediately after the removal of gloves.

6. If you are not sure of the reagent properties, handle it as if it were a hazardous chemical.

7. Discard (in the appropriate manner) all unused, out-of-date chemicals.

8. Examine all procedures for possible replacement of hazardous reagents by less hazardous substitutes.

9. If the use of a hazardous chemical cannot be eliminated or replaced, the use and amount of stock should be minimized.

Hazards in Histopathology lab:

➨Biological or Infection Hazards

1. Tuberculosis exposure
2. HIV, hepatitis C VIRUS (HCV), and HBV
3. **Handling tissue waste**:

All tissue waste and any materials coming in contact with tissue should be disposed of in biohazard bags; sharp objects should be discarded in special "sharps" containers.

➨Mechanical Hazards:

Mechanical hazards primarily consist of sharp instruments (eg, microtome blades, razors, scalpels, and needles), glass.

➨Chemical Hazards

- Employees must prepare an inventory of all hazardous chemicals in the facility, and obtain copies of the Material Safety Data Sheets
- Employers shall ensure that labels on all incoming containers of hazardous chemicals are not removed or defaced.
- Employers must establish a training program in which employees who work with hazardous substances are trained to read and interpret chemical labels
- Employers must establish a means to inform nonemployees or personnel from other departments of the hazards present in the workplace.

➨Corrosive Substances

- Are substances that will cause injury to the skin and eyes by direct contact or severe damage to the tissues of the respiratory and alimentary tracts when inhaled or ingested.
- The effects of corrosive chemicals lead to disruption of cell membranes, coagulation of proteins, and death of essential cellular components.

➨Fire and Explosion Hazards

Although liquid organic compounds are most commonly thought of as fire hazards, certain chemicals such as dry picric acid, benzoyl peroxide, and ammoniacal silver solutions can be explosion hazards

Fire extinguishers should be available in a readily known and accessible places Most fires can be avoided if all laboratory personnel follow good safety practices.

➨Hazardous Chemical Spills and Storage

Laboratory personnel must be trained and prepared to handle spills.

If floor drains are present, spill dikes should be available to prevent hazardous chemicals from entering the sewer system.

Gloves, aprons, and eye protection should be used in the event of a small spill. Spill kits should be available in the laboratory with absorbent pads for the solvents, commercial neutralizers for the acids and alkalis

FIXATIVES USED IN HISTOPATHOLOGY

Mr. Kapil Sharma

Fixation

- This is the process by which the constituents of cells and tissue are fixed in a physical and chemical state so that they will withstand subsequent treatment with various reagents with minimum loss of architecture .This is achieved by exposing the tissue to chemical compounds, call fixatives.

- ✓ The purpose of fixation is to preserve tissues permanently in as life-like a state as possible.

- ✓ The fixative should be 15 – 20 times more in volume then the specimen.

Mechanism of action of fixatives

- Most fixatives act by denaturing or precipitating proteins which then form a sponge or meshwork, tending to hold the other constituents.

Aims of Fixation :

1. It should prevent autolysis & putrefaction of the cell.
2. It should penetrate evenly and rapidly.
3. It should harden the tissues
4. Increase the optical density
5. Should not cause shrinkage or swelling of the cells
6. Must not react with the receptor sites & thus must not interefere with the staining procedure.
7. It must be cheap and easily available.

Contin…..

- ✓ Good fixative is most important factors in the production of satisfactory results in histopathology.

- ❖ Following factors are important:

- ✓ Fresh tissue
- ✓ Proper penetration of tissue by fixatives
- ✓ Correct choice of fixatives

Contin….

- ✓ No fixative will penetrate a piece of tissue thicker than 1 cm.
- ✓ For dealing with specimen thicker than this, following methods are recommended:

1.Solid organ:

Cut slices as necessary as but not thicker than 5 mm.

Continu….

2.Hollow organ:

Either open or fill with fixative or pack lightly with wool soaked in fixative.

3.Large specimen:

It requires dissection, Inject fixative along the vessels or bronchi as in case of lung so that it reaches all parts of the organs.

Properties of an Ideal Fixative

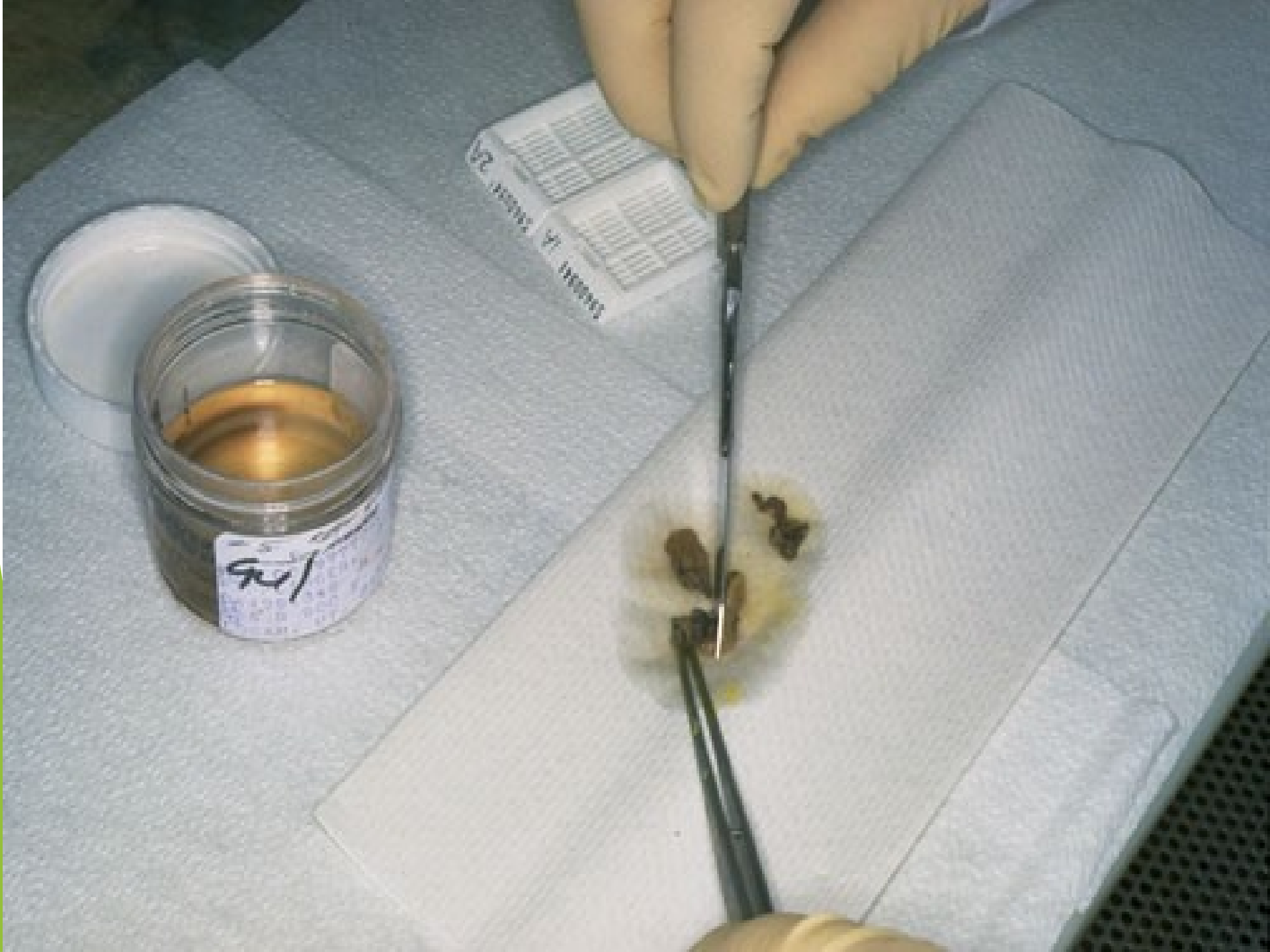
- ✓ Prevents autolysis and bacterial decomposition.
- ✓ Preserves tissue in their natural state and fix all components.
- ✓ Make the cellular components insoluble to reagent used in tissue processing.
- ✓ Preserves tissue volume.

Properties of an Ideal Fixative

- Avoid excessive hardness of tissue.
- Allows enhanced staining of tissue.
- Should be non-toxic and non-allergic for user.
- Should not be very expensive.

Methods of fixation:-

- Heat fixation
- Perfusion
- Immersion
- Vapour method
- Phase partition method



Classification of Fixatives:-

1)Physical fixative

- ✓ Heat
- ✓ Freezing

2)Chemical fixatives

Chemical Fixatives

Simple Fixatives

- Formaline
- Mercuric chloride
- Osmic acid
- Picric acid
- Acetone
- Ethyle alchohol
- Osmium tetroxide
- Osmic acid

Compound Fixatives

Microanatomical

Formal Saline

Neutral buffer Formaline

Zenker's fluid

Bouin's fluid

Cytological

Nuclear

Carnoy's Fluid

Cytoplasmic

Champy's Fluid

Histochemical

Cold acetone

Ethanol

Simple Fixatives

- **Formalin**

- The most commonly used fixative is Formalin .

- It is prepared by mixing 40 % Formaldehyde gas in 100 w/v of distilled water.

- The resultant mixture is 100 % Formalin.

- Routinely, 10 % formalin is used which is prepared by mixing 10 ml of 100 % formalin in 90 ml of distilled water.

MECHANISM OF ACTION

- ✓ It forms cross links between amino acids of proteins thereby making them insoluble.

- ✓ It fixes 4 mm thick tissue in 8 hours .

❖ ADVANTAGES :

1. Rapid penetration
2. Easy availability & cheap
3. Does not overharden the tissue
4. Fixes lipids for frozen sections
5. Ideal for mailing

❖ DISADVANTAGES:

1. Irritant to the nose,the eyes and mucous membranes
2. Formation of precipitate of paraformaldehyde which can be prevented by adding 11- 16 % methanol.
3. Formation of black formalin pigment , Acid formaldehyde hematin.

Other Simple Fixatives

- ✓ Glutaraldehyde
- ✓ Osmium Tetraoxide
- ✓ Pottasium Dichromate
- ✓ Mercuric Chloride

Other Simple Fixatives (contd.)

- ✓ Picric acid
- ✓ Zenker's fluid
- ✓ Zenker’s Formal (Helly’s Fluid)
- ✓ Bouin’s Fluid

Compound Fixatives

- Microanatomical fixatives:

  These are used to preserve the anatomy of the tissue.

- Cytological fixatives:

  These are used to fix intracellular structures.

- Histochemical fixatives :

  These are used to demonstrate the chemical constituents of the cell.

Microanatomical Fixatives

→ 10 % Formal saline :

- ✓ It is a microanatomical fixative.
- ✓ Ideal for fixation of brain.

→ Buffered formalin:

- ✓ Due to the presence of buffer, the pH of the solution remains at neutral or near neutral.
- ✓ As a result, Formalin pigment formation doesn't take place.

- Cytological Fixatives

Nuclear fixatives :

Carnoy’s Fluid

Clarke’s Fluid

Newcomer’s Fluid

Flemming’s Fluid

Cytoplasmic Fixatives :

Champy’s Fluid

Regaud’s Fluid

- Histochemical Fixatives:

  Formal saline

  Cold acetone

  Absolute alcohol

Composition of Fixatives:-

1-Formalin Solution (10%, unbuffered):

Formaldehyde (37-40%) - 10 ml

Distilled water - 90 ml

Mix well.

2-Formalin Solution (10%, buffered neutral):

Formaldehyde (37-40%) - 100 ml

Distilled water - 900 ml

NaH_2PO_4 - 4.0 g

Na_2HPO_4 (anhydrous) - 6.5 g

Mix to dissolve.

3-Zenker's Solution-fixation time 4-24 hours.

Distilled water - 950ml

Potassium dichromate - 25g

Mercuric chloride - 50g

Glacial acetic acid - 50g

Fixed tissue should be washed overnight in running tap water before processing.

4-Bouin's fluid - fixation time 6 hours.

Saturated aqueous solution of picric acid - 75ml

Formalin (~ 40% aqueous solution of formaldehyde) - 25ml

Glacial acetic acid - 5ml

Fixed tissue should be transferred to 70% alcohol.

5-Carnoy's fluid - fixation time 1-3 hours.

Ethanol - 60ml

Chloroform - 30ml

Glacial acetic acid - 10ml

Fixed tissue should be processed immediately or transferred to 80% alcohol.

6-Champy’s fluid – fixation time 12-24 hours.

Methanol, absolute - 60.0 ml

Chloroform - 30.0 ml

Glacial acetic acid - 10.0 ml

6-Helly's fluid - fixation time 12-24 hours.

Potassium dichromate - 25g

Mercuric chloride - 50g

Sodium sulphate - 10g

Distilled water - 1000ml

Stock solution - 100ml

Formalin (~ 40% aqueous solution of formaldehyde) - 5ml

7-Susa Solution:

Stock Solution A:

Mercuric chloride ---------- 4.5 g

Sodium chloride ---------- 0.5 g

Trichloracetic acid ---------- 2 g

Distilled water ---------- 80 ml

Stock Solution B:

Glacial acetic acid ---------- 4 ml

Formaldehyde (37-40%) ---------- 20 ml

Mix Solution A and B. For hard tissues such as inner ear with excellent penetration and little shrinkage.

Factors affecting fixation:-

1 - Temperature

- Affects the morphology of the tissue.
- For electron microscopy and some histochemical procedures, the temperature for fixation is usually 0-4°C.
- It will increase the rate of penetration
- It will also increase the rate of autolysis and diffusion of cellular components.

2 - Size

- Ideal size of the tissue should be 3mm.

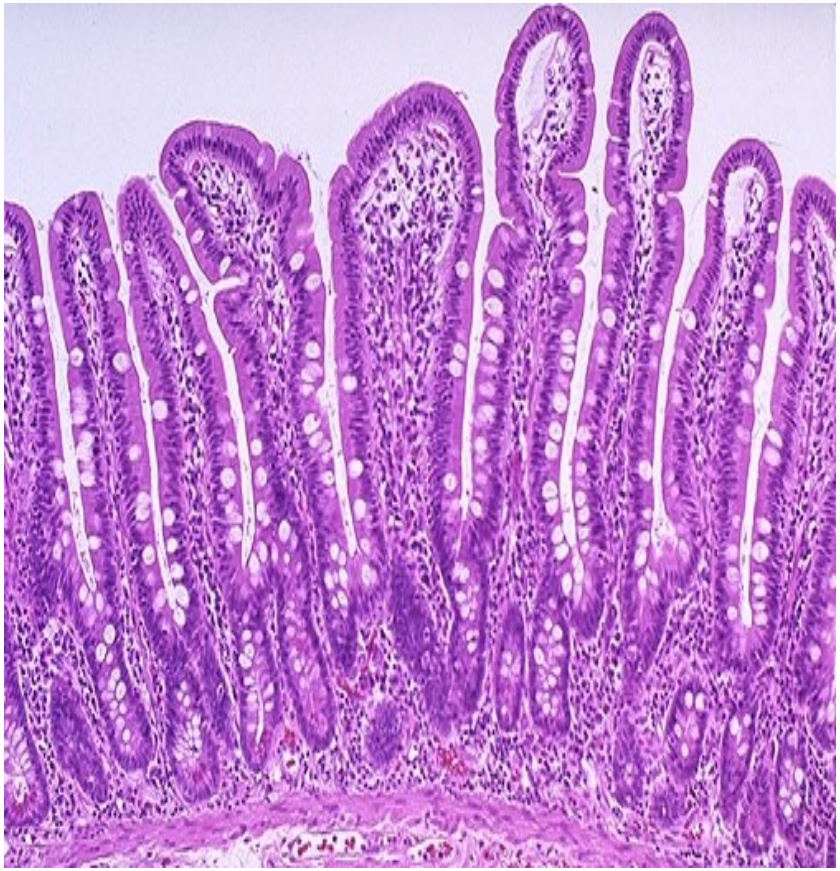
3 - Volume ratio

- Volume of fixative is at least 15 to 20 times greater than volume of tissue.

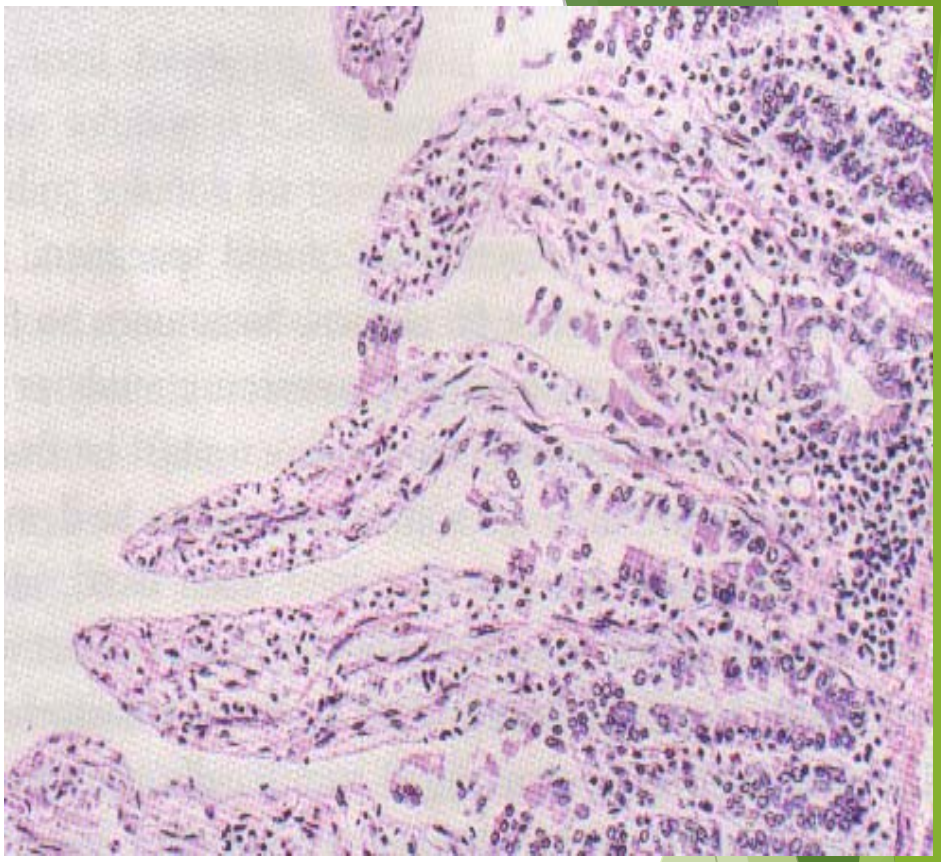
4 – Time

- Minimum fixation time for 5mm tissue is 12hrs.
- For electron microscopy sliced tissue is preserved for

  3 hrs in 3% glutaraldehyde.
- prolonged fixation in aldehydes can cause shrinkage and hardening of tissue and severe inhibition of enzyme activity.



Small intestine well preserved



Autolyzed Small intestine
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Notice how is missing the epithelium

5 - Choice of fixatives

- The method of fixation should be selected immediately once the specimen is presented.
- For Gout a fixative of choice is absolute alcohol.
- Electron Microscopy – the choice is Gluteraldehyde.

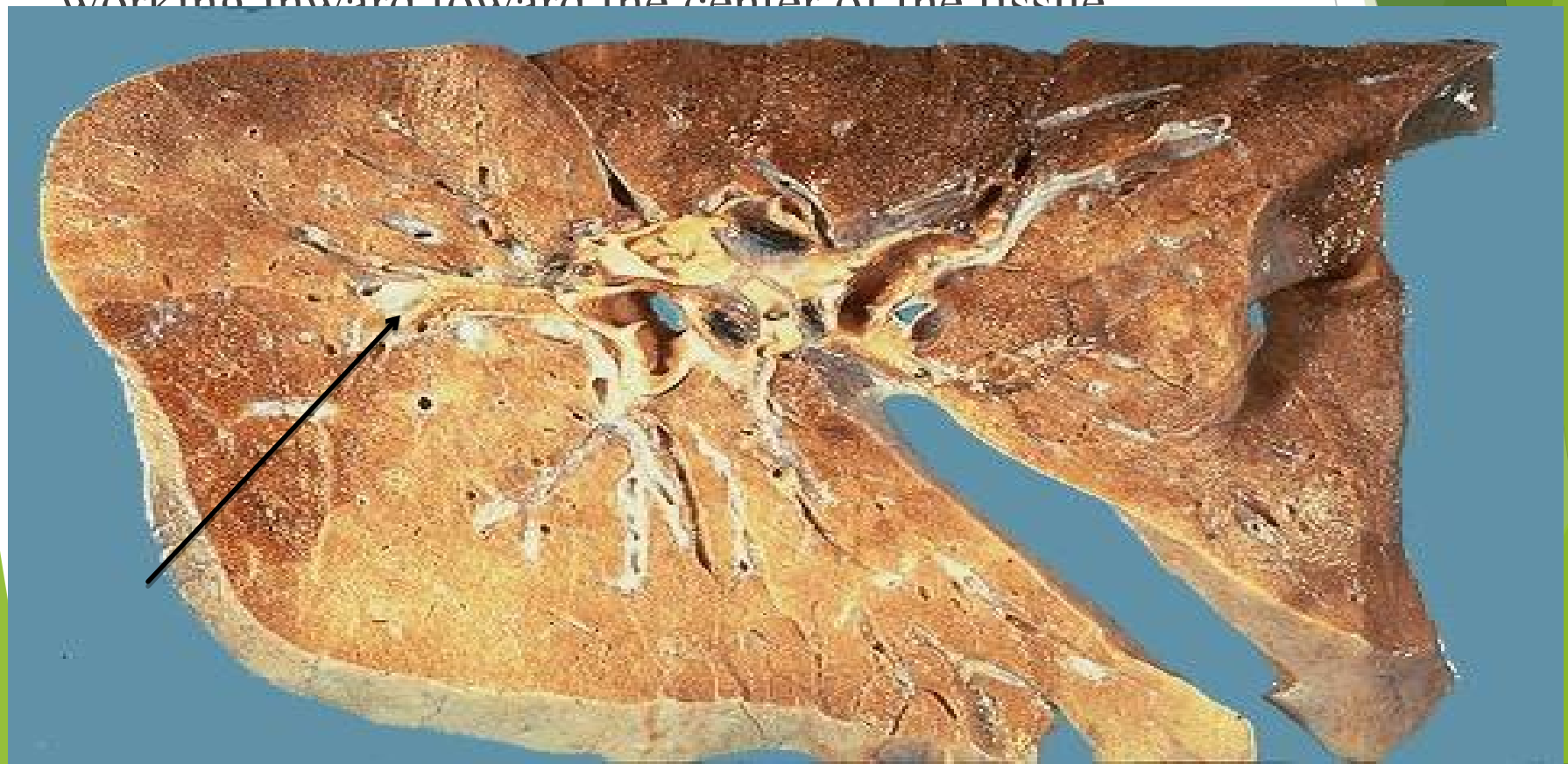
Solutions	Colors	Tissues
Zenker's fluid	Orange	Bone Marrow Biopsies
Helly's fluid	Orange	Bone Marrow Aspirates
B-5	Transparent	Bone Marrow Cores and Tumors
Bouin's fluid	Yellow	GI Biopsies
Hollande's fluid	Green	Small tissue
Orth's fluid	Orange Decals and Bones	Adrenal Medulla

Solutions	Colors	Tissues
Zamboni's	Yellow	EM Fixatives
Carnoy's	Clear	Nuclear Fixatives
10% Formalin	Clear	Routine
10% Formal saline	Clear	Routine
Neutral buffer formalin	Clear	Prevent Pigments
Formalin ammonium Bromide	Clear	Brain Tissues
10% Formal Alcohol	Clear	EM Specimen
Flemming's	Clear	EM Specimen
Gluteraldehyde	Clear	EM Specimen
Schaudinn's	Clear	EM Specimen

6 - Penetration

- Fixatives penetrate the tissue at different rates.

- The tissue is fixed starting at the periphery of the tissue and working inward toward the center of the tissue



7- Tissue Storage

- Storing wet tissue is very important because often the tissue is needed for further studies.
- Tissue fixed in Neutral buffered Formalin are usually safe to use.
- Non fix tissue may remain in 70% methyl alcohol.

8 - pH

- The pH should be kept in the physiological range, between pH 4-9.
- If formalin is allowed to fall to a lower pH this can produce formalin pigments.
- In electron microscopy it is very important.
- The pH for the ultrastructural preservation of great specimen the fixative should be buffered between 7.2 to 7.4.

9 - Osmolality

- The addition of a buffer to the fixative solution may alter the osmotic pressure exerted by the solution.

- Hypertonic solutions give rise to cell shrinkage whereas hypotonic and isotonic fixatives result in cell swelling and poor fixation.

- With electron microscopy, the best results are obtained using slightly hypertonic solutions (isotonic solutions being 340 mOsm) adjusted using sucrose.


Thank You !

Hematoxylin and Eosin staining

Principle

Alum acts as mordant and hematoxylin containing alum stains the nucleus light blue. This turns red in presence of acid, as differentiation is achieved by treating the tissue with acid solution. Bluing step converts the initial soluble red color within the nucleus to an insoluble blue color. The counterstaining is done by using eosin which imparts pink color to the cytoplasm.

Reagents

Harri's Hematoxylin stain

A = 1 gm hematoxylin in 10 ml ethanol
B = 20 gm ammonium alum in hot distilled water
Mix A & B, boil and add 0.5 gm of mercuric oxide and filter.

Eosin solution

Yellow eosin = 1 gm
Distilled water = 80 ml
Ethanol = 320 ml
Glacial Acetic Acid = 2 drops

Procedure

- Deparaffinize the section
- Hydration : Hydrate the tissue section by passing through decreasing concentration of alcohol baths and water. (100%, 90%, 80%, 70%)
- Stain in hematoxylin for 3-5 minutes.
- Wash in running tap water until sections “blue” for 5 minutes or less.
- Differentiate in 1% acid alcohol (1% HCl in 70% alcohol) for 5 minutes.
- Wash in running tap water until the sections are again blue by dipping in an alkaline solution (eg. ammonia water) followed by tap water wash.

- Stain in 1% Eosin Y for 10 minutes.
- Wash in tap water for 1-5 minutes.
- Dehydrate in increasing concentration of alcohols and clear in xylene.
- Mount in mounting media.
- Observe under microscope.

Result and Interpretation

- Nuclei : blue, black
- Cytoplasm : Pink
- Muscle fibres : deep red
- RBCs : orange red
- Fibrin : deep pink.