**Ministry of high Education and scientific research**

**Institute of Medical Technology / Al-Mansour**

**Foundation of Technical Education**

**Training Package**

**In**

# Histological and Cytological Technique

# Theoretical

**For**

**Students of first class**

**Pathology analysis**

**By**

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**1/ Over view:**

1. **Target population:**

For students of first class

Institute of medical technology

Department of pathology analysis

Histological and Cytological Technique

1. **Rationale:**

In general students can prepare permanent slides for different body organs. Specifically students can do the followings:

1. Fix and preserve tissue specimen.
2. Preparing all needed chemical solutions.
3. Permanent stained tissue slides & body fluid smears.
4. **Central idea:**
5. Sciences related to histological and cytological techniques.
6. The steps of tissue processing (paraffin method).
7. Tissue processing for electron microscope.
8. Techniques for exfoliated cytology.

Syllabus for histological and cytological techniques

|  |  |
| --- | --- |
| Week  1  2  3-4  5-6  7-8  9  10-11  12-13  14  15-16  17  18-19  20-21  22-23  24  25-26  28-29  30 | Theoretical lectures  Definition of some terminology that deals with the subject.  Sample collection, fixation, effects of fixation on tissue.  Physical &chemical methods of fixation, compound fixatives.  Mercuric oxide, dichromate, and dehydrant fixatives.  Washing, solutions, dehydration, and dehydrants.  Clearing and clearing agents.  Infiltration, types of waxes, blocking & trimming.  Microtomy, sectioning, mounting & adhesives.  Examination for the first term.  Holiday.  Staining, staining reactions, & theories  Methods of staining, types of staining, terminology of staining.  Hematoxylin, oxidation, & alum hematoxylin.  Examination for the second term.  Iron hematoxylin, eosin.  Bone tissue, decalcification, & frozen sections.  Exfoliative cytology, types of biopsy, terminology.  Final examination. |

**TERMINOLGY**

**Cell**:The building unit of the living body. It consists of nucleussurrounded by cytoplasm & both are enclosed by a membrane.

**Cytology:** Is the study of cells, their origin, structure, function, and pathology

**Histology:** The study of the microscopic anatomy of cells and tissues of animals and plants. It is an essential tool of biology and medicine.

**Pathology:** The science which deals with diseases.

**Cytopathology:** Specialty of pathology on a cellular level with a focus on the diagnosis of diseases through specimens derived from fluids or smears.

**Pathologist:** A physician specializing in rendering medical diagnoses by examination of tissues and fluids removed from the body.

**Histopathology:** The microscopic study of diseased tissue, it is an important tool in anatomical pathology, since accurate diagnosis of cancer and other disease usually requires histopathological examination of samples.

**Histotechnician:** Trained scientists who perform the preparation of histological sections. They are histology technicians, histology technologists, medical scientists, medical laboratory technician, or biomedical scientists.

**Specimen collection:**

Tissue specimen that collected for study is of two kinds:

1. **Biopsy:** Cells or tissues removed from the body for examination.
2. **Autopsy:** Evaluation of a body to determine the cause of death.

**1a/** **Pre-test:**

Tissue specimen should be: a) fixed, b) cut, c) preserved.

**Fixation**

This process is the most important step in the processing of tissue samples; it preserves cells and tissue components with minimal distortion.

It stabilizes proteins, rendering the cells & it's components resistant to further autolysis by inactivating lysosomal enzymes, and changes the tissue receptiveness to further processing.

Fixation is the process by which the biological tissues were

hardened. It preserves tissues and set as closely as possible the structure they had in life.

The mechanisms and principles of specific fixatives act to harden & preserve tissues and prevent the loss of specific molecules; these fixatives fall into broad categories.

Each fixative has advantages & disadvantages, these include molecular loss from "fixed" tissues, swelling or shrinkage of tissues during the process, variation of staining quality, the ability to perform biochemical analysis accurately, and varying capabilities to maintain the structure of cellular organs.

The major objective of fixation in pathology has been to maintain clear and consistent morphological features. Various methods of fixation always produce some artifacts in the appearance of tissue on staining; however, for diagnostic pathology it is important that artifacts are consistent.

A fixative not only interacts initially with the tissue in its

aqueous environment but subsequently the unreacted fixative and the chemical modifications induced by the fixative continue to react.

The most important characteristic of a fixative is to support high quality and consistent staining with HX and E both initially and after storage of the paraffin block for at least a decade.

**Characteristics of good fixative**:

1. The fixative must have the ability to prevent short- and long- term destruction of the micro- architecture of the tissue by stopping the activity of catabolic enzymes and hence autolysis, minimizing the diffusion of soluble molecules from their original locations.

**2.** A good fixative is characterized by the destruction of infectious agents, which helps maintain tissue & cellular integrity.

**3.** It is useful for a wide variety of tissues including fatty , lymphoid, and neutral tissues.

1. It should preserve large & small specimens and support histochemical, immunohistochemical, in situ hybridization and other specialized procedures.
2. It should penetrate & fix tissue rapidly, half a shelf life of at least one year.
3. It should be readily disposable or recyclable and support long-term tissue storage giving excellent microtomy of paraffin blocks, and should be cost effective.

**Effects of fixation on Tissues:**

1. Denaturates proteins to make them insoluble.
2. Consequently the tissue becomes resistant to the effects of the following steps.
3. The tissue will be more permeable to fluid (dead tissue).
4. The tissue will be more acidic or more basic, so staining will be strongly influenced.
5. Some fixatives inhibit or interfere with dye reaction or acts as mordant to enhance staining results

**Methods of fixation**:

Fixation of tissues can be accomplished by physical or chemical methods.

**Physical methods**:

1. Heating; such as boiling egg, frozen section, or warming a slide with water to affix section and partially fix it by heat.
2. Microwaving; it accelerate fixation of gross specimen and tissue sections in a range of 20 min up to 12 hours. But it is hazardous for health because of the vapor of formalin (a hood must be used).
3. Freeze-drying; It is useful technique in studying soluble materials and small molecules. Tissues are cut into thin sections, immersed in liquid nitrogen and the water is removed in a vacuum chamber at -40C.

**1b/ Pre-test**: what is fixative?

**Chemical methods**:

Chemical fixation utilizes organic and non-organic solutions to maintain adequate morphological preservation. These are:

1. **Coagulant fixatives**:

Both organic and non-organic fixatives may coagulate proteins making them insoluble. Coagulating lipoprotein and fibrous proteins such as glycogen will maintain tissue histomorphology at light microscope level. Such fixatives will cause poor preservation of mitochondria and secretory glands are not useful in ultra structural analysis.

The most commonly used coagulating fixatives are alcohol (e.g. ethanol, methanol) and acetone. Methanol is closer to the structure of water than ethanol, ethanol therefore competes more strongly than methanol in the interaction with hydrophobic areas of molecules; thus, at concentration of 50-60% ethanol fixation will begin.

1. **Non-coagulant cross-linking fixatives:**

Chemicals used as fixatives have potential action of forming cross-links within and between proteins and nucleic acids as well as between nucleic acids and proteins. Formaldehyde is one of these chemicals. Aldehyde (i.e. ) are chemically and biologically reactive and are responsible for many histochemical reactions.

1. **Cross-linking fixatives:**

Cell organelles such as cytoplasmic and nuclear membranes, mitochondria, membrane-bound secretory granules, and smooth & rough endoplasmic reticulum need to be preserved carefully for electron microscopy. In these structures fixative used must not solubilize lipids. The preferred fixatives are a strong cross-linking fixatives such as glutaraldehyde, a combination of glutaraldehyde & formaldehyde, or Carson's modified Millonig's followed by post-fixation in an agent that further stabilizes membranes such as osmium tetraoxide.

From these fixatives are mercuric-based fixatives which are toxic and should be handled with care. The chemistry of fixation using mercuric chloride is not understood well. It is known that mercuric chloride reacts with ammonium salts, amines amides, amino acids, and sulfhydryl groups; and hardens tissues. These fixatives penetrate slowly so specimen must be thin. They are no longer used routinely except by some laboratories for fixing hematopoietic tissues.

**Compound fixatives**:

Pathologists use formaldehyde-based fixatives to produce reproducible histomorphometric patterns. Other agents may be added to formaldehyde to produce specific effects not possible with formaldehyde alone. Ethanol can be added to formaldehyde to produce alcoholic formalin, which preserve molecules such glycogen, produce less shrinkage and hardening than pure dehydrants (ethanol).

Compound fixatives are useful for specific tissues:

1. Alcoholic formalin is good fixative for fatty tissue.
2. = = may aid indentifying lymph node embedded in fat.
3. Alcoholic formalin & some compound fixatives are good in preserving antigen immunorecognition.
4. Some compound fixatives such as glutaraldehyde-formaldehyde fixation may increase background staining, and alcoholic formalin may cause non-specific staining of myelinated nerves.

**Formaldehyde fixatives**:

**Neutral buffered 10% formalin (NBF)**

Tap water…………………………………………..900ml

Formalin (37% formaldehyde solution)…………100ml

Sodium phosphate, mono basic, monohydrate..4gm

= = , dibasic, anhydrous…………6.5gm

1. Used for routine histology.
2. It is used for initial fixation and for the first station on tissue processing.

**Formal (10% formalin) saline**

Tap water……………………….900ml

Formaldehyde (37%)………….100ml

Sodium chloride……………….9gm

**Formalin, buffered saline**

Tap water………………………900ml

Formaldehyde (37%)…………100ml

Sodium chloride……………….9gm

Sodium phosphate, dibasic….12gm

**Formal (10% formalin), calcium acetate**

Tap water…………………………900ml

Formaldehyde (37%)……………100ml

Calcium acetate…………………20gm

Good fixative for preservation of lipids.

**Formal (10% formalin), zinc un-buffered**

Tap water……………………………900ml

Formaldehyde (37%)………………100ml

Sodium chloride…………………….4.5gm

Zinc chloride or (zinc sulfate)….1.6gm or (3.6gm)

An excellent fixative, for immunohistochemistry studies.

**Mercuric fixatives**:

A problem with fixatives containing mercury, is that several types of pigments may combine with the mercury and precipitate as black pigments which could be removed by using iodine treatment followed by sodium thiosulfate

**Zinker's solution**

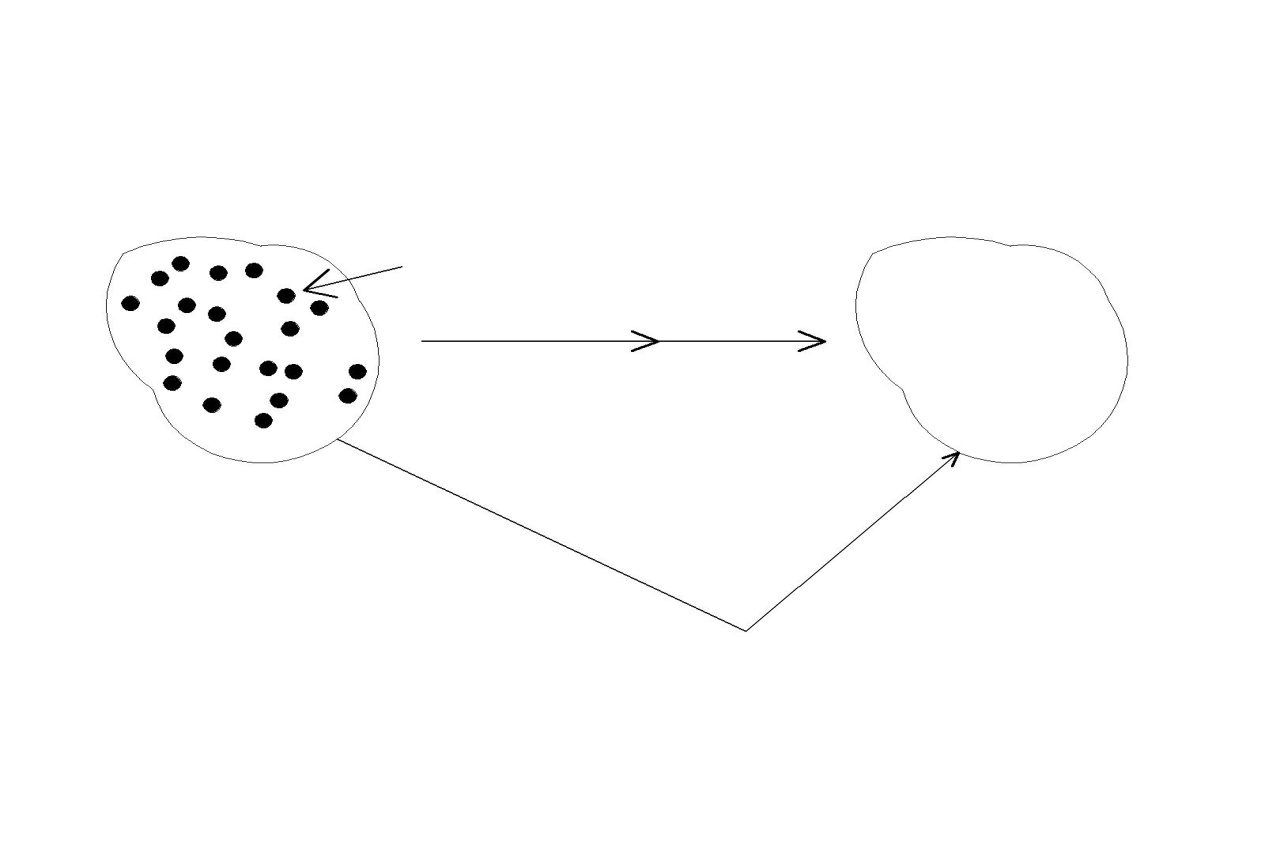
Distilled water……………………………..250ml

Mercuric chloride…………………………12.5gm

Potassium dichromate…………………..6.3gm

Sodium sulfate……………………………2.5gm

Just before use add 5ml of glacial acetic acid to 95ml of the above solution. It is good fixative for bloody (congested) specimens and trichrome stains.



**Using lugols iodine then 5% sodium thiosulphate**

Brown

Lakes

5% na-thio sulfate

Decolorization

**Tissue**

Stain + luguls Iodine

Decolorization

**HgI2 + CL2**

**Hgcl2 + I2**

**(Fixative) (Luguls iodine)**

**Helly's solution**

Distilled water…………………………..250ml

Potassium dichromate…………………6.3gm

Mercuric chloride……………………….12.5gm

Sodium sulfate………………………….2.5gm

Just before use add 5ml of 37% formaldehyde to 95ml of the above solution. It is excellent for bone marrow extra medullary hematopoiesis and intercalated discs.

**B5 fixative**

Stock solution:

Mercuric chloride……………………….12gm

Sodium acetate…………………………2.5gm

Distilled water…………………………..200ml

Add 2ml of formaldehyde (37%) to 20ml of the above solution just before use. It is used for bone marrow, lymph nodes, spleen and other hematopoietic tissue.

**Dichromate fixatives**:

1. It is good for making proteins insoluble without coagulation at pH of 3.5
2. It makes unsaturated lipids insoluble upon prolonged fixation (more than 48hr).
3. It preserves mitochondria well.
4. It is used in fixing endocrine tissues which will be stained, especially normal adrenal medulla and its tumors.
5. Time of fixation is 24hr.; washing is done with water then specimens transferred to 70%ethanol.

**Miller's solution**

Potassium dichromate………………………2.5gm

Sodium sulfate……………………………….1gm

Distilled water………………………………..100ml

**Moller's solution**

Potassium dichromate………………………3gm

Distilled water………………………………...80ml

At time of use add 20ml of 37% formaldehyde.

**Dehydrant fixatives**:

100%ethanol, 95%ethanol, 70%ethanol, these solutions may cause excessive shrinkage of tissue components after more than 3-4hr of fixation. These fixatives could be modified by adding some chemicals to produce specific effects.

Methanol is useful for smears especially blood smears.

Acetone fixation should be short (1hr) at 4C only on small specimens. It causes shrinkage and hardening; it is useful for immunuhistochemistry, enzyme studies, and in the detection of rabies.

**Carnoy's fixative**

Acetic acid…………………………….10ml

Absolute alcohol………………………60ml

Chloroform…………………………….30ml

Useful in:

1. Cytology to clear bloody specimen.
2. Staining of RNA
3. Preservation of glycogen.

**Clarke's solution**

Absolute ethanol……………………….60ml

Glacial acetic acid………………………20ml

1. Short fixation is recommended.
2. Produces good general histological results of HX & E staining.
3. Dissolve lipids.
4. After fixation specimen should be transferred to 95% ethanol.

**Dehydration cross-linkage fixatives:**

Compound fixatives with both dehydrant and cross-linking include alcohol-formalin mixture. These produce excellent results in the immunohistochemical identification of specific antigen, also alcohol-formalin fixation or post fixation is advantageous in large specimen fat (breast). Lymph nodes can be detected much more easily in specimens with alcohol-formalin fixation due to the extraction of lipids texture differences compared with tissue fixed in NBF.

For post-fixation, Carson (1990) recommends the following formula:

Absolute ethanol…………………………..650ml

Distilled water………………………………250ml

Formaldehyde………………………………100ml

Other formulas are used for better fixation, these are:

**Alcoholic formalin**

Ethanol (95%)……………………………895ml

Formaldehyde (37%)…………………...105ml

**Alcoholic-formalin-acetic acid fixative**

Ethanol (95%)……………………………85ml

Formaldehyde (37%)……………………10ml

Glacial acetic acid……………………….5ml

**Alcoholic Bouin's (Gendre's solution)**

95%ethanol saturated with picric acid

(5gm per 100ml)……………….800ml

Formaldehyde (37%)……………………150ml

Glacial acetic acid……………………….50ml

1. This solution is less aqueous than Bouin's fixative.
2. It is good for some carbohydrate retention (glycogen)
3. Fixation should be between 4hr to overnight.
4. Washing with 70%ethanol followed by 95%ethanol (several changes).
5. It is the only fixative that improves with aging.

**Fixation and decalcification**

**Bouin's decalcifying solution**

Saturated aqueous solution of picric acid

(10.5gm per 100ml)………………..500ml

Formaldehyde (37%)…………………………….167ml

Formic acid………………………………………..33ml

**Fixation for fatty acids**

Bouin's solution…………………………….75ml

95%ethanol…………………………………35ml

Fixation may be up to 48hr for good sections of lipomas or well differentiated liposarcoma.

**How to fix tissue:-**

1. Small blocks of tissue must be used (2 cm square) and not more than 4-5 mm thick.
2. Tissue specimen must be fixed immediately after removal, and if it is not possible they should be frozen to retard decomposition and autolysis.
3. Fixative volume should be 10-20 times volumes of tissues.
4. Fixation time depends on:
   1. Size and density of tissue
   2. Rate of penetration of fixative.
   3. Room temperature.
5. The tissue could be fixed in 2 or more fixatives for special studies.

**1/ Post test:**

1. Histopathology deals with a) tissue, b) disease, c) diseased tissue.
2. Mercuric fixatives causes a) black pigments, b) yellow pigments, c) no coloration.
3. Physical methods for fixation are a) 4, b) 3, c) 2.
4. Coagulant fixatives are a) 0rganic, b) non-organic, c) both kinds.

**Note:**

* Check your answers in key answer page (74).

**2/ pre-test**: washing fixed tissue is done by: a) water, b) alcohol, c) either one.

**Washing Tissues after Fixation**

It is the removal of fixative by using different solutions, and it depends on the type of the used fixative.

Tissues fixed in Helly’s or Zinker's fluids should be washed by running tap water (1 ½ -24 hrs) to get rid of the yellow potassium dichromate color.

Tissue fixed in fixatives containing formalin should be washed in running tap water for ½ hr. then transferred to 70% ethanol (formalin moves out of tissue faster).

If tissues fixed in Bouin’s fluid, it should be washed in several changes of 50% or 70% ethanol to remove the yellow color of the fixative; if some of the yellow color remains after sectioning and mounting, tissue sections must be left for a period of time in 70% ethanol (Hydration) during staining procedure.

While tissues fixed in Carnoy’s or alcoholic fluid, it should be transferred into absolute alcohol for dehydration.

**Principles of tissue processing**

Tissue processing is the removal of the extractable water from the tissue, replacing it with supporting medium that provides sufficient rigidity to have good sections. Stages of tissue processing: **1.** Dehydration; removal of water from tissue. **2.** Clearing; removal of dehydrating solution, making tissue components receptive to the infiltrating medium.**3.**Infiltration; permeating the tissue with a supporting medium.**4.** Embedding; Orientation of tissue samples in a supporting medium and allowing it to solidify.

**Dehydration**

It is the removal of unbounded water & aqueous fixatives from tissue. Many dehydrating agents are hydrophilic (water loving), possessing strong polar groups that interact with water molecules in the tissue.

Dehydration should be accomplished slowly through a graded series of reagents of increasing concentrations.

Excessive dehydration may cause the tissue to become hard, brittle, and shrunken. While incomplete dehydration will prohibit the penetration of the clearing agents into the tissue leaving the specimen soft and non-receptive to infiltration.

**Dehydration fluids:**

**Ethanol**

1. It is clear, colorless, inflammable liquid.
2. Miscible with water and other organic solvents.
3. Hydrophilic.
4. Fast acting.
5. It ensures total dehydration by graded concentrations and it may be used for electron microscopy.

**Industrial methylated spirit (denatured ethanol)**

1. It consists of ethanol with the addition of methanol (1%), isopropyl alcohol, or combination of alcohols.
2. It is used in the same manner as ethanol in dehydration.

**Methanol**

1. Clear, colorless, flammable fluid.
2. Highly toxic.
3. Miscible with water & organic solvents.
4. It can be used instead of ethanol.

**Isopropyl alcohol**

1. Miscible with water & organic solvents.
2. Does not cause over-hardening or shrinkage of the tissue.

**Butyl alcohol (butanol)**

1. It is used primarily for plant and animal histology
2. Slow dehydrant.
3. Cause les shrinkage and hardening of the tissue.

**Acetone**

1. Clear, colorless, flammable liquid.
2. Miscible with water, alcohol, and organic solvent.
3. Rapid in action, poor penetration. It is used in two ways, either 4 changes of fresh acetone 20min for each change, or 3 changes of fresh acetone 30min for each change.
4. Prolonged use causes brittleness in tissue.
5. It removes lipids from tissue during tissue processing.

**Dioxane (diethylene dioxide)**

1. It is an excellent reagent since it could be used as dehydrant and clearing agent.
2. It is readily miscible with water, alcohol, xylene and paraffin.
3. It produces less shrinkage than alcohol.
4. Tissue can be left in dioxane for long period of time without affecting tissue.
5. After dehydration tissue transferred to melted paraffin for infiltration.
6. It should be used in a well-ventilated room.
7. It is recommended for processing soft tissues due to their hardening properties.

**3/ Pre-test**: what is clearing?

**Clearing**

This term is related to the appearance of the tissues after they have been treated by special fluids chosen to remove the dehydrating agent; these fluids are called (clearing agents).

**Clearing agents:**

1. They act as an intermediary between the dehydration & infiltration solutions.
2. It should be miscible with the above solutions.
3. Most of these agents are hydrocarbons with refractive indecies similar to protein.

At the end of this process the tissue will have a translucent appearance.

Selection of a suitable clearing agent must be based on:

1. Speed of removal of alcohol.
2. Ease of removal by molten embedding medium.
3. Gentleness towards tissue.
4. Flammability.
5. Toxicity.
6. Cost.

The clearing agent effects both ease of sectioning and the final quality of the sections produced.

Most of clearing agents are flammable, so it must be handled with care. Fluids with a low boiling point are generally more readily replaced by the molten paraffin.

Prolonged treatment with clearing agents causes the tissue to become brittle.

Time for clearing tissue is proportional to the thickness of tissue.

Some of the clearing agents that used:

1. **Xylene:**
   1. The most commonly reagent used for clearing and it is recyclable.
   2. Colorless liquid with petroleum odor.
   3. Relatively rapid in its displacement of alcohol.
   4. Readily miscible with paraffin.
   5. Over exposure during processing will cause over hardening.
2. **Toluene**:
   1. It is similar to xylene.
   2. Does not harden tissue with prolonged treatment.
   3. A good clearing agent in which to leave tissue overnight.
3. **chloroform:**
   1. Slower in action than xylene and toluene.
   2. It does not make tissue brittle as xylene.
   3. Its vapor is dangerous since it affect the liver.
   4. It is used in processing specimens of the central nervous system.
4. **Benzene:**
   1. It is similar to xylene.
   2. It is not recommended for use due to its possible carcinogenic properties.
   3. It is very rapid in clearing.
5. **Cedar wood oil:**
   1. It is very penetrating.
   2. Cause no shrinkage of tissue.
   3. For clearing it must be of thin low viscosity quality.
   4. Tissue may be left in it indefinitely without harm.
   5. After clearing tissue must be immersed in xylene, followed by 3 changes of paraffin to ensure removal of oil.
6. **Citrus fruit oil-limonene reagents**:
   1. Limonene reagents are extracted from orange and lemon rids.
   2. Non-toxic.
   3. Miscible with water.
   4. Disadvantages are: 1.The strong pungent odor. 2. Copper or calcium may dissolve.

**Notice:**

We speak of the clearing in two procedures:

1. In the embedding process, we clear tissue after dehydration.
2. In the mounting procedure, we clear tissue after staining and dehydration.

**2/ Post test:**

1. Washing fixed tissue is done by a) water, b) alcohol, c) according to fixative.
2. Clearing agents are a) acid, b) hydrocarbons, c) alkali.
3. Dehydration is a) removal of unbounded water, b) removal of stain, c) removal of alcohol.
4. Xylol is a) dehydrant, b) fixative, c) clearing agent.

**Note:**

* Check your answer in key answer page (74).

**4/ Pre-test**: infiltration is: a) supporting tissue, b) coloring tissue.

**Infiltration**

After tissues have been thoroughly cleared with a clearing agent, it is necessary to infiltrate the tissue with a supporting medium that holds the cells and intercellular structure in proper relation to each other, so that they may be cut into thin sections.

Infiltrating media also used to support and enclose specimens which are to be subsequently cut into thin sections.

Paraffin wax continues to be the most popular infiltration and embedding medium in the histology laboratories.

Paraffin is solid at normal temperature. The hardness of the paraffin wax used for infiltration is matched to the hardness of the tissue. The hardness of the paraffin wax selected is most usually determined by the temperature in which the tissue blocks are to be sectioned.

Paraffin wax has a wide range of melting points, which is important for use in the different climatic regions of the world.

1. It is inexpensive.
2. Provides quality sections.
3. It can be used for most routine & special stains.
4. Easily adaptable to a variety of uses.

**Types of paraffin wax:-**

1. Soft paraffin wax (low melting point of about 450 - 500 C). It can be used only in cool temperature.
2. Medium (semi-solid) paraffin wax (melting point of 500 -580 C) which is adopted for routine work sometimes.
3. Hard paraffin wax (melting point of 580 - 620 C) this allow thinner sections to be obtained, provides better sectioning of hard objects.

The tissue is submerged in melted paraffin wax which replace the clearing agent in the tissue, two or three changes of molten paraffin wax with time not more than 3 hours. Prolonged immersion of tissue in molten paraffin wax causes hardness. Brain and spinal cord need longer time for infiltration due to their compact nature.

**Method of heating paraffin wax:**

There should be available a thermostatically controlled oven, set at 2 or 3 degrees above the melting point of the wax. Metal jugs or glass beakers containing paraffin wax are stored in this area. In recent years, paraffin wax supplied that there is little need for filter the wax before use. If wax clipping from embedded tissues is to be re-used it is recommended that this wax be filtered. A funnel with No. 1 filler paper may be permanently kept in the wax oven

**Embedding (Casting, Blocking)**

After the tissues have been dehydrated, cleared, and infiltered with the embedding medium, they are ready for external embedding. During this process the tissue samples are placed into molds along with liquid embedding material which is then hardened. This is achieved by cooling in case of paraffin wax and heating in case of epoxy resins.

Certain precautions should be taken when we embed tissue in paraffin wax:

1. The wax must contain no trace of clearing agent.
2. No dust particles must be present.
3. Immediately after tissue casting, the wax must be rapidly cooled to reduce the wax crystal size.

**Orientation of tissues:**

Specimen orientation during embedding is important for the demonstration of proper morphology. Most tissues are embedded flat; the margin of embedding medium around the tissue will assure support of the tissue.

Tissues require special orientation are:

1. Tubular structures: arteries, veins, fallopian tubes, and vas deferens; cut in cross-section of the lumen.
2. Skin, intestine, gallbladder, and other epithelial biopsies; cut in a plane at right angles to the surface.
3. Muscle biopsies are cut in both transverse and longitudinal sections.
4. Multiple pieces of tissue oriented side by side with epithelial surface facing in the same direction.

There are instances where different medium is required instead of paraffin wax if:

1. The impregnation medium is not sufficiently hard to support the tissue.
2. The tissue may be affected by heat.
3. The use of dehydrant and clearing agents may destroy or distort the tissue or tissue components.
4. The adhesion between the paraffin wax and the tissue is inadequate so the tissue will break away from the wax during sectioning.
5. Large crystals in the paraffin wax.
6. The sections cannot be cut thin enough.

Alternative embedding media are:

1. Water soluble: Easter wax, polyester and microcrystalline wax.
2. Alternative resins: acrylic , Epoxy and urea-formaldehyde
3. Other media: agar, Gelatin and Celloidin.

**Trimming**

It is the process of removing the excess of paraffin wax around the embedded tissue, to get a block with regular geometrical figure that can be sectioned to have straight paraffin stripe with good tissue sections which will ease mounting and staining.

**3/ Post test:**

1. The suitable embedding medium for most tissues is a) bees wax, b) paraffin wax, c) celloidin.
2. Trimming is done to get a) single sections, b) curved ribbon, c) straight ribbon.
3. For most tissues the recommended time for infiltrationis a)3hours, b) 4hours, c) 2hours.
4. Easter wax medium is a) resin, b) water soluble, c) alcohol soluble.

**Note**

* Check your answers in key page answer (74).

**5/ Pre-test:** microtome is a) cooler, b) heater.

**Microtomy**

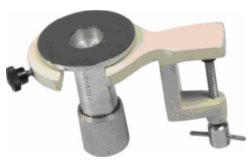
Microtomy is the means by which tissue can be sectioned and attached to a surface for further microscopic examination. The basic instrument used in microtomy, is the microtome. Most microtomy is performed on paraffin-embedded tissue blocks.

The microtome is nothing more than a knife with a mechanism for advancing a paraffin block standard distances across it.

Vertical sectioning perpendicular to the surface of the tissue is the usual method. Horizontal sectioning is often done in the evaluation of hair follicles and pilosebaceous units.

**Types of microtome:-**

1. **Hand microtome**
2. May be used successfully for botanical sections.
3. Its use for animal tissues is limited.



1. **Rocking microtome**
2. It is used for soft tissue**.**
3. The retracting action moves the tissue away from the knife on the upstroke, producing a flat face to the tissue block.

** **

1. **Rotary microtome:**
2. It is easy adaptable for all types of tissue which are embedded in paraffin wax (hard, fragile, or fatty).
3. Its ability to cut thin (2-3µ) sections..

1. **Base slede microtome**
2. The specimen is held stationary and the knife slides across the top of the specimen during sectioning.
3. It is used for large blocks.
4. = = = = hard tissues.
5. = = = = whole mounts.
6. It is ideal for the production of celloidin-embedded sections.
7. Especially useful in neuropathology and ophthalmic pathology.
8. Also used in sectioning of un-decalcified bones.

[](http://en.wikipedia.org/wiki/File:Sledge_microtome.jpg)

1. **Sliding Microtome**
   1. The knife or blade stationary and the specimen slides under it.
   2. It is used with celloidin-embedded tissue blocks. in the



**Mounting & Adhesives**

Once sections are cut, they are floated on a warm water bath that helps remove wrinkles. Then they are picked up on a glass microscopic slide, which is covered by a thin film of adhesive that provides good adhering of the sections.

**Types of adhesives:-**

1. **Albumin:**
2. Mostly used in histopathological labs.
3. Used for more adhering of sections.
4. Source of albumin is egg, bovine, or human albumin.
5. Preservative should be used to prevent putrefaction.
6. Glycerol is added to albumin to increase viscosity & prevent complete drying.
7. **Gelatin:**
8. Gelatin adhesives provide affirmer attachment of sections than albumin.
9. 0.5% in distilled water with preservative is satisfactory before use it should be heated gently to melt the gelatin and then used.
10. **Starch:**
11. Starch adhesives provide greater adhesion than gelatin.
12. Its disadvantage is of interfering with many dyes.
13. Since it is carbohydrate, its use isn't recommended when detecting for these substances.
14. **Cellulose:**
15. 1% methyl cellulose solution found to be a good adhesive.
16. It does not interfere with commonly used dyes.
17. **Sodium silicate:**
18. Used by diluting the commercial syrup up to 10 (1: 100)
19. Good adhesive.
20. Tend to stain with most dyes.
21. **Resins:**
22. A greater adhesion is made by using epoxy resins (araldite).
23. This adhesive, diluted 1in 10 with acetone, it should be painted onto clean slide immediately before use.

**4/ Post test:**

* 1. Rotary microtome cuts blocks of a) paraffin wax, b) celloidin, c) resin.
  2. In mounting we use a) oven, b) water bath, c) microscope.
  3. Rocking microtome is used to cut a) fatty tissue, b) hard tissue, c) soft tissue.
  4. The mostly used adhesive is a) albumin, b) gelatin, c) silica.

**Note**

* Check your answers in key page answer (74).

**6/ Pre-test**: what is staining?

**Staining**

Staining is accomplished by soaking tissue in a solution of one dye or more. Staining is employed to give both contrast to the tissue as well as highlighting particular features of interest.

**Staining Reactions, methods & types**

**A) Staining reactions:**

1. **Direct staining (absorption):**

Stain diffuses into tissue without causing any change, it is simple diffusion (ex: Eosin).

|  |  |
| --- | --- |
| **Stain** | **Tissue** |

**Direct Stain**

**(Eosin Stain)**

1. **Indirect staining:**

Stain needs mordant to colour the tissue, by making a linkage between the dye and the tissue (ex: Haematoxylin, Bests carmine).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Stain** |  | **mordant** |  | **Tissue** |

**Indirect Stain**

**(Haematoxylin Stain)**

1. **Physical staining:-**

It is simple solubility of the dye into cell components as in the staining of fatty tissue (ex: Sudan 3), fatty substance will stain orange.

1. **Chemical staining:**-

A new substance is formed as a result of dye- tissue interaction which is usually irreversible (ex: P.A.S reaction).

1. **Adsorption phenomenon:**

It is an ionic attraction in which the staining is influenced by affinity of acid to base or vice versa. These ions accumulate on the surface of one of cell components.

**B) Methods of staining:-**

1. **Vital staining:-**

It is the injecting of the dye into the living body. This is done for research.

1. **Routine staining:-**

Routine dyes that stain all tissue components with minute differences except nucleus &cytoplasm (ex: Eosin stains cytoplasm with pink colour, Haematoxylin stains nucleus with blue -violet colour).

1. **Special staining:-**

In which dyes stain some tissues, bacteria, fungi, some cell secretion, and components inside and outside the cell.

**C) Types of staining:-**

1. **Regressive staining:**

In this type of staining all the tissue will be stained and then the extra stain removed by using certain solution called: differentiator as 1% acid alcohol used with haematoxylin.

1. **Progressive staining:-**

The stain couldn't be removed from the tissue after staining

as in using eosin.

.**Nature of stains:**

Dyes are divided according to its nature into:

1. Natural stains : These are of natural origin such as

Haematoxylin (plant origin), Carmine (animal origin), and

Orcine (animal origin).

1. Synthetic stains : These are manufactured in factories, such as:

Eosin, Thionine, Light-green, Neutral red, Orange green.

**Staining Terminology**

**1- Stock Solution:-**

This term has two meanings:-

1. The solutions that used in bulk every day. (formalin, alcohol, etc).
2. The solutions that prepared in high concentrations and then diluted on working (on the day of use).

**2- Working solution:-**

The solutions that were prepared from the stock solution on the day of using.

**3- Decolourization:-**

It is a partial or complete removal of stain from the stained sections.

**4- Bleaching:-**

Is a complete removal of stain or any colour from tissue.

**5- Mordant:-**

Is an intermediate agent used to link the regressive dye strongly to the tissue (ex: alums, Fecl3).

**6- Counter stain:-**

Is one dye or more that added to the main stain to bring out the differences between tissues or cell components.

(Ex: eosin HX. Van Gieson Verhoeff's stain)

**7-Acidophilic stain:-**

The dye that stains the acidic components of the cell and it is basic in nature (ex: HX stain).

**8- Basophilic stain:-**

The dye that stains the basic components of the cell, it is acidic in nature (ex: eosin).

**9- Metachromatic stain:-**

A stain that gives a colour different from its original colour

After Stain

(Ex: Methylene blue red colour

After Stain

Toluidine blue red colour

**10-Polychromatic stain:-**

It is a compound of dyes that gives different colours for tissue (ex: acid dyes, fuchsine, basic dyes, alcians, and Masson's trichrome).

**11-Dewaxing:-**

Is the complete removal of paraffin wax from tissue sections by using a clearing agent (xylol, toluene, benzene).

**12-Differentiation:-**

Is a partial removal of the dye from the tissue by using (ex: 1% acid alc. for HX stain; fecl3 for Verhoeff's stain).

**5/ Post Test:**

* 1. Basophilic stain is a) HX, b) eosin, c) methyl blue.
  2. Staining is to a) color, b) clear, c) dehydrate cell component.
  3. HX is a) direct stain, b) indirect stain, c) metachromatic stain.
  4. Dewaxing is the removal of a) water, b) paraffin wax, c) alcohol.

**Note:**

* Check your answer in key answer page (74).

**7/ Pre-test:** what is the difference between natural and chemical?

**Hematoxylin**

The hematoxylin is derived from the old Greek words ***Haimato*** (blood) and ***Xylon*** (wood), referring to its dark red color in the natural state, and to its method of manufacture from wood.

Hematoxylin is a natural dye extracted by boiling the wood of the South American and West Indian logwood tree (***Haematoxylon campichianum***), and partly purified by crystallization. It is sold commercially as crude mixture of hematoxylin and other, unidentified substances. It comes as a brownish tan powder which is poorly soluble in water and somewhat more soluble in ethyl alcohol ( 1gm/100mo water and 30-40gm/100ml alcohol). The active dye is not hematoxylin itself, but its oxidized product, hematin. The later can be bought, but is expensive, and the same results can be had simply by oxidizing the crude hematoxylin.

Oxidation is of two types:

**1) Natural oxidation:**

In the early days oxidation was carried out by making up the hematoxylin solution, plugging the container with cotton, and leaving it exposed to light and air for 6weeks to several months.

**2) Chemical oxidation:**

It was soon found that oxidation (also called ripening) could be achieved much faster by adding a wide variety of oxidizing agents, such as, alum, iodate, and mercuric oxide. With either agent, oxidation not only begins immediately, but apparently continues for some time, causing dye precipitates of uncertain composition, and eventually exhausting the stain.

The addition of glycerin to several formulas is said to guard against over-oxidation and perhaps to retard fungal growth.

Its lifetime is shorter than the naturally oxidized hematoxylin.

**Hematoxylin lakes**

Used alone, hematoxylin is a poor stain, but in combination with various metallic salts (mordants) which link it to the tissue, it is one of the best nuclear stains known. It has also been used to detect metals in tissue, to stain mitotic figures, fibrin, muscle cross striations and other tissue elements.

The combination of hematoxylin plus mordant is called a hematoxylin lake, and lakes with different metals have different colors. The aluminum lake formed with ammonium alum ( aluminum ammonium sulfate) is particularly useful foe staining nuclei. It is purple in acid solution, but blue in alkaline solution. Instead of aluminum ammonium sulfate, either aluminum potassium sulfate (potassium alum) or aluminum sodium sulfate (sodium alum) may be used to create aluminum lake. No appreciable difference in results is found. Hematoxylin recipes using any of these mordants are called "***alum hematoxylin***", or ***"hemalums".***

Other hematoxylin lakes are used for different staining purposes, and have a variety of colors, depending on the metal used.

The staining of nuclei by alum hematoxylin is enhanced by the addition of acetic acid, which apparently reacts with the nuclear chromatin, giving it a somewhat crisper appearance. However, because of the alum content, the pH of alum hematoxylins tend to be around 2.6-2.9 even before the acid is added.

Most mordants are incorporated into the hematoxylin staining solution; but in Heidenhain's iron hematoxylin the tissue sections soaked in the mordant before staining.

Hematoxylines could be classified according to which mordant is used as follows:

**1-** Alum hematoxylin

**2-** Iron hematoxylin

**3-** Tungsten hematoxylin

**4-** Molybdenum hematoxylin

**5-** Lead hematoxylin

**6-** Hematoxylin without mordant.

**1- Alum hematoxylin;**

This group is mostly used in the hematoxylin eosin staining and produce good nuclear stain, the mordant is aluminum in the form of:

**A)** Potash alum (aluminum potassium sulfate)

**B)** Ammonium alum (aluminum ammonium sulfate)

All alum hematoxylins, whatever their formula may be used as either ***progressive*** or ***regressive*** stains.

***Progressive*** staining means that the tissue is left in the stain just long enough to reach the proper end point. Therefore it may be necessary to examine the slides at several different intervals to determine when staining is dark enough but not too dark.

***Regressive*** staining means that the tissue is deliberately over-stained and then de-stained (differentiated) until the proper end point is reached.

The difference between methods is largely one of convenience. Progressive hematoxylins are generally less used, and work slowly to avoid overshooting the end point.

Regressive hematoxylins are more used and many can achieve over-staining in a matter of less than a minute, while differentiation (removal of excess stain) requires only a few seconds. Also, timing is not so important in regressive procedures. As long as the slide is over-stained, it doesn't matter whether it was in the staining dish for 1min or 10.

Regressive procedures are therefore faster and more convenient than the progressive ones, and they have the added advantage that differentiation also removes hematoxylin from the gelatin or other slide adhesive, producing a clear, transparent background.

***Differentiation*** is done in dilute acid (usually acid alcohol because of hematoxylin's greater solubility in alcohol). Differentiation is stopped immediately by simply washing the slides in water.

If too much hematoxylin has been removed, a few more seconds in the stain will correct the problem, and the whole process can be repeated.

If too little has been removed, a few more dips in acid alcohol will produce the correct endpoint.

**Note**: With all hematoxylins, progressive or regressive, the end point is the same.

***Blueing*,** because most alum hematoxylin formulae are fairly acid, the nuclei at first be stained the purplish color of the acid dye. Changing their color to blue gives a much better contrast with the usual red counter stains.

When the endpoint has been reached by either progressive or regressive methods, nuclear color can be changed in one of two ways:

**1.**The slides may be dipped for a few seconds into a weakly alkaline solution such as ammonia water or dilute sodium carbonate. Note that differentiation stopped the second you rinse the slides in water, so the alkaline solution is ***not*** necessary for that.

**2.**They may be washed for 2-5min in tap water. Tap water tends to be slightly acidic, but it is more alkaline than most alum hematoxylins, so bluing results. Tap water has another advantage that is washes out any excess alum, giving a crisper blue-black color to nucleus.

The mostly used hematoxylins are:

**A)** Ehrlich's HX.

**B)** Mayer's HX.

**C)** Harris's HX.

**D)** Cole s HX.

**E)** Delafield's HX.

**F)** Carazzi's HX is used for urgent frozen sections.

**G)**Gill' HX

**A) Ehrlich's HX (1886):**

**a)** An excellent nuclear stain.

**b)** It stains mucins including polysaccharide of cartilage.

**c)** It is recommended for the staining of bone and cartilage.

**d)** It is good for staining tissues which have been stored for a long time in formalin.

**e)** It is useful to stain tissues which were exposed to acid decalcification.

**f)** It is not ideal for frozen sections.

**g)** It ripens naturally for about 2 months, and this period of time could be shortened by placing the stain in un-stoppered bottle, in warm sunny place (window-ledge).

**h)** It lasts for years (in bulk).

**i**) It retains its staining ability in a Coplin jar for some months.

**B) Mayer's HX (1903):**

**a)** This stain chemically ripened with sodium iodate.

**b)** It is useful as a progressive stain when cytoplasmic components are stained with special stain that it may be destroyed or de-colored by differentiation.

**c)** It is used as a counter stain in the demonstration of glycogen and in various enzyme histochemical techniques.

**d)** The time for staining is (5-10 min) until the nuclei are stained, then blued without differentiation.

**C) Harris's HX:**

* 1. It is chemically ripened with mercuric oxide.
  2. Since mercuric oxide is highly toxic, it is replaced by sodium or potassium iodate.
  3. Because it gives clear nuclear staining.
  4. It has been used as a progressive stain in diagnostic exfoliative cytology with an eosin counter stain.
  5. When it is used as a progressive stain, an acetic acid-alcohol rinse provides a more controllable method, (5-10% solution of acetic acid in 70-95% alcohol). This solution detaches dye molecules from the cyt/nucleo-plasm while keeping nucleic acid complexes intact.
  6. Regressively, acid alcohol acts quickly, and it is difficult to be controlled, it can result in a light nuclear stain.
  7. It must be prepared every month.

**D) Cole's HX:**

a) It is artificially ripened with an alcoholic iodine solution.

b) Like Harris's HX, it must be prepared every month.

**E) Delafield's HX:**

**a)** It is naturally ripened.

**b)** It is allowed to stand exposed to light & air for (3-4) months, until the stain is sufficiently dark in color.

**c)** It must be filtered and stored.

**F) Carazzi's HX:**

**a)** It is chemically ripened using potassium iodate.

**b)** It remains stable for about (6 months).

**c)** It could be used as a progressive stain with short time of

staining.

**d)** It is very good for frozen sections when used as a double-

strength solution with a very short staining time.

**G) Gill's HX:**

**a)** Chemically ripened.

**b)** It is more frequently used for routine H&E staining than Mayer's HX.

**c)** It is stable more than Harris's HX.

**d)** Its disadvantage is that it stains gelatin adhesive & even the glass itself.

**Staining time with alum HX depends on:**

**1.** Type of hematoxylin (Ehrlich's HX, 20-45min, Mayer's HX, 10-20min).

**2.** Age of stain. As stain ages, it needs more time for staining.

**3.** Intensity of use of stain. A heavily use of the stain will weaken the power of the stain.

**4.** Using stain regressively or progressively.

**5.** Length of time of fixation, acid decalcifying solution or frozen sections (pre-treatment of the tissue).

**6.** Post-treatment of the sections (subsequent acid stain).

**7.** Personal choice.

**Disadvantages of alum hematoxylins**:

These stains are sensitive to any subsequently applied acid staining solutions (Van-Gieson stain). In this case we can use iron mordant HX, which resists the effect of acidity.

1. **Iron hematoxylins:**

In these hematoxylin solutions, iron salts are used both as the oxidizing and as mordant, these salts are:

**a)** Ferric chloride

**b)** Ferric ammonium sulfate

These iron salts are strong oxidizing agents so it can be used as a differentiating fluid after hematoxylin staining.

The most commonly used iron HX are:

* 1. Weigert's HX.
  2. Heidenhain's HX
  3. Loyez's hematoxylin for myelin
  4. Verhoeff's hematoxylin for elastic fibers

Iron hematoxylins are used in two ways:

1. Prepared separately and then mixed with the mordant and the oxidant immediately before use (as in Weigert's HX).

**OR**

1. Preparing the iron salt solution separately and use before staining with HX (Heidenhain's & Loyez's hematoxylins) .

The iron hematoxylins are capable of demonstrating a much wider range of tissue structures than the alum hematoxylins.

It takes longer staining time than the alum ones.

**Weigert's HX:**

1. Naturally ripened for 4weeks.
2. Just before use, ferric chloride is added to the stain. Iron solution acts as an oxidant and mordant.
3. Time for staining is 15-30min.

**Heidenhain's HX:**

1. Naturally ripened for 4weeks.
2. Ferric ammonium sulfate is used as an oxidant/mordant.
3. The above solution is used as the differentiating fluid.
4. Sections first treated with the iron solution, then stained with HX until it over-stained.
5. Differentiation is done by using the iron solution and controlled under microscope.
6. It can be used to demonstrate many structures according to the degree of differentiation.
7. Time for staining is 1 hour.

**Eosin:**

Is a fluorescent red dye resulting from the action of bromine on fluorescin, It can be used to stain cytoplasm, collagen, and muscle fibers which are called eosinophilic. Eosin is used to differentiate between cytoplasm and different types of connective tissue fibers and matrices by staining the differing shades of red and pink.

Eosin is most often used as a counter stain to HX in H&E staining. This dye could be obtained in these types:

* + - 1. Eosin Y (eosin yellowish, water or alcohol soluble).
      2. Ethyl eosin (eosin S, alcohol soluble).
      3. Eosin B (eosin bluish, erythrosine B).

For staining eosin Y is typically used in concentration of 1-5% w/v, dissolved in water or ethanol. A small concentration (0.5% of acetic acid) usually gives a deeper red color to the tissue. For preventing mold growth in aqueous solution, thymol is sometimes added.

**6/Post test:**

1. HX lake is a) HX + water, b) HX + mordant, c) HX + alcohol.
2. Alum HX contains a) lead, b) iron, c) ammonium alum sulfate.
3. Natural oxidation is a) air + light, b) iron alum, c) mercuric oxide.
4. Ethyl eosin is a) water soluble, b) alcohol soluble, c) acid soluble.
5. Mayer's HX is ripened naturally, b) by KI, c) by NaI.

**Note:**

* Check your answer in key answer page (74).

**Bone tissue**

Some tissues contain calcium deposits which are extremely firm, and will not sectioned properly with paraffin embedding, because of the difference in densities between calcium and paraffin. The bulk of mineral of bone is a crystalline substance" hydroxyapatite", formed mainly of Calcium phosphate and hydroxyl ions (Carbonate, Citrate & fluoride ions, and other minerals such as magnesium, potassium, and strontium).

**Bone specimen processing:**

1. **Biopsy:** is taken by using a good fine saw (jewellery saw). Before sawing bone into slabs, it is advisable to remove connective tissue and tendon because they drag in the blade. Specimen size is 3-5 mm for hard bones & 4-6mm for soft bones.
2. **Fixation:** must be done immediately for (24-48 hours) and during this time specimen should be cleaned. The common & suitable fixative used for most biopsies is 10% neutral or buffered formalin, because it penetrates well and renders the soft tissue resistant to the acids present in the decalcification fluid.
3. **Washing**: is done thoroughly by running tap water to remove the excess fixative.
4. **Decalcification**: In order to obtain satisfactory paraffin or celloidin sections of bone it is necessary to remove the minerals & thus soften the tissue. This is carried out by treatment with reagents which react with calcium. These reagents are:
5. Acids to form calcium salts.
6. Chelating agents to pick up calcium ions.

**Decalcifying agents**

1. **Acid decalcifiers**: there are two groups:
2. **Strong acids:** nitric and hydrochloric acid in (5-10%) aqueous solutions. They decalcify rapidly so fixation time should be not more than (24-48 hours) because of their serious deterioration of stain ability. These acids used only for urgent biopsy specimen with little mineralization.

**Aqueous nitric acid:**

Nitric acid 5 – 10 ml

Distilled water to 100 ml

* **Formalin – Nitric acid:**

Formaldehyde (37-40%) 10 ml

Nitric acid 10 ml

Distilled water 80ml

Decalcification with nitric acid takes short time (1-4days) with changing solution every day or twice a day, but tissue stain ability is less than formic acid which takes long time for decalcification.

1. **Weak acids:**

These are formic, picric and acetic acid is the only weak acid used for decalcification .It s concentration is (5-10%) aqueous solution or with additive, such as formalin or buffer.

* **Aqueous formic acid:**

90% formic acid 5-10 ml

Distilled water to 100 ml

* **Formic acid formalin:**

90% formic acid 5-10ml

Formalin 5 ml

Distilled water to 100 ml

* **Buffered formic acid:**

90% formic acid 35 ml

20% aqueous sodium citrate 65 ml

Washing after acid decalcification is necessary by using running tap water for 3-8hr to remove the last trace of the decalcification.

**2- Chelating agents:**

The mostly used for decalcification is ethylene diamine tetra acetic acid (EDTA), it is nominally acid but it binds metallic ions notably calcium & magnesium ions. It binds faster in pH 7-7.4; EDTA binds to ionized calcium on the outside of the apatite crystal and this layer becomes depleted more calcium ions reform from within; the crystal becomes progressively smaller during decalcification. These processes are slower than acid decalcification and cause little or no effect on tissue elements; it does not affect tissue stain ability. Time required to complete decalcification of dense cortical bone may be as long as (6-8 week).

**Hillman and Lee (1953 )**

EDTA disodium salt 5.5 gm

Distilled water 90 ml

Formalin 10 ml

**Neutral EDTA**

EDTA disodium salt 250 gm

Distilled water 1750 ml

If solution is cloudy, adjust to pH 7 with about 25gm of sodium hydroxide.

**For better decalcification:**

1. Volume of decalcifying solution should be (50-70 times) the specimen size.
2. Bone specimen should be near surface of the decalcifying fluid to ensure settling of salts.
3. Bone specimen should not be left longer than the specified time because it will swell & unstainable.

**Testing the end of decalcification**:

1. **Physical test:**

This test could be done either by inserting a needle into the bone piece. If the needle inters the bone easily, then the bone is ready to be processed; this will injure the tissue.

**OR:** If the bone bends easily & flexible, then it is decalcified.

1. **Chemical test:**

It is used to detect of calcium in the decalcifying fluid by using:

1. Concentrated ammonia
2. Saturated aqueous ammonium oxalate

**Method**:

1. To (5ml) of decalcifying fluid add a small piece of litmus paper.
2. Add concentrated ammonia drop by drop with shaking after each drop until the solution becomes neutral to litmus paper.
3. Add (5ml) of solution (2), shake well and allow to set for 30minutes.

**Result:**

1. If precipitate forms after the addition of ammonia, it means that considerable amount of Calcium is present; so it is unnecessary to proceed further with the test, and the decalcifying fluid should be changed.
2. If precipitation occurs after the addition of ammonium oxalate, it means that less calcium is present, and the decalcifying fluid should be changed.
3. If the solution remains clear after (30min), it means that decalcification is complete.

**Special stain for Bone Tissue**

**(Schmoral's-thionin Stain)**

**Fixative:**

Any fixative other than those containing mercuric chloride is preferred.

**Solutions:**

1. **Stock solution:** 0.25 % aqueous thionine.
2. **Working solution:**  Filter the stock solution and dilute with an equal volume of distilled water. Add ammonia (1or 2drops) immediately before use.

1. Saturated aqueous picric acid.

**Procedure:**

* 1. Dewaxing xylol & heat 10 min
  2. Hydration ethanol Distilled water

100% 90% 70% D.W 2min 1.5min 1.5min 2min

* 1. Staining Thionine 5-20 min
  2. Rinsing Distilled water
  3. Treat with Picric acid 30-60 sec
  4. Rinsing Distilled water
  5. Differentiation 70% ethanol 5-10min or more

Until the bluish –green clouds cease to form

* 1. Dehydration ethanol, 90% (1.5min) 100(1.5min)
  2. Blotting with filter paper
  3. Clearing xylol 10 min
  4. Mounting Canada balsam

**Result:**

Lacunae & canaliculi dark brown –black

Bone matrix red or brownish – yellow

Cells



**7/ post test:**

1. Decalcification is done by a) water, b) formalin, c) acid.
2. Schmoral's stain is used for a) bone, b) elastic fibers, c) glycogen.
3. Physical test for bone decalcification is done by a) alkaline, b) acid, c) bending the bone.
4. Chelation is to a) capture ions, b) release ions, c) keep ions in place.
5. Natural EDTA is weak acid, b) chelating agent, c) strong acid.

Note:

* Check your answer in key answer page (74).

**8/ Pre-test:** how could you cut frozen tissue?

**Frozen sections**

Frozen section is a rapid way to fix and mount histology sections, which helps in diagnosis of a pathological process (during surgery). It is applied in routine histology especially that of central nervous system and histochemistry. Frozen sections; it can be cut from fixed or unfixed tissue.

The principle of cutting frozen sections is by freezing tissue embedded in a freezing medium & cut on a microtome in a cooled machine called cryostat (refrigerated box containing a microtome). The temperature is around (-20 to -30 C).

**Fixation:-**

* 1. 10% formal calcium at 4c is suitable for most enzyme histochemistry & lipids.
  2. Formal ammonium-bromide is used for neuropathology.
  3. Fixative containing mercury, dichromate & alcohol should be avoided, as they tend to make tissue brittle & difficult to cut.

**Impregnation (embedding) medium:**

Some tissues tend to fragment during sectioning so, to avoid this problem gelatine is used as a supporting medium, permitting easier sectioning and handling.

**Gelatine impregnation:**

**Gelatine-glycerine solution:**

Gelatine 16gm

Glycerine 15ml

Distilled water 70ml

Thymol 4 crystal

Store at 4c and warm till liquid before use.

**Method:-**

1. Fix tissue in 10% formal calcium.
2. Wash tissue in running tap water over night.
3. Transfer tissue to the above solution at 370C for 6 hours.
4. Transfer tissue to fresh gelatine- glycerine solution and let embeds in it, allow cooling in a refrigerator.
5. Trim away the excess of gelatine from the block.
6. Harden the block by placing in 10% formal calcium over night.
7. The block stored in the fixative.

**Sectioning:-**

1. The tissue block is fixed on to the microtome stage.
2. Add a drop of water.
3. Blast carbon dioxide, the tissue will be frozen.
4. Sections will be cut when the block is hardened to its optimal temperature.
5. Single sections obtained & collected by finger or wet camel hair brush.
6. Staining could be done by floating sections on surface of staining solutions, or by floating on a slide and drying it by gentle flame to affix the section, then completing the staining procedure.

**Staining:-**

**A) Rapid HE stain:-**

* + - 1. Fix tissue 10% neutral buffered formalin, at room temperature for 20 sec.
      2. Rinse in tap water.
      3. Stain in double strength Carazzi's HX for 1min.
      4. Wash well in tap water for 10-20seconds.
      5. Stain in 1% aqueous eosin for 10 sec.
      6. Rinse in tap water.
      7. Dehydrate, clear, and mount.

**B)** For fat and lipoid material, oil red-o, Sudan III, Sudan black B & fat soluble stains.

**8/ Post test:**

1. Cryostat is used to cut a) paraffin blocks, b) resin, c) frozen specimen.
2. For lipoid materials we use a) Sudan III, b) Alcian blue, c) congo red.
3. Impregnation media for frozen tissue contains a) silica, b) gelatine, c) albumin.
4. Fixative used for lipids is a) Zinker's fluid, b) Bouin's fluid, c) 10% formal calcium.

**Note:**

* Check your answer in key answer page (74).

**9/ Pre-test**: a) what is exfoliation?

b)What is cytology?

**Exfoliative Cytology Techniques**

In the normal life cycle, during growth the cells of the body are continually being renewed by cell division, and the newly formed layer (bottom layer) pushes up the surface layer into the surrounding body fluid , samples are taken from the patient, examined carefully under the microscope.

The size, shape, and staining quality of the cell or any minute abnormality will differentiate normal from diseased cells.

This examination and study is called Exfoliative cytology, which is of important value in the early detection of cancer.

The exfoliated cells into body fluid and secretions are processed in three main techniques which are:-

1. cell blocks
2. Smear preparations
3. Membrane filter methods

If the specimen quantity is sufficient two of the above techniques are used, if it is scant the material is processed as a Papanicolauo smear or method no.3.If the cytological preparations suggest cancer, they do additional test including (biopsy).

Many medical conditions, including all cases of cancer, must be diagnosed by removing a sample of tissue from the patient and sending it to a pathologist for examination.

**Types of biopsies:**

1. **Excisional biopsy**

A whole organ or a whole lump is removed (excised). Enlarged lymph nodes are good example for this kind of biopsies, breast lumps, spleen, and ovary.

1. **Incisional biopsy**

Only a portion of the lump is removed surgically. This type of biopsies is most commonly used for tumors of the soft tissues (muscles, fat, and connective tissue) to distinguish benign conditions from malignant soft tissue tumors (sarcoma).

1. **Endoscopic biopsy**

This is the most commonly performed type of biopsies. It is done through a fiberoptic endoscope the doctor insert into the organ to be checked, it includes these types:

* 1. Alimentary tract endoscopy (for gastrointestinal tract).
  2. Cystoscopy (for urinary bladder).
  3. Laparoscopy (for abdominal cavity).
  4. Arthroscopy (for joint cavity).
  5. Mediastinoscopy (for mid-portion of the chest).
  6. Laryngoscopy and bronchoscopy (for trachea and bronchial system).

The insertion is either through a natural body orifice or a small surgical incision. The endoscopist can directly visualize an abnormal area on the lining of the organ in question and pinch off tiny bits of tissue with forceps attached to a long cable that runs inside the endoscope.

**Fine needle aspiration (FNA biopsy)**

This is an extremely simple technique that has been used for a long time. A needle no wider than that typically used to give routine injection is inserted into a lump (tumor), and a few tens or thousands of cells are drawn up (aspirated) into a syringe. These are smeared on a slide, stained and examined under a microscope by the pathologist. A diagnosis can often be rendered in a few minutes. This technique is done for tumors of deep, hard-to-get-to structures (pancrease, lung, liver, breast, and thyroid).

**Punch biopsy**

This technique is typically used by dermatologist to sample skin rashes and small masses. After a local anesthetic is injected a biopsy punch, which basically a small (3 to 4 mm in diameter) version of cookie cutter, is used to cut out a cylindrical piece of skin.

**Bone marrow biopsy**

In cases of abnormal blood counts, such as unexplained anaemia, high white cell count, and low platelet count, it is necessary to examine the cells of the bone marrow. In adults, the sample is usually taken from the pelvic bone, typically from the posterior superior iliac spine. With the patient lying on his/her stomach, the skin over the biopsy site is deadened with a local anesthetic. The needle is then inserted deeper to deaden the surface membrane covering the bone (the periosteum). A larger rigid needle with a very sharp point is introduced into the marrow space. A syringe is attached to the needle and suction is applied. The marrow cells are then drawn into the syringe. The contents of the syringe, which to the naked eyes looks like blood with tiny chunks of fat floating around in it, is dropped onto a glass slide and smeared out.

1. **Cell block:-**

Body fluids or secretions are collected in special bottles with anticoagulant (e.g., potassium oxalate, heparin, and sodium citrate-citric acid; according to the formula that used).The fluid poured into three 50 ml centrifuge tubes. After centrifugation at medium speed for 15-30 minutes, supernatant is discarded. It is highly recommended to make Papanicolaou smears to assure the results.

A small amount of fixative is poured directly into the tubes, let stand for 15-30 minutes. The residue will coagulate into a soft mass which is wrapped into lens or cigarette paper to prevent its loss. This mass should be fixed for 6-12 hours (time depends on the size of the mass). Then it should go through the routine process of paraffin method.

1. **Smears:-**

The Papanicolauo smears have become an important diagnostic adjunct in daily use in pathology laboratories.

Its purpose mainly for cancer diagnosis, also this technique is used for:-

* 1. Determination of sex chromosome
  2. Effect of estrogen hormones
  3. Presence and type of infection

1. **Nacleopure filter membrane technique:**

This technique is increasingly used in diagnostic cytology.

The filters are designed to isolate or collect cells from body fluids in a concentrated area or a membrane. The membrane then fixed stained and viewed microscopically.

**Advantages:**

* 1. Gives a good examination (1-2ml)
  2. It eliminates the necessity of centrifuging
  3. Close adhering of cells to the filter reduces the possibility of cells washing off
  4. This technique concentrates cells and furnishes more clearly representative sample of cell types

**Fixation:**

The preferable fixative is 95% ethanol with 3% glacial acetic acid added to it.

**Staining:**

With Papanicolauo stain which gives a good detection for any change in the cells.

**9/ Post test:**

1. Punch biopsy is used for a) lung, b) skin, c) bone.
2. Incisional biopsy is used for a) bone, b) liver, c) rashes.
3. Cytoscopy is for a) bladder, b) bone marrow, c) FNA.
4. Papanicolauo technique is used for a) cell blocks, b) Excisional biopsy, c) smears.

**Note:**

* Check your answers in key answer page (74).

**Terminology**

**Clinical Cytology:**

Is the study of normal and abnormal morphological characteristics of human cells.

**Neoplasm (neoplasia)**

A "new growth" of body's own cells which is no longer under normal physiological control. These may be "benign" or "malignant".

**Benign neoplasms** are typically tumors (lumps or masses) that, if removed, never bother the patient again. Even if they are not removed, they are not capable of destroying adjacent organs.

**Malignant neoplasm**, or "cancers", are those whose natural history (i.e., behaviour if not treated) is to cause the death of the patient. Malignancy is expressed by **1)** local invasion, in which the neoplasm extends into vital organs and interferes with their function. **2)** METASTASIS, in which cells from the tumor seed out to other parts of the body and then grow into tumors themselves, and/or **3)** paraneoplastic syndromes, in which the neoplasm secretes metabolic poisons or inappropriately large amounts of hormones that cause problems with functions of various body systems.

**Metastatic**, there are two routes of metastasis, **1)** hematogen- ous, in which the cells travel through the blood vessels, and **2)** lymphogenous, in which the lymphatic vessels conduct the cancer cells.

In the case of lymphogenous metastasis, the metastatic tumors can grow from cancer cells entrapped in the lymph nodes that collect the lymph draining from the organ where the original cancer has developed, causing the nodes to enlarge (breast cancer, cancer of larynx).

Hematogenous metastases tend to deposit in the liver, lungs, and brain. Many cancers metastasize both lymphogenously and hematogenously.

**Carcinoma**

A malignant neoplasm, whose cells appear to be derived from epithelium, Cancers composed of columnar epithelial cells are called (adenocarcinomas). Those of squamous cells are called (squamous cell carcinomas).

**Sarcoma**

A malignant neoplasm, whose cells appear to be derived from those other than epithelium, the connective tissues of the body (fibrous tissue, muscle, bone, cartilage, fat, and lining of joints) tend to give rise to sarcomas. In adults carcinomas are much more common than sarcomas, while in children, sarcomas make up a greater portion of cancers.

**Normal exfoliated cells characteristics:**

1. Size of the cell is fixed (24).
2. Shape of the cell in normal (cytoplasm & nucleus).
3. Cell wall is regular.
4. Nucleus is in centre of the cell.
5. Cytoplasm / Nucleus or N/C ratio is fixed N/C = 1/100
6. Nuclear membrane is fine & regular.
7. Nucleolus (one) only.
8. Chromatin material regularly distributed.
9. The cell stains normally.

**Carcinoma cell characteristics:**

1. Nucleus is large N/C s small = 1/2 
2. Increase of the chromatin material
3. Consequently nucleus stains dark (hyper chromatic)
4. Irregular distribution of chromatin.
5. Abnormal mitosis (polyplax)

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1. Many nuclei inside the nucleus
2. Irregular nuclear membrane
3. Multiduplication more than 2 nuclei after division)

**Inflammatory cell characteristics:-**

1. Infiltration of neutrophils

Neutrophils

Nucleus

1. Vacuolated cytoplasm
2. Perinuclear halo

Nucleus

Halo

1. Degeneration of cytoplasm (Cytolysis)
2. Degeneration of nucleus (Karyolysis)
3. Abnormal staining
4. Piknosis of nucleus
5. Nuclear membrane krenation
6. Karyohexis (fragmentation of nucleus)

**10/ Post test:**

1. Sarcoma arises from a) squamous epithelia, b) columnar epithelia, c) muscles.
2. Hematogenous metastasis deposit in a) lung, b) uterus, c) bone.
3. Normal cell size is a) 34µ, b) 24µ, c) 14µ.
4. Piknosis of nucleus is found in a) inflammatory cells, b) cancer cells, c) dividing cells.

**Note:**

* Check your answers in key answer page (74).

**The differences between Paraffin method & Freezing method**

**Paraffin method Freezing method**

1. The tissue must be fixed 1. Fresh tissue or fixed with 10%

formalin.

2. It takes long time 1-2 days 2. It takes short time, few minutes

or more .

3. Embedding medium is 3. Embedding medium is ice.

paraffin wax.

4. Thickness of sections 4-10 4. Thickness of sections 10-20

5. It is possible to process all 5. It is recommended for fat, lipids,

tissue types. and enzymes. .

6. Produce a ribbon of sections. 6. Produce a single sections.

**Key answers**

1/ Post test:

1. C, 2. a, 3. b, 4. C

2/ Post test:

1. C, 2. b, 3. a, 4. C

3/ Post test:

1. B, 2. c, 3. a, 4. B

4/Post test:

1. A, 2. b, 3. c, 4. A

5/ Post test:

1. B, 2. a, 3. b, 4. B

6/ Post test:

1. B, 2. c, 3. a, 4. b, 5. C

7/ Post test:

1. C, 2. a, 3. c, 4. a, 5. B

8/ Post test:

1. C, 2. a, 3. b, 4. C

9/ Post test:

1. B, 2. a, 3. a, 4. C

10/Post test:

1. C, 2. a, 3. b, 4.

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