Ministry of high education and scientific research

Al-Furat Al-Awsat Technical University

Kufa Technical Institute

Medical laboratory techniques

Training package

Microtechnique

Students of first class

By

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Contents

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Lecture 1

Introduction

Certain cells and tissue may be examined as soon as they are removed from the body as blood , lymph, smears from spleen, scraping from the uterus and connective tissue. Student must remember that fresh preparations when examined in the living condition don't keep long. Only after fixation they may be preserved . We reduce the thickness of the tissue in order to preserve the relationship between the cells and tissues by cutting the tissue into thin slices.

Tissue processing

Tissue processing is a very important subject to be studied in order to have a full knowledge about the steps of preparing pathological tissue for histopathological examination and diagnosis of the disease.

Micro technique :is the preparation and staining of tissue sections from surgical or autopsy material for microscopic study

Histotechnologists: The persons who do the tissue processing and make the glass microscopic slides.

Techniques in pathology include light microscopy, electron microscopy, immunohistochemistry and molecular pathology

1. **Specimen collection**
2. **Tissue processing**

a. Fixation

b. dehydration

c. clearing

d. Impregnation

e. Casting

f. Cutting

g. Staining

1.Specimen collection

Sources of tissue

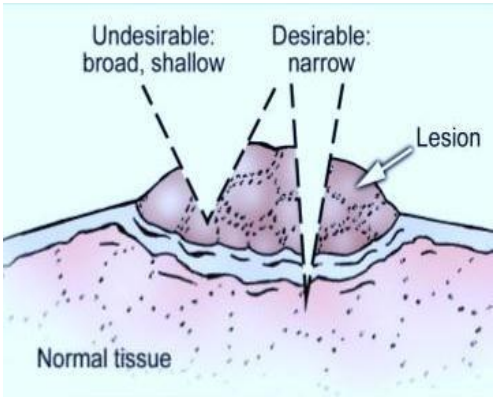
Tissue is taken from human or laboratory animals. Small specimen is taken 0.1 – 10 mm from selective area of tissue that contain significant changes that can be observed macroscopically (by naked eye examination) .

***The specimen of tissue has specific term according to its source:**

1- Biopsy: is the tissue taken from living animal or human being.

2- Autopsy or (necropsy) : is the tissue taken from dead animal or human being.

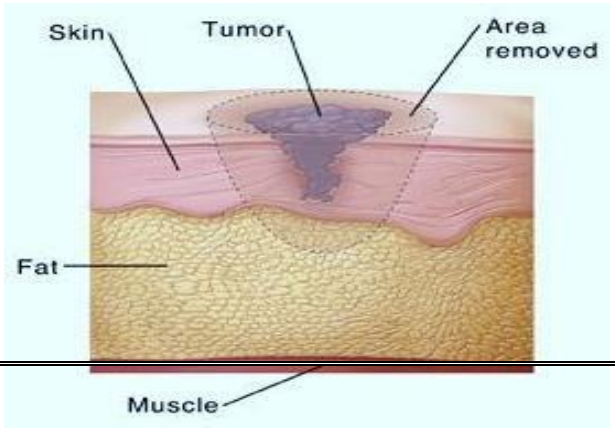
Method of Biopsy Taking:
1. Incisional biopsy
2. Excisional biopsy
3. Punch biopsy
4. Core needle biopsy
5. Curettage biopsy



Incisional biopsy:
In this method only a portion or wedge

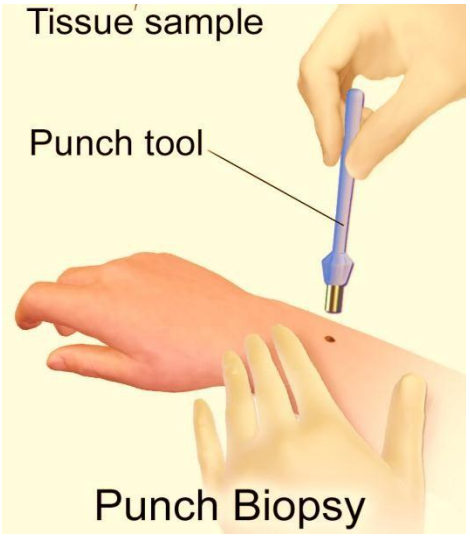
of tissuenhjmu from a large lesion is taken.

Excisional Biopsy:
In this technique, the entire lesion is removed, usually with a rim of normal tissue.



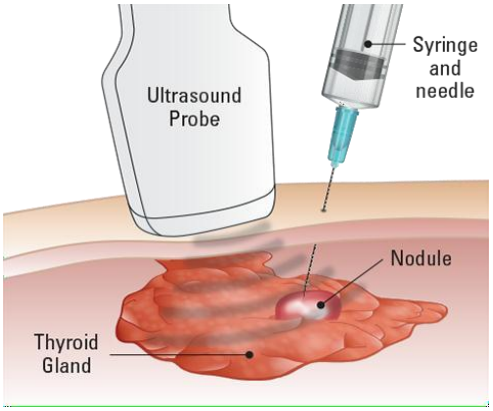
Punch Biopsy:

It is done by biopsy forceps. It is performed in the lesion of uterine cervix, oral cavity, esophagus, stomach, intestine and bronchus.



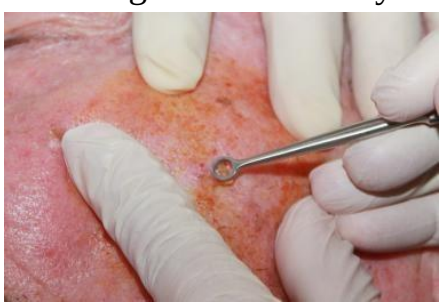
Core Needle Biopsy:

It is done with special type of wide bore biopsy needle.
It permits a percutaneous approach to internal structures. Sampling errors are a significant problem in needle biopsy.



Curettage Biopsy:

Curetting are usually done for diagnosis of endometrial disease



Handling of Specimen

Specimen should be transported in glass, plastic or metal container or in a plastic bag in 10% formalin. If formalin is not available at hand, place the specimen in refrigerator at 4oC to slow down autolysis. The container should have an opening larger enough so that the tissue can be removed easily after it has hardened by fixation.

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Lecture 2

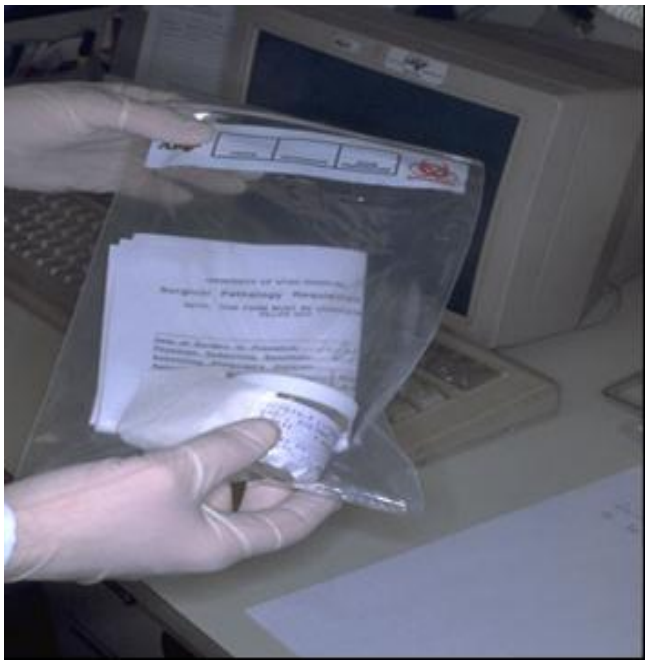
Specimen Reception

A separate room is required for specimen reception, acting as the interface between hospital staff (or other visitors) and the pathological laboratory.

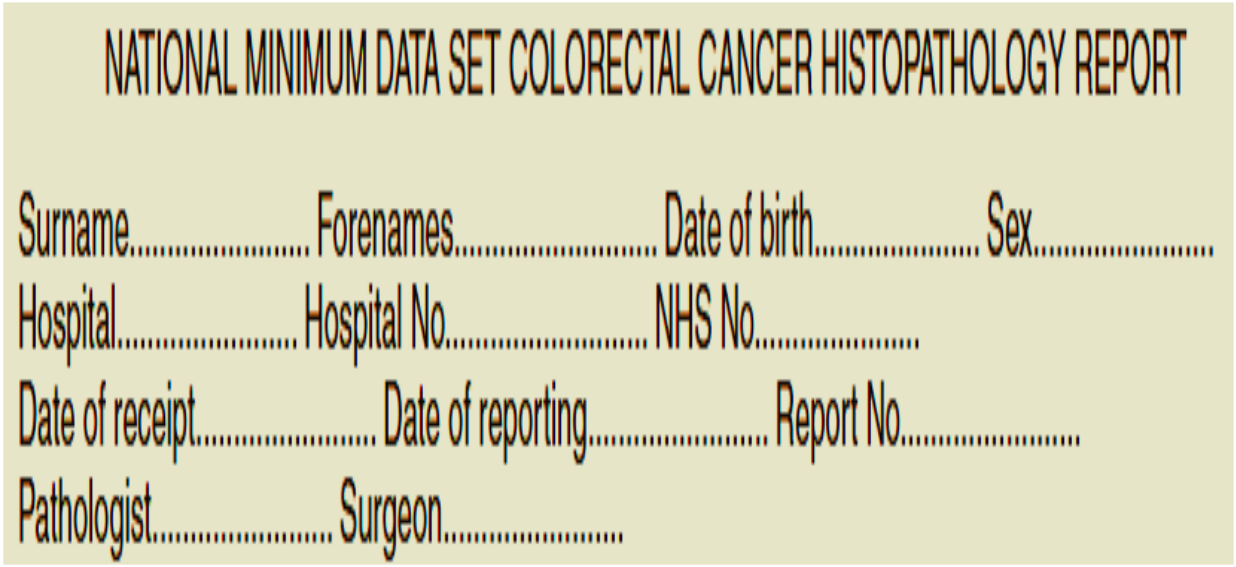
The dissection area must have **good lighting**, **good ventilation**, nonabsorbent wipe-clean surfaces, appropriate **protective clothing** for the laboratory personnel, **gloves.**

General Principle of Gross Examination

1. Labeling of tissues.



2. The unlabelled specimen should never be processed.


NATIONAL MINIMUM DATA SET COLORECTAL CANCER HISTOPATHOLOGY REPORT

Surname..... Forenames..... Date of birth..... Sex.....

Hospital..... Hospital No..... NHS No.....

Date of receipt..... Date of reporting..... Report No.....

Pathologist..... Surgeon.....

3. A properly completed histology requisition form containing the patient's **name, age, sex,** relevant clinical **data,** surgical findings, nature of the operation and name of tissue submitted.

4. Careful search and examination of all the tissue submitted in order.

5. Surgeon should be instructed to submit all the material that they have removed, not the selected portion from it.

6. Place the specimen on the cutting board in an anatomic position and *record the following information:*

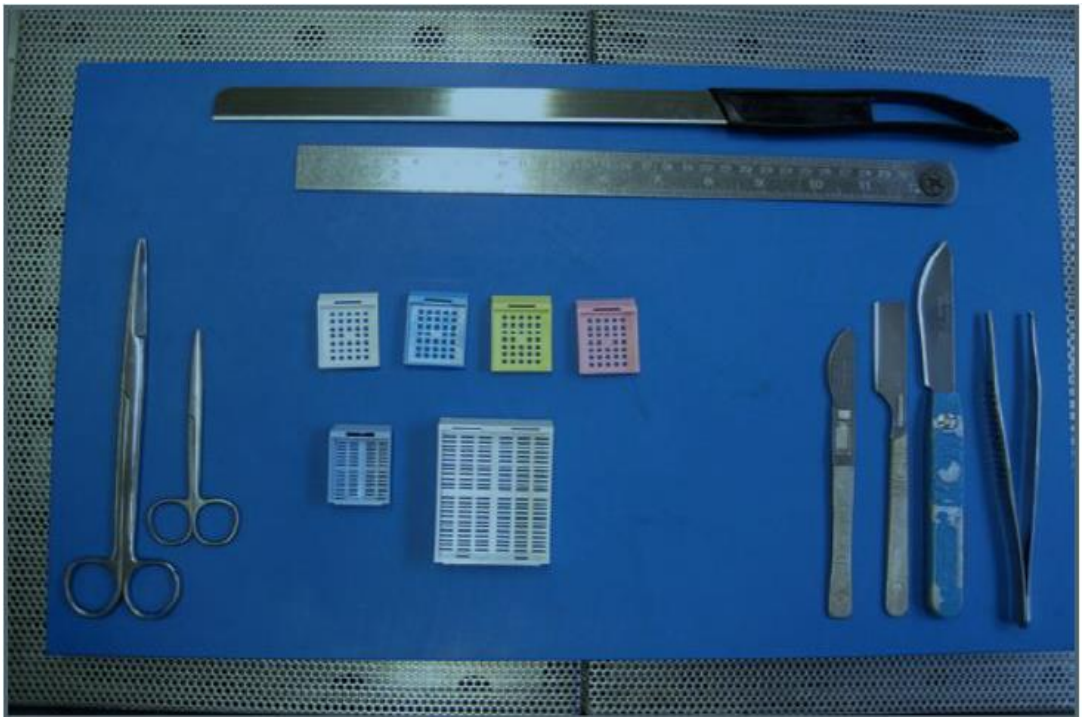
a. Types of specimen b. Structure included.

c. Dimensions d. Weight

e. Shape f. Colour g. Consistency

h. Surgical margin, whether included or not involved by tumor.

7. Measurements are usually given in centimeters unless the specimen is very small in which Millimeter (**mm)** can be used.



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Lecture 3

The Fixation

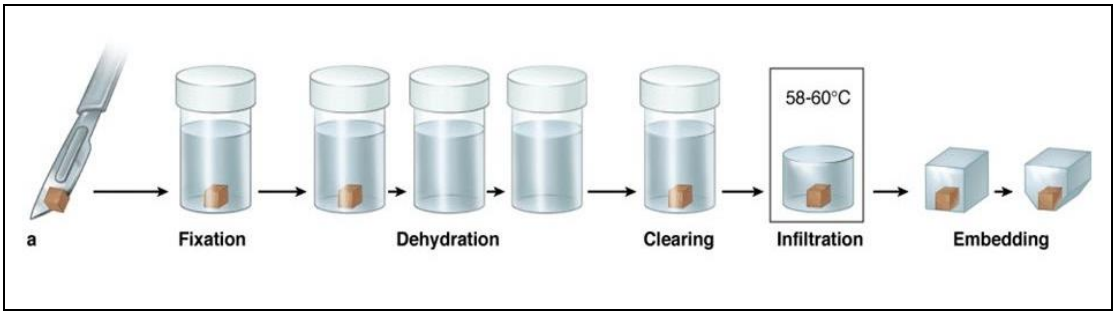
Fixation is preserved tissue structure and prevent degradation by enzymes released from the cells or microorganisms.

Or It is a chemical process by which biological tissues are preserved from decay, either through **autolysis** or **putrefaction**.

Fixation is usually the first stage in a multistep process to prepare a sample of biological material for microscopy or other analysis.

The fixative should penetrate and fix tissues rapidly, have a shelf life of ***at least one year.***

specific fixatives currently used in pathology, histology, and anatomy.



Aim of fixation:

1. Confers chemical stability on the tissue
2. Hardens the tissue (helps further handling)
3. prevent enzyme autolysis
4. Prevent bacterial putrefaction
5. May enhance later staining techniques
6. protect the cells from distortion and shrinkage when they are subjected to alcohols and hot paraffin.

Factors affecting the quality of fixation:

1. Size and thickness of a piece of tissue.

2. Tissue covered by a large amount of **mucous** and **blood** fix slowly.

3. Fatty and lipomatous tissue fixes slowly.

4. pH: Should be kept in the physiological range, between pH 4-9.

The pH for the ultrastructure preservation should be **at pH 7.2–7.4 (i.e. neutral buffered formalin).**

5. Osmolarity: Hypertonic solutions give rise to cell shrinkage, Hypotonic solutions result in cell swelling and poor fixation.

6. Temperature: Hot formalin will fixes tissues faster, care is required to avoid cooking the specimen.

7. Concentration of fixative.

8. Volume of the Fixative: At least 15-20 times greater than tissue volume.

9. **Time interval from the removal of tissues to fixation**: The faster you can get the tissue and fix it, the better.

10- Duration of fixation: Most fixatives, such as NBF, will penetrate tissue to the depth of approximately **1 mm in one hour**.

11- the rate of penetration of the fixing fluid

Properties of an Ideal Fixative:

1. Prevents autolysis and bacterial decomposition.
2. Preserves tissue in their natural state and fix all components.
3. Make the cellular components insoluble to reagent used in tissue processing.
4. Preserves tissue volume.
5. Avoid excessive hardness of tissue.
6. Allows enhanced staining of tissue.
7. Should be non-toxic and non-allergic for user.
8. Should not be very expensive.
9. penetrate rapidly and kill the tissue to prevent post mortem changes.
10. Harden the tissues.

Lecture 4

Methods of fixation:

1-Physical methods such as :

a-Heating

b- Microwaving

c- Freeze-drying

microwaving : Microwave heating speeds fixation and can reduce times for fixation of some gross specimens and histological sections from more than 12 hours to less than 20 minutes. Microwaving tissue in formalin results in the production of large amounts of dangerous vapors.

Freeze-drying is a useful technique for 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 −40°C.

2-Chemical fixation:

1-coagulant, Alcoholic fixatives ethanol , methanol and acetone.

2- Crosslinking, as formaldehyde , glutaraldehyde and osmium tetroxide.

3- Compound fixatives , as ethanol added to formaldehyde to produce alcoholic formalin.

♣ **Crosslinking fixatives act by creating covalent chemical bonds between proteins in tissue. This anchors soluble proteins to the cytoskeleton, and lends additional rigidity to the tissue.**

♣ Precipitating (or *denaturing*) fixatives act by reducing the solubility of protein molecules and (often) by disrupting the hydrophobic interactions that give many proteins their tertiary structure. The precipitation and aggregation of proteins is a very different process from the crosslinking that occurs with the aldehyde fixatives.

Faxative

No single substance or known combination of substances has the ability to preserve and allow the demonstration of every tissue components. Because of this, some fixatives have only special and limit application and most are mixtures of two or more reagents to make use of the special features of each.

A. Agents precipitate protein

1. Mercuric chloride
2. Picric acid
3. Ethyl alcohol

B. Agents not precipitate protein

1. Formaldehyde
2. Potassium dichromate
3. Acetic acid

Agent precipitate protein

1. Mercuric chloride:

a. White crystalline substance , soluble in water at room temperature to about 7% , and in alcohol 33%.

b. It is toxic and corrosive to metals, containers of mercuric chloride should never have metal lids.

c. Precipitate protein, penetrate and harden tissue quickly.

d. It shrink but not distort tissue.

e. fix both nucleus and cytoplasm well.

Advantages and disadvantages of mercuric chloride fixatives (Zenker's & Helly's fluids

Advantages	disadvantages
Is a good mordant	is extremely poisonous and expensive to dispose off
Helly's fluid is excellent fixative for bone marrow and blood containing organs	Prolonged treatment in Zenker's fluid will render the tissue brittle, making sections difficult

	Mercuric chloride crystals will precipitate on the tissue

2. Picric acid:- a. Bright yellow crystalline substance .

b. soluble in water about 1% at room temperature but more in alcohol.

c. precipitate nucleoprotein and cause much shrinkage , but little hardening.

d. precipitate all proteins forming picrate.

Advantages and disadvantages of picric acid fixatives (Bouin's & Gender's fluids)

	advantages	***disadvantages***
1	It provide good penetration and fixation	It precipitate protein , forming protein picrate (water soluble substance)
2	It is stable and can be made up as a stock solution	
3	It does not interfere with staining qualities of the tissue if the fixative is washed out thoroughly	
4	Fix tissue for 4-18 hrs depend on the size and density of the tissue	Does not harden the tissue
5	Is an excellent **mordant** (chemical which increases the staining potential of tissue).	it causes much shrinkage

Lecture 5

3. Ethyl alcohol (Ethanol):-

a. Colorless, inflammable liquid.

b. powerful dehydrating agent.

c. causes shrinkage and hardening.

d.coagulate protein but not nucleoprotein

e. miscible with water.

f. reducing agent, easily oxidized

B. Agents don't precipitate protein

1. Formaldehyde:

a) It is gas , soluble in water to a maximum extent of 40% , so it is termed formalin.

b) This solution is nearly always acid on storage through the production of formic acid , so it must be changed and use fresh solution or neutralized by adding buffer salts.

c) the concentrated solution of formalin some time becomes turbid on keeping through the production of paraformaldehyde, this decreases the strength of the solution, but does not prevent its use if removed by filtration.

2. Potassium dichromate:-

a) Orange crystalline

b) Tissue fixed with it should be washed with tap water better proceeding to alcohol to prevent the formation of an insoluble precipitate.

c) prolong exposure of tissue to this reagent causes most tissue to become brittle with difficulty in sectioning .

3. Acetic acid: (Glacial)

a) colorless fluid with strong odor.

b) swell collagen fibers.

c) precipitate nucleoprotein.

FIXATIVES

1) 10% Formal saline (colorless solution)

40% Formaldehyde …….. 100 ml

Sodium chloride …….. 8.5 gm

Tap water ……. 900 ml

Advantages

1. Penetrate well , causes little shrinkage
2. Blood and fat are well preserved.
3. It doesn't make the tissue brittle in prolong treatment.
4. In fixing large specimen , the normal color is preserved better than with most other fixatives.
5. It permit a large variety of staining methods.

Disadvantages

When specimen is stored in formal saline for many months, formic acid is produced , which destroys the staining properties of the tissues, under this circumstances, it is necessary to change the formal saline every month.

2. 10% Formalin (colorless solution)

40% Formaldehyde ……. 100 ml.

Tap Water …….. 900 ml

Advantages

1. Used for post mortem specimens very widely.

2. Produce very little shrinkage.

3. Large specimen may be fixed for an indefinite period, provide that the solution is changed every 3 months.

4. Fixation with it can be followed by most staining techniques.

***Disadvantages**

a)Formalin has an irritant vapor which may affect the nasal mucosa and cause sinusitis.

b)Dermatitis may be produced by prolong contact of formalin with skin .

3. Mercurials

Mercurials such as Zenker's have an unknown mechanism that increases staining brightness and give excellent nuclear detail. Despite being fast, mercurials penetrate poorly and produce tissue shrinkage. Their best application is for fixation of hematopoietic and reticuloendothelial tissues. Also note that since they contain mercury care must be taken with disposal.

Mercuric chloride fixatives

A) **Zenker's fluid - fixation time 4-24 hours.** is recommended for reticuloendothelial tissues including lymph nodes, spleen, thymus, and bone marrow. Zenker's fixes nuclei very well and gives good detail.

Distilled water -	950ml
Potassium dichromate -	25g
Mercuric chloride -	50g
Glacial acetic acid -	50ml (added before use)
Sodium sulphate	1 gm

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

B) **Helly's fluid - fixation time 12-24 hours.**

Helly's fluid is an excellent fixative for bone marrow and blood containing organs.

Stock solution:-

Potassium dichromate	25g
Mercuric chloride	50g
Sodium sulphate	10g
Distilled water	1000ml

For use:-

Stock solution - 100ml

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

The fixative solution should be made up just before use. Fixed tissue must be washed for 24 hours in running tap water prior to processing.

Advantages and disadvantages of mercuric chloride fixatives (Zenker's & Helly's fluids

Advantages

1. Is a good mordant

2. Helly's fluid is excellent fixative for bone marrow and blood containing organs**.**

3**.** It permits excellent staining of nuclei and connective tissue fibers

Disadvantages

1. is extremely poisonous and expensive to dispose off
2. Prolonged treatment in Zenker's fluid will render the tissue brittle, making sections difficult
3. Mercuric chloride crystals will precipitate on the tissue as black pigment.
4. Penetration is poor
5. Pieces of tissue should not exceed o.5 cm thickness.
2. Washing tissue in running tap water for several hrs. after fixation in Zenkers fluid

4.. Bouin's fluid – (yellow solution) fixation time 6 hours. is sometimes recommended for fixation of testis, GI tract, endocrine tissue and glycogen fixation.

Saturated aqueous solution of picric acid (1- 1.5%) 75ml

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

Glacial acetic acid - …… 5ml

Fixed tissue should be transferred to 70% alcohol.

Wash in several changes of 50% and 70% alcohol for 4-8 hrs. If yellow coloration is exhibited in the sections after mounting on the slide , it should

Advantages and disadvantages of (Bouin's fluids)

	advantages	***disadvantages***
1	It provide good penetration and fixation	It precipitate protein , forming protein picrate (water soluble substance)
2	It is stable and can be made up as a stock solution	
3	It does not interfere with staining qualities of the tissue if the fixative is washed out thoroughly	
4	Fix tissue for 4-18 hrs depend on the size and density of the tissue	Does not harden the tissue
5	Is an excellent **mordant** (chemical which increases the staining potential of tissue).	it causes much shrinkage

5. Carnoy's fluid – fixative (time 1-3 hours)

Carnoy's fluid is recommended for glycogen , since aqueous sol. are to be avoided.

Ethanol - 60ml

Chloroform - 30ml

Glacial acetic acid - 10ml

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

Advantages and disadvantages of Carnoy's fluid

	Advantages	***disadvantages***
1	penetrate and quick acting fixative	
2	Tissue is transferred directly into absolute alcohol , since dehydration has already been initiated during fixation	
3	Thin sections of tissue will be fixed for 1-3 hrs. at room temperature or for 12-18 hrs. at refrigerator temperature	
4	It is good fixative for chromosomes	

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Lecture 6

Frozen Sections

Small pieces of tissue (5X5X3mm) are placed in a cryoprotective embedding medium - OCT, TBS or Cryogel - then snap frozen in isopentane cooled by liquid nitrogen. Tissue is then sectioned in a freezing microtome or cryostat. Sections are then fixed in one of the following fixatives: Absolute acetone for 10-15 minutes, 95% ethanol for 10-15 minutes or Absolute acetone 10minutes followed by 95% ethanol 10minutes

Advantages and Disadvantages of Frozen Sections

Disadvantages	*Advantage*
Lack morphological detail	Give better preservation of antigenicity
Present a potential biohazard	Minimal exposure to fixative
	Not exposed to the organic solvents

- Frozen Sections preserve RNA and Lipids despite poor morphology. Compare to Paraffin sections, synonymous to Chemical Fixatives in the table, which destroy RNA and affect some antigens BUT give good morphology.

Washing tissue after fixation

1. After the tissue is fixed for the proper length of time, excess fixative must be washed out to prevent over-fixation. Washing also removes substances in the fixative which might interfere with the subsequent processing.
2. Since most fixatives are aqueous solutions, the washing is usually carried out for a specific period of time in tap water or isotonic saline solution.

General rules for washing

A. Aqueous fixatives

1.Aqueous solutions containing any of the following :-

Potassium dichromate , and formalin ; wash in water.

2. Aqueous solutions containing picric acid or mercuric chloride such as Zenker's fluid ; wash in 70% alcohol.

3. Boun's fluid : wash in 70% alcohol.

B. Alcoholic fixatives; these are always to be washed with alcohol.

1. Washing in water

This is the most efficient and least troublesome method for objects which are of sufficient size and firmness to withstand such treatment.

Duration of washing

1. Washing is generally continued until all or nearly all of the uncombined fixative has been removed .

2.Tissue should not remain in water longer than is necessary, because they become softened or even partially disintegrated by prolong soaking.

3. The approximate time required for thorough washing in running water 3 -6 hrs . for small and permeable objects , and 6 – 12 hrs. is about slices of tissue up to 5 mm thick and 18 – 24 hrs. for larger or less permeable objects.

4. After fixation with formalin, a few minutes in water will suffice for most purposes, or the alcohol used for dehydration can be counted on to remove the fixative.

2. Washing in alcohol

a. Following alcoholic fixatives, the general rules is to transfer material to alcohol of about the same percentage contained in the fixing fluid or the next lower percentage , change this several times before replacing it with stronger or weaker alcohol.

b. Following Carnoy's acetic acid-alcohol or the modification containing chloroform (Carnoy II) , transfer to 95% alcohol , change this for two to three times at intervals of one to several hrs.

c. For the most formalin acetic acid – alcohol mixture , transfer material to 70% alcohol.

Lecture 7

2. Washing of mercuric chloride

Mercuric chloride forms a deposits on the tissue which consist of minute blackish particles and some large needle_ like crystals with black mass at either end. These deposits will spoil good preparations of tissue.

a. Following fixation with Zenker's and Helly's fluids , wash material in running water for 12 to 24 hrs . , then place it successively in 30 , 50 , and 70% alcohols . leaving it in each for a minimum of one to 6 hrs . according to the size and permeability of the object.

b. The removal of mercury deposits can be accomplish by means of an iodine solution or a mixture of iodine and potassium iodide. The mercury deposits are converted to mercurial iodide , which is soluble in alcohol. Specimens are generally iodized when they placed in 70 or 80% alcohol.

Iodine solutions

1.saturated solution of iodine in 95% alcohol

2.iodine solution consist of:

Iodine	2 gm
Pot. iodide	3 gm
95% alcohol	100 ml

Procedure of mercury deposits removal

1. Add one of iodine solutions drop by drop , until the alcohol containing the specimen is deep umber colour. As the iodine perform its task of removing the precipitate , the alcohol will be decolorized wait for minutes or several hrs.
2. If decolourization is complete, add more iodine _ alcohol.
3. When the rate of decolourization begins to slow down , add iodine in small quantities and discontinue addition once it is apparent that no more decolourization is likely to take place .

4.If the iodine stains the tissue , the higher alcohols into which tissue is placed prior to embedding in paraffin will usually remove it.

3. Washing following aqueous picric acid solution

Picric acid should always be washed out with alcohol not weaker than 50% and preferably in 70% alcohol.

Specimens may be transferred from aqueous picric acid mixture (Bouin's fluid) directly to 50% alcohol .this should be changed once or twice at intervals of one to several hrs. depending on the size of the tissue.

The tissue should be then transferred to 70% alcohol in which the washing may be continued for as long as necessary.

Lecture 8

Dehydration

Dehydration: is removing of excess water from the tissue.

Tissues are dehydrated by using increasing strength of alcohol; e.g. 50%, 70%, 90% and 100%. The duration for which tissues are kept in each strength of alcohol depends upon

1.the size of tissue,

2. fixative used

3. type of tissue; e.g. after fixation in aqueous fixative delicate tissue need to be dehydrated slowly starting in 50% ethyl alcohol directly whereas most tissue specimens may be put into 70% alcohol. Delicate tissue will get high degree of shrinkage by two great concentration of alcohol.

- The volume of alcohol should be 50-100 times that of tissue.

1.Tissues contain much "free" water which does not mix well with the paraffin used later in the procedure. Therefore, water in the tissues must be removed by submerging the tissue in alcohol, a process known as alcohol dehydration.

2.If this is done too rapidly, the large outflow of water can damage the morphology of the cells and tissues.

3.In this step, tissue is placed into a series of gradually increasing concentrations of alcohol, usually ethyl alcohol (30, 50, 70 ,80, 95, and 100%), for specific periods of time.

- **Dehydrating agents**

1.Alcohol

Ethyl and isopropyl alcohol are predominantly used in a routine laboratory for the dehydration of tissue .

- Advantages and disadvantages of alcohol

advantages	***disadvantages***
They are fast acting	Cause shrinkage of tissue
Non toxic	Absolute alcohol causes over hardening of the tissue
Reliable	

2.Acetone

Time recommended for dehydration of tissue is 20 minutes in 4 fresh changes of acetone.

- Advantages and disadvantages of Acetone

Advantages	***disadvantages***
It is very rapid in action	Does not mix readily with paraffin , so the tissue should be transferred to a paraffin solvent to be cleared prior to infiltration
Less expensive than some of other dehydrants	

Notes on dehydration

1. After fixation in aqueous solution , delicate tissue must be dehydrated slowly (ex: brain , spinal cord or embryos) , starting with 50% alcohol, whereas most tissues may be put into 70% alcohol.

2. tissue immersed in too great concentration of alcohol after an aqueous fixatives, will usually show a high degree of shrinkage due to the rapid removal of water.

3. The minimum duration of treatment in graded alcohol will depend on the size and type of tissue , but it will be accepted that long period in diluted alcohol will not harm

tissue . Tissue may be stored in 70% alcohol after fixation because the concentration of water inside the cells equal approximately the concentration of water in alcohol.

4. Giant sections of whole organs are best dehydrated in 50% , 70% , 95% , and 100% for 24 – 28 hrs. in each concentration depend on their thickness , using 3 changes of each strength.

5. Tissue cannot transfer directly to high alcohol concentration , because water will come out of the tissue rapidly which cause shrinkage.

6. Dehydrating of thick pieces of tissue quickly , the paraffin will not penetrate the middle of the pieces and the paraffin block will be soft.

6. preparation of different concentration of alcohol from chemically pure ethanol (98% or 95%)

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Lecture 9

Clearing: (Removal of Alcohol) (De-alcoholization)

During dehydration water in tissue has been replaced by alcohol. The next step alcohol should be replaced by paraffin wax. As paraffin wax is not alcohol soluble, we replace alcohol with a substance in which wax is soluble. This step is called clearing.

Clearing of tissue is achieved by any of the following reagents:

- Xylene
- Chloroform
- Benzene
- Carbon tetrachloride
- Toluene

Xylene is commonly used. Small piece of tissue are cleaned in 0.5 – 1 hour; whereas larger (5 cm or more thick) are cleaned in 2-4 hours.

1.The tissue is placed in several changes of clearing agent for specific periods of time.

2.Clearing also increases hardening of the tissue and makes the tissue translucent.

Clearing agents must meet the following requirements:

1.be miscible with dehydrating agent and embedding medium

2.cause minimal damage to the tissue

3.be easily removed from the tissue by the embedding medium

Clearing agents

1.Xylene: colorless liquid

***Advantages**

1. Rapidly renders thin slices of tissue translucent

2. Quickly eliminated from tissue by molten paraffin wax

Disadvantages

1. Inflammable , Volatile liquid

2. Prolong treatment causes brittleness and distortion

2. Toluene : similar to xylene , but cause less brittleness.

3. Chloroform: colourless liquid

Advantages

a.Causes very little brittleness and distortion

b.Thicker block may be processed

c.inflammable non

Disadvantages

1.Volatile liquid

11.More expensive

111.Slower acting than xylene

IV. Less quickly eliminated from tissue by molten paraffin wax

4. carbon tetrachloride : similar to chloroform , but more toxic.

5. benzene: colorless fluid

***Advantages**

a. It penetrate and clear tissue very rapidly

b. It evaporate from paraffin bath very rapidly

c. It produce minimum of shrinkage

d. Miscible with paraffin

***Disadvantages**

a. Excessive exposure to benzene will damage bone marrow

b. carcinogenic

***Notes on clearing**

1. Clearing with xylene or benzene should take half to three hrs.

2. Prolong treatment in xylene or benzene causes brittleness of the tissue with subsequent difficulty in cutting

3. Sometimes the xylene or benzene takes on a milky appearance when added to the tube containing the tissue. This indicate that dehydration has not been completed , and the tissue must be put into a fresh supply of absolute alcohol for further period of time.

4. If the tissue cleared quickly , the clearing agent don't reach the center , same think happen if the tissues are in close contact with the bottom of the container or with each other.

Lecture 10

Impregnation with Wax (infiltration)

Infiltration: is to impregnate the tissue with supporting medium to facilitate its cutting by microtome knife.

This is allowed to occur at melting point temperature of paraffin wax, which is 54-60oC. Volume of wax should be about 25-30 times the volume of tissues. The duration of impregnation depends on size and types of tissues and the clearing agents employed. Longer periods are required for larger pieces and also for harder tissue like bones and skin as compared to liver kidney, spleen,

lung etc. Xylene is easiest way to remove. Total duration of 4 hours is sufficient for routine impregnation.

Types of Wax employed for Impregnation:

1. Paraffin wax
2. Water soluble wax
3. Other material, like colloidin, gelatin, paraplast etc.

Paraffin wax is used routinely. It has hard consistency, so section of 3-4 micron thickness can be cut.

Causes of infiltration:

1.fixed tissues are not firm or adhesive enough to allow thin sectioning.

2.to hold the cells and intracellular structures in proper relation to each other.

Procedures of infiltration

1.after tissue specimens have been completely dehydrated and cleared , they are immersed in melted paraffin for 2 – 4 hours .

2. two or more changes of paraffin are required to eliminate traces of the solvents which prevent the paraffin from hardening properly.

3. the liquid paraffin infiltrate the tissue , and when cold , it will solidified and provide the supporting necessary for cutting thin sections.

Notes on infiltration

1. Infiltration in over-heated paraffin will produce shrinkage and hardening of tissue. This must be avoided.

2. Duration in paraffin bath depend on the size , thickness , density and nature of the specimen

3. Skin and nervous tissue infiltrate slowly with paraffin. Muscle , fibrous tissue , blood and fibrin will become over-hardened in paraffin if left in this medium for more than three hrs.

4. Tissue from the brain and spinal cord , due to their compact nature, needs relatively longer treatment in paraffin and requires 4-6 hrs impregnation for medium sized sections

5. rapid chilling of the melted paraffin is recommended to provide a fine crystalline structure capable of fitting closely to the individual cells , thus providing adequate support.

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Lecture 11

Embedding. (Blocking)

Embedding: is to make a block of embedding media with the tissue inside it.

Or is the enclosing of the tissue or specimen in a solid mass of the embedding medium .

1. The surface of the section to be cut should be placed parallel to the bottom of the container in which it is cast .
2. To orient the tissue for proper embedding , you must notch with a scalpel or mark with India ink the side of the tissue opposite that to be cut.

Impregnated tissues are placed in a mold with their labels and then fresh melted wax is poured in it and allowed to settle and solidify. Once the block has cooled sufficiently to form a surface skin, it should be immersed in cold water to cool it rapidly.

After the block has completely cooled it is cut into individual blocks and each is trimmed. Labels are made to adhere on the surface of the block by melting the wax with a metal strips sufficiently wormed

Embedding media

Embedding media: are all materials used by histological technique to infiltrate , support and enclose specimens which are to be cut into thin sections.

Characters of embedding media

1.must be capable of being converted readily from liquid to solid form.

2. in the liquid form the embedding media must penetrate easily into the interstices of the tissue , and then is converted into solid.

The conversion into solid may be brought about by:

a. Crystallization (paraffin)
b. Evaporation of solvent (celloidin)
c. Polymerization (plastics)

Paraffin

1. The paraffin used in histology laboratory is a refined , white , filtered paraffin , to which has been added :a. beeswax b. rubber c. plastics , to facilitate riboning:
2. Paraffin is sold at room temperature . Heat renders paraffin fluid , so that it can permeate the tissue.
3. Hardness of paraffin used for infiltration is matched to the hardness of the tissue.
4. Cakes of paraffin are placed in clean metal or enamel pitchers and melted down in a paraffin oven regulated at a temperature just above the melting point of paraffin.
5. The melted paraffin is filtered within the oven by coarse filtered paper and is then ready to use (to reduce injury to the knife edge when sectioning the tissue.

Orientation of specimen

1. Select proper size mold to allow segments of specimen to be embedded all flat to the bottom of the container and still have a margin of few millimeter around all edges . The mold must also be deep enough to allow paraffin to be added to about twice the thickness of the specimen.
2. Specimen without layers : all pieces of tissue should be embedded firmly to the bottom of the container so that the cut section will present a valid representation of the tissue submitted.
3. Stratified tissue like cyst wall , gallbladder wall , and skin section must be carefully oriented on the edge so that the side of the tissue to be sectioned is positioned vertically to the bottom of the mold.
4. Light and loose texture materials such as cell block and scanty endometrial curetting are best stirred up toward the center of the mold with wormed forceps while the paraffin is still worm .
5. It is best not to embed a very small soft specimen in the same mold with a large dense block.

Summary of Paraffin Wax Embedding:

1. Dehydration
    - a. 70% alcohol 1 hour
    - b.90% alcohol I 1 hour
    - c. 90% alcohol II 2 hours
    - d.100% alcohol I 1 hour
    - e.100% alcohol II 2 hours
    - f.100% alcohol III 2 hours
2. Clearing
    - a.Xylene I 2 hours
    - b.Xylene II 2 hours
3. Wax Impregnation
    - a.Paraffin wax 1 1 hour
    - b.Paraffin wax II 1 hour
    - c.Paraffin wax III 1 hour

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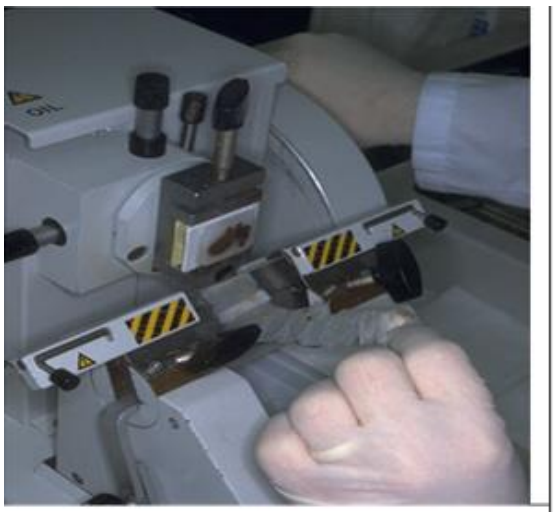
Lecture 12

Cutting (Sectioning.)

Sectioning is accomplished by using a cutting apparatus called a **microtome**. The microtome will drive a knife across the surface of the paraffin cube and produce a series of thin sections of very precise thickness. The objective is to produce a continuous "ribbon" of sections adhering to one another by their leading and trailing edges. The thickness of the sections can be preset, and a thickness between 5 - 10 µm is optimal for viewing with a light microscope. The sections can then be mounted on individual microscope slides.

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



Trimming

The appropriate size mold should be used whenever possible . A margin of paraffin in excess of a few millimeters around the tissue is unnecessary and may cause compression , as the sections are cut . If paraffin surround the tissue in excess of two millimeters around the object , it may be trimmed after it is mounted on the microtome with razor blade.

Instructions of using the Microtome. (reading during Lab.)

1. Stop the fly wheel by using special screw or lever.
2. Insert the block-holder and tight by mean of lever.
3. Insert the cutting knife and fix firmly by mean of two locking levers.
4. Turn the fly wheel and bring the block down carefully , when it reach the level of the knife , leave the block-holder in tight level, then stop the fly wheel.
5. Put the scale at 20 microns which indicate the thickness of the sections.
6. Trim and get rid of the excess paraffin until reach the surface of the tissue .
7. Put the scale at 7 or 8 micron thickness, and continue cutting , until you get a paraffin ribbon.
8. Stop the fly wheel , but notes the block-holder in high level.
9. Collect the paraffin ribbon by using a brush and needle to transfer it to a clean paper.
10. Carry out the knife , clean it with a piece of gauze moistened with xylene, and place it it's wooden container.
11. Remove the block holder.
12. Clean the microtome with brush moistened with xylene.

Cutting sections:- It is essential to insure that:-

1. The knife is sharp and tilted to a suitable angle.

2. The block must be attached firmly to the block holder, which itself must be securely fastened to the microtome.

3. After trimming with the knife and microtome , the block sometimes need to be cooled with ice (remove block from the microtome and place on ice).

4. In order to obtain good ribbon, the front and the back edges of the block should be trimmed parallel to each other, if not ,the ribbon will be curved . The edges

must also be parallel to the knife, otherwise the sections will not adhere to one another .

Lecture 13

Difficulties most commonly encountered during cutting ribbon

Causes	*difficulties*
a. block not parallel to knife edge b. knife dull c. knife tilted too much d. paraffin too hard sections too thick	1.failure of block to ribbon
a. wedge-shaped or irregularly trimmed block b. edge of block not parallel to knife edge c. irregularity in knife edge d. paraffin not homogenous	2.un even and crocked ribbon
a. knife dull b. paraffin block too worm c. paraffin on knife edge d. sections too thin e. microtome screws are loose	3.compressed , Wrinkled sections
a. incomplete fixation of tissue b. incomplete dehydration or clearing of tissue c. incomplete infiltration of tissue with paraffin d. paraffin too hot for bath and or embedding	4. tearing and crumbling of sections

a. nicks in knife edge b. knife edge dirty c. too much knife tilt d. grit , dirt , mercuric chloride crystals, calcium , sutures, or foreign bodies in paraffin or tissue	5. split ribbon or lengthwise scratches in ribbon
a. too vertical knife tilt b. knife dull c. paraffin too soft or room too worm	6. lifting of sections from knife on upstroke
a. static electricity b. knife edge dirty c. knife dull d. knife tilt too vertical	7. section clinging to knife
a. screws on block or knife holder are not tight b. knife not tilted enough to clear bevel c. block too large d. block too hard e. microtome not adjusted correctly	8. varying thickness of sections

Notes:-

1. Microtome knifes are provided with a fitted back , to insure the proper angle when hand sharpening , and a handle that is screwed into the base of the knife to facilitate handling while honing and stropping.
2. Great care must be taken to protect the edge of the knife at all times.
3. T keep the knife in good working , it must be honed to remove nicks and stropped to sharpen it.

How to care for the rotary microtome

1. After you finish cutting sections , brush away with a soft brush all accumulated paraffin and tissues.
2. You may wipe clean with xylene all metal parts.
3. Dry carefully the microtome , with particular attention to the area under the stage (knife holder)
4. Keep the area under the stage well-oiled to prevent rust formation , which will interfere with it's performance.
5. Keep the moving parts of the microtome oiled with a light oil.

Lecture 14

Tissue flattening

Technique of floating out paraffin wax sections

1. The instrument used for this purpose is called (water bath for mounting sections), it's temperature must not exceed the melting point of the paraffin used (usually 45c^{o}) . Note: the melting point of hard paraffin is 60c^{o}.
2. Clean the slide with 70% alcohol to get rid of grease and dust, let dry.
3. Put one drop of (adhesive mixture) on the slide.
4. With a clean finger or hand-palm spread this drop to get a thin film of this mixture, let dry.
5. Put a few drops of 20% alcohol on a clean slide, and place 4-5 sections.
6. Make sure the temperature of the water bath is 45c^{o} by using thermometer.
7. Carefully get rid of excess 20% alcohol pour it in a clean beaker , then lower the slide with sections on to the surface of the water , and withdraw the slide slowly and carefully.
8. As soon as the sections flattens out , separate each section or two sections by using forceps horizontally to the water level.
9. Introduce the albumenized slide into the water, the slide should not be vertical in the water, but should be held obliquely with the sections, lift the floating sections.
10. The slides must be dried , first by blotting them stand vertically for nearly 1-2 minutes , then place them in the oven , with temperature less than the paraffin melting point by 2-5c^{o} .
11. After 5-10 minutes, collect all slides in a clean container.

Drying slides in drying oven

Lecture 15

Staining

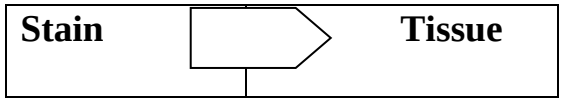
Staining: is accomplished by soaking tissue in a solution of one dye or more. Staining is employed to give both contrasts to the tissue as well as highlighting particular features of interest.

Staining Reactions, methods & types

A) **Staining reactions:**

1-Direct staining (absorption):

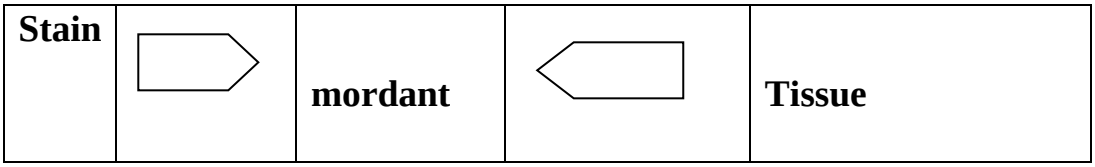
Stain diffuses into tissue without causing any change, it is simple diffusion (ex: Eosin).



Direct Stain (Eosin Stain)

2-Indirect staining:

Stain needs mordant to color the tissue by linking the dye and the tissue (ex: Haematoxylin, Bests carmine).



Indirect Stain (Haematoxylin Stain)

3-Physical staining:-

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

4-**Chemical staining-:**

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

5-**Adsorption phenomenon :**It is an iconic attraction in which the staining is influenced by the affinity of acid to the base or vice versa. These ions accumulate on the surface of one of the 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.

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

3-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 a certain solution called: differentiator as 1% acid alcohol used with hematoxylin .

2-**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 their nature into:

a) Natural stains: These are of natural origin such as Haematoxylin (plant origin), Carmine (animal origin), and

Orcine (animal origin).

b) Synthetic stains: These are manufactured in factories, such as:

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