Unit-1
Introduction and definitions

Histology

- **It** is the scientific study of biological tissues**.**
- **Histology** is the microscopic study of the structure of biological tissues using special staining techniques combined with light and electron microscopy.
- **Histology** is the study of the microscopic structures of cells and tissues of plants and animals. It is often carried out by examining a thin slice (called a 'section') of tissue under a light microscope or an electron microscope. In order to distinguish different biological structures more easily and accurately histological stains are often used.

Histopathology

- **Histopathology** is the microscopic examination of biological tissues to observe the appearance of **diseased cells and tissues** in very fine detail.

The word 'histopathology' is derived from a combination of three Greek words:

  - ***histos*** meaning tissue,
  - ***pathos*** meaning disease or suffering, and
  - ***logos*** which refers to study in this context*.

- Hence **histopathology** is the study of microscopic changes or abnormalities in tissues that are caused as a result of diseases.

Biopsy

- A biopsy is a medical procedure that involves taking a small sample of tissue so that it can be examined under a microscope.
- A tissue sample can be taken from almost anywhere on, or in the body, including the skin, stomach, kidneys, liver and lungs.
- The term biopsy is often used to refer to both the act of taking the sample and the tissue sample itself.
- An examination of tissue removed from a living body to discover the presence, cause, or extent of a disease.

Autopsy

- An **autopsy** (post-mortem examination, necropsy) is a surgical procedure that consists of a thorough examination of a corpse by dissection to determine the cause, mode, and manner of death or to evaluate any disease or injury that may be present for research or educational purposes.
- Autopsies are performed by pathologists, medical doctors who have received specialty training in the diagnosis of diseases by the examination of body fluids and tissues.
- In academic institutions, autopsies sometimes are also requested for teaching and research purposes.
- Forensic autopsies have legal implications and are performed to determine if death was an accident, homicide, suicide or a natural event.

Autolysis

- When a body dies, there is an organized process of decomposition that begins almost immediately. One part of this process is **autolysis** (auto = self and lysis = breakdown), which is cellular self-digestion. This self-destruction of cells occurs as **endogenous** or internal cellular enzymes (endo = inside and genous = originating from) are released and work to break down cellular material.
- Autolysis is the breakdown of cell component or organism by its own enzyme.

Putrefaction

- Putrefaction is the decay of the organic matter by the action of microorganisms resulting in the production of a foul smell. It occurs between 10 to 20 days of the death of an organism. It is the fifth stage of death.
- Putrefaction involves the decomposition of proteins, breakdown of cohesiveness between the tissues, and liquefaction of most organs. The body is decomposed by the action of putrefying bacteria and fungi which releases certain gases that infiltrate and deteriorates the body tissues and organs. Putrefying bacteria play a major role in recycling nitrogen from the dead organism.

- Cytology is a study of structure, composition and function of cells.
- Cytopathology is a study of abnormal or diseased cells
- Autolysis is a cell death due self enzymes digestion
- Fixation is a process of preserving tissue or cells using chemical agent(s) in as life-like manner as possible.
- Fixative is a chemical agent that is used to preserve tissue or cells.
- Decalcification is a process of removing calcium from bone and calcified tissue.
- Dehydration is a process of removing free water (not molecularly bound water) from the tissue.

- Clearing is a process of replacing the dehydrating agent with a reagent that is miscible with paraffin wax.
- Infiltration/impregnation is a processing of filling in tissue, intracellular and extracellular, spaces with a medium which supports it during sectioning.
- Tissue blocking/ embedding/ casting is a process of enclosing the tissue in the infiltration medium used for processing and then allowing the medium to solidify.
- Microtomy/section cutting is a cutting of thin sections of tissue for microscopic examination using a microtome.
- Adhesive materials are materials which are thinly smeared on the microscope glass slided before mounting for the purpose of increasing adherence of tissue section to slide. E.g. Mayer's egg-albumin.

- Stain is a substance used to impart colour to tissue or cells, facilitate microscopic study and identification.
- Staining is a process of imparting colour to tissue or cells so as to facilitate microscopic study and identification.
- Mounting/coverslipping is a process of covering the stained slide with coverslip using special media in between them.
- Mountants are special media with gluing property used to facilitate adhering of coverslip to stained slide.
- Cytological aspirates is a specimen obtained by sucking fluid from the body for the purpose of harvesting cells for investigation.

Unit-2
Preparation of Tissue

Unfixed Tissue preparations

- **Imprint preparation:-**

These are prepared by touching a freshly cut piece of tissue with the surface of clean microscope slide. This way, cells are transferred and adhere to the slide. The smear can be examined with the phase contrast microscope or by using vital stain.

- **Impression smears:-**

Smearing a piece of fresh specimen of tissue evenly on the surface of microscope slide is an acceptable practice in histopathology. The making of such smears depends on the type of tissue to be examined. The smear can be examined fresh in which case it is stained as for teased preparation or by using supravital stain in conjunction with a warm stage. The preparation is never permanent.

- **Teased preparation –**

The fresh specimen of tissue, immersed in saline or. Ringer's solution, is dissected with mounted needles. Pieces of the tissue are picked onto a microscope slide and mounted as a wet preparation under a coverslip. The slide is then examined by the ordinary light microscope or better still by phase contrast microscope.

- **Frozen section:-**

Fresh tissue frozen on microtome with CO_2 can be cut into sections of about 10 to 15 um in thickness. The section are transferred to a dish and attached onto the slide before staining or from the dish carried on a glass rod through staining solution.

- **Squashed preparation:-**

Small pieces of tissue not more than 1mm in diameter are placed in the Center of a microscope slide. A coverslip is forcibly pressed down on them. Vital staining can be done by placing a drop of the stain at the junction of the slide and the coverslip. The stain 1s drawn in by capillary action and absorbed by the tissues.

Fixed Tissue preparations

- **Paraffin embedding**

  Paraffin is not miscible with water, but tissues are mostly water. In addition, most fixatives are aqueous solutions.

  This means that water must be removed from tissues before they are infiltrated with paraffin.

  It is done by dehydration of tissues with alcohol, mostly ethanol, from a graded series of alcohols from 50º to 100º (absolute o pure alcohol). All the water needs to be removed for a good embedding.

  After dehydration, samples are transferred to an intermediary liquid, like xylene, benzene, propylene oxide, or toluene, which are miscible with both absolute alcohol and paraffin.

  These are clearing substances and we can check their infiltration in the sample by observing how translucent the sample is.

The immersion of the sample in the intermediary liquid, like xylene and toluene, must not last very long because they harden the samples and getting sections might be more difficult.

The last step of the embedding procedure is to plunge the sample in melted paraffin.

It is done in an oven at a temperature properly set for the paraffin type we are working with. For a complete replacement of the intermediary liquid with paraffin, three changes in fresh paraffin are recommended.

How long the samples are incubated in paraffin depends on the intermediary liquid, size of the sample, and type of paraffin.

- **Celloidin embedding**

  Celloidin is dissolved in equal parts of absolute alcohol and ether. The tissue is dehydrated in alcohol in the same way as for paraffin except that it is transferred from absolute alcohol to a dilute solution of celloidin. As the alcohol and ether evaporate, they are replaced by more concentrated celloidin. It is finally hardened in chloroform and stored in 80 percent alcohol. It is a much longer process than paraffin but causes much less shrinkage and distortion. It is used especially in examination of the eye and brain.

- **Gelatin embedding**

  A method is described for embedding tissues in gelatin which makes it possible to cut thin sections for electron microscopy. With this method it is possible to embed the tissue without passing it through organic solvents extracting the lipid soluble components.

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Unit-3
Reception of Specimen

Reception

- Specimen Reception is the first point of contact with Laboratory Medicine and is the department where specimens requiring analysis are received.
- Specimen reception plays one of the most important roles in the pathology department.
- It is here that patient samples from many different wards, clinics, departments, other hospitals and GPs arrive so they can be sorted and sent, with relevant information, to the appropriate laboratory including immunology for testing.

- Reception staff provide support to the Biomedical Scientists (BMS’s), Clinical Scientists and Medical Staff and also carry out the pre-analytical preparation of samples.
- This includes the inputting of patient demographics and investigations into the laboratory computer system, labelling and sorting of pathological samples including blood, urine, Cerebrospinal Fluid (CSF), faeces and other body fluids.

Recording

- The number of specimen received daily may be small or large depending on whether the laboratory caters to a small or large hospital.
- However, it is essential that a records are kept from the outset. This is best done by having a reception book in which all specimens are recorded, including all the relevant details.
- These consist of the name, age, and sex of the patient, the OPD number, with hospital ward and bed number of the inpatient, the name of the clinician and the organ biopsied or excised with the clinical diagnosis.

- Give an identification number as lab record to every specimen. All the detail from request form which is sent by doctor with the specimen is recorded in the lab record as name of doctor and patient, date and time of collection, size of the specimen and name of fixative etc. are recorded in personal record of laboratory.
- On arrival each specimen is given an accession number. This is followed by the year of entry, e.g. 1/85, continuing
- throughout the year and starting again as 1/86. The specimen will carry this number until it is processe sectioned, reported and field.

labeling

- It is the process in which is done by the technician after receiving the sample in the histopathology lab. It can be done to every specimen after grossing for correct resulting and easy working.
- Once tissue have been selected for processing they are accompanied through all stage by a label bearing the number given to the specimen. The label is retained as a permanent record during sectioning and storage of tissue blocks.
- Very small biopsies like needle biopsies of kidney and liver, small curetting, etc. may be wrapped in filter paper soaked in formalin before being put in the capsules. Printed, graphite penciled, type written or India ink written labels are satisfactory. Ordinary ink should not be used as this may be dissolved in the reagents used during processing.

- Remains of all specimens are preserved in formalin until the reported on are discarded. This may be indicated by writing SK (stock kept) at the end of grossing notes.
- All specimen kept on the shelves are to be identified by legibly written number for future. All specimen of potential teaching value may be photographed and if considered worthy of display in the museum may be mounted.

Preservation:

- The act or process of preserving, or keeping safe; the state of being preserved, or kept from injury, destruction, or decay; security; safety; as, preservation of life, fruit, game, etc.; a picture in good preservation.
- The specimen is placed in a liquid fixing agent (fixative) such as formaldehyde solution (formalin). This will slowly penetrate the tissue causing chemical and physical changes that will harden and preserve the tissue and protect it against subsequent processing steps.

Unit-4
Fixation (Histological Specimens)

Fixation & Fixative

- **Fixation**: It is the preservation of biological tissues from decay due to autolysis or putrefaction. It terminates any ongoing biochemical reactions and may also increase the treated tissues' mechanical strength or stability.
- Fixation is done to maintain the structure of tissues in almost lifelike conditions before they are ready to be examined under the microscope.

Fixation also serves the following important functions.

- It prevents the autolysis and bacterial decomposition/ Putrefaction. Autolysis is most rapid in brain and Kidney.
- It coagulates the tissue to prevent the loss of diffusible substances. It fortifies the tissue against the deleterious effect of various stages in the preparation of the section, like Dehydration, Clearing and Wax impregnation.

CLASSIFICATION OF FIXATIVES

Fixatives can be functionally classified into two major groups:

- ***Simple Fixatives*** **–** These fixatives are made up of simple chemical compounds and take more time for the fixation of tissues. For example, Formalin, Picric acid, Mercuric oxide, osmic acid, Osmium tetroxide etc.
- ***Compound Fixatives*** **–** These are the mixtures of a number of fixatives in definite proportion and require a lesser amount of time for fixation. For example, Susa fluid, Carnoy's fluid, Bouin's Fluid, Formal saline, buffered formalin etc.

The compound fixatives can further be classified into three types as follows:

1. **Micro anatomical fixatives:** These fixatives are used for routine work of normal and histopathological study. For example, buffered formalin, Zenker's fluid, Bouin's fluid etc.
2. **Cytological fixatives**: These are intended to preserve the constituents elements of the cells themselves.
3. **Histochemical fixatives: These** are used for the Histochemical studies of the tissues where the minimum or no changes in the components to be demonstrated are required. for example, Buffered formalin or vapor fixatives include Formaldehyde, Glutaraldehyde, Acrolein etc.

SIMPLE FIXATIVES

- **FORMALDEHYDE:**
  - Commercial formaldehyde is saturated solution of formaldehyde (H.CHO) gas in water, approximately 40% gas by weight.
  - 10% of formalin used for fixation is prepared by adding 10ml of formalin to 90ml of saline.

ADVANTAGES:

i.) It fixes the proteins without precipitation.
ii.) Has no effect on Carbohydrates.
iii.) Preserves Glycogen and Lipids.

DISADVANTAGES

i.) It causes little Shrinkage.
ii.) Over hardens the tissue if left for a long time in formaldehyde solution.

- **GLUTARALDEHYDE**
  - Stable at 0 to 4^{o}c and at PH 3.0 to 5.0
  - To remove the impurities in Glutaraldehyde which are polymers of glutaraldehyde (eg Acrolein, Ethanol, Glutaric acid etc) Charcoal is added.
  - For fixation 2.5 % to 4% conc. is required.
- **Advantages**
  1. Formation of more cross linkages with better preservation of cellular & fluid proteins
  2. Resists acid hydrolysis
  3. Causes less shrinkage than formalin
  4. More pleasant & less irritant
  5. Does not cause dermatitis

- **Disadvantages:**
  1. Expensive
  2. Less stable
  3. Penetrates tissue more slowly from formalin
  4. Inferior formalin for PAS satin.

- **Formal Mercuric chloride**
  - Mercuric chloride -30g
  - Distilled water -900ml
  - Formalin -100ml

ADVANTAGES:

i.) It precipitates the proteins and hardens the tissue.
ii.) Has beneficial effect on staining.
iii.) Causes neither Shrinkage nor Swelling.

DISADVANTAGES

i.) It damages the tissue lipids.
ii.) It is difficult to make frozen sections after fixing with Mercuric Chloride.

- **OSMIUM TETROXIDE**
  - Used in electron microscopy
  - Used in fixing material for ultrathin sections for electron microscopy

ADVANTAGES:

i.) It fixes fats, conjugated lipids and mitochondria.
ii.) It preserves all the details of tissues.
iii.) Excellent fixative for the Electron microscopy.

DISADVANTAGES:

i.) May produce Black coloration on the tissue.
ii.) It is very expensive.
iii.) Its vapors are irritating and can cause Conjunctivitis.

- ***PICRIC ACID***
  - It gives better preservation of alcohol
  - Picric acid forms protein picrates, some of which are water soluble until treated with alcohol

ADVANTAGES:

i.) It precipitates & combines with proteins to form picrates.
ii.) Preferred fixative for connective tissues.
iii.) Prevents over hardening of tissue during dehydration.
iv.) Preserves glycogen well & does not shrink the tissues.

DISADVANTAGES:

i.) It does not fix the carbohydrates.
ii.) Picric acid is Highly explosive.

- **Chromic acid:**
  - Chromic acid is a strong oxidizer hence used with other fixatives, but not alcohols and formalin.
  - Coagulate proteins and fixes carbohydrates.
  - If tissue is not washed well after fixation in chromic acid, an insoluble precipitates will be formed.
- **Potassium dichromate**
  - Potassium dichromate is an orange crystalline substance used at 2% solution with water, fixes tissue by oxidizing proteins.
  - If mixed with ethanol it forms insoluble lower oxide that can not be removed from issue.
  - Tissue fixed in potassium dichromate must be washed thoroughly in water before commencing dehydration in alcohols.

COMPOUND FIXATIVES

- **10% of buffered neutral formalin**
  - Water -900ml
  - NaH2Po4 (anhydrous) -3.5gm
  - Na2HPo4 (anhydrous)-6.5gm
  - Formalin -100ml
  - Hydrated salts –
    - NaH2Po4.H2O-4.02g
    - Na2HPo4 .12H2O-16.37g/l

ADVANTAGES:

i.) It fixes proteins without precipitation.
ii.) Fats are preserved and can be stained by suitable methods.
Formalin pigment is not formed.

DISADVANTAGES:

i.) High strength of formalin can causes the shrinkage of tissues.
ii.) Over-exposure may over-hardens the tissue.

- **HEIDENHAIN SUSA**
  - HgCl2 -45gm
  - Nacl – 5gm
  - Formalin (40% formaldehyde solution)- 200 ml
  - Glacial acetic acid – 40 ml
  - Trichloroacetic acid – 20 gm
  - Distilled water – 800 ml

ADVANTAGES:

i.) Tissues are fixed quickly.
ii.) Gives Rapid and even penetration with minimum Shrinkage.

DISADVANTAGES:

i.) Over exposure can bleaches the tissue and over hardens it.
ii.) Tissue requires a treatment with iodine to remove mercury pigments.

- **CARNOY's FLUID**
  - Absolute ethylalcohol – 60 ml
  - Choloform – 30ml
  - Glacial acetic acid -10ml

ADVANTAGES:

i.) It is one of the most penetrating fixative.
ii.) It rapidly fixes the tissue.
iii.) After fixation the tissues can be directly transferred to 90-100% Alcohol.

DISADVANTAGES:

i.) It causes lysis of Red blood cells and much shrinkage.
ii.) Some cytoplasmic granules may be preserved.

- **BOUIN's FLUID**
  - 1.2% aqueous picric acid -75ml
  - Formalin – 25ml
  - Glacial acetic acid – 5ml

ADVANTAGES:

i.) It penetrates evenly and rapidly.
ii.) Causes less shrinkage and can be used to demonstrate glycogen.
iii.) Tissues may be left in it for months without any harm.

DISADVANTAGES:

i.) It is not suitable for tissues containing mucin, since it becomes greatly swollen.
ii.) The cortex of Kidney is badly preserved.
iii.) It is necessary to remove excess picric acid by washing or by alcohol treatment.

- **ZENKER's FLUID**
  - HgCl2 – 50gm
  - Potassium dichromate – 25gm
  - Sodium sulphate – 10gm
  - Distilled water – 1000ml
  - Add 50ml glacial acetic acid before use (5 ml/dl of stock)

ADVANTAGES:

i.) It rapidly and evenly penetrates the tissue.
ii.) It is a good routine fixative.

DISADVANTAGES:

i.) It is unstable after the addition of Acetic acid, hence acetic acid (or formalin) should be added just before use.

Unit-5
Processing (by Paraffin Technique)

- **Tissue processing:** A procedure which need to take place after gross examination between tissue fixation and the embedding and then sectioning of paraffin blocks is called tissue processing.

There are some basic steps for tissue processing:

- Dehydration
- Clearing/Dealcoholization
- Infilteration and impregnation
- Paraffin embedding
- Sectioning
- staining

DEHYDRATION

- The first stage in tissue processing is dehydration (the removal of water). In tissues, water is present in both free and bound forms and needs to be removed before processing can continue.
- Dehydration is usually carried out using alcohols (such as ethanol) but these can dissolve certain cellular components such as lipids.
- Although dehydration can also cause tissue shrinkage, the stage is necessary in all infiltration methods, except where tissues are supported by an aqueous embedding medium (such as water-soluble waxes).
- In paraffin wax processing, dehydration from aqueous fixatives such as formalin is usually initiated in 70% alcohol before progressing through 90%-95% to absolute alcohol before proceeding to the clearing stage.

CLEARING

- Clearing is the transition step between dehydration and infiltration with the embedding medium.
- The term clearing arises because some solvents have a high refractive index. When dehydrated tissues are placed into these reagents, they are rendered transparent.
- This property is used to determine the endpoint and duration of the clearing step since the presence of opaque areas indicates incomplete dehydration. Clearing agents are fat solvents and therefore remove fat from the tissue.
- It must be noted that shrinkage occurs when tissues are transferred from the dehydrating agent to the clearing agent and from the clearing agent to wax.

- In the final stage shrinkage may result from the extraction of fat by the clearing agent.
- Xylene is the most popular clearing agent and several changes of it are required to completely displace the ethanol.
- The choice of a clearing agent depends upon the type of tissue processor used, the processing conditions such as temperature, safety factors and cost.

Infiltration

- This is the saturation of tissue cavities and cells by a supporting substance which is generally the medium in which they are finally embedded.
- The most common agent of choice is paraffin wax which is molten when hot and solid when cold.
- An infiltrating and embedding medium should ideally be molten between 30°C and 60°C and suitable for sectioning.
- Additionally, the properties of the medium should be similar to those of the tissues to be sectioned with regard to density and elasticity.
- Various substances have been used to infiltrate and embed tissues in readiness for eventual section cutting or microtomy.

Embedding

- Paraffin embedding is the standard method used in histology laboratories to produce blocks of tissue for section cutting (microtomy).
- After tissue have been dehydrated, cleared and infiltrating with embedding material like paraffin ,agar ,gelatin, which is then hardened.
- This is achieved by placing tissue in a metalic angle or leuckharts moulds and cooling in case of paraffin and heating in case of epoxy resin.
- In case of automated tissue processor tissues are still in the casettes and pick the tissue out of the casettes and pour molten paraffin over them.

MICROTOMY

- A microtome is a tool used to cut extremely thin slices or sections of tissue for light microscopy studies.
- The most commonly used microtomes in the histology laboratory are the rotary and sledge varieties (see images below). Microtomes use steel, glass, or diamond blades depending upon the specimen and thickness of the section required.
- Nowadays, disposable steel blades are generally used to prepare paraffin sections of tissues for light microscopy histology.

AUTOMATIC TISSUE PROCESSOR MACHINE (ATPM)

- A tissue processor is a device that prepares tissue samples for sectioning and microscopic examination in the diagnostic laboratory.
- Microscopic analysis of cells and tissues requires the preparation of very thin, high quality sections (slices) mounted on glass slides and appropriately stained to demonstrate normal and abnormal structures.
- The ATP machine plays a big role in the preparation of the tissue by passing them through various chemicals; a major process called TISSUE PROCESSING.

TISSUE PROCESSING

- The ATPM works by following through an already established processing steps.
- Tissues to be processed are cut into small pieces to ensure the tissue fits into the **tissue cassettes.**
- Smaller tissues (2-4 um) will be processed faster than the whole tissue or organ.
- These tissue cassettes are packed into the **oscillating tissue basket** to tissue prior to fixation.

- **FIXATION** – this is the process of preserving or fixing tissues by passing them through chemicals called fixatives. The fixatives will help protect the tissue from decay and autolysis. Routine fixative of use is 10% formalin.
- **DEHYDRATION** – this is the process of removing water molecules from the tissue by passing the tissue through ascending grades of alcohol. E.g methanol, acetone, 70-100% alcohol.
- **CLEARING** – this is the process of removing alcohol from the tissue by passing it through chemicals that will remove the alcohol molecules. These agents are called clearing agents. Xylene is mostly used for clearing.

- **INFILTRATION** – this is the process of filling intracellular spaces left in the tissue by paraffin wax. This will help confer a bit of rigidity to the processed tissue.
- **EMBEDDING-** this last step is manually done. This has to do with immersing the processed tissue into a mould containing liquid paraffin wax. This is for external support so that the tissue won’t crumble during microtomy

PARTS OF THE ATPM

- Oscillating tissue basket
- 10 beakers or jars
- 2 thermostatically controlled beakers
- An electric rotor at the base
- Lifting mechanism
- Time disc and alarm system
- Control unit - with display screen and control buttons

WORKING PRINCIPLE OF AUTOMATIC TISSUE PROCESSOR MACHINE

- The tissue basket oscillates up and down in each station at three-second intervals to ensure thorough and even mixing of the reagents and optimum tissue infiltration.
- Infiltration time is separately programmable for each station. Up to nine programs may be run with immediate or delayed starting times.
- When it’s time for tissue to be transferred to the next beaker or jar, the cover of the machine is raised up, and the lifting mechanism carefully removes the tissue basket and gently transfers it to the next beaker.

- When the infiltration time for any particular station is exceeded, a warning message will display, indicating the station number and excess time.
- Controls are arranged by functionality with an LCD to indicate operational parameters. Reagent container lids have seals to minimize operator exposure to hazardous fumes.
- Tissue basket immediately immerses in a station in the event of power loss to protect samples from drying out.
- When power is restored, program will resume. In the event of long-term power failure, wax is liquified. Capacity of tissue basket is 80 cassettes.

- Vacuum configurations hasten infiltration, allowing pressure to be applied to any station in either manual or automatic operation.
- Fume control configurations extract fumes with a fan and pass them through an internal carbon filter.
- For added efficiency, these models feature a two-part containment shield surrounding the reagent container platform.

ADVANTAGES OF ATPM

- It’s very efficient
- Saves time and energy to operate
- Cost effective and user friendly
- Can process different tissues same time
- The machine does the transfer of tissue from one bath to another.

MICROTOMY

Microtomy :

- Is the means by which tissue can be sectioned and attached to a surface for further microscopic examination.

Microtome:

- Basic instrument used in microtomy.
- Mechanical device for cutting thin uniform slices of tissue – sections.

Types of microtomes

- There are 5 basic types of microtomes named according to the mechanism-

- Rocking microtome
- Rotary microtome
- Base sledge microtome
- Sliding microtome
- Freezing mictotome.

Rocking microtome:

- Name derived from the rocking action of the cross arm.
- Oldest in degisn, cheap , simple to use.
- Extremely reliable.
- Very minimum maintenance.

Mechanism of action:

- Knife is fixed, the block of the tissue moves through an arc to strike the knife.
- Between strokes the block is moved towards the knife for the required thickness of sections by means of a ratchet operated micrometer thread.
- Steady backward and forward movement of the handle gives ribbons of good sections.

Disadvantage:

- Size of the block that can be cut is limited.
- Sections are cut in a curved plane:
  (Microtomes designed to cut perfectly flat sections; the block moving through an arc at right angles to the knife edge are available.)
- Light instrument : advisable to fit it into a tray which is screwed to the bench , or to place it on a damp cloth to avoid movement during cutting.

Rotary microtome

- First machine designed by Professor Minot,hence often referred to as the "Minot Rotary".

Mechanism:

- The hand wheel rotates through 360 degree moving the specimen vertically past the cutting surface and returning it to the starting position.
- Block holder is mounted on a steel carriage which moves up and down in grooves and is advanced by a micrometer screw- cutting perfectly flat sections.

- Manual (completely manipulated by the operator).
- Semi-automated (one motor to advance either the fine or coarse hand –wheel)
- Fully automated (two motors that drive both the fine and the coarse advace hand-wheel)
- Mechanism of block advancement: retracting or non retracting.
- Retracting action moves the tissue block away from the knife on upstroke, producing a flat face to the tissue block.

Advantages:

- Ability to cut thin 2-3 mm sections.
- Easy adaptation to all types of tissues (hard, fragile, or fatty) sectioning.
- Ideal for cutting serial sections: large number of sections from each block.
- Cutting large blocks
- Cutting angle of knife is adjustable.
- Large and heavier knife used-less vibration when cutting hard tissue.
- Heavier and more stable .

Sledge microtome

- Originally designed for cutting sections of very large blocks of tissue (eg. whole brains)
- Used primarily for
- Large blocks,hard tissues,whole mounts.
- Especially useful in neuropathology and ophthalmic pathology.

Mechanism of action:

- The block holder is mounted on a steel carriage which slides backwards and forwards on guides against a fixed horizontal knife.

Advantages:

- Heavy , very stable, not subject to vibration.
- Knife large(24 cm in length) and usually wedge shaped –less vibration .
- Adjustable knife holding clamps allow tilt and angle of the knife to the block to be easily set
  – used for cutting celloidin sections by setting the knife obliquely
  - paraffin wax embedded sections are more easily cut .

Disadvantages

- Slower in use than rocker or rotary microtome- true only when change from one instrument to another is made .
- With practice, sections from routine paraffin blocks can be cut as quickly as on any other type of microtome.

Sliding microtome

- Designed for cutting celloidin-embedded tissue blocks.
- The knife or blade is stationary, specimen slides under it during sectioning.
- Also used for paraffin –wax embedded sections.

Freezing microtome

- Gives best results for cutting frozen sections.
- Machine is clamped to the edge of a bench and connected to a cylinder of CO_2 by means of a specially strengthened flexible metal tube.

Freezing microtome

- Knife freezing attachment is supplied with most machines.
- Separately controlled flow of CO2 on the edge of the knife - to delay the thawing of sections on the knife and make it possible to transfer them directly from knife to slides.
- Sections thickness gauge is graduated in units of 5 micrometer instead of 1micrometer.

Vibrating microtome

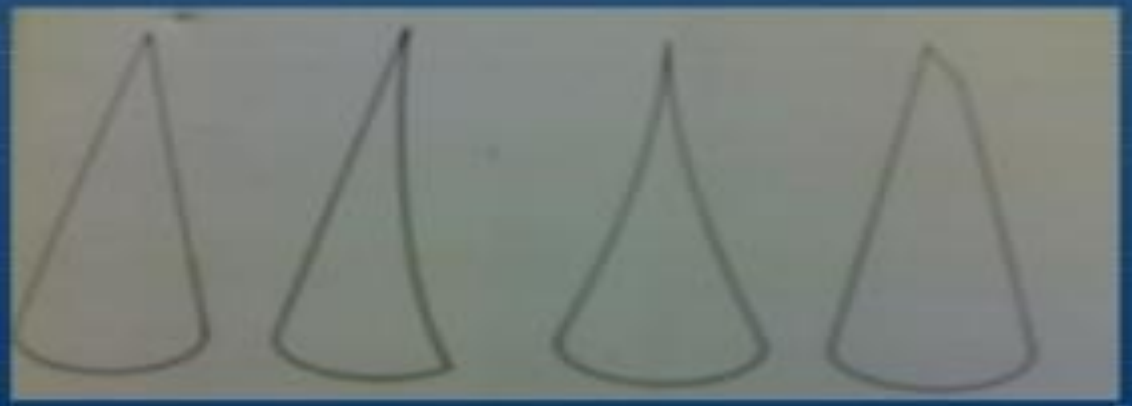
- Designed to cut tissue which has not been fixed, processed or frozen.
- Used in enzyme histochemistry ,ultrastructural histochemistry.
- During sectioning, the tissue is immersed in either water ,saline or fixative.
- It is cut by a vibrating razor blade , at thickness generally greater than used for paraffin wax.
- Tissues are cut at a very slow speed to avoid disintegration.

Microtome knives

- Developed to fit specific types of microtomes and cope with different degrees of hardness of tissues and embedding media.
- Paraffin-wax embedded tissues knives are made of steel.
- Resin-embedded tissue is normally cut using glass knives.

Knives are classified according to their shape when viewed in profile as:

- Wedge.
- Planoconcave.
- Biconcave.
- Tool edge or D profile.



Wedge :

- Originally designed for cutting frozen sections
- Gives great rigidity to the knife
- Used for cutting all types of section on any microtome.

Plano-concave:

- Used primarily for cutting nitrocellulose – embedded tissues.
- Available with varying degrees of concavity.

Biconcave :

- Classical knife shape introduced by Heiffor.
- Used with the rocking microtome.
- Relatively easy to sharpen.
- Less rigid , prone to more vibrations.
- With gradual adoption of more substantial microtomes , this knife design has lost popularity.

Tool edge(D-profile):

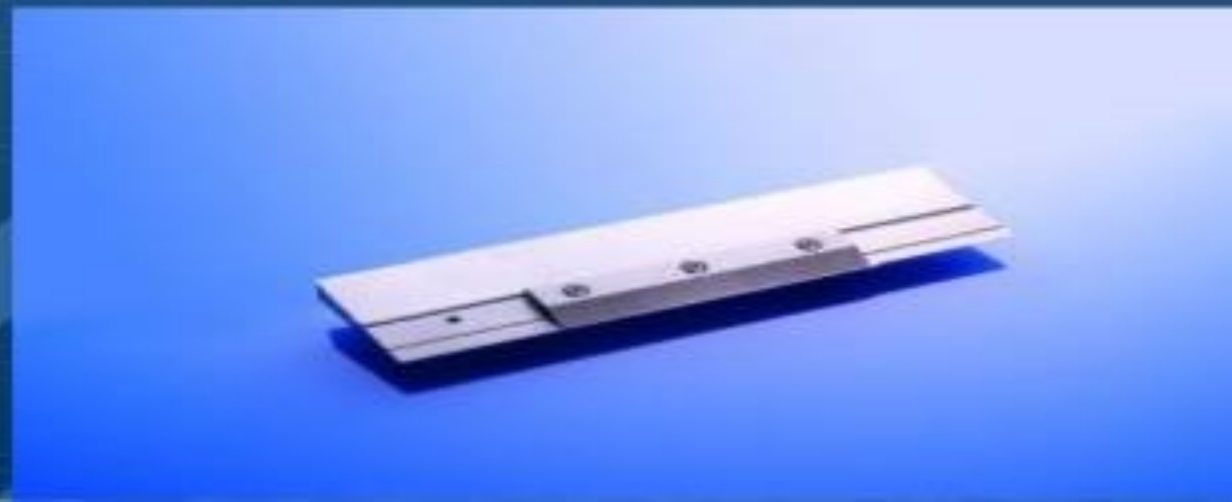
Called 'chisel edge', similar to a woodworker's chisel.

Used primarily to section exceptionally hard tissue.

- Decalcified dense cortical bone.
- Undecalcified bone.
- Stouter than conventional knives to give added rigidity.
- Edge may be coated with tungsten-carbide for increased life.

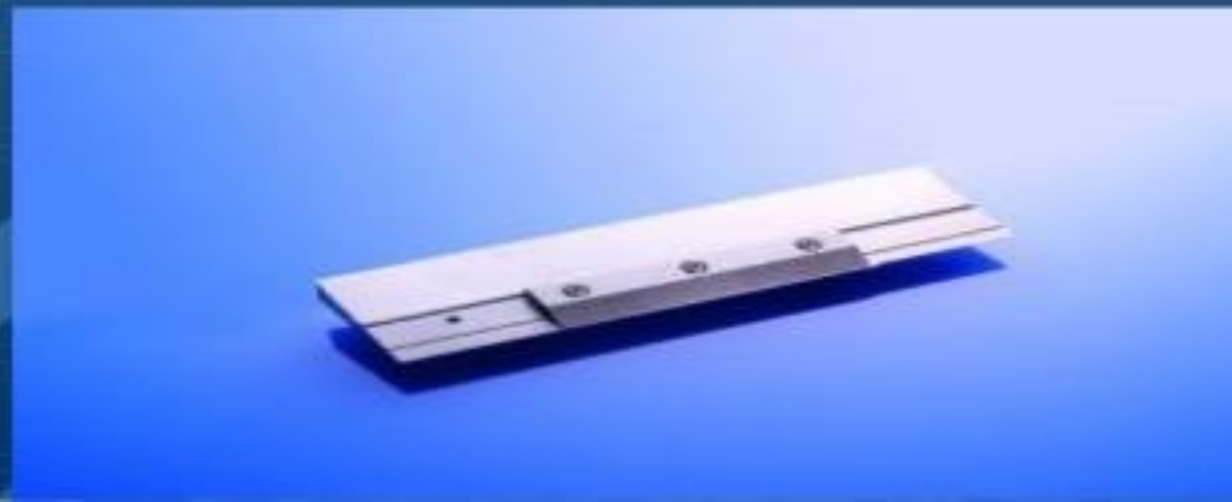
Disposable blades

- Used for routine microtomy and cryotomy.
- Provide a sharp cutting edge, produce flawless 2-4 mm sections.
- Disposable blade holders incorporated into the microtome or an adapter.



Disposable blades

- Blade is coated with PTFE (polytetrafluoroethylene) allowing ribbons to be sectioned with ease.
- Over-tightening the disposable blade in the clamping device may cause cutting artifact such as thick and thin sections.



Glass and diamond knives

- Used in electron microscopy and with plastic resin-embedded blocks.

Microtome knife sharpening

- Manual procedure or automatic procedure.

- 1) Abrasive grinding of the facets [HONING]
- 2)Polishing [STROPPING]

Abrasive grinding of the facets [HONING]

Glass plates:

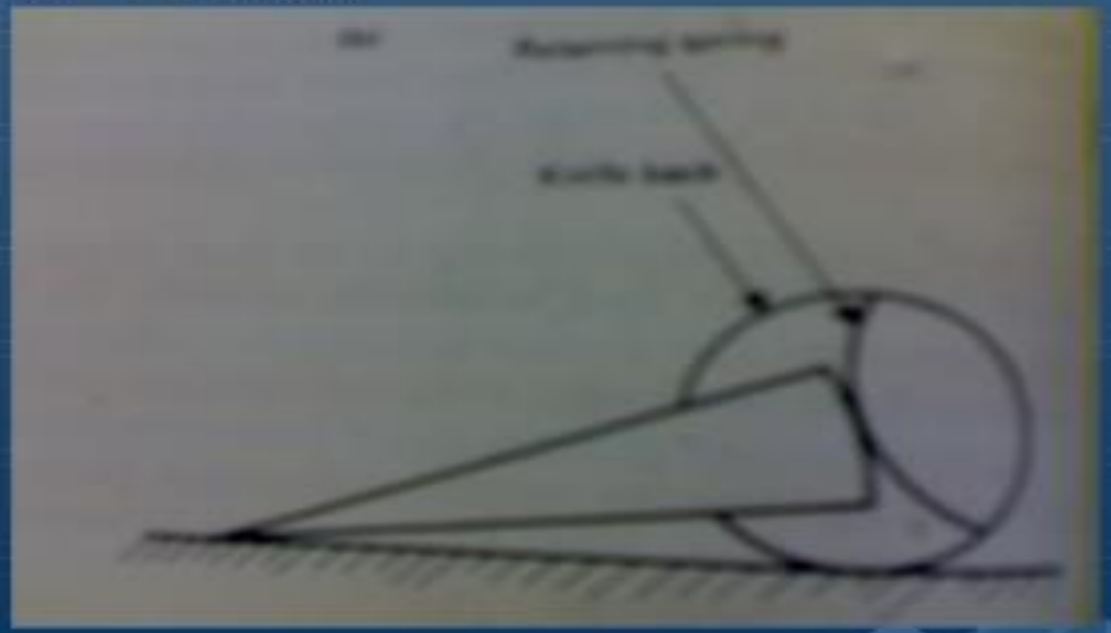
- hand sharpening
- Readily available ,cheap
- Surface roughened to enable particles of abrasive to adhere to the glass .
- Easily cleaned after use.

Copper and bronze plates: automatic knife sharpening machines.

- Expensive , superior properties

Manual method

- Knife with a 'back' effectively raises the non cutting edge up off the hone.
- Back of the knife is ground simultaneously with the edge , hence reserved for use only with that particular knife.



Manual method

- Hone is placed on the bench on a non-skid surface (damp cloth) to prevent moving during honing.
- Small quantity of light oil or soapy water applied to the hone and smeared over the surface.
- Abrasive is applied to the glass or metal plate.
- Knife with handle and backing sheath is laid on the hone with cutting edge facing away from the operator , heel roughly in the centre of the nearest end of the hone.

Manual method

- Handle of the knife is held between the thumb and the forefinger .
- Thumb and forefinger of other hand rest on the other end of the knife to ensure even pressure along the whole edge of the knife.
- Knife is pushed forward diagonally from heel to toe ,turned over on its back and moved across the hone until the heel is in the centre with the cutting edge leading and then brought back diagonally.
- It is then turned over on its back and moved across the hone to its original position completing figure of eight movement.

Stropping

- Process of polishing an already fairly sharp edge.
- Types of strop: best strops made from hide from the rump of the horse marked ‘shell horse’.
- 2 types: flexible(hanging) and rigid.
- Flexible type:
- Back of the strop is made of canvas and is intended to support the leather during stropping.
- Strops should be kept soft by applying a small quantity of vegetable oil into the back of the leather

Stropping

- Strops should be kept free from grit and dust.
- Rigid type:
- Single leather strop stretched over a wooden frame to give a standard tension or a block of wood about 12x2x2 inches in size having a handle at one end with four grades of leather or even a soft stone cemented on each side.
- The sides of these strops are numbered and the knife is stropped on No1, then No2 and so on finishing on the finest leather.

Automatic knife sharpners

Automatic knife sharpners

- Two basic designs available.

  1) knife is held vertically with revolving sharpening wheels grinding the cutting edge.

  2) knife is held horizontally against the surface of a slowly rotating flat plate.

Automatic knife sharpners

- Plates – glass , copper or bronze charged with an abrasive.
- Glass plates need to be roughened before use to allow the abrasive particles to be held more easily in place.
- Copper and bronze plates used in conjunction with diamond paste, 6micrometer particle size being most appropriate for rough sharpening, and 1 micrometer for fine polishing.

Stropping Technique:

- Knife is laid on the near end of the strop with the cutting edge towards the operator (opposite direction to that used in honing.)
- Knife held with forefinger and thumb to facilitate easy rotation at end of each stroke.
- Action is exact opposite to that used in honing,using full length of the strop and stropping evenly the whole of the blade.

Paraffin section cutting

- Equipment required:
- Microtome.
- Flotation(water bath)
- Slide drying oven or hot plate
- Fine pointed or curved forceps.
- Sable or camel haired brush.
- Scalpel.
- Slide rack.
- Clean slides.
- Teasing needle.
- Ice tray.
- Chemical resistant pencil or pen.

Cutting technique

- Insert apprropriate knife in the knife-holder of the microtome and screw it tightly in position.
- Correctly set the adjustable knife angles.
- Fix the block in the block holder of the microtome
- Move the block holder forward or upward until the paraffin wax is almost touching the knife edge.
- Ensure that the whole surface of the block will move parallel to the edge of the knife,

Cutting technique

- Trim the excess wax from the block surface and expose the tissue,advance the block by setting the thickness to about 15 micrometer.
- Care should be taken not to trim too coarsely as

A)Small biopsies may be lost.

B) tissue in the block may be torn giving rise to considerable artefact.

C) unsuspected small foci of calcification may cause tears in the tissue and nicks in the knife.

- Once the surface of tissue has been revealed proceed to trim the next block.
- Replace the trimming edge by a sharp one and check it is tightly secured.
- Reset the thickness gauge to 4-5 micrometer.
- Insert the block to be cut and tighten securely.
- Bring the block face up until it nearly touches the knife edge.

- Paraffin-wax embedded tissue , sections are normally cut at a thickness of 4-5 micrometer.
- Thicker sections(10-20 micrometer) :demonstrate certain features of the central nervous system.
- Thin sections(1-2 micrometer): for examining highly cellular tissue such as lymph nodes.
- The amount of advance is operator determined most commonly in graduated 1 micrometer stages.

- Paraffin wax embedded tissue: the properties of the wax causes each section to adhere by its edge to the previous forming a ribbon of sections Ribbons should be held gently with a fine moistened brush or with a pair of fine forceps.
- Holding the ribbons with the finger is to be discouraged : section and water bath may become contaminated with the operator's exfoliated squames.
- Before being attached to the slides the creases must be removed and the sections flattened.
- This is achieved by floating them on warm water.

Flotation (water bath)

Flotation(water bath)

- Thermostatically controlled water baths for floating out tissue ribbons after sectioning.
- To remove the creases and flatten the sections.
- Temperature of water in the bath should be 10 degree celcius below the melting point of paraffin employed.
- Distilled water may be used to prevent water bubbles from being trapped under the sections.
- Alcohol or a small drop of detergent may be added to the water to reduce the surface tension-to flatten out the sections with ease.

Flotation(water bath)

- Sections which are curled will flatten on warm water, creases removed.
- To remove air bubble, thick sections of wax which curl into a roll during trimming are used. Hold one roll in the end of a pair of forceps and bring the end of the wax roll up under the section to touch the air bubble.The bubble will adhere to the wax roll and come away with it when removed.

Mounting the section on a slide:

- A clean slide is half submerged in water and brought into contact with the edge of the section.
- Section approached from the side, straight approach will push the section away.
- Section oriented on wet slide using the edge of the forceps or dissecting needle.
- Section should be centrally positioned on the slide.
- Slide should be identified by inscribing the appropriate no.on the slide with a diamond pencil.

Section adhesives

Sections may detach from the slides:

- Exposure to strong alkali solutions during staining.
- Cryostat sections for immunofluorescense, immunohistochemistry ,or intraoperative diagnosis.
- Central nervous system tissues.
- Decalcified tissues.
- Tissues containing blood and mucus.
- Sections submitted to extreme temperatures.

- For sections from ester or polyester –wax embedded tissue , adhesives are mandatory.

Albumen

- Equal parts of glycerin,distilled water and egg white are mixed filtered through coarse filter paper.
- A crystal of thymol is added to inhibit the growth of moulds, solution kept in refrigerator.
- Small quantity of the solution is smeared over the surface of the slide immediately before mounting sections from the water bath.

Gelatin :

- May be used as a 0.5% solution in distilled water.
- Liable to be contaminated with moulds ,needs to be melted with gentle heat before use.

Araldite:

- Clean slides are coated with 1 in 10 dilution of the resin in acetone immediately before use.
- As section dries ,resin polymerizes forming a rigid bond between tissue and slide.

Starch:

- Successful adhesive .
- Lost popularity due to staining reactivity with many dyes.

Poly-L-lysine :

- As 0.1% solution, diluted further for use 1 in 10 with distilled water.
- Effectiveness diminishes in few days.

Serial sections

Necessary to cut and preserve every section from a piece of tissue or from a specific area .

Required:

- To identify small ulcer
- Presence of malignant cells tracking along a lymphatic or neural sheath.
- Scarce organisms such as acid fast bacilli.
- In embryology.

Problems and solutions for paraffin section.

Problem:
Ribbon/consecutive sections curved.

1) Block edges not parallel
2) Dull blade edge.
3) Excessive paraffin.
4) Tissue varying in consistency

Solutions

1) Trim block until parallel.
2) Replace balde.
3) Trim away excess paraffin.
4) Re-orient block

Problem: Thick and thin sections

1) Paraffin too soft for tissue
2) Insufficient clearance angle
3) Faulty microtome mechanisms
4) Blade or block loose in holder.

Solutions

1) Remove excess paraffin
2) Increase clearance angle.
3) Check for faults in microtome.
4) Tighten block and blade

Problem:

Sections will not form ribbons

1) Paraffin too hard for sectioning.
2) Debris on knife edge.
3) Clearance angle incorrect.

Solutions

1) Re-embed in lower melting point paraffin.
2) Warm surface of block
3) Clean blade and back of blade holder
4) Adjust to optimal angle.

Problem: sections attach to block on return stroke

1) Insufficient clearance angle.
2) Debris on blade edge.
3) Debris on block edge.
4) Static electricity on ribbon.

Solutions

1) Increase clearance angle.
2) Clean blade edge.
3) Trim edges of block
4) Humidify the air around the microtome.
5) Place static guard or dryer sheets near microtome.

Unit-7
Theory of staining (Routine)

H&E Staining

- For routine diagnosis, the use of Hematoxylin and Eosin (H&E) is by far preferred for viewing cellular and tissue structure detail by pathologists.
- The variation of stain intensity is often driven by the pathologist's learning experience and personal preference.
- Because this stain demonstrates such a broad range of cytoplasmic, nuclear, and extracellular matrix features, nearly all teaching texts use H&E images.
- In a high quality H&E there are subtle differences in the shades of color produced by the stains, particularly eosin, and this aids in the detection and interpretation of morphological changes associated with disease.

The staining procedure for H&E follows a basic protocol

- Dewaxing
- Dehydration
- Hematoxylin
- Differentiation
- Bluing
- Eosin
- Dehydration
- Clearing
- Cover-slipping

Remove the Wax

- Following the preparation of a paraffin section, all the elements are infiltrated with and surrounded by paraffin wax which is hydrophobic and impervious to aqueous reagents.
- The majority of cell and tissue components have no natural color and are not visible.
- The first step in performing an H&E stain is to dissolve all the wax away with xylene (a hydrocarbon solvent).

Hydrate the Section

- After thorough de-waxing, the slide is passed through several changes of alcohol to remove the xylene, then thoroughly rinsed in water. The section is now hydrated so that aqueous reagents will readily penetrate the cells and tissue elements.

Apply the Hematoxylin Nuclear Stain

- The slide is now stained with a nuclear stain such as Harris hematoxylin, which consists of a dye (oxidized hematoxylin or hematein) and a mordant or binding agent (an aluminium salt) in solution. Initially this stains the nuclei and some other elements a reddish-purple color.

Complete the Nuclear Stain by “Blueing”

- After rinsing in tap water, the section is “blued” by treatment with a weakly alkaline solution.
- This step converts the hematoxylin to a dark blue color.
- The section can now be rinsed and checked to see if the nuclei are properly stained, showing adequate contrast and to assess the level of background stain.

Remove Excess Background Stain (Differentiate)

- On most occasions when Harris hematoxylin is employed, a differentiation (de-staining) step is required to remove non-specific background staining and to improve contrast.
- A weak acid alcohol is used.
- After this treatment, blueing and thorough rinsing is again required.
- Staining methods that include a de-staining or differentiation step are referred to as “regressive” stains.

Apply the Eosin Counterstain

- The section is now stained with an aqueous or alcoholic solution of eosin (depending on personal preference).
- This colors many non-nuclear elements in different shades of pink.

Rinse, Dehydrate, Clear and Mount (Apply Cover Glass)

- Following the eosin stain, the slide is passed through several changes of alcohol to remove all traces of water, then rinsed in several baths of xylene which “clears” the tissue and renders it completely transparent.
- A thin layer of polystyrene mountant is applied, followed by a glass cover slip. If the stain and all the subsequent steps have been properly performed, the slide will reveal all the important microscopic components and be stable for many years.

Unit-8
Mountants

- **Mountant** any substance in which a specimen is suspended between a slide and a cover glass for microscopic examination.
- The **mounting medium** is the solution in which the specimen is embedded, generally under a cover glass. It may be liquid, gum or resinous, soluble in water, alcohol or other solvents and be sealed from the external atmosphere by non-soluble ringing media.
- The main purpose of mounting media is to physically protect the specimen; the mounting medium bonds specimen, slide and coverslip together with a clear durable film. The medium is important for the image formation as it affects the specimen's rendition.

Properties of an Ideal Mounting Media (Mountant)

- It should be colorless and transparent.
- It should not cause stain to diffuse or fade.
- It should be dry to a non-stick consistency and harden relatively quickly.
- It should not shrink back from the edge of cover-glass.
- It should be able to completely permeate and fill tissue interstices.
- It should have no adverse effect on tissue components.
- It should be resistant to contamination (particularly microorganism growth).
- It should protect the section from physical damage and chemical activity.
- It should be completely miscible with dehydrant or clearing agent.

Classification of Mounting Media

1. Resinous media
2. Aqueous media

Resinous media

- These are natural resins such as Canada balsam and gum dammar. For many years these were used for mounting sections.
- These natural resins usually dissolve in xylene.
- They are inherently acidic and caused fading of some stains after the sections were stored for several years.
- They also set very slowly. Sometimes taking months to harden to non-stickiness. They also tend to yellow with age.
- Resinous media consists of solid resins which are dissolved in an appropriate solvent.

- The viscosity of the medium should be such that the solution will enter the tissue spaces and flow readily between the slide and the cover glass.
- Air bubbles should be removed quickly. Most resinous media are dissolved in toluene. Because slides are usually mounted from xylene, xylene should be the solvent for the mounting media.
- Toluene is more volatile than xylene so bubbles are more likely to appear.

AQUEOUS MOUNTING MEDIA

- Aqueous mounting media are used when dehydrating and clearing will adversely affect the stain.
- They can be classified for use in histology as simple syrups, gum arabic media, and glycerol gelatins.
- Both gum arabic and glycerol gelatins media cause, or allow diffusion of basic aniline dyes into the surrounding medium. This can be prevented by adding large amounts of sugar (sucrose), fructose, or D-sorbitol, to the gum Arabic or glycerol gelatin media.
- The syrups remain wet and sticky in most climates and will only serve as temporary mounting media. Aqueous mounting media have an index of the fraction that differs greatly from that of the tissue.

Unit-9
Various Terms associated with staining

- **Solvent:** A **solvent** is a substance that becomes a solution by dissolving a solid, liquid, or gaseous solute. A **solvent** is usually a liquid, but can also be a solid or gas. The most common **solvent** in everyday life is water. Most other commonly-used **solvents** are organic (carbon-containing) chemicals.
- **Mordant:** A mordant or dye fixative is a substance used to set dyes on fabrics by forming a coordination complex with the dye, which then attaches to the fabric. It may be used for dyeing fabrics or for intensifying stains in cell or tissue preparations.
- **Progressive staining:** It **stain** to a desired intensity and no more. Therefore they do not require differentiation in a dilute acid alcohol.
- **Regressive staining:**It means that the tissue is deliberately over **stained** and then de-**stained** (differentiated) until the proper endpoint is reached.

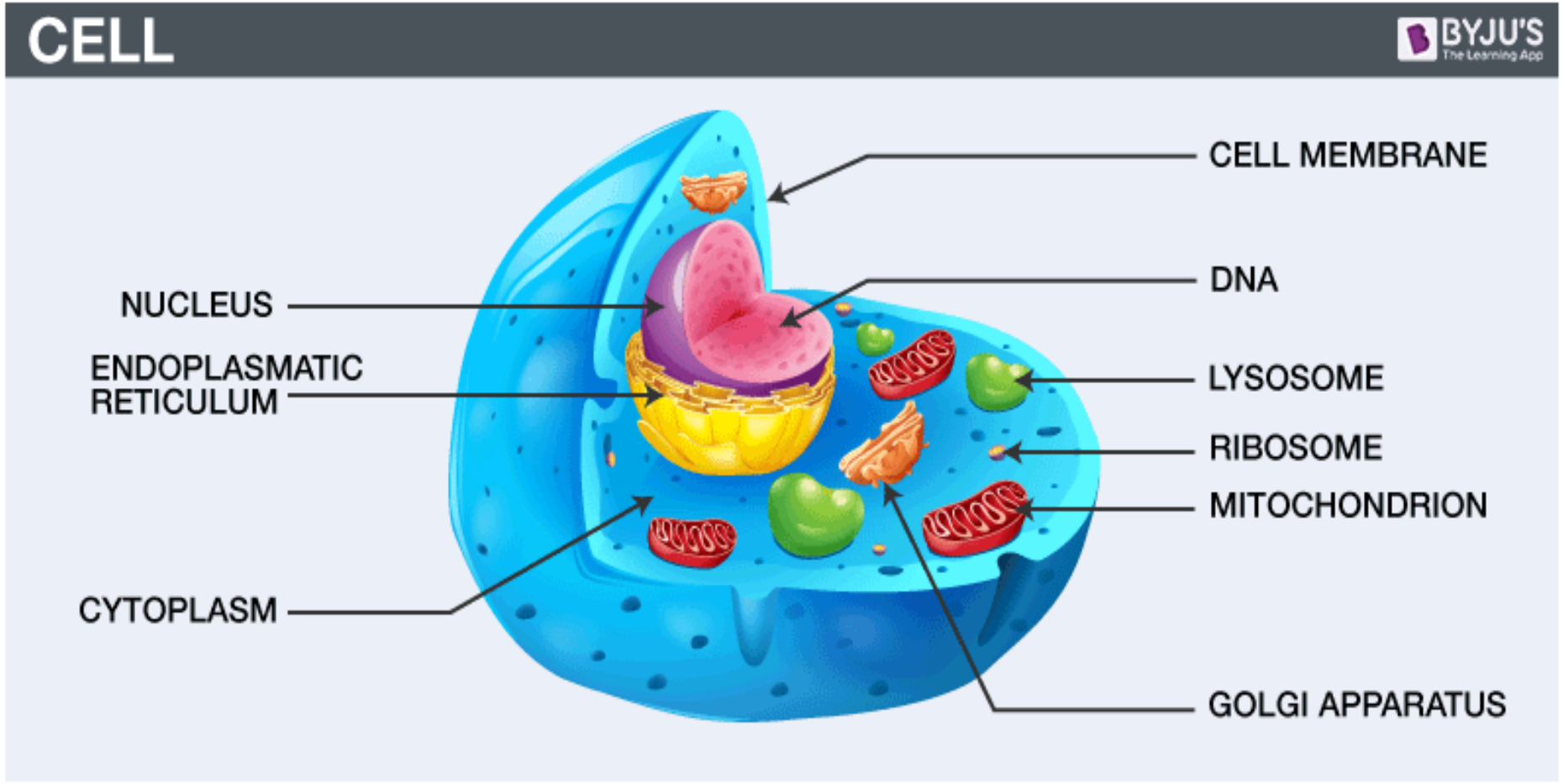
- **Accelerators:** An accentuator is any chemical which facilitates the staining process. Usually the purpose is to intensify staining, and accentuation with this meaning is obviously the derivation of the term. However, it should be noted that inhibition of staining can also accentuate a structure in comparison to the background staining.
- **Metachromasia:** It is a characteristical change in the color of staining carried out in biological tissues, exhibited by certain dyes when they bind to particular substances present in these tissues, called chromotropes. For example, toluidine blue becomes dark blue when bound to cartilage.

Unit-10
Cell

Cell:

- “A cell is defined as the smallest, basic unit of life that is responsible for all of life’s processes.”
- Cells are the structural, functional, and biological units of all living beings. A cell can replicate itself independently. Hence, they are known as the building blocks of life.
- Each cell contains a fluid called the cytoplasm, which is enclosed by a membrane. Also present in the cytoplasm are several biomolecules like proteins, nucleic acids and lipids.
- Moreover, cellular structures called cell organelles are suspended in the cytoplasm.

Structure of cell:



1. Cell Membrane

- The cell membrane supports and protects the cell. It controls the movement of substances in and out of the cells. It separates the cell from the external environment. The cell membrane is present in all the cells.
- The cell membrane is the outer covering of a cell within which all other organelles, such as the cytoplasm and nucleus, are enclosed. It is also referred to as the plasma membrane.
- By structure, it is a porous membrane (with pores) which permit the movement of selective substances in and out of the cell. Besides this, the cell membrane also protects the cellular component from damage and leakage.
- It forms the wall-like structure between two cells as well as between the cell and its surroundings.
- Plants are immobile, so their cell structures are well-adapted to protect from them from external factors. The cell wall helps to reinforce this function.

2. Cell Wall

- The cell wall is the most prominent part of the plant’s cell structure. It is made up of cellulose, hemicellulose and pectin.
- The cell wall is present exclusively in plant cells. It protects the plasma membrane and other cellular components. The cell wall is also the outermost layer of plant cells.
- It is a rigid and stiff structure surrounding the cell membrane.
- It provides shape and support to the cells and protects them from mechanical shocks and injuries.

3. Cytoplasm

- The cytoplasm is a thick, clear, jelly-like substance present inside the cell membrane.
- Most of the chemical reactions within a cell take place in this cytoplasm.
- The cell organelles such as endoplasmic reticulum, vacuoles, mitochondria, ribosomes, are suspended in this cytoplasm.

4. Nucleus

- The nucleus contains the hereditary material of the cell, the DNA.
- It sends signals to the cells to grow, mature, divide and die.
- The nucleus is surrounded by the nuclear envelope that separates the DNA from the rest of the cell.
- The nucleus protects the DNA and is an integral component of a plant’s cell structure.

5. Nucleolus

- The nucleolus is the site of ribosome synthesis. Also, it is involved in controlling cellular activities and cellular reproduction

6. Nuclear membrane

- The nuclear membrane protects the nucleus by forming a boundary between the nucleus and other cell organelles.

7. Chromosomes

- Chromosomes play a crucial role in determining the sex of an individual. Each human cells contain 23 pairs of chromosomes

8. Endoplasmic reticulum

- The endoplasmic reticulum is involved in the transportation of substances throughout the cell. It plays a primary role in the metabolism of carbohydrates, synthesis of lipids, steroids, and proteins.

9. **Golgi Bodies**

- Golgi bodies are called the cell's post office as it is involved in the transportation of materials within the cell

10. **Ribosome**

- Ribosomes are the protein synthesisers of the cell

11. **Mitochondria**

- The mitochondrion is called "the powerhouse of the cell." It is called so because it produces ATP – the cell's energy currency

12. **Lysosomes**

- Lysosomes protect the cell by engulfing the foreign bodies entering the cell and helps in cell renewal. Therefore, it is known as the cell's suicide bags.

13. **Vacuoles**

- Vacuoles stores food, water, and other waste materials in the cell

Function of Cells

A cell performs these major functions essential for the growth and development of an organism. Important functions of cell are as follows:

- Provides Support and Structure

All the organisms are made up of cells. They form the structural basis of all the organisms. The cell wall and the cell membrane are the main components that function to provide support and structure to the organism. For eg., the skin is made up of a large number of cells.

- Facilitate Growth Mitosis

In the process of mitosis, the parent cell divides into the daughter cells. Thus, the cells multiply and facilitate the growth in an organism.

Allows Transport of Substances

Various nutrients are imported by the cells to carry out various chemical processes going on inside the cells. The waste produced by the chemical processes is eliminated from the cells by active and passive transport.

Small molecules such as oxygen, carbon dioxide, and ethanol diffuse across the cell membrane along the concentration gradient. This is known as passive transport. The larger molecules diffuse across the cell membrane through active transport where the cells require a lot of energy to transport the substances.

Energy Production

Cells require energy to carry out various chemical processes. This energy is produced by the cells through a process called photosynthesis in plants and respiration in animals.

Aids in Reproduction

A cell aids in reproduction through the processes called mitosis and meiosis. Mitosis is termed as the asexual reproduction where the parent cell divides to form daughter cells.

Meiosis causes the daughter cells to be genetically different from the parent cells. Thus, we can understand why cells are known as the structural and functional unit of life.

This is because they are responsible for providing structure to the organisms and performs several functions necessary for carrying out life’s processes.

Cells division

There are two types of cell division:

1. **Mitosis**
2. **Meiosis.**

- Mitosis: It is a fundamental process for life. During mitosis, a cell duplicates all of its contents, including its chromosomes, and splits to form two identical daughter cells.

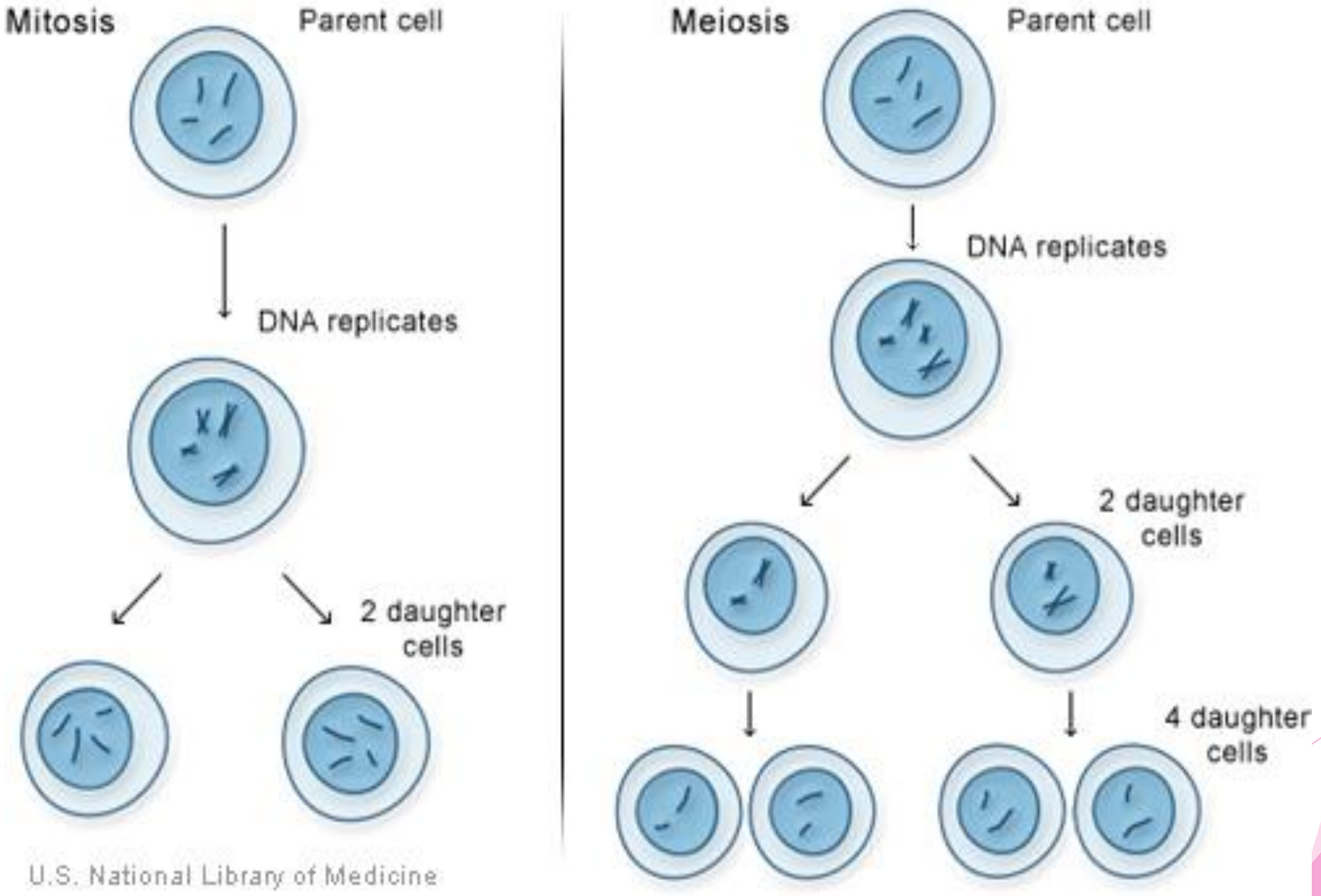
  Because this process is so critical, the steps of mitosis are carefully controlled by a number of genes. When mitosis is not regulated correctly, health problems such as cancer can result.

- Meiosis: The other type of cell division, meiosis, ensures that humans have the same number of chromosomes in each generation.

  It is a two-step process that reduces the chromosome number by half—from 46 to 23—to form sperm and egg cells.

  When the sperm and egg cells unite at conception, each contributes 23 chromosomes so the resulting embryo will have the usual 46.

  Meiosis also allows genetic variation through a process of DNA shuffling while the cells are dividing.


Mitosis
Parent cell
DNA replicates
2 daughter cells
Meiosis
Parent cell
DNA replicates
2 daughter cells
4 daughter cells
U.S. National Library of Medicine

Unit-11
Exfoliative Cytology

Exfoliative Cytology:

- It is the study of cells that have been shed or removed from the epithelial surface of various organs.
- Cells from all organs, which communicate with the exterior of the body, are suitable for study.
- These cells can be recovered either from natural secretions such as urine, sputum and vaginal or prostate fluids or by artificial means such as paracentesis or lavage.
- The cells can be collected from the epithelial surfaces by lightly scraping the surface, by swabbing, aspirating or washing the surfaces.

Collection and Processing of specimen for cytology:

- **1. Cervical smears:-**

Cervical smears are made from material collected with help of a speculum (a metal or plastic device) which is inserted into the vagina and allows the uterine cervix to be readily visible. A specialized spatula known as the Ayre spatula or cervical spatula is used for collection. The collection is made at the junction of the columnar epithelium by visualizing the cervix, the spatula is inserted via the speculum into the cervical os and rotated through 360 degrees.smeared over a pre-labelled microscope slide and fixed immediately. It is ideal for detection of cervical carcinoma.

- **2. Aspiration from the posterior fornix:-** With the aid of a speculum, cellular material is collected from the posterior fornix, using a disposable plastic pipette with a suction bulb. Following aspiration, smears are prepared and fixed immediately.
- **3. Vaginal smears:-** Vaginal smears are valuable for the assessment of hormonal function. Cellular material is collected by scraping the upper third of the lateral wall of the vagina with a wooden spatula. The cells are evenly and thinly smeared over a clean pre-labelled microscope slide and fixed.

- 4. **Endocervical smears:-** This is used mainly for follow up cases where a surgical treatment has been used after a cone biopsy has been taken for assessment of dysplasia and malignancy or as a curative procedure. A cotton tip swab is inserted into the endocervix and rotated gently to cover a wide area of the endocervix. The material collected is smeared on a clean pre-labelled microscope slide and fixed.
- **5. Endometrial aspiration:-** This procedure has to be performed under strict aseptic conditions so as not to introduce infection into the patient. A cannula is inserted into the uterine cavity and the cellular material is aspirated using a syringe. Thin smears are made on clean pre-labelled slides and fixed.

NON.GYNAECOLOGICAL CYTOLOGY:-

This aspect of cytology involves the study of cells suspended in body fluids. The specimen are varied and taken from various parts of the body.

- **1. Sputum:-**
  - Sputum specimen is valuable for the study of respiratory tract disorders. It is used in the dingnose of the following abnormal conditions:

    A) Malignant disease of the lower respiratory tract.

    B) Pulmonary asbestosis.

    C) Pulmonary inflammatory conditions due to fungal infection, bacterial infection, viral infection or parasitic infection.

- It is normally collected as early morning deep cough specimens and is preferably submitted on three consecutive days. It is not advisable to collect sputum specimen after a recent bronchoscopy has been done.
- **Preparation of smears:-**
  - Sputum must be processed in a biological safety cabinet. Purulent or blood stained particles are selected from the sputum with a microbiological wire loop and used to make thin smears.
  - Bronchial washings are usually submitted in sterile containers. They are centrifuged without delay and smears made from the sediment. They can also be spun at 150 rpm for 10 minutes in a cytocentrifuge directly onto a clean prelabelled microscope slides and fixed immediately.
- **Fixation:-** Fixation should be carried out while the smear are still wet.3% acetic acid in 95%alcohol is used.

- **2) Pleural fluid and ascitic fluid:-**
  - These are serious fluids that normaly lubricate the wall of pleural, pericardial fluid, synovial fluid. CSF and peritoneal cavities. They increase in volume and contain cells under certain pathological conditions. Cytological examination of these fluids reveal malignant cells which may arise from tumours of the surrounding mesothelium or they be metastatic deposits.
- **Collection and preparation of smears:**
  - By means of a needle or canula with an attached syringe, the specimens are aspirated from the pleural or peritoneal cavities. The aspirated material is transferred into a sterile container and sent to the laboratory. The specimens are centrifuged at 800 rpm for 10 minutes or cytospun at 1500 rpm for 10 minutes and thin smears made, at least two smears from each specimen. Any clots that are formed are fixed and histological lab.

- **Fixation:-** The choice of stain is Romanowskv, then smear should be air dried and then fixed with methanol.
- **3. Urine:-**
  - Urine cytology is of great value in the diagnosis of urethral tumours, urinary bladder carcinoma, carcinoma of the kidney and carcinoma of the prostate in males. Normal urine contains few or no cells; but under certain pathological conditions, the urine contains many abnormal cells. Early morning specimens of urine are preferred because they give larger concentration of cells due to relatively long residence in the bladder.
- **Fixation:-** Urine tends to wash of slide during fixation and staining due to the low protein content.

Unit-12
Fixation (Cytological Specimen)

Fixation of Cytology Specimens

- Fixation means prevention of degeneration of cells and tissue by the autolytic enzymes present in the cells and preservation of cells as close as possible to the living state.
- To achieve this smears are placed in the fixative solutions for specific periods of time before the staining procedure is started.
- Fixation changes the physical and chemical state of the cells and determines the subsequent staining reactions that could be carried out on the smears.

VARIOUS TYPES OF CYTOLOGICAL FIXATIVES:

- These fixatives can be subdivided into:
  1. (A) Nuclear fixative and
  2. (B) Cytoplasmic fixatives.

1. Nuclear fixatives:

- **1. Carnoy's fluid**

  a) Absolute alcohol = 60 ml

  b) Chloroform 30 ml

  c) Glacial acetic acid = 10 ml

- **Specific features :-**
  - It penetrates very rapidly and gives excellent nuclear fixation.
  - Nissl substance and glycogen are preserved.
  - Good fixative for carbohydrates.
  - It causes considerable shrinkage.
  - It destroys or dissolves most cytoplasmic elements.
  - It causes haemolysis of erythrocyte.
  - It is used for urgent biopsy.
  - Fixative is usually complete in 12 hours. (small pieces 23 mm thick require 15 minutes for fixation).

2) Clarke's fluid :-

- Absolute alcohol = 75 ml
- Glacial acetic acid = 25 ml

Specific features :-

- This fixative penetrates rapidly, gives good nuclear fixation and effects preservation of cytoplasmic elements.
- It is an excellent fixative for smears or coverslip preparations of cell cultures for general fixation and chromosome analysis.

3. Newcomer's fluid:

- Isopropanol = 60 ml
- Propionic acid = 40 ml
- Petroleum either = 10 m)
- Acetone = 10
- Dioxane = 10 ml

SPECIFIC FEATURES

- This fixative penetrates rapidly and preserves the chromatin better than Carnoy's fluid.
- It is a good fixative for the preservation of mucopolysaccharides.

- **4. Alcohol — ether (Equal volumes of 95% alcohol and ether):-**
  - This is the routinely used cytological fixative for wet smears.
  - It is specially recommended for use with the Papanicolaou staining technique.
  - Smears are fixed within 30 minutes but can be left in the fixative for longer period.
  - Smears are rinsed in water before staining.
- **1) Alcohol-ether fixative :-**
  - Absolute ethyl alcohol — 50 ml
  - Ether 50 ml

Mix and place in a jar with a tight stopper. Fixation is carried out for about 30 minutes, Followed by a rinse in alcohol and then the section is taken to water.

5. Schaudinn's Fluid:-ltis prepared as follows:

- Saturated mercuric chloride solution 60 ml
- Absolute alcohol 33ml
- Glacial acetic acid I ml

Fixation is carried out for about 2 minutes.afterwashing in distilled water, the mercuric chloride black clumps are removed by adding a few drops of saturated alcoholic iodine solution. After rinsing in water the smear is taken for staining

2. Cytoplasmic fixative:

- **1) Champy's fluid** :-
  - 3 g/dl potassium dichromate = 7 ml
  - 1% (v/v) chromic acid = 7 ml
  - 2 g/dl, osmium tetroxide = 4 ml
- **Specific features :-**
  - This fixative cannot be stored, hence should be prepared fresh before use. It penetrates poorly and unevenly.
  - It preserves mitochondria, fat, yolk and lipids.
  - Tissue must be washed overnight after fixation.

- **2) Flemming's fluid :-**
  - Osmium tetroxide= 2gm

    Distilled water = 100 ml
  - Chromium trioxide = 1 gm

    Distilled water = 100 ml
- Working solution:-
  - Solution A = 16 ml
  - Solution B =60ml
  - Acetic acid = 4 ml

- This perhaps is the most widely used fixative for the preservation of nuclear structures, especially chromosomes.
- the omission of acetic acid, the solution becomes a cytoplasmic fixative. Small pieces of tissue not more n 2 mm in thickness, are adequately fixed in 12-24 hours.
- It preserves fat permanently. It is a costly fixative.
- Flemmings fluid minus acetic acid is very good for mitochondria and other cytoplasmic structures.

Unit-13
Cytological Staining

Hematoxylin and eosin stain

- Hematoxylin and eosin staining technique functions to recognize different types of tissues and their morphological changes, especially in cancer diagnosis.
- Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex, incompletely understood reaction.
- Eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining.
- Hematoxylin and eosin are both dyes have a high affinity for tissues, depending on the Acidity and/or alkalinity of the dyes.

- **Principle**

  Eosin dye is acidic dye hence it as a negative charge. Therefore it stains the basic structures of a cell (acidophils), giving them a red or pink color, for example, the cytoplasm is positively charged, and therefore it will take up the eosin dye, and appear pink.

  Hematoxylin dyes are basic dyes, hence they are positively charged.Therefore it will stain the acidic structures of tissues and cell structures purplish-blue.

  Hematoxylin is not basic by itself. It has to be conjugated with a mordant (aluminum salt) before it is used so as to strengthen its positive charge for efficiency in binding to the tissue components.

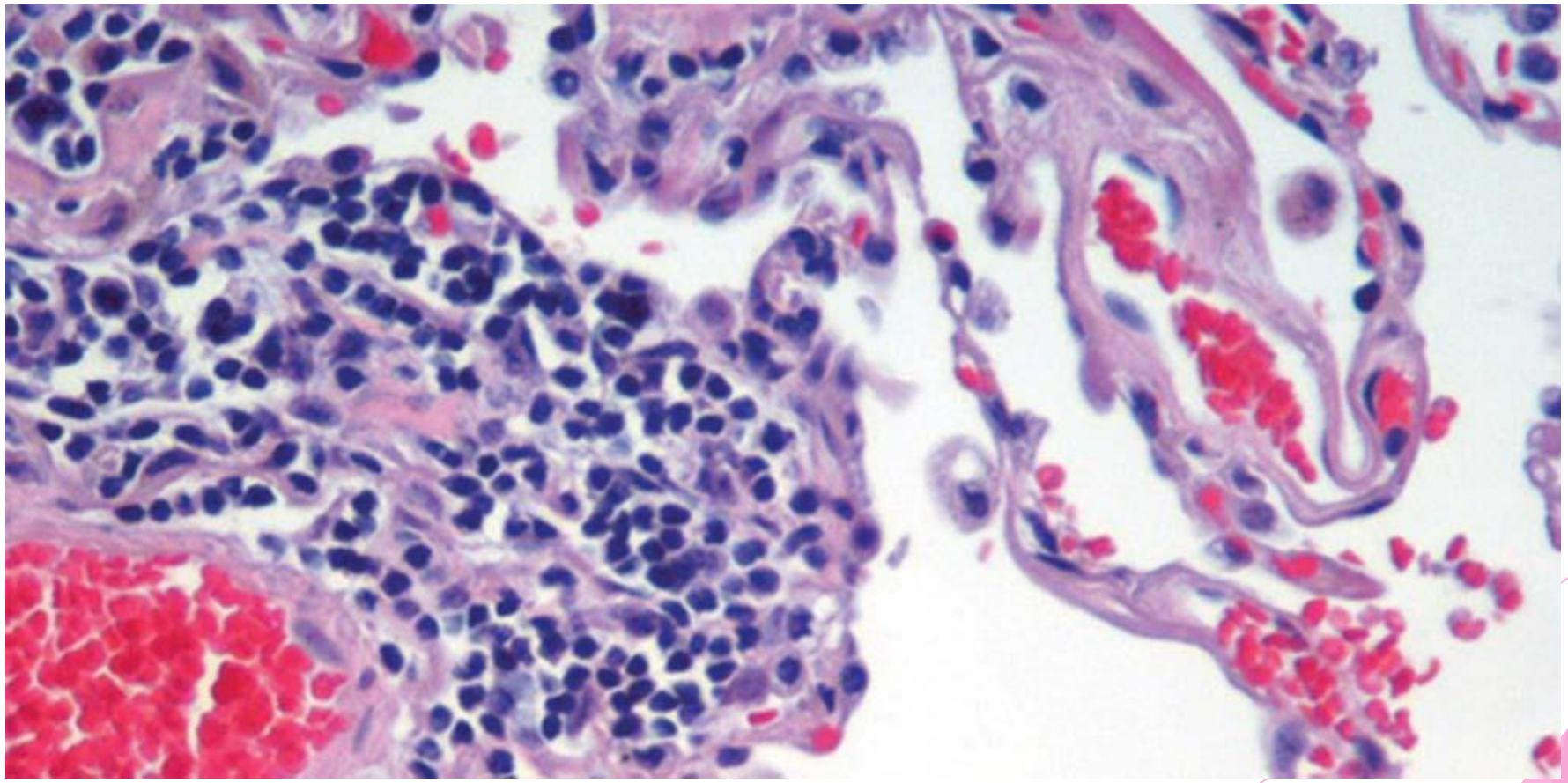
  The mordant, which also defines the color of the stain, will bind to the tissue, then the hematoxylin will bind to the mordant to form a tissue-mordant-hematoxylin complex link. This will stain the nuclei and chromatin bodies purple.

- **Reagents**
  - Distilled water
  - Alum hematoxylin
  - Acid alcohol
  - Scott’s tap water
  - Eosin dye

Procedure

1. Clean the sections to distilled water.
2. Then stain nuclei with the alum hematoxylin (Mayer's) to fix the tissue, for about 5 minutes.
3. Rinse the stain with smoothly running tap water
4. Using the differentiator, 0.3% acid alcohol, and note the endpoint i.e the correct endpoint is after bluing up, the background becomes colorless.
5. Rinse the stain in smoothly running tap water.
6. Rinse the satin in Scott's tap water substitute which shortens the time for the correct end-point.
7. Rinse with running tap water
8. Flood the smear with eosin for 2 mins, and since eosin is highly soluble in water, use enough quantify of it. The over stained eosin can be removed or washed off with running tap water.
9. Dehydrate the smear, clear, and mount using a clean coverslip.

- **Results and Interpretation**
  - Nuclei are stained blue
  - cytoplasm and extracellular matrix have varying degrees of pink staining.



MGG stain

- **Introduction**

May Grunwald Giemsa stain is one of many stains under the Romanowsky staining procedure. It is a combination of two stains, May Grunwald stain and Giemsa stain. Like other Romanowsky stains, the principle is the same. It is used for bone marrow smear staining.

- **Materials**

  May Grunwald dye

  Absolute methanol

  Giemsa dye

  Glycerol

  Phosphate buffer pH 6.8

- **Method**

  Preparation of May Grunwald stain

  - Dissolve 0.3 g of May Grunwald dye in 100 mL absolute methanol in a 250 mL conical flask.
  - Warm the mixture to 50°C in a water bath for a few hours and allow it to cool to room temperature.
  - Stir the mixture on a magnetic stirrer and leave it stirring for 24 hours.
  - Filter the mixture and stain is ready for use.

- **Preparation of Giemsa stain**
  - Add 1.0 g of Giemsa dye into 66 mL of glycerol and warm the mixture in a conical flask for 1-2 hours at 50°C.
  - Cool the mixture to room temperature and add 66 mL of absolute methanol.
  - Leave the mixture to dissolve for 2-3 days, mixing it at intervals.
  - The stain is then ready for use after filtering.

Staining

1. Fix BM smears in absolute methanol for 10-15 minutes.
2. Prepare an equal volume of May Grunwald stain and phosphate buffer pH 6.8. Mix well and pour onto the slides to fully flood the slides. Stain for 10 minutes.
3. Prepare a 1:10 dilution of Giemsa stain with phosphate buffer pH 6.8. Mix well.
4. After 10 minutes of May Grunwald staining, pour away the May Grunwald stain off the slides.
5. Then pour the Giemsa mixture onto the slides and stain for another 15 minutes.
6. After 15 minutes, pour off stain and flush the slides with running tap water.
7. Clean excess stain with kim wipes.
8. Air dry the slides. Place the long cover slip on the area of interest.
9. The slide is now ready for examination.

- Results :

1. methylene blue stains blue the acidic components of the cell
2. eosin stains orange-red the alkaline components of the cell
3. Azure stains red and purple the basic cellular components

PAP stain

- Papanicolaou stain (also Papanicolaou's stain or PAP stain) is the most important stain utilized in the practice of Cytopathology. It is a polychromatic stain containing multiple dyes to differentially stain various components of the cells.
- This technique was developed by George Papanicolaou, the father of Cytopathology. This method is used to differentiate cells in the smear preparation of various gynecological specimens (pap smears), materials containing exfoliative cells and material from fine needle aspiration.

Principle of PAPANICOLAOU stain

- Papanicolaou stain includes both acidic and basic dyes. Acidic dye stains the basic components of the cell and basic dye stain the acidic components of the cell.
- The polychromatic PAP stain involves **five dyes in three solutions**.
- **Hematoxylin** : Natural dye hematoxylin is the nuclear stain which stains cell nuclei blue. It has affinity for chromatin, attaching to sulphate groups on the D.N.A. molecule. Harris' hematoxylin is the commonest cytologically although Gills' hematoxylin and Hematoxylin S can be used.
- **Orange Green 6** : This is the first acidic counterstain (cytoplasmic stain) which stains matured and keratinized cells. The target structures are staine d orange in different intensities.

- **Eosin Azure** : This is the second counterstain which is a polychrome mixture of eosin Y, light green SF and Bismarck brown. **Eosin Y** gives a pink colour to cytoplasm of mature squamous cells, nucleoli, cilia and red blood cells. Staining solutions commonly used in cytology are EA 31 and EA 50, while EA 65
- **Light green SF** stains blue to cytoplasm of metabolically active cells like parabasal squamous cells, intermediate squamous cells and columnar cells.
- **Bismarck brown Y** stains nothing and sometimes it is often omitted.

Composition and preparation of reagents

- **Harris' hematoxylin** :

  Hematoxylin = 5g
  Ethanol = 50ml
  Potassium alum = 100g
  Distilled water (50°C) = 1000ml
  Mercuric oxide = 2-5g
  Glacial acetic acid = 40ml

- **Orange G 6** :

  Orange G (10% aqueous) = 50ml
  Alcohol = 950ml
  Phosphotungstic acid = 0-15g

- **EA 50** :

  0.04 M light green SF = 10ml
  0.3M eosin Y = 20ml
  Phosphotungstic acid = 2g
  Alcohol = 750ml
  Methanol = 250ml
  Glacial acetic acid = 20ml

- Filter all stains before use.

Procedure of PAPANICOLAOU staining

1. 95% Ethanol 15 minutes (fixation)
2. Rinse in tap water
3. Harris or Gill Hematoxylin 1-3 minutes (Time vary with selection of hematoxylin solution)
4. Rinse in tap water or Scott's tap water
5. 95% Ethanol 10 dips
6. OG-6 stain for 1.5 minutes.
7. 95% Ethanol 10 dips
8. EA-50, or Modified EA-50, or EA-65 stain for 2.5 minutes.
9. 95% Ethanol 10 dips, 2 changes
10. 100% Ethanol 1 minute
11. Clear in 2 changes of xylene, 2 minutes each
12. Mount with permanent mounting medium

Results and interpretation of PAPANICOLAOU Staining

- Nuclei : Blue
- Acidophilic cells : Red
- Basophilic cells : Blue Green
- Erythrocytes : Orange-red
- Keratin : Orange-red
- Superficial cells : Pink
- Intermediate and Parabasal Cells : Blue Green
- Eosinophil : Orange Red
- Candida : Red
- Trichomonas : Grey green

Unit-14
Role of Laminar airflow and cytotechnician in cytology

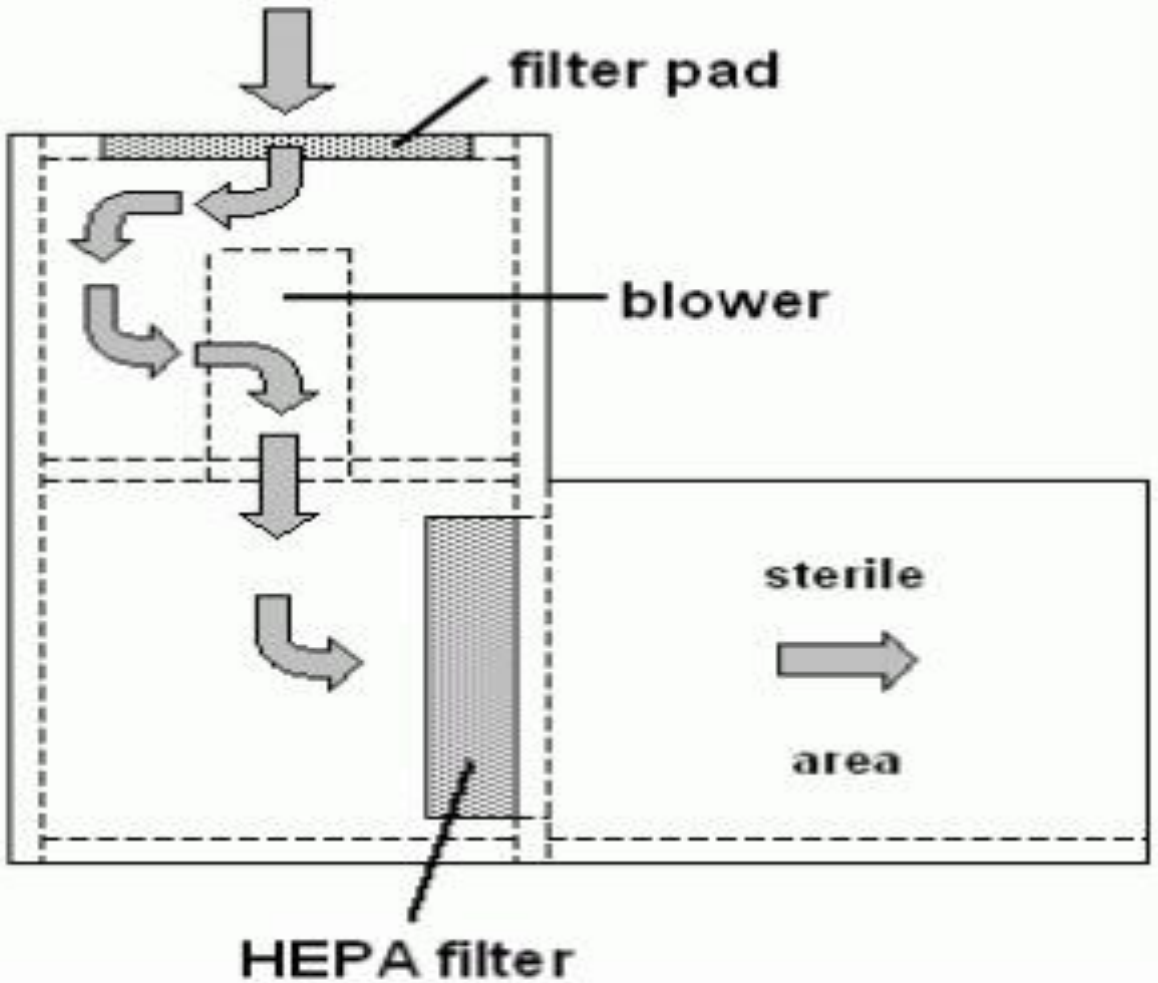
Laminar flow cabinet

- A **laminar flow cabinet** or **laminar flow closet** or **tissue culture hood** is a carefully enclosed bench
- designed to prevent contamination of semiconductor wafers, biological samples, or any particle sensitive materials
- air is passed through a HEPA (High Efficiency Particulates Air) filter which removes all airborne contamination to maintain sterile conditions

Parts of Laminar Air Flow

- A laminar flow hood consists of a filter pad, a fan and a HEPA (High Efficiency Particulates Air) filter
- The fan sucks the air through the filter pad where dust is trapped
- After that the prefiltered air has to pass the HEPA filter where contaminating fungi, bacteria, dust etc are removed
- sterile air flows into the working (flasking) area where you can do all your flasking work without risk of contamination.

Side view of a laminar flow hood



HEPA fliter

Uses

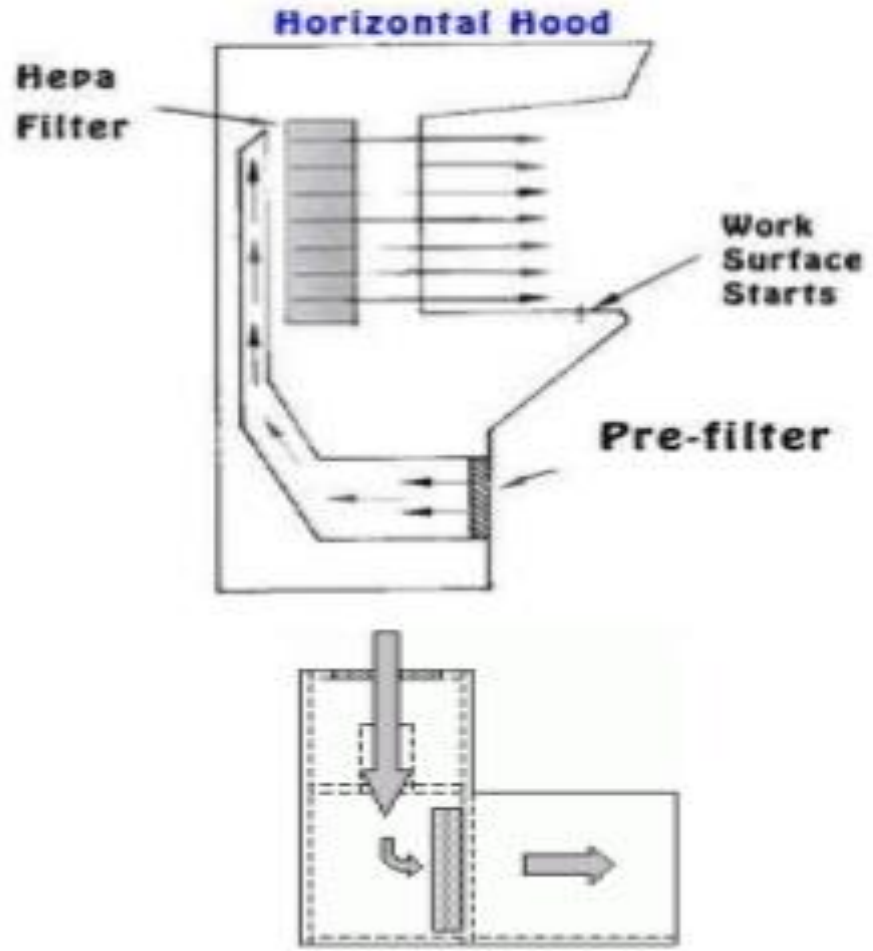
- Laminar Flow Cabinets are suitable for a variety of applications
- where an individual clean air environment is required for smaller items, e.g. particle sensitive electronic devices.
- In the laboratory, Laminar Flow Cabinets are commonly used for specialised work.
- Laminar Flow Cabinets can be tailor made to the specific requirements of the laboratory
- ideal for general lab work, especially in the medical, pharmaceutical, electronic and industrial sectors.

Types of Laminar Flow Cabinets

- Laminar Flow Cabinets can be produced as both horizontal and vertical cabinets
- There are many different types of cabinets with a variety of airflow patterns for different purposes
  - Vertical Laminar Flow Cabinets
  - Horizontal Laminar Flow Cabinets
  - Laminar Flow Cabinets and Hoods
  - Laminar Flow Benches and Booths

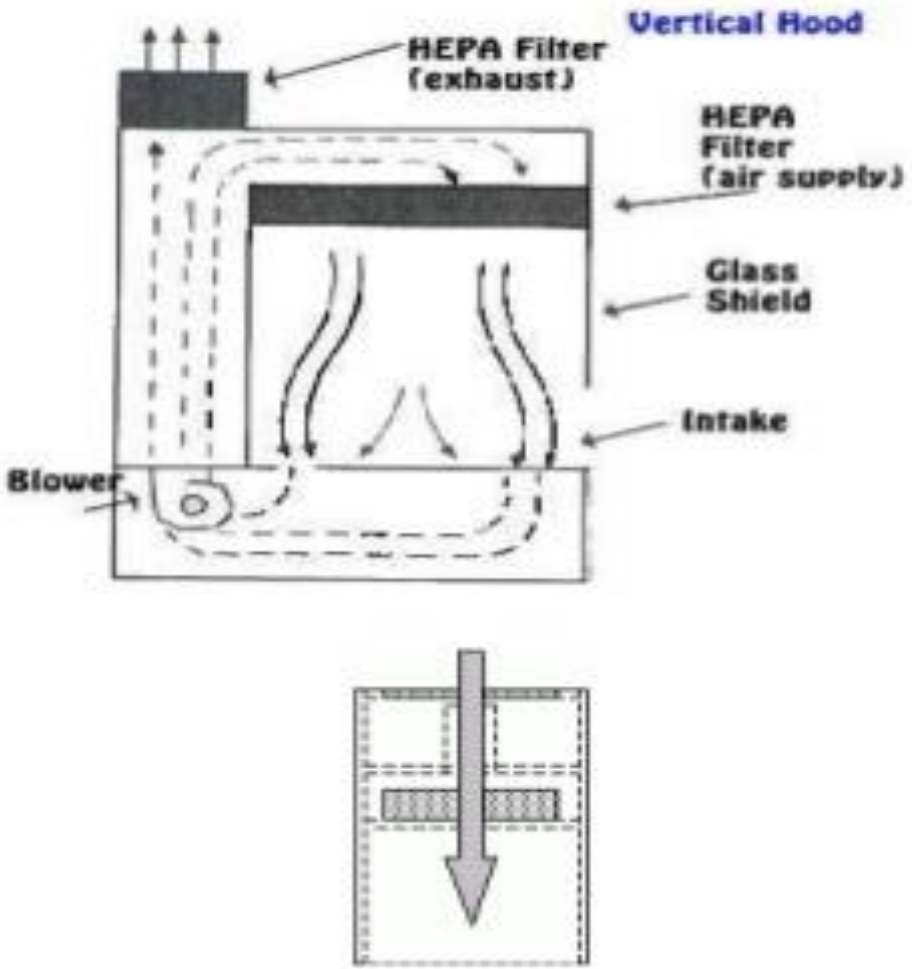
Horizontal Laminar Flow Cabinets

- direction of air flow which comes from above
- then changes direction and is processed across the work in a horizontal direction.
- The constant flow of filtered air provides material and product protection.



Vertical Laminar Flow Cabinets

- function equally well as horizontal Laminar Flow Cabinets
- laminar air directed vertically downwards onto the working area
- The air can leave the working area via holes in the base
- Vertical flow cabinets can provide greater operator protection.



Efficient functioning

- Important parameters to make sure that the hood works efficiently:
  - the HEPA filter has to remove all airborne materials
  - the air speed in the working area has to be about 0,5 m/s

How to use a laminar flow hood

- Before you start flasking in your laminar flow hood you should do the following actions.
  - Turn on the blower and wipe out the sterile area with an alcohol soaked piece of kitchen paper.
  - Let the blower run continuously for 30 minutes.
  - When this time has passed, wipe out of the sterile area with an alcohol soaked piece of kitchen paper.