# VU Medical Zone

## Admin: Amaan Khan

## Biological Techniques

## *ZOO101 Midterm* ppt

## Lecture 1 to 53

# Microscopy

## Lesson 2: MAGNIFICATION, RESOLUTION and PRINCIPLE of a microsope

# Types of microscopes

- Depending on the number of lenses, there are two i. e

1. **Simple light microscopes**: use a single lens to magnify an object and cannot reach high magnification.

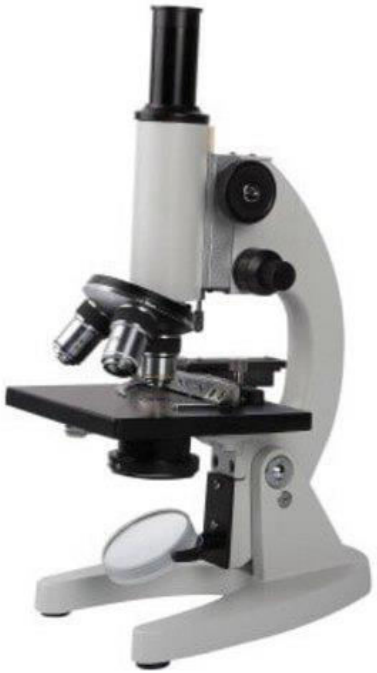
2. Compound light microscope (it has a higher magnification compared to the simple microscope because it uses at least two sets of lenses, an objective lens, and an eyepiece).

- The lenses are aligned in that, they can be able to bend light for efficient magnification of the image.

Logan's Simple Microscope (circa 1871)

- The simplest microscope of all is a magnifying glass made from a single convex lens, which typically magnifies by about 5–10 times.

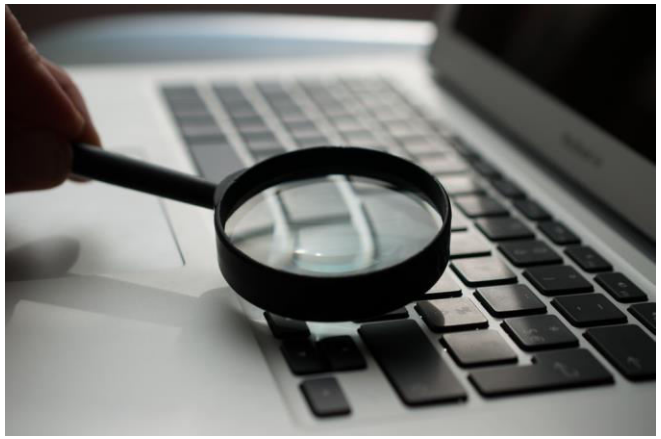
- Microscopes used in homes, schools, and professional laboratories are actually **compound microscopes** and use at least two lenses to produce a magnified image.
- Most compound microscopes can magnify by 10, 20, 40, or 100 times



# MAGNIFICATION

- Degree of enlargement.
- No of times the length, breadth or diameter, of an object is multiplied.
- depends upon 3 factors

1. **Optical tube length**
2. **Focal length of objective**
3. **Magnifying power of eye piece**



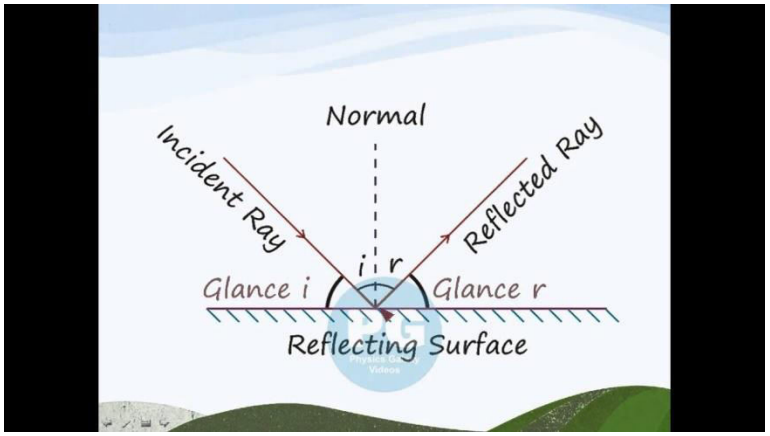
**Total magnification=magnification of the eyepiece x magnification of the objective.**

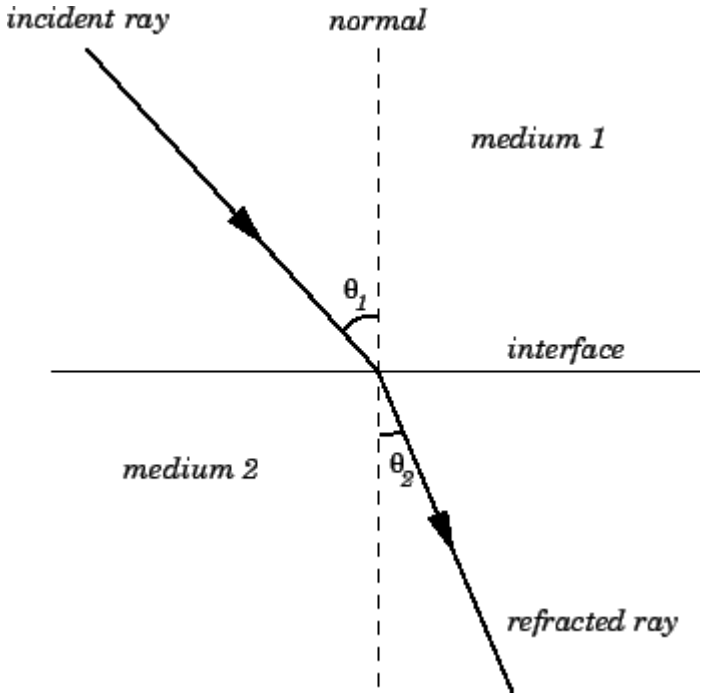
=10x40 = 400

=10x100 = 1000

# Principle of Microscope

- Generally.. microscopes visualize an image by using a glass lens and magnification is determined by, **the lens's ability to bend light and focus it on the specimen,** which forms an image.
- When a ray of light passes through one medium into another, the ray bends at the interface causing **refraction.**
- The bending of light is determined by the **refractive index**, which is a measure of how great a substance slows the speed of light. The direction and magnitude of the **bending of the light are determined by the refractive indexes** of the two mediums that form the interface.




- A medium with a lower refractive index such as glass to air, it normally speeds up the light penetration and making light bend away from the normal and when light is passed through a medium with a greater refractive index such as air to glass, it normally slows down and bends towards the normal, perpendicularly to the surface.
- If an object is put between these two mediums i.e between water and air, in this case, a prism, the prism will bend the light at an angle. This is how the microscopic lenses work, they bend the light at an angle.

- **Refraction** is the change in direction of a wave passing from one medium to another.
- **Refraction of light waves** is the most commonly observed phenomenon, but other waves such as sound waves and water waves also experience **refraction**.
- **Refractive index**, also called **index** of **refraction**, measure of the bending of a ray of light when passing from one medium into another.

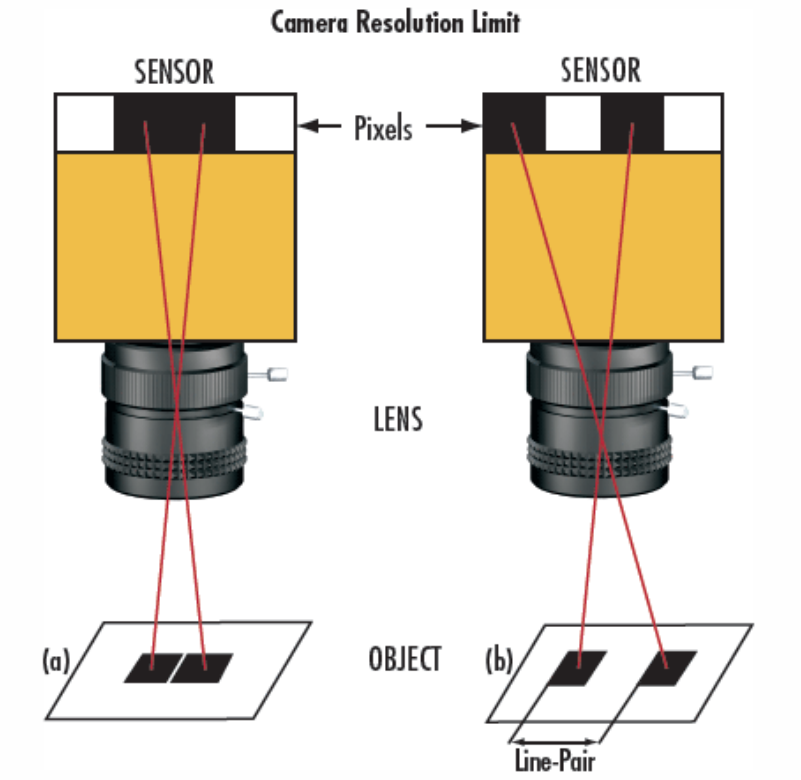
# Microscopy

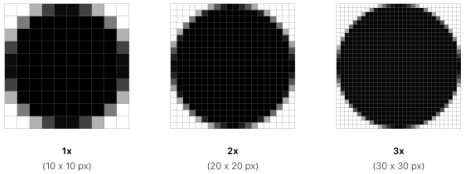
## Lesson 3: MAGNIFICATION, RESOLUTION and PRINCIPLE of a microsope..continued…

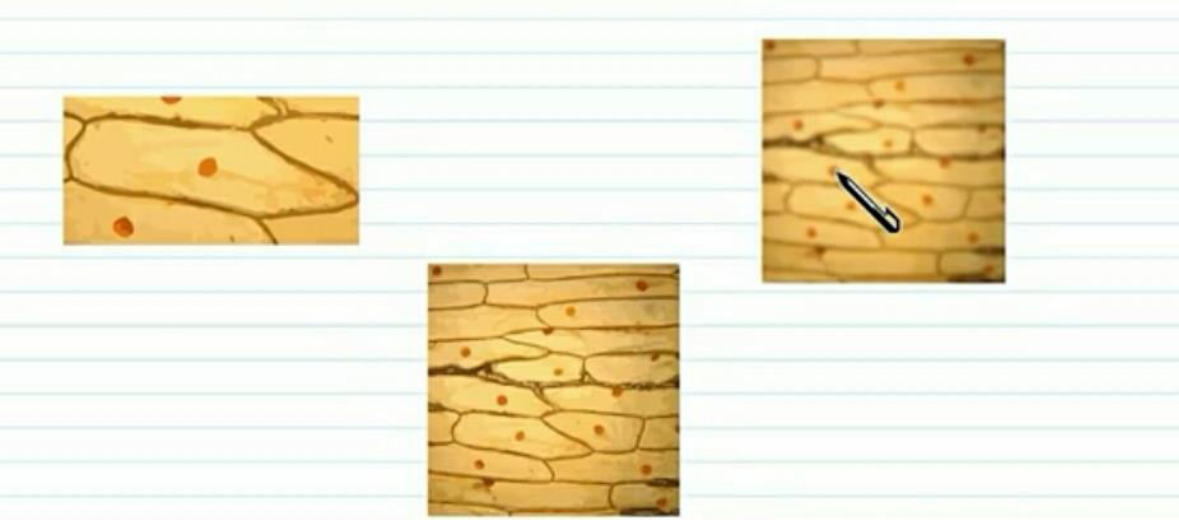
## …2

# RESOLUTION/resolving power

- In microscopy, the term ‘resolution’ is used to describe the ability of a microscope to distinguish details of given specimen. This is the resolving power of microscope
- In other words, this is the shortest distance (d) at which two distinct points of a specimen can still be distinguished - either by the observer or the microscope camera - as separate entities

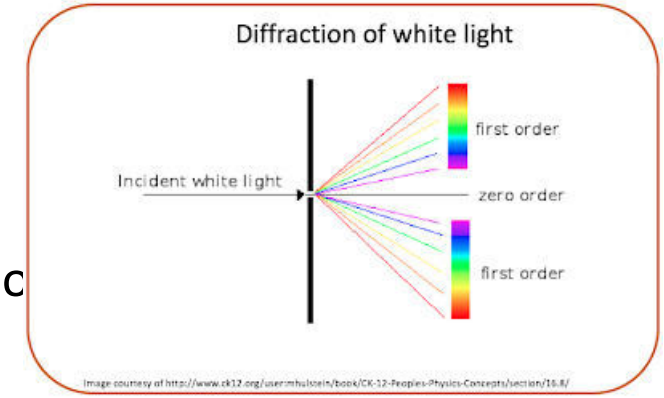




## Magnified vs resolved image

- **Diffraction:**
- **Diffraction** refers to various phenomena that occur when a wave encounters an obstacle or opening. It is defined as the bending of waves around the corners of an obstacle or through an aperture into the region of geometrical shadow c the obstacle/aperture.




- **airy disks**: In optics, the Airy disk (or Airy disc) and Airy pattern are descriptions of the best-focused spot of light that a perfect lens with a circular aperture can make, limited by the diffraction of light.

# Rayleigh criterion

- gives the smallest possible angle θ between point sources, or the best obtainable resolution.
- The **Rayleigh criterion** for the resolution states that two images are just resolvable when the center of the diffraction pattern of one is directly over the first minimum of the diffraction pattern of the other.

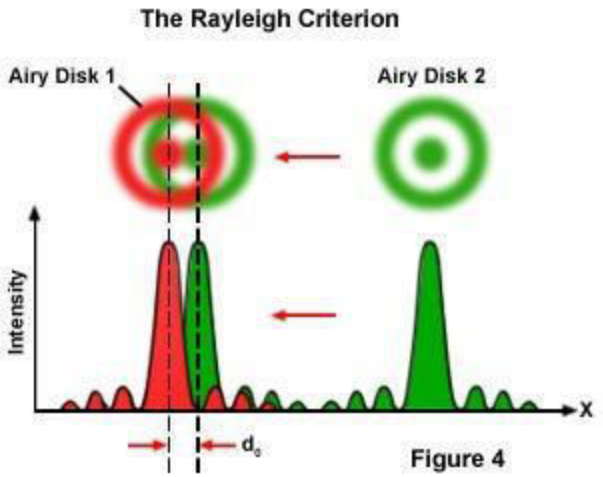
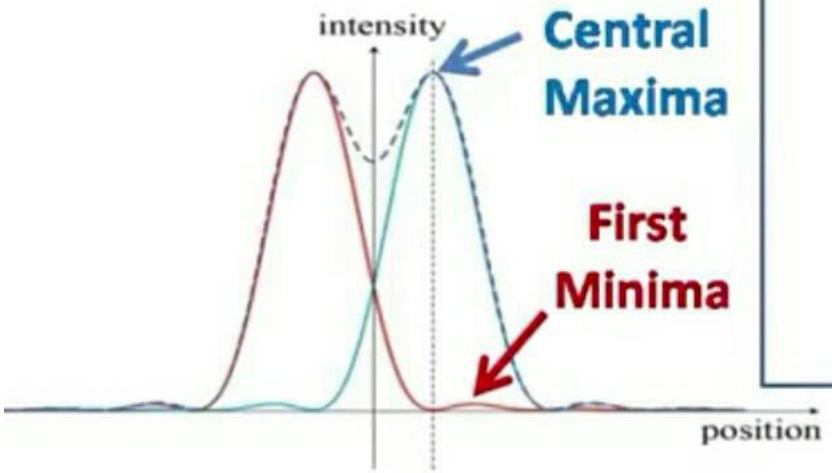


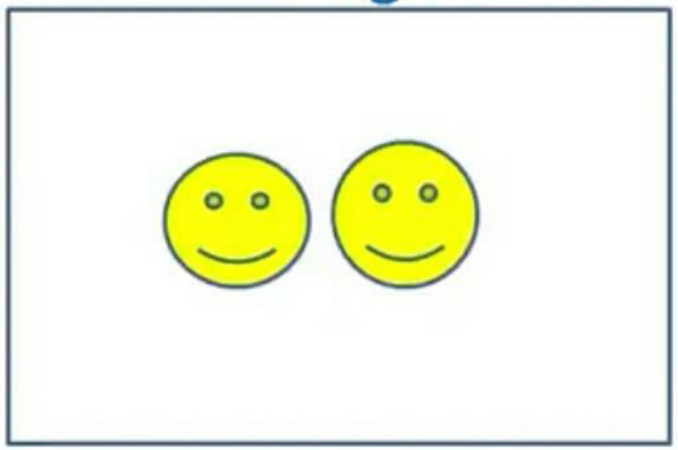
Figure 4

# The Rayleigh Criteria




Two closely spaced objects

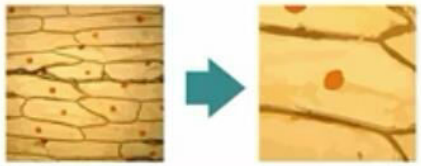
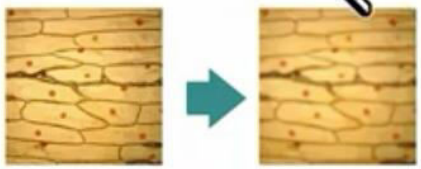
Image



## The Rayleigh Criteria

Rayleigh Criteria: Not Followed
Two closely spaced
Objects will be seen as one

Image

| Magnification | Resolution |
| --- | --- |
| How many times larger an image appears | Smallest distance between two distinguishable points |
|  |  |

# Factors affecting RESOLUTION

- **d= λ/2 NA**

- The resolution of a microscope is a function of two factors as given below:

1. numerical aperture (NA) of the optical components
2. wavelength of light (λ)which is used to examine a specimen.

- **LIMIT OF RESOLUTION (LR):** The minimum distance between any two closest points on the microscopic object, which can be resolved into two separate and distinct points on the enlarged image.

- **$d = \lambda / 2\ NA$**

# Lesson 4: Nuclear aperture of microscope

# Factors affecting RESOLUTION

- **d= λ/2 NA**

- The resolution of a microscope is a function of two factors as given below:

1. numerical aperture (NA) of the optical components
2. wavelength of light (λ)which is used to examine a specimen.

# wavelength of light and RESOLUTION

- The smaller is the wavelength of light (λ), the greater is its ability to resolve the points on the object into distinctly visible finer details in the image.
- Thus, the smaller is the wavelength of light, the greater is its resolving power.

# 1.Effect of NA on Resolution

- **Numerical aperture is a number that expresses the ability of a lens to resolve fine details in an object being observed.**
- In MICROBIOLOGY, the **numerical aperture** of a microscope objective is a measure of its ability to gather light and resolve fine details of the specimen at a fixed object distance. light waves that are forming the Image pass through the specimen and enter the objective **in an inverted cone** as shown in fig 1
- Numerical aperture is concerned with the diameter of the objective lens in relation to its **focal length.**
- Thus, it is related to the size of the lower aperture of the objective, through which light enters into it.

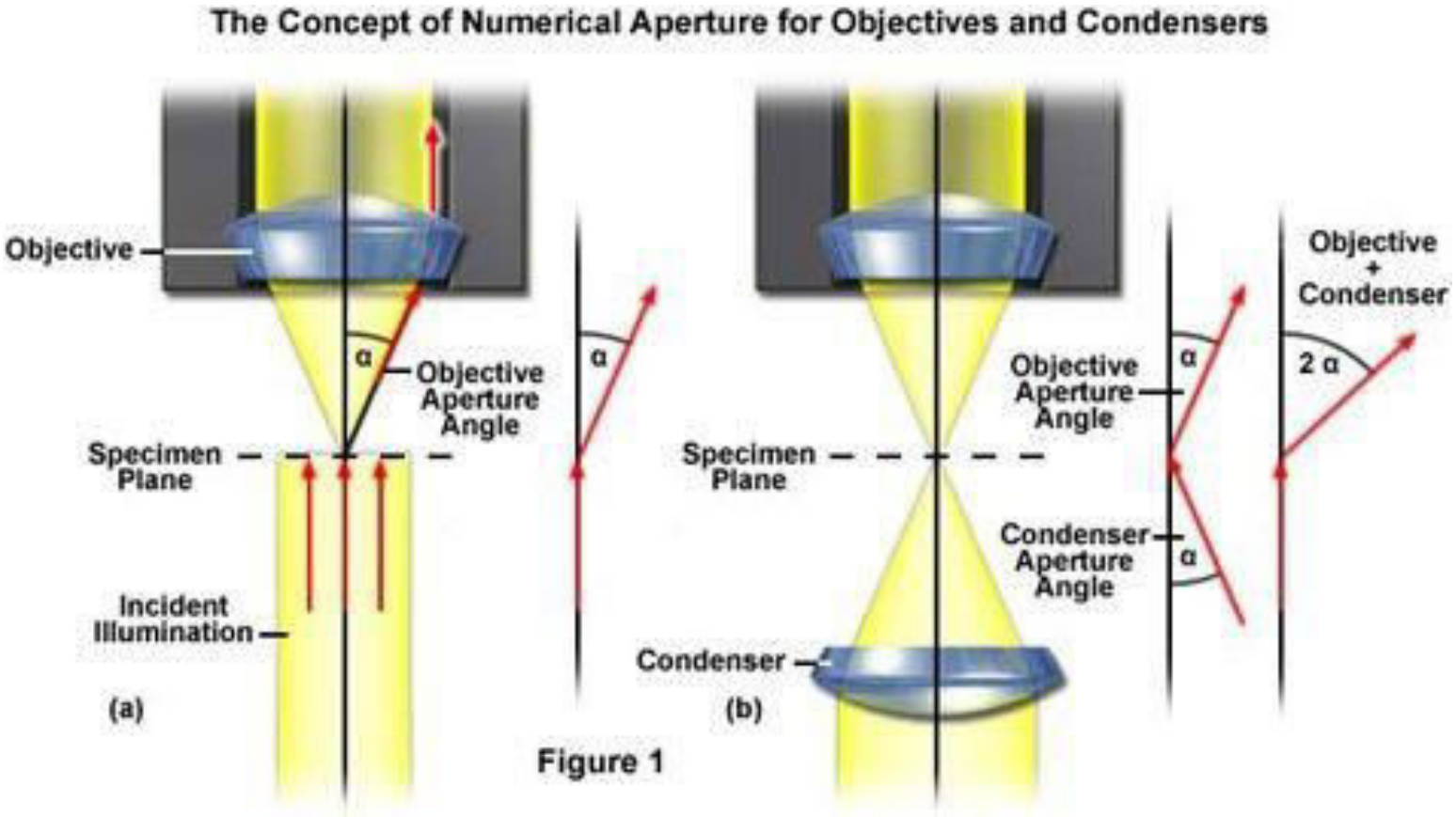



Figure 1

- In a microscope, light is focused on the object as a **narrow pencil of light**, from where it enters into the objective as a diverging pencil
- Light diffracted by the specimen is presented as an inverted cone of half-angle (α), which represents the limits of light that can enter the objective. In order to increase the effective aperture and resolving power of the microscope, a condenser (Figure 1(b)) is added to generate a ray cone on the illumination side of the specimen. This enables the objective to gather light rays that are the result of larger diffraction angles, increasing the resolution of the microscope system.

# Effect of NA on Resolution …

- Higher values of numerical aperture permit increasingly oblique rays to enter the objective front lens, which produces a more highly resolved image and allows smaller structures to be visualized with higher clarity.

- **Numerical Aperture (NA) = n x sin(α)....** equation 1
- where
- n = Refractive index of the medium between the object and the objective (or the objective and the cover slip )
- α = Half aperture angle (equals one-half of the objective's opening angle)
- (n = 1 for air; n = 1.51 for oil or glass).
- By examining Equation (1), it is apparent that the refractive index is the limiting factor in achieving numerical apertures greater than 1.0.
- Therefore, in order to obtain higher working numerical apertures, the refractive index of the medium between the front lens of the objective and the specimen cover slip must be increased.

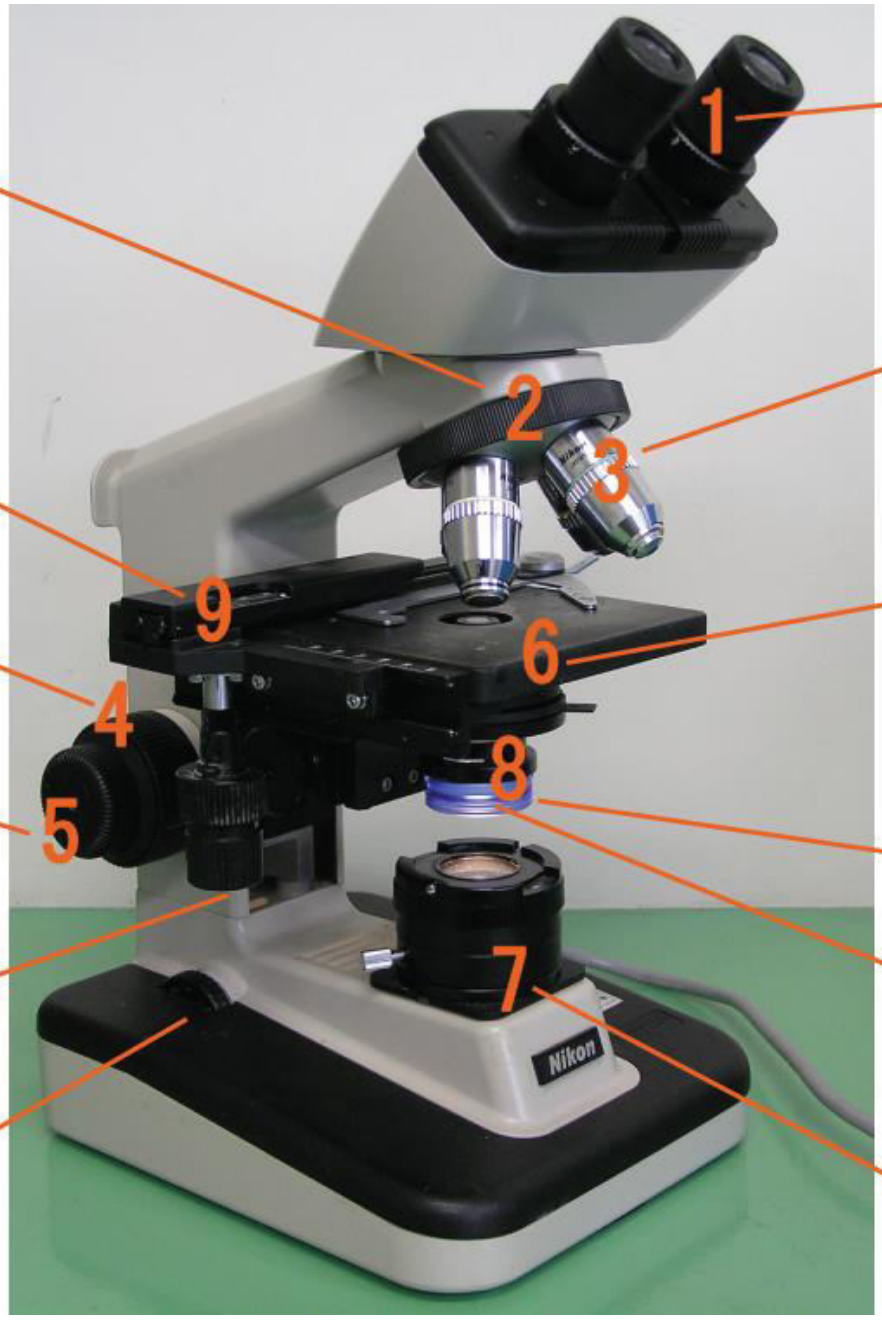
# Immersion Media

- One way of increasing the optical resolving power of the microscope is to use immersion liquids between the front lens of the objective and the cover slip.
- Most objectives in the magnification range between 60x and 100x (and higher) are designed for use with immersion oil.
- Good results have been obtained with oil that has a refractive index of

n = 1.51, which has been precisely matched to the refractive index of glass. All reflections on the path from the object to the objective are eliminated in this way. If this trick were not used, reflection would always cause a loss of light in the cover slip or on the front lens in the case of large angles

- **Aperture** can be defined as the opening in a lens through which light passes to enter the camera. It is expressed in f-numbers like f/1.4, f/2, f/2.8 and so on to express the size of the lens opening, which can be controlled through the lens or the camera.
- **focal length**
- For a thin lens in air, the **focal length** is the **distance** from the center of the lens to the principal foci (or **focal** points) of the lens.
- For a converging lens (for example a convex lens), the **focal length** is positive, and is the **distance** at which a beam of light will be focused to a single spot.

# Lesson 4: Bright-field microscopy




1. eyepiece (ocular lens)
2. revolving nose piece (to hold multiple objective lenses)
3. objective lenses
4. coarse focus (larger knob)
5. fine focus (small knob)
6. stage (to hold the specimen)
7. illuminator
8. diaphragm
9. mechanical stage

x-y mechanical stage knobs (to move slide)

condenser

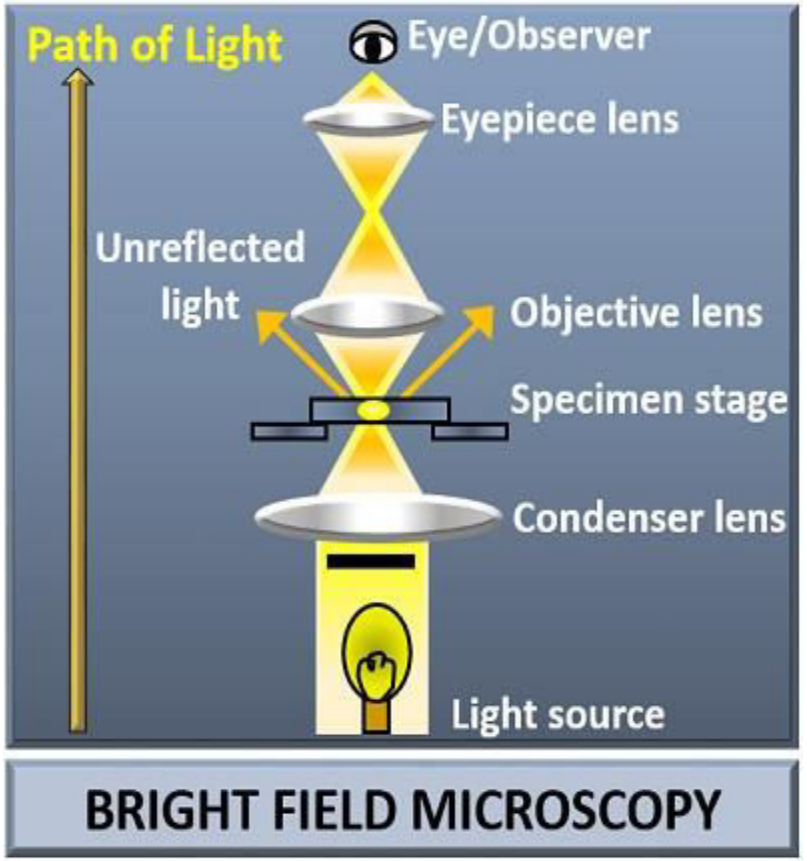
rheostat (to adjust light intensity)

# Bright-field microscopy

- In Bright-field microscopy the Sample is illuminated from below and observed from above through the eye piece)
- white light and contrast in the sample is caused by absorbance of some of the transmitted light in dense areas of the sample.
- Staining is often required to increase contrast, which prevents use on live cells in many situations
- Bright field illumination is useful for samples which have an intrinsic colour, for example chloroplasts in plant cells.

# The light path consists of:

1. a transillumination light source, commonly a **halogen lamp** in the microscope stand;
2. a **condenser lens** which focuses light from the light source onto the sample;
3. objective lens which collects light from the sample and magnifies the image.
4. Oculars and/or a camera to view the sample image



# Performance

- STAINING is often required to increase contrast, which prevents use of **live cells** in many situations.
- Bright-field illumination is useful for samples that have an intrinsic color, for example **RBCs in animal cells.**

# Limitations

- Very low contrast of most biological samples.
- The practical limit to magnification with a light microscope is **around 1300X.** Although higher magnifications are possible, it becomes increasingly difficult to maintain image clarity as the magnification increases
- Low apparent **OPTICAL RESOLUTION** due to the material used.
- Samples that are naturally colorless and transparent cannot be seen well, e.g. many types of mammalian cells. These samples often have to be stained before viewing. Samples that do have their own color can be seen without preparation

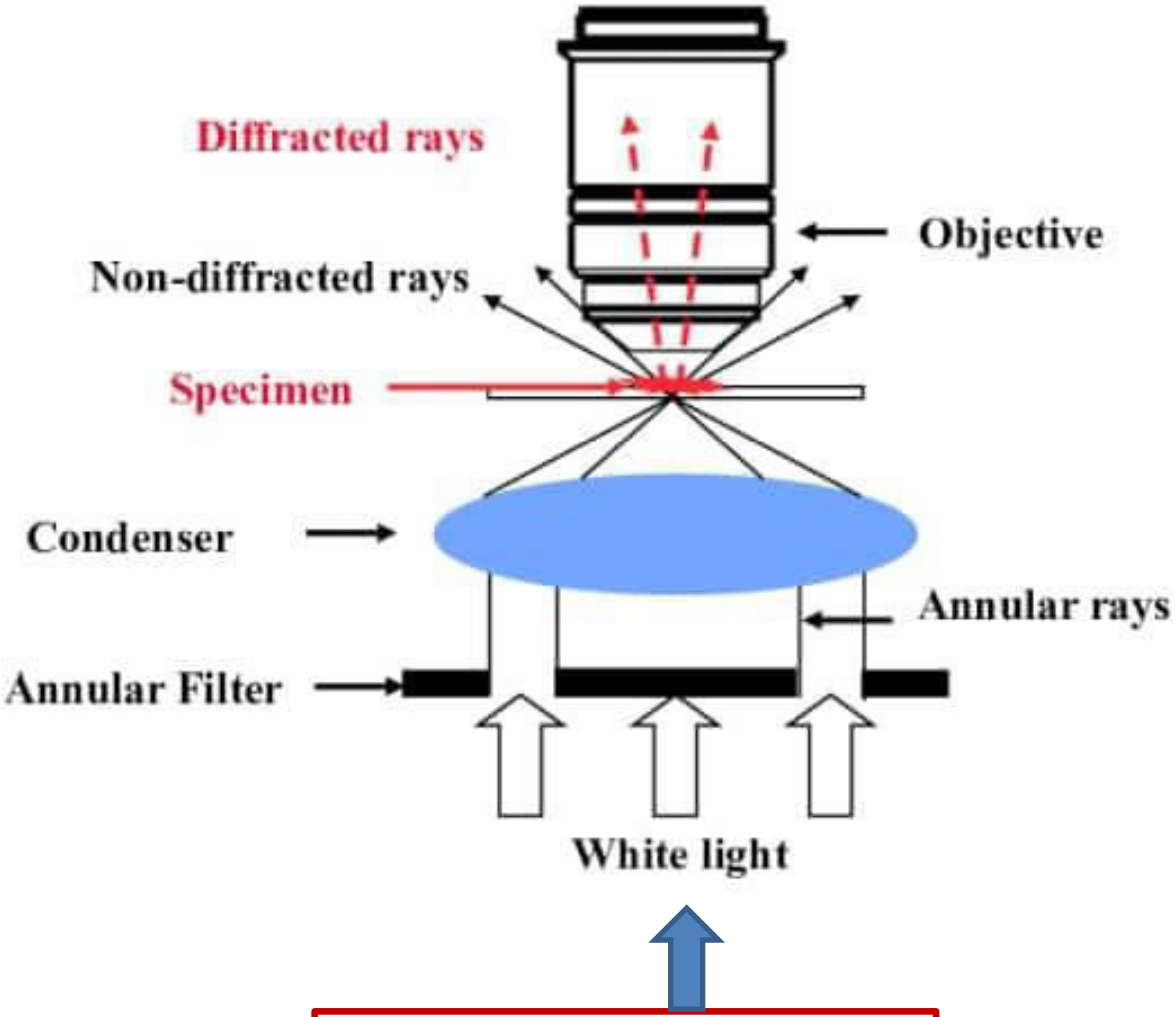
# Enhancements

- Reducing or increasing the amount of the light source by the iris diaphragm.
- Use of an OIL IMMERSION LENS (OBJECTIVE) lens and a special immersion oil (cedar wood oil) placed on a glass cover over the specimen. Immersion oil has the same refraction as glass and improves the resolution of the observed specimen.
- Use of sample-staining methods for use in microbiology, such as simple stains (methylene blue, safranin, crystal violet) and differential stains (negative stains, flagellar stains, endospore stains).
- Use of a colored (usually blue) or polarizing filter on the light source to highlight features not visible under white light.

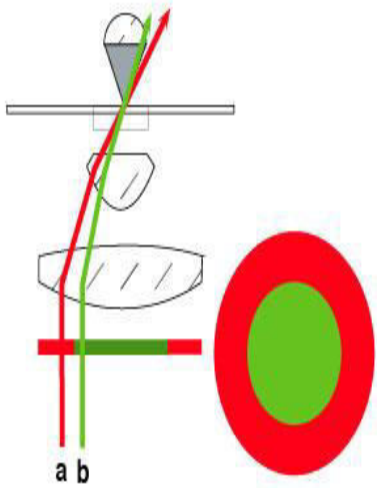
# Dark-field microscopy

# Dark Field Microscopy

- Dark field microscopy is a type of microscopy technique that is used in **both light and electron microscopy**, where only the specimen is lit by a light or electron beam, and the rest of the specimen field is dark.
- As a result, the field around the specimen (i.e., where there is no specimen to scatter the beam) is generally dark.
- It's also called **dark ground microscopy**, and is cheaper yet **higher contrast and resolution as compared to the** alternative technique , i-e, phase contrast microscopy
- Darkfield illumination requires blocking out of the central light that ordinarily passes through and around the specimen, allowing only those rays converging at oblique angles to strike the specimen. This results in a brightly illuminated specimen appearing on a very dark background, which greatly increases contrast and visibility of the specimen.








Annular filter

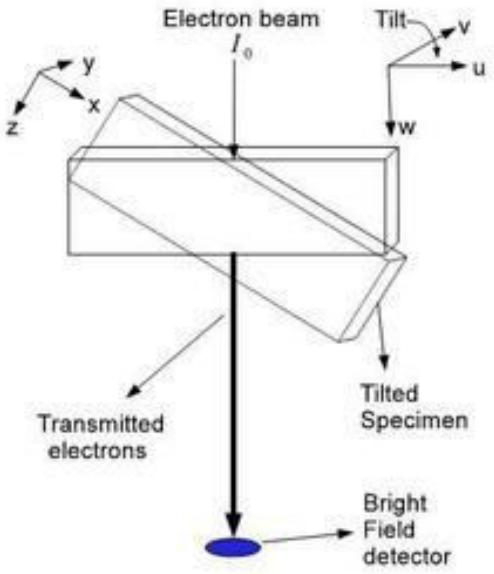
# The light's path

1. Light enters the microscope for illumination of the sample.
2. A specially sized disc, the patch stop (see figure) blocks some light from the light source, leaving an outer ring/slit for entry of light. A wide phase annulus can also be reasonably substituted at low magnification.
3. The condenser lens focuses the light towards the sample.

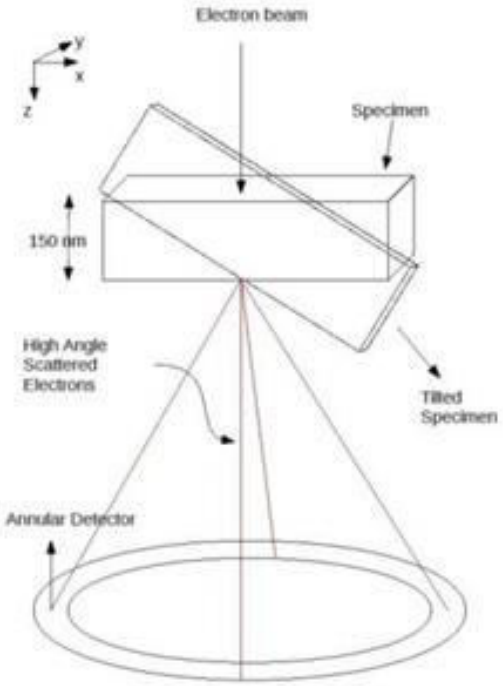
4. The light enters the sample. Most is directly transmitted, while some is scattered from the sample.
5. The scattered light enters the objective lens, while the directly transmitted light simply misses the lens and is not collected due to a direct illumination block (see figure).
6. Only the scattered light goes on to produce the image, while the directly transmitted light is omitted.

- As a result, the entire field of view is dark by default, ( when the sample is not there )and when a specimen is placed on the path of this light cone, only the sample appears bright against a clear, almost black background, therefore making its details stand out.

# Bright Field (BF) vs. Dark Field Imaging




Bright Field:
Image is **bright** when sample is removed



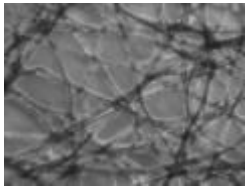

Dark Field:
Image is **dark** when sample is removed

# Uses of dark filed microscopy

- Simple and popular method for making ***unstained objects*** clearly visible.
- This mode of illumination is perfectly adequate for examining transparent stained specimens with transmitted light or for reflected light observation of opaque specimens such as insects, integrated circuits, plants, and other dense objects.
- Many specimens, including living cells and small aquatic organisms such as algae and protozoans, are difficult to visualize with brightfield microscopy because their refractive index is very close to water, the surrounding medium. Other specimens difficult to detect with brightfield transmitted light are diatoms, fibers, hair and fur, mineral thin sections, and small insects

Bright-field illumination, sample contrast comes from attenuation of light in the sample

Cross-polarized light illumination, sample contrast comes from rotation of polarized light through the sample



Phase-contrast illumination, sample contrast comes from interference of different path lengths of light through the sample

# Microscopy

## Lesson 7 : Phase contrast microscopy

# History

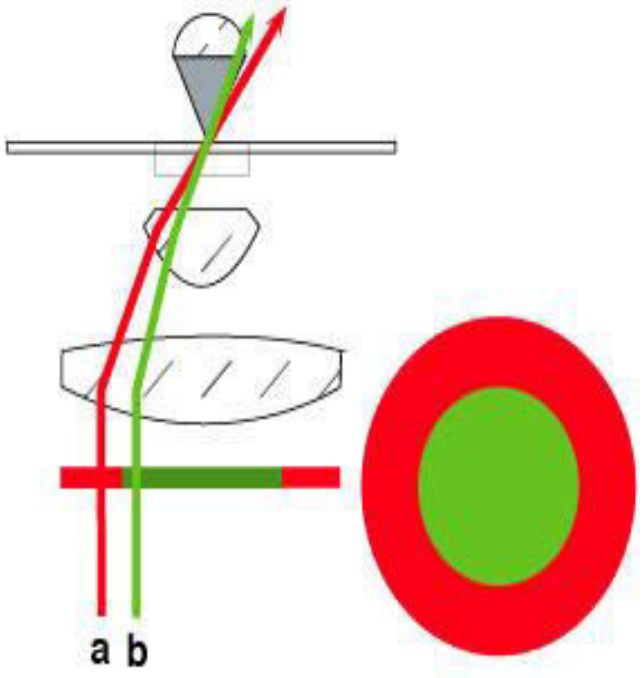
- Phase contrast microscopy first described in 1934 by Dutch physicist **Fritz Zernike,** Who was awarded **Nobel prize in physics in 1953.**
- A phase contrast microscope makes it possible by utilizing **two characteristics of light, diffraction and interference, specimens based on brightness differences.**
- It requires additional specialized structure annular diaphragm and phase contrast ring.

# Phase contrast microscopy

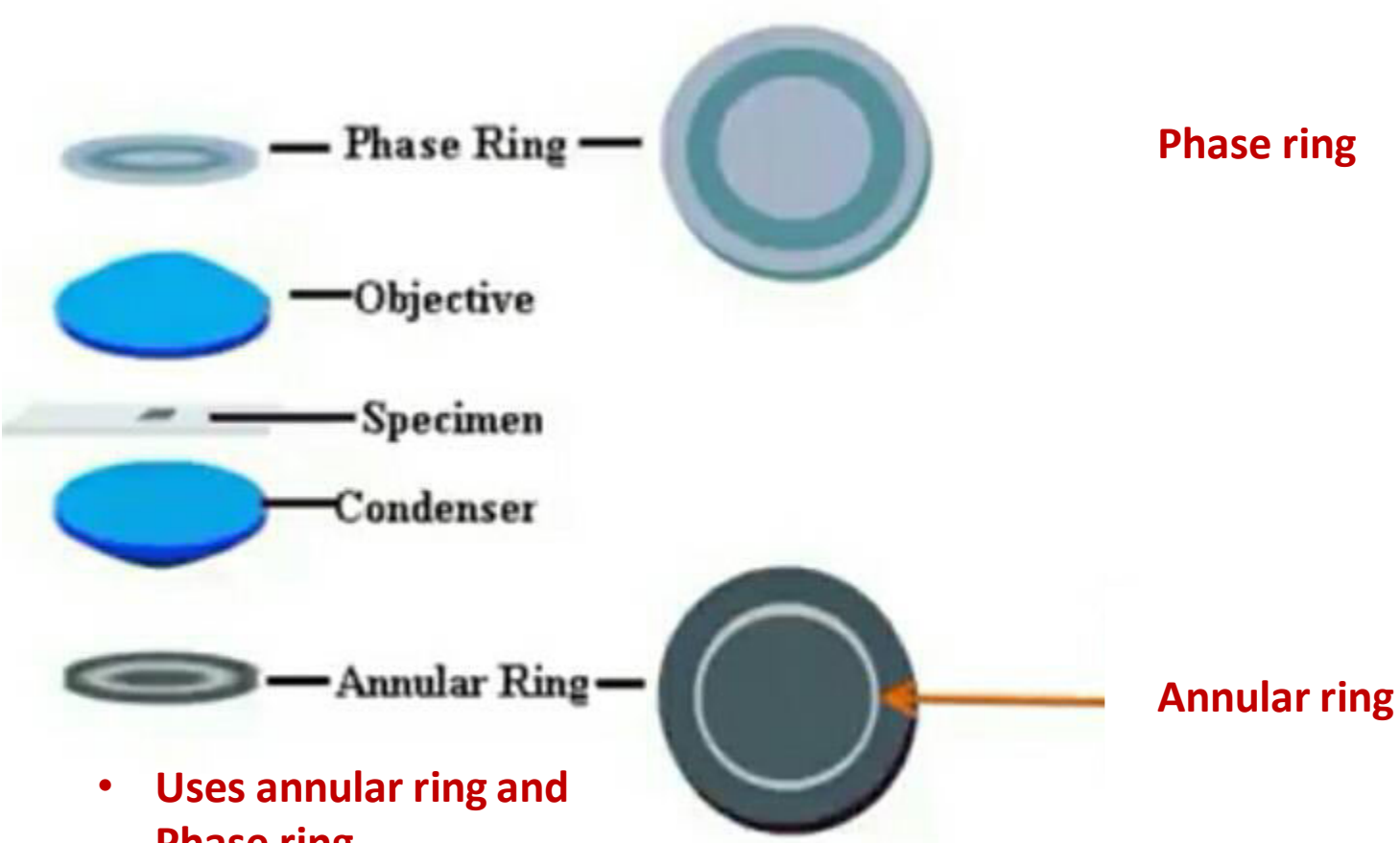
- Phase-contrast microscopy is particularly important in biology.
- It reveals many **cellular structures** that are invisible with a **Bright-field microscope**
- In Bright-field microscopy the Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light and contrast in the sample is caused by absorbance of some of the transmitted light in dense areas of the sample
- Bright-field microscopy typically has low contrast with most biological samples as few absorb light to a great extent.
- Staining is often required to increase contrast, which prevents use on live cells in many situations.
- Bright field illumination is useful for samples which have an intrinsic colour, for example chloroplasts in plant cells or that are stained.

The phase contrast microscopy is a special adaptation of the light microscopy & helps to obtain a clear picture of living or unstained cells.

- The adaptors convert minute difference in phase changes in transmitted light due to refractive indices of all cell organelles in to perceptible shades of grey
- This allow organelles of the living cell to become visible with fair contrast in them.




**Annular filter in dark field microscope**




Phase ring

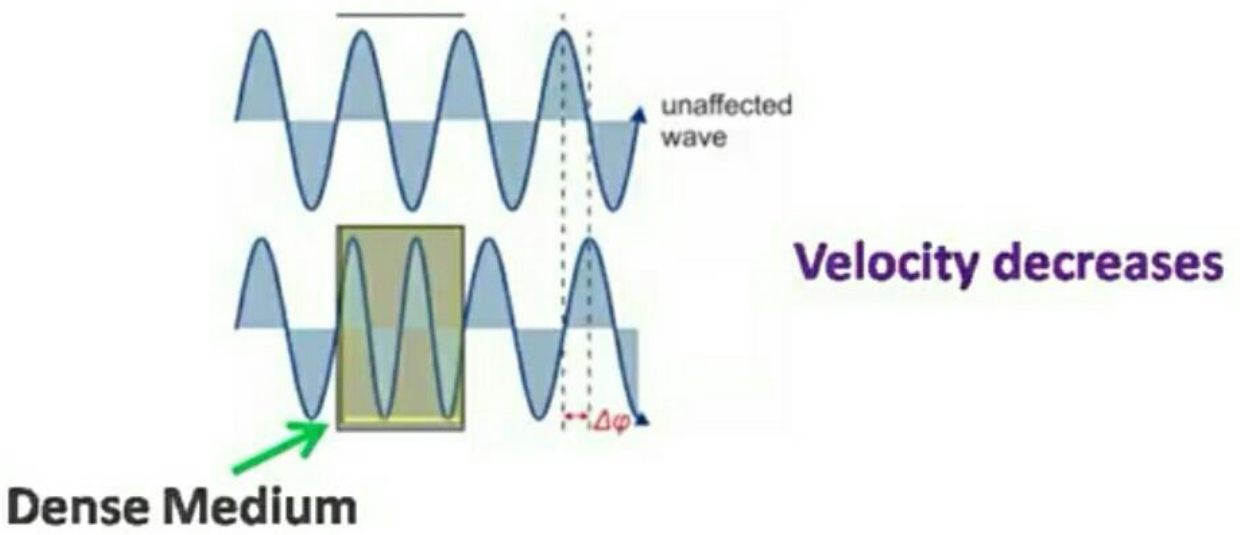
Annular ring

- Uses annular ring and Phase ring
- Annular ring:
- Phase ring:

# **Principle:** Phase Contrast Microscope

- It based on the wavelength (nature) of light rays and the fact that light rays can be **in phase or out of phase.**
- Different shade of grey are distinguished to our eyes due to differences in amplitude of light rays.
- PCM converts invisible small phase changes caused by the cell component in to visible intensity changes.

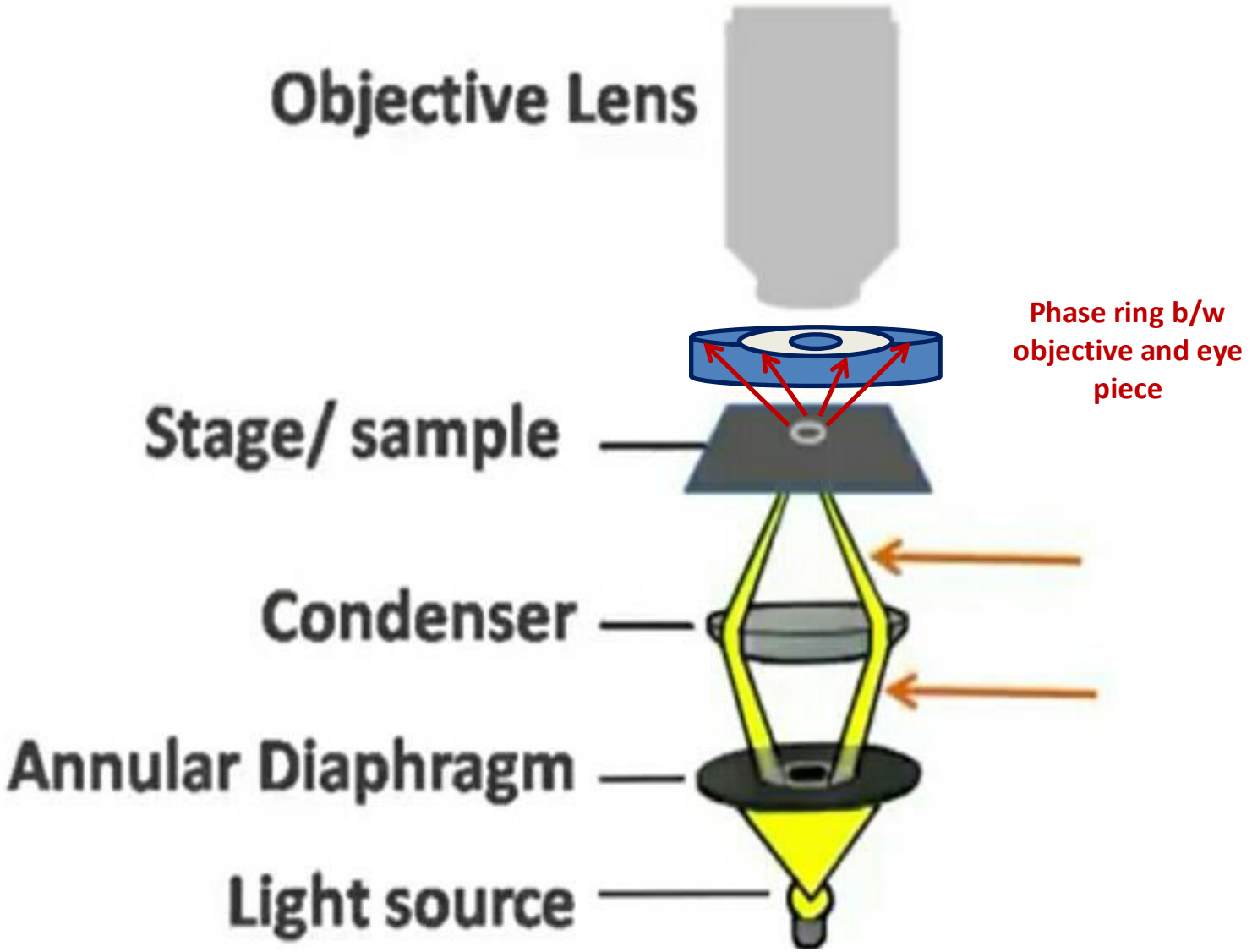
## What is phase shift ?




**Out of phase:** when diffracted and surrounding waves do not over lap

**In Phase:** when diffracted and surrounding waves over lap

# Light path


Objective Lens
Phase ring b/w objective and eye piece
Stage/ sample
Condenser
Annular Diaphragm
Light source

# Microscopy

## Lesson 8 : Phase contrast microscopy-2

## How it works

# How does it work ?

- Regions of different composition likely to have different Refractive indices. Normally such differences cannot be detected by our eyes. However, PCM depicts them in terms of differences in intensity of brightness and darkness, which are visible to eye
- In a Phase contrast microscope, one set of light rays comes directly from the light sources **(surround waves – S waves).**
- The other set comes from light that is reflected or diffracted from a particular structure in the specimen **(diffracted waves – d waves).**

- **The images differences in refractive index of cellular structure. Light passes through thicker parts of cell is held up relative to the light that passes through thinner parts of cytoplasm**

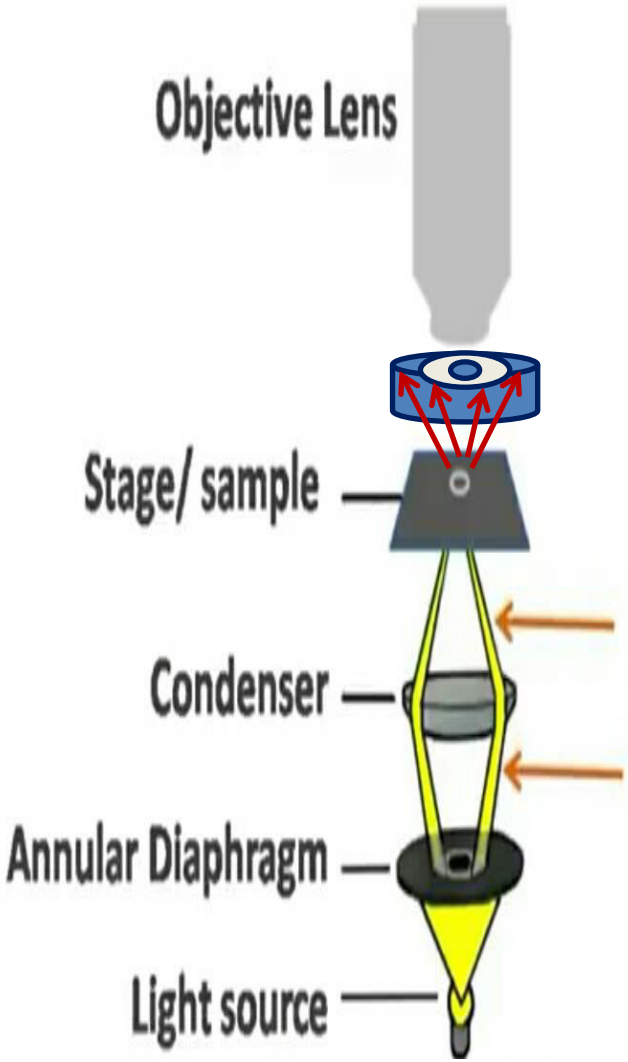
- Light passing through one material & into another material of slightly different refractive index or **thickness will undergo a change in phase.**
- This change is translated into variations in brightness of the structures.
- Phase contrast is obtained with the help of the **Phase ring** by separating the central & direct ray from the diffracted ray

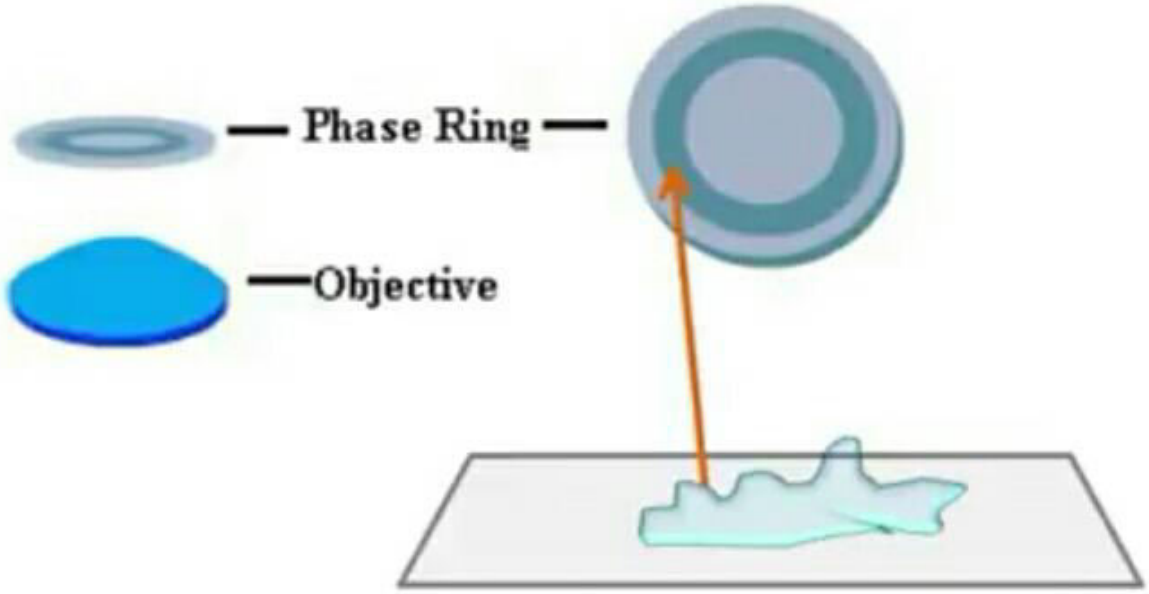
# How does it work ?

- The ring shaped illuminating light that passes the condenser annulus is focused on the specimen by the condenser
- Some of the illuminating light is scattered by the specimen .
- The remaining light is unaffected by the specimen and forms the background light.

# How does it work ?

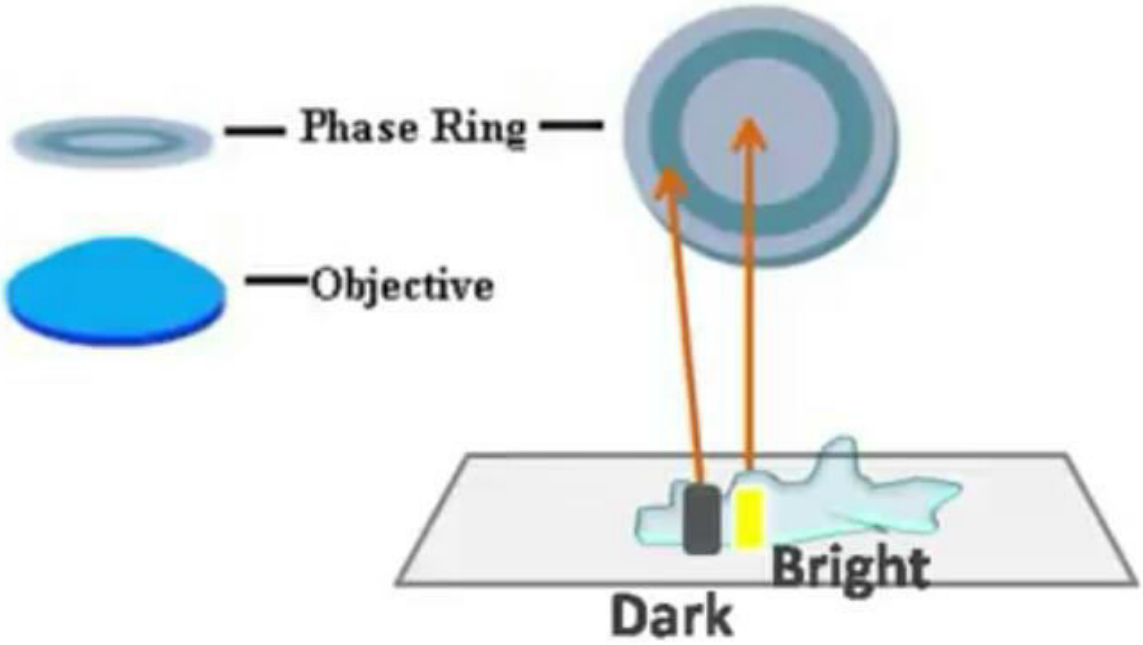
- Light that does not interact with the speciman is collected by the objective passes through the phase ring, and is regarded exactly ¼ wavelength.
- The Phase shifted is not detectable by the eye so the resulting image on the image plane in the microscope appears as a normal bright background






**Refraction in different directions**

**Light passing through the specimen is phase shifted**



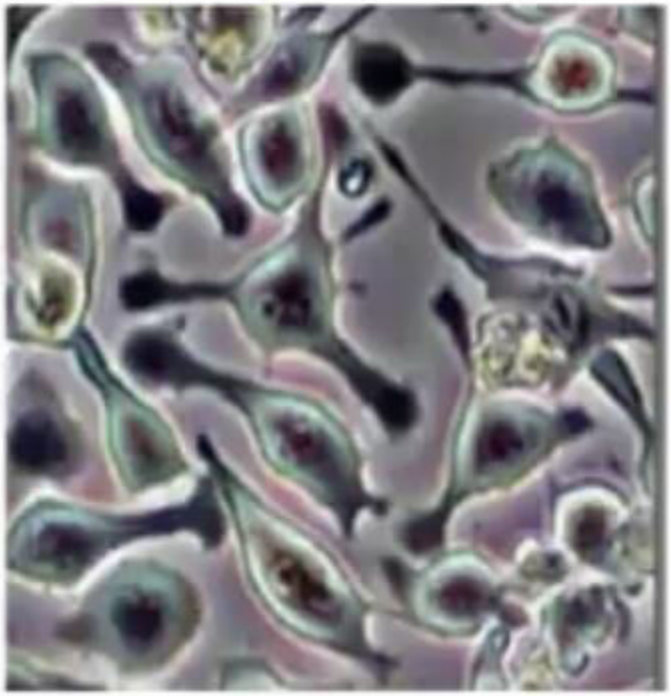

**Refraction in different directions**

**Light passing through the specimen is phase shifted**

- **If a microscope can delineate change of phase as a change in brightness or color, the eye** or the camera**, or photocell will be able to detect the microscopic areas causing the phase changes**

**Bright field**

**Phase contrast**



# Applications of Phase contrast microscopy

- Most commonly used to provide contrast of transparent specimens such as living cells or small organisms.
- Useful in observing cells cultured in vitro during mitosis.
- Phase contrast enables visualization of internal cellular components.
- It’s used in examination of growth, dynamics, and behaviour of a wide variety of living cells in cell culture.

- **Refractive index (μ):** It is the ratio of Velocity of light in air to that of in given medium
- **Diffraction:** The process by which a beam of light is spread out as a result of passing through a narrow aperture or across an edge typically accompanied by interference b/w the wave forms produced

# Two main types of Phase contrast microscopy

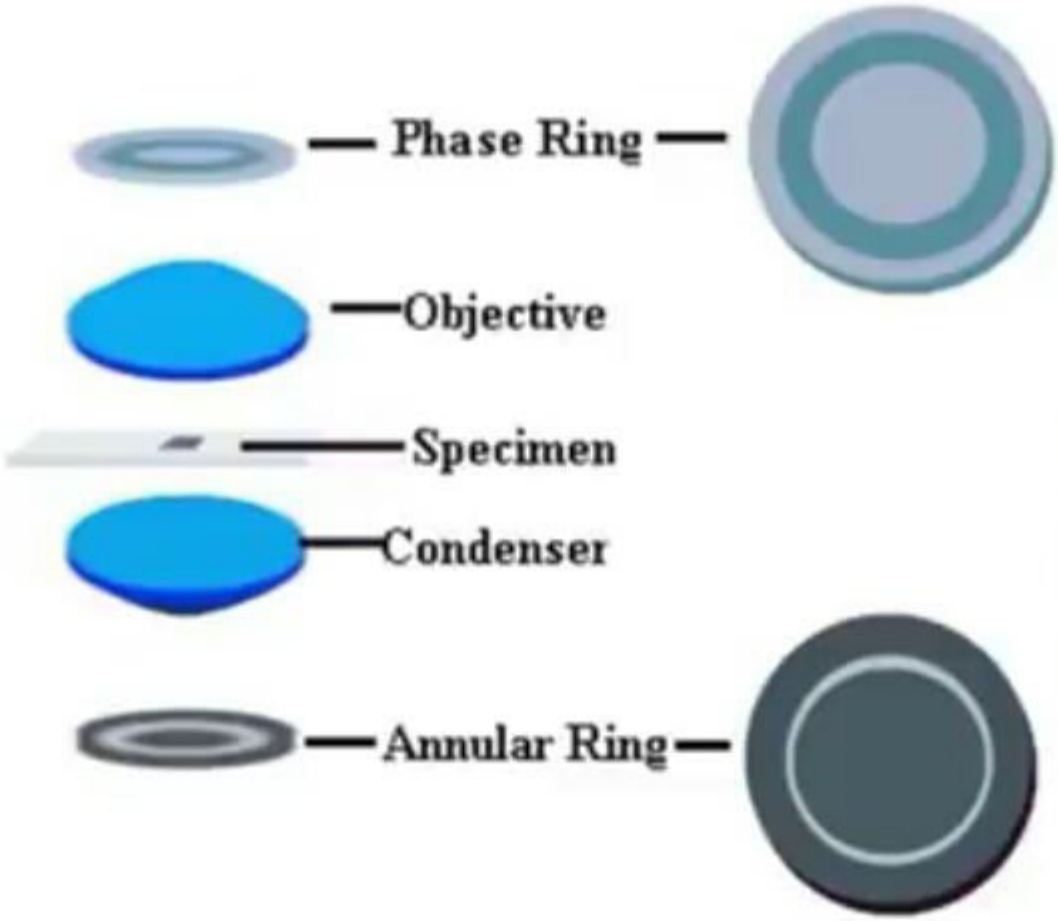
- Today, two main types of phase contrast are positive and negative. Since the observed particles are usually thin and transparent, these polar contrasts provide strikingly different images.
- **Positive phase contrast** reveals medium to dark gray images on a lighter grey background; these images often have a bright halo along the edge of the sample.
- **Negative phase contrast** is the opposite. The **specimen appears lighter** with a dark background; they also have a dark halo outlining the image.

**Negative Phase contrast:**

The background light is phase shifted -90° by passing it through a Phase shift ring. This eliminates the phase difference between the background and the scattered light. • To further increase contrast, the background is dimmed by a gray filter ring

Positive phase contrast:

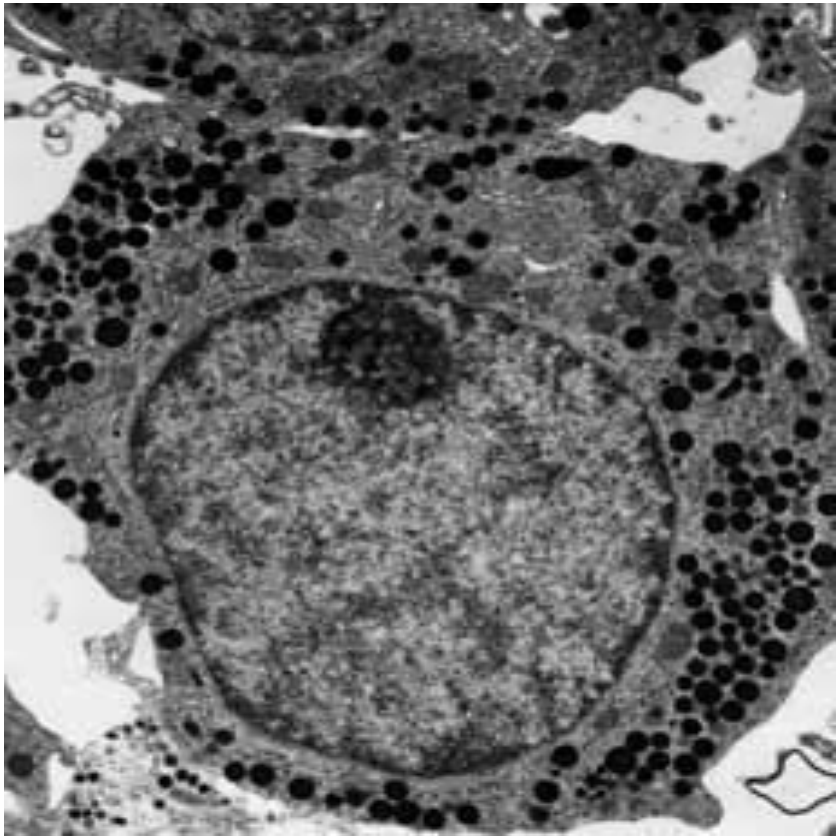
the background light is instead phase shifted by +90°. The background light will thus be 180° out of phase relative to the scattered light. • Leads to formation of  an image where the foreground is darker than the background.


Phase Ring
Objective
Specimen
Condenser
Annular Ring

# Phase contrast microscopy makes these phase difference visible

- The **details of many biological and industrial specimens are characterized by differences in refractive index rather than by differences in light absorption**.
- Under an ordinary microscope such details are invisible, unless the aperture of the condenser or objective is made so small that the resolving power suffers a serious deterioration with resultant loss in the observer's ability to interpret what he sees.
- **Light can be considered a form of wave motion consisting of sinusoidal waves.** When a light wave traverses a medium of different optical path the phase of the light wave is altered.
- This alteration may be visualized as simply a displacement of the wave in its direction of propagation.

# Lesson Electron microscope



# Electron microscope

- Electron microscopy (EM) is a technique for obtaining high resolution images of biological and non-biological specimens.
- It is used in biomedical research to investigate the detailed structure of tissues, cells, organelles and macromolecular complexes.
- **The high resolution of EM images results from the use of electrons (which have very short wavelengths) as the source of illuminating radiation.**
- Electron microscopy is used in conjunction with a variety of ancillary techniques (e.g. thin sectioning, immuno-labeling, negative staining) to answer specific questions.
- EM images provide key information on the structural basis of cell function and of cell disease.

# History of Electron microscope

- In 1926 **HANS BUSCH** developed the electromagnetic lens.
- According to DENNIS GABOR , the physicist **LEO SZILARD** tried in 1928 to convince him to build an electron microscope, for which he had filed a patent.
- The first prototype electron microscope, capable of four-hundred-power magnification, was developed in 1931 by the physicist ERNST RUSKA and the electrical engineer MAX KNOLL The apparatus was the first practical demonstration of the principles of electron microscopy.
- In May of the same year, REINHOLD RUDENBERG, the scientific director of **Siemens-Schuckertwerke**, obtained a patent for an electron microscope.
- In 1932, Ernst Lubcke of **SIEMENS & HALSKE** built and obtained images from a prototype electron microscope, applying the concepts described in Rudenberg's patent.
- In the following year, 1933, Ruska built the first electron microscope that exceeded the resolution attainable with an optical (light) microscope.
- Four years later, in 1937, Siemens financed the work of Ernst Ruska and Bodo von Borries, and employed **HELMUT RUSKA**, Ernst's brother, to develop applications for the microscope, especially with biological specimens
- Also in 1937, MENFRED VON ARDENNE pioneered the **Scanning EM (SEM).**
- Siemens produced the first commercial electron microscope in 1938.
- The first North American electron microscope was constructed in 1938, at the **UNIVERSITY OF TORONTO**, by **ELI FRANKLIN BURTON** and students **Cecil Hall**, **JAMES HILLIER, and Albert Prebus.**
- Although current transmission electron microscopes are capable of two million-power magnification, as scientific instruments, they remain based upon Ruska's protype

The first practical TEM, originally installed at IG Farben-Werke and now on display at the Deutsches Museum in Munich, Germany

**A transmission electron microscope (1976).**

# Types of electron microscope

- Transmission EM (TEM) and
- Scanning EM (SEM).

# Types of electron microscope

- **Transmission EM (TEM)**
- The **transmission electron microscope** is used to view thin specimens (tissue sections, molecules, etc) through which electrons can pass generating a projection image.

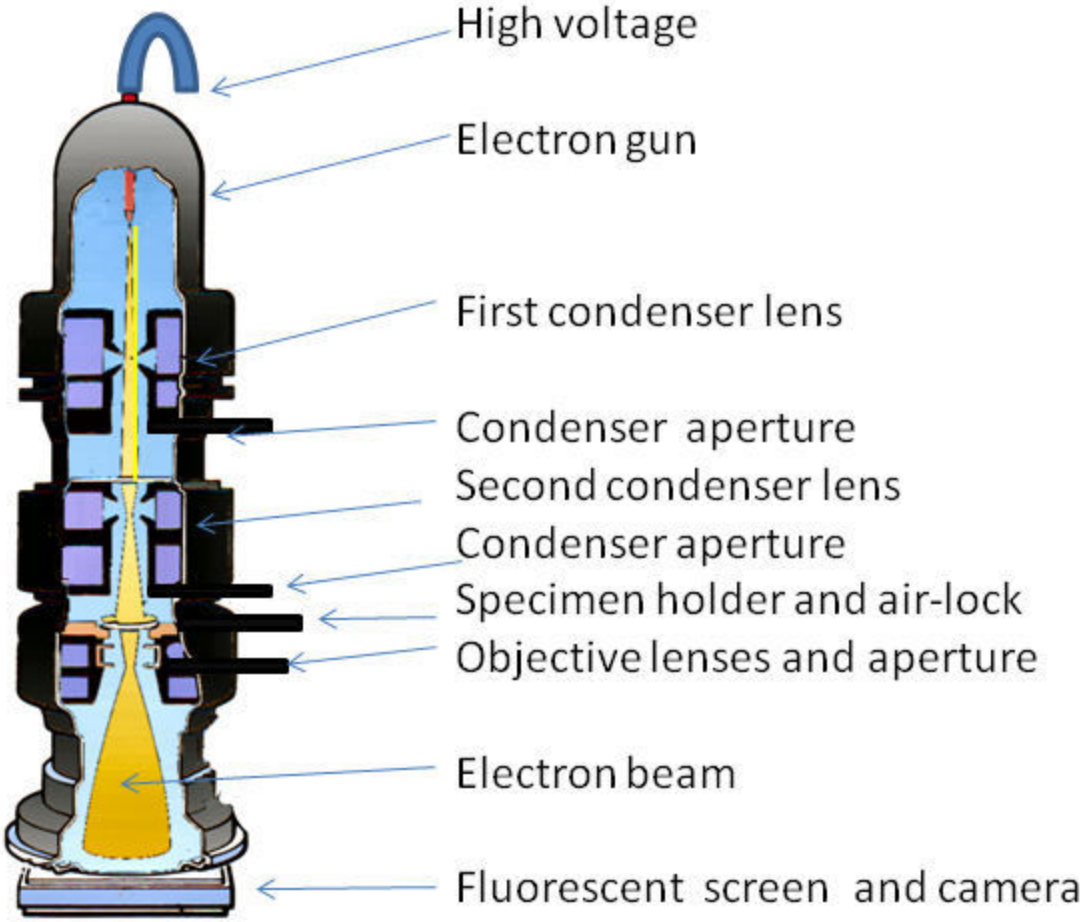
- **Scanning EM (SEM).**
- A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons annd is used to view the 3d structure of sample
- Specimen preparartion for TEM and SEM is done in different ways.

# USES oF ELECTRON MICROSCOPY

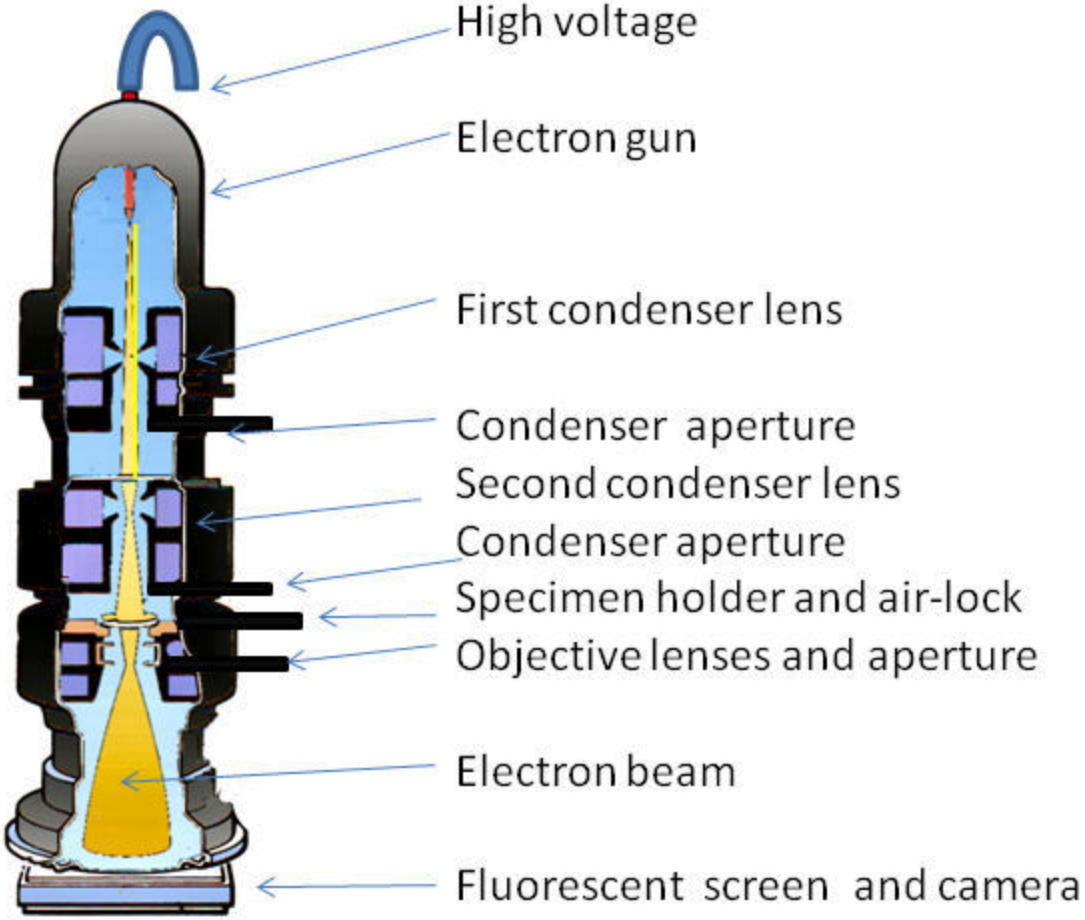
- Even the very best light microscopes have a resolution limit of about 0.2 µm, which greatly compromises their usefulness for detailed studies of many microorganisms.
- Viruses are too small to be seen with light microscopes. Prokaryotes can be observed, but because they are usually only 1 m to 2 m in diameter, just their general shape and major morphological features are visible.
- The resolution of a light microscope increases with a decrease in the wavelength of the light it uses for illumination.

- **Electrons replace light as the illuminating beam. They can be focused, much as light is in a light microscope, but their wavelength is around 0.005 nm, approximately 100,000 times shorter than that of visible light**.
- Therefore, electron microscopes have a practical resolution roughly 1,000 times better than the light microscope; with many electron microscopes, points closer than 0.5 nm can be distinguished, and the useful magnification is well over 100,000X

# Lesson 10: Transmission electron microscope




Transmission Electron Microscope



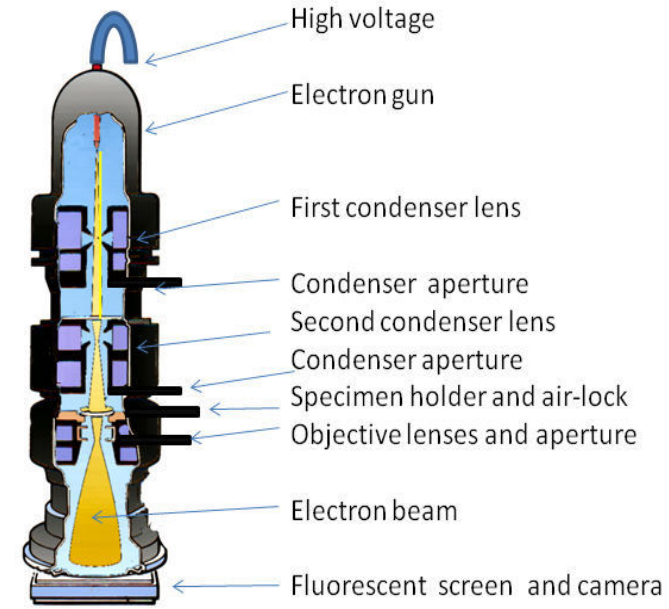

Transmission Electron Microscope

# Sample Preparation for TEM

- Since electrons are deflected by air molecules and are easily absorbed and scattered by solid matter, only extremely thin slices (20 to 100 nm) of a microbial specimen can be viewed in the average TEM. Such a thin slice cannot be cut unless the specimen has support of some kind; the necessary support is provided by plastic.
- After fixation with chemicals like glutaraldehyde or osmium tetroxide to stabilize cell structure, the specimen is dehydrated with organic solvents (e.g., acetone or ethanol). Complete dehydration is essential because most plastics used for embedding are not water soluble.
- .

- Next the specimen is soaked in unpolymerized, liquid epoxy plastic until it is completely permeated, and then the plastic is hardened to form a solid block.
- Thin sections are cut from this block with a glass or diamond knife using a special instrument called an ultramicrotome

# Lesson 11: Transmission electron microscope ..continued




Transmission Electron Microscope

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# Staining

- The probability of electron scattering is determined by the density (atomic number) of the specimen atoms.
- Biological molecules are composed primarily of atoms with low atomic numbers (H, C, N, and O), and electron scattering is fairly constant throughout the unstained cell.
- Therefore specimens are prepared for observation by soaking thin sections with solutions of heavy metal salts like **lead citrate and uranyl acetate.**
- The **lead and uranium ions** bind to cell structures and make them more electron opaque, thus increasing contrast in the material.
- Heavy osmium atoms from the osmium tetroxide fixative also "stain" cells and increase their contrast
- The stained thin sections are then mounted on tiny copper grids and viewed.

# Shadowing

- Two other important techniques for preparing specimens are **negative staining and shadowing.**
- **In negative staining,** the specimen is spread out in a thin film with either phosphotungstic acid or uranyl acetate. Just as in negative staining for light microscopy, heavy metals do not penetrate the specimen but render the background dark, whereas the specimen appears bright in photographs. Negative staining is an excellent way to study the structure of viruses, bacterial gas vacuoles, and other similar objects
- **In shadowing,** a specimen is coated with a thin film of **platinum or other heavy metal by evaporation at an angle of about 45°** from horizontal so that the metal strikes the microorganism on only one side. In one commonly used imaging method, the area coated with metal appears dark in photographs, whereas the uncoated side and the shadow region created by the object is **light**
- This technique is particularly useful in studying virus morphology, prokaryotic flagella, and DNA.

# Freeze-etching

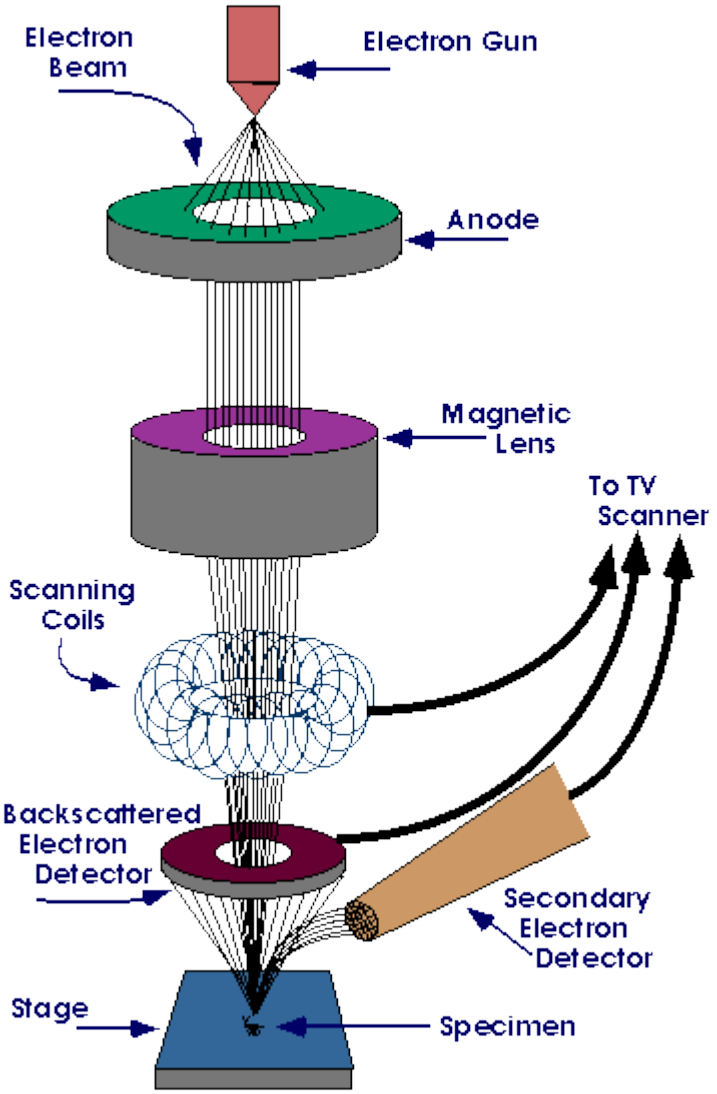
- The TEM will also disclose the shape of organelles within microorganisms if specimens are prepared by **the freezeetching procedure**.
- First, cells are rapidly frozen in liquid nitrogen and then warmed to 100°C in a vacuum chamber. Next a knife that has been precooled with liquid nitrogen **(-196°C)** fractures the frozen cells, which are very brittle and break along lines of greatest weakness, usually down the middle of internal membranes
- The specimen is left in the high vacuum for a minute or more so that some of the ice can sublimate away and uncover more structural detail. Finally, the exposed surfaces are shadowed and coated with layers of platinum and carbon to form a replica of the surface. After the specimen has been removed chemically, this replica is studied in the TEM and provides a detailed, three-dimensional view of intracellular structure
- An advantage of freeze-etching is that it minimizes the danger of artifacts because the cells are frozen quickly rather than being subjected to chemical fixation, dehydration, and plastic embedding.

# Lesson 12: Scanning electron microscope (SEM)

# Scanning electron microscope

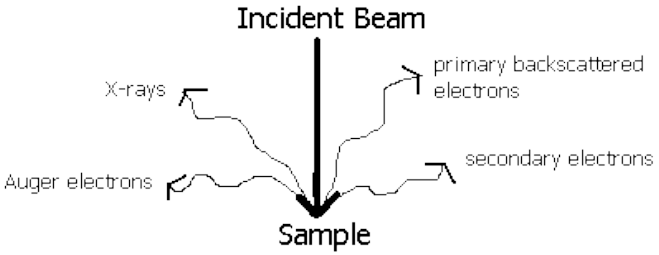
- A **scanning electron microscope** (**SEM**) is a type of **electron microscope** that produces images of a sample by scanning the surface with a focused beam of **electrons**
- The SEM has allowed researchers to examine a much bigger variety of specimens.
- The electrons interact with atoms in the sample, producing various signals that contain information about the surface TOPOGRAPHY and composition of the sample.
- Specimens are observed in high vacuum in a conventional SEM, or in low vacuum or wet conditions in a variable pressure or environmental SEM, and at a wide range of cryogenic or elevated temperatures with specialized instruments

# How does SEM work?



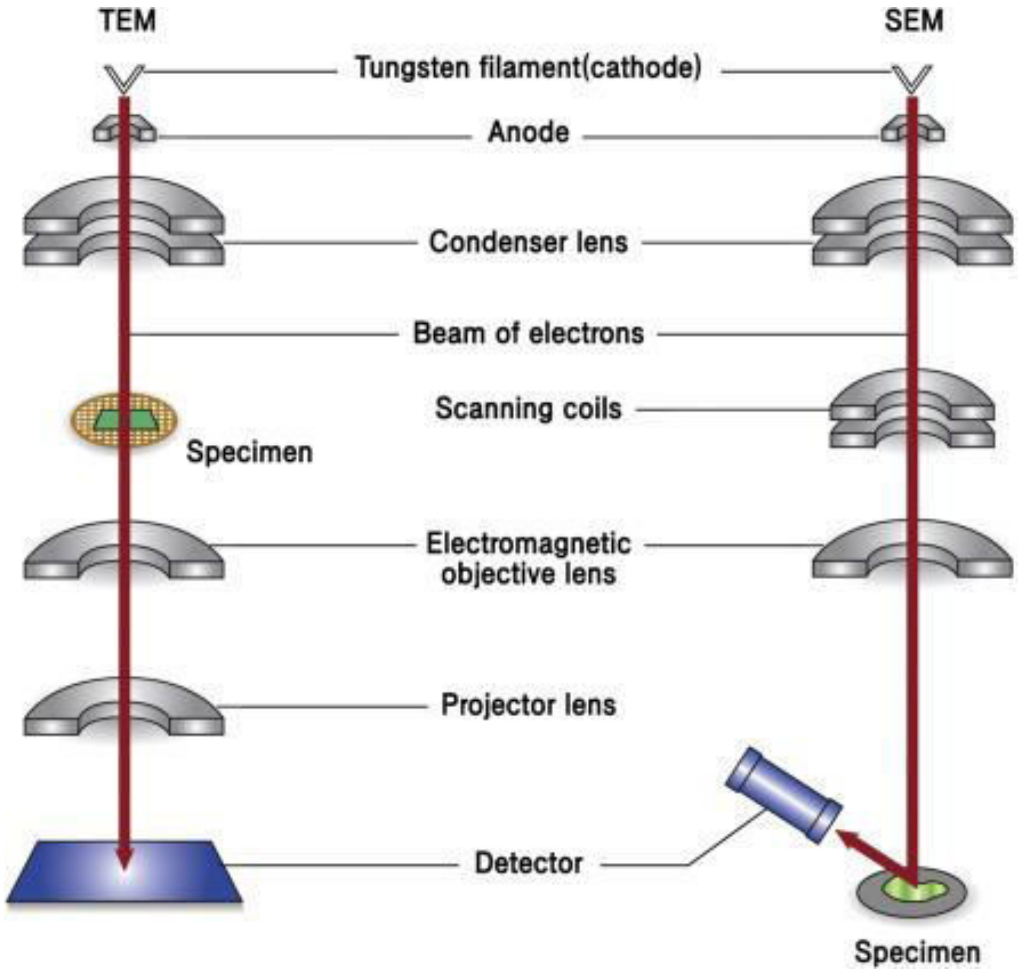
## How does SEM work?

- The SEM is an instrument that produces a largely magnified image by using electrons instead of light to form an image.
- A beam of electrons is produced at the top of the microscope by an electron gun.
- The electron beam follows a vertical path through the microscope, which is held within a vacuum.
- The beam travels through electromagnetic fields and lenses, which focus the beam down toward the sample.
- Once the beam hits the sample, electrons and X-rays are ejected from the sample.
- Detectors collect these X-rays, backscattered electrons, and secondary electrons and convert them into a signal that is sent to a screen similar to a television screen. This produces the final image.



# Advantages of SEM

- The scanning electron microscope has many advantages over traditional microscopes.
- The SEM has a large depth of field, which allows more of a specimen to be in focus at one time.
- The SEM also has much higher resolution, so closely spaced specimens can be magnified at much higher levels.
- Because the SEM uses electromagnets rather than lenses, the researcher has much more control in the degree of magnification.
- Provides strikingly clear images, make the scanning electron microscope one of the most useful instruments in research today.


TEM
SEM
Tungsten filament(cathode)
Anode
Condenser lens
Beam of electrons
Scanning coils
Specimen
Electromagnetic objective lens
Projector lens
Detector
Specimen

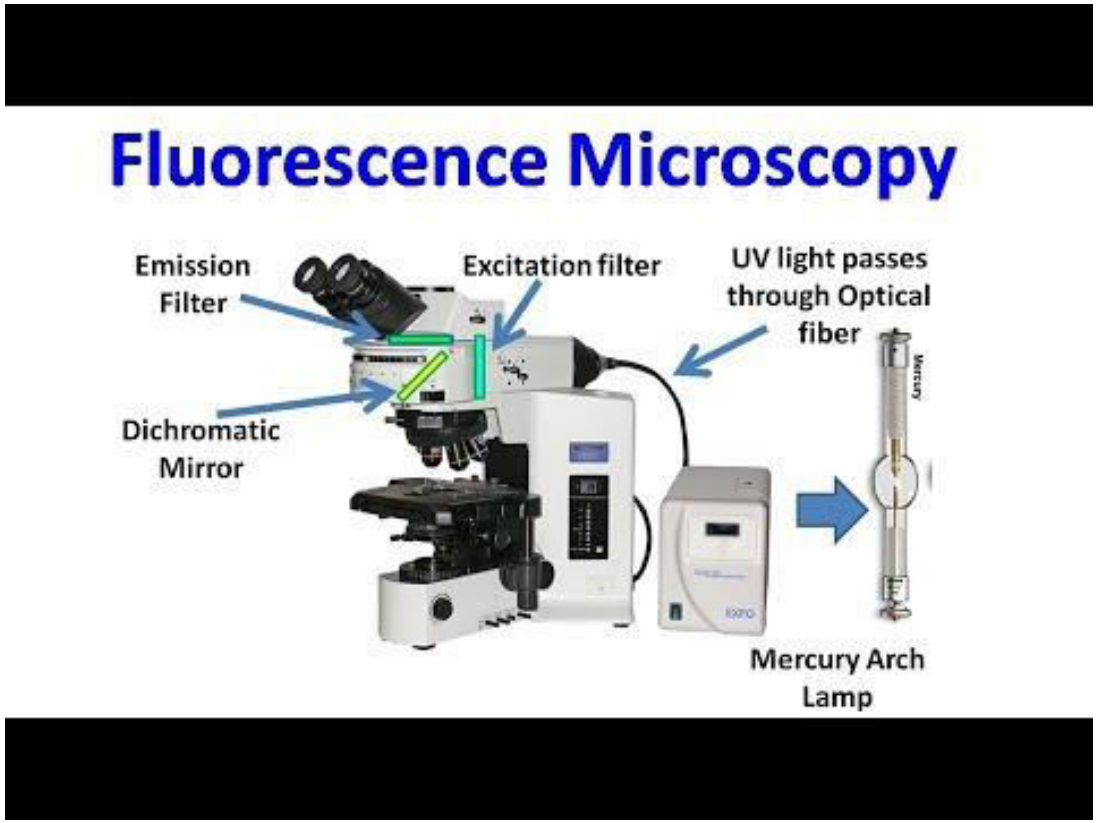
# Lesson : Fluorescence Microscope

# Fluorescence Microscope

- Fluorescent microscope is much the same as a conventional light microscope with added features to enhance its capabilities.
- **This microscope additionally requires an excitation filter, a barrier and a dichromatic mirror, fluorescent stain.**
- A specific wavelength of light is used to excite fluorescent molecule in specimen.
- Light of higher wavelength is then imaged.

- **The key feature of fluorescent microscopy is that it employs reflected rather than transmitted light**, which means transmitted light techniques such as **phase contrast microscope** can be combined with fluorescent microscopy.

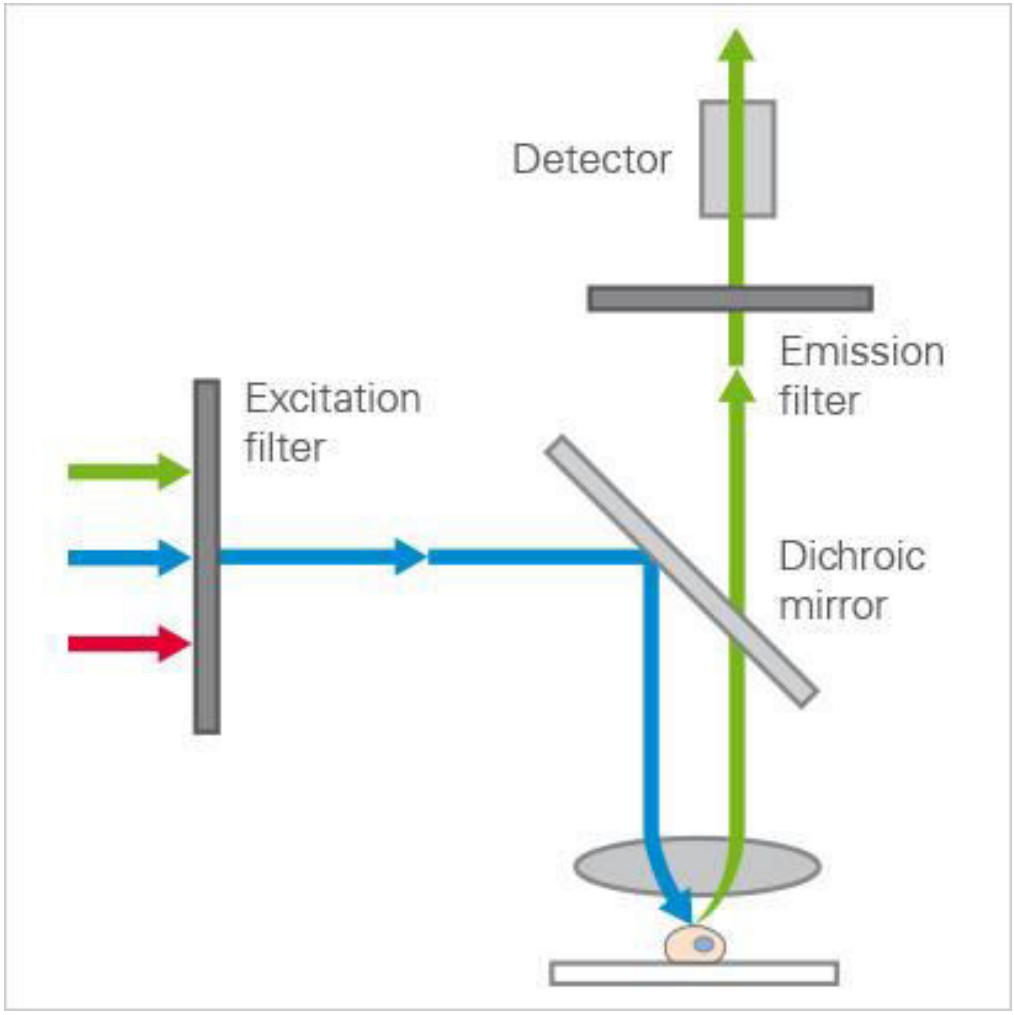
- This is achieved by using powerful light sources, such as lasers, that can be focused to a pinpoint.
- This focusing is done repeatedly throughout one level of a specimen after another.
- Most often an image reconstruction program pieces the multi level image data together into a 3-4 D reconstruction of the targeted sample.




An upright fluorescence microscope (Olympus BX61) with the fluorescence filter cube turret above the objective lenses, coupled with a digital camera.

# Components of a Fluorescence Microscope

- **Light source: Xenon arc lamp or mercury-vapor lamp** are common; power LED and lasers are used in more advanced forms.
- **A set of optical filters:** Optical filters include a set of a compatible excitation filter, emission filter, and dichroic beam splitter;
  - **An excitation filter** selects the wavelengths to excite a particular dye within the specimen.
  - **A dichroic beam splitter/ dichroic mirror** reflects light in the excitation band and transmit light in the emission band, enabling the classic epifluorescence incident light illumination.
  - An emission filter serves as a kind of quality control by letting only the wavelengths of interest emitted by the fluorophore pass through.
- **Darkfield condenser**: It provides a black background against which the fluorescent objects glow.
- The filters are often plugged in together in a filter cube (compound microscopes)

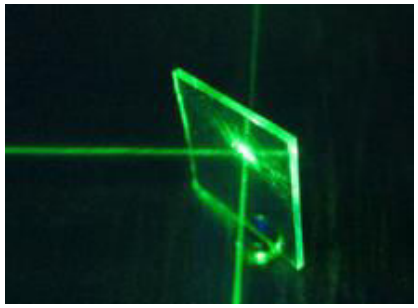

Detector
Emission
filter
Excitation
filter
Dichroic
mirror

# Types of Fluorescence Microscopes

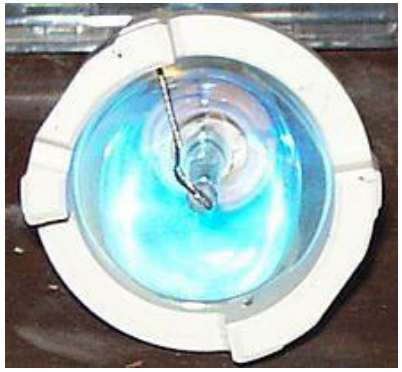
There are various types of fluorescence microscopes. Some of the common types are:

- **Epifluorescence microscopes**: The most common type of fluorescence microscope in which, excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective).
- **Confocal microscope**: In this type of fluorescence microscope, high-resolution imaging of thick specimens (without physical sectioning) can be analyzed using fluorescent-labeled dye.
- **Multiphoton microscope:** In this type of microscope, multiphoton fluorescence excitation results in the capture of high-resolution three-dimensional images of specimen tagged with highly specific fluorophores.
- **Total internal reflection fluorescence (TIRF) microscope**: Total internal reflection fluorescence microscopy (**TIRFM**) exploits the unique properties of an induced evanescent wave or field in a limited specimen region immediately adjacent to the interface between two media having different refractive indices.

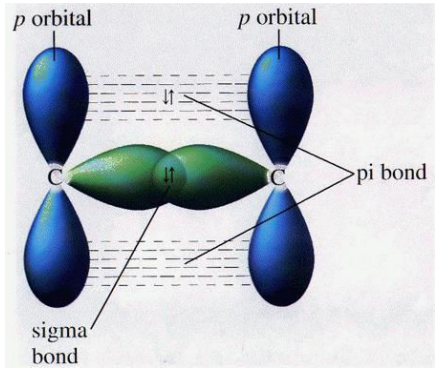
- A **beam splitter** is an **optical device** that splits a beam of light in two. It is a crucial part of many optical experimental and measurement systems, as fluorescent mcicroscope or fibre optic telecommunications.



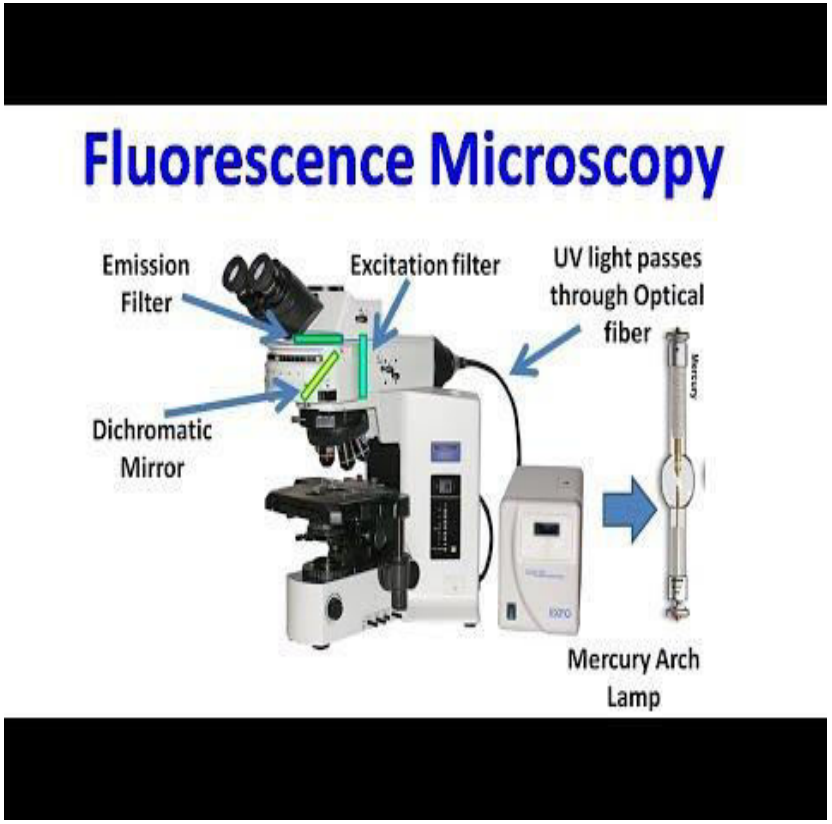
- A **xenon arc lamp** is a highly specialized type of **GAS discharge lamp**, an electric light that produces light by passing electricity through IONIZED XENON gas at high pressure. It produces a bright white light that closely mimics natural sunlight.

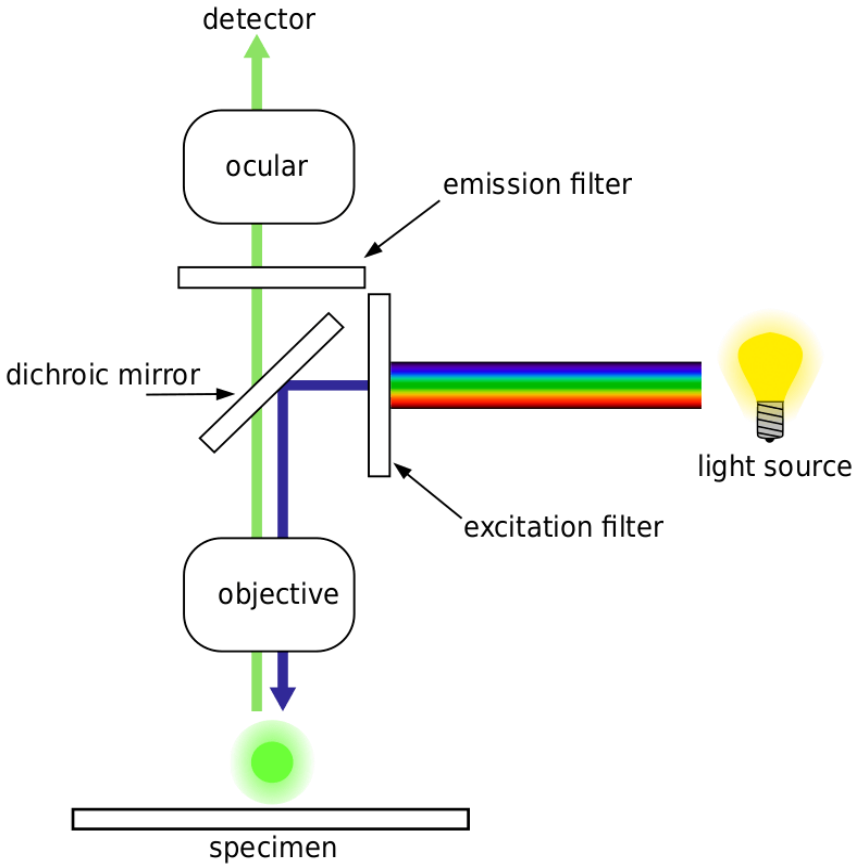


- **Sigma and pi bonds** are chemical covalent **bonds**. **Sigma and pi bonds** are formed by the overlap of atomic orbitals. **Sigma bonds** are formed by end-to-end overlapping and **Pi bonds** are when the lobe of one atomic orbital overlaps another. ... Generally **sigma bonds** are stronger than **pi bonds**.



# Lesson : Fluorescence Microscope-2




An upright fluorescence microscope (Olympus BX61) with the fluorescence filter cube turret above the objective lenses, coupled with a digital camera.

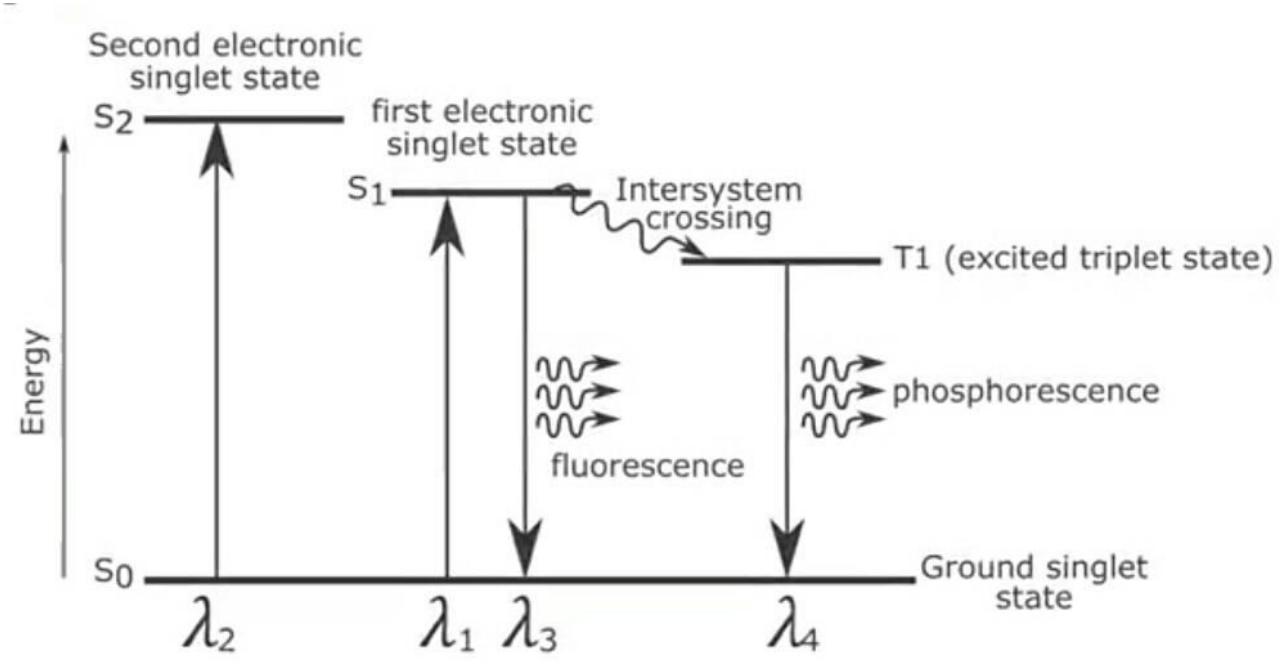
**Light path**

# Principle/How does it work ?

- To observe the sample through a fluorescence microscope, it should be first labeled with a fluorescent dyes/substance known as a fluorophore.
- Higher energy light shorter wavelength of lights (UV rays or blue light) generated from mercury vapor arch lamp passes through the **excitation filter** which allows only the short wavelength of light to pass through and removes all other non-specific wavelengths of light.
- The filtered light is reflected by the **dichroic filter** and falls on the **sample** (i.e. fluorophore-labeled).
- The fluorochrome **absorbs shorter wavelength rays** and emits rays of longer wavelength (lower energy) that passes through the **emission filter.**
- The emission spectrum is usually sharper than the excitation spectrum, and it is of a longer wavelength and correspondingly lower energy.
- The emission filter blocks (suppresses) any residual excitation light and passes the desired longer emission wavelengths to the **detector.** Thus the microscope forms glowing images of the fluorochrome-labeled microorganisms/ cellular structure against a dark background

# How does it work ?

- The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the **fluorophores**, causing them to emit longer wavelength of light (or a different colour then the absorbed light ).



# Applications and uses of Fluorescence microscope

- **Fluorescence microscopy** is a critical tool for academic and pharmaceutical research, pathology, and clinical medicine.
- This method is used for demonstration of naturally occurring fluorescent material and other **non- fluorescent substances** or microorganisms after staining with some **fluorescent dyes**. e.g.; Mycobacterium tuberculosis, amyloid, lipids, elastic fibers etc.

# Applications and uses of Fluorescence microscope

- It helps to identify the specific molecules with the help of the fluorescence substances.
- Tracing the location of a specific **protein** in the specimen.
- Imaging structural components of small specimens, such as cells.
- Conducting viability studies one cell Imaging the genetic material within a cell (DNA & RNA ).
- Viewing specific cells within a larger populations with techniques such as FISH.
- View different proteins located in various portions of cells
- The Fluorescence Microscopy allows the researchers to identify various different molecules in the targeted specimen or sample at the same time.

# Lesson 7: Confocal Microscopy

# Marvin Minsky-the inventor

- Confocal microscopy was pioneered by Marvin Minsky in **1955** while he was a Junior Fellow at Harvard University.
- Minsky's invention would perform a point-by-point image construction by focusing a point of light sequentially across a specimen and then collecting some of the returning rays.
- By illuminating a single point at a time **Minsky** avoided **most of the unwanted scattered light (unwanted fluorescence from background)** that obscures an image when the entire specimen is illuminated at the same time.
- Additionally, the light returning from the specimen would pass through a **second pinhole aperture** that would reject rays that were not directly from the focal point.

# Confocal Microscopy

- A confocal microscope creates sharp images of a specimen that would otherwise appear blurred when viewed with a conventional microscope.
- This is achieved **by excluding most of the light from the specimen that is not from the microscope's focal plane**.
- The image **has less haze and better contrast** than that of a conventional microscope and represents a thin cross-section of the specimen.
- Thus, apart from allowing better observation of fine details it is possible to build three-dimensional (3D) reconstructions of a volume of the specimen by assembling a series of thin slices taken along the vertical axis.

- The remaining **“desirable”** light rays would then be collected by a photomultiplier and the image gradually reconstructed using a long-persistence screen.
- **To build the image, Minsky scanned the specimen** by moving the stage rather than the light rays. This was to avoid the challenge of trying to maintain sensitive alignment of moving optics.
- **Using a 60 Hz solenoid** to move the platform vertically and a lower-frequency solenoid to move it horizontally, Minsky managed to obtain a frame rate of approximately one image **every 10 sec**

- Laser is used as energy source that excites the fluorophore on the specimen
- The fluorophore emits light with another wavelength, detected by the detector and the image is formed

# Confocal Microscopy Vs Fluorescent microscopy

- Laser helps to locate the specific structure and in 3D modeling while eliminating all background staining, even sub cellular organelles can be located
- **Laser can penetrate cells to give exact details of cell, that can be changed according to requirement, therefore multiple optical planes can be used ..**
- **These multiple images can be combined to get a 3D structure by the help of software (Z-stack is a software used)**
- **pinhole aperture is used that does not allow any fluorescent signals coming from surrounding structures,**
- **second pinhole aperture (before the detector)to eliminate any unrequired beam to reach the detector because only very specific reflected light can pass through, that is not the case in fluorescence microscope**
- **Image is sharper, and comparatively well defined**

# Lesson 15-16: Micrometry

# Micrometry

- **Meaning of Micrometry:**
- Micrometry is the science in which we have some measurement of the dimensions of an object being observed under the microscope. The method employs some special types of measuring devices which are so oriented that these can well be attached to or put into the microscope and observed. The object, to be measured, is calibrated against these scales.
- Once we are observing an object under a microscope by the 5X objective and the 10X eyepiece we say that the image that we are able to perceive is 5 × 10 = 50 times of the object.

# Micrometry

- We get the magnified view, but to find out the exact size of the object will need precision and that is achieved through the application of some small scales called micrometers.
- **Types of Micrometers:**
- There are usually two types of micrometers, i.e.

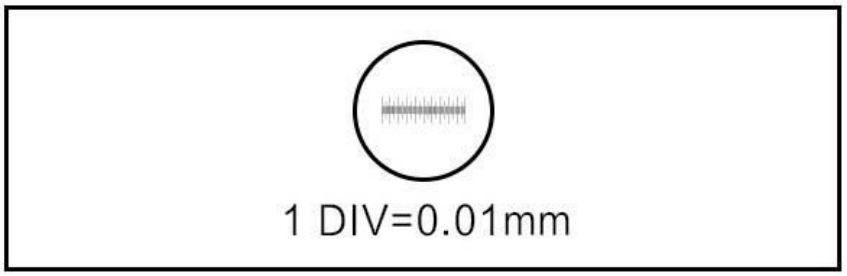
1. stage micrometer and
2. ocular meter or ocular micrometer

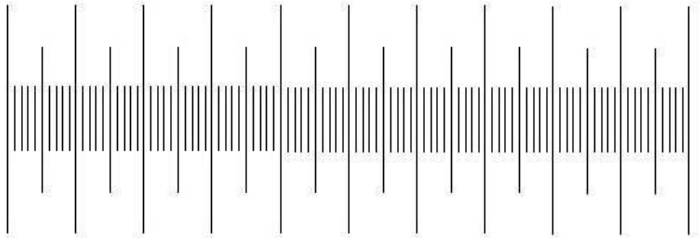
# 1. Stage Micrometer:

- **As is clear from its name it is for the measurement on the stage of the microscope where an object is to be kept.**
- This micrometer is of **a slide's sh**ape and size and has a mount of very finely graduated scale.
- The scale measures only 1 mm and has a least count of 0.01 mm, i.e. 1 mm region is divided into 100 divisions.
- It is used to calibrate the Ocular micrometer.
- It looks like a microscope slide but has a standard scale etched into it.
- It is just like a tiny ruler.
- As 1 mm has 1000µ, one division of stage micrometer is equivalent to 10µ.
- 0.01mm=10 micro m

# ii. Ocular Meter

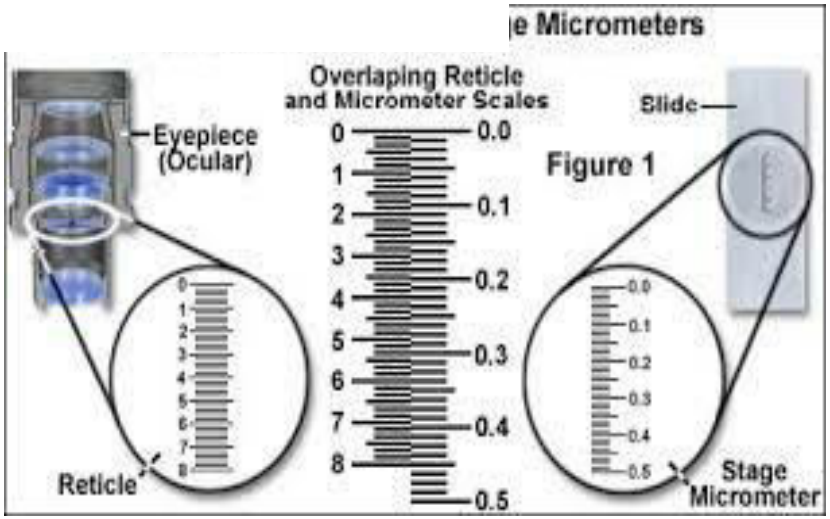
- The ocular micrometer is a glass disc with 100 equal divisions or lines on it but with no absolute value
- This micrometer is used inside the eyepiece.
- The upper eye lens is unscrewed and the ocular meter is put into the tube of eyepiece, and the eye lens is again replaced in its original position.
- 100 equal divisions in the ocular meter are engraved on the glass
- The physical length of the marks on the scale depends on the degree of magnification.



# C1 stage micrometer

DIV=0.01

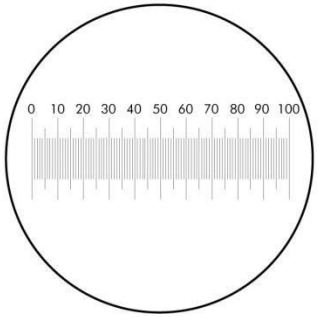


# Lesson 17: Micrometry -calibration

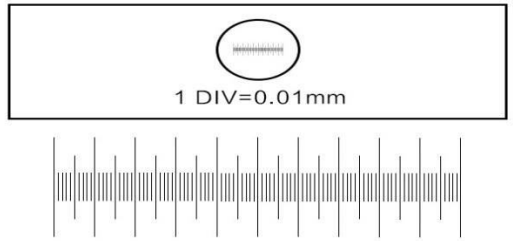
# calibration

- **In measurement technology and metrology, calibration is the comparison of measurement values delivered by a device under test with those of a calibration standard of known accuracy.**
- The calibration procedure for the ocular micrometer requires the graduations on both micrometers to be superimposed on each other.
- The number of ocular divisions that corresponds to the known distance in the stage micrometer is determined.
- When the microscope is calibrated, then the object or organism to be measured is kept on the stage of the microscope and is observed through the eyepiece with ocular. The object is measured in the particular magnification by ocular divisions and then is changed into microns by multiplying ocular divisions with calibrated value of one ocular division in that particular magnification.

- The disc has numbered lines on it as is shown :

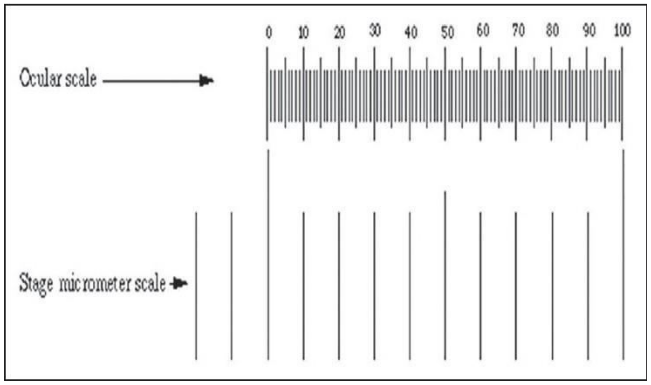


- The units might be different on different ocular micrometers; i.e., some go up to 10 or 50 while others go up to 100. Our main concern here is to determine the length of one unit of the ocular micrometer. For this purpose, we need to calibrate this unit against a known length. There are prepared slides on the market called stage micrometers which have a scale of known length etched in the glass. The scale when observed under the microscope looks as below:

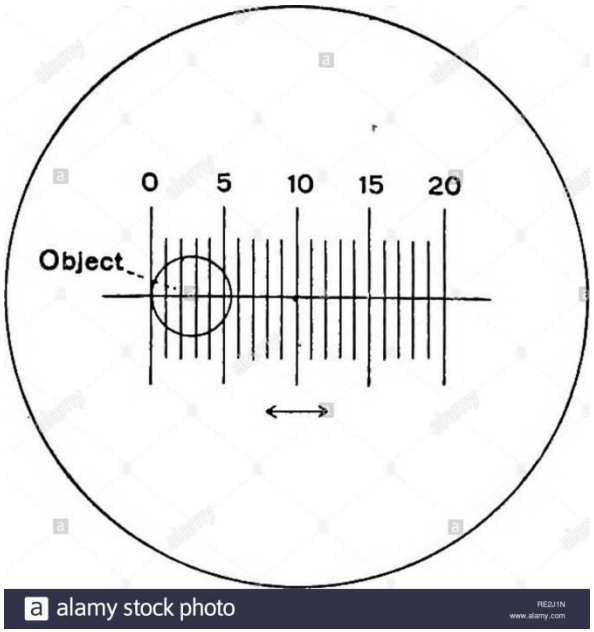


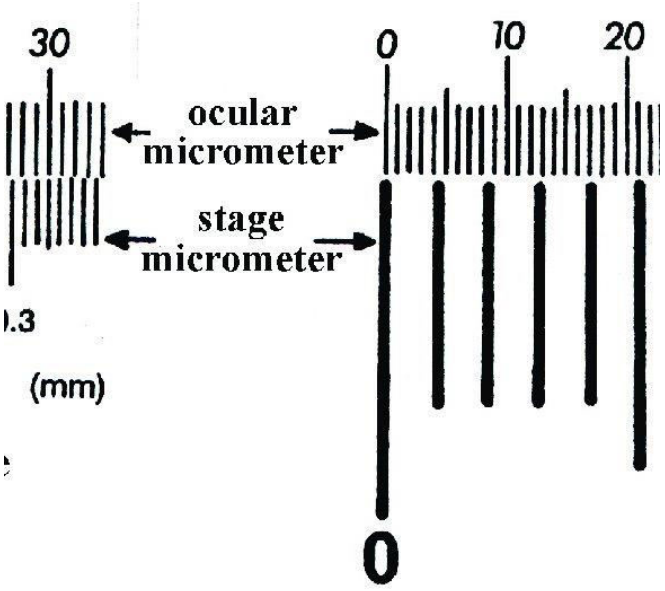

**C1 stage micrometer**
DIV=0.01

1. To be able to measure the size of microorganisms, an ocular micrometer disc is placed in one of the oculars.
2. After calibration, the ocular micrometer measures the size of various microbes including its length, breadth, and diameter.
3. **First put the stage micrometer on stage and let 1 end of 2 scales coincide with each other**
4. **As an example, suppose that for a certain microscope, after lining up the micrometers, we get the following image under the 10X objective:**




$$\text{One division of ocular micrometer} = \frac{\text{No. of divisions on stage micrometer}}{\text{Number of divisions on ocular micrometer}} \times 10$$


0
5
10
15
20
Object
alamy stock photo
RE2J1N
www.alamy.com


30
0
10
20
ocular micrometer
stage micrometer
.3
(mm)
0

**5. Suppose that 80 ocular units are equal to 1.0 mm (1000 microns) for this particular microscope and objective lens.**

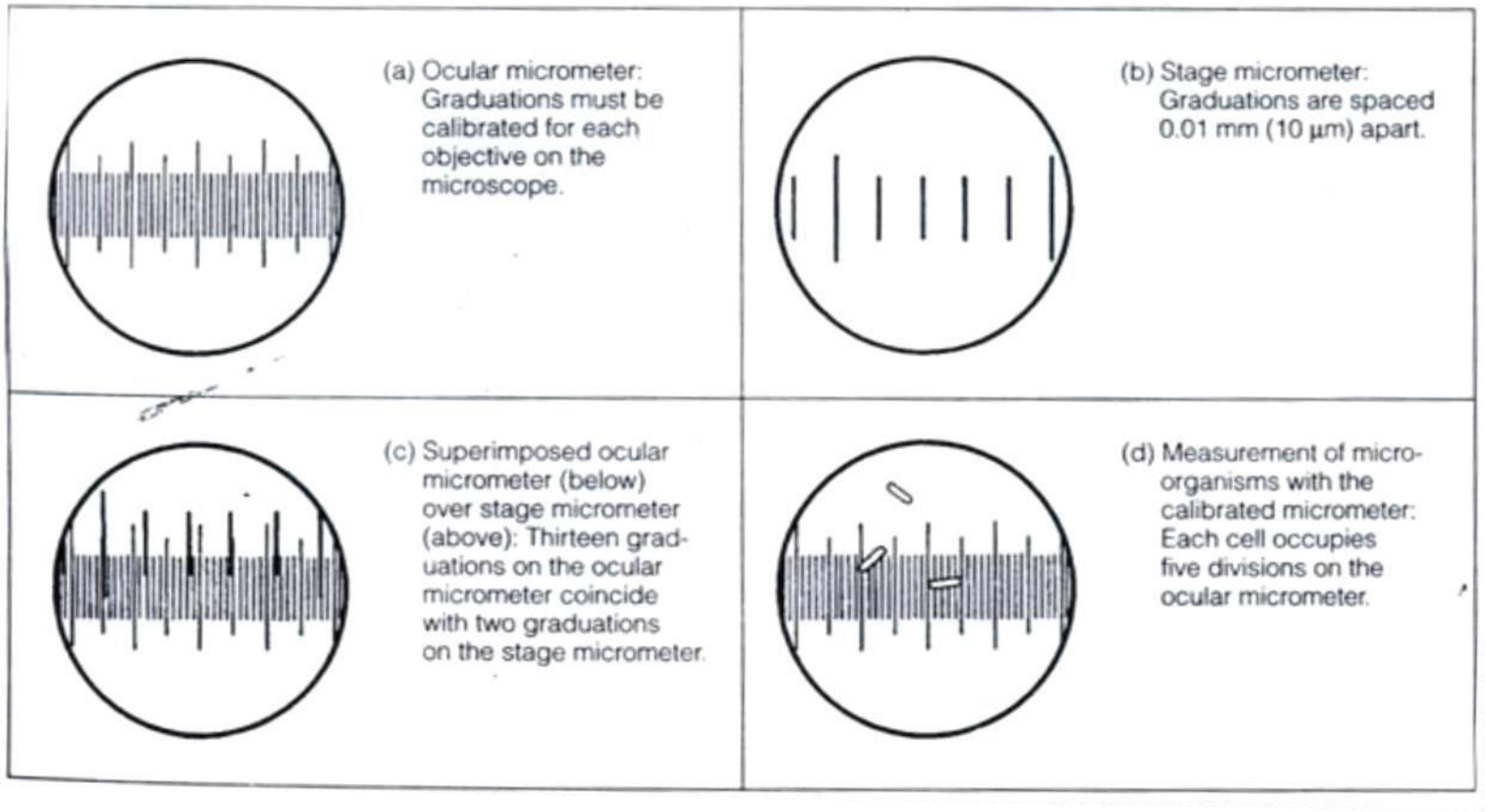
**6. This corresponds to each ocular unit being 12.5 µ long at this specific magnification (10X).**

**1 div of ocular = 12.5 µ**

**7. Then remove the stage micrometer and put the slide and with bacteria/ sample on the stage and count the number of spaces occupied by the organism on ocular micrometer graduations , eg, 10 divisions of ocular.**

**8. As 1 div of ocular = 12.5 µ**

**10 div of ocular = 12.5 x 10 = 125 µ**




**The size of the given microbial cell determined as (length, breadth) in 10x/40x/100x.**

- **5 div of ocular = 12.5 x 5=** 62.5 µ
- **Size of microbe=62.5 µ**

# Microtomy-Method (fixation and processing)

# Method (fixation and processing)

## Method

- It consists of following main steps
- Fixation
- Processing
  - Dehydration
  - Clearing
- Embedding
- Section cutting
- Staining
  - Deparaffinization

# Sample collection

- Small piece of tissue (as early as possible) Piece is removed with sharp knife
- At the time of tissue collection, it should be kept in mind that the representative tissue piece should include the part of lesion and a part of normal tissue,
- Tissues should be collected directly in the fixative and not in any other pot or water.
- The tissue pieces from hollow organs like intestines, oviduct etc., should be cut transversely.

# Method (fixation and processing)

## Fixation

- **It is the most important step in histological studies**
- **The histological details will only be demonstrated if the tissue is promptly and adequately fixed**
- **It is the process of preserving the tissues in the natural condition**
- In the fields of histology, pathology, and cell biology, **fixation** 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.
- **Poorly fixed specimens are more difficult to section than the well fixed ones**

# Method (Commonly used fixatives )

- **Commonly used fixatives are alcohol, formalin, glutaraldehyde, etc.**
- Formalin –routine
- Glutaraldehyde – electron microscopy
- Picric acid(Bouin's solution) – renal & testicular tissue
- Alcohol (Carnoy's fixative) – cytologic smears, endometrial sampling
- Osmium tetraoxide – CNS tissues & electron microscopy

# COMMONLY USED FIXATIVES

- **Factor affecting fixation are temperature, change in pH, penetration of the fixative, volume, time, etc.**
- **The lowest concentration of the fixative is preferred than the higher one**
- **10% formalin or 2.5% glutaraldehyde is used**

# Significance of fixation

Fixation should be carried out as soon as possible

- prevents autolysis and putrefaction.
- Large pieces of tissues cannot be examined under the microscope for ongoing molecular or morphological alterations
- Rapid and even penetration.
- To preserve cells and tissues in a life like manner as possible.
- Stabilize labile elements.
- Must be rigid to allow sectioning.
- Must allow staining.
- Optical contrast must be induced for morphological examination.
- Allow long storage of tissues.

# References

https://en.wikipedia.org/wiki/Zenker%27s_fixative
https://en.wikipedia.org/wiki/Fixation_(histology)#Purposes
https://en.wikipedia.org/wiki/Mutagen
Slide share

# Microtomy- Method (fixation and processing)-3

# Cross-linking **fixative**

- Crosslinking fixatives act by creating COVALENT BONDS between proteins in tissue.
- This anchors soluble proteins to the CYTOSKELETON, and lends additional rigidity to the tissue.
- Preservation of transient or fine cytoskeletal structure is best achieved by a pretreatment using microwaves before the addition of a cross linking fixative

# Cross-linking fixative

- Examples; Formaldehyde, Glutaraldehyde and other aldehydes. e.g. Chloral hydrate and Glyoxal.
- Metal salts such as Mercuric and Zinc chloride. and other metallic compounds such as Osmium tetroxide.
- **Formaldehyde:** Powerful reducing agent.
- Most common fixative -fixation of biopsy specimen.
- Formalin: 40% formaldehyde in water.

# Neutral Buffered Formalin

- Neutral Buffered Formalin. Buffer prevents acidity (promote autolysis and cause precipitation of formol - heme pigment in the tissues).
- Formaldehyde fixes tissue by cross-linking the proteins, primarily the residues of the basic amino acid LYSINE.
- Its effects are reversible by excess water and it avoids formalin pigmentation.
- It is usually used as a 10% neutral buffered formalin (NBF), that is approx. 3.7%–4.0% formaldehyde in phosphate buffer, pH 7.

# Neutral Buffered Formalin

Advantages Cheap, easy to prepare, relatively stable
Good preservation of cell morphology
Good penetration properties.
Do not cause excessive hardening.
Best fixative for nervous system
Disadvantages
Slow fixation reaction.
Dermatitis of hand.
Fumes irritating to nostrils.
In tissue containing blood, dark brown pigment granules are formed

# Paraformaldehyde

- **Paraformaldehyde** is also commonly used and will depolymerise back to formalin when heated, also making it an effective fixative.
- Other benefits to paraformaldehyde include long term storage and good tissue penetration.
- It is particularly good for immunohistochemistry techniques.
- The formaldehyde vapor can also be used as a fixative for cell smears.

# Oxidizing agents

- The oxidizing fixatives can react with the side chains of proteins and other biomolecules, allowing the formation of crosslinks that stabilize tissue structure.
- they cause extensive denaturation despite preserving fine cell structure and are used mainly as secondary fixatives.
- OSMIUM TETRAOXIDE is often used as a secondary fixative when samples are prepared for ELECTRON MICROSCOPY.
- (It is not used for light microscopy as it penetrates thick sections of tissue very poorly.)
- POTASSIUM DICHROMATE, CHROMIC ACID, and POTASSIUM PERMANGANATE all find use in certain specific histological preparations.

# Mercurials

- Mercurials such as Zenker's fixative have an unknown mechanism that increases staining brightness and give excellent nuclear detail.
- They do fixation fast
- mercurials penetrate poorly and produce tissue shrinkage.
- Their best application is for fixation of hematopoietic and reticuloendothelial tissues.
- Fixatives containing mercuric chloride or potassium dichromate are toxic, making disposal as HAZARDOUS WASTE costly

- **Zenker's fixative**
- This fixative is named after Konrad Zenker, a German histologist, who died in 1894
- **Zenker's fixative** contains MERCURIC CHLORIDE (corrosive ) POTASSIUM DICHROMATE, SODIM SULPHATE, WATER , and ACETIC ACID.
- It is employed to prepare specimens of ANIMAL OR VEGETABLE tissues for microscopic study.
- It provides excellent fixation of nuclear CHROMTIN, CONNECTIVE TISSUE fibers and some CYTOPLASMIC features but does not preserve delicate cytoplasmic organells as MITOCHONDRIA.
- Mercuric chloride can be replaced with the same weight of less toxic ZINC CHLORIDE but the resulting "zinc-Zenker" may not give the same quality of fixation as the original mixture.

# References

https://en.wikipedia.org/wiki/Zenker%27s_fixative
https://en.wikipedia.org/wiki/Fixation_(histology)#Purposes
https://en.wikipedia.org/wiki/Mutagen
Slide share

# Microtomy- Tissue embedding

# Tissue embedding

Introduction

• Embedding is the process in which the tissues or the specimens are enclosed in a mass of the embedding medium using a mould.

• Since the tissue blocks are very thin in thickness they need a supporting medium in which the tissue blocks are embedded.

• This supporting medium is called embedding medium.

Fixation

Decalcification

Grossing

Enclosing

## The Choice of Embedding medium depends upon

- 1. Type of Microscope
- 2. Type of Microtome
- 3.Type of Tissue. Eg : Hard tissue like Bone or Soft tissue liver biopsy.

## TYPES OF EMBEDDING MEDIUMS/ AGENTS

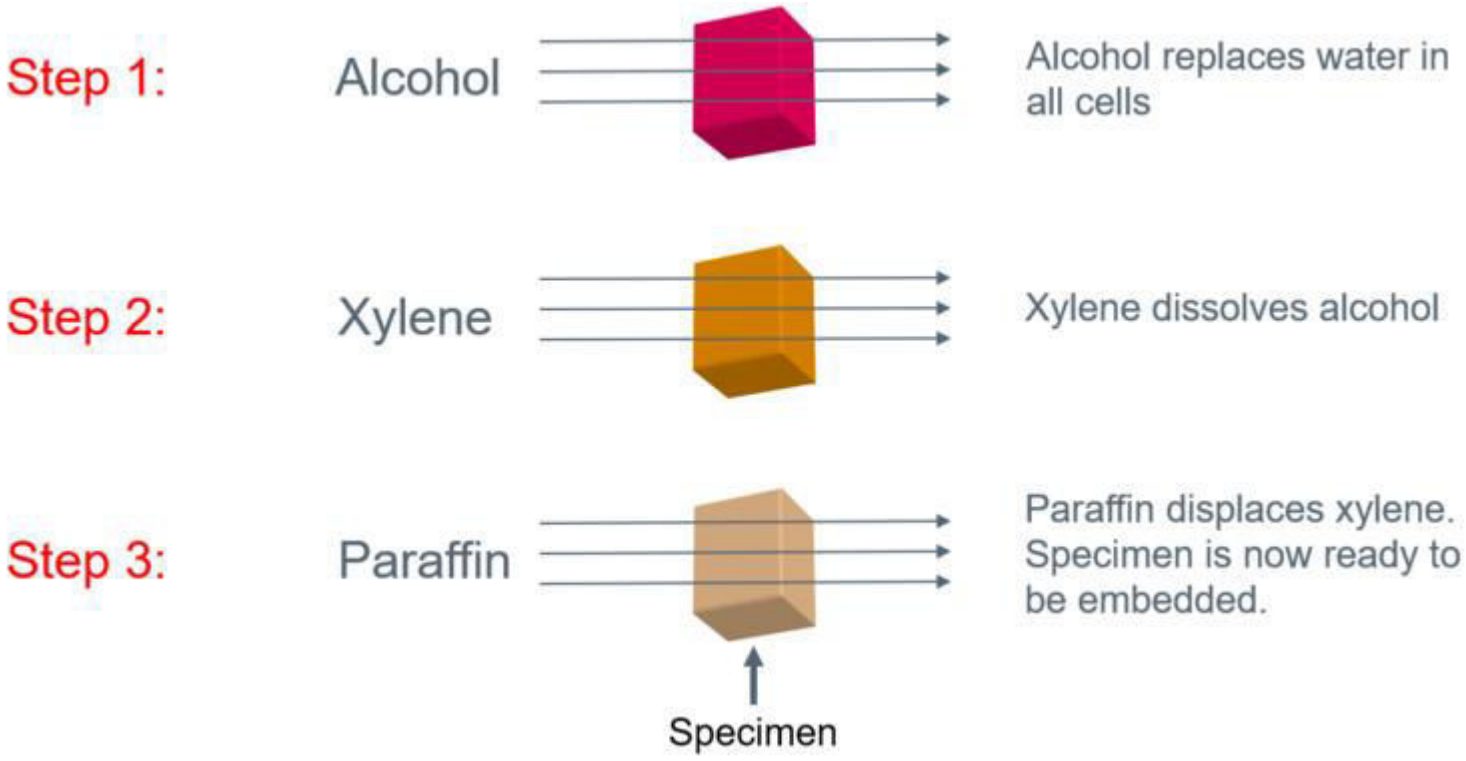
1.Paraffin wax
2.Celloidin
3.Resin
4.Agar
5.Gelatin
6.Carbowax
7.Methacrylate

# Tissue embedding in PARAFFIN WAX

- Paraffin wax with higher melting point (56-62 C) is used for embedding.
- The molten wax is filtered inside the oven through a course filter paper into another container.
- This will protect the knife edge.

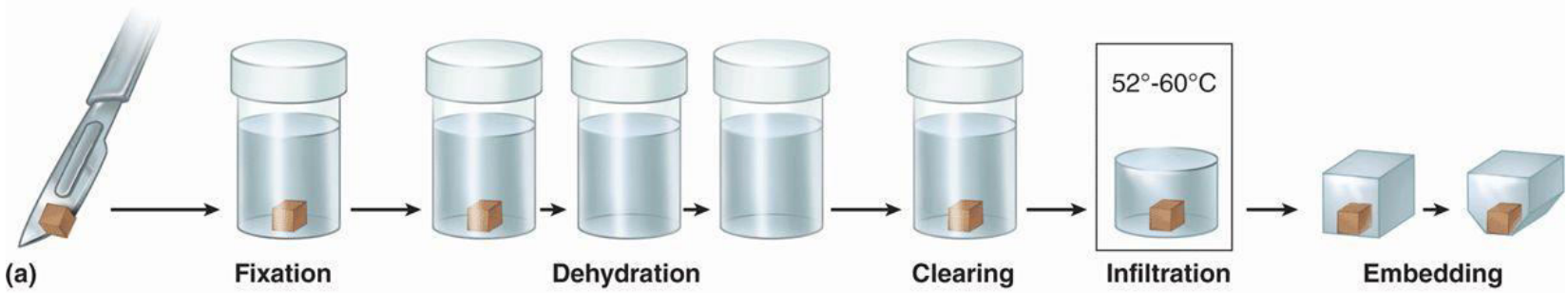
# Tissue Processing

- **It is a process in which tissues are treated to make the thin sections**
- **A specimen is generally processed as follows**



# Tissue Processing

- **1. Dehydration.** It is the removal of water from the tissues by immersing serially in 50%, 70%, 85% and 100% alcohol for some time.
- Since paraffin is hydrophobic (immiscible i.e. not mixable with water), water inside a specimen must be removed before it can be infiltrated with paraffin. This process is carried out by immersing specimens in a series of alcohol.
- Alcohol progressively replaces water in all the cells of the specimen.
- A series of increasing (typically from 70% to 100%) alcohol concentrations are used to avoid excessive distortion of the tissue.


52°-60°C
(a)
Fixation
Dehydration
Clearing
Infiltration
Embedding


Drive wheel
Block holder
Paraffin block
Tissue
Steel knife
b

# 2. Clearing

**2. Clearing** Since alcohols and paraffins are not miscible, an intermediate solvent that is fully miscible with both (such as xylene), must be used.

1. This solvent displaces the alcohol in the tissue through the process called “clearing”.
2. “Clearing” relates to how clearing agents impart an optical clarity or transparency to the tissue due to their relatively high refractive index.
3. Another important role of the clearing agent is to remove a substantial amount of fat from the tissue which otherwise presents a barrier to paraffin infiltration.
4. To make sure that all traces of alcohols are removed from tissues being processed, multiple changes of fresh xylene, clear of carried-over alcohol, are required.

# 3. Infiltration

3. Infiltration The specimen can now be infiltrated with paraffin.

Molten paraffin infiltrates tissues and when cooled solidifies to a consistency that allows sectioning on a microtome.

The amount of structural support given by solidified paraffin can be regulated by choosing different paraffin formulations.

Multiple changes of histological paraffin are required to completely displace the clearing agent.

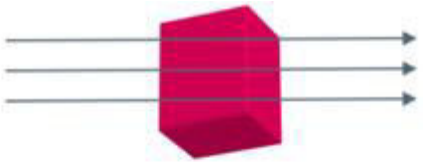
Paraffin infiltration is greatly enhanced by vacuum.

https://www.leicabiosystems.com/knowledge-pathway/an-introduction-to-specimen-processing/

https://www.google.com/search?q=alcohol+series+while+paraffin+mebedding+&tbm=isch&ved=2ahUKEwjPhrDf7OrsAhUCEhoKHeMmBbYQ2-cCegQIABAA&oq=alcohol+series+while+paraffin+mebedding+&gs_lcp=CgNpbWcQA1CACVieIWDNI2gAcAB4AIABzwKIAbYhkgEIMC4xLjE3LjGYAQCgAQGqAQtnd3Mtd2l6LWltZ8ABAQ&sclient=img&ei=faWjX4_UFYKkaOPNlLAL&bih=880&biw=1920&rlz=1C1GCEU_enPK869PK870#imgrc=FkNUCdqinfoEEM

# Microtomy- Tissue embedding-2

Step 1: Alcohol

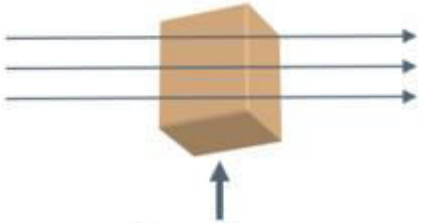


Alcohol replaces water in all cells

Step 2: Xylene

Xylene dissolves alcohol

Step 3: Paraffin

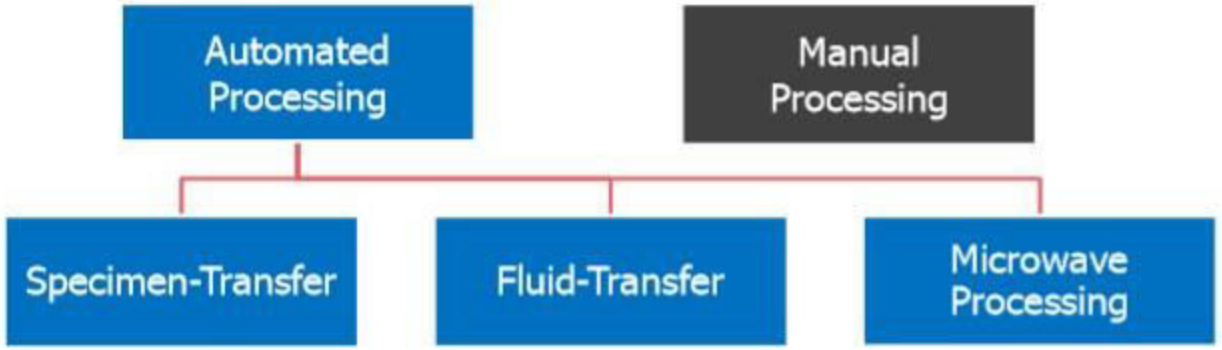



Paraffin displaces xylene. Specimen is now ready to be embedded.

# IMPREGNATION

- Empty spaces in tissues and cells , after removal of clearing agent, are taken by molten wax
- Hardens the tissue – helps in section cutting
- Melting point of wax –62 degree C

# Processing Methods



# Processing Methods

## Manual Processing

Slow, most labor intensive method since transfer of specimens or changing reagents is done by hand. With an advance of automation this method is almost obsolete.

## Automated Processing

Specimen-transfer or “dip and dunk” processors: instruments which transfer cassettes from station to station in a rotary or linear configuration.

Fluid-transfer or “enclosed” instruments hold the specimens in a process chamber or retort and the reagents are pumped in and out during processing.

Microwave assisted processing: might require manual transfer of specimen or reagents, it accelerates processing by heating reagents.

# TISSUE PROCESSOR

- Tissue processing is routinely done on an instrument called Tissue Processor.
- “Tissue processing” describes the steps required to take animal or human tissue from fixation to the state of complete infiltration with a histological paraffin.
- Subsequently, the processed tissue is made into a paraffin block so it can be sectioned on the microtome Dehydration + clearing +impregnation
- **Automated tissue processor**

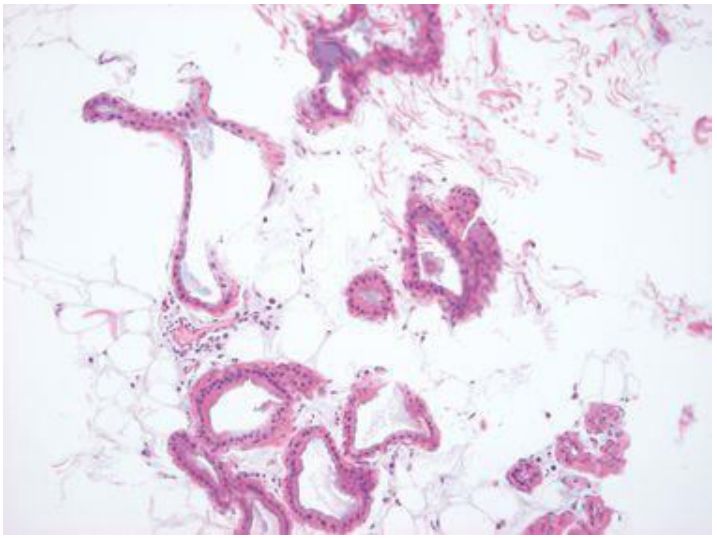
1. Open (hydraulic )
2. Closed (vaccum)

## OPEN / HYDRAULIC PROCESSOR

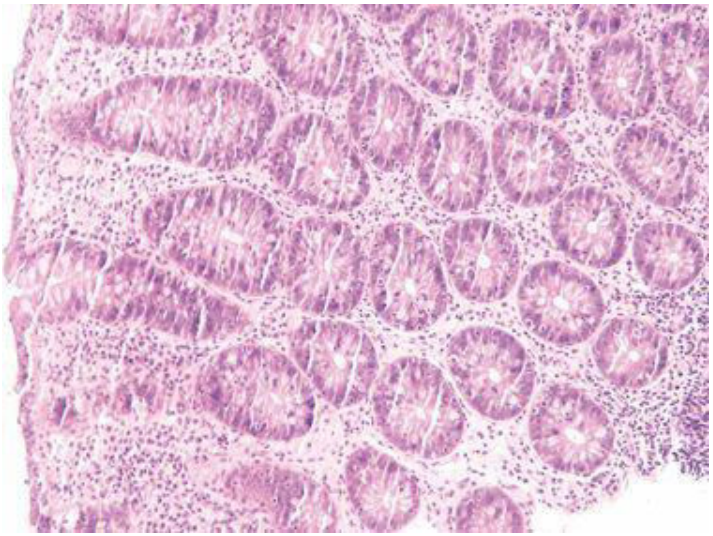
- 12 stations
- 1 jar – formalin
- 6 jars – grades of alcohol
- 3 jars – xylene
- 2 jars – molten paraffin wax

## CLOSED / VACCUM PROCESSOR

Different processing fluids are moved in and out of a single station sequentially



This micrograph of a small area of subcutaneous tissue from a large, fatty specimen shows the effects of under-processing. The fibro-fatty tissue is poorly supported and therefore fragmented while the epithelial tissue of the glands shows a lack of nuclear definition and peculiar staining due to retained solvent (H&E)



This endoscopic biopsy has been over-processed and has become very brittle. As a consequence many fine cracks are visible through out the section. Poor microtomy technique will exacerbate the problem (H&E).

# TYPES OF MOULDS

- A variety of moulds are used for embedding .
- Plastic moulds
- Casettes
- Metal moulds

# PLASTIC MOULDS

- Most of the laboratories use plastic embedding rings now.
- These are relatively inexpensive Convenient & Support the block during sectioning And are designed to fit it on the microtome.
- This eliminates the step of mounting or attaching the block on a holder

# Plastic casettes

- Since the cassette is processed with the tissues and afterwards used for embedding, the writing has to be done once.
- Cassettes are thin so less wax is required.
- The space required for filing the blocks is less
- The cassettes are shallow hence thin sections should be taken for processing

# Metal moulds

- Made from stainless steel for optimal thermal conductivity they have a well polished surface for easy paraffin block removal.
- All of the corners are rounded for good paraffin ribboning.
- These Base Molds are for all applications in specimen embedding

https://www.leicabiosystems.com/knowledge-pathway/an-introduction-to-specimen-processing/

https://www.google.com/search?q=alcohol+series+while+paraffin+mebedding+&tbm=isch&ved=2ahUKEwjPhrDf7OrsAhUCEhoKHeMmBbYQ2-cCegQIABAA&oq=alcohol+series+while+paraffin+mebedding+&gs_lcp=CgNpbWcQA1CACVieIWDNI2gAcAB4AIABzwKIAbYhkgEIMC4xLjE3LjGYAQCgAQGqAQtnd3Mtd2l6LWltZ8ABAQ&sclient=img&ei=faWjX4_UFYKkaOPNlLAL&bih=880&biw=1920&rlz=1C1GCEU_enPK869PK870#imgrc=FkNUCdqinfoEEM

# Lesson: Microtomy-

# Microtomy

- The means by which tissue can be sectioned and attached to a surface for further microscopic examination
- A microtome (derived from the Greek mikros, meaning “small”, and temnein, meaning “to cut”) is a mechanical device for cutting thin uniform slices of tissue sections.
- **Microtomy** is a method for the preparation of thin sections for materials such as bones, and biological tissue.
- Microtome sections can be made thin enough to section a human hair across its breadth, with section thickness between 50 nm and 100 µm.
- These sections are stained with appropriate dyes and observed under the microscope.

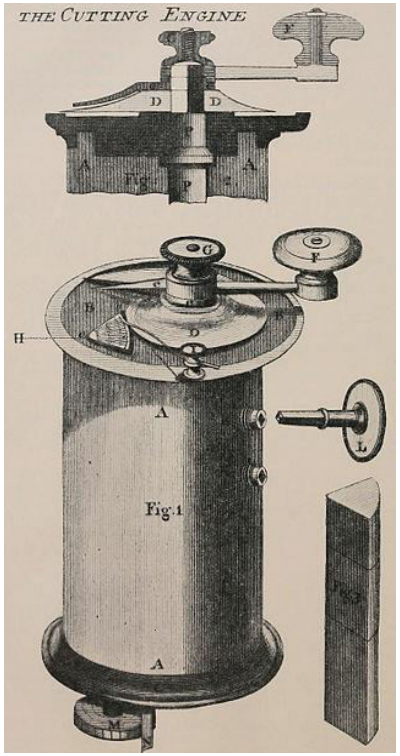
# Principle

- Microtomy is a sectioning instrument that allows the cutting of extremely thin slices of a material known as section .
- Microtomes are used in microscopy , allowing for the preparation of sample for observation under transmitted light or **electrons** radiation

# History

- In the beginnings of light microscope development, sections from plants and **animals were manually prepared using razor blades.**

- One of the first devices for the preparation of such cuts was invented in 1770 by George Adams, Jr. (1750–1795) later it was further developed by Alexander Cummings.




A diagram of a microtome drawn by Cummings in 1770

# History

- The table-top based **microtome** model was developed by Andrew Prichard in 1835.
- In 1865 **Wilhelm His** invented the most improved version of microtome, a mechanical device used to slice thin tissue sections for microscopic examination.

Table-top based **microtome**

**Wilhelm His**

# Types of microtome

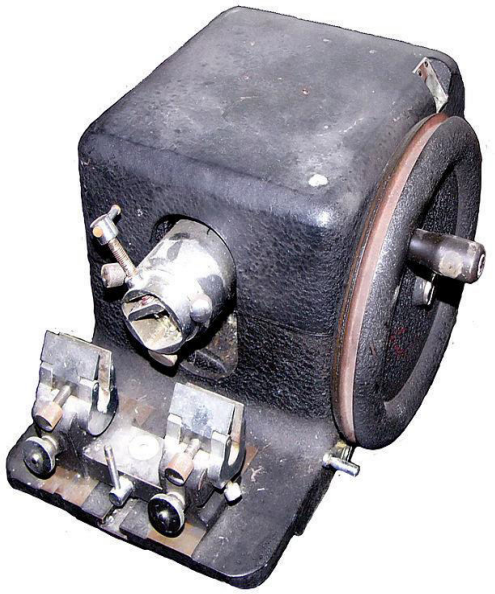
- Rocking
- Rotary
- Rotary Rocking
- Base-sledge
- Sliding
- Freezing
- Vibrating
- Saw
- Cryostat
- Ultra
- Laser

# Rocking microtome

- The rocking microtome was developed at the end of 19$^{th}$ century.
- It is an old model but it is still very effective for producing series of good quality 6-20 micron thin paraffin sections.
- It produces very long ribbons of 5-20 micron sections without interruptions.

# Rotary microtome

- The Rotary microtome is so-called because of a Rotary action of the handwheel responsible for the cutting moment.
- This device operates with a staged rotary action such that the actual cutting is part of the rotary motion.
- The typical cut thickness for a rotary microtome is between 1 and 60 µm.



# Rotary rocking microtome

- This is highly developed than the rocking microtome.
- It produces a flat face to the tissue block.
- It is also called Minot microtome, after its inventor Professor Minot

# Base sledge microtome

- It has a fixed knife beneath which the object moves mounted on a heavy sliding base.
- It contains the feed mechanism used primarily for cutting the sections of cellulose nitrate embedded tissues with an obliquely set knife.
- It is used to section very large samples. This microtome is capable of sectioning samples as large as 10cm * 15cm.

# Sliding microtome

- Microtome in which the tiss ue being sectioned slides alo ng a track.
- The knife or blade is stationary, the specimen slides under it during sectioning.
- It was designed mainly for cutting celloid in embedded blocks of tissue.
- It can also be used for paraffin wax embedded sections.

# Vibrating microtome

- It uses a vibrating blade to cut through tissues.
- High speed vibration produced in a safety razor blade to provide the cutting power.
- The cut thickness is usually around 30–500 µm for live tissue and 10–500 µm for fixed tissue.

# Ultra Microtome

- It produces extremely thin sections.
- The typical thickness of these cuts is between 40 and 100 nm for transmission electron microscopy.
- It is used for multiple types of samples, including biological specimens and industrial materials, e.g. polymers (rubber and plastics) and ductile, hard, or brittle materials (metals or ceramics).

# Lesson: Microtomy-types-II

# History

- In 1865 **Wilhelm His** invented the most improved version of microtome…
- **Father of microtomy**

**Wilhelm His**

# Saw microtome

- It is especially used for hard materials such as teeth or bones.
- The microtome of this type has a recessed rotating saw, which slices through the sample.
- The minimal cut thickness is approximately 30 µm and can be made for comparatively large samples.

# Cryostat microtome “Cryotome”

- A **cryostat** is a **microtome** machine for cutting tissue at low temperatures (typically around −15 to −30°C).
- frozen sections in a liquid-nitrogen chamber.
- Specimens are frozen and cut at 4-8 µm thickness in an cryo-microtome.
- A major advantage of this type is that the tissue requires little preparation.

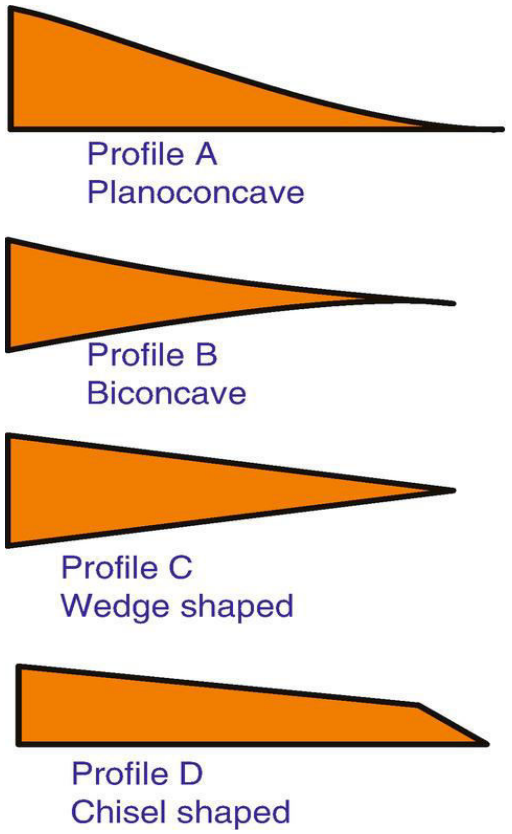
# Laser microtome

- It cuts tissue or other material with the help **of photons instead of steel blades**.
- The method is contact-free and enables to cut tissue in its native state.
- Special preparation techniques are not required.
- Cutting by optical breakdown.
- The thickness achievable is between 10 and 100 µm.

Figure 11 Laser Microtome

# Knife profile

- **Planar concave microtome** knives are extremely sharp, but are also very delicate and are therefore only used with very soft samples.
- **Biconcave knives** are classical knives shape with concavity on both sides. They are very sharp
- The **wedge shaped knives** are somewhat more stable and find use in moderately hard materials, such as in epoxy or cryogenic sample cutting.
- The **chisel shaped knife** with its blunt edge, raises the stability of the knife, whilst requiring significantly more force to achieve the cut.



# references


- https://en.wikipedia.org/wiki/Microtome

# **Lesson: Microtomy-** Features & Parts of the Microtome

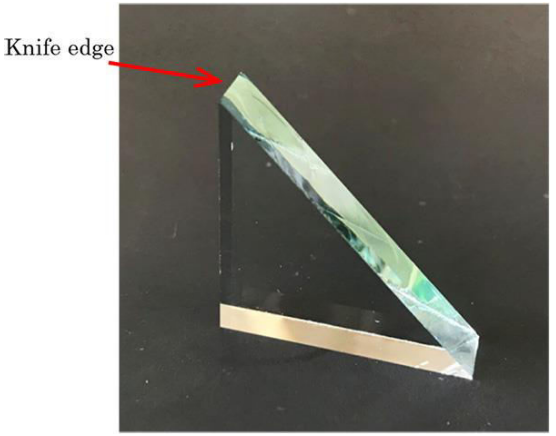
# Types of Knives

- STEEL KNIVES
- GLASS KNIVES
- DIAMOND KNIVES
- SAPPHIRE KNIVES


# Types of Knives

- **STEEL KNIVES:** Stainless Steel, Disposable Microtome Blades which fit most microtomes and cryostats. **Steel** blades are used to prepare sections of animal or plant tissues for light microscopy histology.

- GLASS KNIVES : **Glass knives** are used in an ultramicrotome to cut ultrathin slices of samples for electron and light microscope applications. For resin and for cryosections the **knife** edge must be extremely sharp, strong and stable. Precautions for use; When the knife is applied to hard materials or the same position of the blade is repeatedly used, the blade tends to spill and can damage the surface of a specimen. Thus, the knife needs to be used while changing the position of the blade.



- **DIAMOND KNIVES** A **diamond knife** is a very sharp **knife** in which the edge is made from **diamond**, invented by Humberto Fernández-Morán in 1955. **Diamond knives** are **used for** medical and scientific applications where an extremely sharp and long-lasting edge is essential. These **knives** are available in 35, and 45 degree angles. The optimal thickness range for cutting with these **knives** is between 30-150nm.


- **SAPPHIRE KNIVES** Although **sapphire** is not as hard as diamond or tungsten carbide, it is hundreds of times harder than steel razor **blades**.


- Can be Used in vibratome

# Features & Parts of the Microtome (Rotary microtome)

1. **Microtome base plate or stage:** A platform which has rails that secure the knife holder base.
2. **Knife holder base:** A part that anchors the knife holder to the microtome stage. The knife holder base can be moved toward or away from the block, but **MUST** be stationary and locked during microtomy.
3. **Knife holder:** This part is comprised of several components including the **blade clamp** that holds the blade, the **knife tilt** for adjusting the knife angle, and the **face plate** that guides that ribbons away from the blade and towards the operator.
4. **Cassette clamp or block holder:** Holds the paraffin block in place. Typically, the block moves up and down with each revolution while the blade is stationary. The block holder may have knobs that allow the user to manipulate the block face in various directions to bring the tissue in alignment with the blade.


Source: Mescher AL: Junqueira's Basic Histology: Text and Atlas, 12th Edition: http://www.accessmedicine.com

# … Parts of the Microtome

**5. Coarse handwheel:** Moves the block holder either toward the knife or away from the knife.
**6. Advancement handwheel:** Turns in one direction and advances the block toward the knife at the specified microns. Most handwheels are equipped with a safety lock to prevent the wheel from releasing and having the block holder come down towards the blade while a block is inserted or removed. The safety lock should be used anytime the microtomist is not actively sectioning paraffin blocks.
**7. Micron adjustment:** Micron settings for section thickness can range from 1 to 60 microns on most microtomes.

## Leica RM2125 RTS (from the right)


## Leica RM2125 RTS (from left)

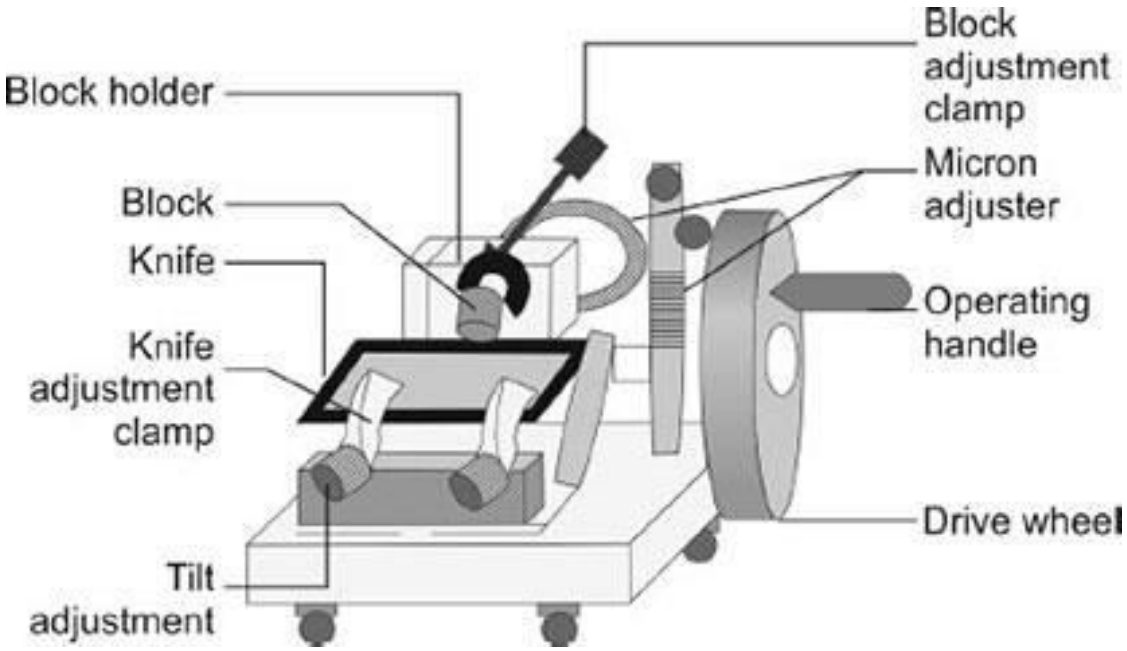
https://www.labce.com/spg605374_instrumentation_for_microtomy_rotary_microtome_par.aspx

# Lesson 27: Microtomy-method

# Method (sectioning)

## Sectioning

- **It is process of cutting the embedded/processed samples into thin sections**
- **It requires great care as tissues of the diagnostic importance can easily be lost or the block surface may be damaged.**
- This can be done with a microtome, an apparatus for cutting the paraffin embedded blocks using an ultra sharp blade with micron level precision.
- Then the term "microtome" was coined in 1839 by Chevalier.


Block holder
Block
Knife
Knife adjustment clamp
Tilt adjustment
Block adjustment clamp
Micron adjuster
Operating handle
Drive wheel

# Rotary microtome

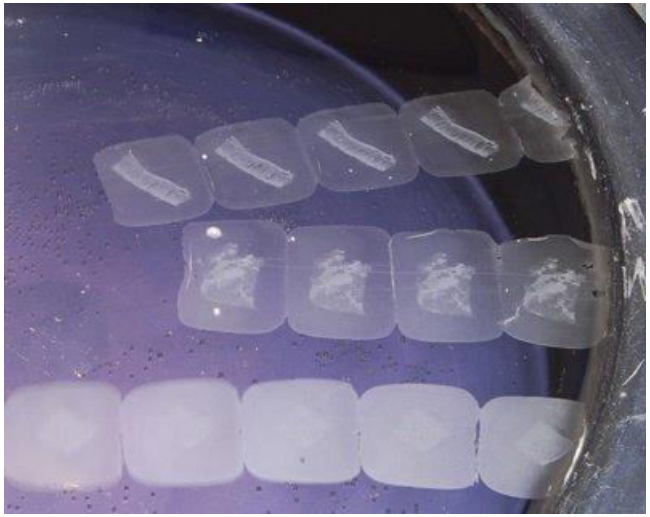
- **The most common type of the microtome**
- **Rotary action involves the sectioning process at predetermined thickness on every rotation of the drive wheel**
- Creating great paraffin sections using a rotary microtome takes a great deal of skill and experience.
- "Microtomy and Paraffin Section Preparation" is a great training aid for new microtomists and is an excellent refresher for experienced operators.

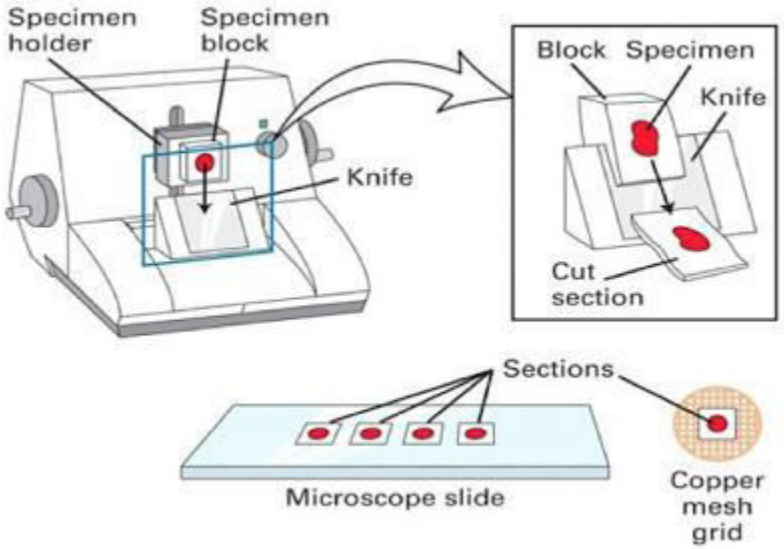
# Procedure to use Rotary microtome

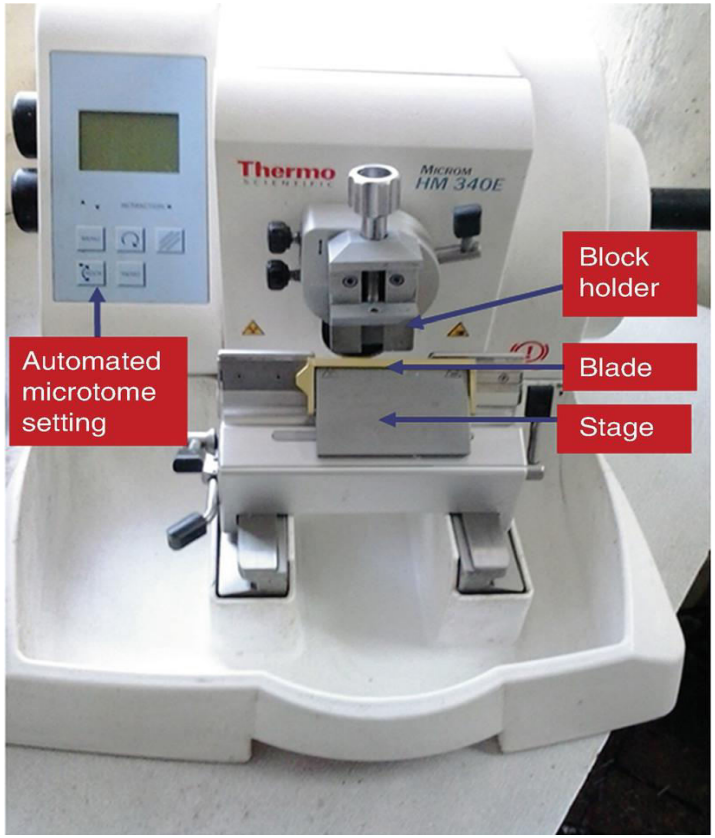
- **Blade is fixed at horizontal position**
- **The sample holder moves the sample ahead by the fixed distance for cutting**
- **Drive wheel of the instrument may be automatic or manual**
- **Section thickness may vary from 1 µm to 60 µm**
- **The cut sections are floated on the top of a liquid**
- **These are then mounted on a copper, nickel, gold, or other metal grid**
- **The cuts sections are then floated on warm water bath that helps to remove wrinkles**
- **These are then placed on the glass**

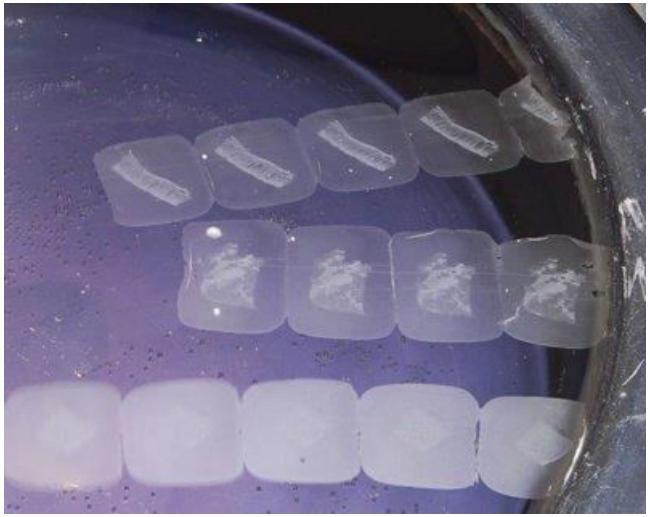
**microscopic slides or some other**

**support for staining, etc.**

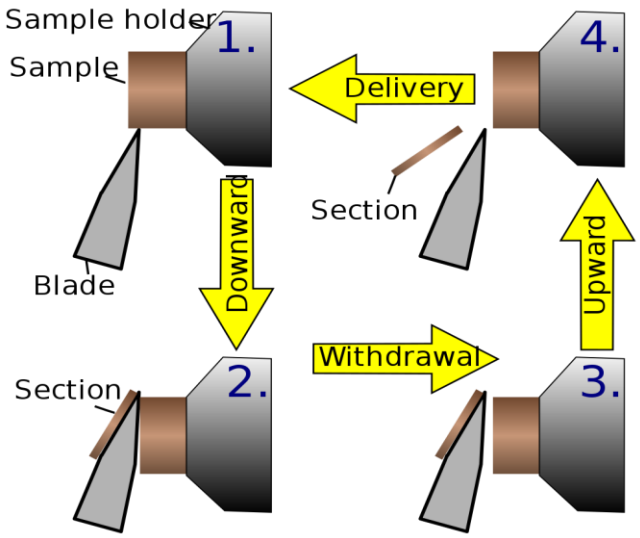



Specimen holder
Specimen block
Knife
Block
Specimen
Knife
Cut section
Sections
Microscope slide
Copper mesh grid


Thermo
Microm HM 340E
Block holder
Blade
Stage
Automated microtome setting

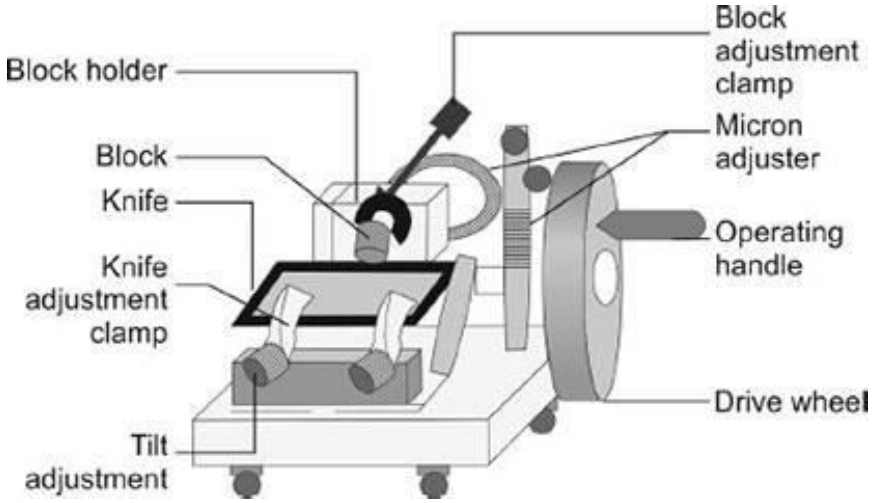


- The knife is motionless, remaining in a horizontal position.
- The machine body **itself rotates around the blade, slicing as it goes. For example, the machine holds the sample just above the knife, then it moves downward over the** knife, slicing a piece of the tissue off, then the machine pulls back and moves upwards before starting again.
- This produces tissue slices of 1-60 micrometres (adjustable)




**Principle of sample movement for making a cut on a rotary microtome**

- Glass slides are coated with some adhesives to facilitate sticking of slice onto the slide
- Typical adhesives for this purpose include starch, albumen, resins and combinations.
- The adhered sections are then ready for further processing.

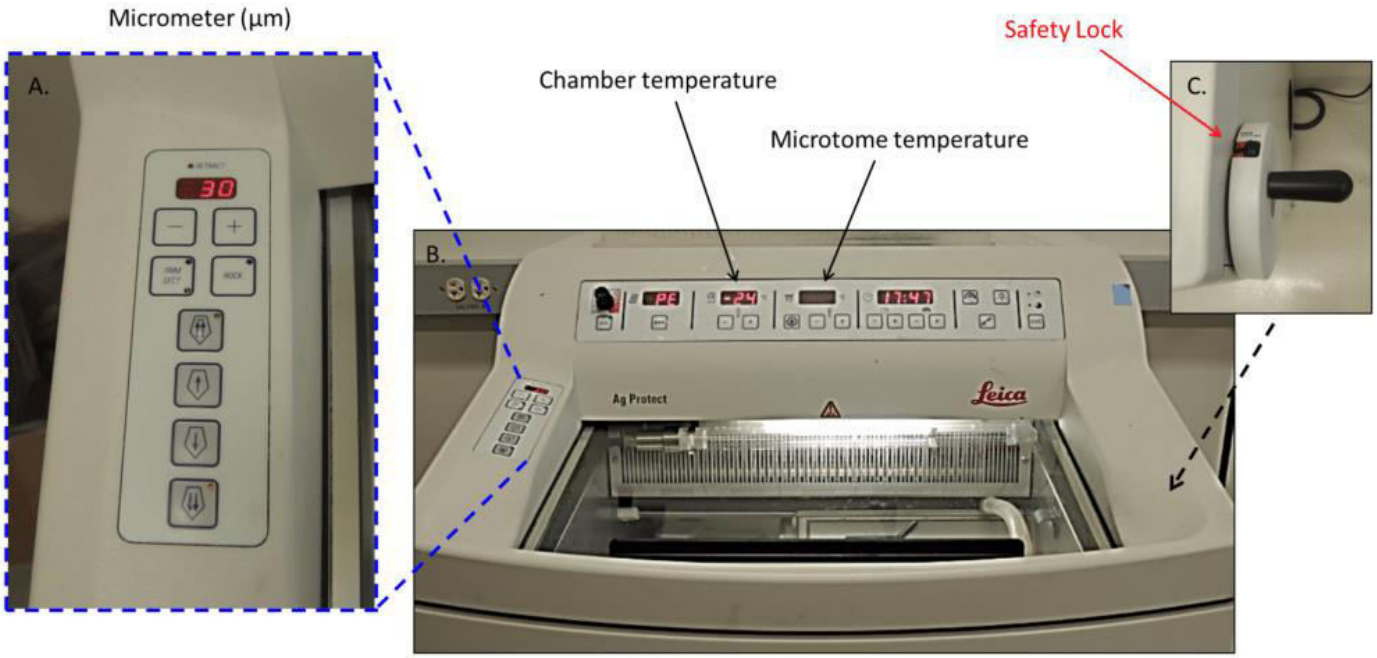


- https://conductscience.com/mastering-the-microtome/
- https://www.leicabiosystems.com/knowledge-pathway/steps-to-better-microtomy-flotation-section-drying/
- https://medsynapses.blogspot.com/2012/02/microtome.html
- https://www.deakin.edu.au/__data/assets/pdf_file/0003/1037460/Microtome-Basic-Operation-and-Safety-Procedures.pdf

# Lesson 28:
# cryomicrotome

# cryomicrotome

- **cryomicrotome is used** for cutting frozen tissues.
- This is a refrigerated box containing a microtome.
- Any microtome arranged in a cryostat and used to prepare thin sections of frozen tissue for microscopic examination.
- This is a rotary **microtome**: in which wheel action is translated into a back-and-forth movement of the specimen being sectioned.
- For the cutting of frozen samples, many **rotary microtomes can be adapted to cut in a cold chamber**
- The reduced temperature allows the hardness of the sample to be increased, (it does not melt ) such as by undergoing a glass transition, which allows the preparation of semi-thin samples.




**This is the micrometer display. This is where you see how thick or thin your slices of tissues are, expressed in µm.**
**B. This is the actual cryostat chamber with visible chamber and microtome temperature settings.**
**C. This is the handle and your all important safety lock.**

# Cleaning Your Cryostat

- You will need to clean the cryostat after every session, and likely a few times during.
- But never clean components inside the chamber with water! If you do the components will **ice over and freeze in place**, rendering them immobile.
- To clean, simply wipe down with dry **Kim wipes** or paper towels to brush tissue and medium off the metal surfaces.
- And be sure you ONLY use Kim wipes for the glass surfaces and near the blade.
- Otherwise you will get fibers on your equipment that can cause fracturing in your sections.

# Significance of cryomicrotome

- For rapid medical diagnosis when it is necessary to perform a rapid analysis of a sample, a frozen section is required
- The piece(s) of tissue to be studied are snap frozen in a cold liquid or a cold environment (-20° to -70° Celsius).
- Freezing makes the tissue solid enough to section with a microtome.
- Frozen sections are performed with an instrument called a cryostat, The temperature inside the cryostat is about -20° to -30° Celsius.
- The tissue sections are cut and picked up on a glass slide.
- The sections are then ready for staining.
- If not immediately required.. They should be kept in freezer at -20 C.

https://bitesizebio.com/28466/can-stand-cold-cryosectioning-beginners/
https://en.wikipedia.org/wiki/Microtome

# Lesson 29: Preparation of tissue for cryomicrotome

Dr. Ayesha Maqbool

# Tissue Preparation for cryomicrotomy

- The cryostat is the instrument that has the arrangement to freeze the tissue and also to cut the frozen tissue for microscopic section.
- The rapid freezing of the tissue sample converts the water into ice.
- The firm ice within the tissue acts as embedding media to cut the tissue.
- Lowering the temperature makes the tissue more firm, whereas increasing temperature makes the tissue softer.

- **Specimen holder:** The specimen holder or chuck is supplied by the manufacturers in different sizes and shapes. Usually these are round metal structures

**Specimen holder:**

- **Embedding medium:** This medium is used to
- hold the tissue over the chuck. Presently **optimum cutting temperature (OCT) compound (Tissue-Tek)** is used as embedding medium. The OCT is made of water-soluble glycols and resin.
- **Tissue embedding in the mould**

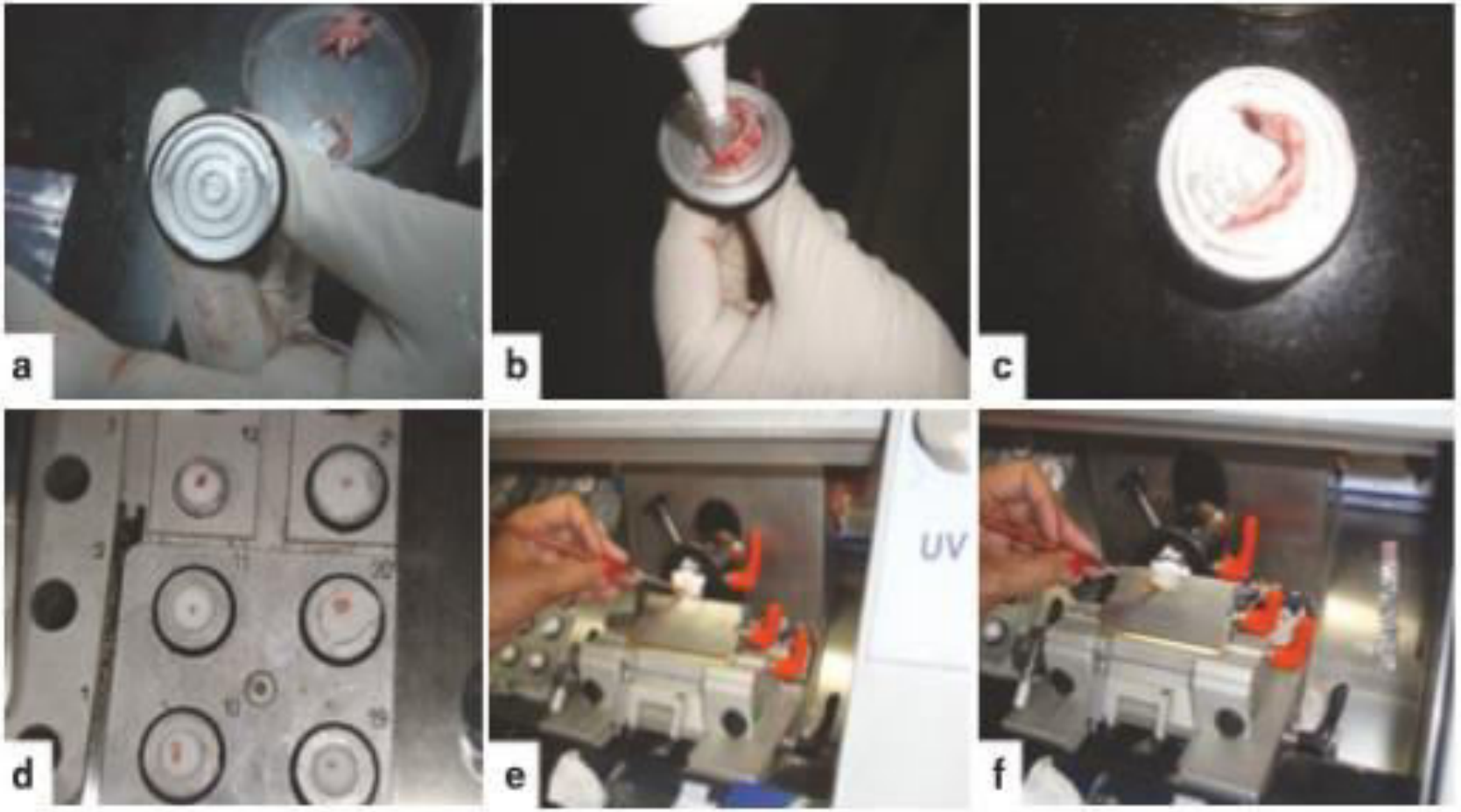
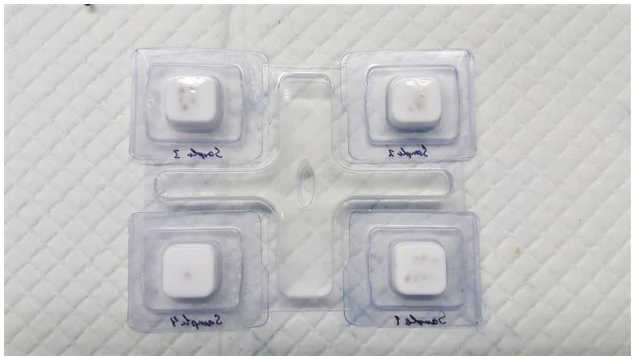


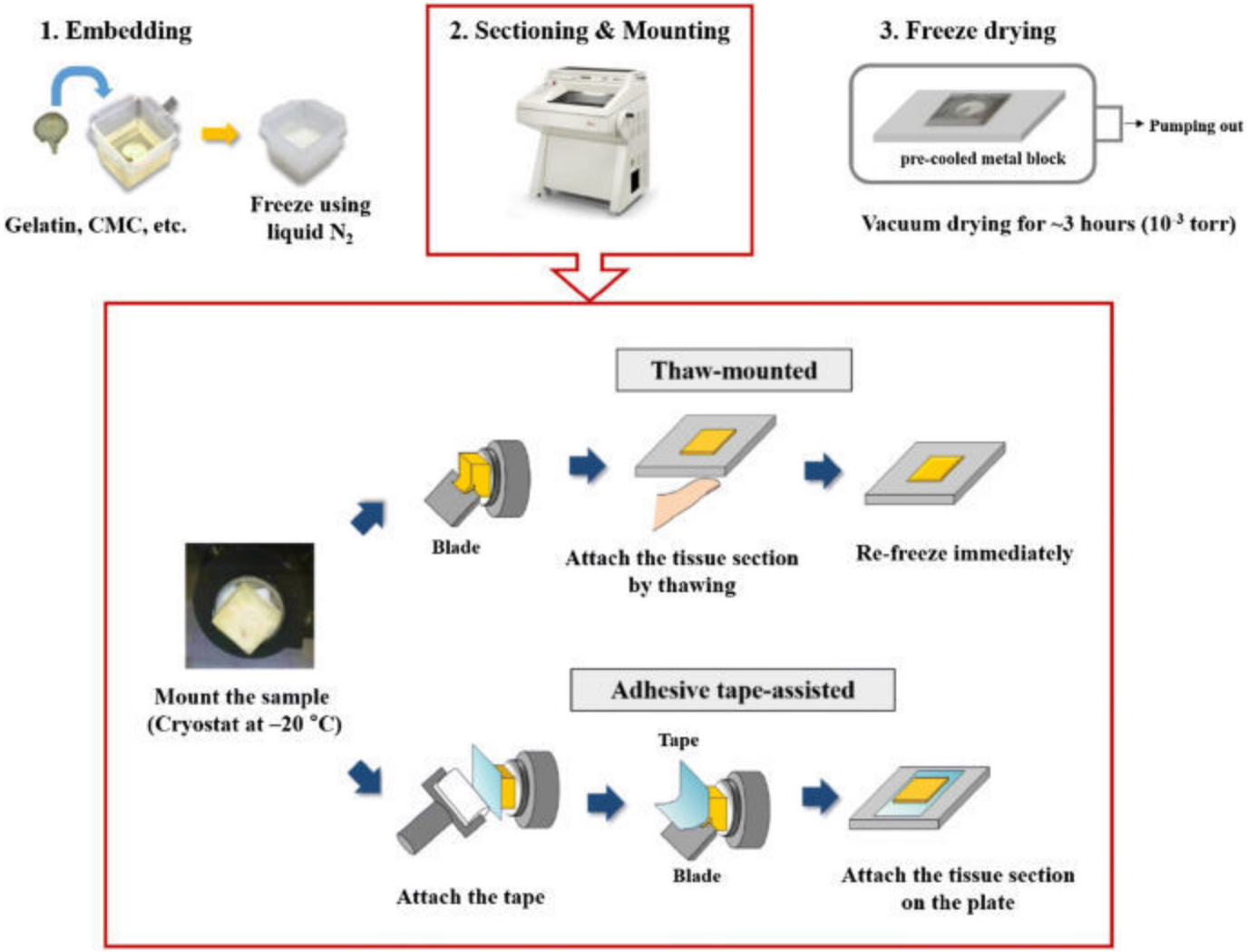

**Fig. 6.3** Cryostat processing: (**a**) mould is covered with OCT, (**b**) the tissue is now put on the block, (**c**) OCT is flooded over the tissue, (**d**) the tissue now is put in the cooling chamber, (**e**) the brush guides the tip of the tissue, (**f**) the tissue section is gently spread over the antiroll plate and later picked up by touching a glass slide

- **Tissue embedding in the mould**
- The small piece of the tissue is kept in the centre of the mould, and then the OCT is poured over it in excess.

cryo-moulds

Peel away mould

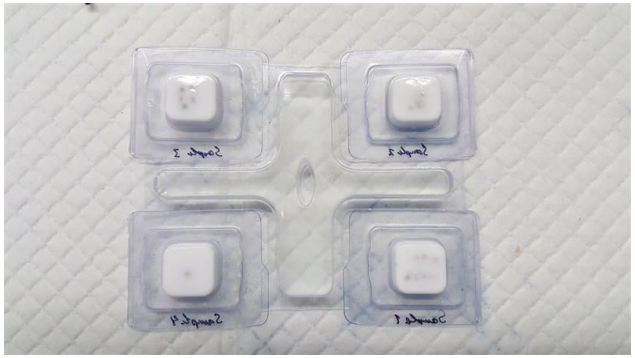

1. Embedding
Gelatin, CMC, etc.
Freeze using liquid $N_2$
2. Sectioning & Mounting
3. Freeze drying
pre-cooled metal block
Pumping out
Vacuum drying for ~3 hours ($10^{-3}$ torr)
Thaw-mounted
Blade
Attach the tissue section by thawing
Re-freeze immediately
Mount the sample (Cryostat at –20 °C)
Adhesive tape-assisted
Tape
Attach the tape
Blade
Attach the tissue section on the plate

https://bitesizebio.com/28466/can-stand-cold-cryosectioning-beginners/
https://en.wikipedia.org/wiki/Microtome
https://nanoconvergencejournal.springeropen.com/articles/10.1186/s40580-018-0157-y
Dey, P. (2018). Frozen Section: Principle and Procedure. In *Basic and Advanced Laboratory Techniques in Histopathology and Cytology* (pp. 51-55). Springer, Singapore.

# Lesson 30: Preparation of tissue for cryomicrotome -II

Dr. Ayesha Maqbool

- **Specimen holder:** The specimen holder or chuck is supplied by the manufacturers in different sizes and shapes. Usually these are round metal structures
- **Tissue embedding in the mould**
- The small piece of the tissue is kept in the centre of the mould, and then the OCT is poured over it in excess.

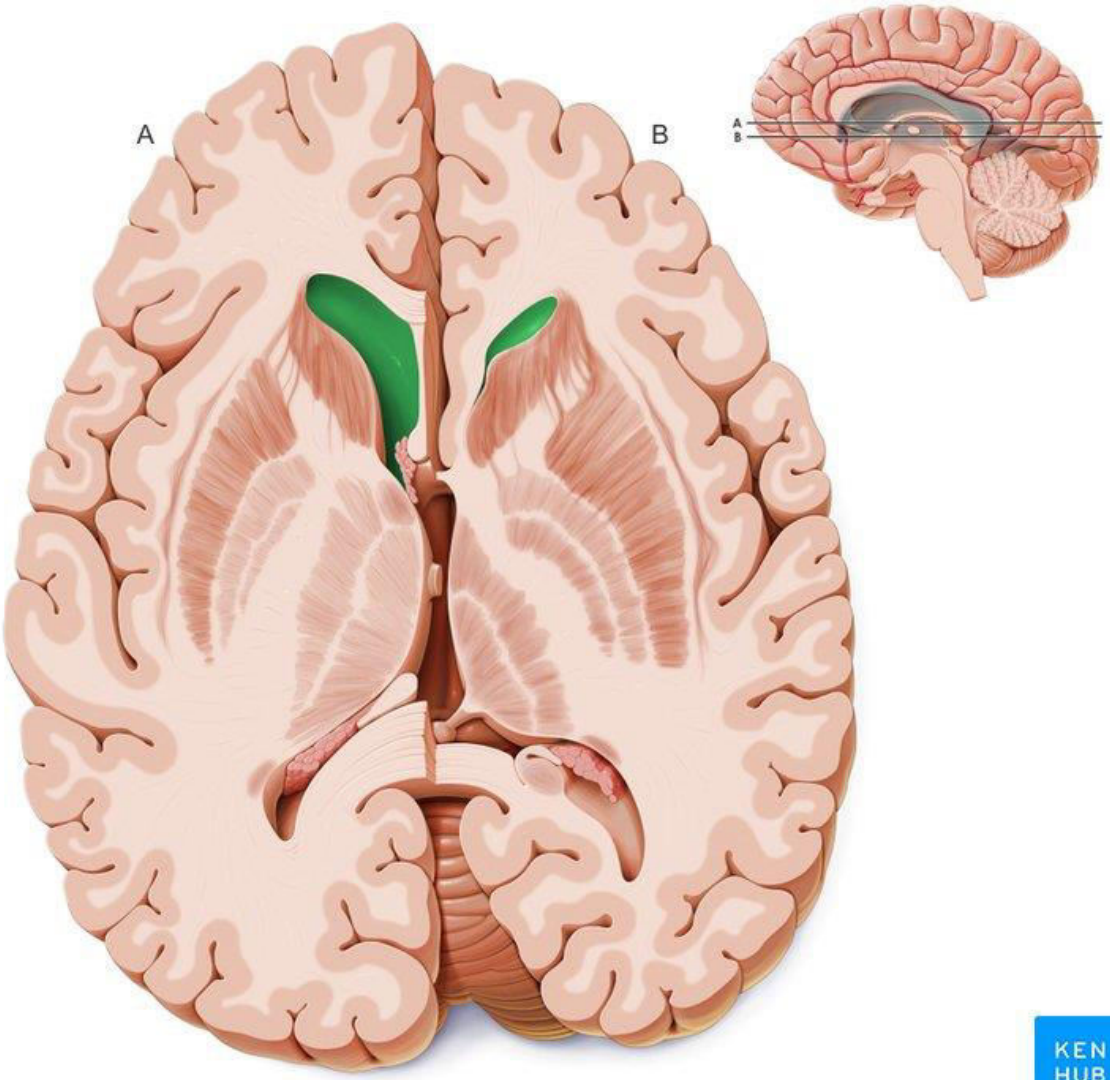



cryo-moulds

Peel away mould

# Grossing for Frozen Section Tissue

- Identify the tissue sample of the patient. Clinical information: provides possible differential diagnosis.
- Tissue appearance: colour, texture, nodule, any suture.
- Anatomy of the tissue: identify the resection planes and margins.
- Colour the resection planes and margins.
- Section cutting: – Use sharp blade.
- Always ensure you know the orientation of your tissue block, particularly if you have multiple tissue samples within one block.
- It is crucial to mark one spot on your tissue block after mounting onto the cryostat chuck to know the orientation.
- The edges of your block on round or square chucks will become indistinguishable once mounted.
- Now that you have chosen how to prepare and freeze your tissue, you are ready to move on to cryosectioning.

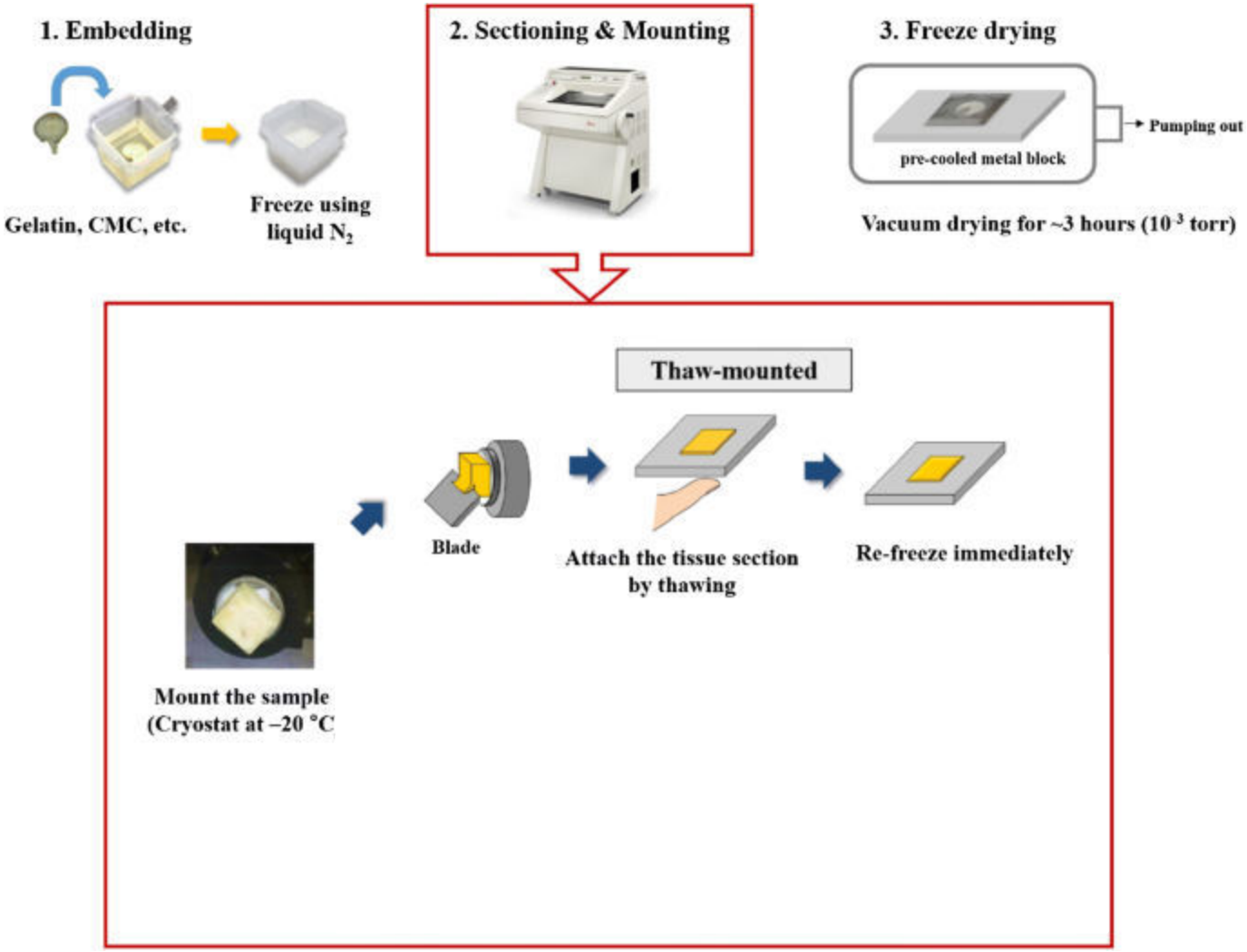


# Sectioning by cryomicrotome

1. Place prepared tissue block within the cryostat chamber for 30-60 minutes prior to beginning your sectioning, to allow the tissue to acclimate to -20$^0$C.
2. Tissue loading in the frozen section chamber:
3. Begin your cryosectioning practice with either non-essential tissue
4. Learning to cryosection with essential tissue will only lead to heartache.
5. when sectioning maintain your patience and stay calm. Otherwise your hot temper may melt your tissue!
6. Start your sectioning practice by sectioning your tissue at a thickness of ~50µm.
7. Then, as you begin to have more success with mounting your tissue without problems, gradually decrease the thickness (40µm, 30µm, 25µm, and 20µm).
8. Section lifting: The glass slide of normal room temperature is pressed firmly over the tissue section, and normally the tissue sticks immediately
9. Tissue folds, tears, and bubbles are common.

# .. cont

- use a fine-tip paintbrush to carefully flip the tissue over so that it will naturally uncurl upward towards the glass plus slide.
- do **not** press the glass slide all the way down to the metal plate (stage) with too much force, and do not let your glass slide out of your grasp onto the tissue otherwise the tissue will likely freeze and stick to the metal plate.
- Avoid the curling of tissue When mounting your tissue to the glass slide, always pay close attention to how close your hands are to the blade – you do not want to cut yourself!


1. Embedding
Gelatin, CMC, etc.
Freeze using liquid $N_2$
2. Sectioning & Mounting
3. Freeze drying
pre-cooled metal block
Pumping out
Vacuum drying for ~3 hours ($10^{-3}$ torr)
Thaw-mounted
Mount the sample (Cryostat at –20 °C
Blade
Attach the tissue section by thawing
Re-freeze immediately

https://bitesizebio.com/28466/can-stand-cold-cryosectioning-beginners/
https://en.wikipedia.org/wiki/Microtome
https://nanoconvergencejournal.springeropen.com/articles/10.1186/s40580-018-0157-y
Dey, P. (2018). Frozen Section: Principle and Procedure. In *Basic and Advanced Laboratory Techniques in Histopathology and Cytology* (pp. 51-55). Springer, Singapore.

# Lesson 31: Vibratome

Dr. Ayesha Maqbool

# Vibratome

- A **vibratome** is an instrument used to cut thin slices of material (although, usually thicker slices than those cut in paraffin-embedded samples using a microtome).
- **It is similar to a microtome but uses a vibrating blade to cut through tissue.**
- The vibrating microtome operates by cutting using a vibrating blade, allowing the resultant cut to be made **with less pressure** than would be required for a stationary blade.
- This machine produces slices from 30-50 micrometres thick.

# Vibratome-Advantages

1. It cuts sections directly from fixed material, without embedding.
2. **No PARAFFIN embedding required…** No need to deparaffinise and rehydrate sections prior to immunostaining
3. Sometimes, samples need to preserve particular features that are lost during embedding in paraffin or epoxy resins. During embedding, samples undergo dehydration and heating, which may cause damage in those molecules or molecular regions that we are interested in. For example, to detect them with antibodies by immunohistochemistry or in situ hybridization. No need to dehydrate tissues prior to embedding, thus decreased loss of cell constituents
4. No high temperatures or harsh chemical treatments that may lead to antigen instability
5. Furthermore, some tissue or cellular features need to be studied in thick sections of about 100 µm or larger. For example, the morphology of neuron dendritic trees is difficult to image in thin sections (4 to 10 µm), and we may also need to visualize dendrites after immunohistochemistry.

6. **Vibratome can do these two things:** thick sections and avoid embedding. The other way to get thick sections without embedding is using the freezing microtome, but cryoprotection and sample freezing are needed.

7. No special microtome blades required

8. Avoids artifacts caused by paraffin embedding or freezing

9. Decreased tissue autofluorescence due to avoidance of formalin-fixation and paraffin embedding

10. Less wait period from tissue sampling to time of immunolabelling

11. Allows for direct creation of free-floating sections for immunohistochemistry

# Vibratome-DisAdvantages

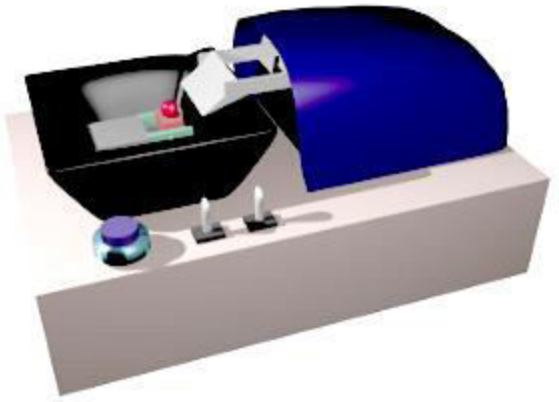
1. Only some samples are suitable for being sectioned with the vibratome. Those samples too soft or having hard or elastic parts are usually dragged by the blade, even if the blade speed is decreased and lateral vibration of the blade is ample.
2. It means that the sample should be consistent and as homogeneous as possible if good quality sections want to be obtained
3. Sections are generally thicker than those obtained with paraffin methods
4. penetration of antibodies and other reagents may be slower and thus longer incubation times may be necessary.
5. Also, thick sections may be difficult to image with the microscope. (However, thick sections are compatible and sometimes even desirable if using confocal microscope by z stack pictures.
6. Securing vibratome sections to glass slides can be difficult or impossible, due to the thickness of the sections
7. Once cut by vibratome, cannot be use for traditional stainings

# Vibratome-DisAdvantages

7. Another feature of the vibratome is that all the cutting is done in an aqueous solution, usually a buffer or saline solution. … Both the sample and the blade edge are immersed and the sections remain in the solution. They are known as floating sections, i.e., not attached.

9. These sections can be processed all the way as floating sections or they can be attached to a slide before the histological procedure.

10. processed as floating section and only before the observation they are placed onto slides, dehydrated and coverslipped with mounting medium.



- ncbi.nlm.nih.gov/pmc/articles/PMC7417605/pdf/fpls-11-01180.pdf
- https://www.tedpella.com/microslicer_html/10110.htm
- http://mmegias.webs.uvigo.es/02-english/6-tecnicas/4-vibratomo.php

# Gel Electrophoresis

## Introduction

# Introduction

## Electrophoresis

- near the heart of molecular cloning to separate, identify, and purify DNA fragments.
- simple, rapid, and capable of resolving fragments of DNA that cannot be separated by other procedures, such as density gradient centrifugation.

# Introduction

## Gel staining

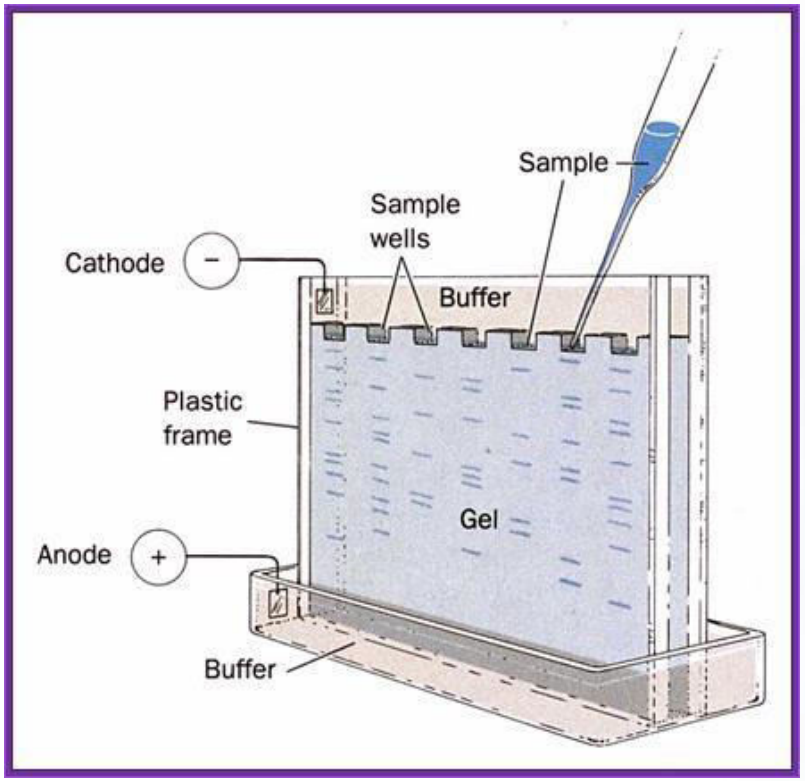
- DNA location within the gel can he determined by staining with low conc. of fluorescent intercalating dyes, such as ethidium bromide
- Bands containing as little as 20 pg of double-stranded DNA can be detected under UV.

# Introduction

## Types of Gels

- Agarose Gel
- Polyacrylamide gels
- variety of shapes, sizes, and porosities
- can be run in a number of different configurations.
- depend primarily on the sizes of the fragments being separated.

# Introduction




## Polyacrylamide Gel

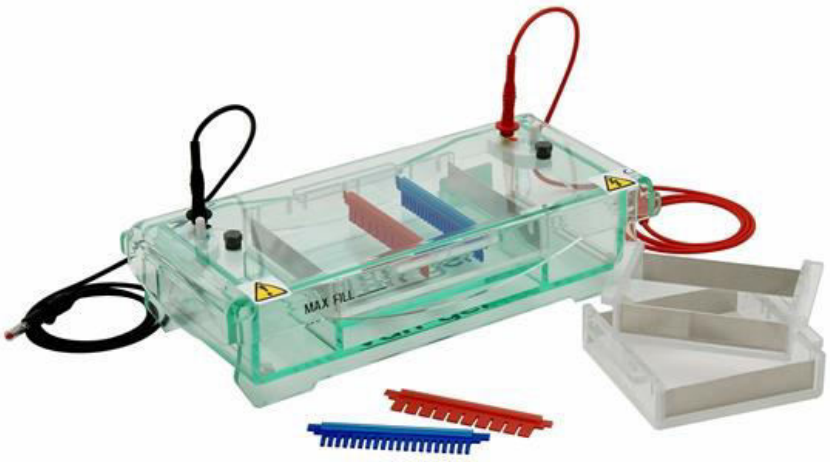
- Polyacrylamide gels are most effective for separating small fragments of DNA (5-500 bp ).
- resolving power is extremely high,
- Separate the DNA that differ in size by as little as 1 bp in length.

# Introduction

## Advantage/Disadvantage of Polyacrylamide

- Run very rapidly
- Accommodate comparatively large quantities of DNA,
- run in a vertical configuration in a constant electric field.
- However, more difficult to prepare and handle than agarose gels.

# Introduction




## Agarose Gel

- lower resolving power
- have a greater range of separation.
- DNAs from 50 bp to several megabases in length can be separated on agarose gels of various concentrations and configurations.

# Introduction

## Agarose Gel

- Small DNA fragments (50-20,000 bp) are best resolved in agarose gels run in a horizontal configuration in an electric field of constant strength and direction.
- As size of DNA fragment increases the percentage of gel composition have to be decreased.

# Introduction

## Gel composition

- The greater the pore size of the gel, the larger the DNA that can be sieved.
- Thus, agarose gels cast with low concentrations of agarose (0.1-0.2% w/v) are capable of resolving extremely large DNA molecules.

# Introduction

## Low percentage Agarose

- such gels are extremely fragile and must be run for several days.
- Even then, they are incapable of resolving linear DNA molecules larger than 750 kb in length.

# Introduction

## Pulsed field gel Electrophoresis

- In 1984, when Schwartz and Cantor developed pulsed-field gel electrophoresis (PFGE).
- In this method, alternating orthogonal electric fields are applied to a gel.

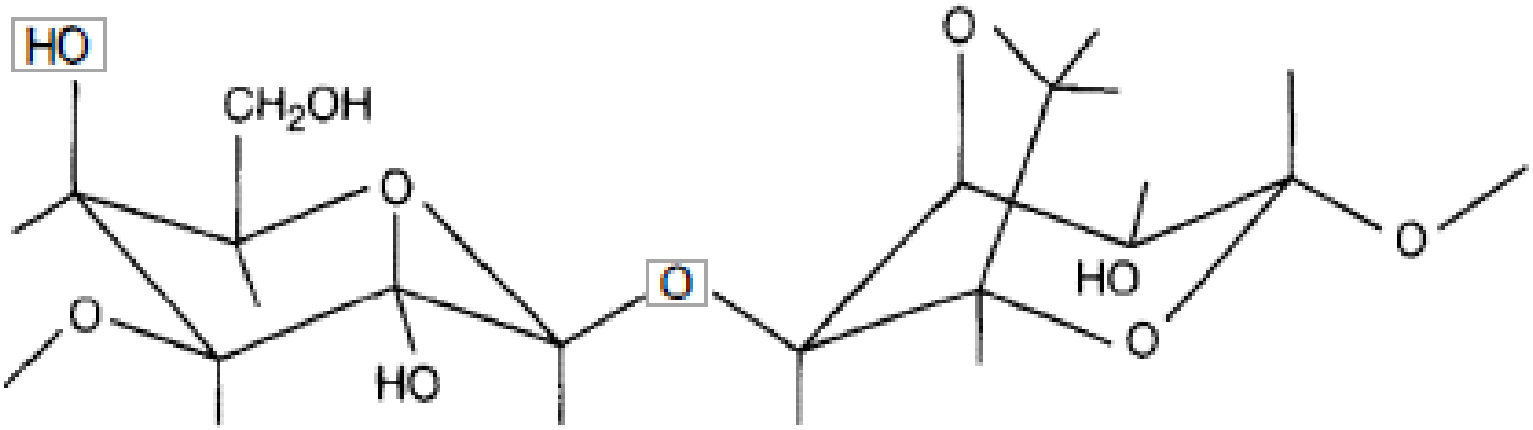
# Agarose Gel electrophoresis

# Agarose

## Agarose chemical composition

- A linear polymer composed of alternating residues D-and and L-galactose joined by α(1 3) and β-(1 4) glycosidic linkages.

# Agarose



# Reagent and Supplies

## Agarose Polymers

- Commercially prepared agarose polymers are believed to contain -800 galactose residues per chain.
- Agarose is not homogeneous: The average length of the polysaccharide chains varies from batch to batch and from manufacturer to manufacturer.

# Reagent and Supplies

## Low grade agarose

- Lower grades of agarose may be contaminated with other polysaccharides, salts and proteins.

# Reagent and Supplies

## Low grade agarose

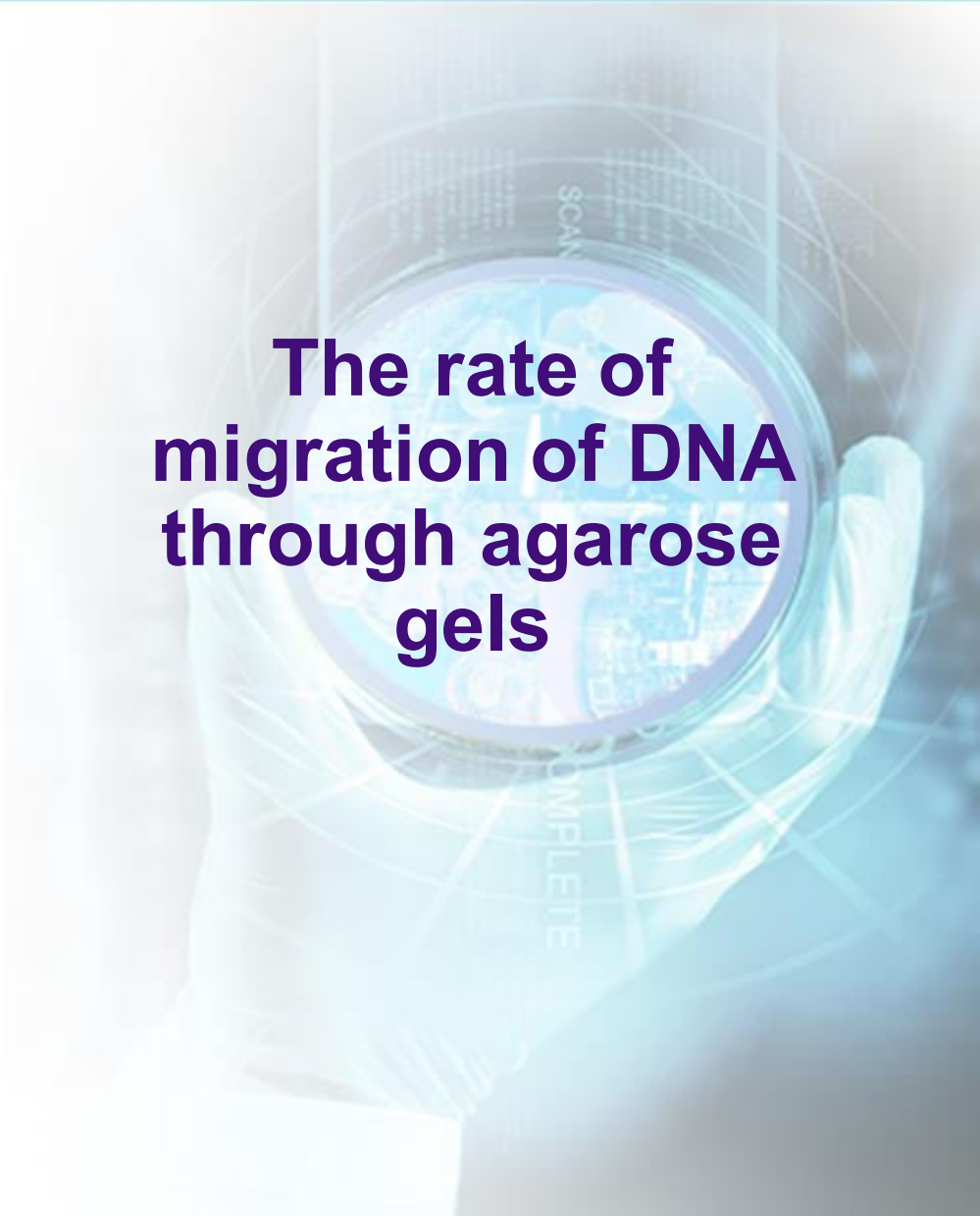
- This variability can affect the
- gelling/melting temperature of agarose solutions,
- the sieving of DNA, and
- the ability of the DNA recovered from the gel to serve as a substrate in enzymatic reactions.

# Reagent and Supplies

## Low grade agarose

- These potential problems can be minimized by using special grades of agarose that are screened for the presence of inhibitors and nucleases and for minimal background fluorescence after staining with ethidium bromide.

# Agarose Gel electrophoresis



**The rate of migration of DNA through agarose gels**

# Rate of migration of DNA

## Factors affecting the rate of DNA migration

Different factors determine the rate of migration of DNA through agarose gels.

# Rate of migration of DNA

## Molecular Size of DNA

- Molecules of double-stranded DNA migrate through gel matrices at rates that are inversely proportional to the log10 of the number of base pairs.

# Rate of migration of DNA

## Molecular Size of DNA

- Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules.

# Rate of migration of DNA

## Concentration of Agarose

- A linear DNA fragment of a given size migrates at different rates through gels containing different concentrations of agarose.

# Rate of migration of DNA

## Conformation of the DNA

- Super helical circular (form I),
- Nicked circular (form II),
- Linear (form III) DNAs migrate through agarose gels at different rates

# Rate of migration of DNA

## Conformation of the DNA

- The relative mobilities of the three forms depend primarily
  - on the concentration and type of agarose
  - strength of the applied current
  - the ionic strength of the buffer
  - density of superhelical twists in the form I DNA

# Rate of migration of DNA

## Conformation of the DNA

- Under some conditions, form I DNA migrates faster than form III DNA; under other conditions, the order is reversed.

# Rate of migration of DNA

## Ethidium Bromide

- Intercalation of Et. br. causes a decrease in the negative charge of the ds DNA and an increase in both its stiffness and length.
- The rate of migration of the linear DNA-dye complex through gels is retarded by a factor of ~15%.

# Rate of migration of DNA

## Voltage

- At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied.
- However, as the strength of the electric field is raised, the mobility of high-molecular-weight fragments increases differentially.

# Rate of migration of DNA

## Voltage

- Thus, the effective range of separation in agarose gels decreases as the voltage is increased.
- To obtain maximum resolution of DNA fragments >2 kb in size, agarose gels should be run at no more than 5-8 V/cm.

# Rate of migration of DNA

## Type of Agarose

- Standard agaroses
- Low-melting temp.
- Intermediate melting/gelling temperature agaroses, exhibiting properties of each of the two major classes.

# Rate of migration of DNA

## Electrophoresis Buffer

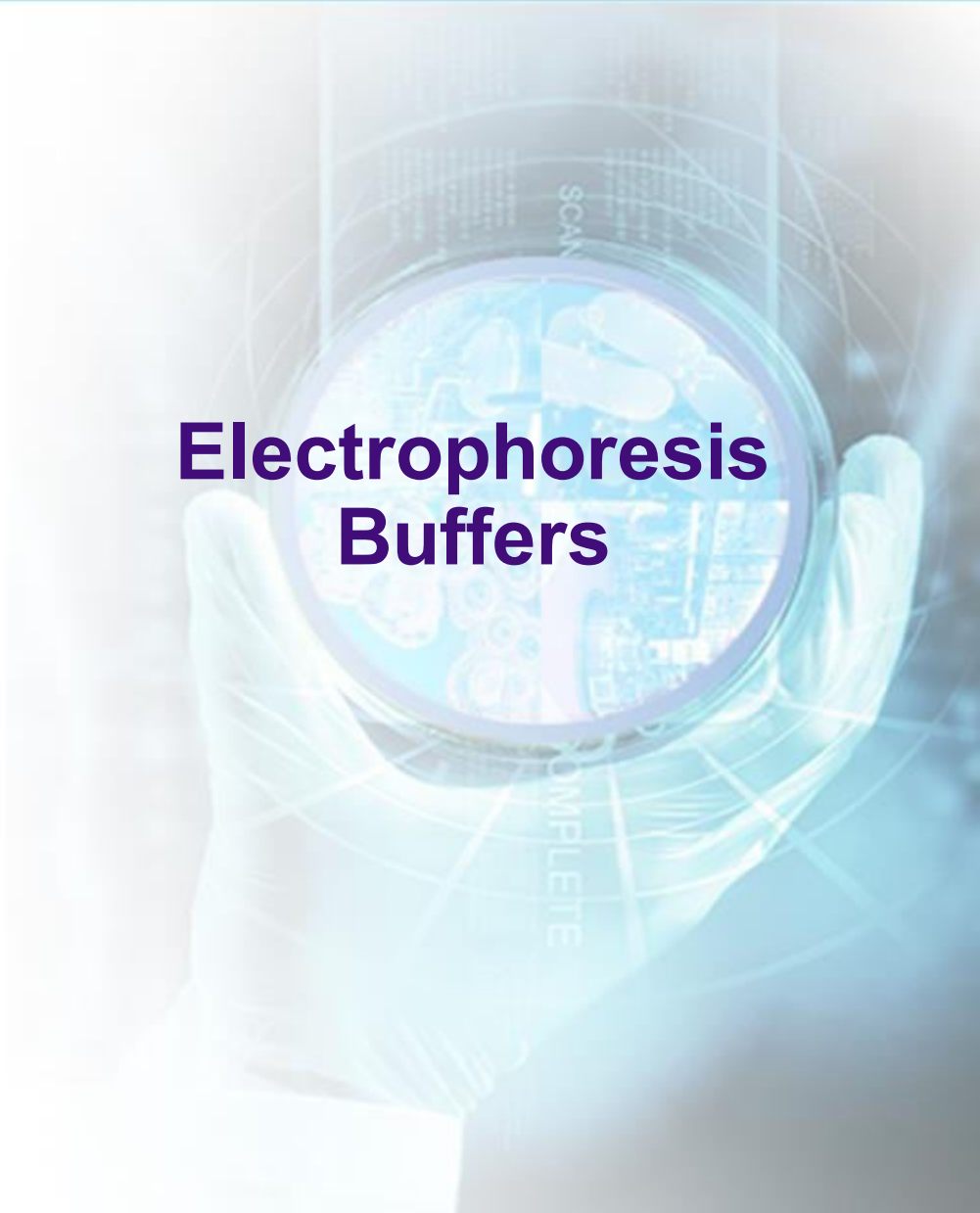
- In the absence of ions (e.g., if water is substituted for electrophoresis buffer in the gel or in the reservoirs), electrical conductivity is minimal and DNA migrates slowly, if at all.

# Rate of migration of DNA

## Electrophoresis Buffer

- In buffer of high ionic strength, electrical conductance is very efficient and significant amounts of heat are generated, even when moderate voltages are applied.
- In the worst case, the gel melts and the DNA denatures.

# Agarose Gel electrophoresis



## Electrophoresis Buffers

# Electrophoresis Buffers

## Buffers

- Several different buffers are available for electrophoresis of native, double-stranded DNA.
  - Tris-acetate and EDTA (pH 8.0; TAE) (also called E buffer),
  - Tris-borate (TBE)
  - Trisphosphate (TPE) at a concentration of 50 mM (pH 7.5-7.8).

# Electrophoresis Buffers

## Concentrated Buffers

- Electrophoresis buffers are usually made up as concentrated solutions and stored at room temperature

# Electrophoresis Buffers

## TAE

- has the lowest buffering capacity
- becomes exhausted if electrophoresis for prolonged electrophoresis periods

# Electrophoresis Buffers

## TAE

- In such situation
- the anodic portion of the gel becomes acidic
- bromophenol blue migrating through the gel toward the anode changes in color from bluish-purple to yellow
- This change begins at pH 4.6 and is complete at pH 3.0.

# Electrophoresis Buffers

## TAE

- Exhaustion of TAE can be avoided by periodic replacement of the buffer during electrophoresis

# Electrophoresis Buffers

## TBE & TPE

- Are slightly more expensive than TAE,
- have significantly higher buffering capacity.
- ds linear DNA fragments migrate 10% faster through TAE than through TBE or TPE

# Electrophoresis Buffers

## TBE & TPE

- The resolving power of TAE is slightly better than TBE or TPE for high-molecular-weight DNAs and worse for low-molecular-weight DNAs.

# Electrophoresis Buffers

## Difference in the results of buffers

- This difference probably explains the observation that electrophoresis in TAE yields better resolution of DNA fragments in highly complex mixtures such as mammalian DNA.

# Electrophoresis Buffers

## Difference in the results of buffers

- Gels for southern blots are prepared and run with TAE as the electrophoresis buffer.
- The resolution of supercoiled DNAs is better in TAE than in TBE.

# Electrophoresis Buffers

| BUFFER | WORKING SOLUTION | STOCK SOLUTION/LITER |
|---|---|---|
| TAE | 1x<br>40 mM Tris-acetate<br>1mM EDTA | 50x<br>242 g of Tris base<br>57.1 ml of glacial acetic acid<br>100 ml of0.5 M EDTA (pH 8.0) |
| TPE | 1x<br>90mM Tris-phosphate<br>2mM EDTA | 10X<br>108 g of Tris base<br>15.5 ml of phosphoric acid (85%, 1.679 g/ml)<br>40 ml of 0.5 M EDTA (pH 8.0) |
| TBE | 0.5x<br>45 mM Tris-borate<br>1mM EDTA | 5x<br>54 g of Tris base<br>27.5 g of boric acid<br>20 ml of 0.5 M EDTA (pH 8.0) |

# Agarose Gel electrophoresis

## Reagents, Supplies and Equipment

# Reagent and Supplies

## Buffers and Solutions

- Agarose solutions
- Electrophoresis buffer (usually 1x TAE or 0.5x TBE)
- Ethidium bromide
- 6x Gel-loading buffer

# Reagent and Supplies

## DNA Samples

- DNA samples
- DNA size standards

# Reagent and Supplies

## Special Equipment

- Equipment for agarose gel electrophoresis
- Clean, dry horizontal electrophoresis apparatus with chamber and comb,
- or clean dry glass plates with appropriate comb.

# Reagent and Supplies

## Gel-sealing tape

- Common types of lab tape, such as Time tape or VWR lab tape, are appropriate for sealing the ends of the agarose gel during pouring.

# Reagent and Supplies

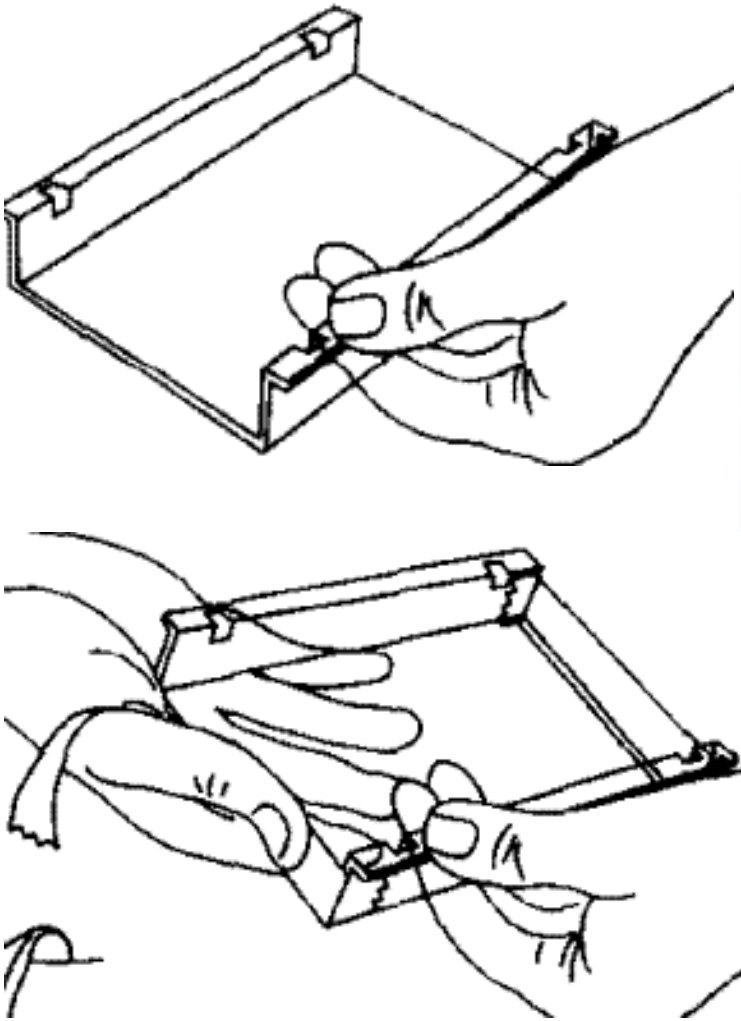
## Special Equipment

- Microwave oven or Boiling water bath
- Power supply device capable of up to 500 V and 200 mA.
- Water bath preset to 55°C

# Agarose Gel electrophoresis

## Procedure

# Procedure



## Gel casting mold

- Seal the edges of a clean, dry glass plate (or the open ends of the plastic tray supplied with the electrophoresis apparatus) with tape to form a mold (figure 5-3).
- Set the mold on a hori-zontal section of the bench.

# Procedure

## Electrophoresis buffer

- Prepare sufficient electrophoresis buffer (usually 1x TAE or 0.5x TBE) to fill the electrophoresis tank and to cast the gel.

# Procedure

## Agarose Gel preparation

- Prepare a solution of agarose in electrophoresis buffer at an appropriate required concentration appropriate for separating the particular size fragments expected in the DNA sample(s).

# Procedure

## Agarose Gel preparation

- Add the correct amount of powdered agarose (please see Table 5-5) to a measured quantity of electrophoresis buffer in an Erlenmeyer flask or a glass bottle.

# Procedure

| Range of separation in Agarose Gels (Low-EEO) | |
|---|---|
| Agarose Concentration (%[w/v]) | Range of separation of Linear DNA molecule (kb) |
| 0.3 | 5-60 |
| 0.6 | 1-20 |
| 0.7 | 0.8-10 |
| 0.9 | 0.5-7 |
| 1.2 | 0.4-6 |
| 1.5 | 0.2-3 |
| 2.0 | 0.1-2 |

# Procedure

## Agarose Gel preparation

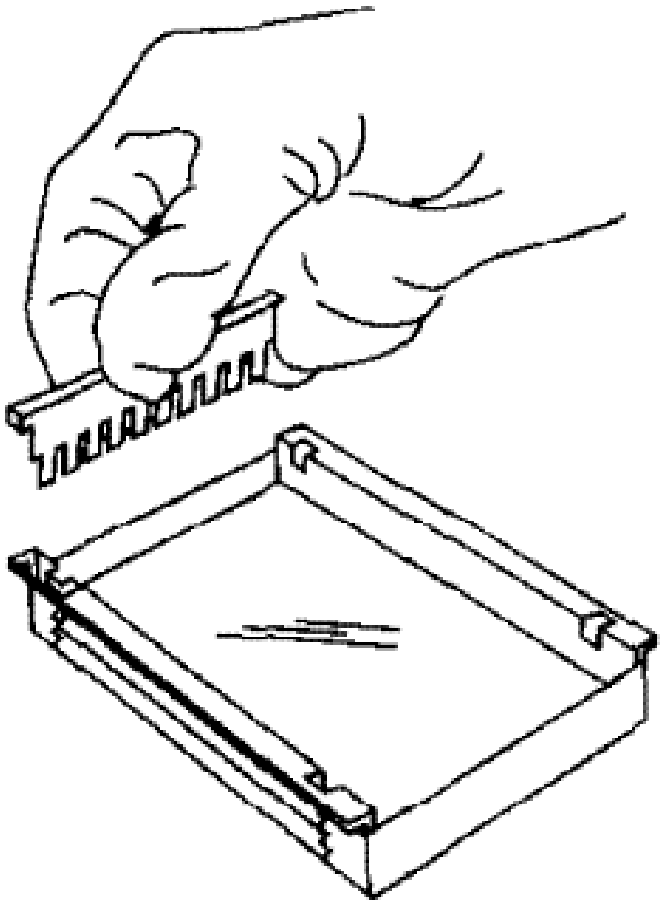
- Loosely plug the neck of the flask with Kim wipes.
- If using a glass bottle, make certain the cap is loose.
- Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves .

# Procedure

## Agarose Gel preparation

- Use insulated gloves or tongs to transfer the flask/bottle into a water bath at 55°C.
- When the molten gel has cooled, add et. br. to a final concentration of 0.5µg/ml.
- Mix the gel solution thoroughly by gentle swirling .

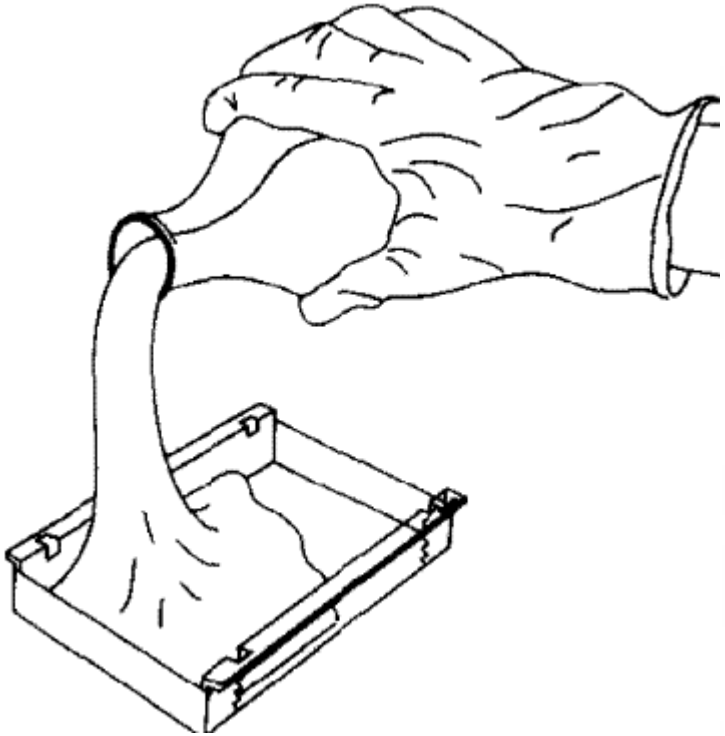
# Procedure



## Gel Casting

- While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel.
- Position the comb 0.5-1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold.

# Procedure



## Gel pouring

- Pour the warm agarose solution into the mold.

# Procedure

## Waiting period

- Allow the gel to set completely (30-45 minutes at room temperature)
- Then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb.

# Procedure

## Waiting period

- Pour off the electrophoresis buffer and carefully remove the tape. Mount the gel in the electrophoresis tank.
- Add just enough electrophoresis buffer to cover the gel to a depth of -1 mm.

# Procedure

## DNA sample

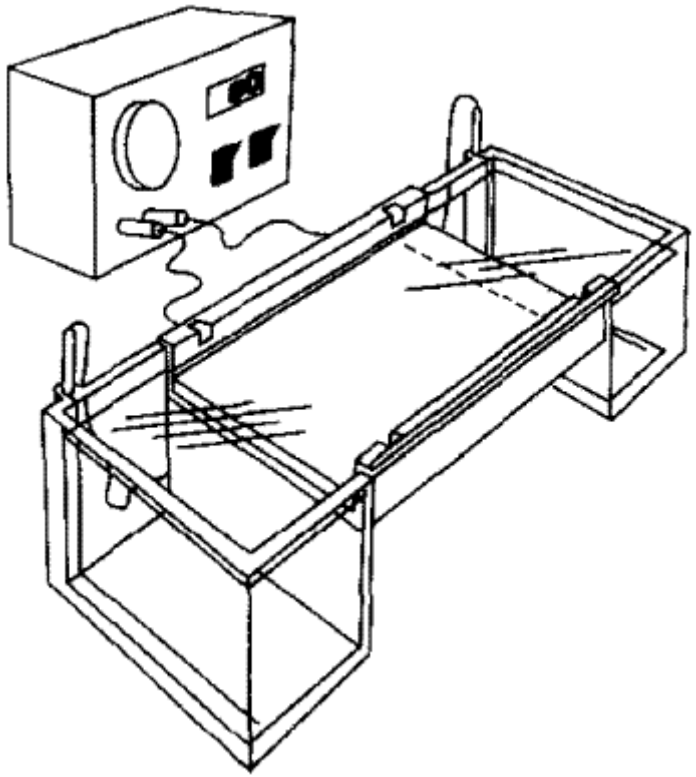
- Mix the samples of DNA with 0.20 volume of the desired 6x gel-loading buffer.

# Procedure

## Sample loading

- Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipette.
- Load size standards into slots on both the right and left sides of the gel.

# Procedure



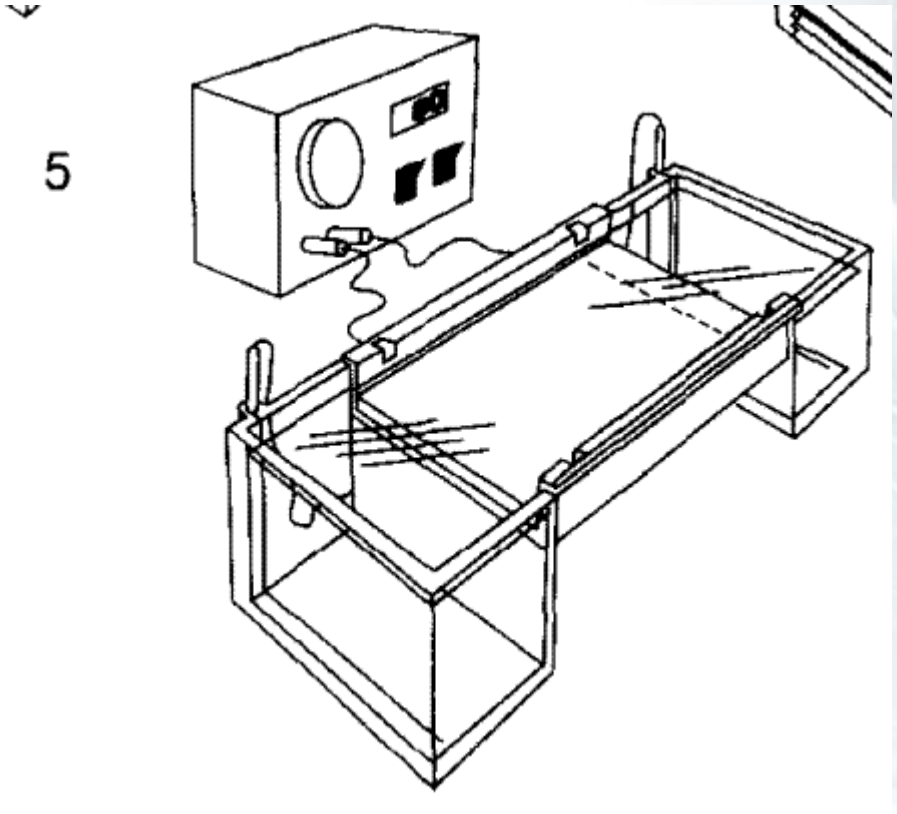
## Electrophoresis

- Close the lid of the gel tank
- attach the electrical leads so that the DNA will migrate toward the positive anode (red lead).
- Apply a voltage of 1-5 V/cm (measured as the distance between the positive and negative electrodes).

# Procedure

## Maxi-preps

- L



# Procedure

## Electrophoresis

- If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis),
- Within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel.

# Procedure

## Electrophoresis

- Run the gel until the bromophenol blue and xylene cyanol FF have migrated an appropriate distance through the gel.
- Turn off the electric current and remove the leads and lid from the gel tank.

# Procedure

## Gel Staining

- If ethidium bromide is present in the gel and electrophoresis buffer, examine the gel by UV light and photograph the gel as described in
- Otherwise, stain the gel by immersing it in electrophoresis buffer or $H_2O$ containing et. br. (0.5 µg/ml) for 30-45 minutes at RT.

# Agarose Gel Electrophoresis

## Detection of DNA in Agarose gel

# Detection of DNA in Agarose gel

## Gel Staining

- Nucleic Acids separated through agarose gel electrophoresis may be detected by
  - Staining
  - Visualized by illumination with 300-nm UV light.

# Detection of DNA in Agarose gel

## Methods

Two methods

- Ethidium bromide
- SYBR Gold

# Detection of DNA in Agarose gel

## Ethidium Bromide

- is a fluorescent dye
- most convenient and commonly used
- contains a tricyclic planar group that intercalates between the stacked bases of DNA.
- binds to DNA with little or no sequence preference.

# Detection of DNA in Agarose gel

## Rate of incorporation

- At saturation approximately one ethidium molecule intercalats per 2.5 bp.
- After insertion, the dye lies perpendicular to the helical axis and makes van der Waals contacts with the base pairs above and below.

# Detection of DNA in Agarose gel

## UV light

- UV radiation at 254 nm is absorbed by the DNA and transmitted to the dye and at 302 nm and 366 nm is absorbed by the bound dye itself.
- In both cases, the energy is re-emitted at 590 nm in the red-orange region of the visible spectrum

# Detection of DNA in Agarose gel

## ssDNA &dsDNA

- Et. Br can be used to detect both single- and double-stranded nucleic acids (both DNA and RNA).
- The affinity of the dye for single-stranded nucleic acid is relatively low and the fluorescent yield is comparatively poor.

# Detection of DNA in Agarose gel

## Et. Br. stock

- Prepared as a stock solution of 10 mg/ml in $H_2O$
- stored at room temperature in dark bottles or bottles wrapped in aluminum foil.
- For agarose gels and electrophoresis buffers at a concentration of 0.5µg/ml

# Detection of DNA in Agarose gel

## Ethidium bromide in Gels

- Polyacrylamide gels cannot be cast with ethidium bromide
- Et. Br. inhibits polymerization of the acrylamide.
- Stained with the ethidium solution after the gel has been run.

# Detection of DNA in Agarose gel

## Advantage over Disadvantage

- electrophoretic mobility of linear dsDNA reduced by ~15% in the presence of the dye
- the ability to examine the agarose gels directly under UV illumination during or at the end of the run is a great advantage.

# Detection of DNA in Agarose gel

## Advantage over Disadvantage

- Sharper DNA bands are obtained in the absence of et. br.
- For accurate size of DNA fragment, the gel should be run in the absence of et. br.
- stain after electrophoresis is complete.

# Detection of DNA in Agarose gel

## Et. Br. Staining

- immerse the gel in electrophoresis buffer or $H_2O$ containing et. br. (0.5 µg/ml) for 30-45 minutes at room temperature.
- Destaining is not usually required.

# Detection of DNA in Agarose gel

## Et. Br. Staining

- For detection of very small amounts ( < 10 ng) of DNA is made easier if the background fluorescence caused by unbound ethidium bromide is reduced by soaking the stained gel in $H_2O$ or 1mM $MgSO_4$ for 20 minutes at room temperature.

# Agarose Gel Electrophoresis

## Staining with Sybr Gold

# Staining with Sybr Gold

## Ultrasensitive dye

- high affinity for DNA
- fluorescence enhancement upon binding to nucleic acid.
- the fluorescence enhancement is > 1000 times greater than equivalent et. br.-DNA complex.

# Staining with Sybr Gold

## Detection limit

- Can detect <20 pg of ds DNA in an agarose gel
- 25 times less than the amount visible after ethidium bromide staining
- Can reveal 100 pg of ss-DNA in a band or 300 pg of RNA.

# Staining with Sybr Gold

## Staining

- Separation of the DNA fragments through electrophoresis
- Soak the gel, in a 1:10,000-fold dilution of the stock dye solution.

# Staining with Sybr Gold

## Precaution

- SYBR Gold should not be added to the molten agarose or to the gel before electrophoresis,
- Causes severe distortions in the electrophoretic properties of the DNA and RNA.

# Staining with Sybr Gold

## Photography

- greatest sensitivity at 300nm UV.
- Photography is carried out with green or yellow filters.

# Agarose Gel Electrophoresis

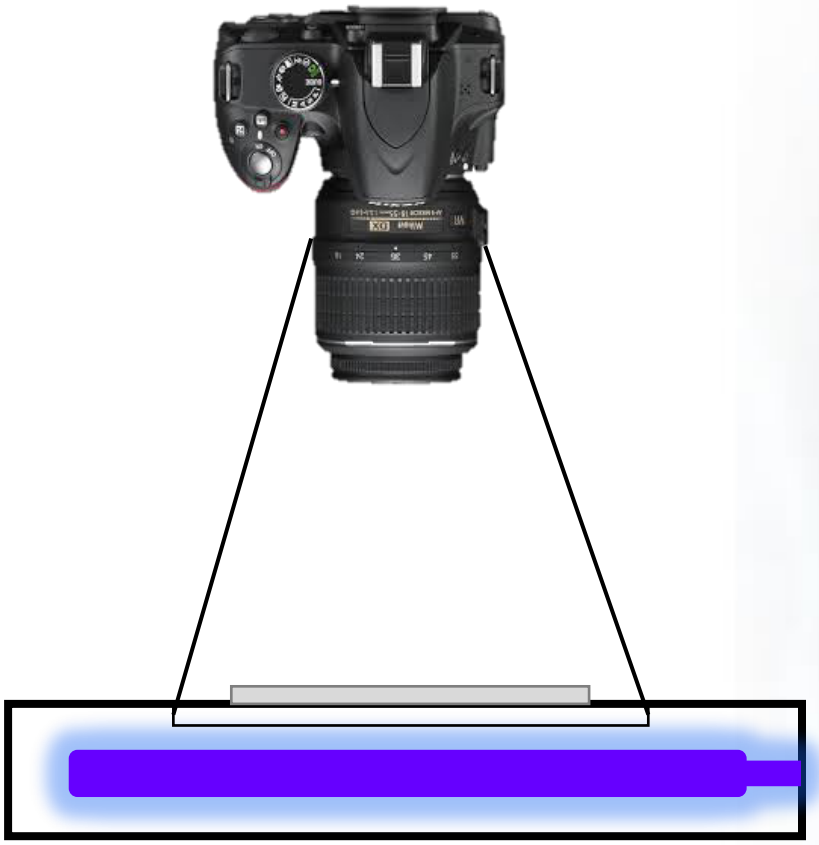
**Photography of DNA in Gel**

# Photography of DNA in Gel

## Transmitted vs Incident UV light

- Photographs of ethidium-bromide-stained gels may be made using transmitted or incident UV light
- Most commercially available devices (transilluminators) emit UV light at 302 nm.

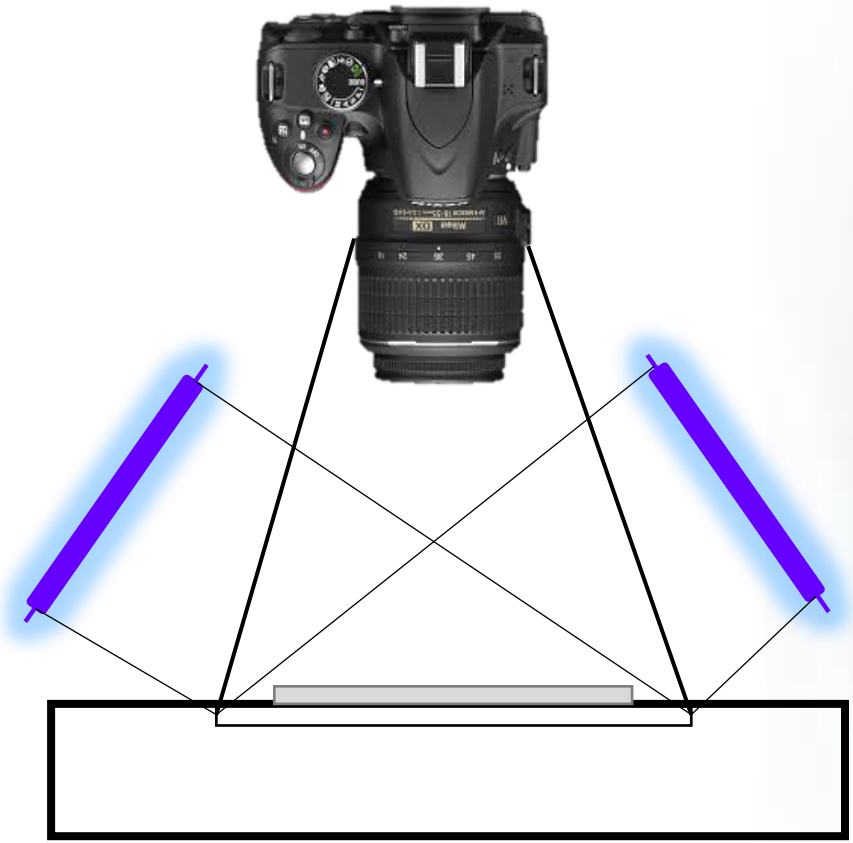
# Photography of DNA in Gel



## Transmitted UV light

- the arrangement of the
  - UV light source,
  - the gel,
  - the camera

# Photography of DNA in Gel



## Incident UV light

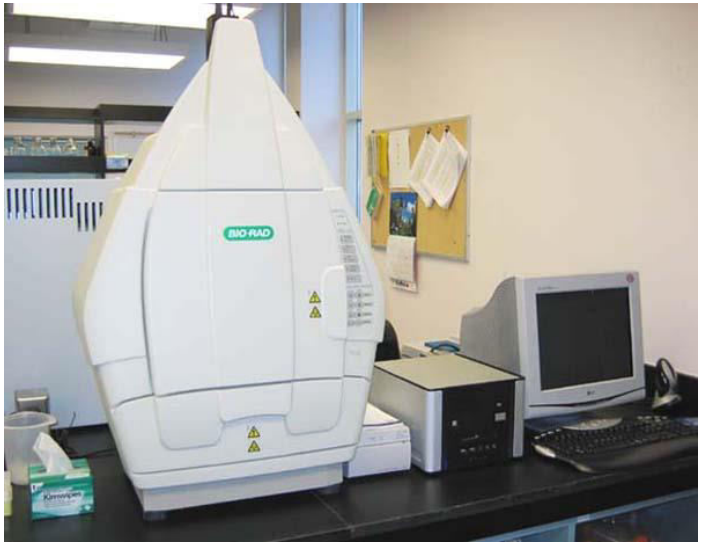
- the arrangement of the
  - UV light source,
  - the gel,
  - the camera

# Photography of DNA in Gel


## Gel Documentation Systems

- Today, images of ethidium-bromide-stained gels may be captured by integrated systems containing light sources, fixed-focus digital cameras, and thermal printers.

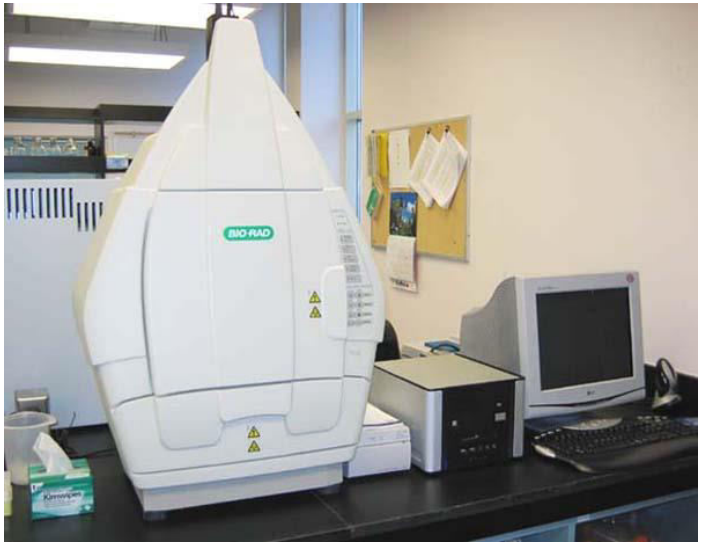
# Photography of DNA in Gel




## Gel Documentation Systems

- Images are directly transmitted to a computer and visualized in real time.
- manipulated on screen with respect to field, focus, and cumulative exposure time prior to printing.

# Photography of DNA in Gel




## Gel Documentation Systems

- images can be
- printed,
- Saved
- stored electronically in several file formats
- further manipulated with image analysis software programs.

# Photography of DNA in Gel

## Improved sensitivity

- A further 10-20-fold increase in the sensitivity of conventional photography can be obtained by staining DNA with SYBR Gold
- However cost difference is too much 100 USD vs 5 cents

# LESSON 41
# **Separation Techniques**

**Dr. Aneela Ulfat**

# Separation Techniques

- Separation techniques are those techniques that can be used to separate two different states of matter such as liquids and solids.
- Separation processes or a separation method or simply a separation is methodology to attain any mass transfer phenomenon that convert a mixture of substances into two or more distinct product mixtures.
- Separation is an important asset to purify component of interest from a mixtures.

# Need of Separation Techniques:

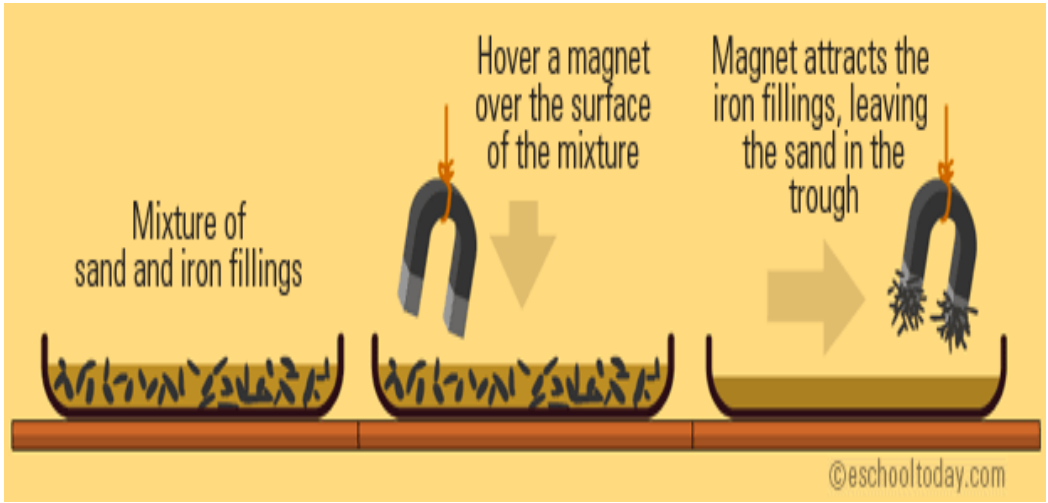
- To identify what to be separated from mixture.
- To obtain Important and pure substances.
- To remove unwanted particles

# Classification of Separation Techniques

| Basis Of Separation | Separation Technique(s) |
| --- | --- |
| Size | Filtration; Dialysis; Size-exclusion Chromatography |
| Mass Or Density | Centrifugation |
| Complex Formation | Masking |
| Change In Physical State | Distillation; Sublimation; Recrystallization |
| Change In Chemical State | Precipitation; Electrode position; Volatilization |
| Partitioning Between Phases | Extraction; Chromatography |

# Magnetic Separation

- This method involves the separation of magnetic substances from non-magnetic substances by means of magnet.
- Takes advantage of physical property of magnetism, so it useful only for certain substances such as ferromagnetic (materials strongly affected by magnetic fields) and paramagnetic (materials that are less affected, but the effect is still noticeable).
- This method involves the separation of magnetic substances from non-magnetic substances by means of magnet

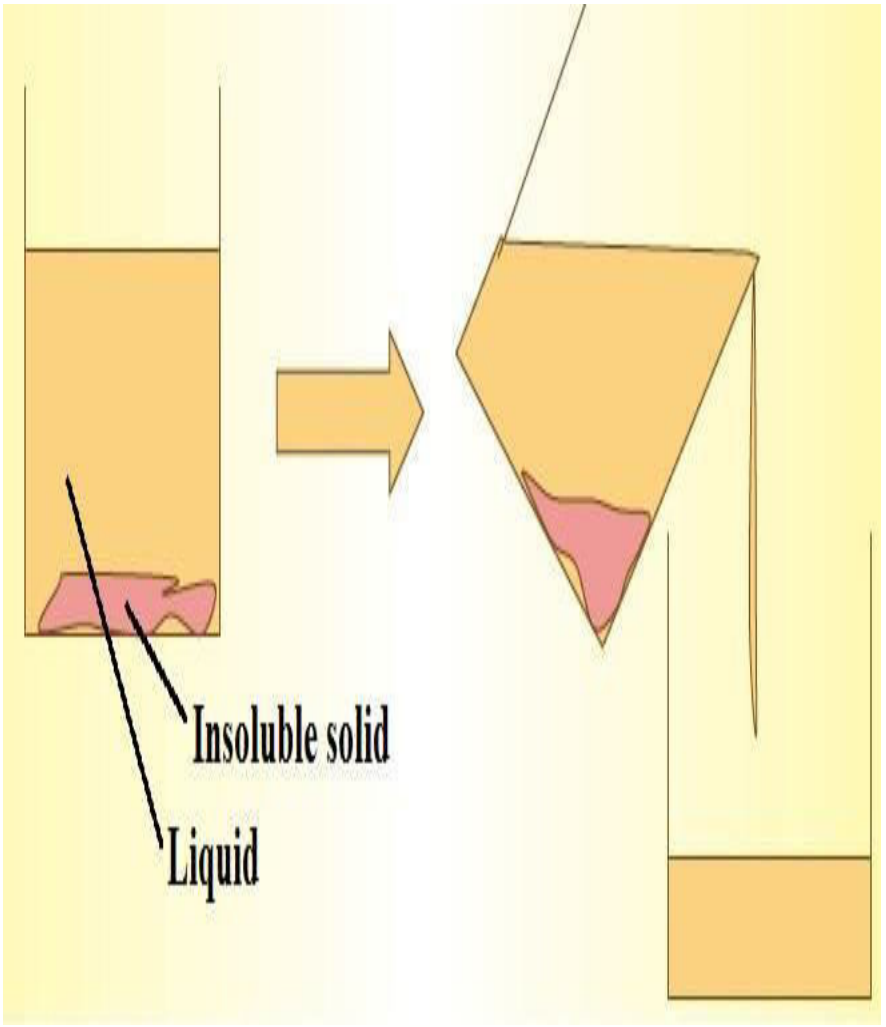


# Applications

- Waste management, low-magnetic field separation in water purification and separation of complex mixtures.
- To remove metal contaminants from pharmaceutical product streams.
- Magnetic cell separation. It is currently being used in clinical therapies, more specifically in cancer and hereditary diseases researches.
- These techniques are combined with PCR (polymerase chain reaction), to increase sensitivity and specificity of results.

# DECANTATION

- Decantation is a process for the separation of mixtures of immiscible liquids or of a liquid and a solid mixture such as a suspension. Immiscible liquid separation:
- Takes advantage of differences in density of the liquids.
- A separator funnel is an alternative apparatus for separating liquid layers. It has a valve at the bottom to allow draining off the bottom layer. It can give a better separation between the two liquids.
- Example: separation of mixture of oil and water. Takes advantage of gravity/sedimentation of solids in case solid-liquid separation.



- Sedimentation: The tendency of particles in suspension to settle down in the fluid due to certain forces like gravity, centrifugal acceleration, or electromagnetism is called as sedimentation.
- The solid that gets settled down is called as sediment.
- In laboratory it can be done in test tubes.
- To enhance productivity test tubes should be placed at 45° angle to allow the sediments to settle at the bottom of the apparatus.
- A decanter centrifuge may be used for continuous solid-liquid separation.

# Applications

- Decantation is frequently used to purify a liquid by separating it from a suspension of insoluble particles.
- Decantation is also present in nanotechnology. In the synthesis of high-quality silver nanowire (AgNW) solutions and fabrication process of high-performance electrodes, decantation is also being applied which greatly simplifies the purification process.
- Fat is determined in butter by decantation.
- In sugar industry, processing of sugar beets into granular sugar many liquid - solid separations are encountered.

# FILTRATION

- Takes advantage of physical property of state of matter, its size and solubility in liquid. The substance which is left behind in the filtration medium is called **residue**.
- The liquid which passes through the filtration medium is called **filtrate**. Mechanical, physical or biological operations that separates solids from fluids (liquids or gases ) by adding a medium through which only the fluid can pass can be called as **filtration**.
- **Applications**

Most important techniques used by chemists to purify compounds. HEPA filters in air conditioning to remove particles from air. In the laboratory, a Buchner funnel is often used, with a filter paper serving as the porous barrier.

# Centrifugation

- Centrifugation is one of the techniques that scientists can use to separate mixtures.
- In its simplest terms, a centrifuge is a mechanical device designed to separate particles from a solution by way of centrifugation. Centrifugation involves subjecting a sample to Relative Centrifugal Force (RCF). The success of the separation will depend on the size, shape, density, viscosity of the medium and the rotor speed.
- If a solution has some particles that are a higher density than others, then the lighter particles will separate from the heavier ones. The user will see the heavier substances forced to the bottom of the container (tube, bottle, bag etc.) and the lighter ones will remain suspended at the top.
- If there is no difference in the density of the particles (known as isopycnic conditions), then the sample will not separate.

# Applications

- Micro centrifuges are used to process small volumes of biological molecules, cells, or nuclei.
- Used in diagnostic laboratories for blood and urine tests.
- Aids in separation of proteins using purification techniques such as salting out.
- Differential Centrifugation used to separate organelles and membranes found in cells.

# References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- https://www.slideshare.net/SivaYarasi/separation-techniques-introduction-162887276
- https://henderson-biomedical.co.uk/blog/understanding-centrifugation/
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# Lesson 42

# Separation Techniques

# Separation Techniques

- Separation is an important asset to purify component of interest from a mixtures

# Evaporation

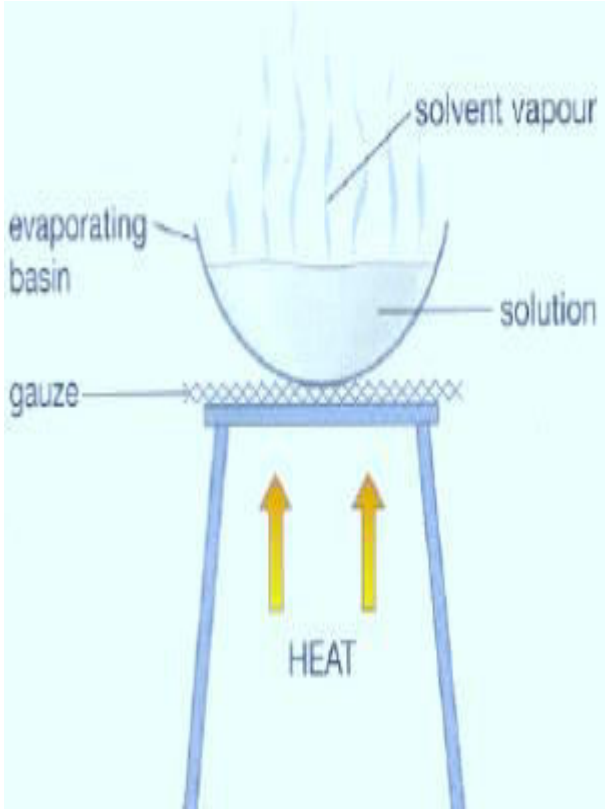
- Evaporation is type of vaporization that occurs on the surface of liquid as it changes into gas phase.
- This separation technique can be used to separate solutes that are dissolved in solvent, by boiling the solution. The solvent gets vaporized leaving back the solute.
- Factors affecting:

1. Concentration of the substance evaporating in the air.
2. Flow rate of air, pressure, temperature.
3. Surface area. Intermolecular forces.

- **Evaporation equipment:**
- Natural circulation evaporators: Evaporating still and pans. Evaporating short tubes. Forced circulation evaporators.
- Film evaporators: Climbing film evaporators. Horizontal film evaporators.

# Applications/examples

- Recovering salts from solution.
- The use of evaporation to dry or concentrate samples is a common preparatory step for many laboratory analyses such as spectroscopy and chromatography.
- Demineralization of water
- In the case which we do not need to collect the solvent. The solvent is boiled off and escape into the air while the solute is left behind in the holding container. Note that this method is not suitable for use on solutes which can decomposed by heating (e.g. Copper II sulfate).



# Distillation

- Distillation is an effective method to separate mixtures comprised of two or more pure liquids (called “components”). The separation of a mixture of liquids based on the physical property of boiling point. Distillation is a purification process where the components of a liquid mixture are vaporized and then condensed and isolated.
- The mixture is heated until one of the components boils (turns to a vapor). The vapor is then fed into a condenser, which cools the vapor and changes it back into a liquid that is called distillate. What remains in the original container is called the "residue".

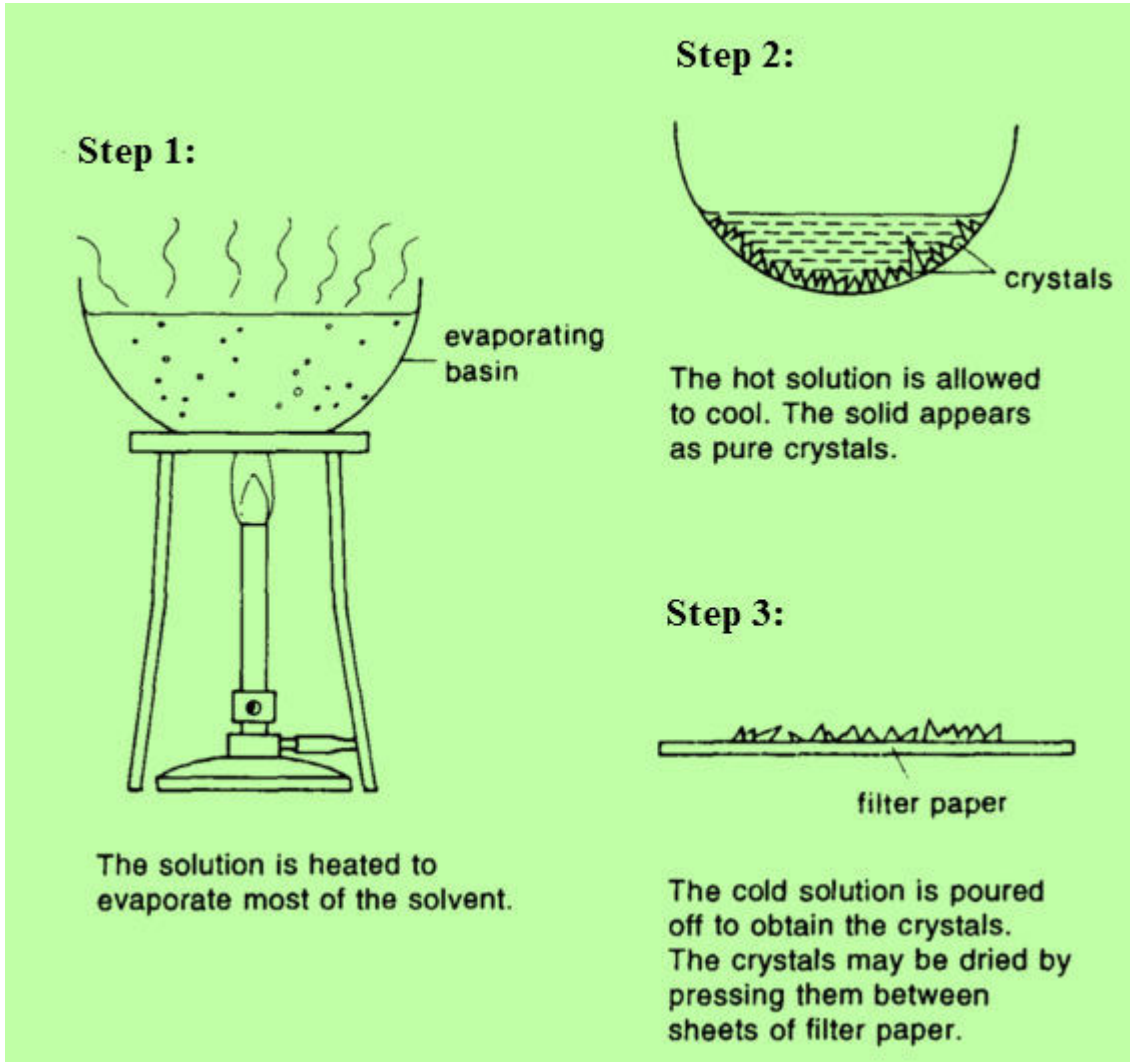
# Applications

- Separation of different fractions from petroleum products.
- Separation of a mixture of methanol and ethanol.
- Separation of volatile oils.
- Separation of drugs obtained from plant or animal sources – vitamin A from fish liver oil.
- Purification of organic solvents.
- Manufacture of official preparations – spirit of nitrous ether, spirit of ammonia, distilled water, and water for injection.
- Refining petroleum products.

# Crystallization

- Used to separate a dissolved heat-liable (will decompose upon heating and hence can sublime) solid (solute) from a solution.
- You will need a saturated solution to being with.
- A **saturated solution** is a solution that contains the maximum amount of solute dissolved in a given volume of solvent at a particular temperature.
- Do not mix this up with a **concentrated solution**, which is a solution that contains lots of solute dissolved in it.
- The amount of solute in a concentrated solution may/may not be the maximum amount which can be dissolved in the solution.

- First, you will need to heat to evaporate off most of the solvent from a solution to make a hot and nearly saturated solution.
- Else, if you already have a saturated solution, heat it up slightly such that the solution becomes hot.
- After which, allow the hot solution to cool naturally. The solubility of the solute decreases as the solution is cooled, and the excess solute which can no longer be dissolved in the saturated solution crystallizes out of the solution.
- The crystals which are formed can be separated from the remaining solution by filtration.

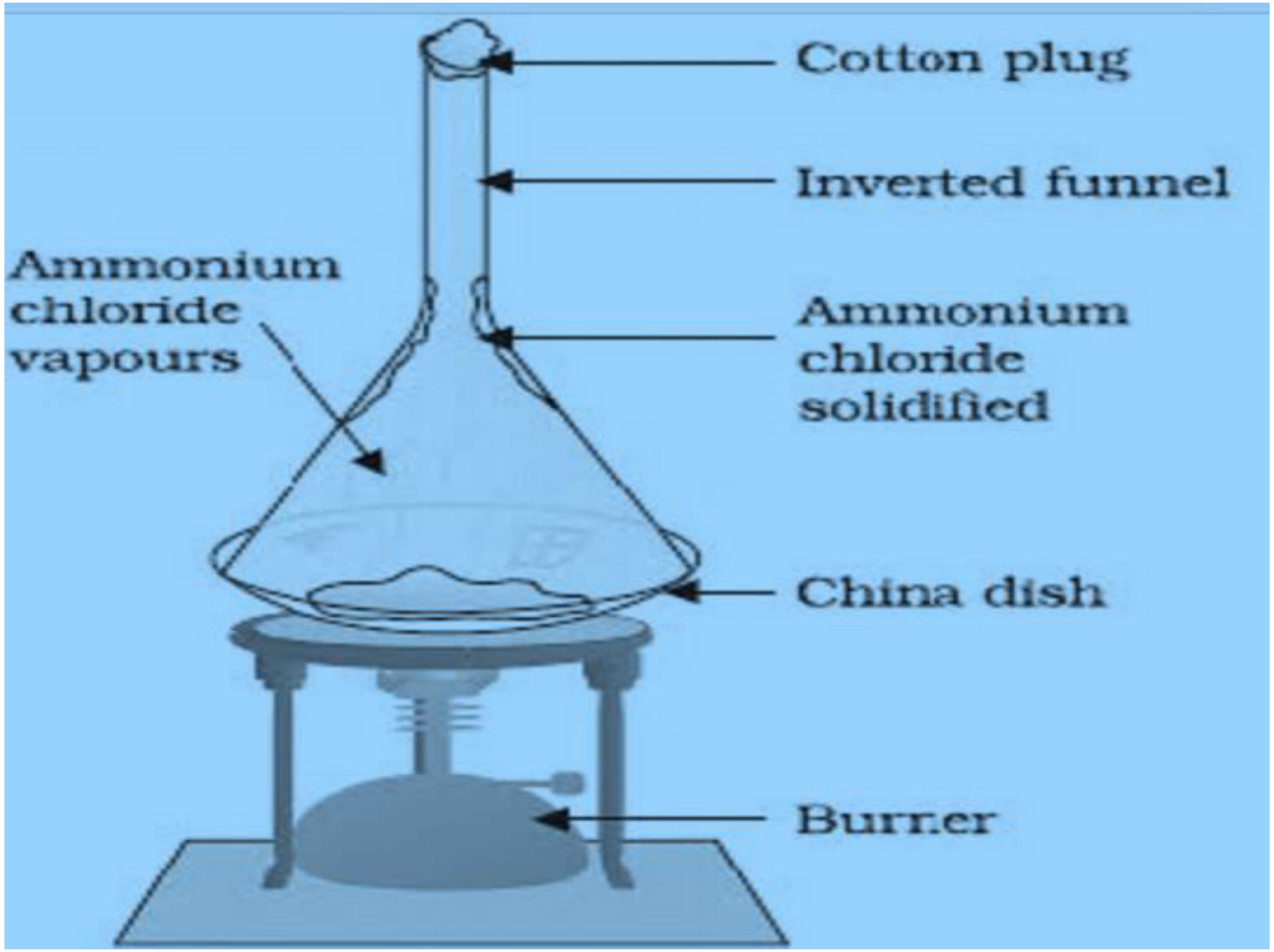


# Sublimation Separation

- Sublimation is the transition of a substance directly from the solid to the gas phase, without passing through the intermediate liquid phase.
- This technique takes the advantage of substance’s sublimely property.
- Separate a mixture of solids containing one which sublimes and one (or more than one) which does not, by heating the mixture.

# Experiment showing the sublimation process

This experiment shows the separation of ammonium chloride from ammonium chloride salt.



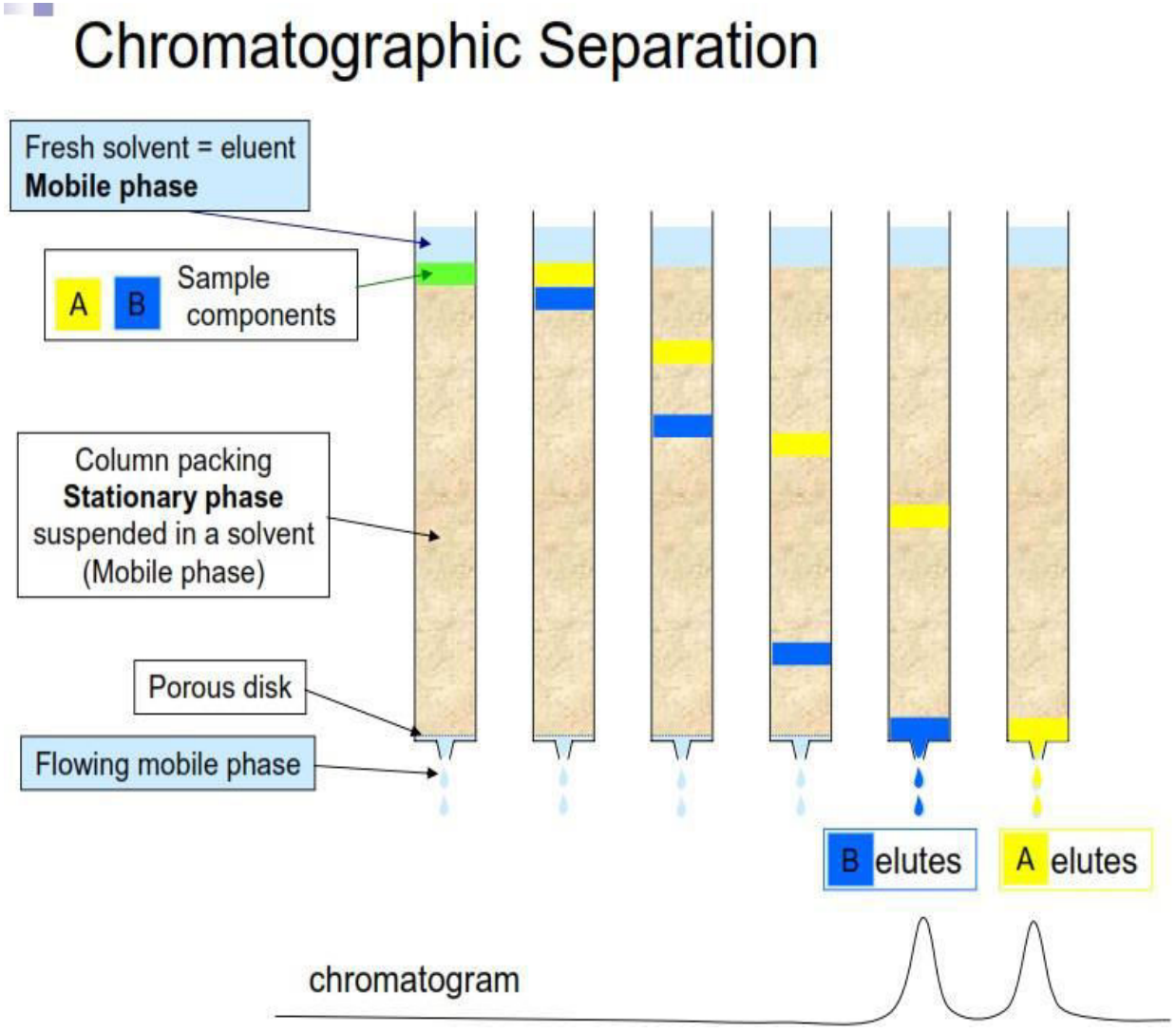
# Applications

- Separate iodine from sand.
- Dye-sublimation printers help in rendering digital pictures in a detailed and realistic fashion which helps in the analysis of substances.
- Sublimation finds practical application in forensic sciences.
- Chemists usually prefer sublimation as a purification method to purify volatile compounds.

# Chromatographic separation

- Chromatography is a separation technique used to separate the different components in a liquid mixture.
- The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase.
- Chromatography is vast separation technique which has many methods or has different principles of separation involved.

This picture shows the chromatographic separation of two samples showing yellow and blue color, stationary phase, mobile phase and porous disk allowing the elutes to move down for collection.



# Applications

1. Food industry:

- Spoilage detection
- Additive detection
- Determining nutritional quality

2. Forensic

- Crime scenes testing
- Forensics pathology

3. Molecular biology studies

- Metabolomics and proteomics
- Nucleic acids research

# References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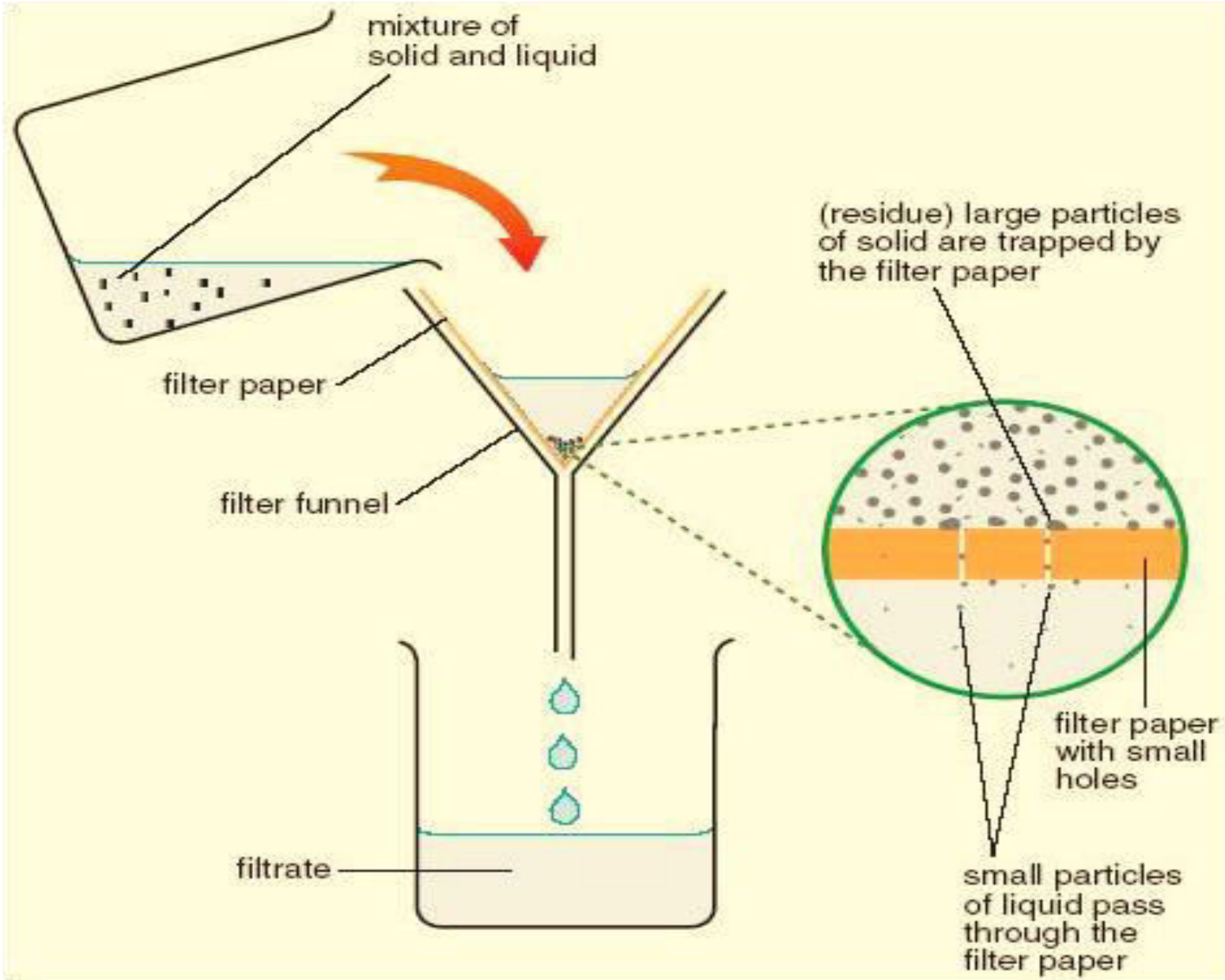
- https://www.slideshare.net/SivaYarasi/separation-techniques-introduction-162887276
- https://henderson-biomedical.co.uk/blog/understanding-centrifugation/
- https://www.azolifesciences.com/article/Life-Science-Applications-of-Chromatography.aspx

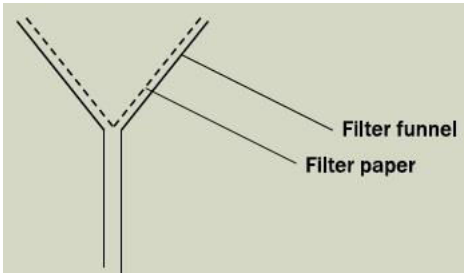
# Lesson 43
# FILTRATION

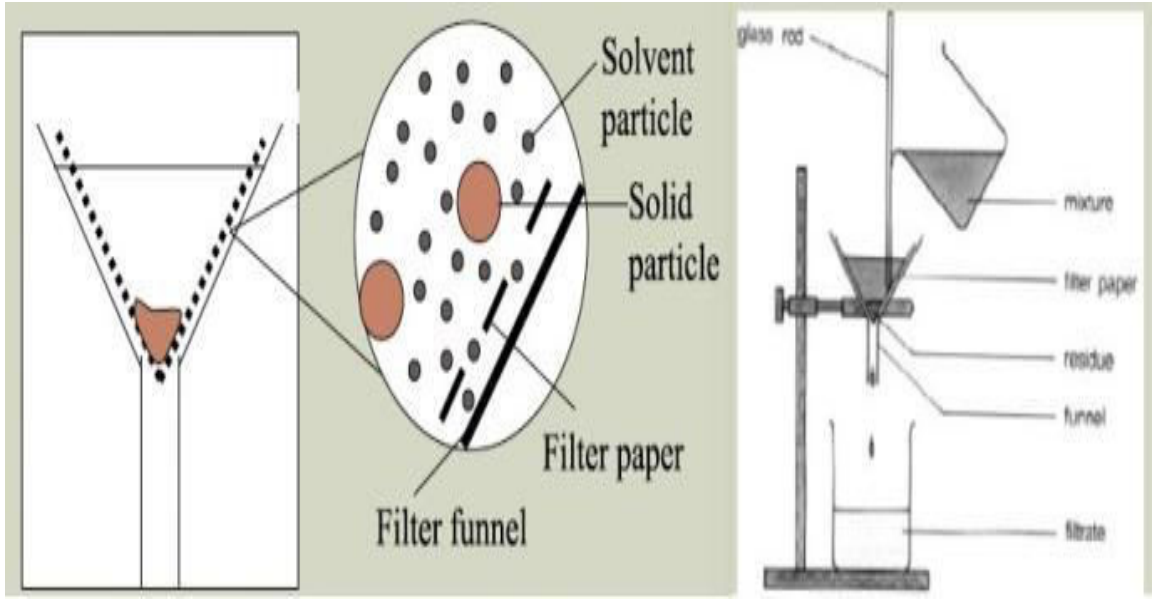
# Filtration

- Filtration is the process of separating substances based on their different physical and chemical qualities. In this process, we refer to the collected solid material as the residue and the fluid material as the filtrate. Usually, a tool that contains some form of pores is used, which allows the fluid portion, but not the solid portion, to pass through. Different materials are used for the purpose of filtration, including paper, sand, and cloth. Filtration also occurs naturally in our bodies, for example in the kidneys, where the blood is filtered in a process called glomerular filtration.


mixture of
solid and liquid
filter paper
filter funnel
filtrate
(residue) large particles
of solid are trapped by
the filter paper
filter paper
with small
holes
small particles
of liquid pass
through the
filter paper

- The medium which we are using over here is the filter paper. The filter paper is folded and placed onto the filter funnel.
- The liquid-solid mixture is poured onto the filter paper. Using a filter paper with pores of a smaller size than the solid particles (and is larger than the size of the liquid molecules), the liquid (or solvent) should pass through the filter paper, and is collected by a collection container placed at the bottom of the filter funnel.
- The liquid that passes through the filter paper is called the filtrate while the solid left on the filter paper is called the residue

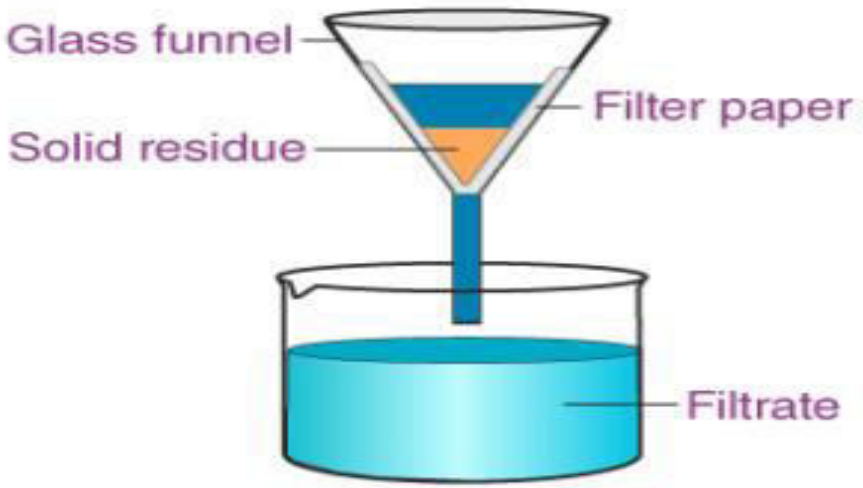


# Filtration Examples

- The most common example is making tea. While preparing tea, a filter or a sieve is used to separate tea leaves from the water. Through the sieve pores, only water will pass. The liquid which has obtained after filtration is called the filtrate; in this case, water is the filtrate. The filter can be a paper, cloth, cotton-wool, asbestos, slag- or glass-wool, unglazed earthenware, sand, or any other porous material. Filtration is used in water treatment and sewage treatment.

# Filtration Process




Filtration process

# Filtration Process

- The mixtures are of two main types: homogeneous mixtures and heterogeneous mixtures. A homogeneous mixture is a mixture that is uniform throughout. A heterogeneous mixture is a mixture that is not uniform throughout, i.e., ingredients of the mixture are distributed unequally. Air is a homogeneous mixture of different gases, including oxygen, nitrogen, carbon dioxide, and water vapour.

# Continue

- Homogeneous mixtures are sometimes also called solutions; especially when it is a mixture of a solid dissolved in a liquid. An example of a heterogeneous mixture is the mixture of sand in water. On shaking, sand will stay undissolved and are distributed unevenly. The sand particles floating around which will eventually settle to the bottom of the bottle makes it a heterogeneous mixture. Different types of filters are used to purify and for separation of mixtures from the contaminants. Based on the type of contaminant-large or small, filters of different pore sizes can be used, even at home.

# REFERENCES


- https://www.thoughtco.com/filtration-definition-4144961

LESSON 44

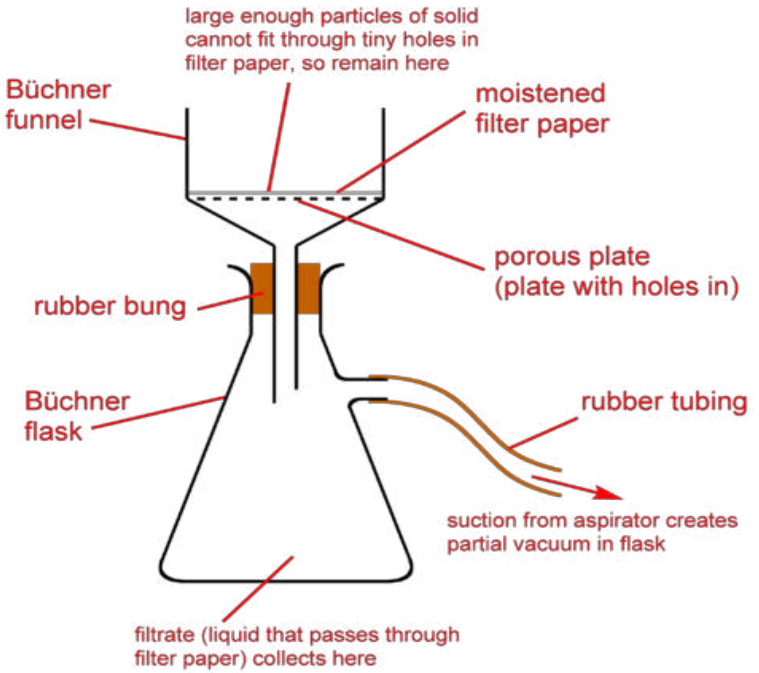
# TYPES OF FILTERATION

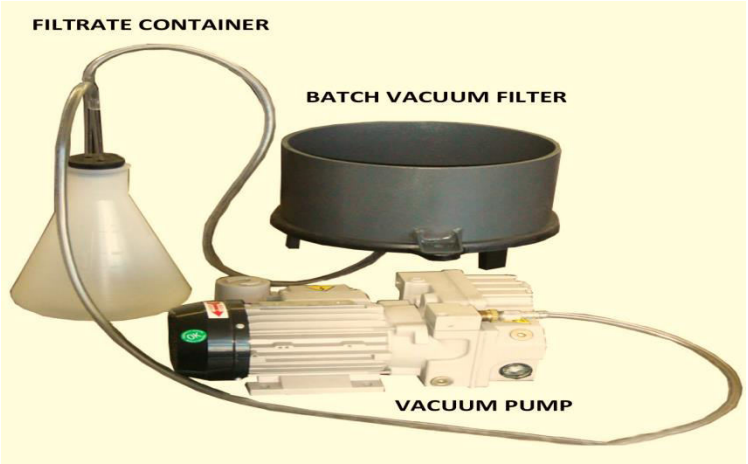
# Types of filtration

- Vacuum Filtration
- Centrifugal filtration
- Gravity filtration
- Cold filtration
- Hot filtration
- Multilayer filtration

# Vacuum Filtration

- In vacuum filtration, a vacuum pump is used to rapidly draw the fluid through a filter.
- Hirsch funnels and Buchner funnels, which are the same kind of funnel in two different sizes, are used along with filter paper.
- The funnels have a plate with holes in it, as we can see below, and they are usually used when the substance to be filtered is small in volume.



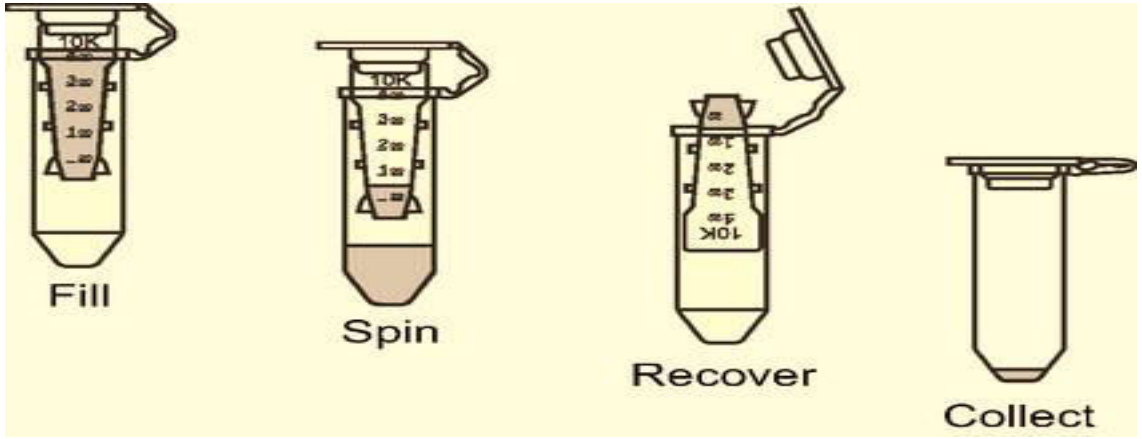



# Multilayer filtration

- This can refer to multiple layers of different material, including sand, gravel, or charcoal, where the different layers contain different particle sizes of that material. In this type of filtration, a mixture of liquid and insoluble solid particles is poured over the layers, and the solid particles are caught throughout, resulting in a filtered liquid.

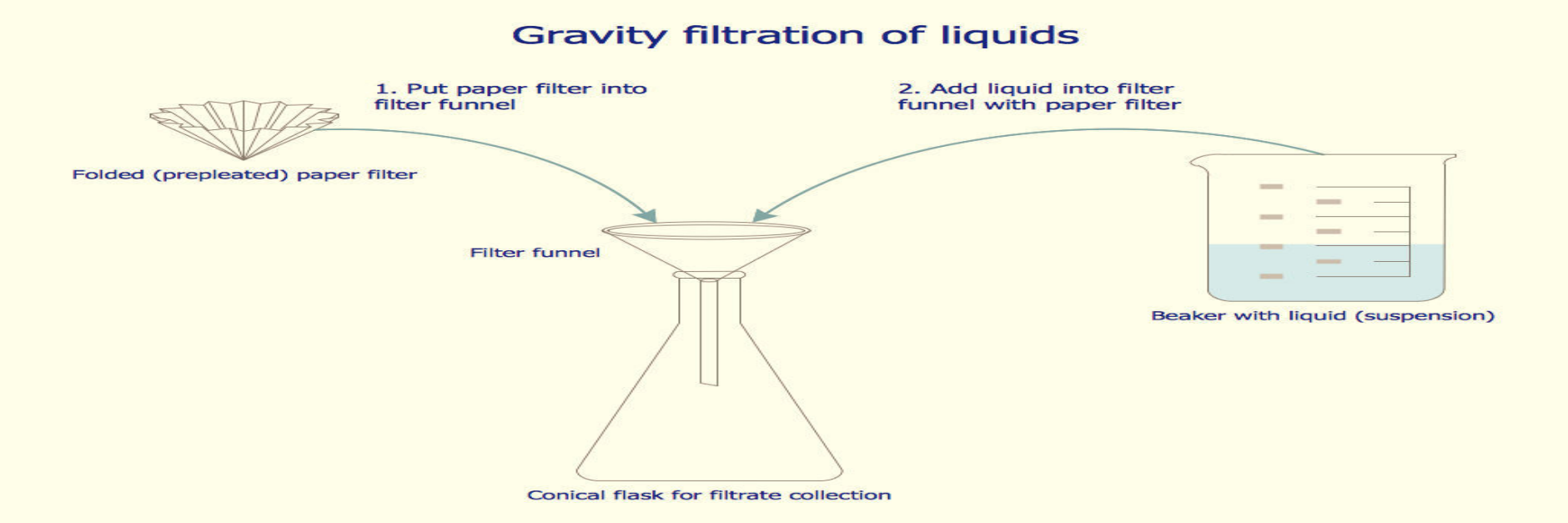
# Centrifugal Filtration

- This kind of filtration is done by rotating the substance to be filtered at very high speed.
- Due to the horizontal rotation, the more dense matter is separated from the less dense matter.



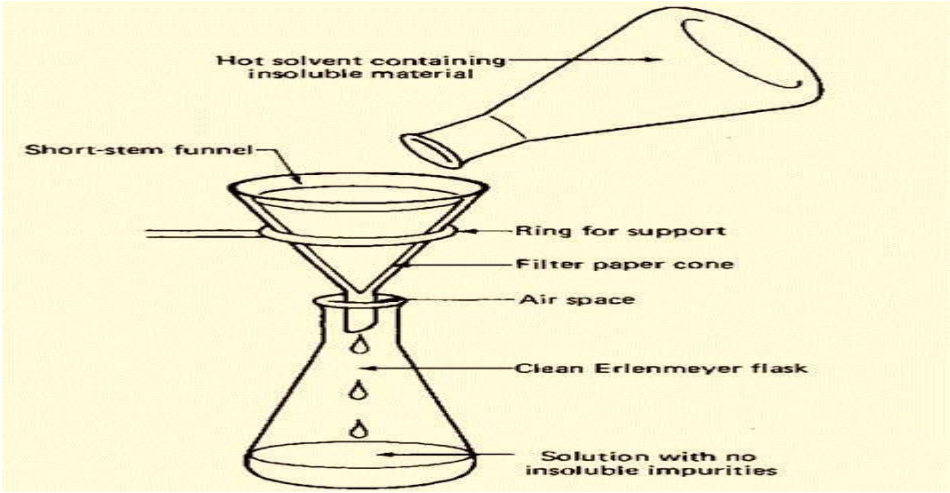

# Gravity filtration

- This is where the mixture is poured from a higher point to a lower one. It is commonly done through simple filtration, using filter paper in a glass funnel, where the insoluble solid particles are captured by the filter paper and the liquid goes right through by gravity's pull. Depending on the volume of the substance at hand, filter cones, fluted filters, or filtering *pipets* can be used.



# Cold filtration

- Cold filtration makes use of very low temperatures, often by using an ice bath. Some substances, such as fatty acid particles, become suspended in the mixture as they cool down, which then allows us to filter them out more easily.



# Hot filtration

- This is often used for crystalline compounds that contain impurities. The way this filtration is done is by melting down the crystalline compound, removing the impurities as the substance is still in liquid form, and finally recrystallizing the now pure substance. Often, it is recommended that the apparatus used in this filtration be heated up so that the filtered substance doesn't crystallize in the funnel and block the flow.

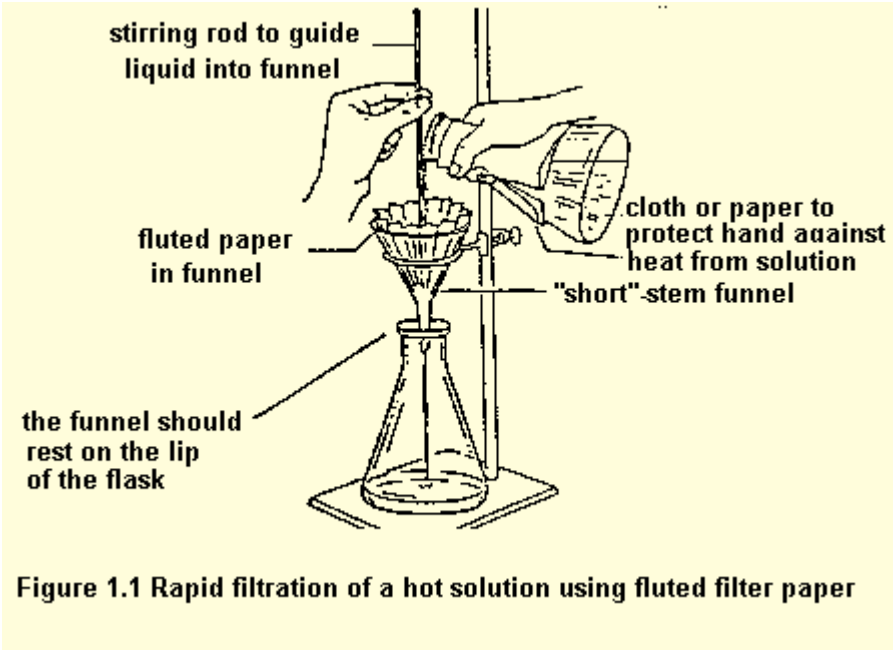



Figure 1.1 Rapid filtration of a hot solution using fluted filter paper

# References


- https://biologydictionary.net/filtration/

# LESSON# 45
# Distillation

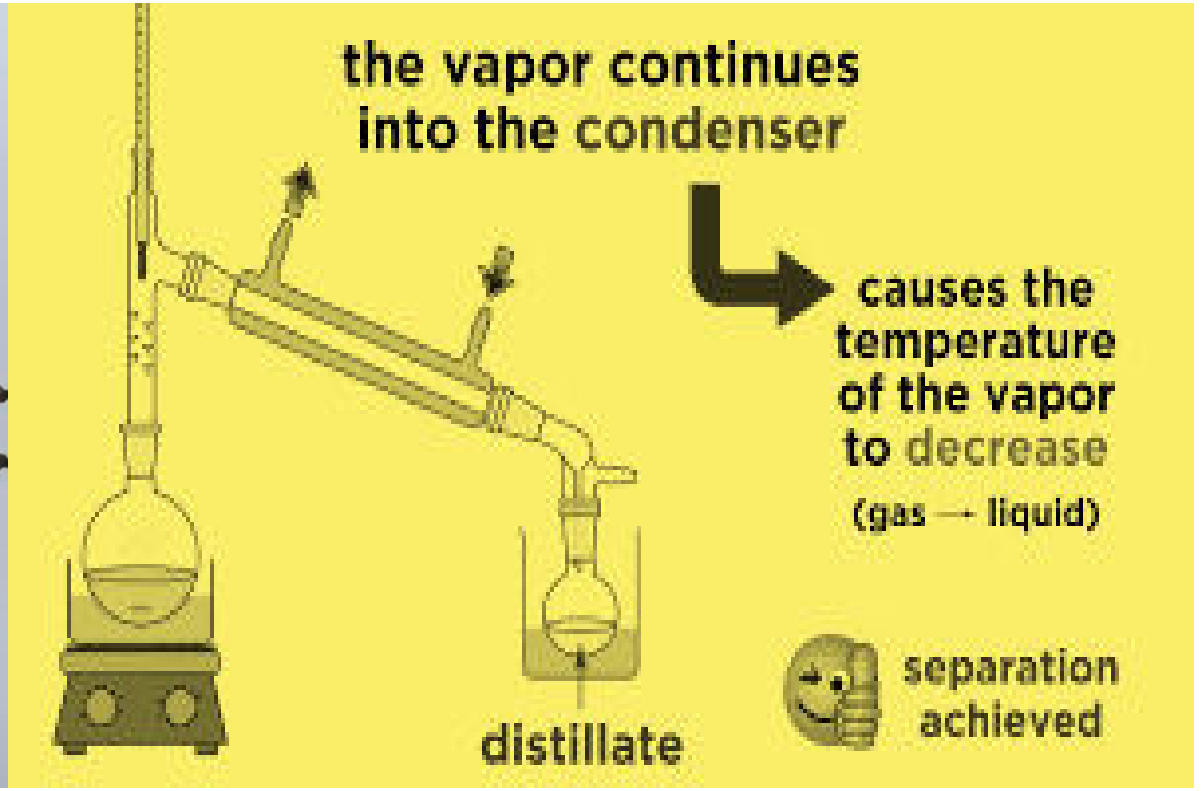
# What is distillation?

- Distillation is a widely used method for separating mixtures based on differences in the conditions required to change the phase of components of the mixture.
- To separate a mixture of liquids, the liquid can be heated to force components, which have different boiling points, into the gas phase. The gas is then condensed back into liquid form and collected. Repeating the process on the collected liquid to improve the purity of the product is called **double distillation**.

Although the term is most commonly applied to liquids, the reverse process can be used to separate gases by liquefying components using changes in temperature and/or pressure.

A plant that performs distillation is called a distillery. The apparatus used to perform distillation is called a still.

# Plant for distillation



# Applications of Distillation

Some important applications of distillation are listed below.

- Distillation plays an important role in many water purification techniques. Many desalination plants incorporate this method in order to obtain drinking water from seawater.
- Distilled water has numerous applications, such as in lead-acid batteries and low-volume humidifiers.
- Many fermented products such as alcoholic beverages are purified with the help of this method.
- Many perfumes and food flavorings are obtained from herbs and plants via distillation.

- Oil stabilization is an important type of distillation that reduces the vapor pressure of the crude oil, enabling safe storage and transportation.
- Air can be separated into nitrogen, oxygen, and argon by employing the process of cryogenic distillation.
- Distillation is also employed on an industrial scale to purify the liquid products obtained from chemical synthesis.

# Processes involved in distillation

Distillation refining consists of two processes of distilling and condensation reflux. Distillation is usually carried out in distillation column, the gas-liquid two-phase flow through the countercurrent contact, the phase heat and mass transfer.

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LESSON 46

# Types of Distillation

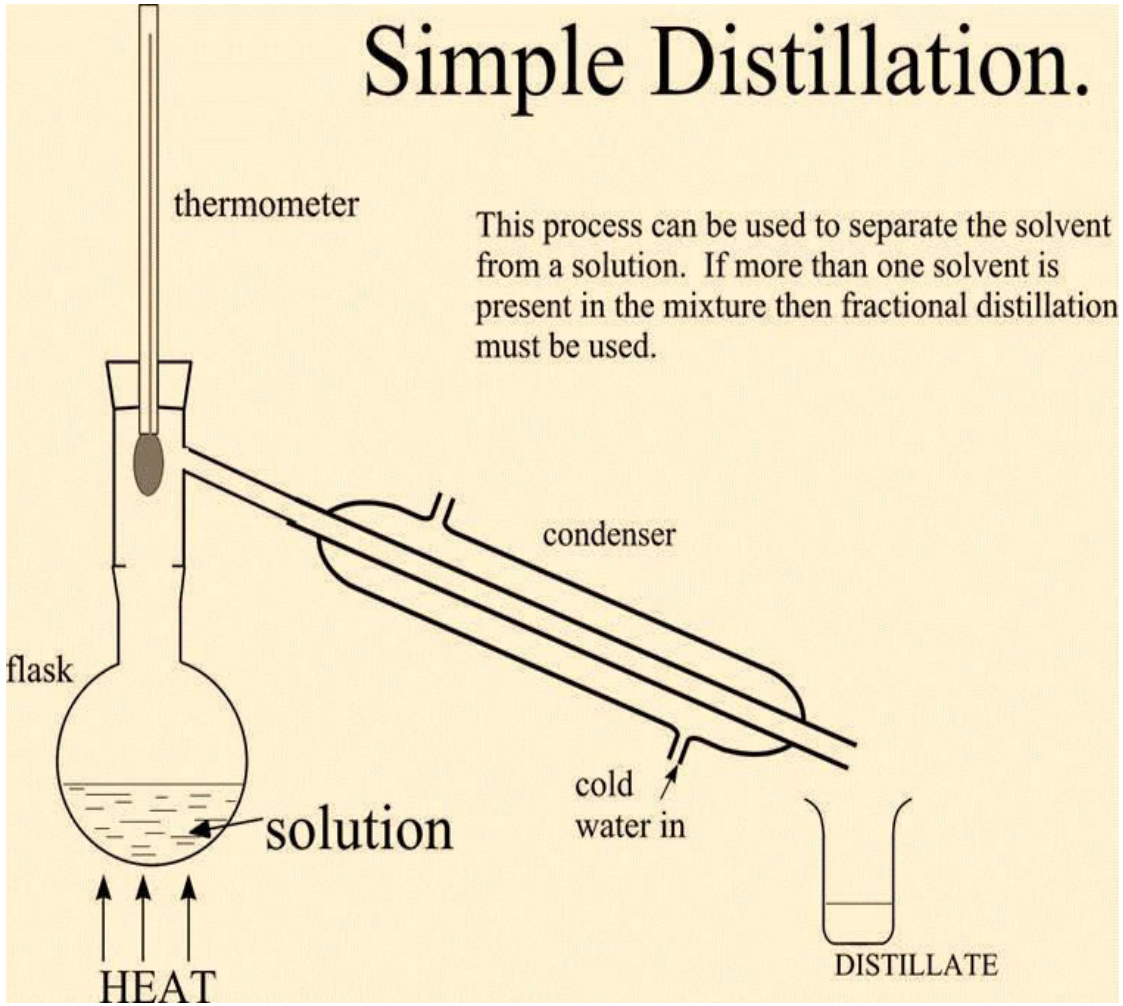
# Types of Distillation

Types of distillation include simple distillation,

- Fractional distillation (different volatile 'fractions' are collected as they are produced), and
- Destructive distillation (usually, a material is heated so that it decomposes into compounds for collection).

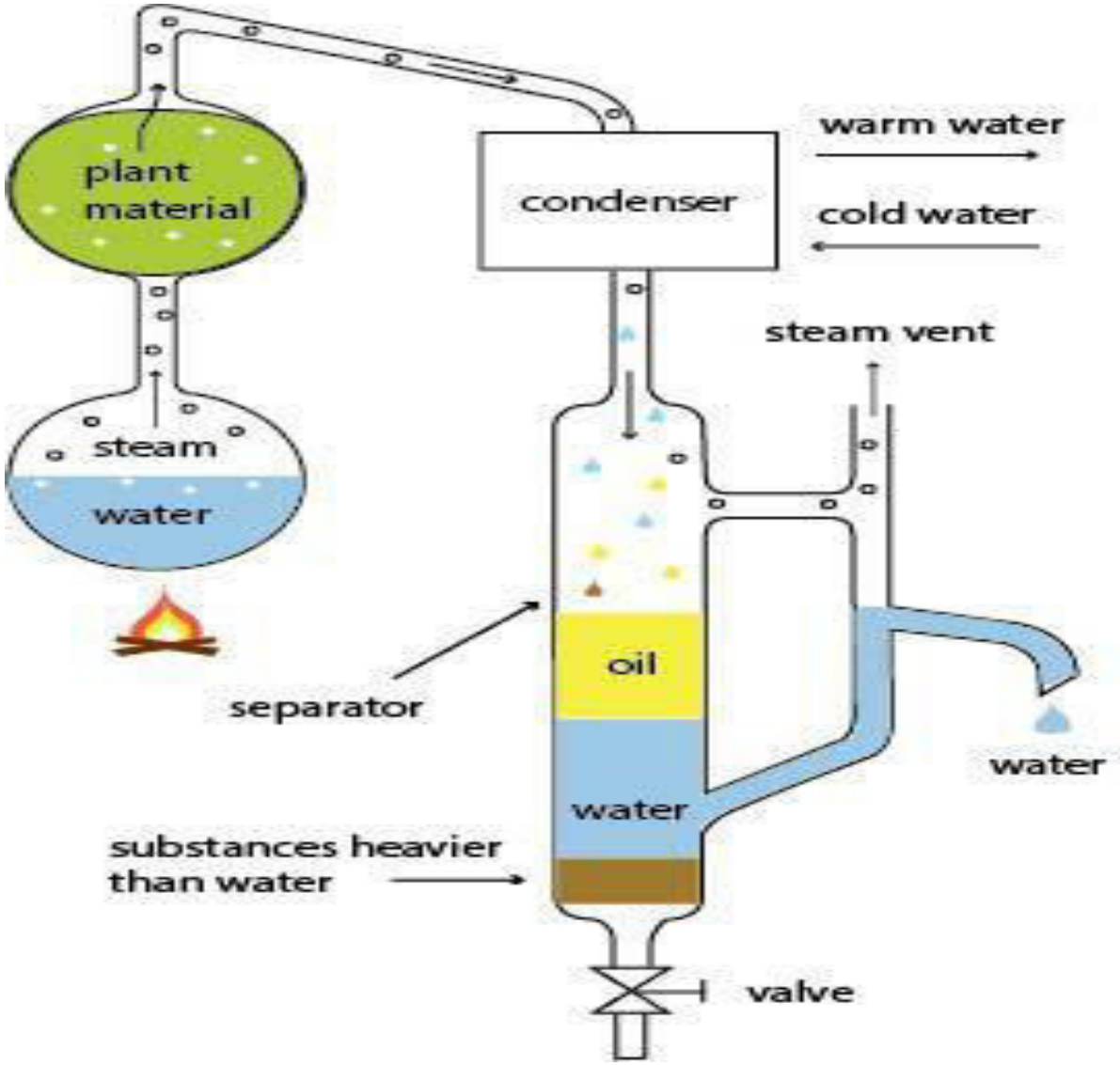
# Simple Distillation

- Simple distillation may be used when the boiling points of two liquids are significantly different from each other or to separate liquids from solids or nonvolatile components.
- In simple distillation, a mixture is heated to change the most volatile component from a liquid into vapor. The vapor rises and passes into a condenser.
- Usually, the condenser is cooled (e.g., by running cold water around it) to promote condensation of the vapor, which is collected.



# Steam Distillation

- Steam distillation is used to separate heat-sensitive components. Steam is added to the mixture, causing some of it to vaporize.
- This vapor is cooled and condensed into two liquid fractions.
- Sometimes the fractions are collected separately, or they may have different density values , so they separate on their own.
- An example is steam distillation of flowers to yield essential oil and a water-based distillate as shown here.



# Fractional Distillation

Fractional distillation is often used to separate mixtures of liquids that have similar boiling points. It involves several vaporization-condensation steps (which takes place in a fractioning column). This process is also known as rectification. The apparatus required to perform a fractional distillation on a mixture is listed below.

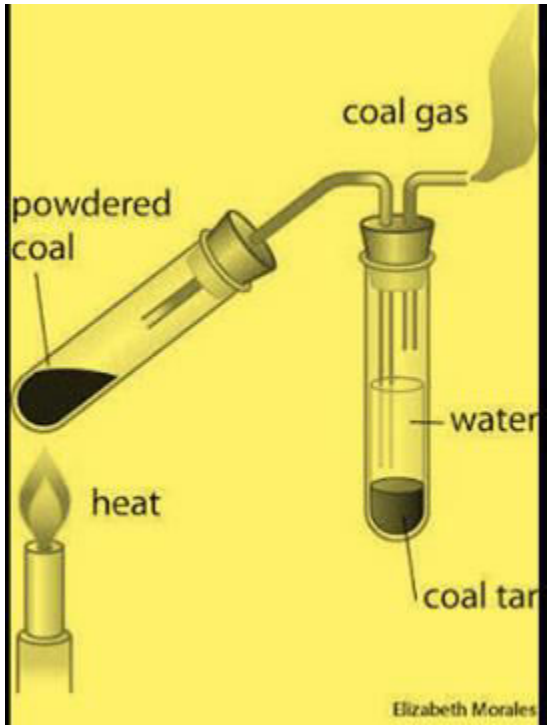
- Round-bottom flask or distilling flask
- A source of heat, which can be a fire or a hot bath.
- Receiving flask to collect the condensed vapors
- Fractioning column
- Thermometer to measure the temperature in the distilling flask
- Condenser

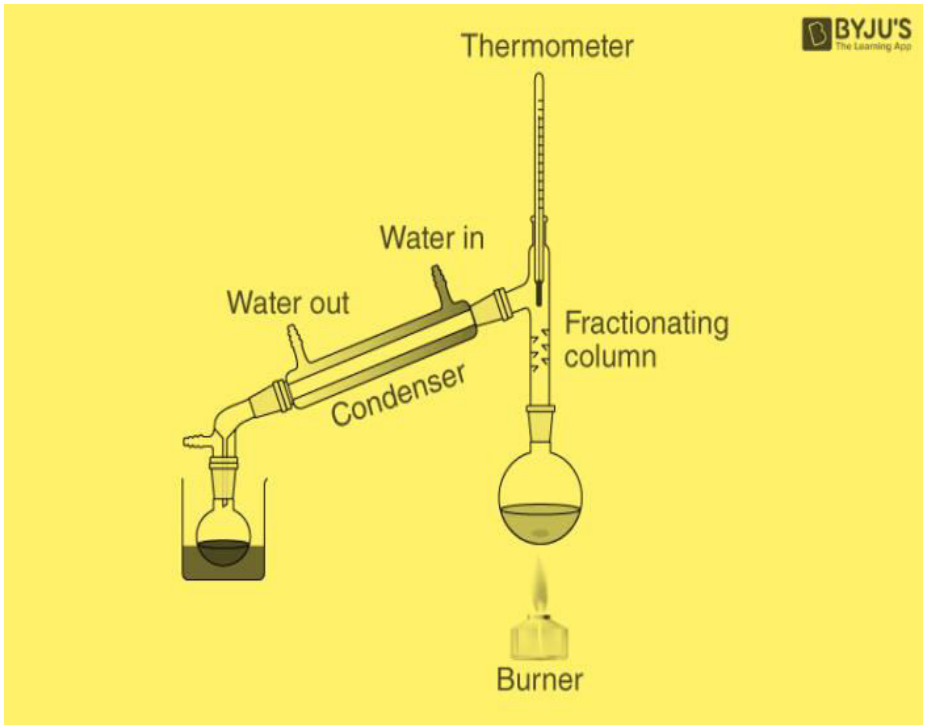
- Standard Glassware.

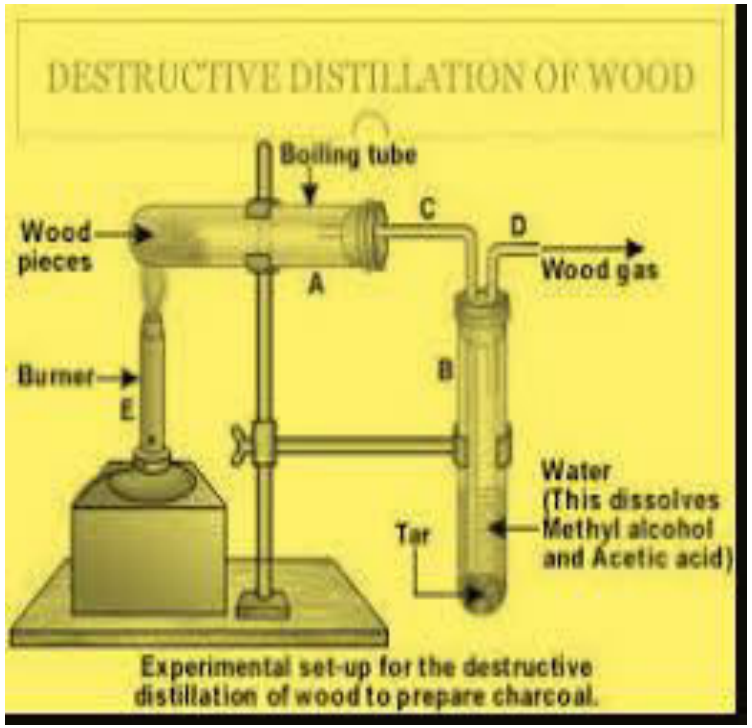
- When heated, the liquid mixture is converted into vapors that rise into the fractioning column. The vapors now cool and condense on the walls of the condenser. The hot vapors emanating from the distilling flask now heat the condensed vapor, creating new vapors.

- Many such vaporization-condensation cycles take place and the purity of the distillate improves with every cycle. An illustration depicting a fractional distillation setup is provided below.

# Pictorial Presentation of Fractional Distillation

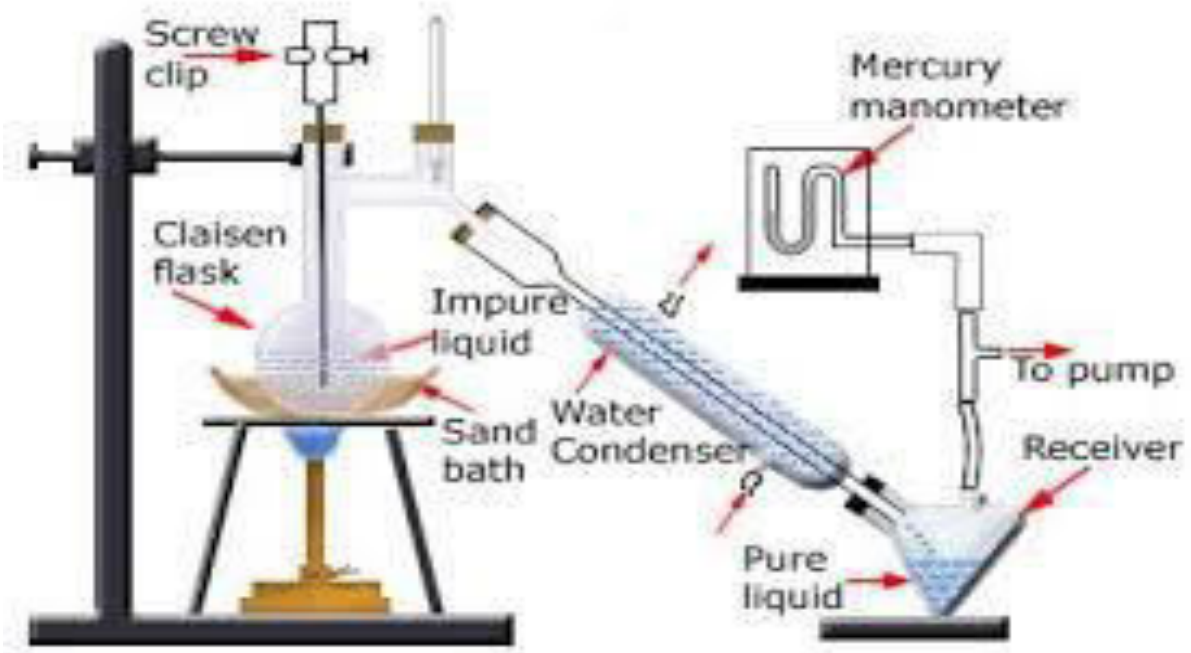


# Vacuum Distillation

- Vacuum distillation is used to separate components that have high boiling points. Lowering the pressure of the apparatus also lowers boiling points.
- Otherwise, the process is similar to other forms of distillation.
- Vacuum distillation is particularly useful when the normal boiling point exceeds the decomposition temperature of a compound.



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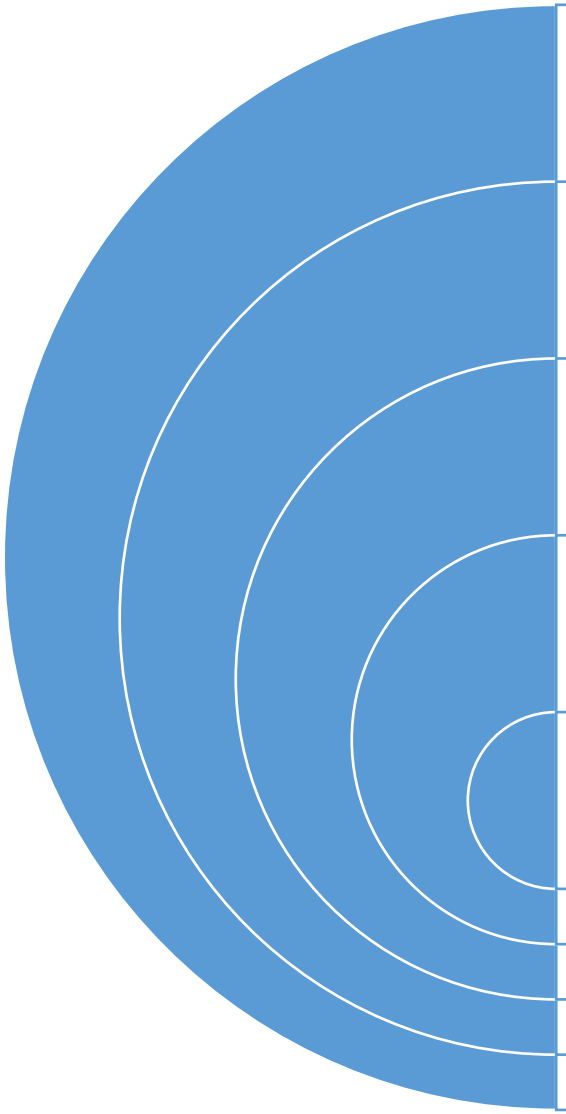
# LESSON 47

**Chromatography, Types and their Principle**

# What is chromatography?



Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.

The Russian botanist Mikhail Tswett coined the term chromatography in 1906.

The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of fatty acid mixtures.

A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties to separate materials.

It is a powerful separation tool that is used in all branches of science and is often the only means of separating components from complex mixtures

# History

Chromatography gets its name from a technique first used in the late 19th century to separate pigments in a complex mixture.

If a sheet of paper or cloth contacts a container filled with water or alcohol in which a complex pigment is dissolved, capillary action will carry the mixture up the paper or cloth, but the components of the pigment will not all travel at the same rate.

The largest molecules of the mixture will travel more slowly while the smallest ones race ahead, causing the stationary phase to develop discrete bands of color corresponding to each component of the mixture. This gives the technique the name "chromatography" or "writing color.

# Principle of Chromatography (how does chromatography work)

•Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase.

•The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights.

•Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into the mobile phase, and leave the system faster.

Three components thus form the basis of the chromatography technique.

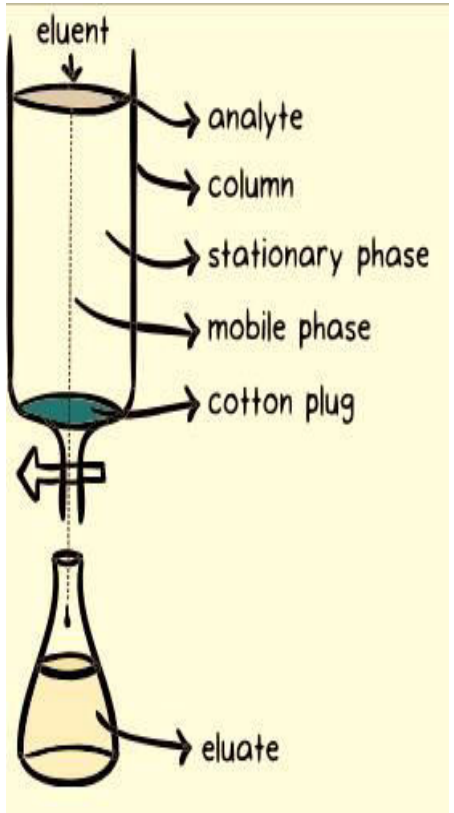
**1.Stationary phase:** This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface solid support".

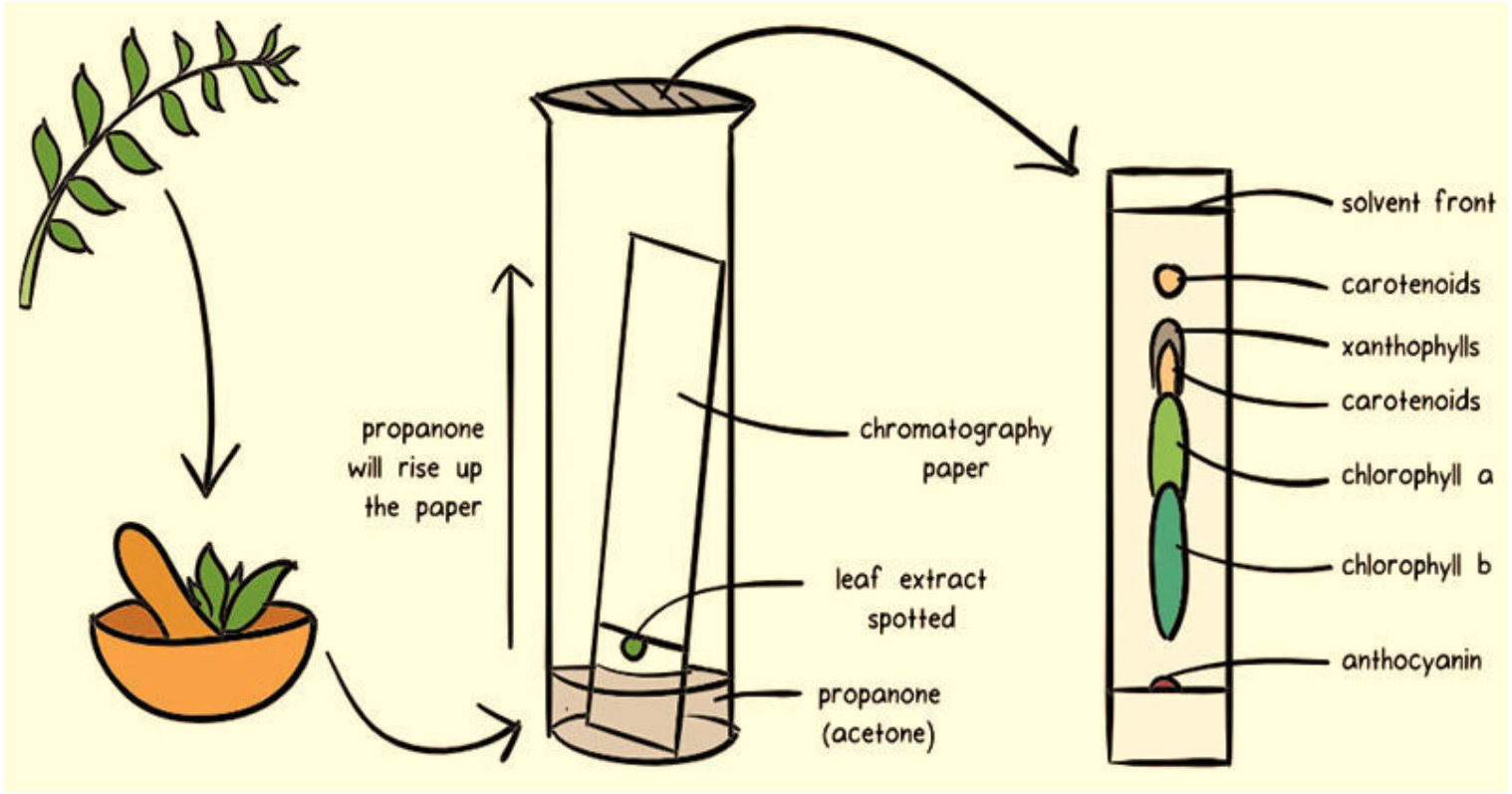
**2.Mobile phase:** This phase is always composed of "liquid" or a "gaseous component."

**3.Separated molecules**

The type of interaction between the stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on the separation of molecules from each other.

# Experimental elaboration of chromatography



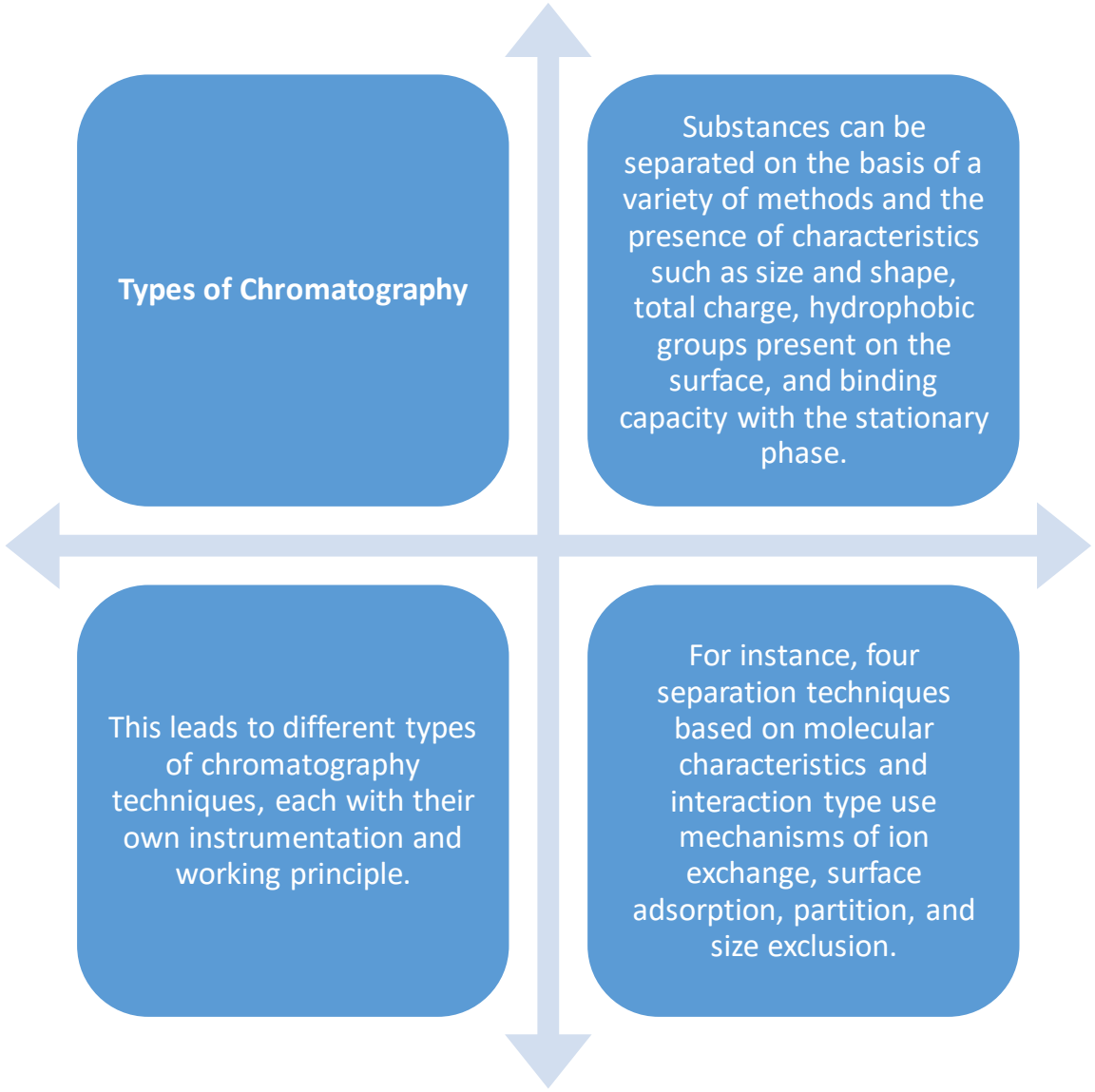



# Components of Chromatography

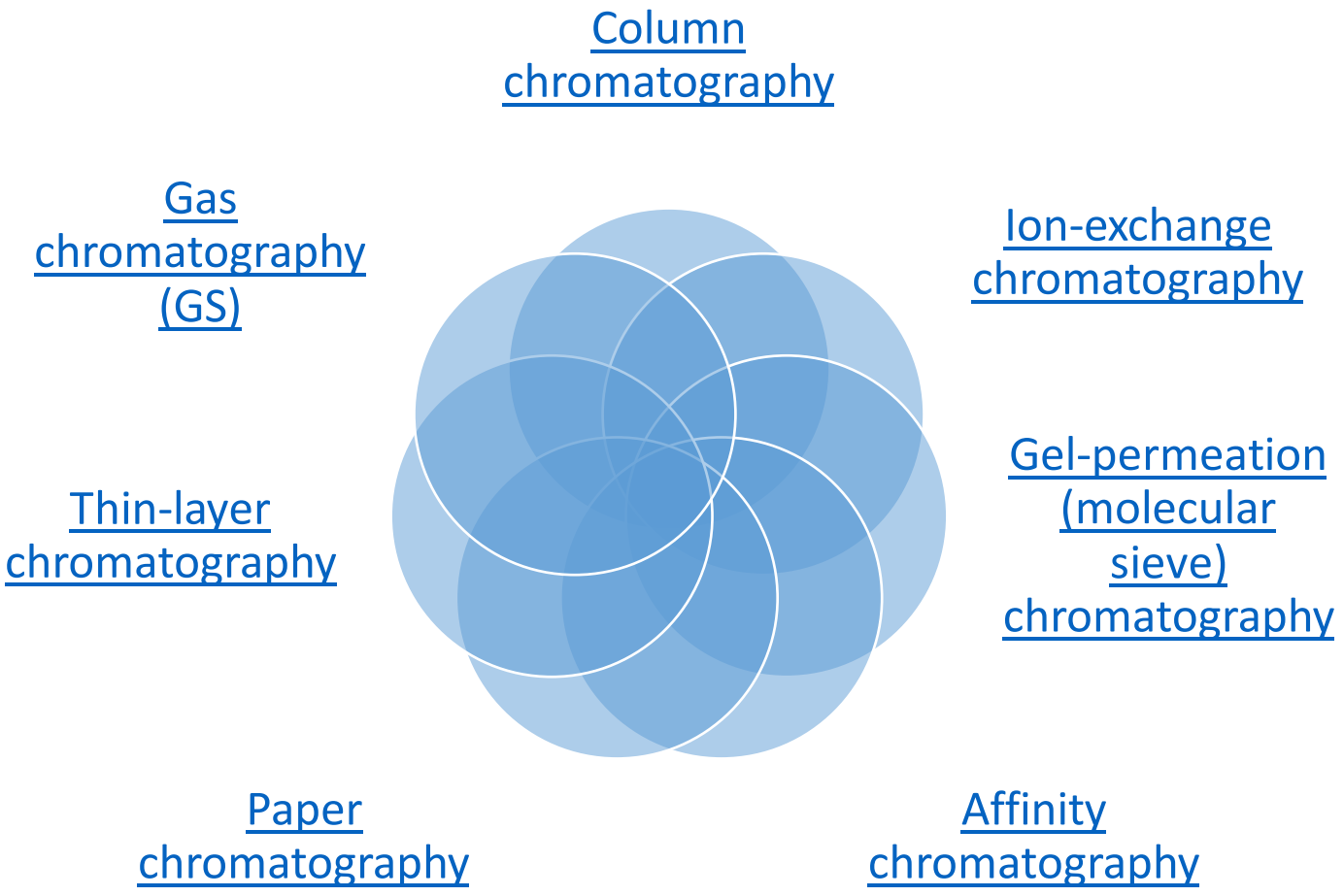
- Three components thus form the basis of the chromatography technique.
- **Stationary phase:** This phase is always composed of a “solid” phase or “a layer of a liquid adsorbed on the surface solid support”.
- **Mobile phase:** This phase is always composed of “liquid” or a “gaseous component.”
- **Separated molecules**
- The type of interaction between the stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on the separation of molecules from each other

# Types of chromatography

- Gas chromatography
- Liquid chromatography
- Thin layer chromatography
- Ion-exchange chromatography


Types of Chromatography
Substances can be separated on the basis of a variety of methods and the presence of characteristics such as size and shape, total charge, hydrophobic groups present on the surface, and binding capacity with the stationary phase.
This leads to different types of chromatography techniques, each with their own instrumentation and working principle.
For instance, four separation techniques based on molecular characteristics and interaction type use mechanisms of ion exchange, surface adsorption, partition, and size exclusion.

# Commonly employed chromatography techniques include:

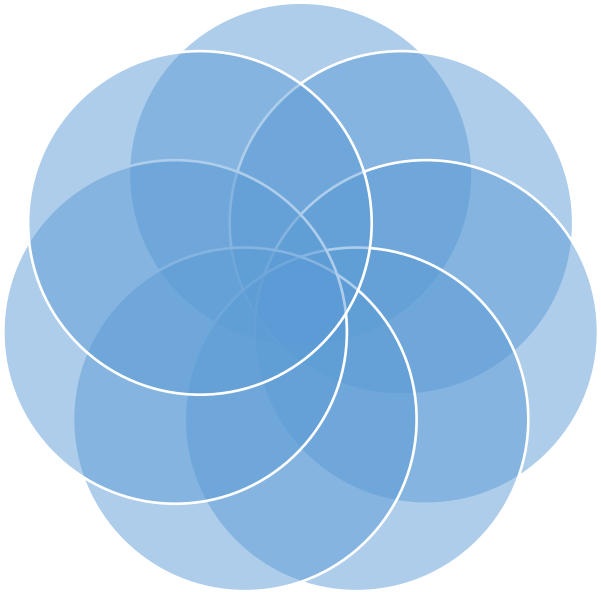


# Applications of Chromatography

**Pharmaceutical sector**

To identify and analyze samples for the presence of trace elements or chemicals.

In testing water samples and also checks air quality.



Separation of compounds based on their molecular weight and element composition.

**Chemical industry**

In drug development.

Detects the unknown compounds and purity of mixture.

**Forensic Science**

•In forensic pathology and crime scene testing like analyzing blood and hair samples of crime place.

**Molecular Biology Studies**

•Various hyphenated techniques in chromatography such as EC-LC-MS are applied in the study of metabolomics and proteomics along with nucleic acid research.

•HPLC is used in Protein Separation like Insulin Purification, Plasma Fractionation, and Enzyme Purification and also in various departments like Fuel Industry, biotechnology, and biochemical processes.

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# LESSON 48
# **PAPER CHROMATOGRAPHY**

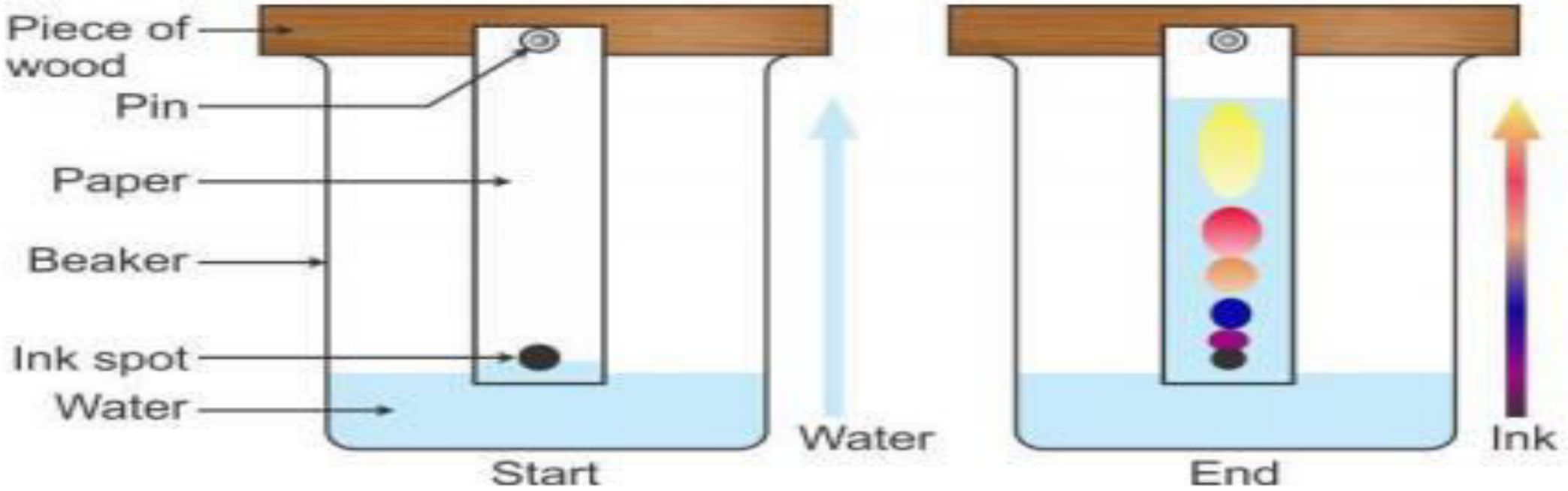
# PAPER CHROMATOGRAPHY

- Chromatography technique that uses paper sheets or strips as the adsorbent being the stationary phase through which a solution is made to pass is called paper chromatography. It is an inexpensive method of separating dissolved chemical substances by their different migration rates across the sheets of paper. It is a powerful analytical tool that uses very small quantities of material. Paper chromatography was discovered by Synge and Martin in the year 1943.

# Paper Chromatography Principle

- The principle involved can be partition chromatography or adsorption chromatography. Partition chromatography because the substances are partitioned or distributed between liquid phases. The two phases are water held in pores of the filter paper and the other phase is a mobile phase which passes through the paper. When the mobile phase moves, the separation of the mixture takes place. The compounds in the mixture separate themselves based on the differences in their affinity towards stationary and mobile phase solvents under the capillary action of pores in the paper. Adsorption chromatography between solid and liquid phases, wherein the solid surface of the paper is the stationary phase and the liquid phase is the mobile phase.

# Paper Chromatography Diagram




Paper Chromatography

# Types of paper chromatography

- Ascending Paper Chromatography – The techniques goes with its name as the solvent moves in an upward direction.
- Descending Paper Chromatography – The movement of the flow of solvent due to gravitational pull and capillary action is downwards hence the name descending paper chromatography.
- Ascending – Descending Paper Chromatography – In this version of paper chromatography movement of solvent occurs in two directions after a particular point. Initially, the solvent travels upwards on the paper which is folded over a rod and after crossing the rod it continues with its travel in the downward direction.

# Types

Radial or Circular Paper Chromatography – The sample is deposited at the centre of the circular filter paper. Once the spot is dried, the filter paper is tied horizontally on a Petri dish which contains the solvent.

Two Dimensional Paper Chromatography – Substances which have the same rf values can be resolved with the help of two-dimensional paper chromatography.

# Applications of paper chromatography

- There are various applications of paper chromatography. Some of the uses of Paper Chromatography in different fields are discussed below:

•To study the process of fermentation and ripening.

•To check the purity of pharmaceuticals.

•To inspect cosmetics.

•To detect the adulterants.

•To detect the contaminants in drinks and foods.

•To examine the reaction mixtures in biochemical laboratories.

•To determine dopes and drugs in humans and animals

# LESSON 49
# **Thin-layer chromatography**

# Thin-layer chromatography

Thin-layer chromatography is a “solid-liquid adsorption” chromatography. In this physical method of separation, the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction. Depending upon the stationary phase and mobile phase chosen, they can be of different types.

Thin Layer Chromatography can be defined as a method of separation or identification of a mixture of components into individual components by using finely divided adsorbent solid / (liquid) spread over a plate and liquid as a mobile phase.

# Principle of Thin Layer Chromatography (TLC)

- Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase.
- After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.
- It is thus based on the principle of adsorption chromatography or partition chromatography or combination of both, depending on adsorbent, its treatment and nature of solvents employed. The components with more affinity towards stationary phase travels slower. Components with less affinity towards stationary phase travels faster.
- Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or character is identified by means of suitable detection techniques.

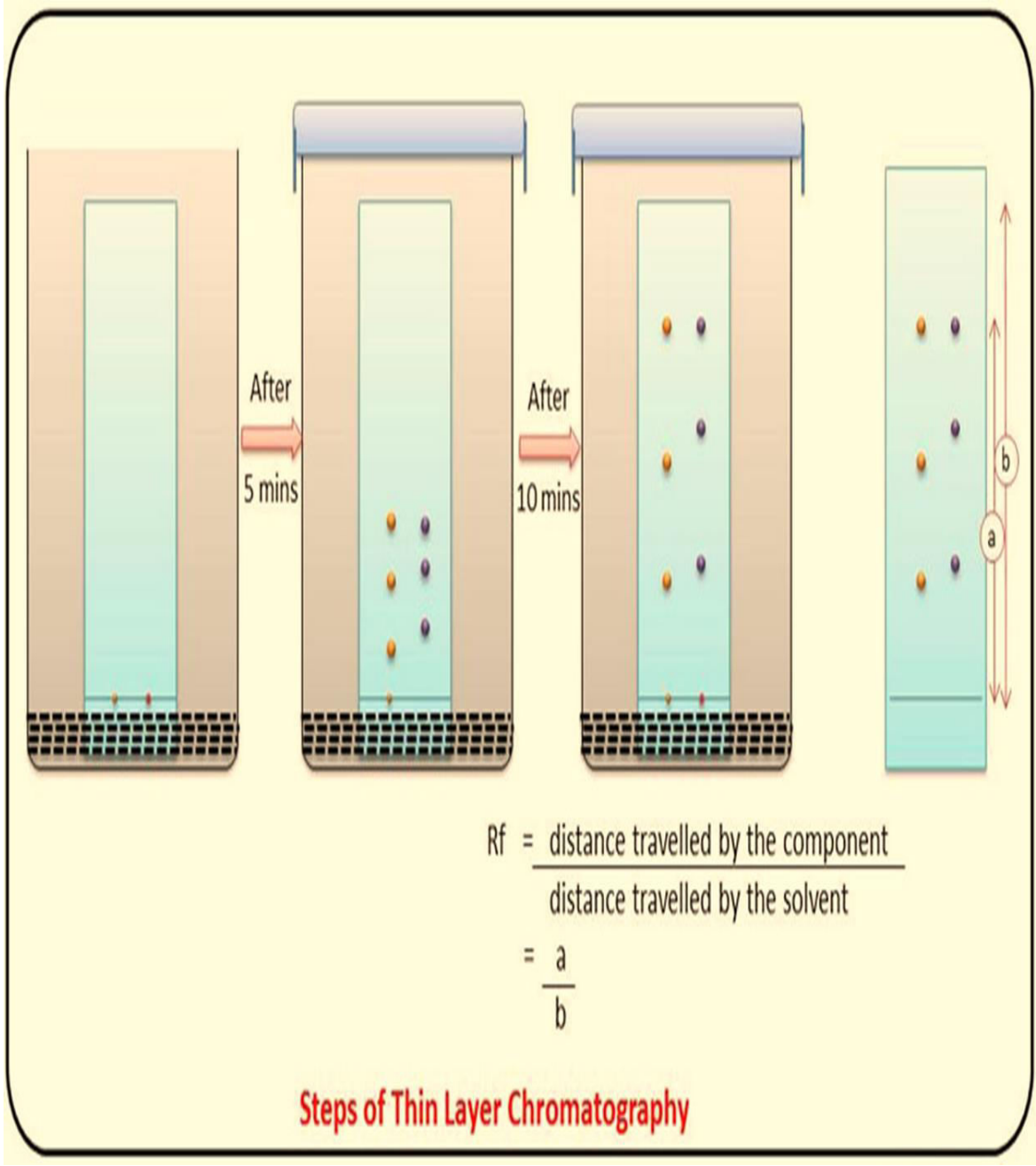
# TLC components

- TLC system components consists of:
- **TLC plates,** preferably ready made with a stationary phase: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is of uniform thickness and is in a fine particle size.
- **TLC chamber-** This is used for the development of TLC plate. The chamber maintains a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.
- **Mobile phase-** This comprises of a solvent or solvent mixture The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.
- **A filter paper-** This is moistened in the mobile phase, to be placed inside the chamber. This helps develop a uniform rise in a mobile phase over the length of the stationary phase.

# Procedure of Thin Layer Chromatography (TLC)

- The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are more commonly used.
- With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
- Then, samples solutions are applied on the spots marked on the line in equal distances.
- The mobile phase is poured into the TLC chamber to a leveled few centimeters above the chamber bottom.
- A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect).
- Now, the plate prepared with sample spotting is placed in TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.

- The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent) for development.
- Sufficient time is given for the development of spots.
- The plates are then removed and allowed to dry.
- The sample spots are then seen in a suitable UV light chamber, or any other methods as recommended for the given sample.



# Some common techniques for visualizing the results of a TLC plate include

| |
|---|
| UV light |
| Iodine Staining: is very useful in detecting carbohydrates since it turns black on contact with Iodine |
| KMnO4 stain (organic molecules) |
| Ninhydrin Reagent: often used to detect amino acids and proteins |

# Retention Factor ($R_f$) Value

| |
|---|
| The behaviour of a compound on a TLC is usually described in terms of its relative mobility or Rf value. |
| Rf or Retention factor is a unique value for each compound under the same conditions. |
| The Rf for a compound is a constant from one experiment to the next only if the chromatography conditions below are also constant: |
| solvent system |
| adsorbent |
| thickness of the adsorbent |
| amount of material spotted |
| temperature |
| Since these factors are difficult to keep constant from experiment to experiment, relative Rf values are generally considered. |
| Relative Rf" means that the values are reported relative to a standard. |

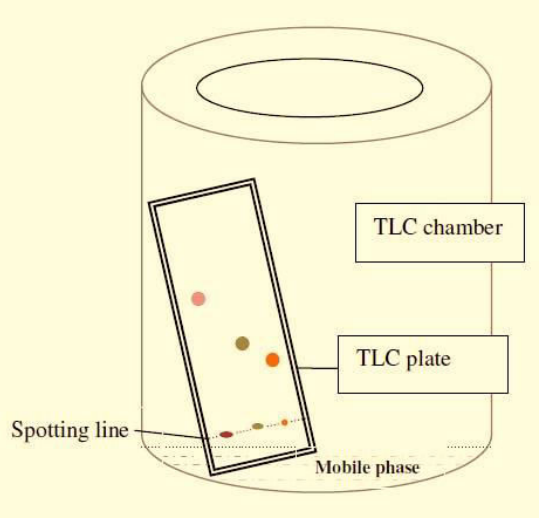
# Applications of Thin Layer Chromatography (TLC)

| |
|---|
| In monitoring the progress of reactions |
| Identify compounds present in a given mixture |
| Determine the purity of a substance. |
| Analyzing ceramides and fatty acids |
| Detection of pesticides or insecticides in food and water |
| Analyzing the dye composition of fibers in forensics |
| Assaying the radiochemical purity of radiopharmaceuticals |
| Identification of medicinal plants and their constituents |

In cases where molecules of the sample are colorless, florescence, radioactivity or a specific chemical substance can be used to produce a visible colored reactive product so as to identify their positions on the chromatogram.

Formation of a visible color can be observed under room light or UV light. The position of each molecule in the mixture can be measured by calculating the ratio between the distances travelled by the molecule and the solvent.

This measurement value is called relative mobility, and expressed with a symbol $R_f$. $R_f$. value is used for qualitative description of the molecules

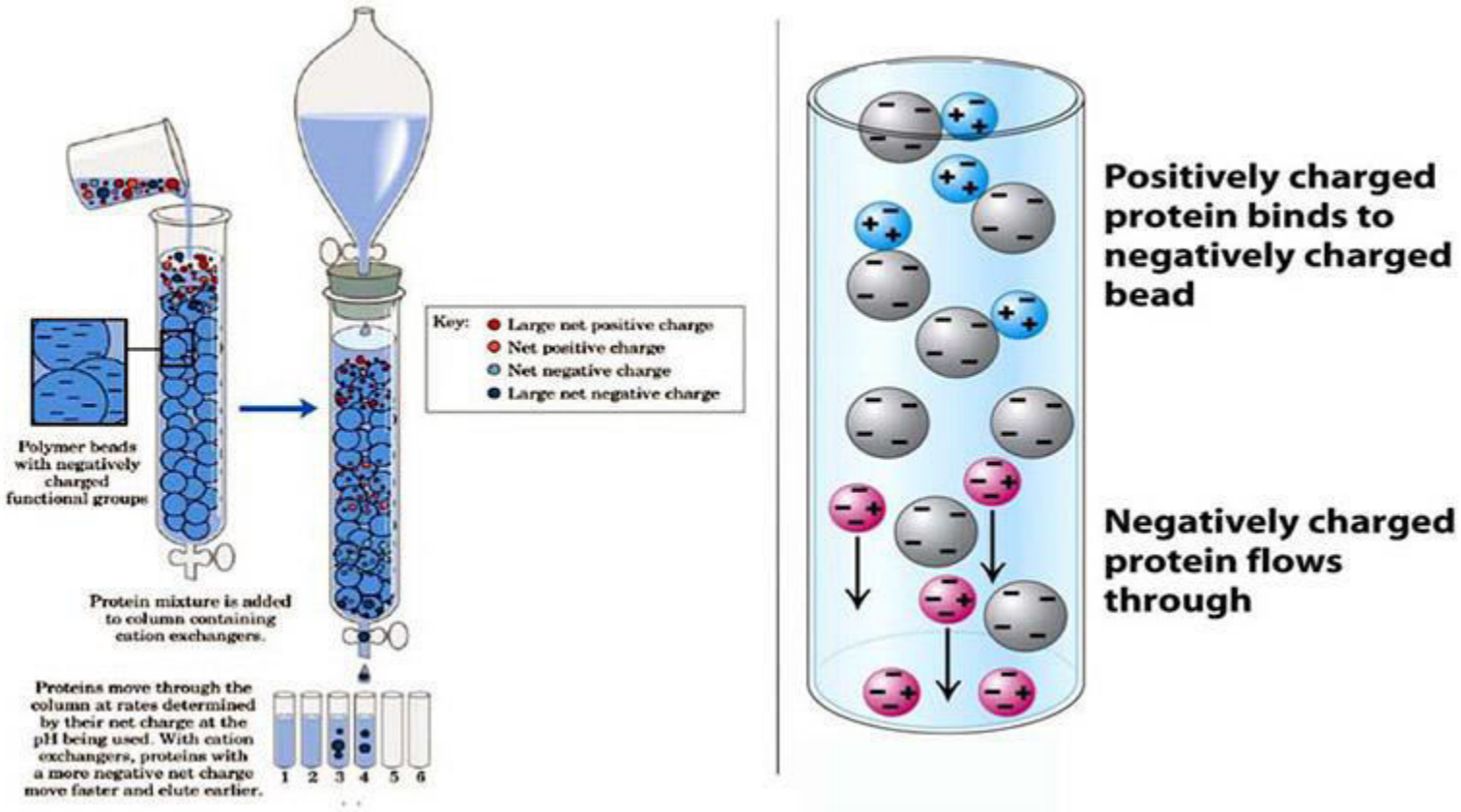


# LESSON 50

# Ion exchange chromatography

Ion exchange chromatography (or ion chromatography) is a process that allows the separation of ions and polar molecules based on their affinity to ion exchangers. The principle of separation is thus by reversible exchange of ions between the target ions present in the sample solution to the ions present on ion exchangers. In this process two types of exchangers i.e., cationic and anionic exchangers can be used.

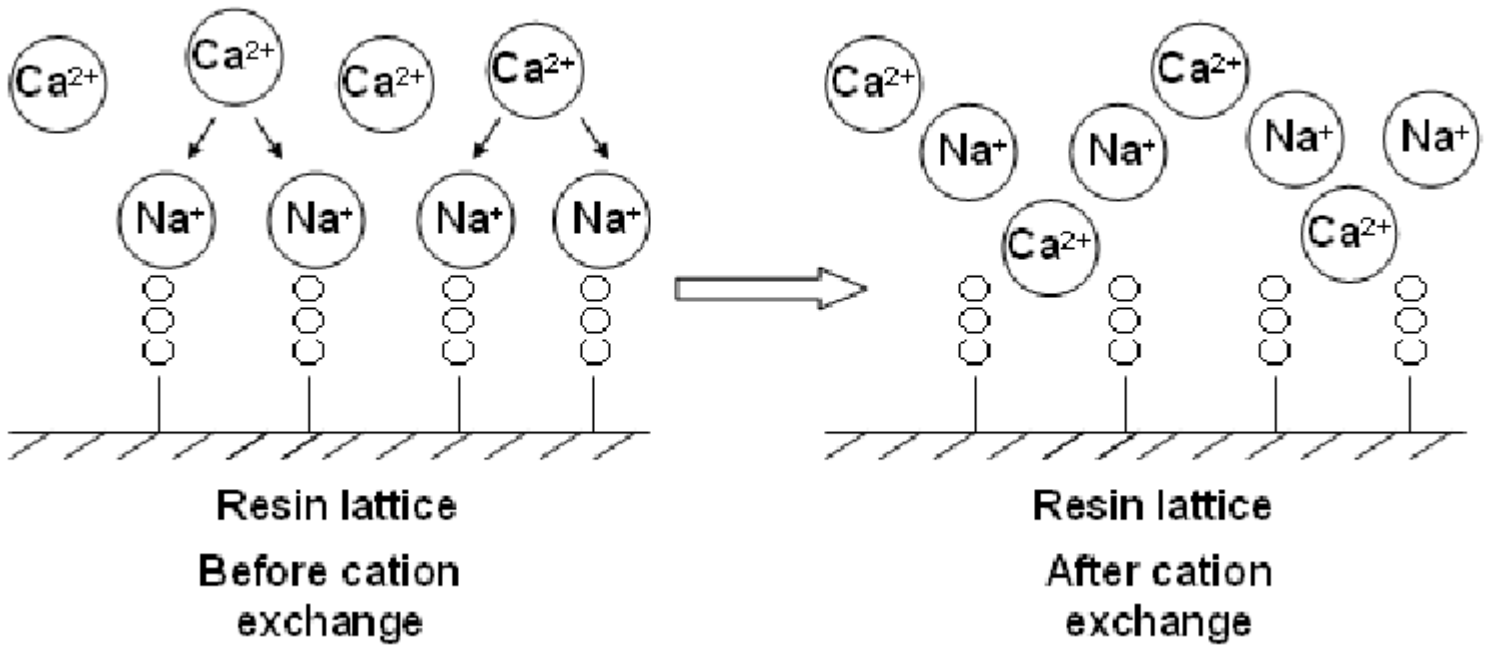
# Ion exchange chromatography



# Cationic exchangers

- Cationic exchangers possess negatively charged group, and these will attract positively charged cations. These exchangers are also called “Acidic ion exchange” materials, because their negative charges result from the ionization of acidic group.

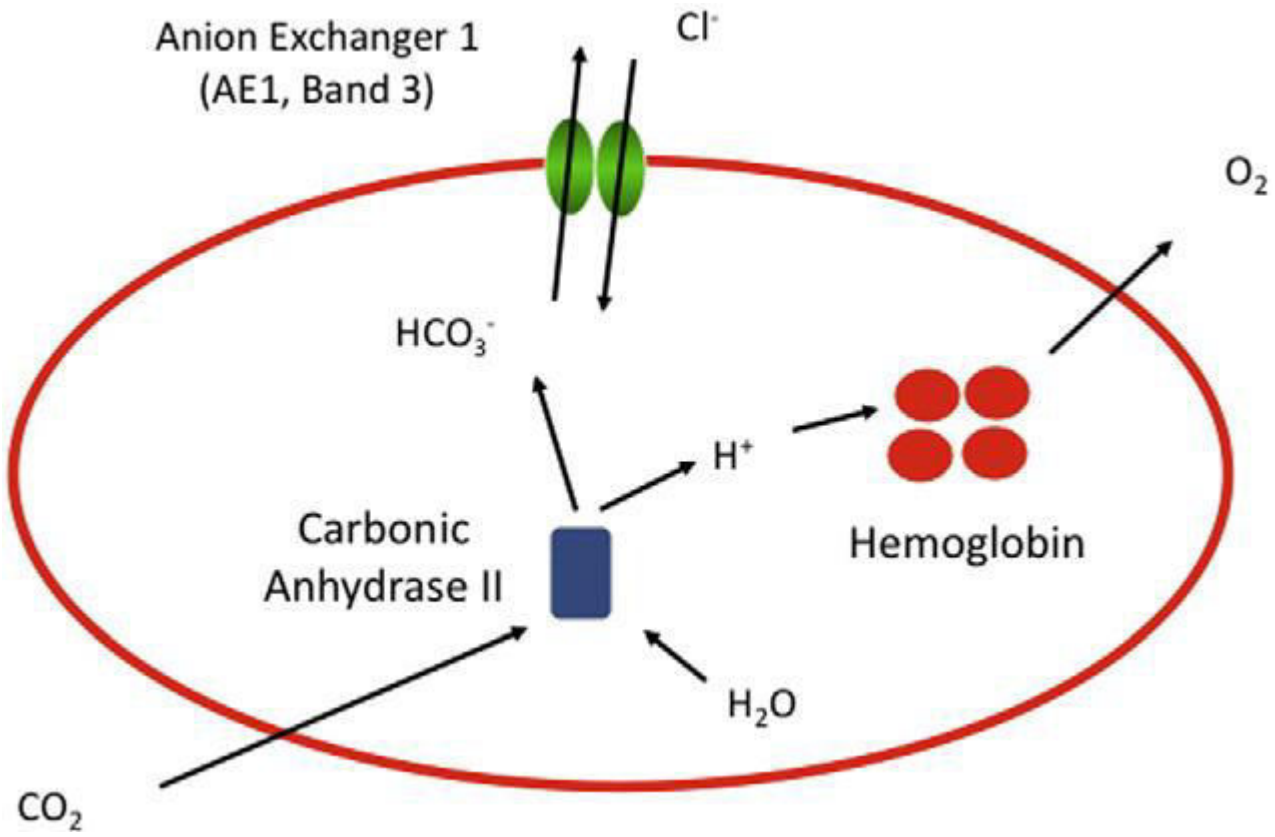
# Mechanism of cation exchange



# Anionic exchangers

Anionic exchangers have positively charged groups that will attract negatively charged anions. These are also called “Basic ion exchange” materials. Ion exchange chromatography is most often performed in the form of column chromatography. However, there are also thinlayer chromatographic methods that work basically based on the principle of ion exchange.

# Mechanism of cation exchange



# Working Principle of ion exchange chromatography

This form of chromatography relies on the attraction between oppositely charged stationary phase, known as an ion exchanger, and analyte. The ion exchangers basically contain charged groups covalently linked to the surface of an insoluble matrix.

The charged groups of the matrix can be positively or negatively charged.

When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite charge.

In this “ion cloud”, ions can be reversibly exchanged without changing the nature and the properties of the matrix

# Applications of ion exchange chromatography

- An important use of ion-exchange chromatography is in the routine analysis of amino acid mixtures.
- The 20 principal amino acids from blood serum or from the hydrolysis of proteins are separated and used in clinical diagnosis.
- This is most effective method for water purification. Complete deionization of water (or) a non-electrolyte solution is performed by exchanging solute cations for hydrogen ions and solute anions for hydroxyl ions. This is usually achieved by method is used for softening of drinking water.

# Applications

- In the analysis of products of hydrolysis of nucleic acids. In this way, information is gained about the structure of these molecules and how it relates to their biological function as carriers of hereditary information.
- Chelating resins are used to collect trace metals from seawater.
- To analyze lunar rocks and rare trace elements on Earth.

# Limitations of ion exchange chromatography

- Only charged molecules can be separated
- Buffer Requirement

# Lesson 51
# High performance liquid chromatography (HPLC)

# High performance liquid chromatography

- HPLC stands for “High performance liquid chromatography”(sometimes referred to as High pressure chromatography).

- High performance liquid chromatography is a powerful tool in analysis, it yields high performance and high speed compared to traditional columns chromatography because of the forcibly pumped mobile phase.

- HPLC is a chromatographic technique that can separate a mixture of compounds.
- It is used in biochemistry and analytical chemistry to identify, quantify and purify the individual.

# HPLC

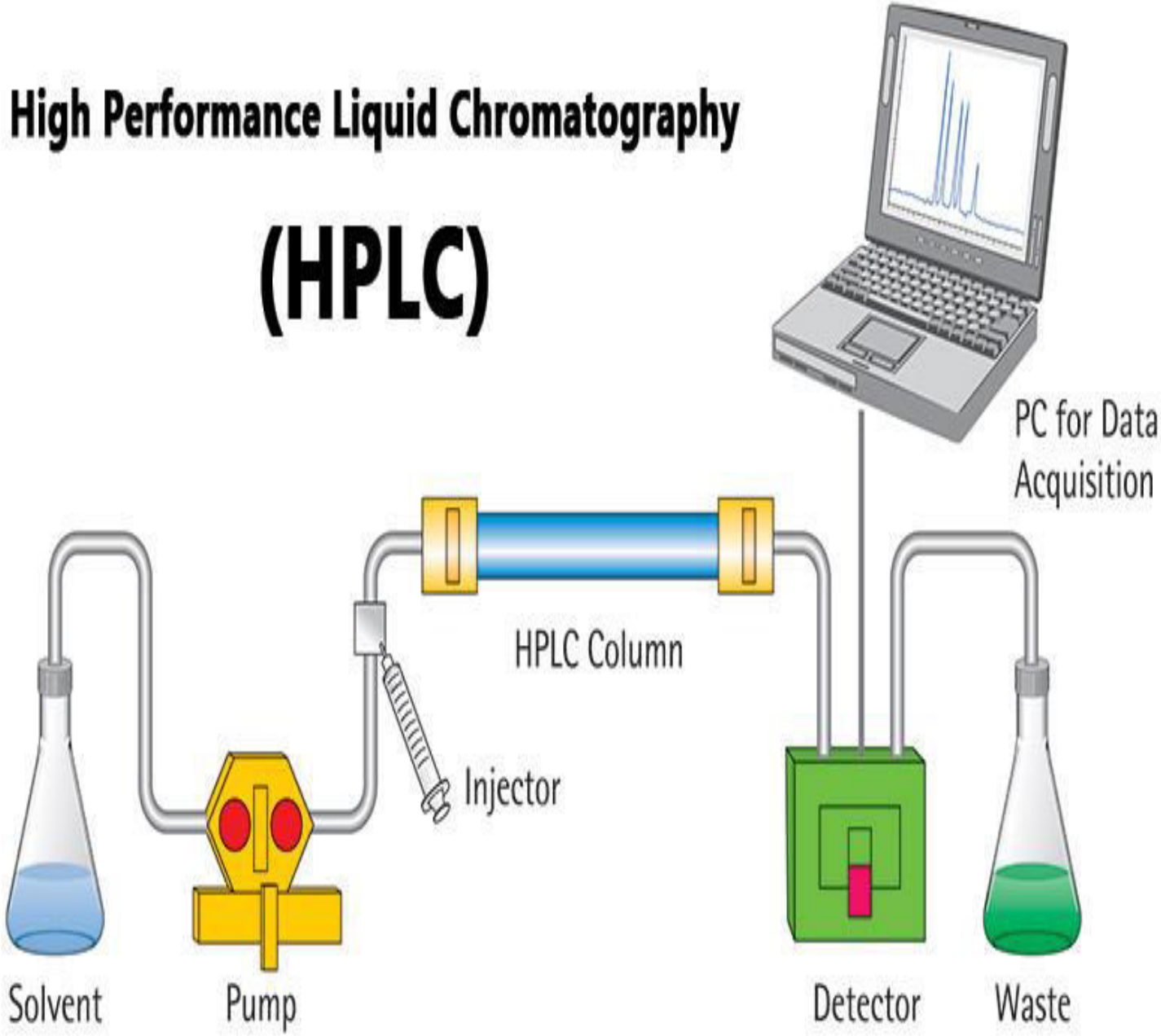
- **Chromatography :** physical method in which separation of components take place between two phases –a stationary phase and a mobile phase.

- **Stationary phase:-** The substance on which phase adsorption of analyte(the substance to be separated during chromatography ) take place.IT can be a solid ,a gel, or a solid liquid combination

- **Mobile phase:-** Solvent which carries the analyte (a liquid or a gas)

# High Performance Liquid Chromatography

# (HPLC)



# Principle of High-Performance Liquid Chromatography (HPLC)

- The purification takes place in a separation column between a stationary and a mobile phase.
- The stationary phase is a granular material with very small porous particles in a separation column.
- The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.

# Principle

❖Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.

❖Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.

❖After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.

❖At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.

# Types of High-Performance Liquid Chromatography (HPLC)

- **Normal phase :** Column packing is polar (e.g. silica) and the mobile phase is non-polar. It is used for water-sensitive compounds, geometric isomers, cistrans isomers, and chiral compounds.
- **Reverse phase :**The column packing is non-polar (e.g. C18), the mobile phase is water+ miscible solvent (e.g. methanol). It can be used for polar, non-polar, ionizable and ionic samples.

# Types

❖**Ion exchange:** Column packing contains ionic groups and the mobile phase is buffer. It is used to separate anions and cations.

❖**Size exclusion :** Molecules diffuse into pores of a porous medium and are separated according to their relative size to the pore size. Large molecules elute first and smaller molecules elute later.

# Applications of High-Performance Liquid Chromatography (HPLC)

- The HPLC has developed into a universally applicable method so that it finds its use in almost all areas of chemistry, biochemistry, and pharmacy.
- Analysis of drugs
- Analysis of synthetic polymers
- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Product purity and quality control of industrial products and fine chemicals

# Applications

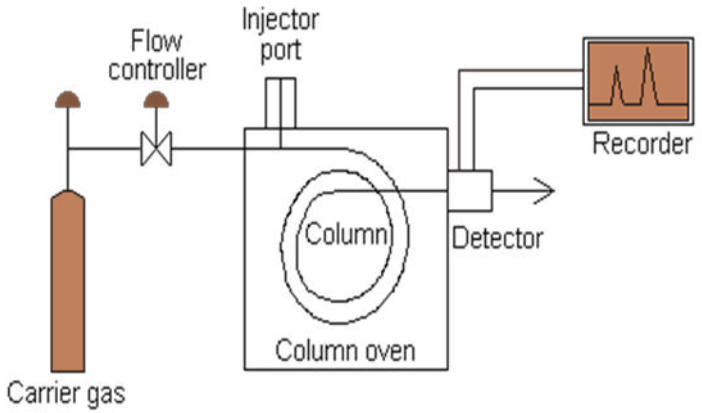
- Separation and purification of biopolymers such as enzymes or nucleic acids
- Water purification
- Pre-concentration of trace components
- Ligand-exchange chromatography
- Ion-exchange chromatography of proteins
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides

LESSON 52

# Gas Chromatography

# Gas Chromatography

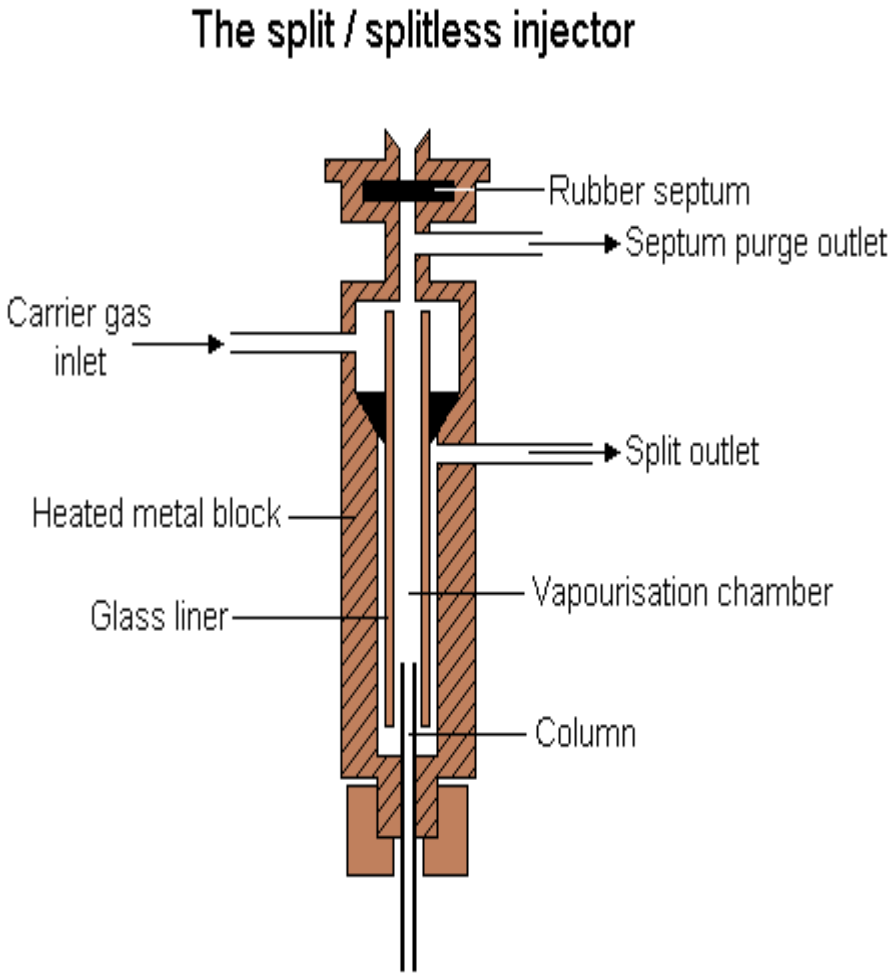
- In this method stationary phase is a column which is placed in the device, and contains a liquid stationary phase which is adsorbed onto the surface of an inert solid. Gas chromatography is a "gas-liquid" chromatography.
- Its carrier phase consists of gases as He or $N_2$. Mobile phase which is an inert gas is passed through a column under high pressure. The sample to be analyzed is vaporized, and enters into a gaseous mobile phase .
- The components contained in the sample are dispersed between mobile phase, and stationary phase on the solid support.
- Gas chromatography is a simple, multifaceted, highly sensitive, and rapidly applied technique for the extremely excellent separation of very minute molecules.



# Have a look at this schematic diagram of a gas chromatograph

- **Instrumental components**
- **Carrier gas:** The carrier gas must be chemically inert. Commonly used gases include nitrogen, helium, argon, and carbon dioxide. The choice of carrier gas is often dependent upon the type of detector which is used. The carrier gas system also contains a molecular sieve to remove water and other impurities.
- **Sample injection port:** For optimum column efficiency, the sample should not be too large, and should be introduced onto the column as a "plug" of vapor - slow injection of large samples causes band broadening and loss of resolution.
- The most common injection method is where a micro syringe is used to inject sample through a rubber septum into a flash vaporizer port at the head of the column. The temperature of the sample port is usually about 50°C higher than the boiling point of the least volatile component of the sample. For packed columns, sample size ranges from tenths of a microliter up to 20 microliters.

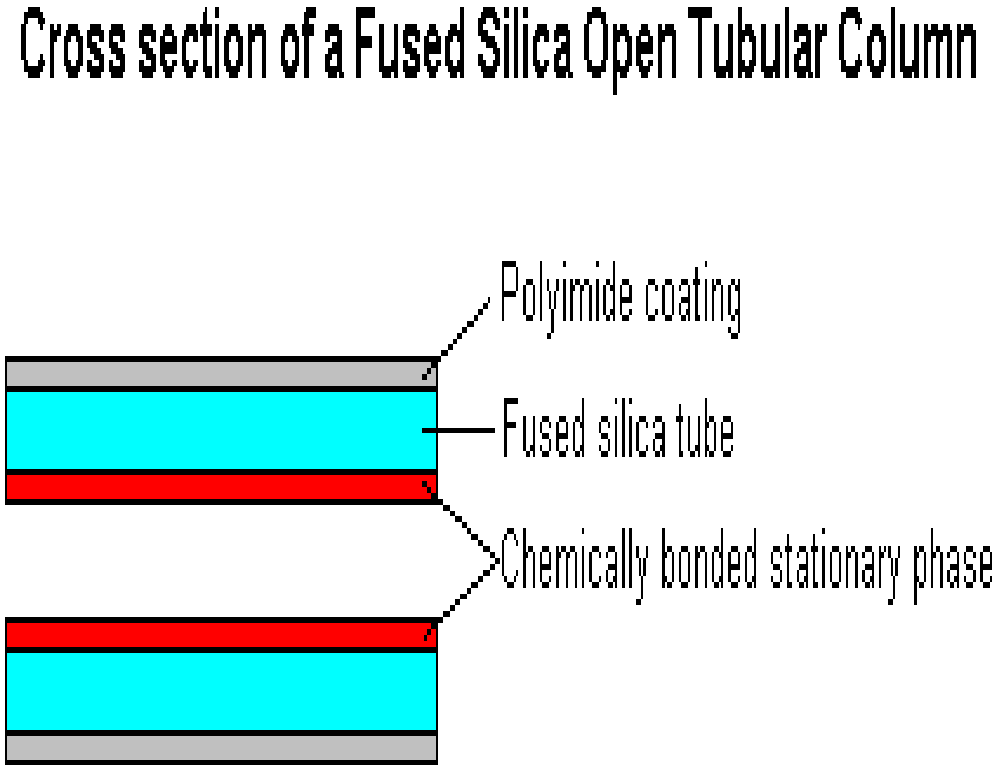
- Capillary columns, on the other hand, need much less sample, typically around $10^{-3}$ ml. For capillary GC, split/split less injection is used. Have a look at this diagram of a split/split less injector.
- The injector can be used in one of two modes; split or split less. The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum.
- The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). The sample vaporizes to form a mixture of carrier gas, vaporized solvent and vaporized solutes.
- A proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column.



# Columns

- There are two general types of column, packed and capillary (also known as open tubular). Packed columns contain a finely divided, inert, solid support material (commonly based on diatomaceous earth) coated with liquid stationary phase.
- Most packed columns are 1.5 - 10m in length and have an internal diameter of 2 - 4mm.
- Capillary columns have an internal diameter of a few tenths of a millimeter. They can be one of two types; wall-coated open tubular (WCOT) or support-coated open tubular (SCOT).
- Wall-coated columns consist of a capillary tube whose walls are coated with liquid stationary phase.
- In support-coated columns, the inner wall of the capillary is lined with a thin layer of support material such as diatomaceous earth, onto which the stationary phase has been adsorbed.
- SCOT columns are generally less efficient than WCOT columns. Both types of capillary column are more efficient than packed columns.

- These have much thinner walls than the glass capillary columns, and are given strength by the polyimide coating. These columns are flexible and can be wound into coils. They have the advantages of physical strength, flexibility and low reactivity.



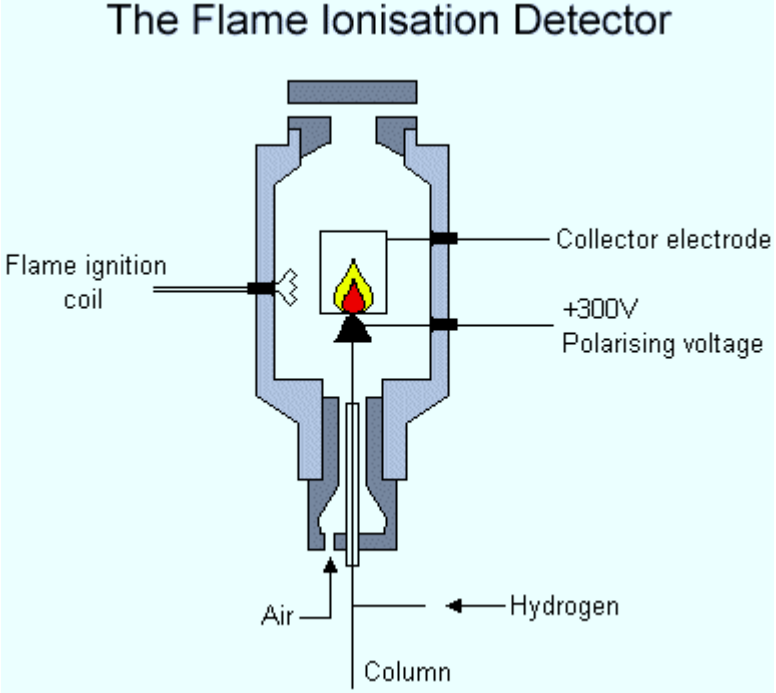
# Column temperature

- For precise work, column temperature must be controlled to within tenths of a degree.
- The optimum column temperature is dependant upon the boiling point of the sample. As a rule of thumb, a temperature slightly above the average boiling point of the sample results in an elution time of 2 - 30 minutes.
- Minimal temperatures give good resolution, but increase elution times. If a sample has a wide boiling range, then temperature programming can be useful.
- The column temperature is increased (either continuously or in steps) as separation proceeds.

# Detectors

- There are many detectors which can be used in gas chromatography. Different detectors will give different types of selectivity. A non-selective detector responds to all compounds except the carrier gas, a selective detector responds to a range of compounds with a common physical or chemical property and a specific detector responds to a single chemical compound. Detectors can also be grouped into concentration dependant detectors and mass flow dependant detectors.

- The signal from a concentration dependent detector is related to the concentration of solute in the detector, and does not usually destroy the sample Dilution of with make-up gas will lower the detectors response.
- Mass flow dependent detectors usually destroy the sample, and the signal is related to the rate at which solute molecules enter the detector. The response of a mass flow dependent detector is unaffected by make-up gas.



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# LESSON 53
# Use of Soxhalet and Rotary evaporator for extraction

# Definition of Soxhlet extractor

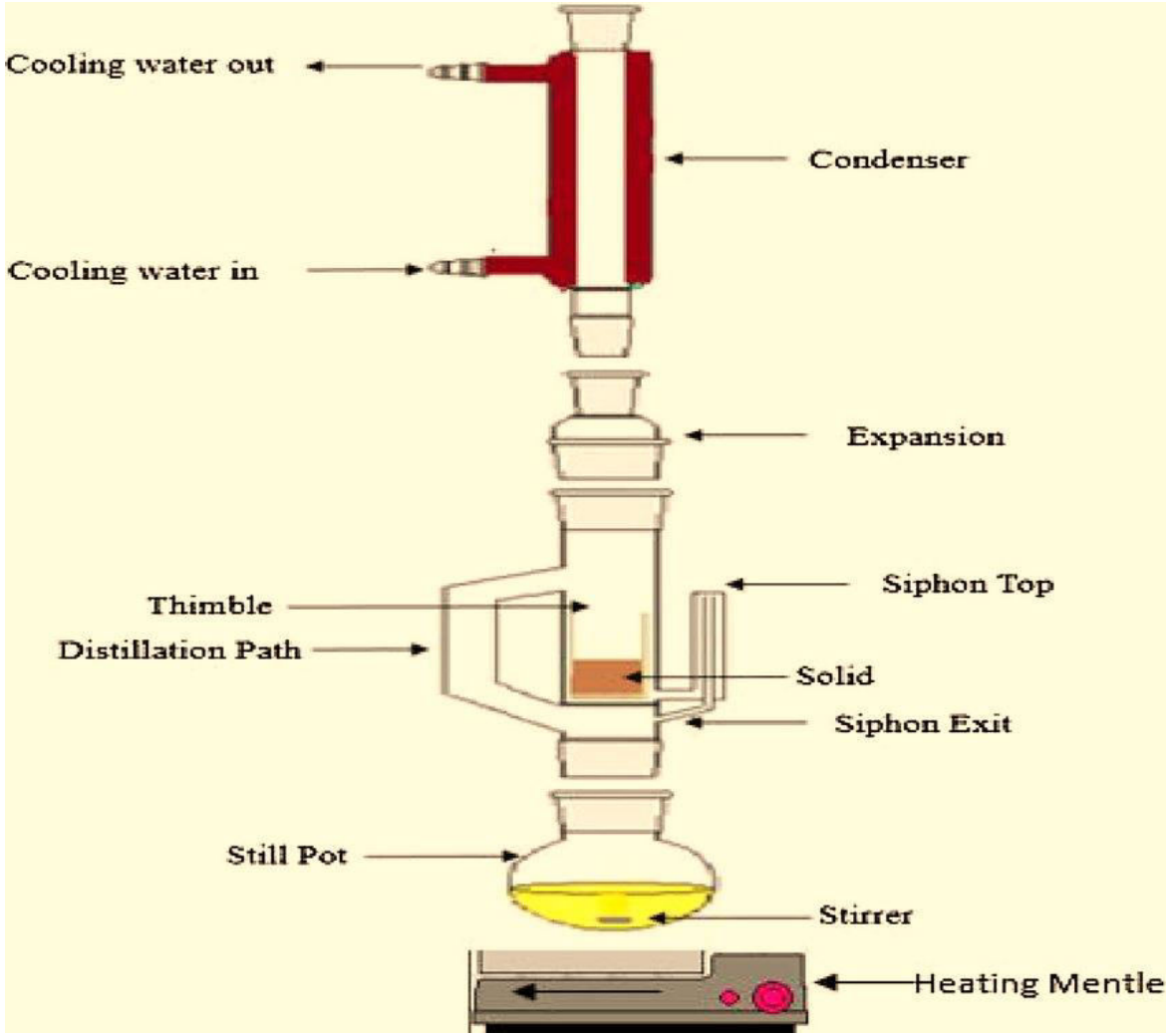
- A **Soxhlet extractor** is a piece of laboratory apparatus invented in 1879 by Franz von Soxhlet.
- It was originally designed for the extraction of a lipid from a solid material. Typically, Soxhlet extraction is used when the desired compound has a *limited* solubility in a solvent, and the impurity is insoluble in that solvent.
- It allows for unmonitored and unmanaged operation while efficiently recycling a small amount of solvent to dissolve a larger amount of material.

# Description

A Soxhlet extractor has three main sections:

1. a percolator (boiler and reflux) which circulates the solvent,
2. a thimble (usually made of thick filter paper) which retains the solid to be extracted,
3. a siphon mechanism, which periodically empties the thimble.

- The source material containing the compound to be extracted is placed inside the thimble.
- The thimble is loaded into the main chamber of the Soxhlet extractor.
- The extraction solvent to be used is placed in a distillation flask. The flask is placed on the heating element.
- The Soxhlet extractor is placed atop the flask.
- A reflux condenser is placed atop the extractor.



# Working Principle

- The solvent vapors travels up a distillation arm and floods into the chamber housing the thimble of solid.
- The condenser ensures that any solvent vapors cool, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent.
- Some of the desired compound will then dissolve in the warm solvent.
- When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask.
- The thimble ensures that the rapid motion of the solvent does not transport any solid material to the still pot. This cycle may be allowed to repeat many times, over hours or days.

- During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask.
- The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled.
- After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound.
- The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded.

# VU Medical Zone

## Admin: Amaan Khan

## Biological Techniques

## *ZOO101 Midterm*

## ***Syllabus Finished***