Virtual University of Pakistan

200101 – Biological Techniques

For Online Classes & Study Help

Umar Ghafoor Wattu

MSC-Zoology / MA-Islamiyat (*Lecturer / Entrepreneur*)

Contact: +92 - 335 - 4232497 (9**am** – 5**pm**)

Email ID: UmarGhafoorWattu@gmail.com

Skype: live:umarghafoorwattu

Table of Contents

BIOLOGICAL TECHNIQUES

Lesson 1: Microscopy

What is microscope???

- MICROSCOPE: Is an instrument for viewing objects that are too small to be seen by the naked or unaided eye.
- **A microscope (Greek: mikron = small and skopein = to look).**
- MICROSCOPY: The science of investigating small objects using a microscope

History of microscopy

- 1590 - Hans Janssen and his son Zacharias Janssen, developed first microscope.
- 1609 - Galileo Galilei
- 1625 – **Giovanni Faber** coined the name *microscope* for the compound microscope Galileo submitted to the Accademia dei Lincei

Microscope Parts

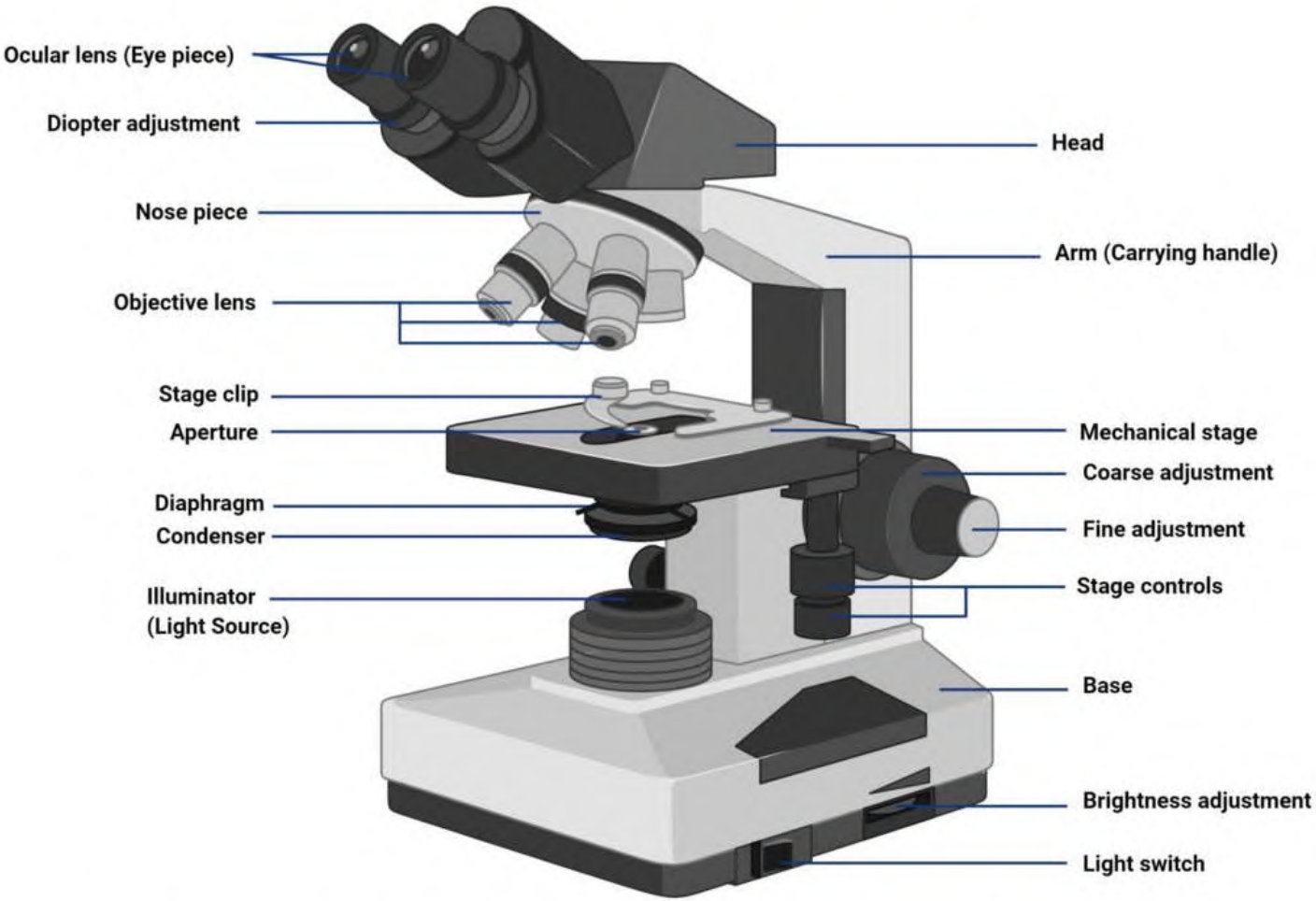


Figure: Parts of a microscope, Image Copyright © Sagar Aryal, www.microbenotes.com

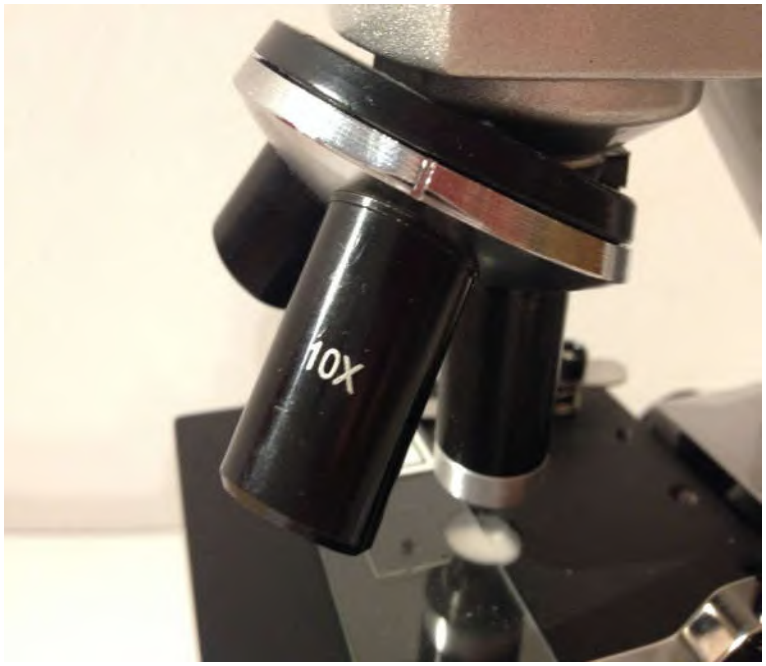
Ocular / Eyepiece

- An **eyepiece** is that part of an optical system, which is directed to the viewer. It is a construction of at least one or more lenses. The function of the eyepiece in a microscope is to convert the real- enlarged-intermediate-image from the objective into an enlarged-virtual-image.
- **Lens tube** The lens tube is connected with the eyepiece and it´s main task is to hold it.

- The **diopter adjustment** is a control knob on your binocular. It is designed to let you compensate for differences between your own two eyes. Once you set the **diopter**, then the two barrels should stay in proper relation.

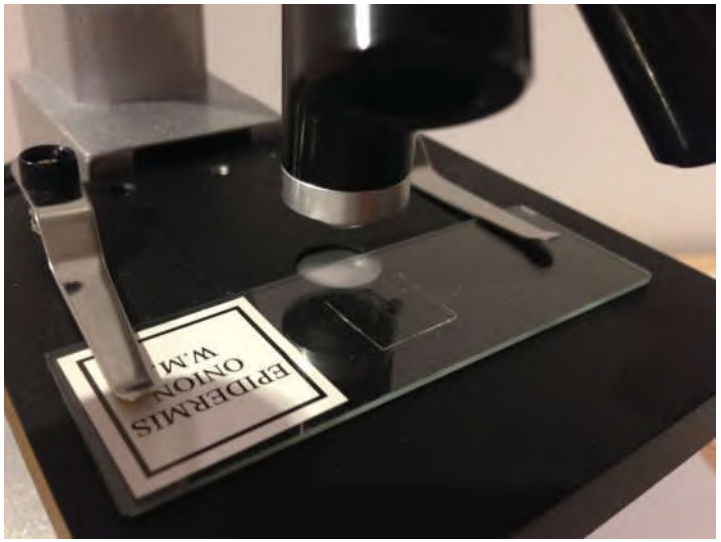
Objective revolver/Nose piece

- Objective revolvers are used in microscopes with multiple objective lenses, that have different magnification factors.



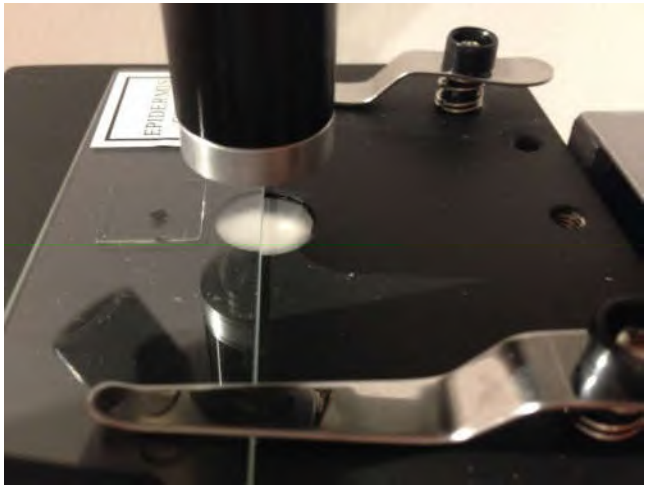
Objective lens

- An objective (lens) is that part of an optical system, which is directed to the object. It´s task is to collect the light rays, that are reflected from the observed item. The objective generates a real-optical image
- **Clip**
- The clip serves as a holder for the object plate and makes sure, that it doesn´t get out of its place unintentionally



Microscope stage

- On the stage, one can place the object on slide with the cover glass on it.
- one the part of the object, which one wants to look at can be chosen by moving the stage by the help of screws and knobs.
- This allows to shift the object plate via adjusting screw, instead of doing it by hand. “Cross table” is technical term.
- There are adjusting screws, which can be used to move the table very precisely. The screws are provided with a measuring scale, so one can always find again a certain point of the object.



Condenser

- A **condenser** is an optical **lens** which renders a divergent beam from a point source into a parallel or converging beam to illuminate an object.
- Thus, every part of the object is illuminated on the same brightness level.
- Condensers normally consist of one or two lenses. These lenses fractionize the light and all the rays leave as parallel beams. **An aspheric lens avoids chromatic aberration.** This guarantees a better image quality. Their production costs are higher than those of normal lenses. They can be another criteria that differentiates high-performance microscopes from cheap microscopes.

A condenser (right) and its respective diaphragm (left)

Fine focus & Coarse focus

- With the fine focus one can regulate the distance between object and objective, to achieve the necessary sharpness. The fine focus moves the stage only minimally – like the name already says.
- Like the fine focus, the coarse focus also moves the stage to regulate the difference between object and objective. its task is, to catch the right distance roughly and quickly. The optimal sharpness can be adjusted with the fine coarse afterwards.

Light Source

The early microscopes used concave mirrors to refrect light on the objects. Later, they used light bulbs.

Most microscopes operate with LED light. The light source´s task is to illuminate the object evenly.

Base

- The base guarantees the necessary stability to the microscope.

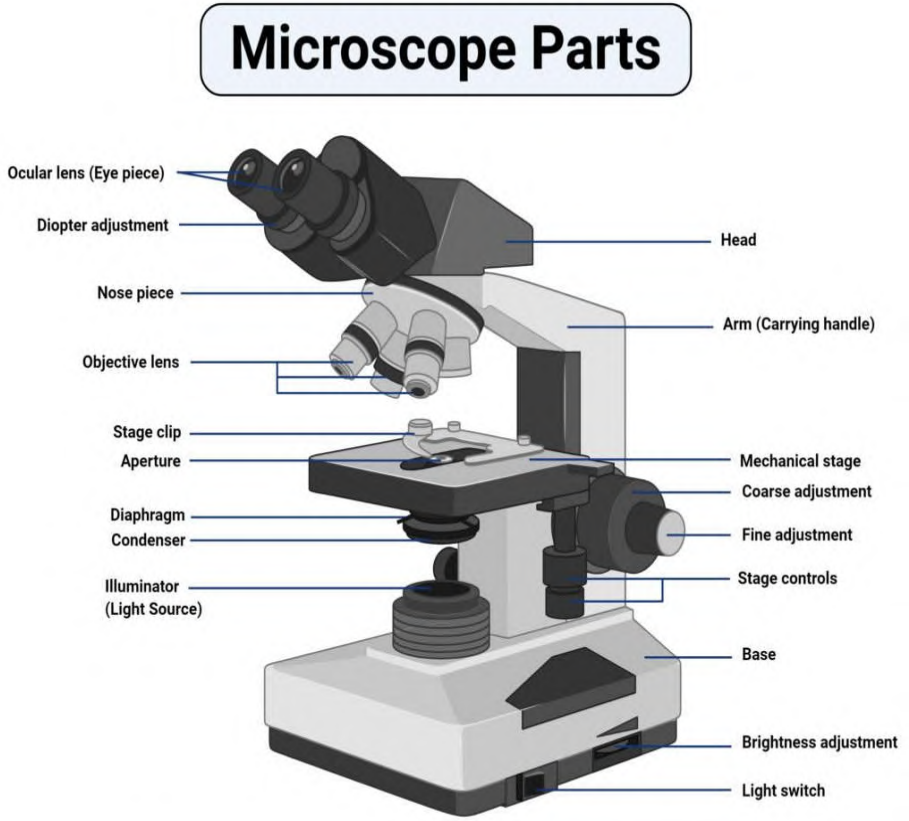
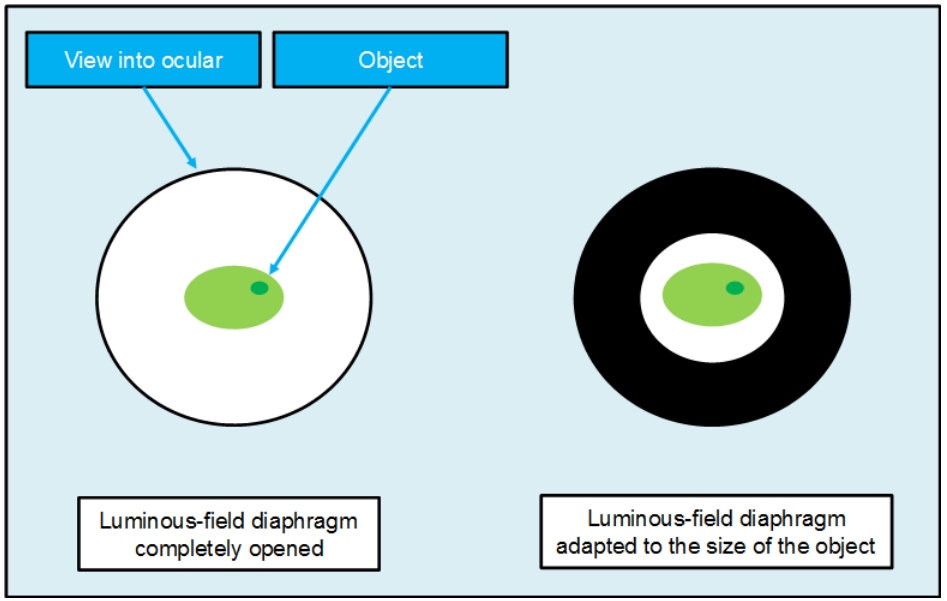


Figure: Parts of a microscope, Image Copyright © Sagar Aryal, www.microbenotes.com

Luminous-field diaphragm

- Luminous-field diaphragm: cam be used to reduce the illumination and adapt it to the size of the object
- This can prevent the object from being outshined.



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.

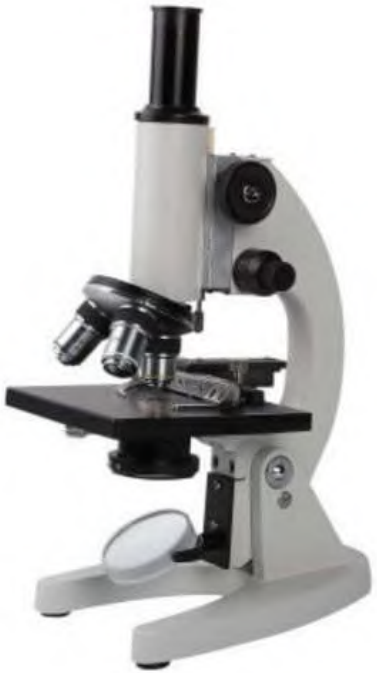
2. Compound light microscope (it has a higher magnification compared to the simple microscope because it uses at least two sets of lenses, an objective lens, and an eyepiece).

- 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**

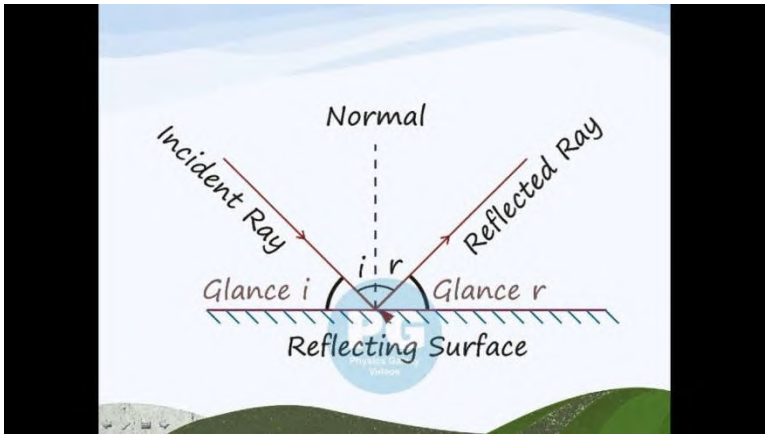
Total magnification=magnification of the eyepiece x magnification of the objective.

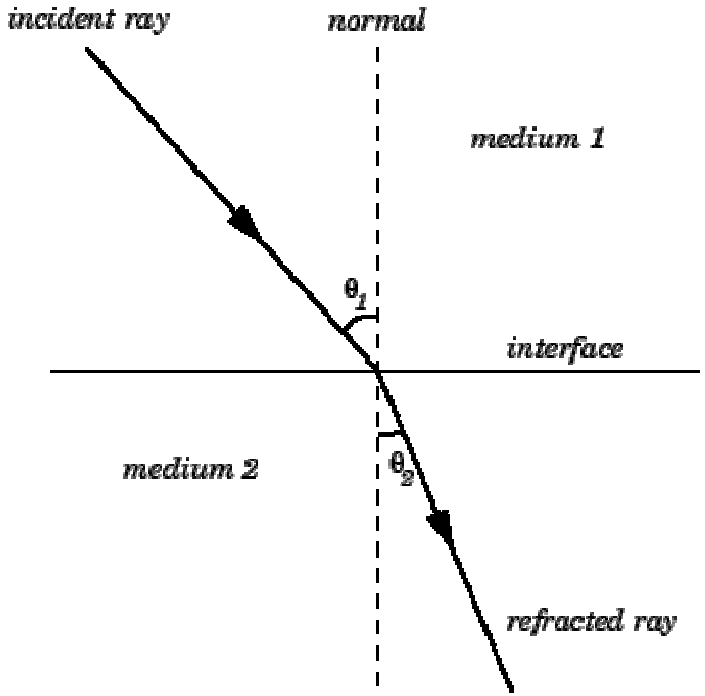
=10x40 = 400

=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.

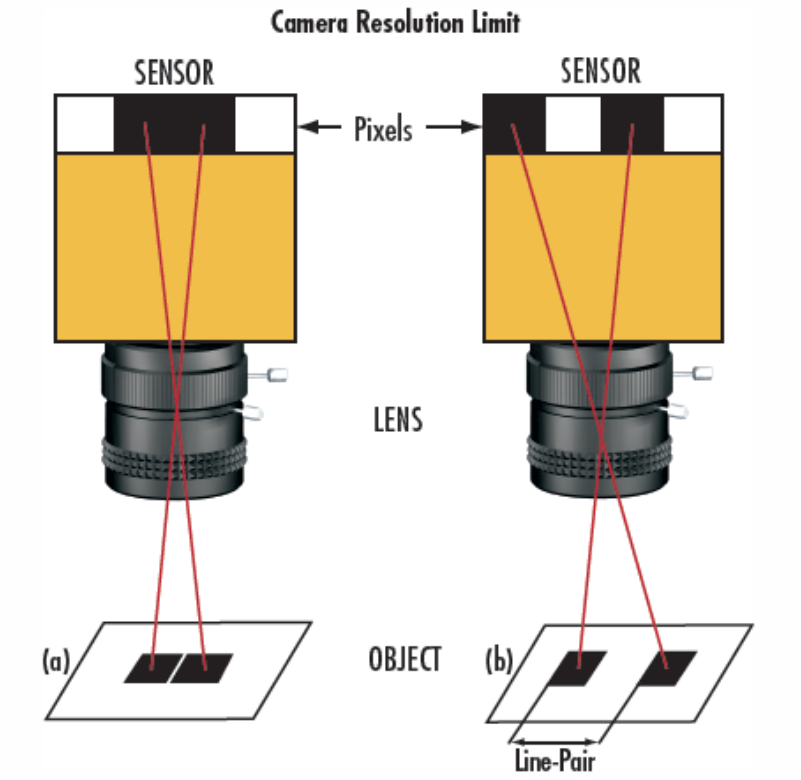
Microscopy

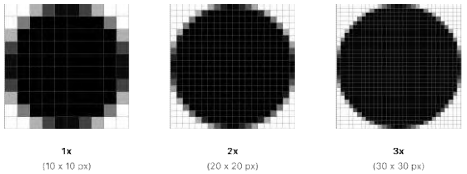
Lesson 3: MAGNIFICATION, RESOLUTION and PRINCIPLE of a microsope..continued…

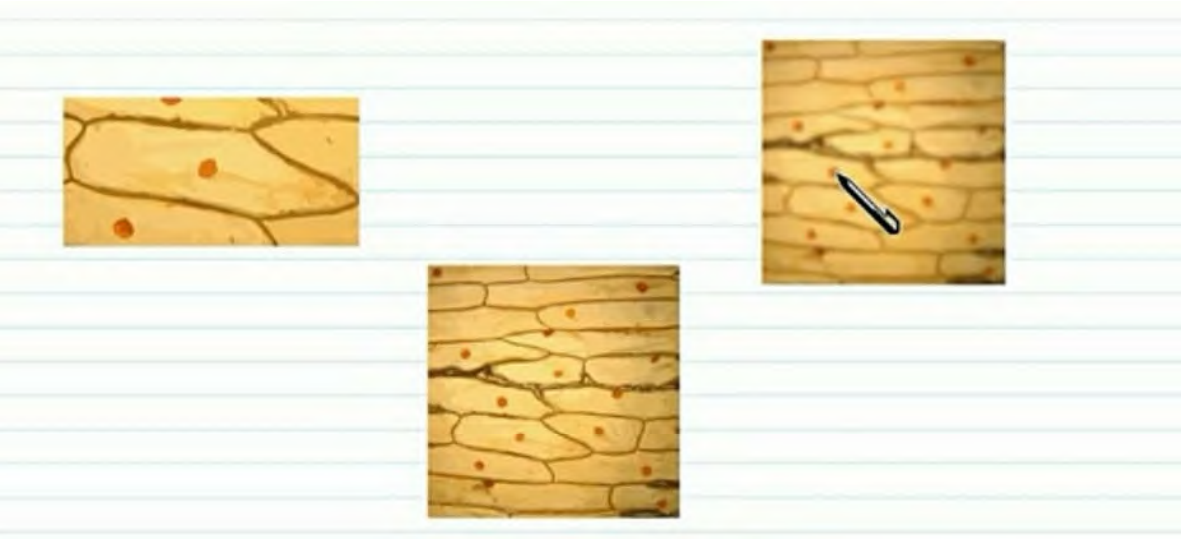
…2

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

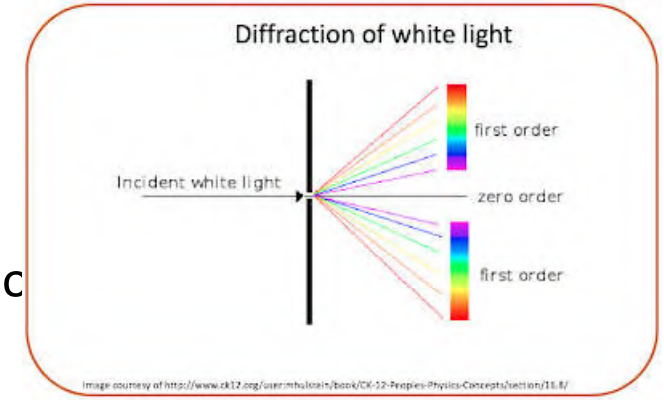






Magnified vs resolved image

- **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.

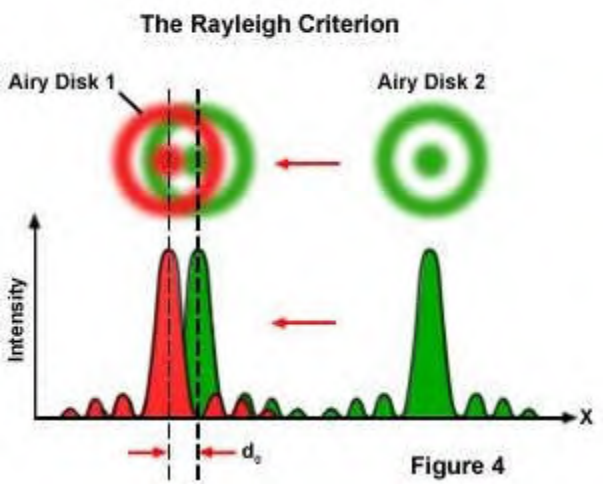
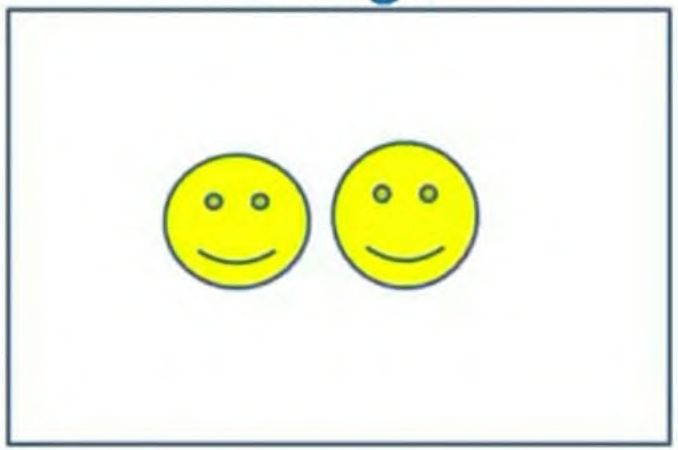


Figure 4

The Rayleigh Criteria

Two closely spaced objects

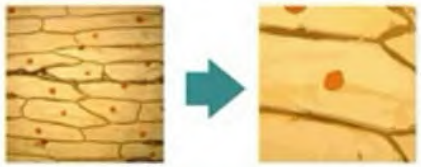
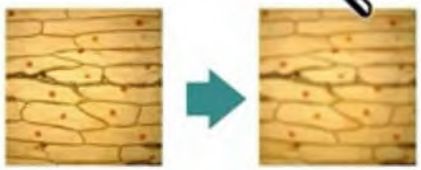
Image



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.

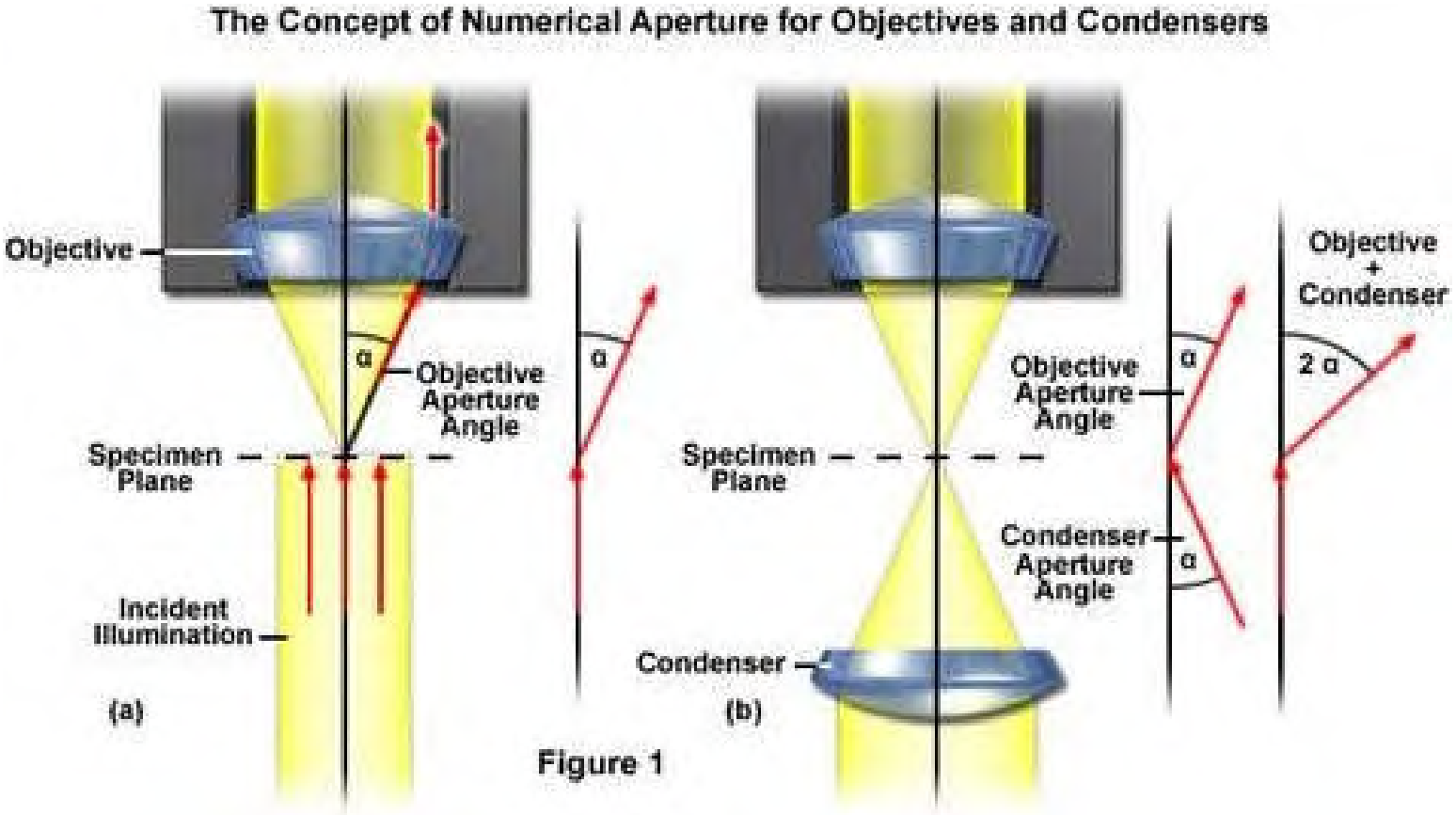


Figure 1

- 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.

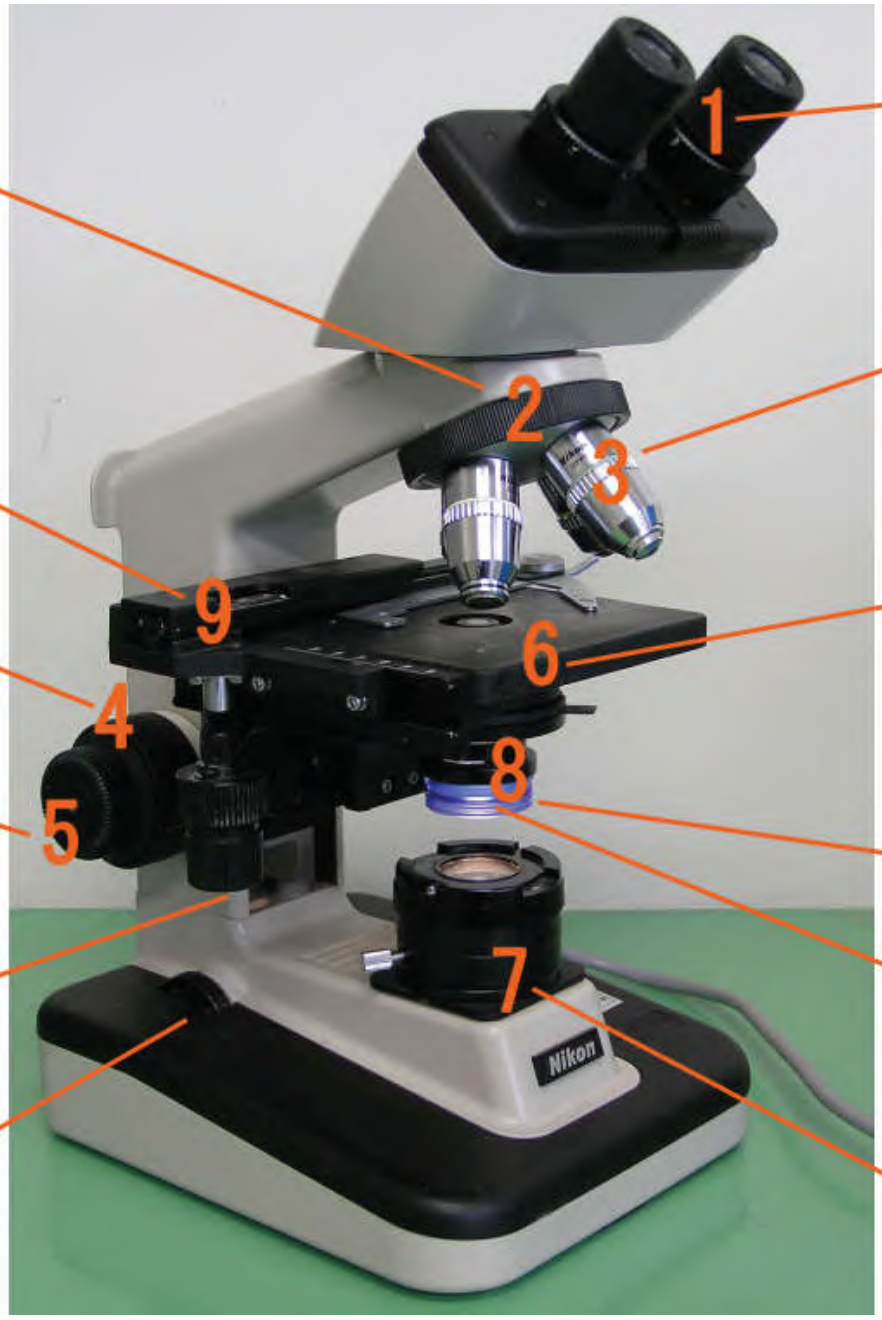
Immersion Media

- 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

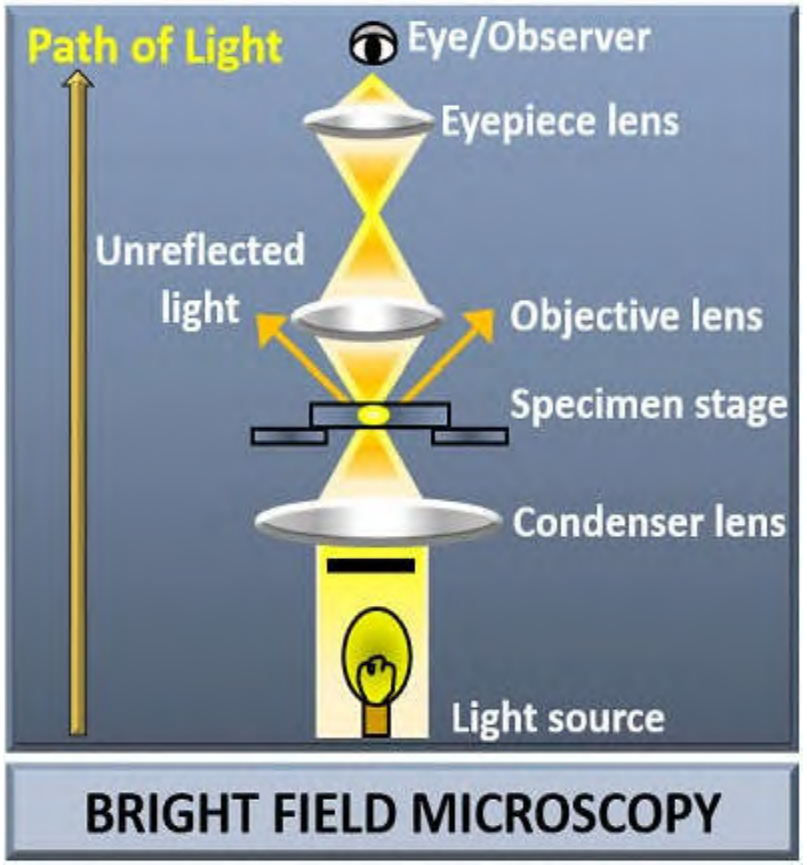
rheostat (to adjust light intensity)

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

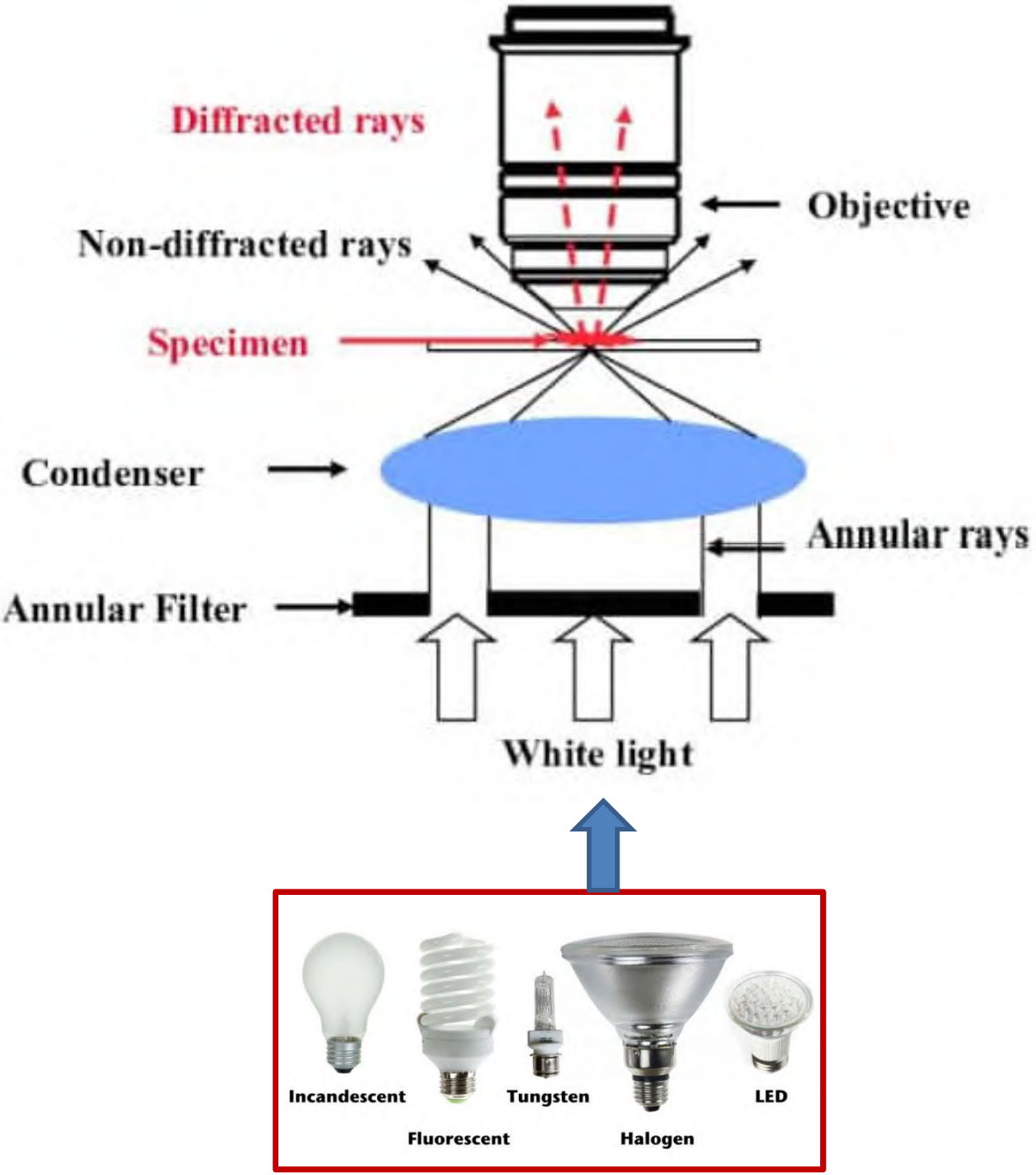
Enhancements

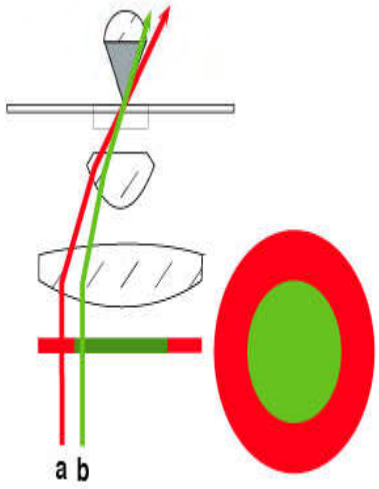
- Reducing or increasing the amount of the light source by the iris diaphragm.
- Use of an OIL IMMERSION LENS (OBJECTIVE) lens and a special immersion oil (cedar wood oil) placed on a glass cover over the specimen. Immersion oil has the same refraction as glass and improves the resolution of the observed 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

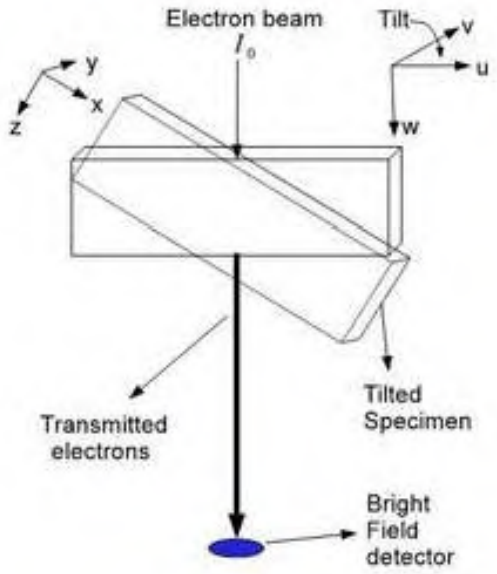
The light's path

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.

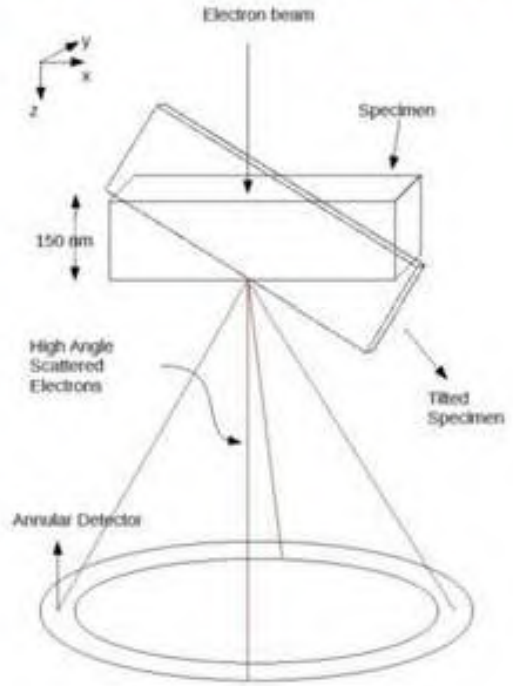
4. The light enters the sample. Most is directly transmitted, while some is scattered from the sample.
5. The scattered light enters the objective lens, while the directly transmitted light simply misses the lens and is not collected due to a direct illumination block (see figure).
6. Only the scattered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



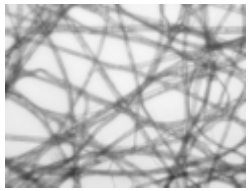
Bright Field:
Image is **bright** when sample is removed



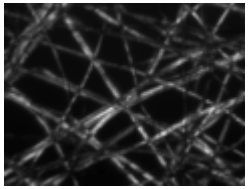
Dark Field:
Image is **dark** when sample is removed

Uses of dark filed microscopy

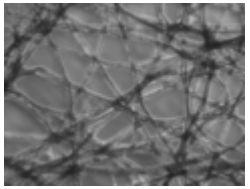
- Simple and popular method for making ***unstained objects*** clearly visible.
- This mode of illumination is perfectly adequate for examining transparent stained specimens with transmitted light or for reflected light observation of opaque specimens such as insects, integrated circuits, plants, and 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

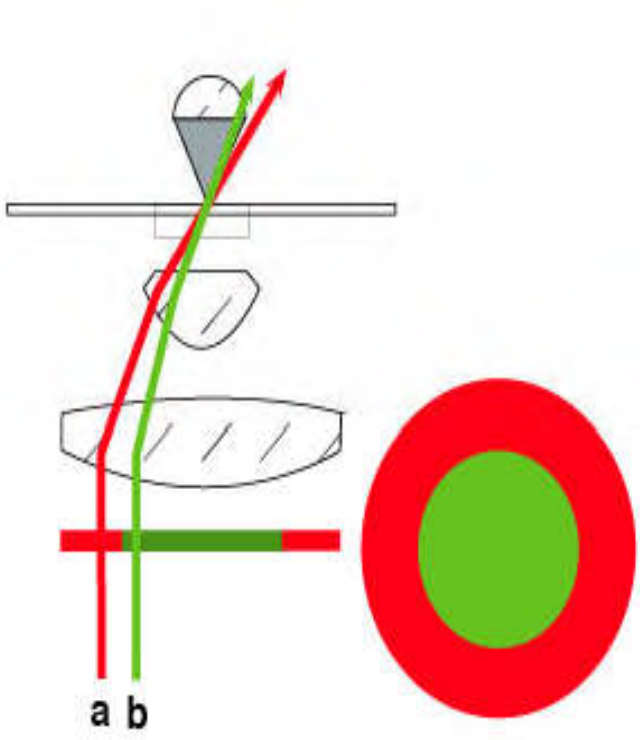
- Phase contrast microscopy first described in 1934 by Dutch physicist **Fritz Zernike,** Who was awarded **Nobel prize in physics in 1953.**
- A phase contrast microscope makes it possible by utilizing **two characteristics of light, diffraction and interference, specimens based on brightness differences.**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

Phase contrast microscopy

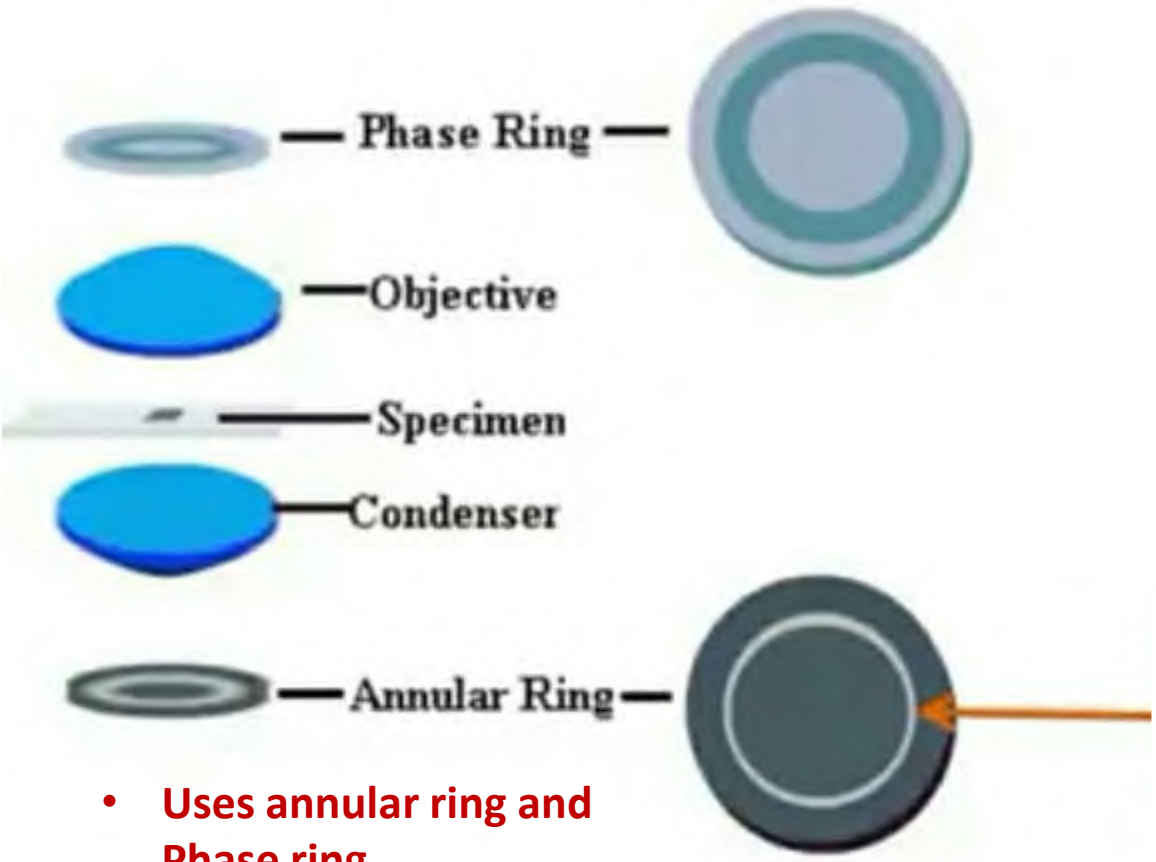
- Phase-contrast microscopy is particularly important in biology.
- It reveals many **cellular structures** that are invisible with a **Bright-field microscope**
- In Bright-field microscopy the Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light and 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

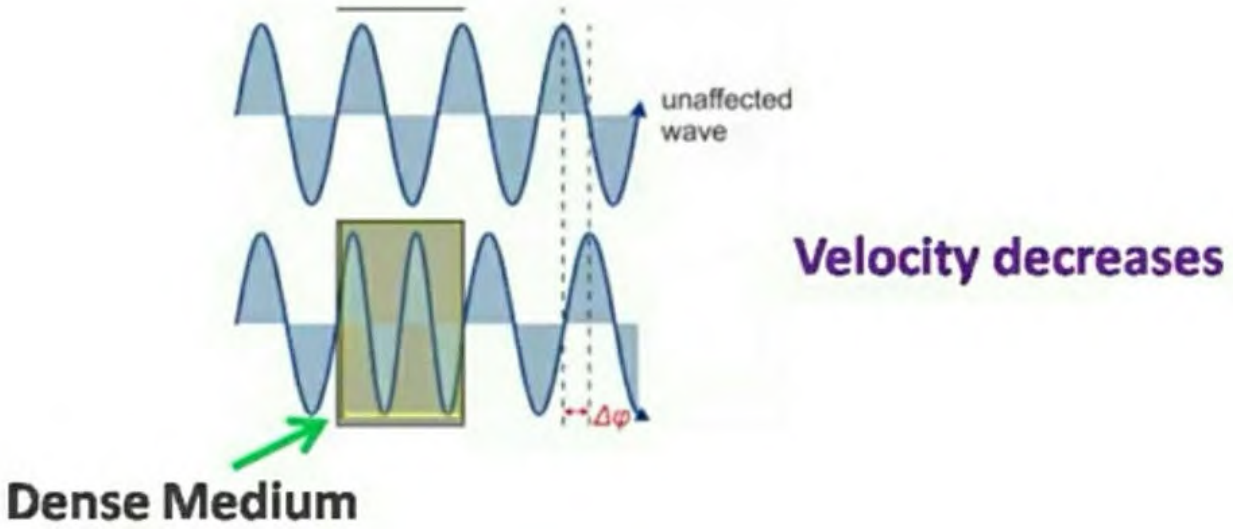
Annular ring

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

Principle: Phase Contrast Microscope

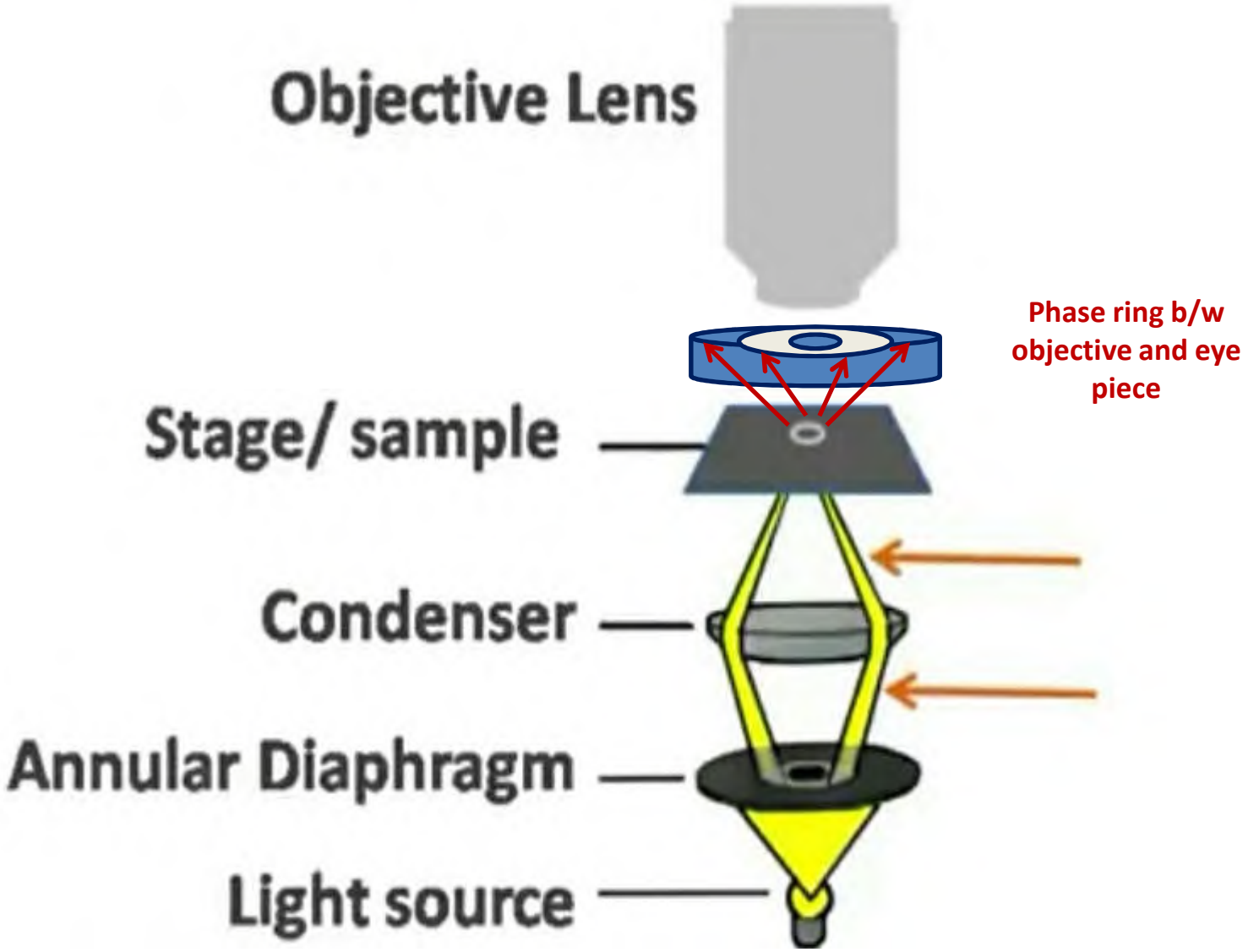
- It based on the wavelength (nature) of light rays and the fact that light rays can be **in phase or out of phase.**
- Different shade of grey are distinguished to our eyes due to differences in amplitude of light rays.
- PCM converts invisible small phase changes caused by the cell component in to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

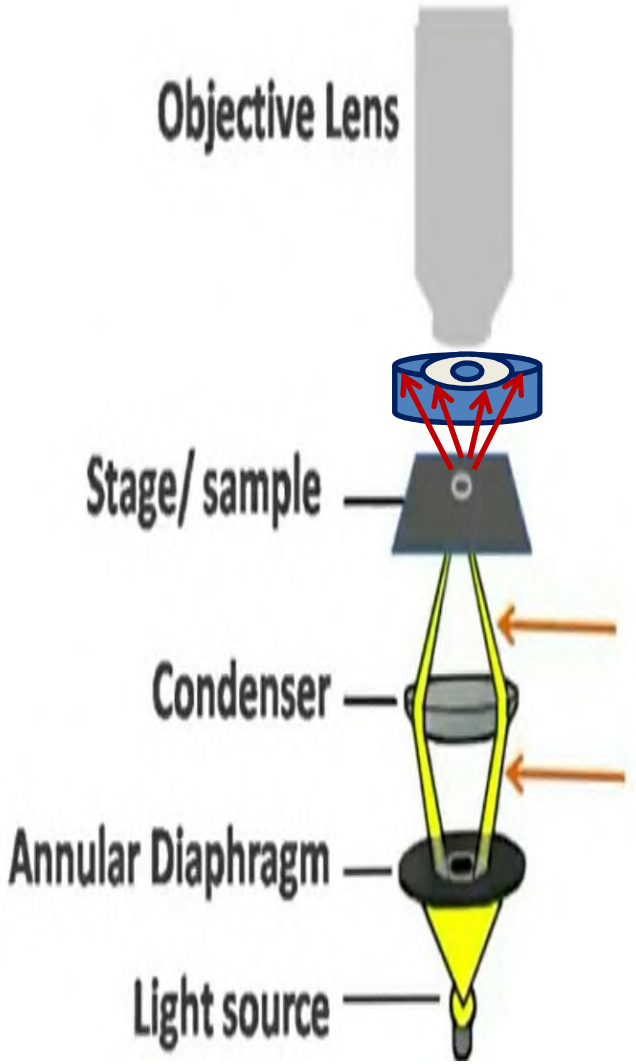
- Light passing through one material & into another material of slightly different refractive index or **thickness will undergo a change in phase.**
- This change is translated into variations in brightness of the structures.
- Phase contrast is obtained with the help of the **Phase ring** by separating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

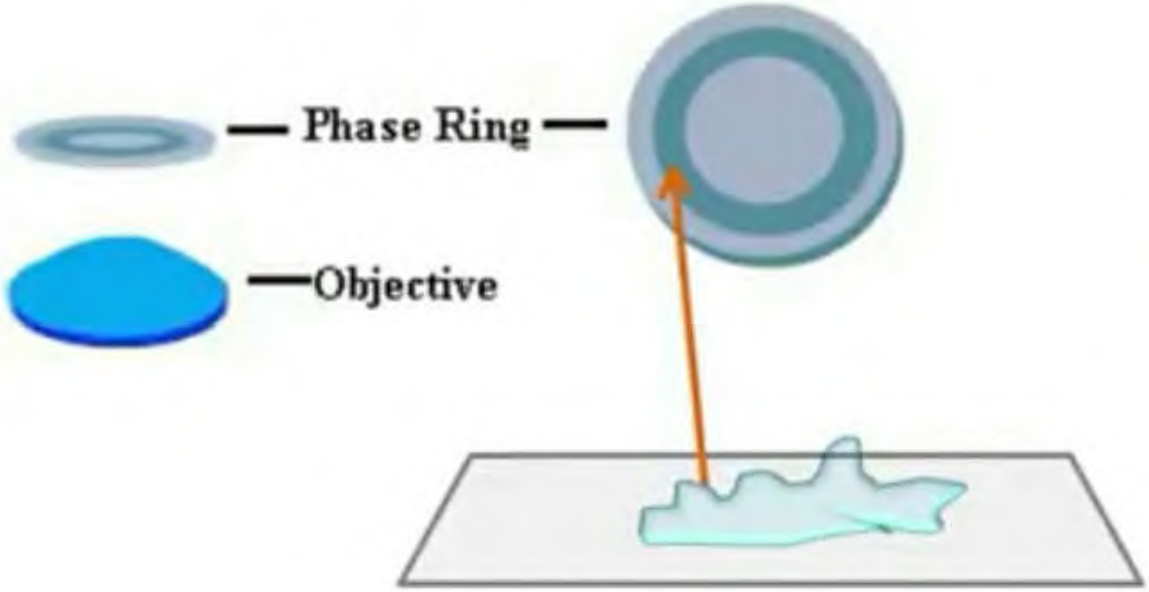
How does it work ?

- 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 ?

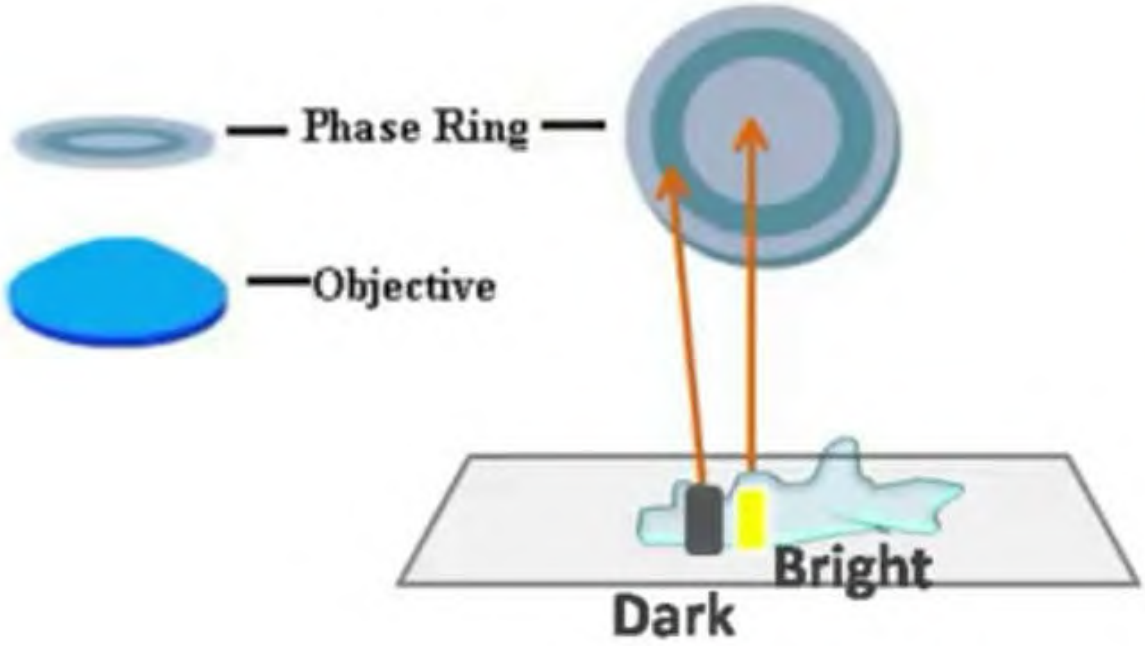
- Light that does not interact with the speciman is collected by the objective passes through the phase ring, and is regarded exactly ¼ wavelength.
- The Phase shifted is not detectable by the eye so the resulting image on the image plane in the microscope appears as a normal bright background





Refraction in different directions

Light passing through the specimen is phase shifted



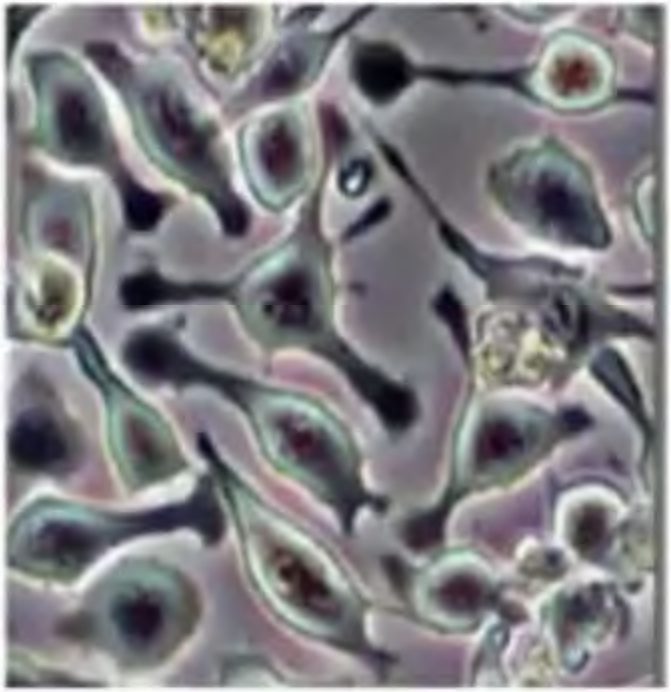
Refraction in different directions

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

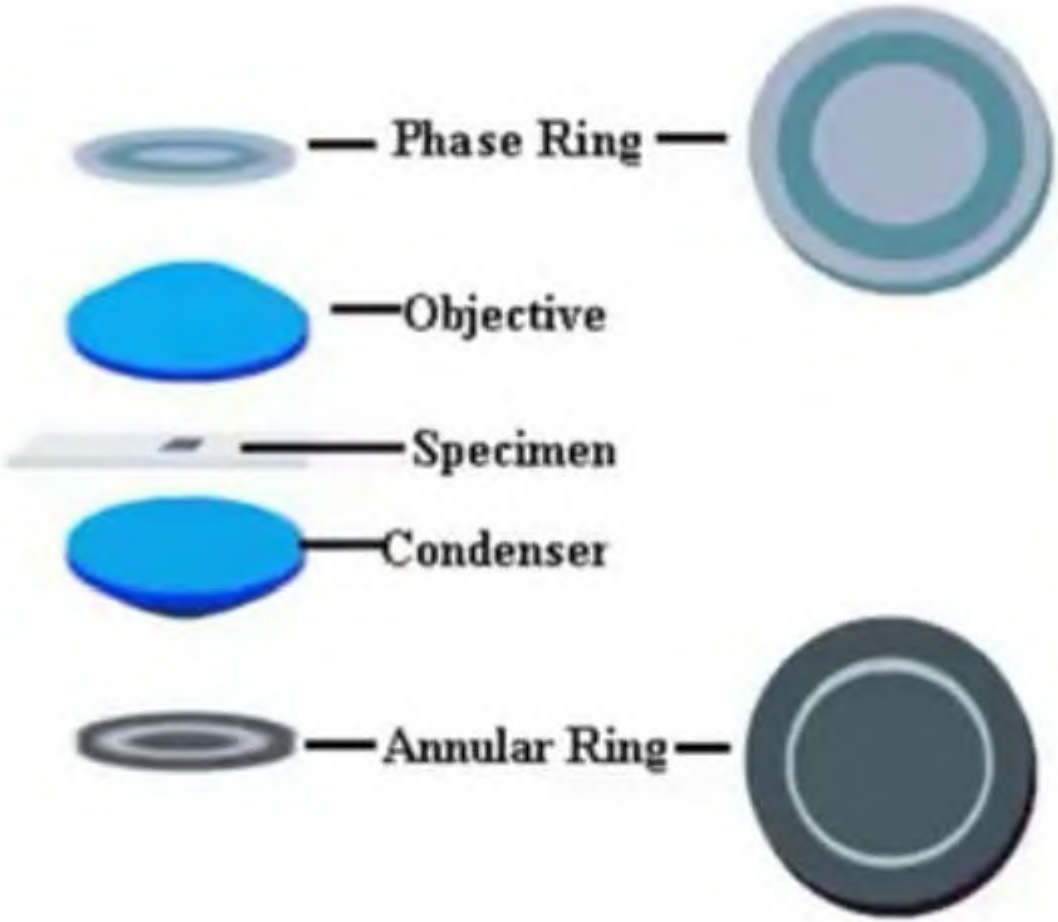
- Today, two main types of phase contrast are positive and negative. Since the observed particles are usually thin and transparent, these polar contrasts provide strikingly different images.
- **Positive phase contrast** reveals medium to dark gray images on a lighter grey background; these images often 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:

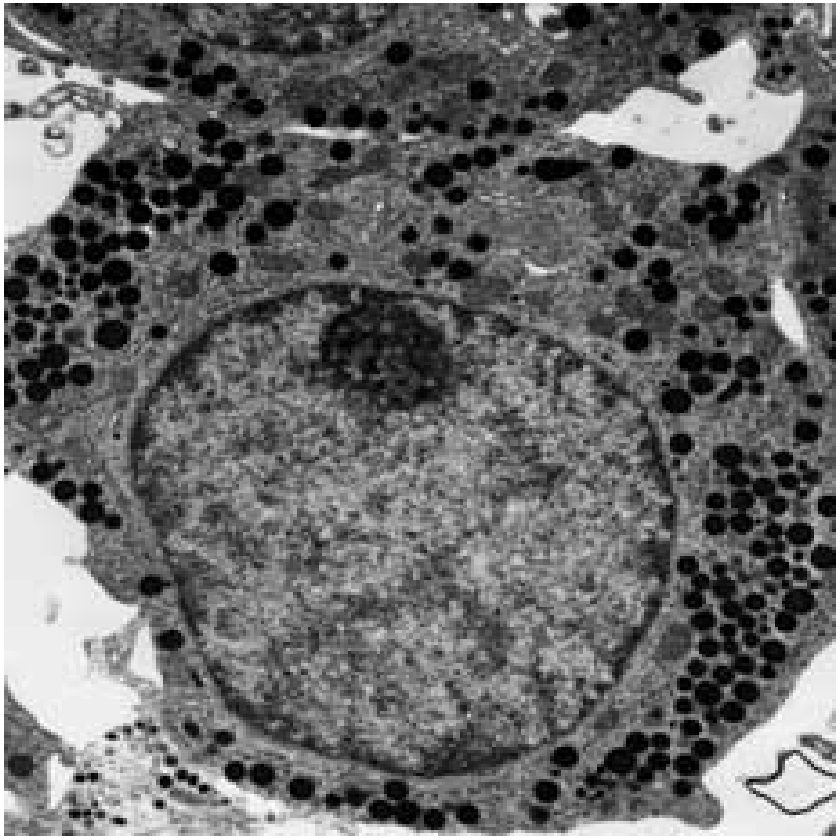
the background light is instead phase shifted by +90°. The background light will thus be 180° out of phase relative to the scattered light. • Leads to formation of an image where the foreground is darker than the background.


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.

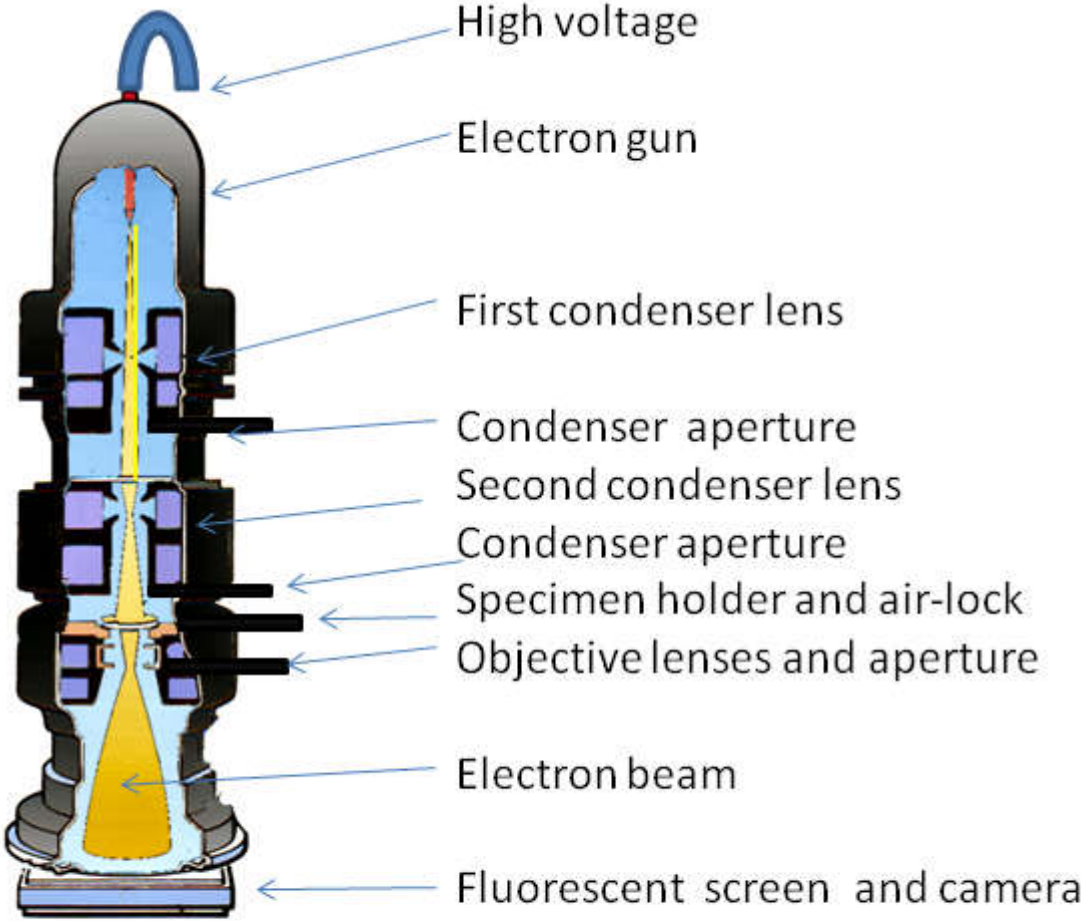
- **Scanning EM (SEM).**
- A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons annd is used to view the 3d structure of sample
- Specimen preparartion for TEM and SEM is done in different ways.

USES oF ELECTRON MICROSCOPY

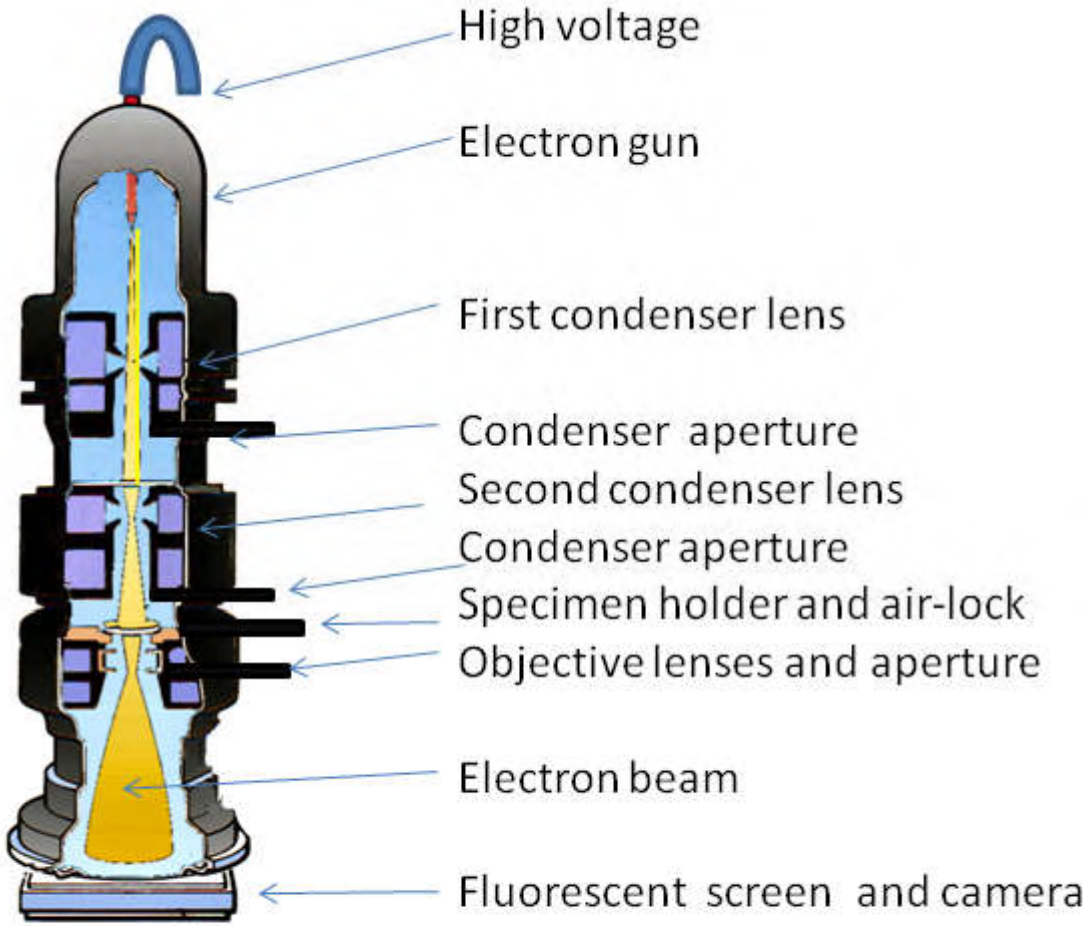
- Even the very best light microscopes have a resolution limit of about 0.2 µm, which greatly compromises their usefulness for detailed studies of many microorganisms.
- Viruses are too small to be seen with light microscopes. Prokaryotes can be observed, but because they are usually only 1 m to 2 m 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



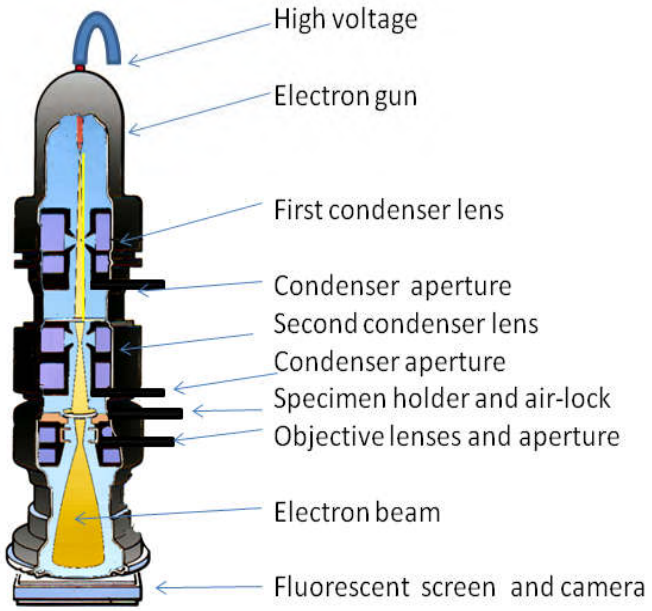
Transmission Electron Microscope

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



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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

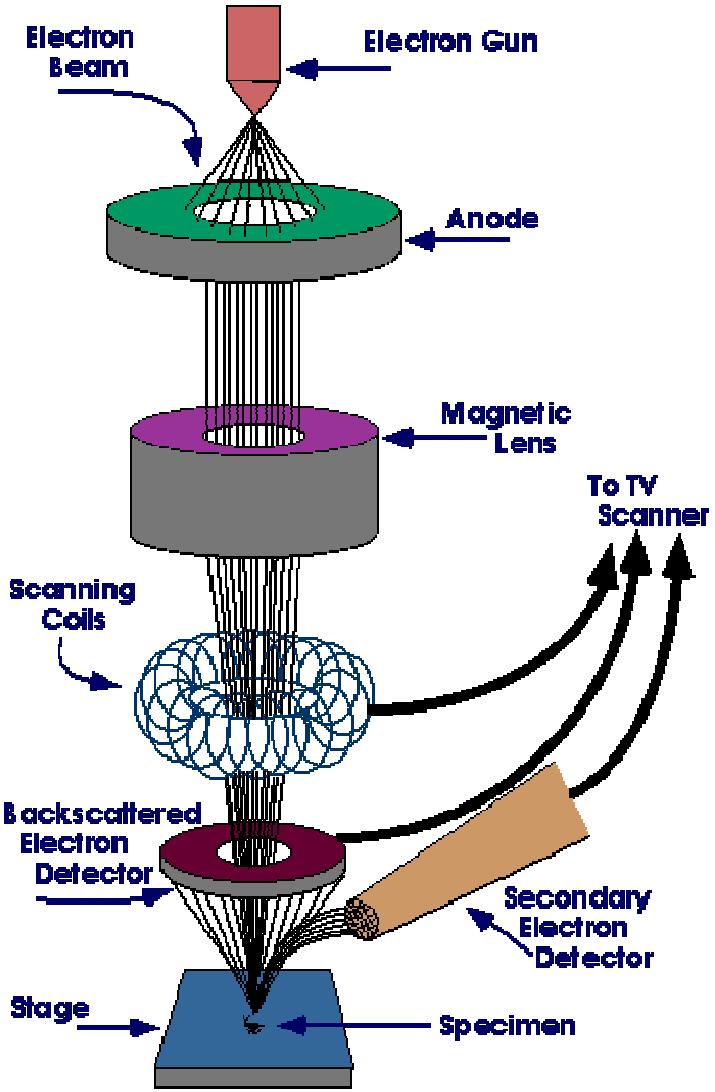
- The TEM will also disclose the shape of organelles within microorganisms if specimens are prepared by **the freezeetching procedure**.
- First, cells are rapidly frozen in liquid nitrogen and then warmed to 100°C in a vacuum chamber. Next a knife that has been precooled with liquid nitrogen **(-196°C)** 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

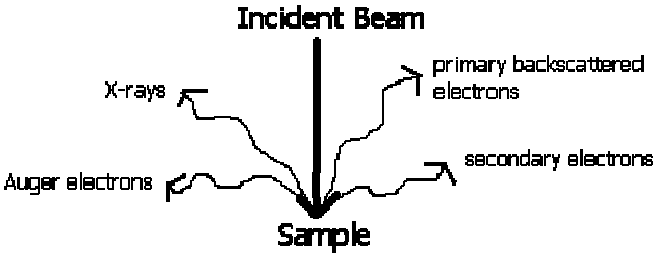
- A **scanning electron microscope** (**SEM**) is a type of **electron microscope** that produces images of a sample by scanning the surface with a focused beam of **electrons**
- The SEM has allowed researchers to examine a much bigger variety of specimens.
- The electrons interact with atoms in the 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?



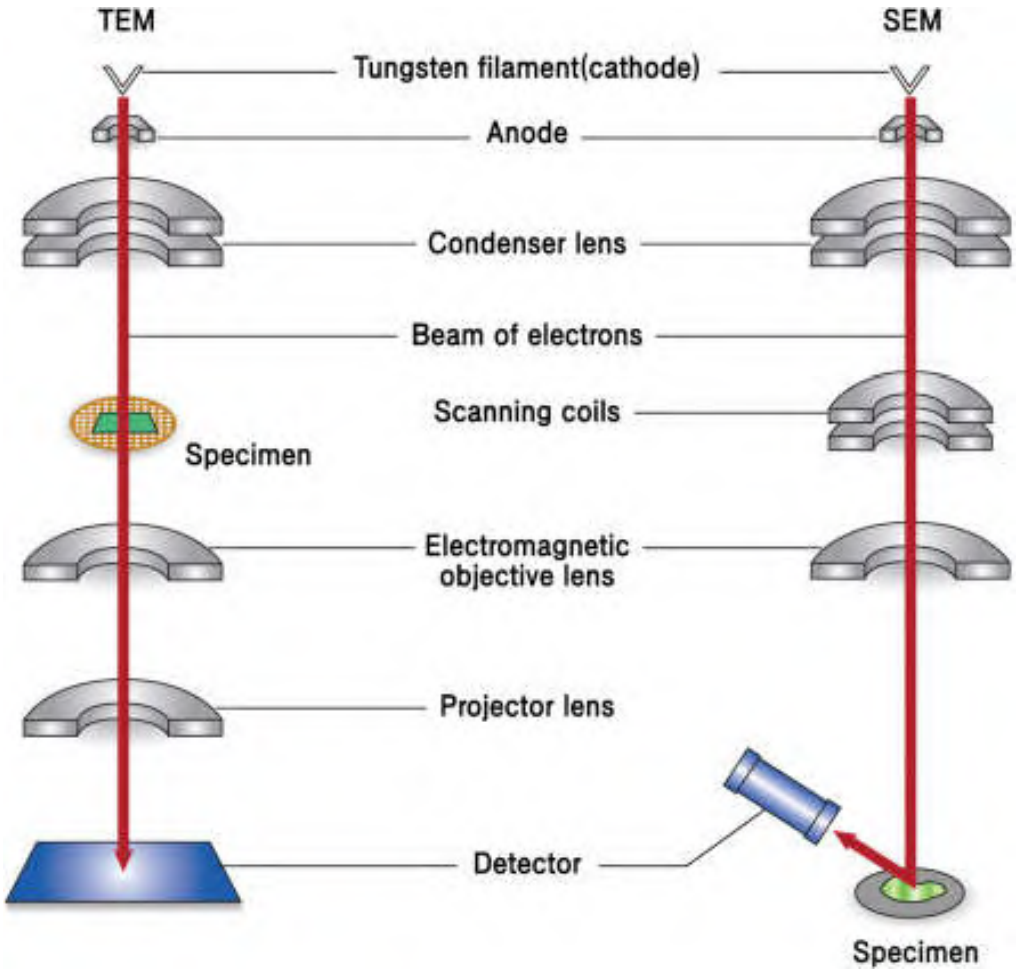
How does SEM work?

- 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

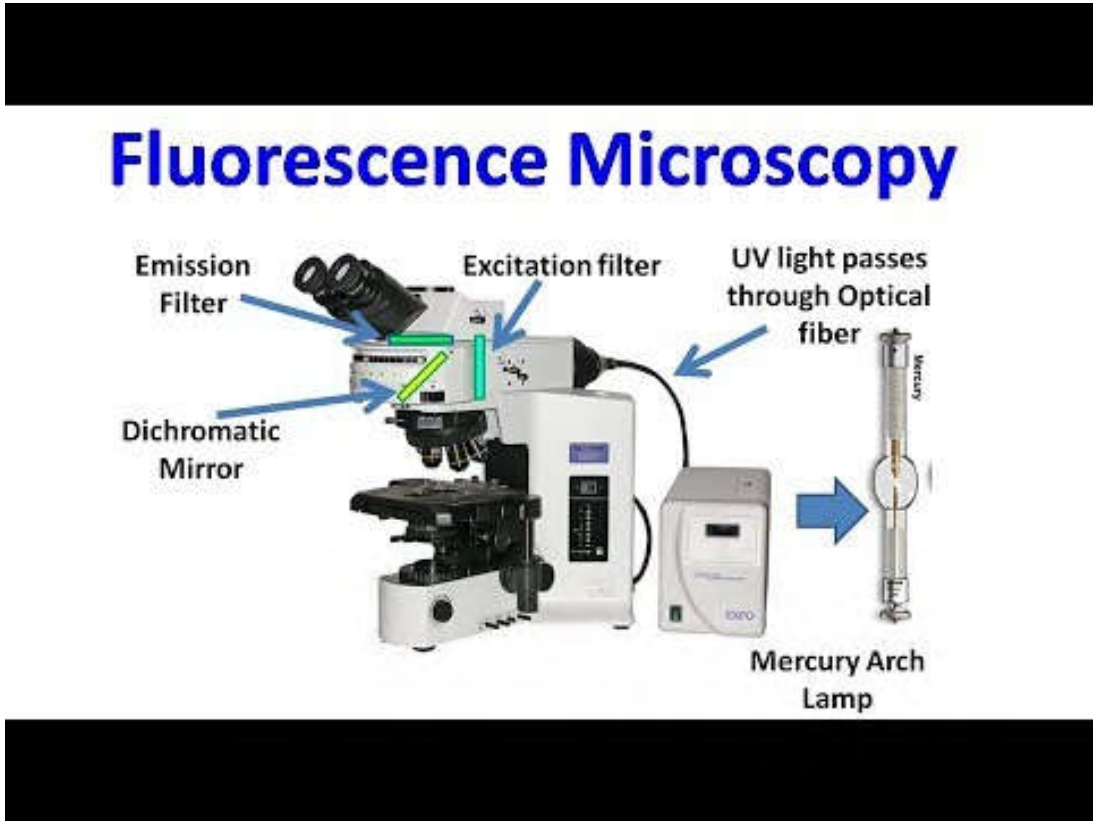
Lesson : Fluorescence Microscope

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.

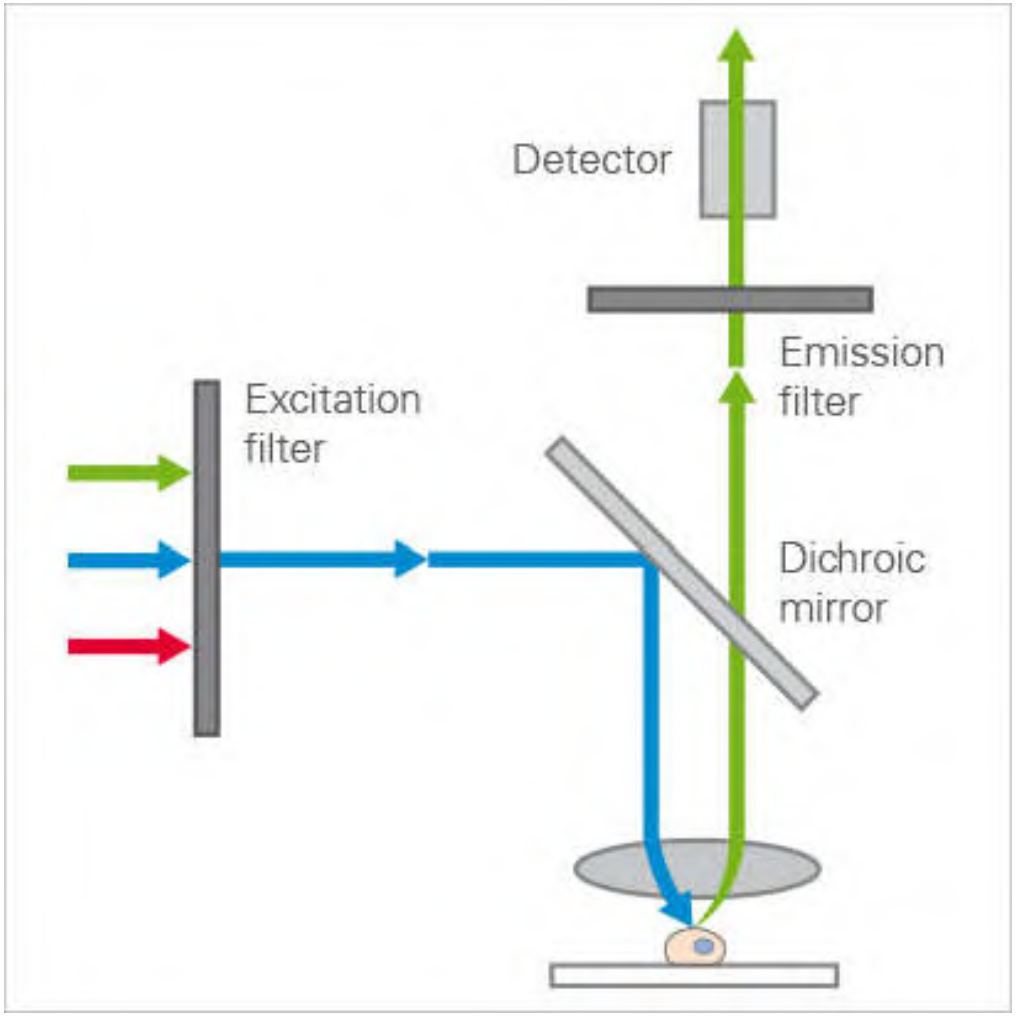
- This is achieved by using powerful light sources, such as lasers, that can be focused to a pinpoint.
- This focusing is done repeatedly throughout one level of a specimen after another.
- Most often an image reconstruction program pieces the multi level image data together into a 3-4 D reconstruction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

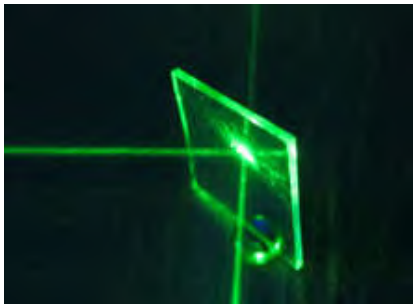

Detector
Emission filter
Excitation filter
Dichroic mirror

Types of Fluorescence Microscopes

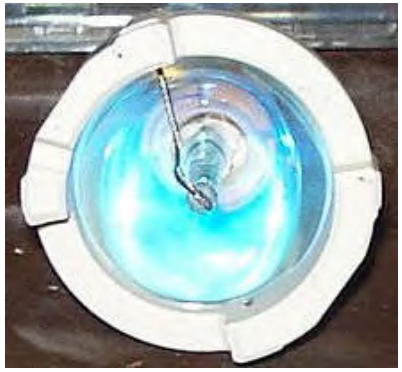
There are various types of fluorescence microscopes. Some of the common types are:

- **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.

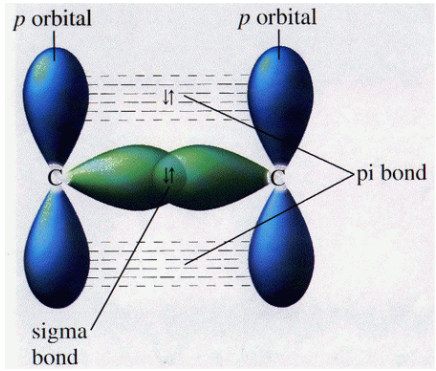
- A **beam splitter** is an **optical device** that splits a beam of light in two. It is a crucial part of many optical experimental and measurement systems, as fluorescent mcicroscope or fibre optic telecommunications.



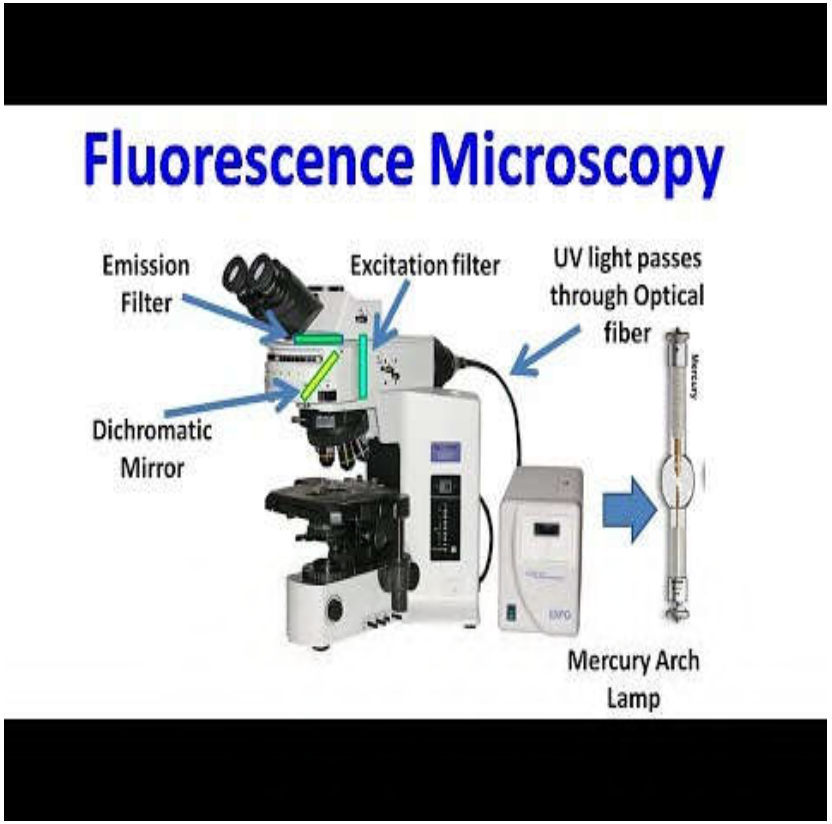
- A **xenon arc lamp** is a highly specialized type of **GAS discharge lamp**, an electric light that produces light by passing electricity through IONIZED XENON gas at high pressure. It produces a bright white light that closely mimics natural sunlight.



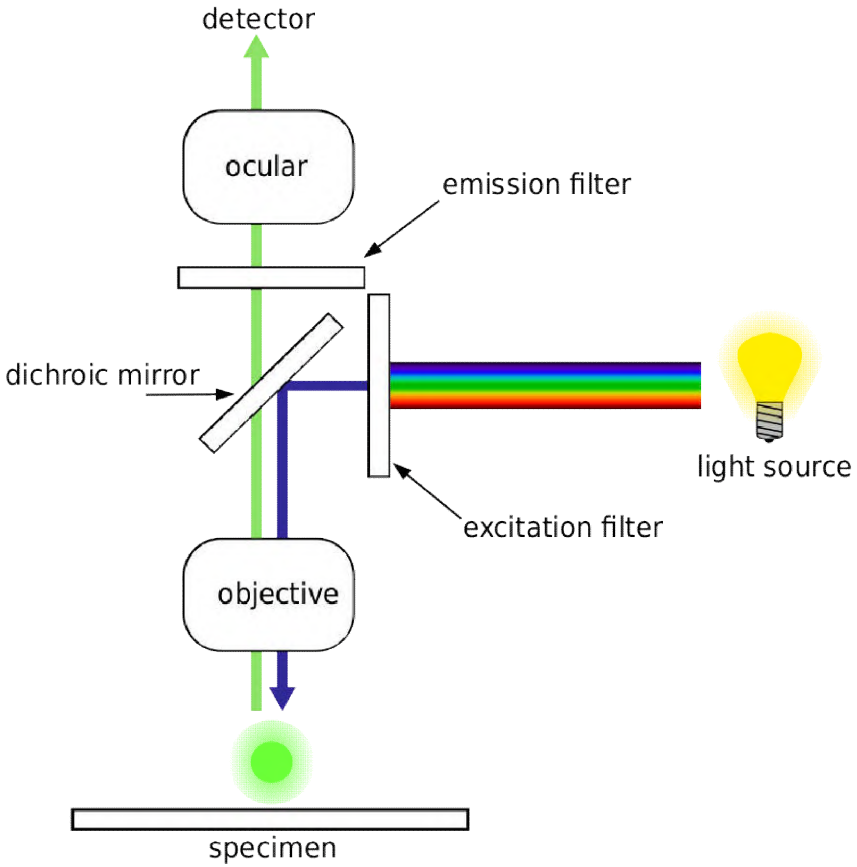
- **Sigma and pi bonds** are chemical covalent **bonds**. **Sigma and pi bonds** are formed by the overlap of atomic orbitals. **Sigma bonds** are formed by end-to-end overlapping and **Pi bonds** are when the lobe of one atomic orbital overlaps another. ... Generally **sigma bonds** are stronger than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



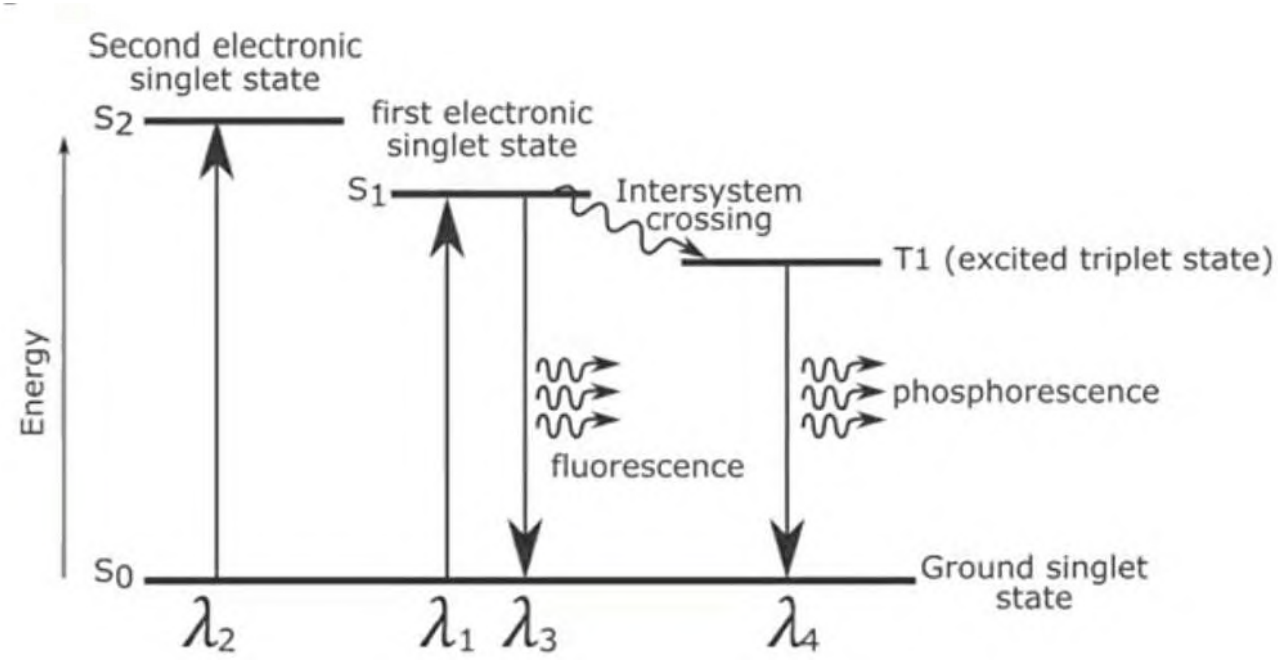
Light path

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 obser-ved. 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 eye-piece 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.

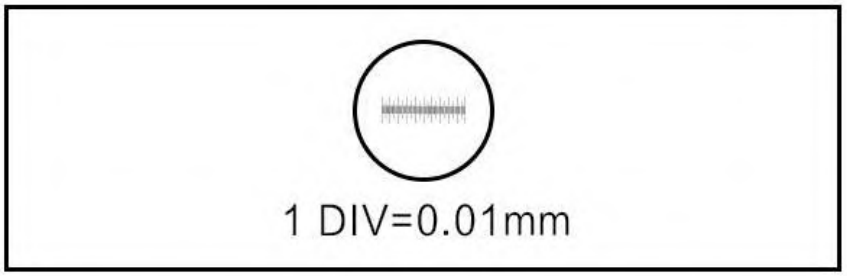
1. stage micrometer and
2. ocular meter or ocular micrometer

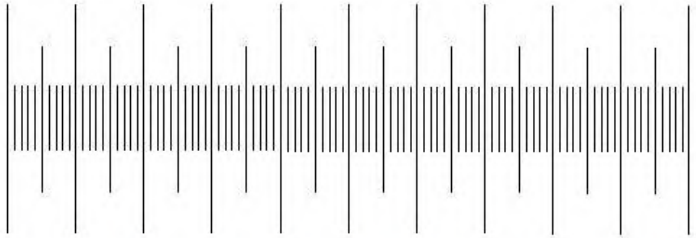
1. Stage Micrometer:

- **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

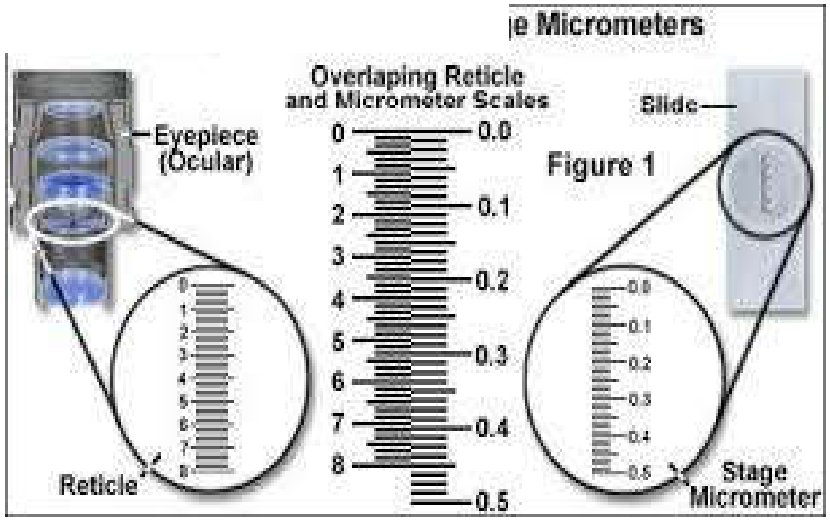
- The ocular micrometer is a glass disc with 100 equal divisions or lines on it but with no absolute value
- This micrometer is used inside the eyepiece.
- The upper eye lens is unscrewed and the ocular meter is put into the tube of eyepiece, and the eye lens is again replaced in its original position.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

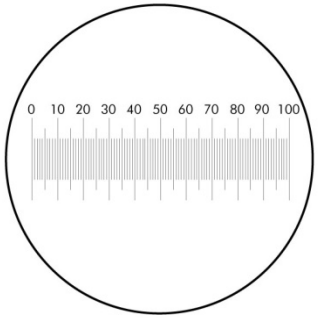


Lesson 17: Micrometry -calibration

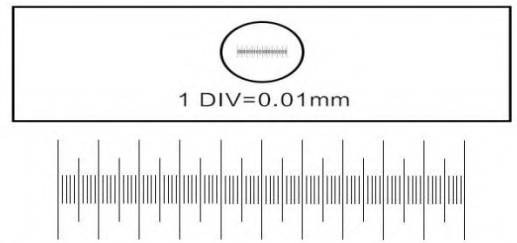
calibration

- **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.

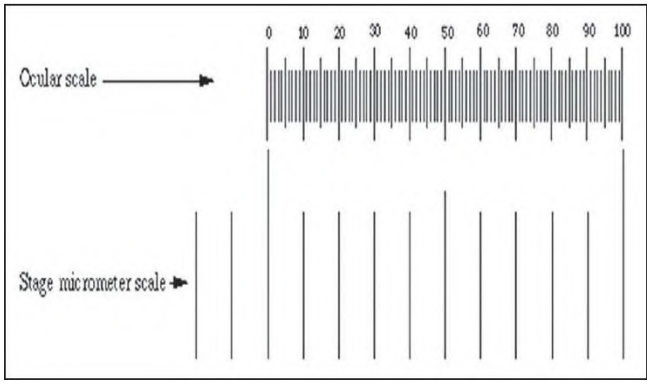
- The disc has numbered lines on it as is shown :



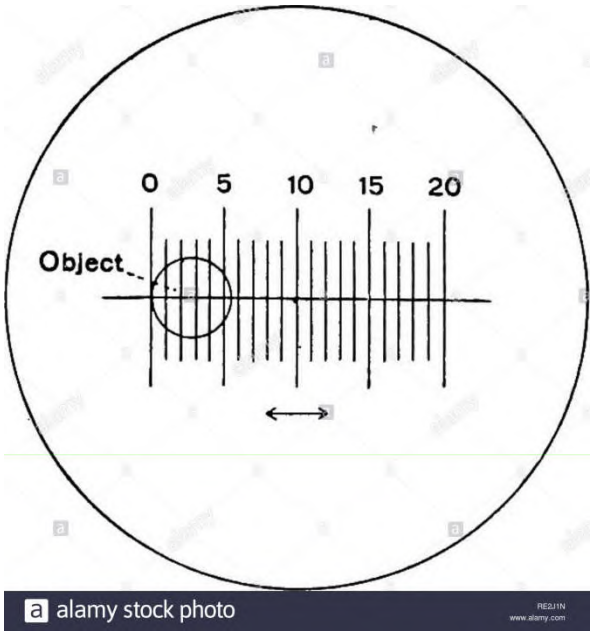
- The units might be different on different ocular micrometers; i.e., some go up to 10 or 50 while others go up to 100. Our main concern here is to determine the length of one unit of the ocular micrometer. For this purpose, we need to calibrate this unit against a known length. There are prepared slides 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:

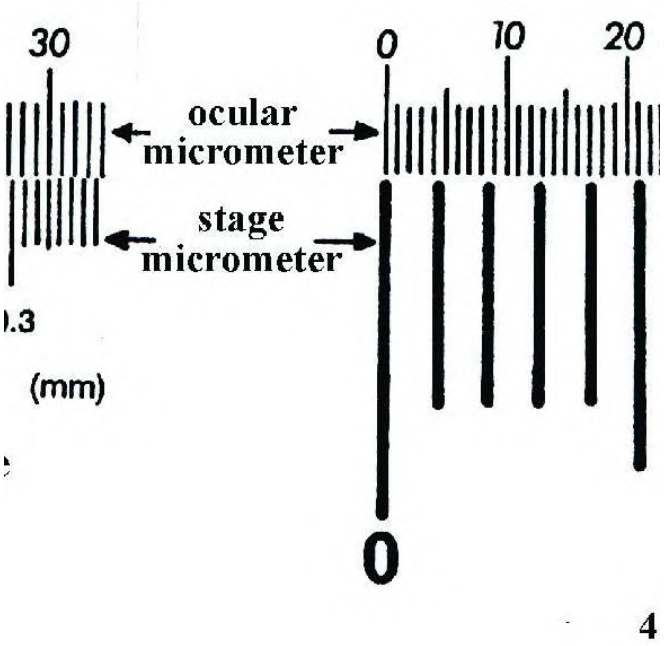


1. To be able to measure the size of microorganisms, an ocular micrometer disc is placed in one of the oculars.
2. After calibration, the ocular micrometer measures the size of various microbes including its length, breadth, and diameter.
3. **First put the stage micrometer on stage and let 1 end of 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:**



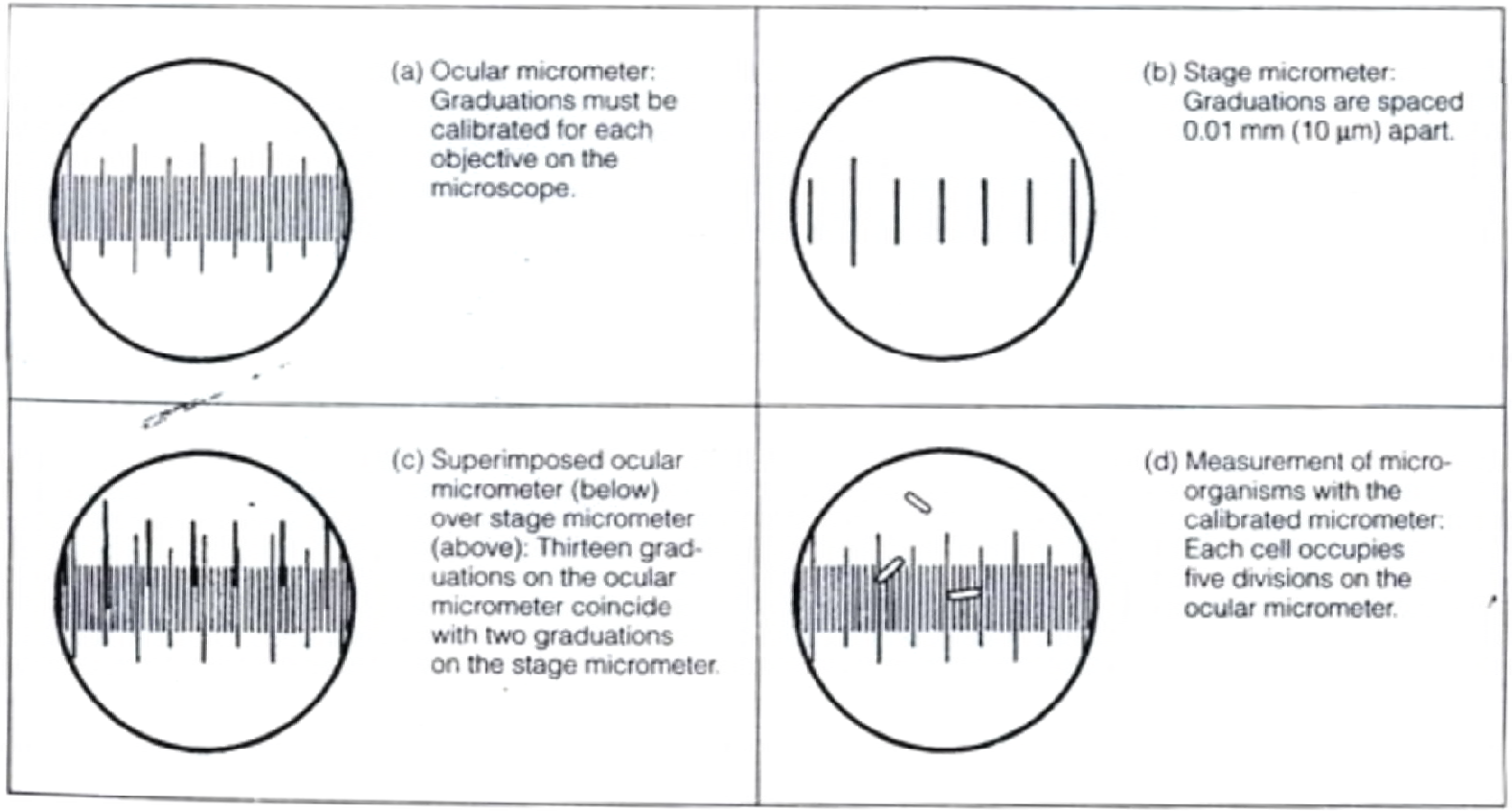
$$\text{One division of ocular micrometer} = \frac{\text{No. of divisions on stage micrometer}}{\text{Number of divisions on ocular micrometer}} \times 10$$


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)-2

Properties of a fixative

- Support high quality and consistent staining with (H&E) both initially and after storage of the paraffin blocks.
- Prevent short- and long-term destruction of the micro-architecture of the tissue by stopping the activity of catabolic enzymes and hence autolysis,.
- Destruction of infectious agents, -maintain tissue and cellular integrity.
- Important to have good toxicological and flammability profiles that permit the safe use of the fixative.
- Versatile
- Preserve small & large specimens and support histochemical, immuno-histochemical, in situ hybridization and other specialized procedures.
- Penetrate and fix tissues rapidly, -shelf life of at least one year. Readily disposable/recyclable and support long-term tissue storage
- Cost effective.
- Non-toxic and non-allergenic (hypoallergenic)

Coagulant fixative

- Cellular architecture maintained- lipoproteins and fibrous proteins (collagen)

- Coagulation of proteins-makes them insoluble.
- Coagulant fixatives -cytoplasmic flocculation
- Not useful -ultrastructural analysis.
- Commonly used (e.g. 50-60% ethanol, 80% methanol).
- Remove and replace free water from tissue.
- Coagulant fixatives

- Molecules of water participate in hydrogen bonding
- Removal of water destabilizes hydrogen bonding.
- Other types of coagulant fixative Acidic coagulants such as picric acid and trichloroacetic acid Change the charges on the ionizable side chains of protein- disruption electrostatic and hydrogen bonding.

Coagulant fixative

- Trichloroacetic acid (Cl3CCOOH) can penetrate hydrophobic domains of proteins and the anion produced reacts with charged amine groups- precipitates proteins.
- Picric acid forms salts with basic groups of proteins coagulate
- Fixation by picric acid produces brighter staining.

Bouin solution

- **Bouin solution** is a preparation of 5% acetic acid, 9% formaldehyde, and 1.5% picric acid in aqueous **solution**.
- The picric acid in **Bouin solution** presents challenges for safe handling and disposal due to its mutagenic and explosive potential.

mutagenic chemicals:

a mutagen is a physical or chemical agent that changes the genetic material, usually DNA, of an organism and thus increases the frequency of mutations above the natural background level. As many mutations can cause cancer, mutagens are therefore also likely to be CARCINOGENS, although not always necessarily so.

explosive chemicals

An **explosive** (or **explosive material**) is a reactive substance that contains a great amount of potential energy that can produce an explosion if released suddenly, usually accompanied by the production of LIGHT, HEAT and sound, and pressure.

An **explosive charge** is a measured quantity of explosive material, which may either be composed solely of one ingredient or be a mixture containing at least two substances.

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
https://fas.org/man/dod101/navy/docs/es310/chemstry/chemstry.htm

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
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Microtomy- Types of fixatives And methods of tissue fixation

Types of fixatives

There are several methods for fixating tissues that are selected according to the type of fixative, the structure to be fixed, and what we want to observe.

Fixation methods can be grouped in two:

1. **Physical methods**
   a. **Quick freezing**
   b. **Fixation by heating**

2. **chemical methods.**

1. Quick freezing

- Quick freezing is a suitable method for preserving molecular features of samples because they are nor affected by any external chemical compound.
- Freezing must be very fast to prevent autolysis of the cells and formation of large ice crystals that may destroy the tissue structure.
- it is convenient to process sample sizes not larger than 2 mm so that freezing is homogeneous through all the tissue thickness, including the inner parts.
- before freezing, the samples should be pretreated by a cryoprotectant that prevent cellular freezing damages.

- **Fast freezing chemicals**
- A very fast freezing is achieved by immersing the samples in to the following
- isopentane (-170 ºC)
- liquid nitrogen (-196 ºC)
- in dry ice
- acetone (-70 ºC)
- liquid helium (-268 ºC).

Cryoprotectants for Quick freezing

- Cryoprotection is always a good practice, although it is not always possible.
- Dimethyl sulfoxide
- Glycerol
- sucrose or mixed solutions containing some of these substances are the most common cryoprotectants

2.Fixation by heating

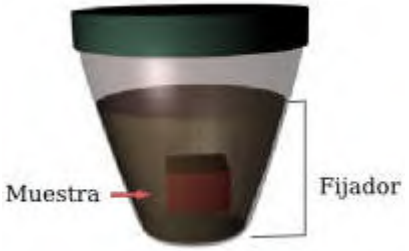
- Fixation by heating is not common in histology because it introduces damages in the tissues.
- Heat coagulates proteins and dissolves lipids.
- However, it is a good fixative method for microorganisms because their morphology is preserved, and that helps in the identification.
- In Some protocols, heating fixation is combined with chemical fixation. For example, samples are immersed in a chemical fixative and microwaved at 55 ºC. (tissue immersed in citrate buffer and heated in microwave while staining)
- This temperature does not produce artifacts, increases the fixation speed, and decreases the fixation time from hours or days to minutes.
- Microwave is a good heater because temperature rises quite homogeneously and immediately through the sample. Microwaves are sometimes used in later steps of the histological processing, particularly during staining.

Chemical methods

- Chemical methods use aqueous solutions containing fixative substances that make bridges between tissular molecules and results in the immobilization of the tissular compounds and prevents degradation of the tissue sample.
- affect tissues both chemically and physically.
- physical effects include Retraction, distension, and hardening. There are two major chemical methods:

1. immersion
2. perfusion.

1.immersion



Tissues are plunged into fixative solutions.

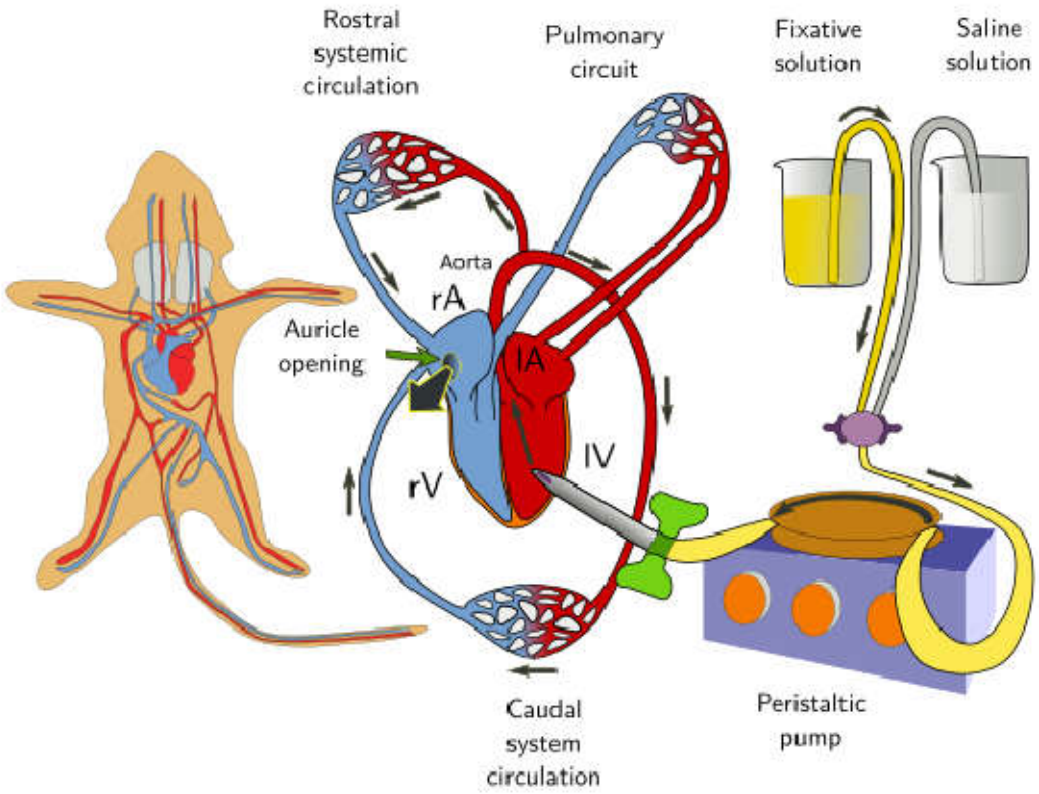
Precautionary measures include:

1. The piece of tissue should not be larger than 0.5 cm in thickness to allow the fixative enters the deeper part of the sample before the cells start to get damaged.
2. Penetration speed depends on the fixative type and the tissue to be fixed.
3. The size of the sample should be considered according to the fixative type.
4. Slow diffusion fixatives are recommended in samples not larger than 0.2 cm.
5. The volume of the fixative needs to be 10 to 20 times larger than the volume of the sample.
6. Osmolarity of the sample and the fixative solution need as similar as possible.
7. The pH of the fixative solution should be close to the physiological pH of the sample.
8. The fixation time must be enough for a proper fixation, but not excessive because it may cause artifacts in the sample. A gentle agitation of the sample during fixation reduces the fixation time.
9. common duration for most of the fixatives is 24 hours

2. Perfusion

- In this fixation method, the fixative solution is introduced through the vascular system and reach all the cells of the tissue via capillaries
- if the fixative solution enters through the left ventricle of the heart, a whole animal can be fixed.
- **Then, the fixative solution is driven through the vascular branches of the arteries coming from this ventricle. If we want the fixative in the lungs, the fixative solution is introduced through the right ventricle.**
- Fixation by perfusion is not in many biopsies or in plants.
- Fixation by perfusion is more effective than fixation by immersion because the fixative solution gets in contact with all cells of the perfused structure very quickly.
- Before the introduction of the fixative in the vascular system, the blood should be removed with an oxygenated saline solution.
- Otherwise, the fixative may fix blood, coagulates it and produces thrombus.
- As in the fixation by immersion, the pH and osmolarity of the fixative solution, and the fixation time, must be set properly.

2. Perfusion



Fixation by perfusion of a complete animal. The fixative solution is introduced through the vascular system. Pressure is provided by a peristaltic pump. The fixative solution enters via the left ventricle (lV) and reach the aorta. This artery and its branches distribute the fixative through the body (excepting the pulmonary circuit). The fixative arrives to and fills the capillary net, where most of the fixation process occurs. After that, fixative solution is gathered by venous system that converges in the right auricle (rA). This heart chamber is opened with a small cut to open the circuit and allow the fixative solution to leave the vascular system and the body. lA: left auricle; rV; right ventricle.

References

https://mmegias.webs.uvigo.es/02-english/6-tecnicas/2-metodos-fijacion.php

Lesson: Microtomy- Features & Parts of the Microtome

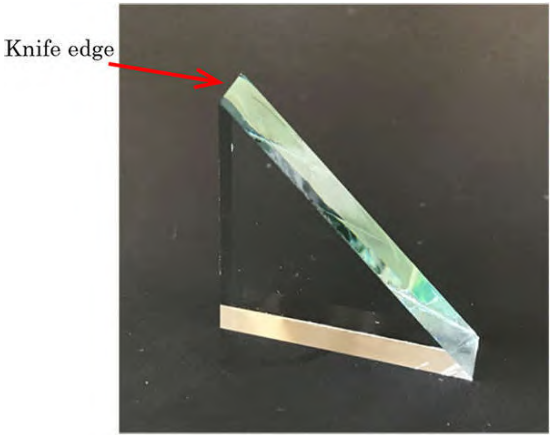
Types of Knives

- 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)

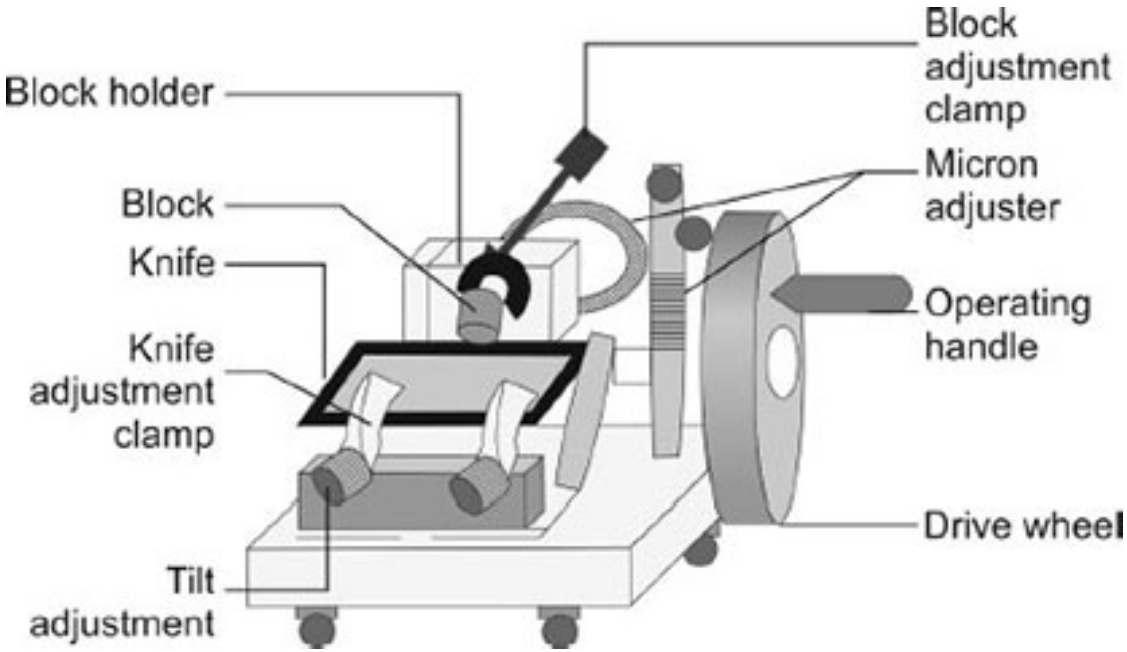
https://www.labce.com/spg605374_instrumentation_for_microtomy_rotary_microtome_par.aspx

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

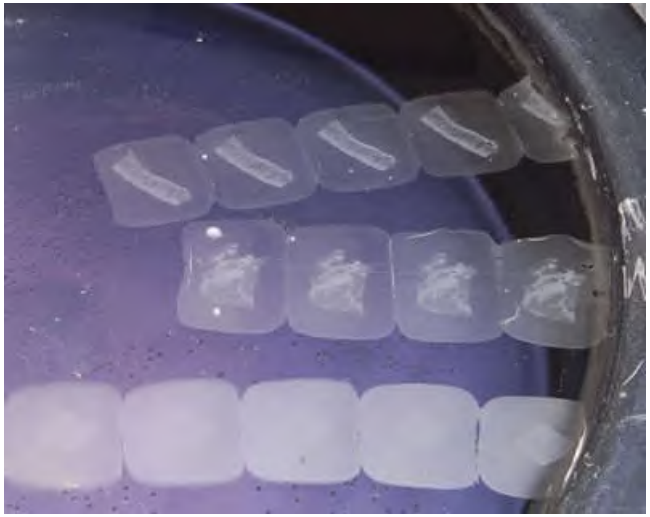
- **The most common type of the microtome**
- **Rotary action involves the sectioning process at predetermined thickness on every rotation of the drive wheel**
- Creating great paraffin sections using a rotary microtome takes a great deal of skill and experience.
- "Microtomy and Paraffin Section Preparation"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

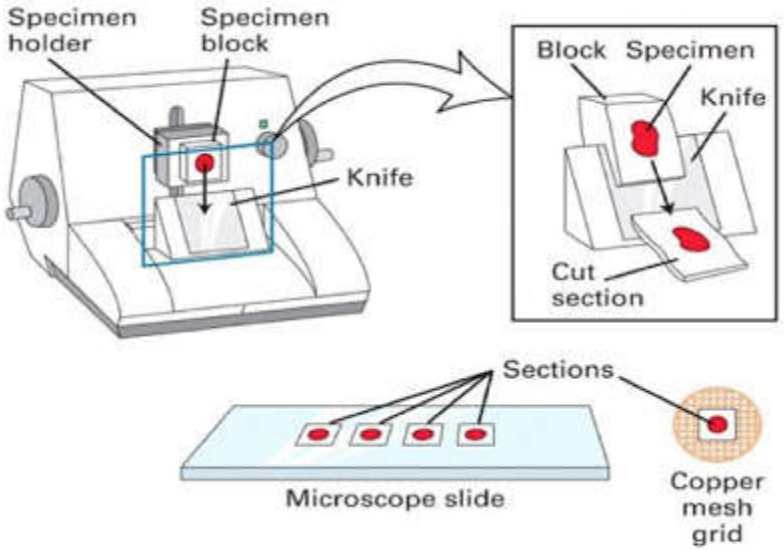
Procedure to use Rotary microtome

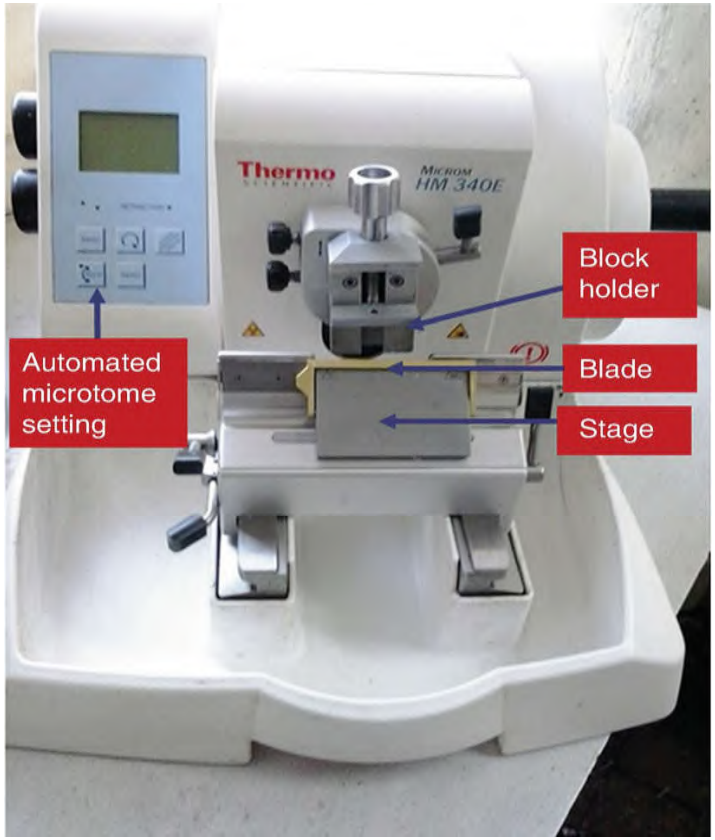
- Blade is fixed at horizontal position
- The sample holder moves the sample ahead by the fixed distance for cutting
- Drive wheel of the instrument may be automatic or manual
- Section thickness may vary from 1 µm to 60 µm
- The cut sections are floated on the top of a liquid
- These are then mounted 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

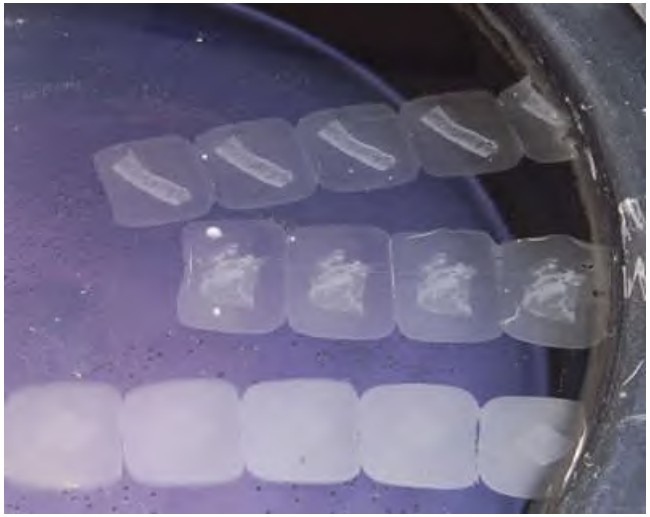
microscopic slides or some other

support for staining, etc.



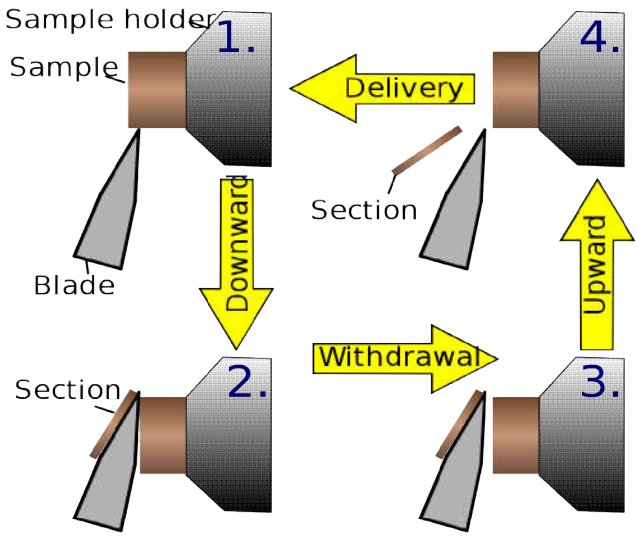

Specimen holder
Specimen block
Knife
Block
Specimen
Knife
Cut section
Sections
Microscope slide
Copper mesh grid


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

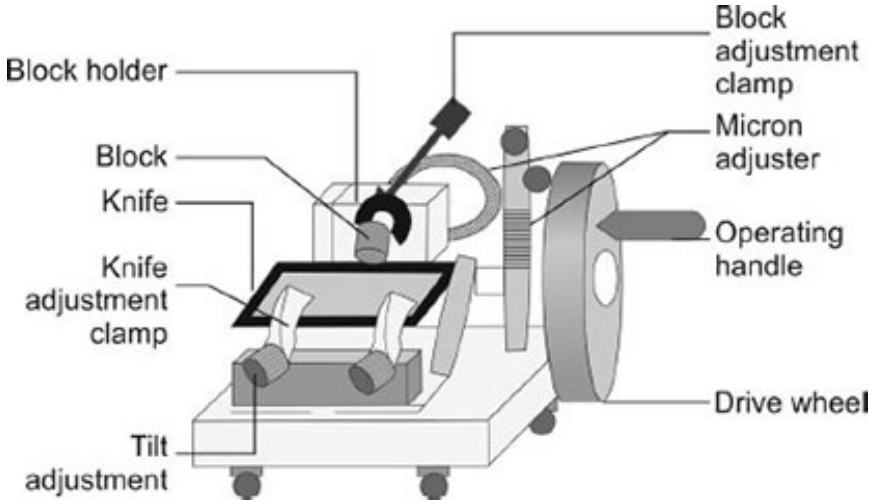


- The knife is motionless, remaining in a horizontal position.
- The machine body **itself rotates around the blade, slicing as it goes. For example, the machine holds the sample just above the knife, then it moves downward over the** knife, slicing a piece of the tissue off, then the machine pulls back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

- 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.



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

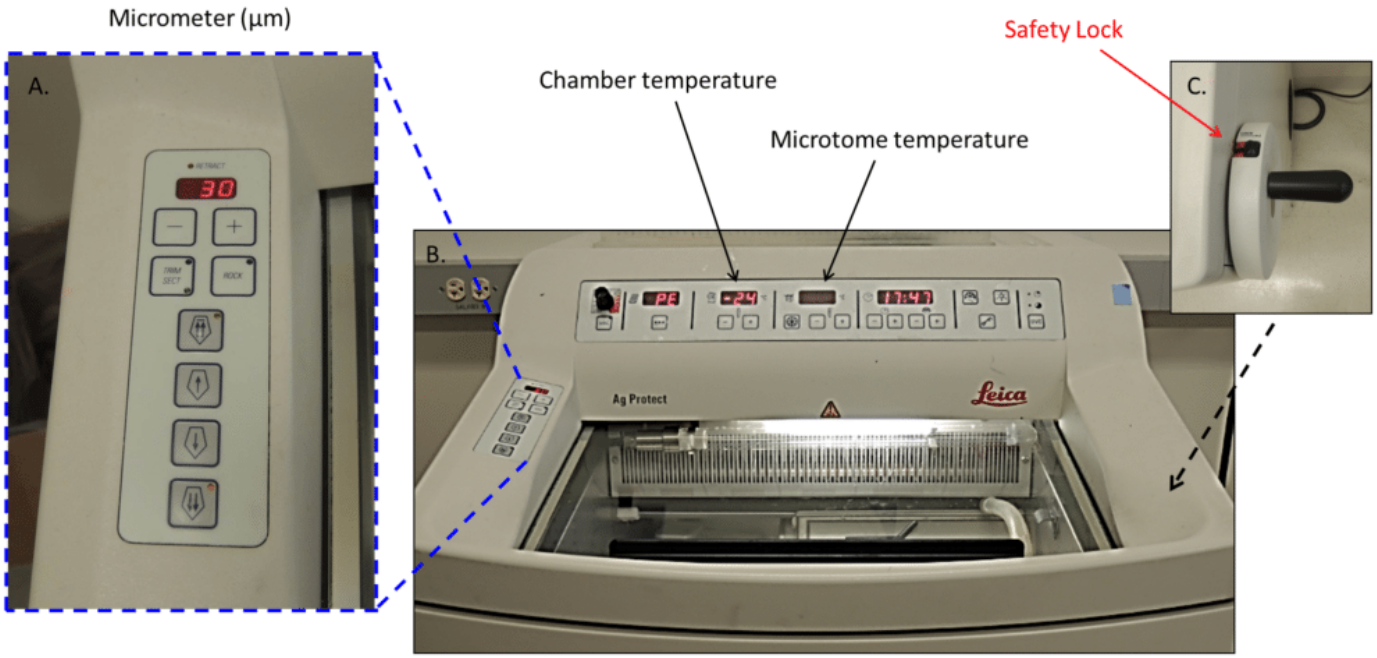


- https://conductscience.com/mastering-the-microtome/
- https://www.leicabiosystems.com/knowledge-pathway/steps-to-better-microtomy-flotation-section-drying/
- https://medsynapses.blogspot.com/2012/02/microtome.html
- https://www.deakin.edu.au/__data/assets/pdf_file/0003/1037460/Microtome-Basic-Operation-and-Safety-Procedures.pdf

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**

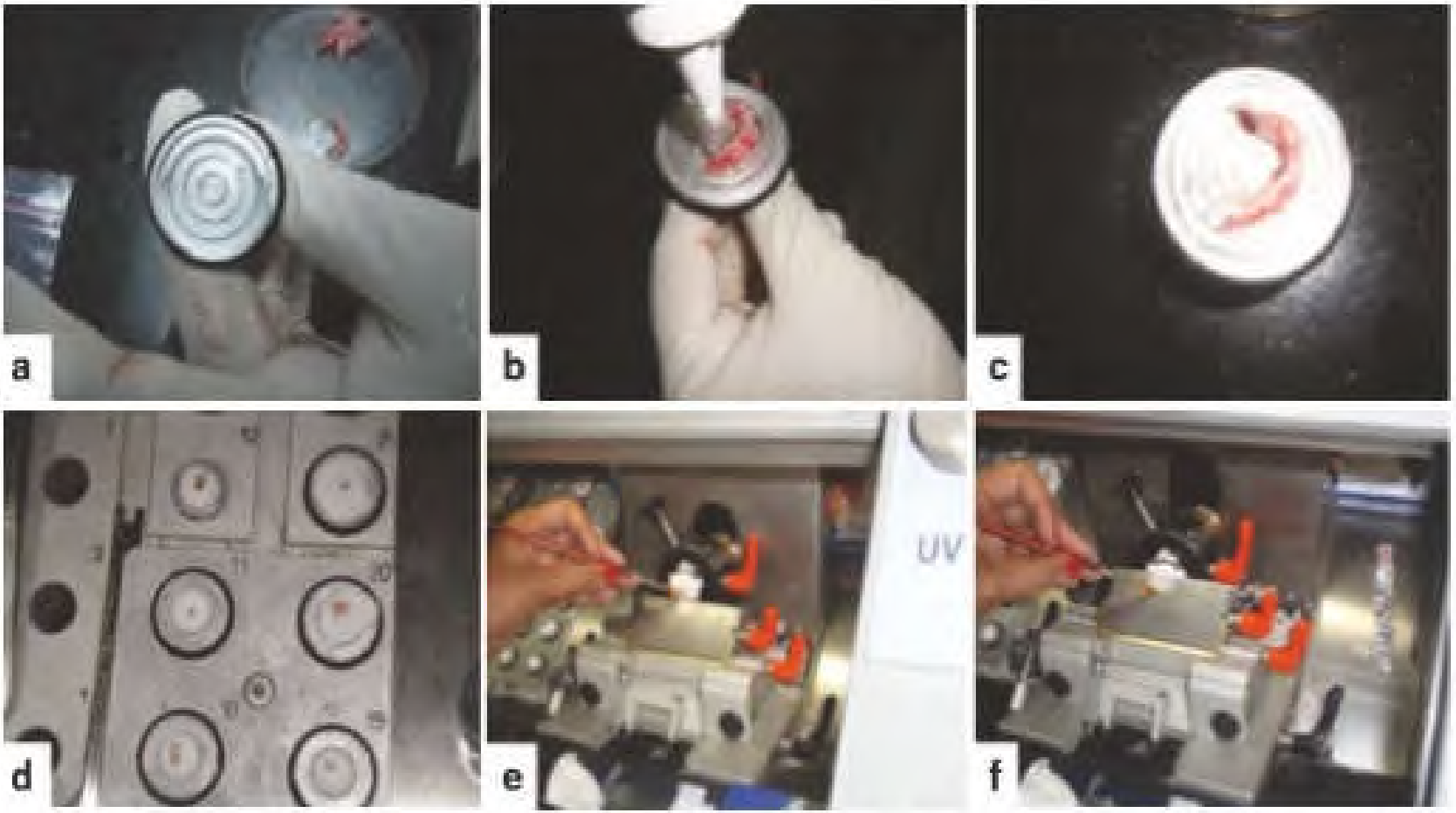
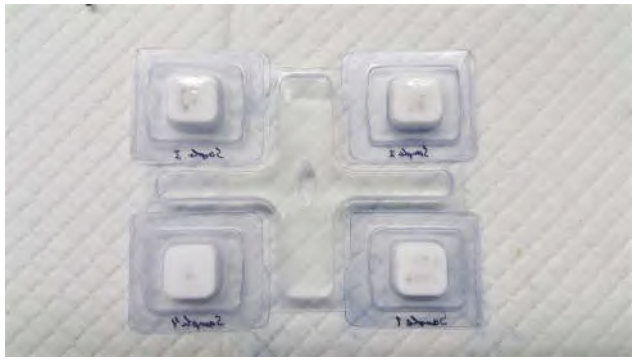


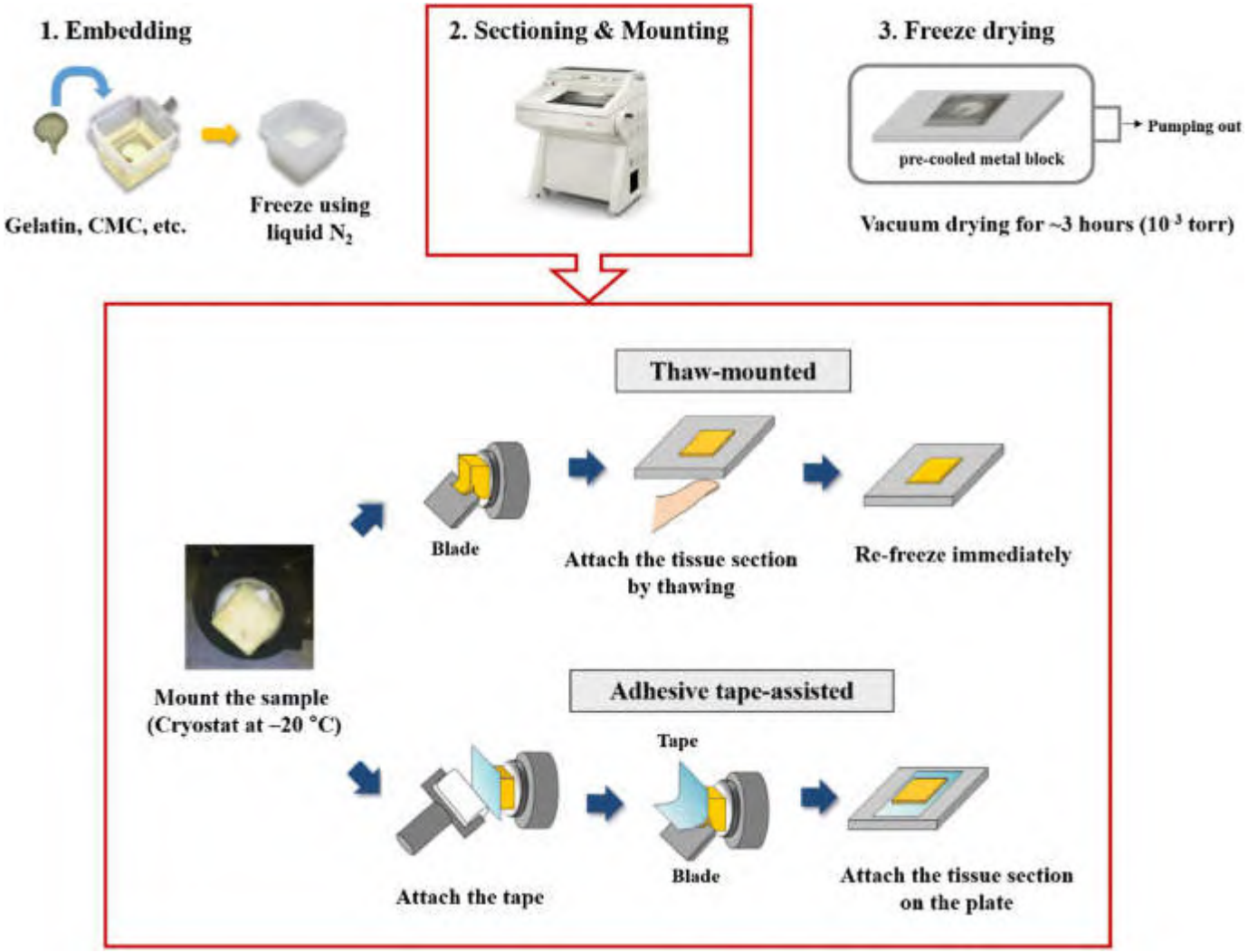
Fig. 6.3 Cryostat processing: (a) mould is covered with OCT, (b) the tissue is now put on the block, (c) OCT is flooded over the tissue, (d) the tissue now is put in the cooling chamber, (e) the brush guides the tip of the tissue, (f) the tissue section is gently spread over the antiroll plate and later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

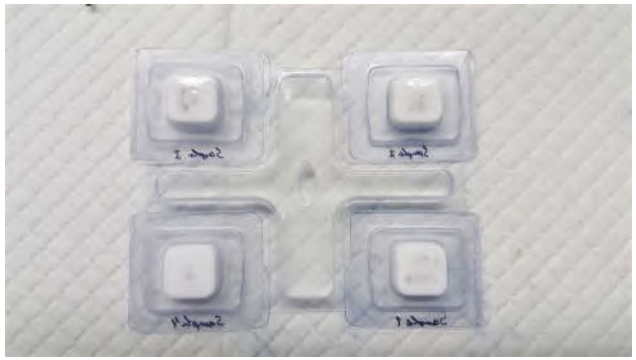

1. Embedding
Gelatin, CMC, etc.
Freeze using liquid N_2
2. Sectioning & Mounting
3. Freeze drying
Pumping out
pre-cooled metal block
Vacuum drying for ~3 hours (10^{-3} torr)
Thaw-mounted
Blade
Attach the tissue section by thawing
Re-freeze immediately
Mount the sample (Cryostat at –20 °C)
Adhesive tape-assisted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

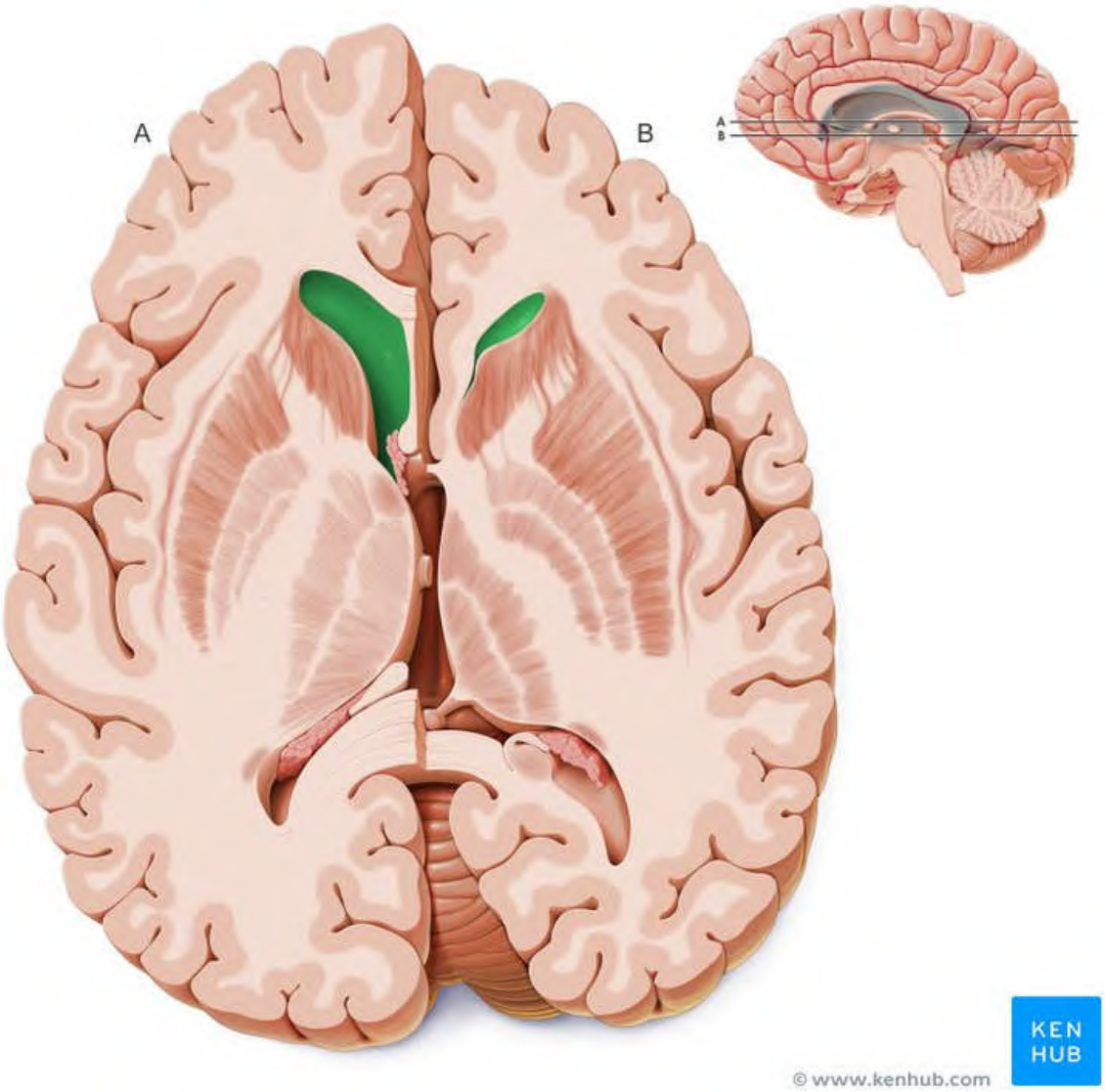


cryo-moulds

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.

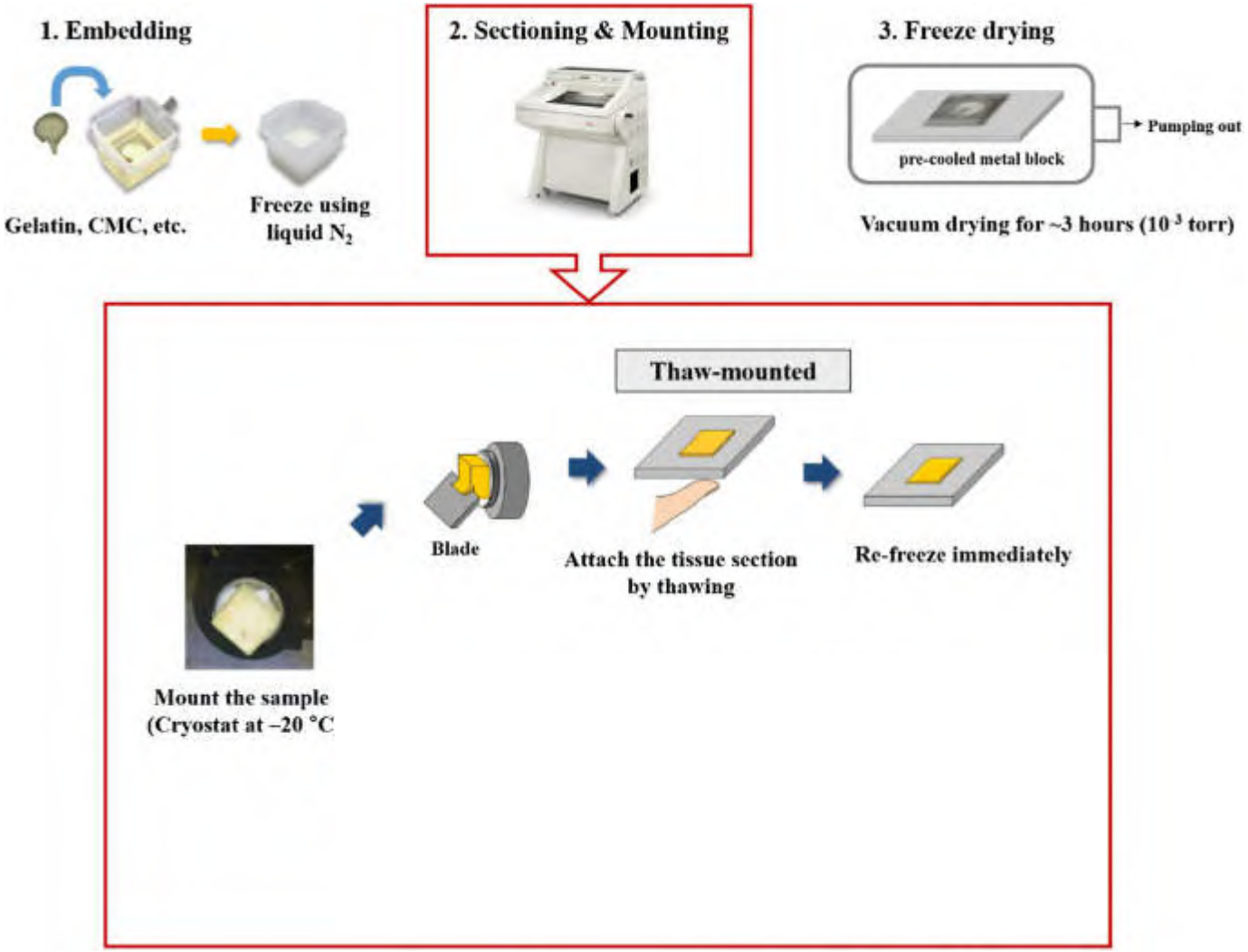


Sectioning by cryomicrotome

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!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Freeze using liquid N_2
2. Sectioning & Mounting
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pre-cooled metal block
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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

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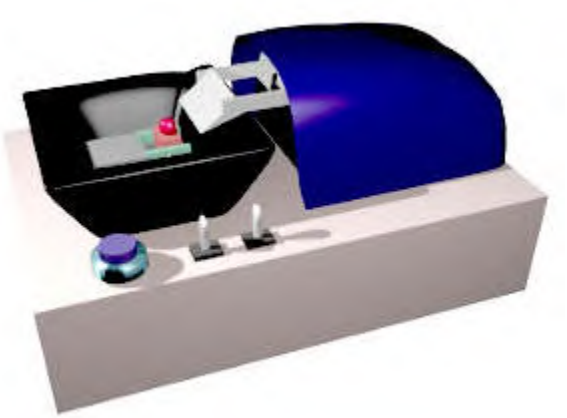
Lesson 31: Vibratome

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

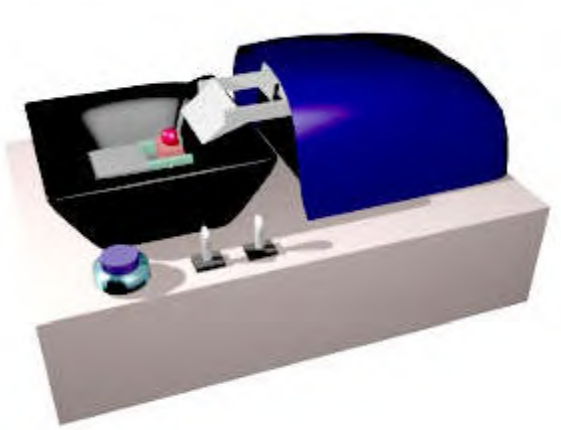
1. Only some samples are suitable for being sectioned with the vibratome. Those samples too soft or having hard or elastic parts are usually dragged by the blade, even if the blade speed is decreased and lateral vibration of the blade is ample.
2. It means that the sample should be consistent and as 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

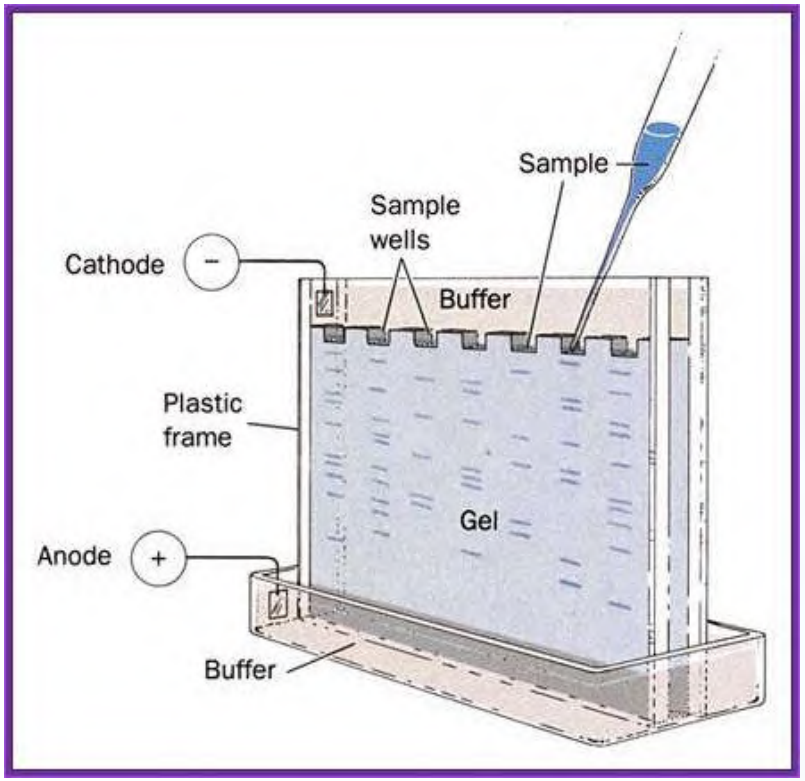
- DNA location within the gel can he determined by staining with low conc. of fluo-rescent intercalating dyes, such as ethidium bromide
- Bands containing as little as 20 pg of double-stranded DNA can be detected under UV.

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

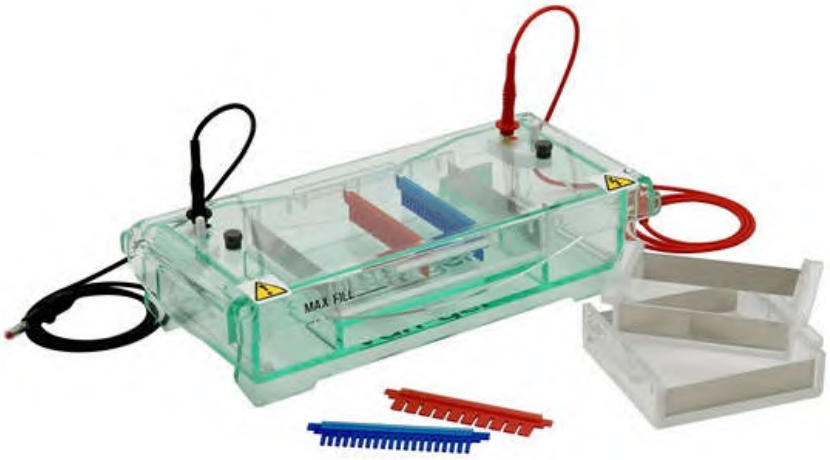
- Polyacrylamide gels are most effective for separating small fragments of DNA (5-500 bp).
- resolving power is extremely high,
- Separate the DNA that differ in size by as little as 1 bp in length.

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.

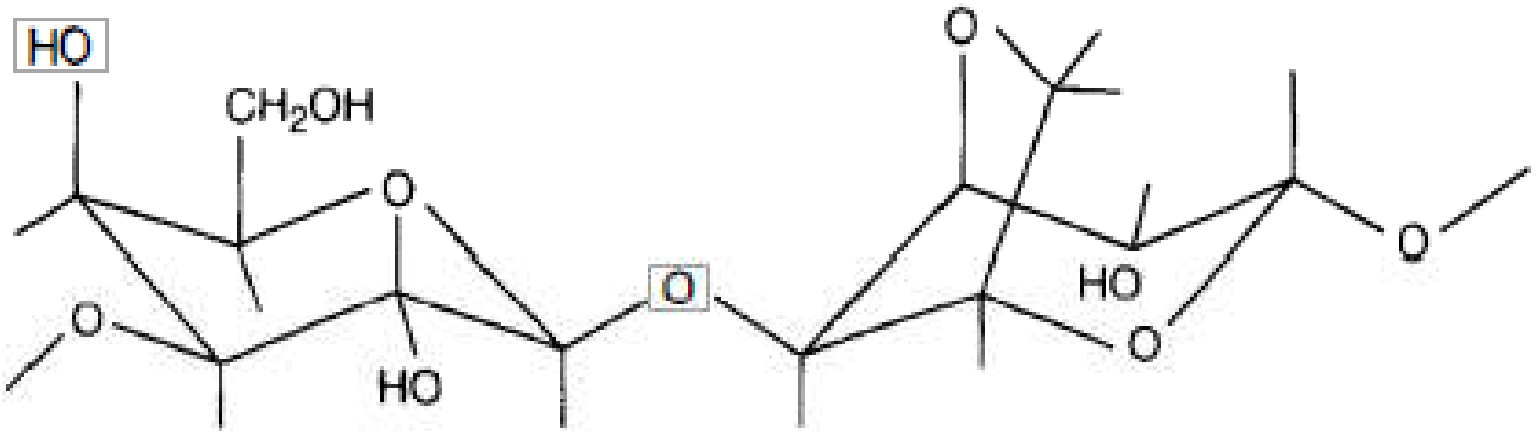
Agarose Gel electrophoresis

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

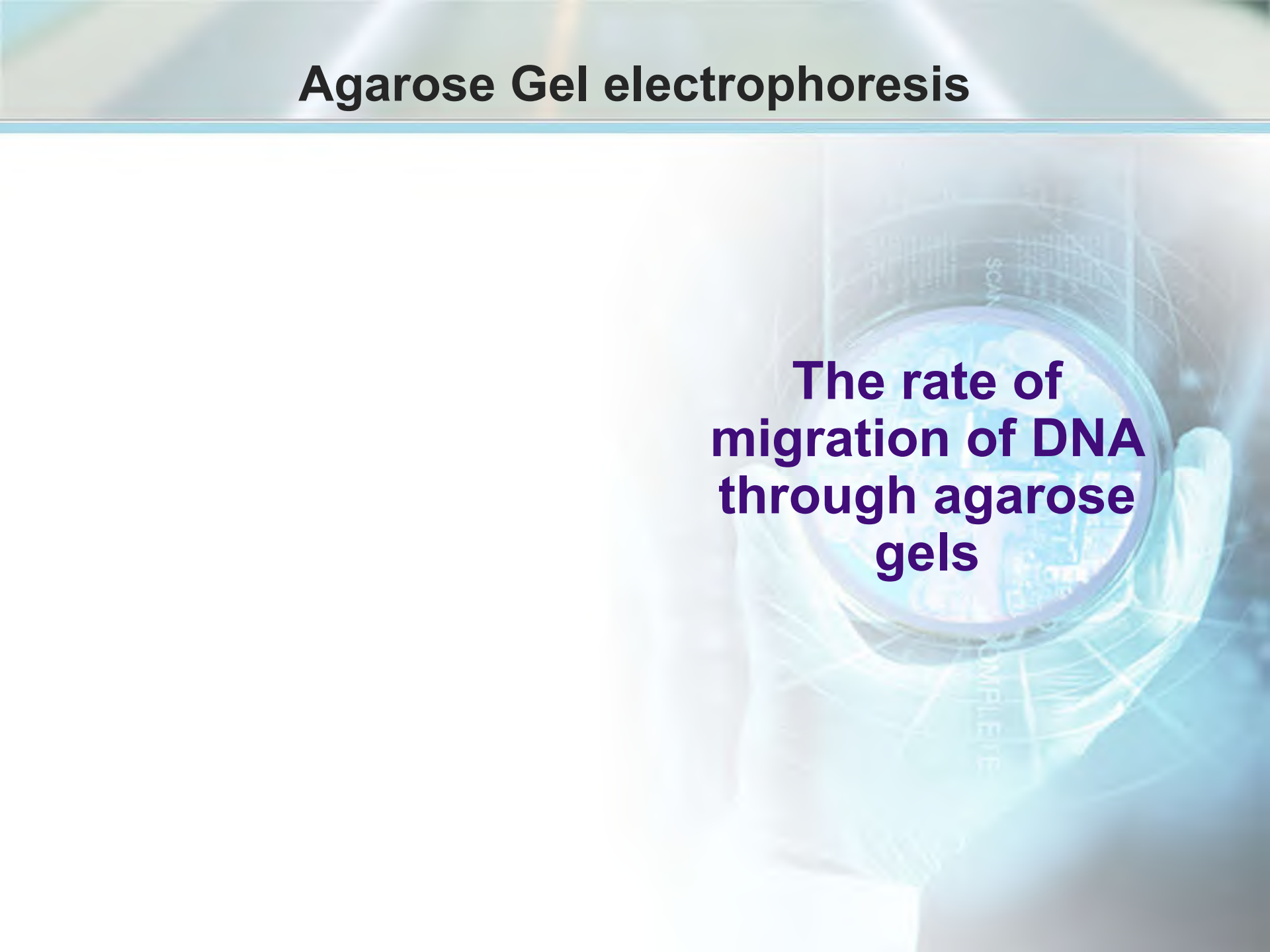
- This vari-ability can affect the
- gelling/melting temperature of agarose solutions,
- the sieving of DNA, and
- the ability of the DNA recovered from the gel to serve as a substrate in enzymatic reactions.

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 cur-rent
  - 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 pro-portional 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 resolu-tion 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

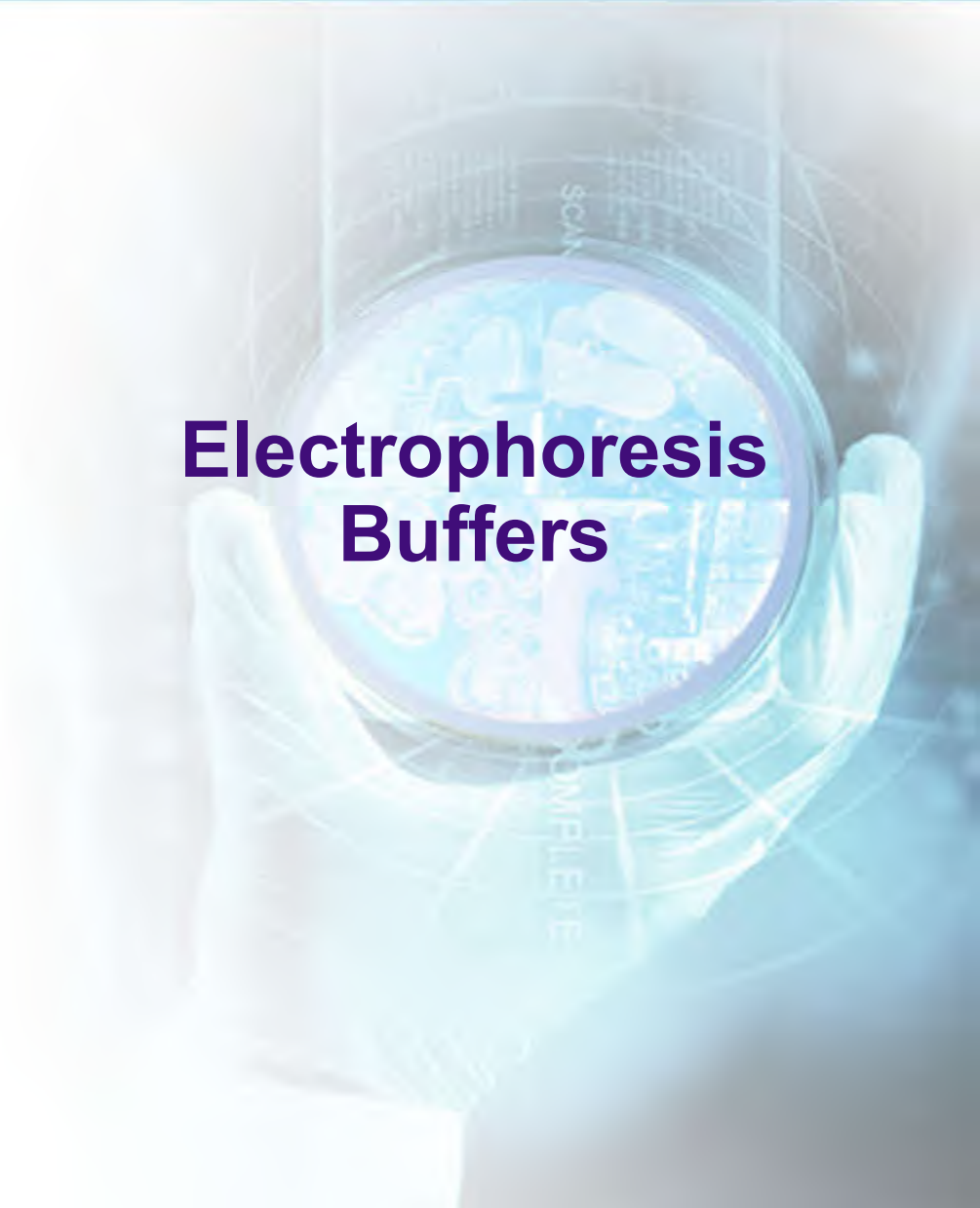
- In the absence of ions (e.g., if water is substi-tuted for electrophoresis buffer in the gel or in the reservoirs), electrical conductivity is minimal and DNA migrates slowly, if at all.

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)
  - Tris-phosphate (TPE) at a concentration of 50 mM (pH 7.5-7.8).

Electrophoresis Buffers

Concentrated Buffers

- Electrophoresis buffers are usual-ly 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 elec-trophoresis

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 res-olution of supercoiled DNAs is better in TAE than in TBE.

Electrophoresis Buffers

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

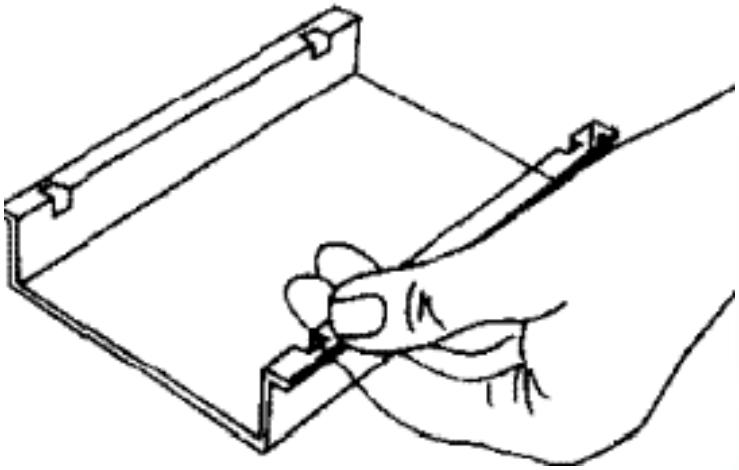
Special Equipment

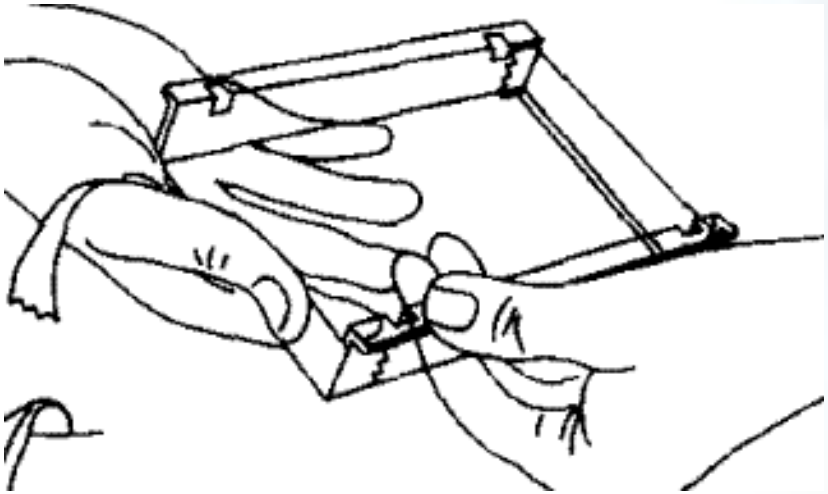
- Microwave oven or Boiling water bath
- Power supply device capable of up to 500 V and 200 mA.
- Water bath preset to 55°C

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

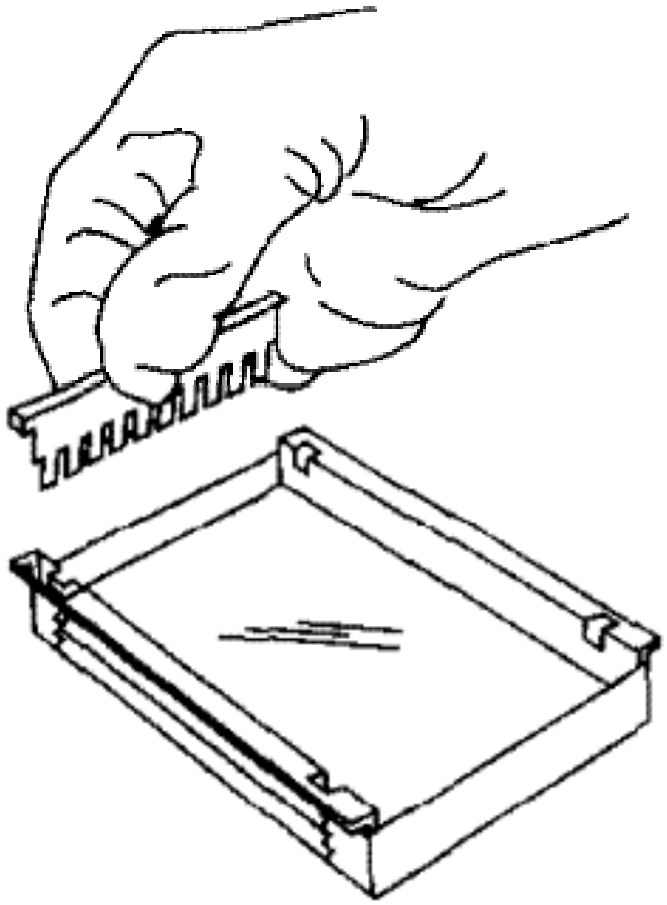
- Loosely plug the neck of the flask with Kim wipes.
- If using a glass bottle, make certain the cap is loose.
- Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves .

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 .

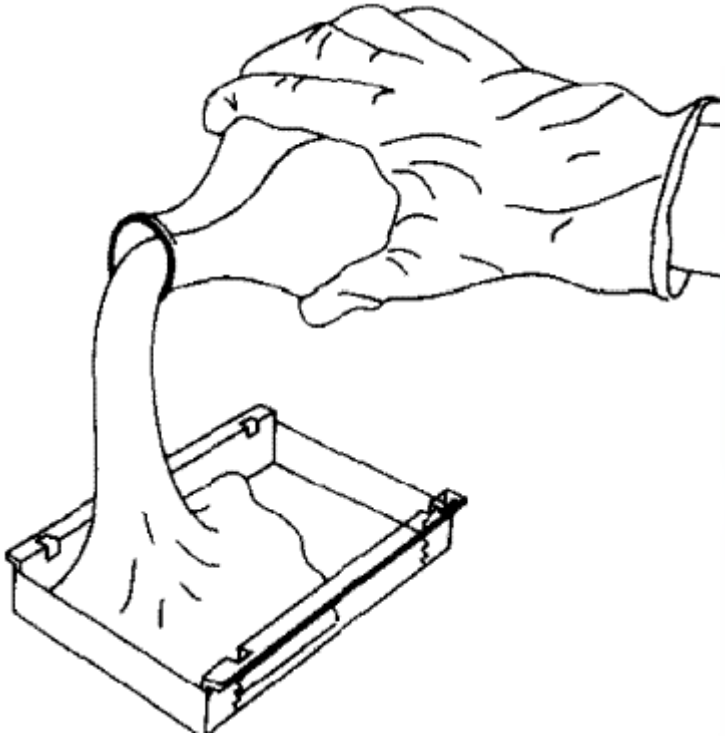
Procedure



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

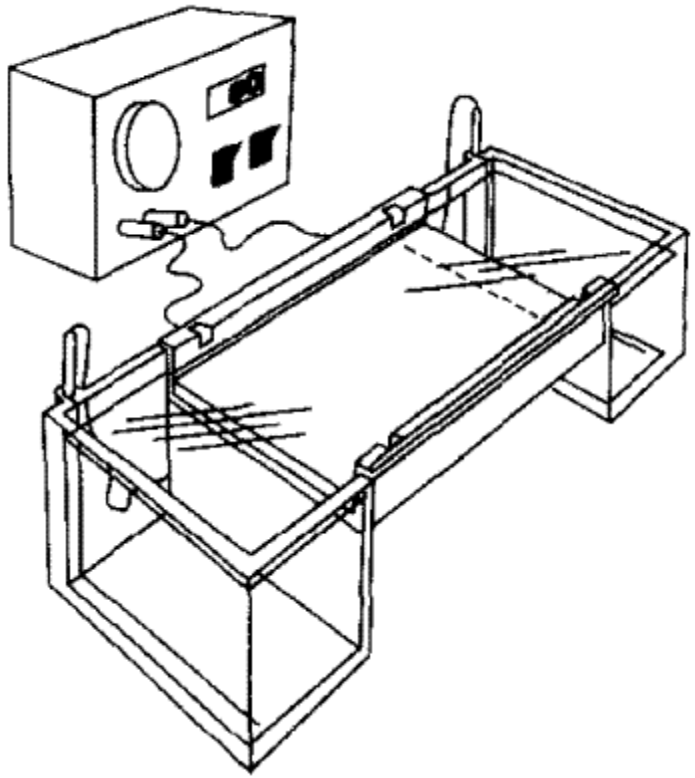
- Mix the samples of DNA with 0.20 volume of the desired 6x gel-loading buffer.

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



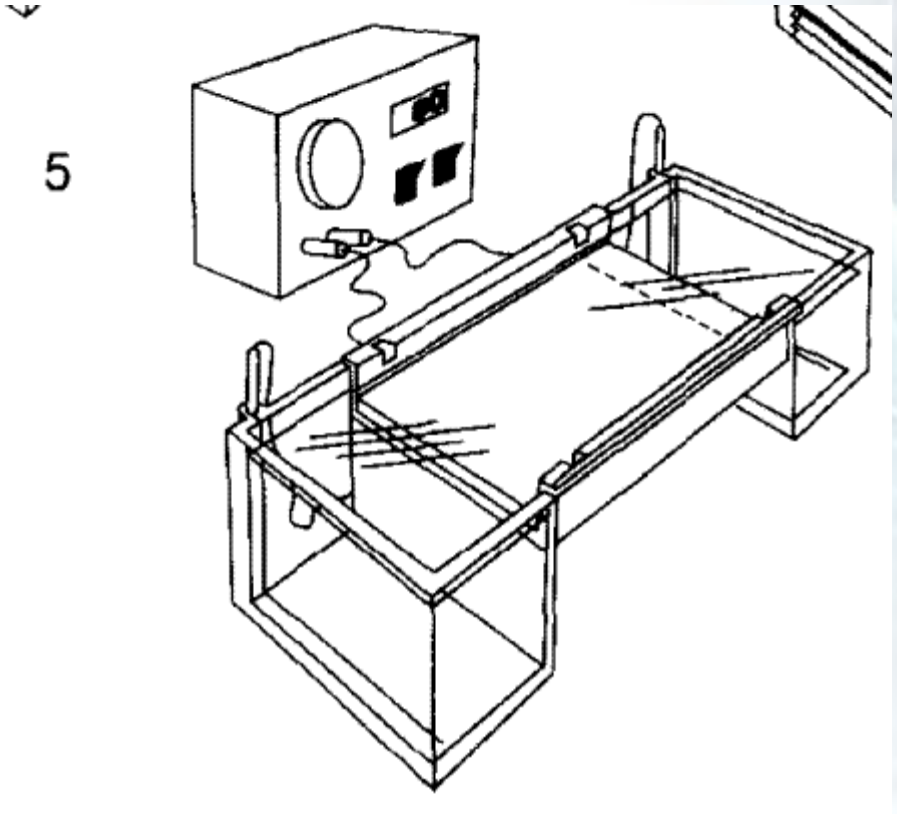
Electrophoresis

- 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, bub-bles 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 dis-tance 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 immers-ing 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 rela-tively 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 bro-mide 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 frag-ments through electrophoresis
- Soak the gel, in a 1:10,000-fold dilution of the stock dye solution.

Staining with Sybr Gold

Precaution

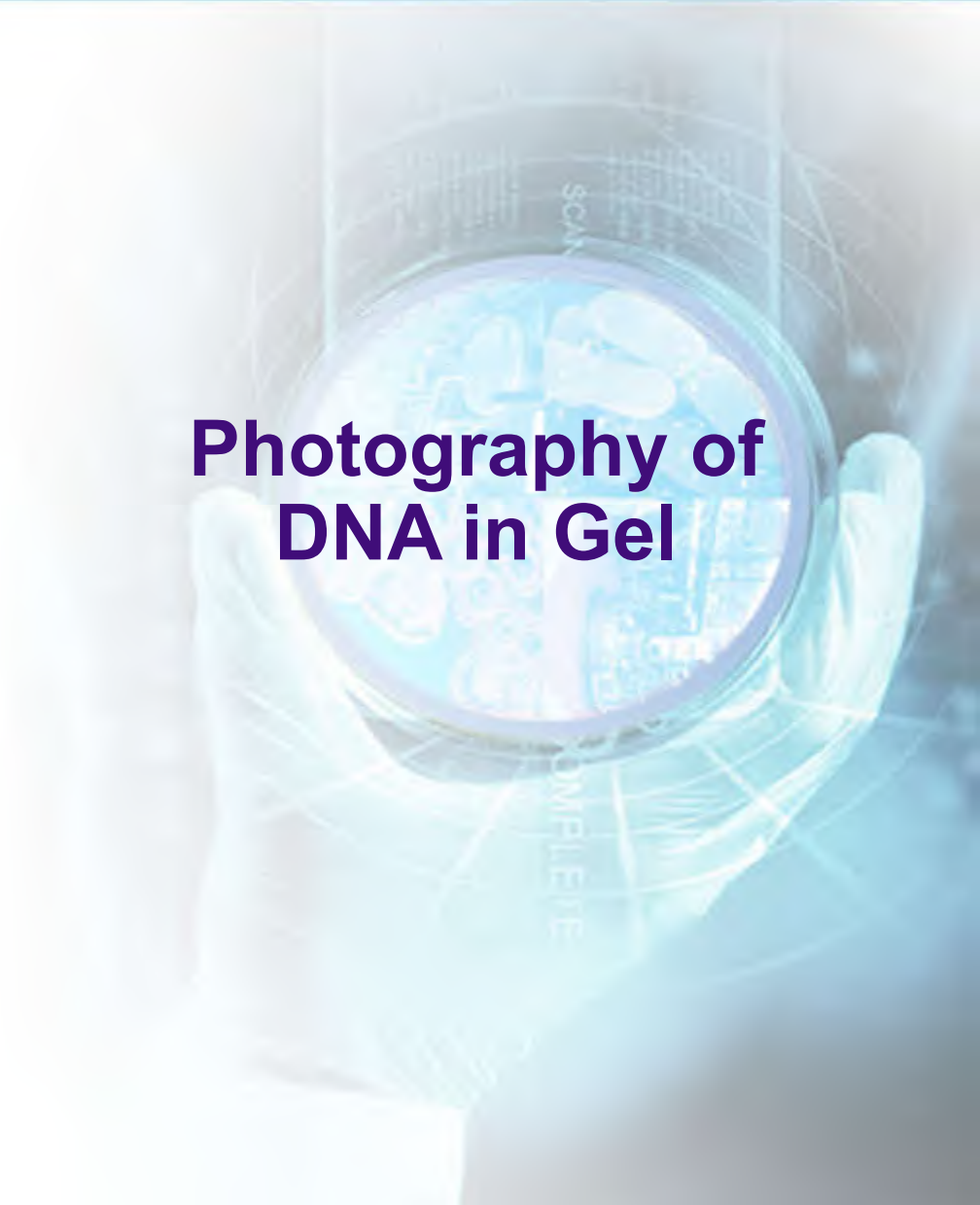
- SYBR Gold should not be added to the molten agarose or to the gel before electrophoresis,
- Causes severe distortions in the electrophoretic properties of the DNA and RNA.

Staining with Sybr Gold

Photography

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

Agarose Gel Electrophoresis



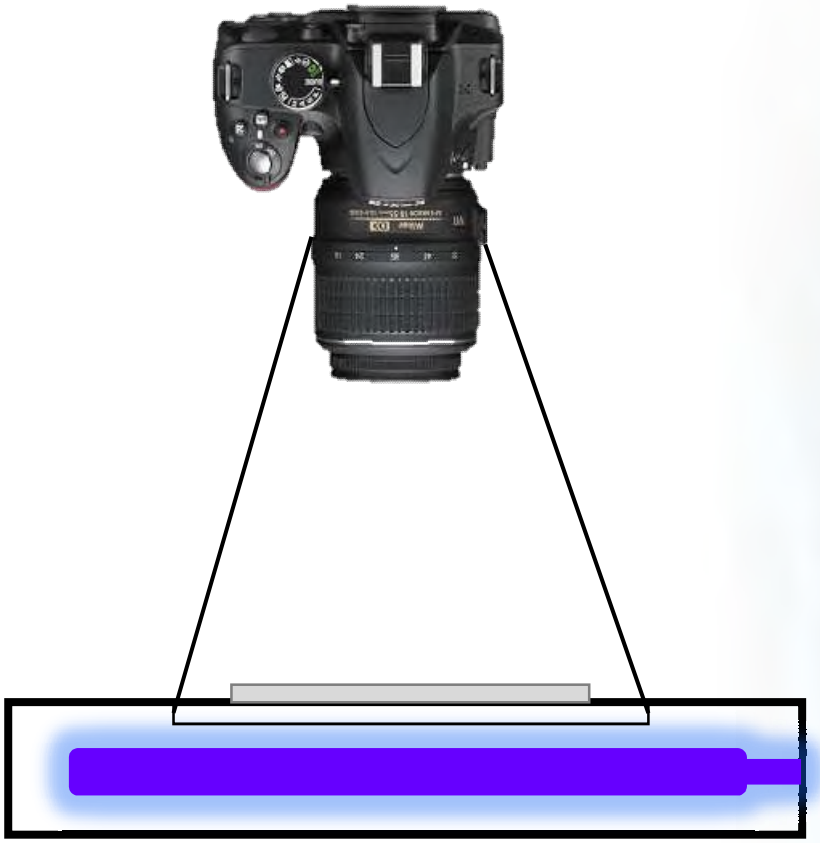
Photography of DNA in Gel

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.

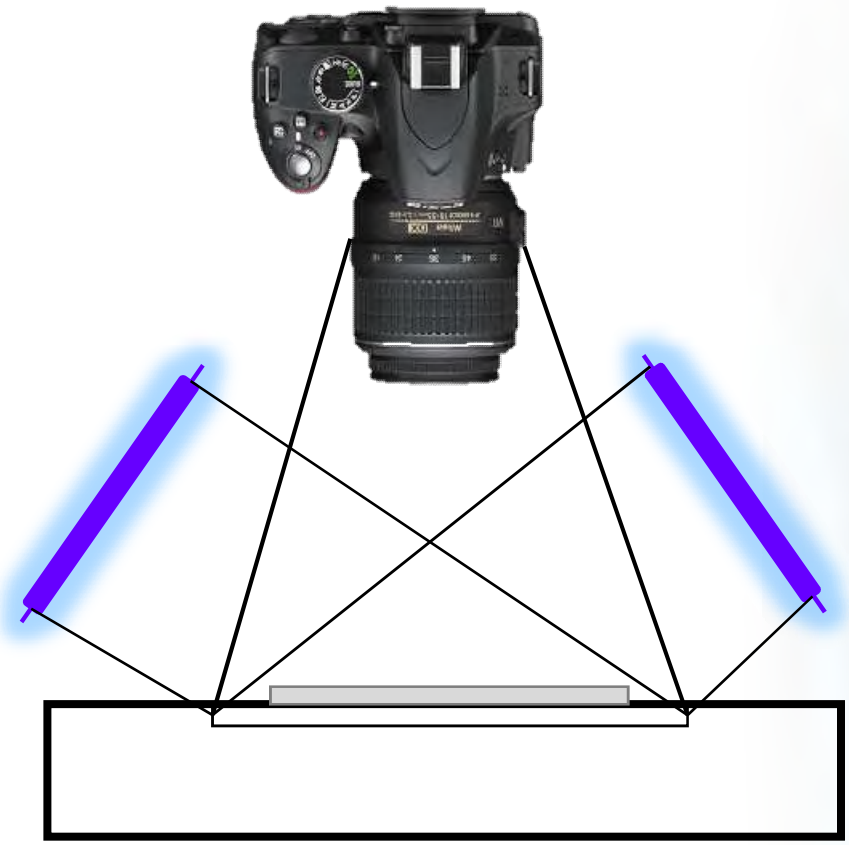
Photography of DNA in Gel



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