M. SAMUVEL.

P. Rathika

OK L. ASHOKKUMAR M.Sc., M.Phil.

Unit: 3

MICROSCOPES.

Microscopes can be categorized into two groups (i) Light microscope, in which magnification is obtained by a system of optical lenses using light waves. it includes (1) bright field (2) dark field (3) fluorescence (4) phase contrast (ii) Electron microscope where a beam of ~~light~~ Electrons is used in the place of light waves and it includes scanning electron microscope and transmission electron microscope.

Each microscope possesses a definite limit of visibility eg. Light microscope has a lower visible limits of 0.2 μm whereas electron microscope has lower visible limit 0.4 n.m.

BRIGHT FIELD MICROSCOPE:

✓ The ordinary microscope is called a bright field microscope because it forms a dark image against a brighter back ground

PARTS OF A MICROSCOPE:

✓ It consists of a metal body or stand composed of a base and an arm to which the other parts are attached.

→ The light source is either a mirror or an electric illuminator which is located in the base.

→ Two focusing knobs, the fine and coarse adjustment knobs are located on the arm which can either move the nose piece or stage.

→ The stage is positioned in halfway up the arm, it holds the microscopic slide either with simple slide clips or mechanical slide clip.

→ Stage control knobs provided in the stage helps the viewer to move the slide around.

→ The substage condenser is mounted within or beneath the stage and it focuses a cone of light on the slide and it can adjusted vertically. There are different types of condenser Abbe condenser, aplanatic condenser and achromatic condensers. Generally condenser also

incorporate iris diaphram which is used to control the light intensity and a filter holder.

→ The curved upper part of the arm holds the body assembly to which a nose piece and the eyepiece or the ocular is attached.

→ The main purpose of the eyepiece or the ocular is to magnify the intermediate image and to correct certain abberation produced by the objective. It is composed of two or more lenses of the upper component or eye lens is the magnifier while the lower component called the field lens. They are available in various magnification like 5X, 10X, 15X.

→ Objectives are considered to be the most important parts since they affect the quality of image formation. Generally three to five objectives with different magnifying power. They are low, high power and oil immersion objectives according to their degree of correction chromatic aberration it is categorized into three classes (i) achromate (ii) fluorites and (iii) apochromate.

The objective lens forms an enlarged real image within the microscope and the eyepiece lens further magnifies this primary image. When one looks into a microscope the enlarged specimen image called virtual image appears to lie beyond the stage at about 25 cm away. The total magnification is calculated by multiplying the objective and eyepiece magnification together. Eg: 10X × 45 = 450

MICROSCOPE RESOLUTION:

The obvious purpose of the microscope is not only magnification but also the resolving power, the ability to distinguish two adjacent points as distinct and seperate. Limit of resolution or minimum distant of between two objects that reveal them seperate

or distinct depends on the wavelength of light used and Numerical aperture. It is given by the equation

light wave length 400nm - 700nm

$d = \frac{\lambda}{NA_{condenser} + NA_{objective}}$

The shorter the wavelength of the light used and lower the Numerical aperture, the greater is the resolving power.

NUMERICAL APERTURE:

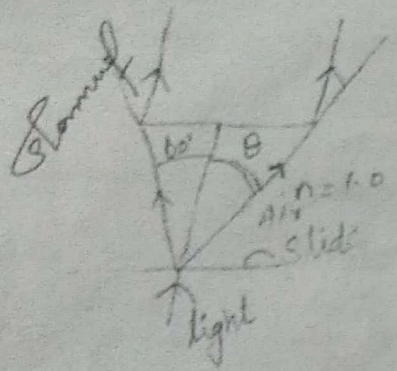
The numerical aperture $n \sin\theta$ where n is the refractive index of the medium between the object and objective.

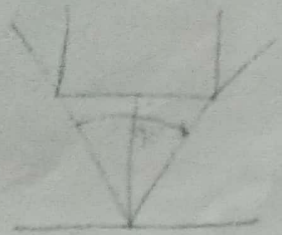
refractive index(η) = $\frac{\text{Velocity of light in vaccum}}{\text{Velocity of light in medium.}}$

Theta θ is defined as $\frac{1}{2}$ the angle of the cone of light entering an objective. Light that strikes the most after passing through a condenser is cone shaped!

When this cone has a narrow angle and tappers to a sharp point, it does not seperate out much after leaving the slide and therefore doesnot adiquately seperate images of closely packed objects. So resolution is low. If the cone of light has a very wide angle and seperates out rapidly after passing through a specimen, the resolution is good. The angle of the cone of light depends on the refractive index of the medium on which the lens works.

Refractive index of air is 1.00. Since $\sin\theta$ cannot be greater than 1.00 (maximum θ is 90°, $\sin 90°$ is 1.00) no lens working in air can have a numerical aperture greater than 1.00. So to increase the resolution, the refractive index of the medium through which the light enters is to be (increased with oil immersion) or changed from air to oil immersion.





Refractive indices of certain mounting media

Dis H_2O – 1.33, Eucalyptus oil – 1.46, Carbon tetrachloride 1.46, olive oil, glycerol – 1.47, Euparal – 1.48, Sandal wood oil – 1.51, Cedarwood oil – 1.51, (↳ refractive index is same as glass) Canada balsam – 1.54, polystyrene – 1.59.

$$d = \frac{(0.5)(530\,nm)}{1.25} = 212\,nm \text{ or } 0.2\,\mu m.$$

or $5 - \frac{\lambda}{n}$

Resolution power of bright field microscope is 0.2 μm apart.

Working distance of an objective is the distance between the front surface of the lens and surface of the slide.

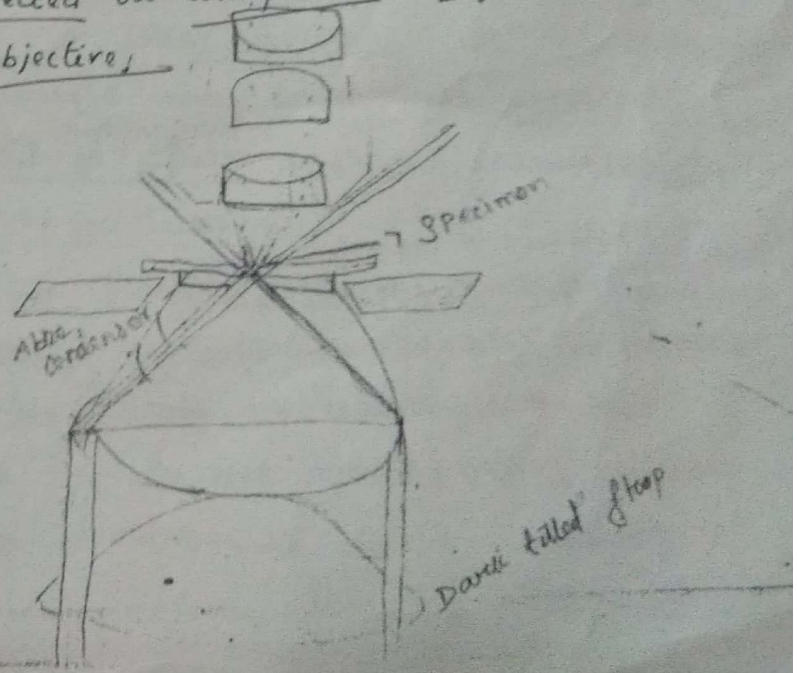
low power – 4–8 m·m oil → 0.1 m·m.

high power – 0.5–0.7 m·m

- Magnification: See R. C. Dubey book P. No: 87

DARK FIELD MICROSCOPE:

The effect produced by the dark field technique is that of a dark back ground against which objects are illuminated (appear) brightly. This can be accomplished by fitting the light microscope with a special kind of condenser that transmits a hollow cone of light from the source of illumination. Most of light directed through the condenser does not enter the objective, the field is essentially dark. Unreflected or unrefracted rays don't enter the objective.



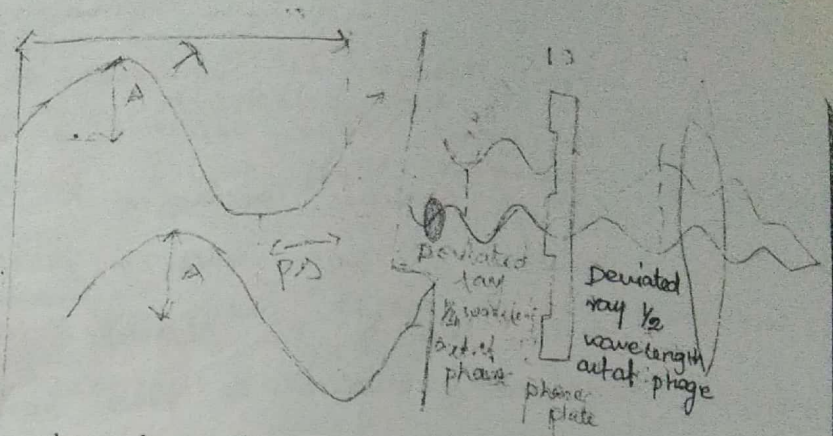
only light reflected or refracted light by the specimen forms an image.

[If light passes from one medium to another having different refractive index refraction takes place. so that the direction of propagation will change at the boundary of the two media]

[If light travels from a dense medium to light medium it strikes at an incident angle greater than critical value $\overline{C}$ with and the light rays will be reflected back.] used to view living, unstained cells.

PHASE CONTRAST MICROSCOPE:

It is widely used in studying the unstained cells and theotical biological studies. This phase contrast principle was introduced by Fritz Zernike. According to this, light has variable character for frequency and amplitude. Human eye cannot understand a phenomeron when two light rays have similar amplitude and frequency but from different phase.



unpigmented living cells are not clearly visible in bright field microscope because there is a little difference in contrast between the cells and water.

→ Phase contrast Microscopy is a kind of microscopic observation in which two light rays having similar amplitude and frequency, but differering in phase are used for illumination.

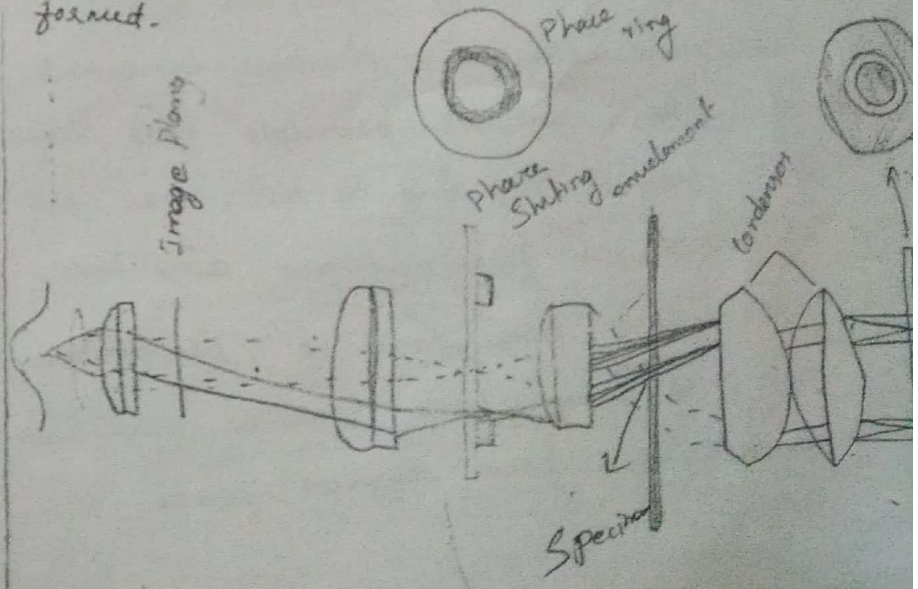
Phase contrast microscope is an ordinary bright field microscope with two additional plates, namely annular diaphram and phase shifting plate, which enables some of the image forming rays to be phase shifted with respect to others.

Annular diaphram is attached in place of iris diaphram. It allows only a ring of light to

Pass through the condenser, and then to the object. Each objective requires different size of annulus according to its numerical aperture. The phase shifting plate is attached at the back of the objective which is composed of a disc of glass having circular trough etched in it and of such a depth that light after passing through it creates a phase difference of a quater of a wave length as compared with the light from rest of the plate.

In phase contrast Microscope when a single ray of incident light falls on a specimen, two rays develop, one of these is direct or transmitted light ray from annular and passing through the object and is focused on the phase shifting plate which will either delay or advance this rays.

The second ray is a scattered or diffracted ray, as a result of passing through the margin of the specimen & not striking the phase shifting element, therefore its phase is not affected. When these two beams unite, they are not in phase and the phase difference becomes clear. Two sets of rays emanating from the same point of the specimen return to the same point in the image having phase difference with respect to one another interfere to produce variation in intensity of illumination, and therefore contrast in image formed.



Depending upon the phase shifting element employed the specimen may appear lighter against dark background (dark contrast) or darker against light background (bright contrast)

FLUORESCENCE MICROSCOPY:

Many chemical substance absorb light of a particular wavelength and energy and, some substances will then emit light of a longer wavelength and lesser energy. Such substance are called fluorescent and the phenomenon is termed fluorescence. In Microscopes so far considered only image from light the passes through a specimen is seen, whereas in fluorescente Microscope the object can also be seen.

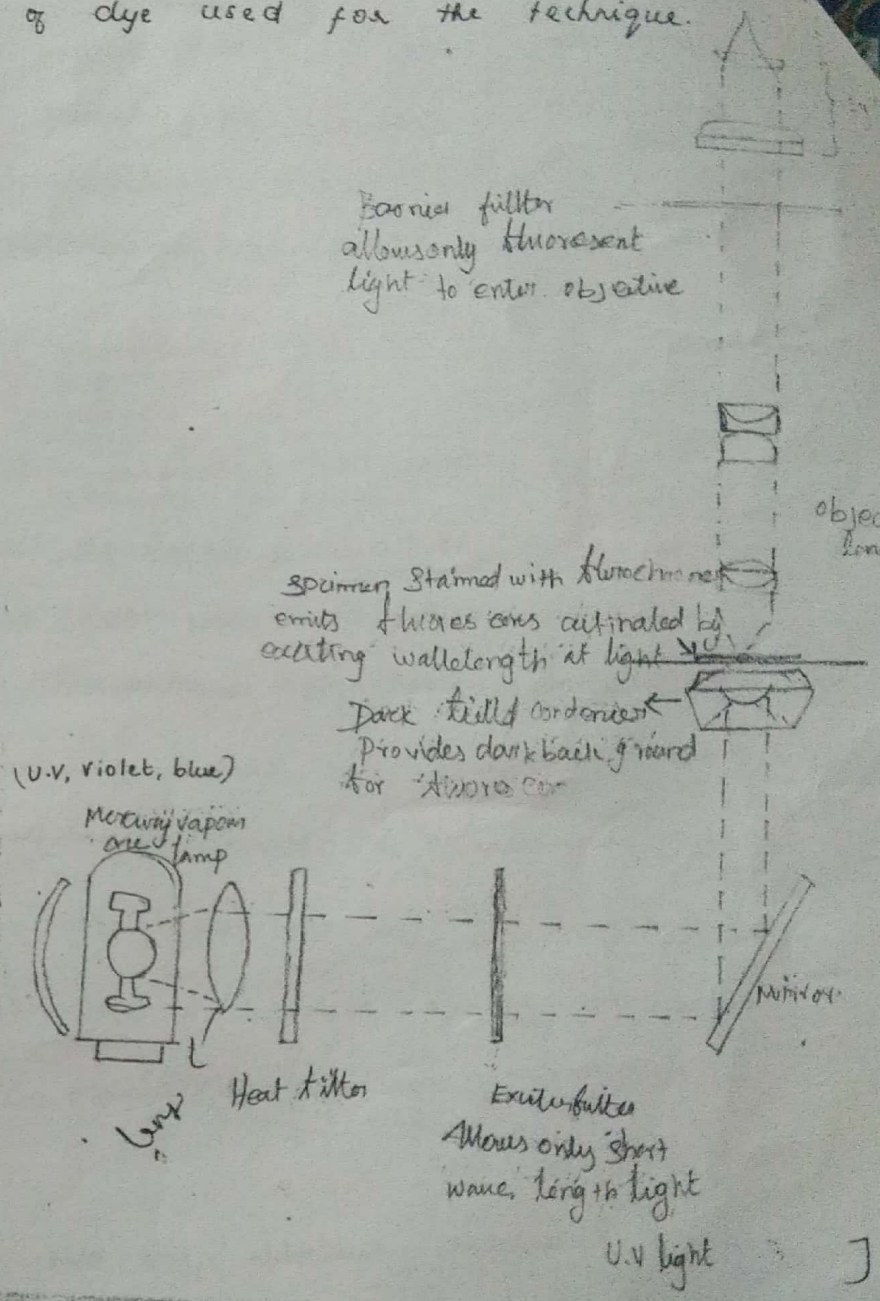
Microscopes suitable for this purpose are readily available, but it is possible to convert bright field microscope for fluorescent work.

The main object of this instrument is to transmit as much as possible the fluorescence emitted by the object, since this fluorescence is of low intensity. To avoid loss of intensity monocular microscope is preferred. Ultraviolet light 365 n.m is preferred, and this should be available to the object with maximum intensity. The substage condenser should be made of ultraviolet transmitting glass. When fluorescent dye is used to detect the organism they appear larger than the normal. As a result low power objective can be used effectively. This facilitates the inspection of larger area of

specimen is shorter time.

The use of oil immersion objective posses a problem because usual immersion oils become fluorescent after exposure to air and more particularly to U.V light. It is better to use highest available magnification with dry objective.

Two filter systems are necessary in this microscopy. primary or exciter filter which is placed between the illumination source and the objective. This filter is usually blue and allows ultra violet light to pass. and blocks out all other colour. secondary or barrier filter is placed in the eyepiece or anywhere between the eye and objective. It allows only passage of light from fluorescing specimen. It depends upon the type of dye used for the technique.



* ELECTRON MICROSCOPE.

It was realised that the resolving power of light Microscope cannot be increased more than 0.2 μm, because of the limitation with light as source of illumin Max KROll and Ernst Ruska in 1931, constructed and demonstrated the use of electron Microscope, Although the source of illumination in electron microscope is electron beam and not light, the optical principles and its constructio are closely related to those of light Microscope. Electron gun generates electron beam. These electrons are concentrated by other components of electron gun producing a fast moving narrow beam of electrons. Elec are focussed by electromagnetic lenses which consists of wire encased in soft iron casing When electric current is passed through the coil, it generates an electromagnetic field through which they are focussed.

with resolution power of 0.5 n.m.

There are three general types of electromagnetic lenses. The one is placed between the source of illumination and the specimen. This focuses the beam of electrons on the specimen and functions in a similar manner as the condenser in light microscop The other two lens systems are on the other

side of the specimen which magnify the image in similar fashion as the objective and ocular lens in light microscope. The final image is made visible on screen coated with a phosphorous compound which fluoresces upon irradiation by electrons.

Air molecules in the microscope interfere with the movement of electrons. To prevent this, the interior of the microscope is needed to keep in the state of high vaccum, around 10^{-4} _ 10^{-6} m.m. Hg. It is also necessary to have specimen ultrathin. These requirements do not allow observation of living material through electron microscope.

The electron beam has very poor penetration power and so only very thin (20-100nm thick) sections of specimen can be examined under the electron microscope. If the objective the study is to reveal the intracellular details, the usual mode of approach involves preparation of thin sections of fixed material.

PREPARATION OF SECTION:

Heavy suspension of bacteria or virus is required for this type of work which can be prepared by centrifuging thera pellet into small amount of suitable medium.

(1) FIXATION:

Objective of fixation is rapid killing and preservation of the specimen. Many fixatives like chromium and uranium salts, lead compounds Osmium tetraxide are available, fixation is usually carried at low temperature

PROCEDURE:

Mix 30 ml of suitable suspension with

3 ml of kellenberger's fixative and centrifuge at 1800 rpm for five minutes. Resuspend the pallet in 1 ml of kellenberger's fixative(S_1) containing 0.1 ml of tryptone medium (S_2). Incubate overnight at room temp. and add 8 ml of kellenberger's buffer (S_3) and centrifuge at 1800 RPM for 5 min. After removing the supernatent, resuspend the pallat in dis H_2O and use this for direct electron microscopy.

Glutaraldehyde - osmium tetroxide is also a commonly used fixative. Prepare 4% solution of glutaraldehyde and 2% solution of osmium tetroxide in phosphate buffer (pH-7). Take 10 ml glutaraldehyde fixative in a centrifuge tube and keep specimen for 2-15 hr. Remove the material by centrifugation and wash with buffer three times. To the sediment add osmium tetroxide solution to cover it and incubate at 0-5°c for five min. Remove the cells, wash with buffer and dehydrate it with series of alcohol.

EMBEDDING, preparation of grid, ultramicrotomy: less than 0.1 μm thick sections have been obtained when high polymer plastics and resins are used. The presence of plastic embedding material Epon, Araldite, Maraglas are used which prevents the collapse of specimen. The specimen is soaked in the unpolymerised plastic until it is completely and then the plastic is hardened to form a solid block. Then thin sections are cut from this block with a glass or diamond knife using a special instrument called an ultramicrotome.

CONTRAST DEVELOPMENT:

In order to demonstrate the cellular and subcellular components the basic anatom

and intracellular developments of viruses. Some form of contrast enhancement is essential. Biological materials are composed of element carbon, nitrogen, oxygen and hydrogen. They have low electron scattering power, have hardly any contrast is observed which in turn results in a poor quality of image production. So techniques are now available which increases contrast.

(i) Staining → (a) Negative (b) Positive staining.

(ii) Shadow casting ↓

Viruses are bacteria are suspended in 1% ammonium acetate and then add 2% phosphotungetic acid.

(3) Surface replicas

(4) Freeze etching.

SCANNING ELECTRON MICROSCOPE

Scanning Electron microscope p... surface views of whole structure of specim... The application of SEM are enormously incre... It is mainly due to the availability of appreciable contrast and the preparation specimen is fairly rapid and simple. SEM very useful in studies associated with surface characteristics of viruses.