Paper 10: Techniques in Biophysics II (Based on Spectroscopy)

Module 2

Optical (Light) Microscopy

Objectives

- **The present module is designed to offer an overview of Microscopy and its applications**
- **Introduction to Optical microscopy and fluorescence microscopy**
- **To compare a typical Light microscope (LM) and Electron microscope**
- **Biophysical principle, instrumentation and their applications in biology**

1 Introduction :

Why do you need to study microscopic techniques ?

Microscopy finds plethora of applications in biology and medicine, in addition to other subjects like geography etc. Some of the important applications are given below: Uses of microscopes in biology

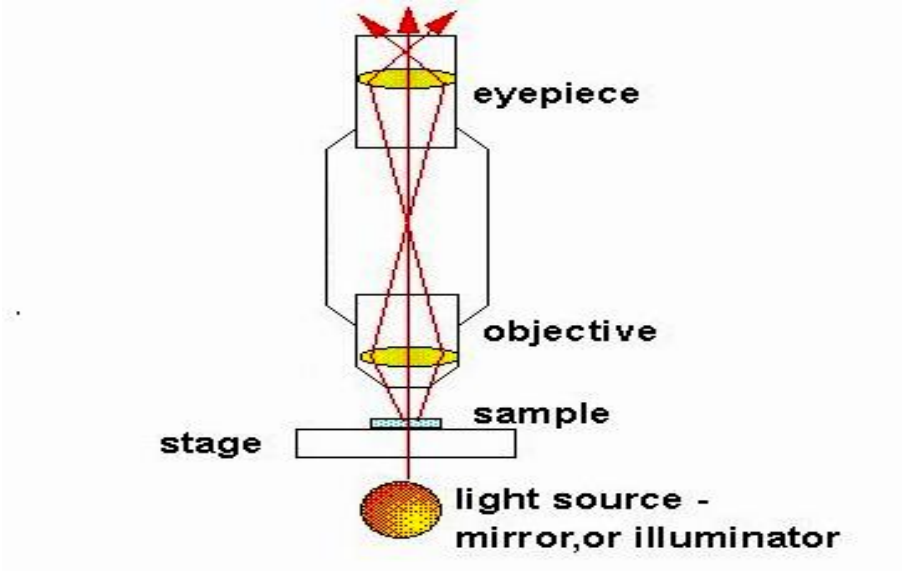
Basic unit observable	**Method of observation of basic unit**	**Resolution**
Organs & Tissues	Eyes and simple lenses	0.1mm
Cells, bacteria etc.	Light/Phase contrast/ UV	Up to 10μm
Cell organelles virus macromolecules	Electron microscope	Up to 1nm
Molecular level	Indirectly localized by EM	Up to 1 Å

2 Light or Optical Microscope :

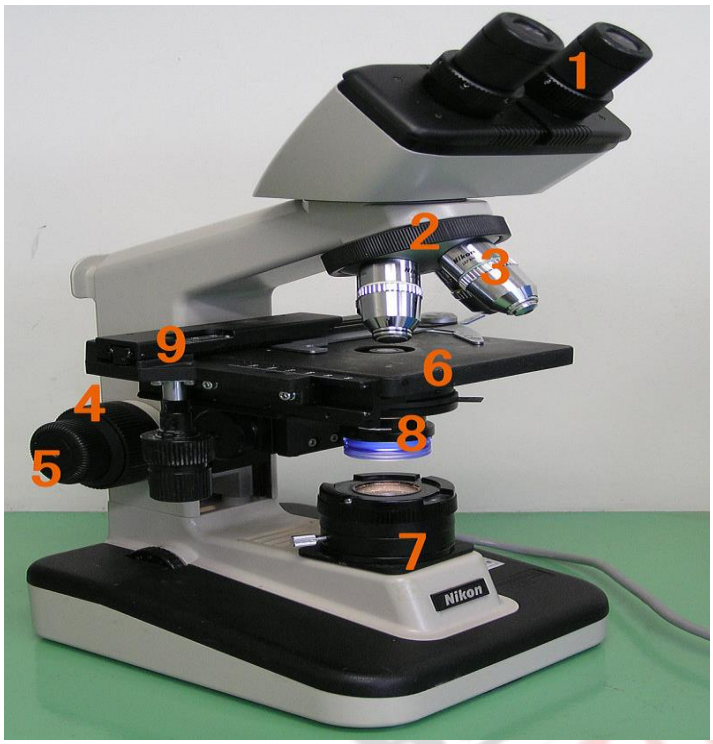
An Optical (**light) microscope** works like a refracting telescope except that the object is very close to the objective lens. An object to be studied, for example a tiny organism so small it looks like just a dot, is put on a slide, which is usually a flat piece of glass. The clips on the microscope's flat stage hold the slide in place. The stage can be adjusted to add more light. It also moves to allow different layers of the object to be in focus. The user looks through the microscope eyepiece. A mirror at the bottom of the microscope reflects light rays up to the object through a hole in the stage. Objective lenses magnify the image which is made even larger when it is seen through the eyepiece lenses. Some light microscopes are actually digital cameras, made to photograph small things but having no eyepiece.

Many microscopes, often used in colleges and high schools, normally have a top magnification of 40x with the option of having 4x and 8x. This lets the microscope show basic cells and other items. Others can magnify hundreds of times, or thousands.

All modern optical microscopes designed for viewing samples by transmitted light share the same basic components of the light path, listed here in the order the light travels through them. Also almost all microscopes have the same 'structural' components: Schematic representation of the basic components of a compound microscope:



Below is the microscope which is used routinely



1 Eyepiece)
2 Objective turret or Revolver or Revolving nose piece (to hold multiple objective lenses)
3 *Objective*
4 Focus wheel to move the stage (4 – coarse adjustment,
5 fine adjustment
6 Frame
7 Light source, a light or a mirror
8 Diaphragm and condenser lens
9 Stage (to hold the sample)

The compound light microscope focuses visible light through a specimen. Different regions of the object scatter the light differently, producing an image.

3 Limitations of Light Microscope :

The use of light microscope is limited. The limitations are :

1. **The user can view only two dimensional plane of the specimen at a time.**
2. **The optical microscope has the resolution of about 0.2 µ (200 nm),.**
3. **It is good enough to see cells , but not cell organells.**

4. **Specimen has to be stained with a dye to be visible in the visible light**
5. **Different dyes are needed to stain DNA, lipids etc**
6. **Methyleneblue for staining animal cells**
7. **I2 / KI for staining plant cells**
8. **The compound microscpe uses different lenses to get high magnfication**

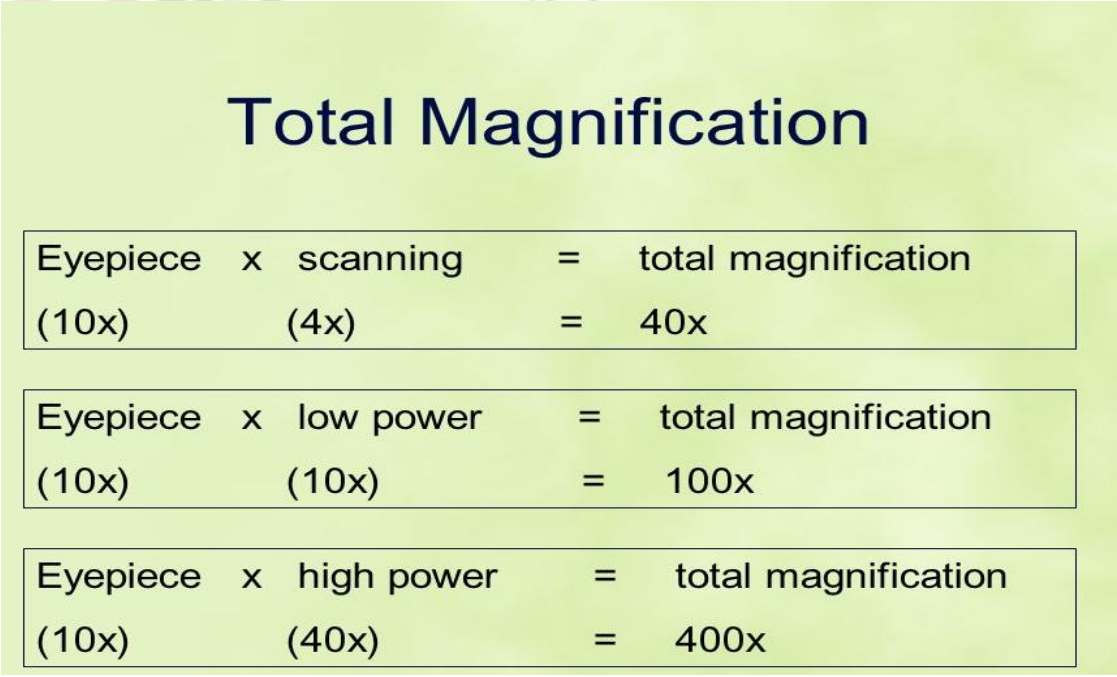
What is Magnification? and how is it different fro\m Resolution? :

The term magnification is often confused with the term "Resolution". Magnification is the process of enlarging something only in appearance, not in physical size. This enlargement is quantified by a calculated number also called "Magnification"

5 Magnification :

It is the enlargement of an image. **Magnification is the ability to make small objects seem larger, such as making a microscopic organism visible.** Which is expressed as x or 4x or 8x or 10x etc.

Overall Magnification = Objective lens x Eyepiece lens



Total Magnification:

X

= 40 X

4X Scanning Objective 10X Eyepiece

X

= 100 X

10X Objective 10X Eyepiece

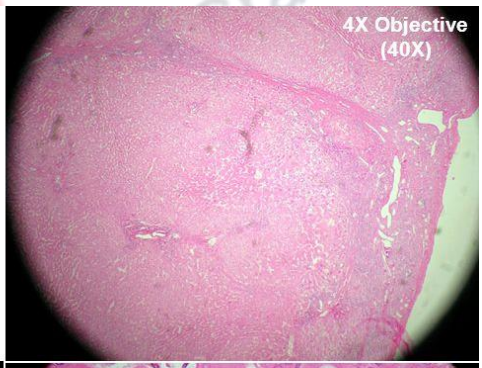
X

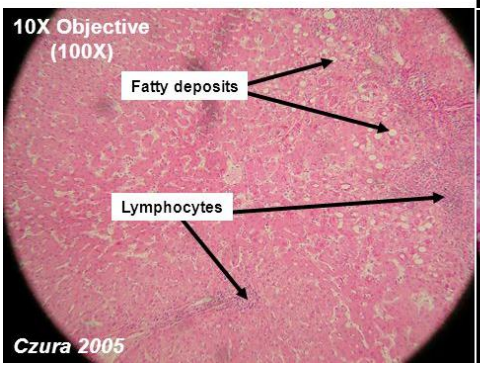
= 400X

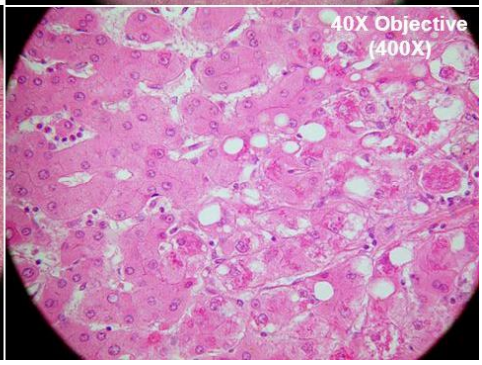
40X Objective 10X Eyepiece

Cirrhosis of the Liver

Two pathological conditions are visible in this specimen: Fatty deposits (white holes) which destroy/replace hepatocytes, and a chronic inflammation as evidenced by dense collection of lymphocytes (purple clustered nuclei are visible)







Example : The same sample at different magnifications, 4X, 10X and 40X (Ref : czura2 005)

6 Resolution :

Resolution is the ability to distinguish two objects from each other**. The resolution of an optical microscope is defined as the shortest distance between two points on a specimen that can still be distinguished by the observer or camera system as separate entities.** The resolution of a microscope is intrinsically linked to the numerical aperture (NA) of the optical components as well as the wavelength of light which is used to examine a specimen.

6.1 Resolution of a microscope:

You have studied in Paper 1, the principle of Heisenberg's uncertainity principle was explained with an example of the microscope and its limitations on deciding the resolution power.

Taking all of the above theories into consideration, it is clear that there are a number of factors to consider when calculating the theoretical limits of resolution. Resolution is also dependent on the nature of the sample.

$$NA = n \times \sin \alpha$$

where n is the refractive index of the imaging medium and α is half of the angular aperture of the objective. The maximum angular aperture of an objective is around 144º. The sine of half of this angle is 0.95. If using an immersion objective with oil which has a refractive index of 1.52, the **maximum NA of the objective will be 1.45**. If using a 'dry' (non-immersion) objective the maximum NA of the objective will be 0.95 (as air has a refractive index of 1.0).

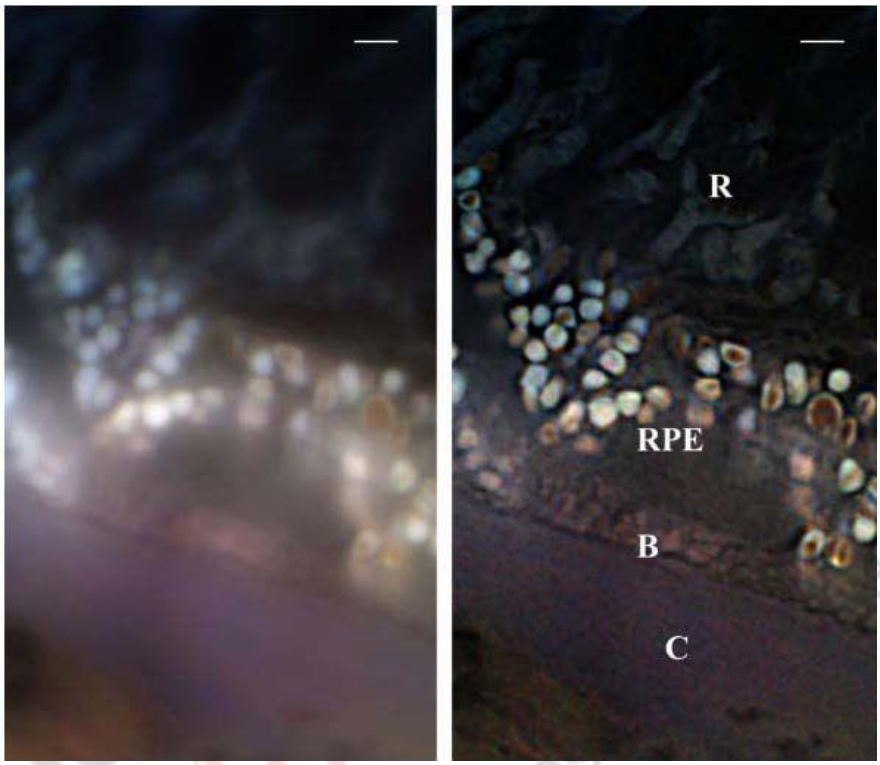
Abbe's diffraction formula for lateral (i.e. XY) resolution is:

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

Where λ is the wavelength of light used to image a specimen. If using a green light of 514 nm and an oil immersion objective with an NA of 1.45, **then the (theoretical) limit of resolution will be 177 nm.**

To achieve the maximum (theoretical) resolution in a microscope system, each of the optical components should be of the highest NA available (taking into

consideration the angular aperture). In addition, using a shorter wavelength of light to view the specimen will increase the resolution. Finally, the whole microscope system should be correctly aligned.



The above figure reveals the Aging Tissue and Vision Loss: These are micrographs of a section of a human eye. Right has a higher resolution and is therefore clearer. It should be noted that both panels ***are at the same magnification,*** *yet the panel on the right has a higher resolution and gives more information on the sample. The labels represent various parts of the human eye: Bruch membrane (B); choroid (C); retinal pigment epithelium (RPE); and retinal rod cells (R). The scale bar is 2um.*

Ref :https://www.boundless.com/microbiology/textbooks/boundless-microbiology-textbook/microscopy-3/looking-at-microbes-28/magnification-and-resolution-241-6418/)

7 ELCTRON MICROSCOPY

The first breakthrough in the development of the electron microscope came when Louis de Broglie advanced his theory that the electron had a dual nature, with characteristics of a particle or a wave. The demonstration, in 1923 by Busch, that a beam of electrons could be focused by magnetic or electric fields opened the way for

the development of the first electron microscope, in 1931 by Knoll and Ruska. Electrons travel in straight lines and have a wavelength, which is about 100,000 times smaller than of light. Dr. Ernst Ruska at the University of Berlin combined these characteristics and using two magnetic lenses, built the first electron microscope (EM), a Transmission electron microscope, in 1931 and could get a resolution of 100nm. Today, by using 5-7 magnetic lenses in the imaging system, a re An **electron microscope** is a microscope that uses a beam of accelerated electrons as a source of illumination. As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher resolving power than light microscopes and can reveal the structure of smaller objects. A scanning transmission electron microscope has achieved better than 50 pmresolution in annular dark-field imaging mode and magnifications of up to about 10,000,000x whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x.

Electron microscopes have electron optical lens systems that are analogous to the glass lenses of an optical light microscope. Electron microscopes are used to investigate the structure for a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, electron microscopes are often used for quality control and failure analysis. Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the image.

Electron microscope is a device is designed to offer greatly magnified images of objects by means of electrons. Electron microscope serves primary two purposes:

i) The visual examination of structures too fine to be resolved with ordinary, or light microscopes, and

ii) The study of surfaces that emit electrons. The first function made transmission electron microscopes essential research tools in biology

2.3 Resolution of an Electron Microscope

The resolving power of the Electron microscope is approximately three orders of magnitude (10,000) greater than that of the light microscope. The very short wavelength of electrons makes it possible a resolution of 3 nm on an Electron microscope. The resolving power decreases as the wavelength increases. The

resolution also improves with increase in the voltage because the voltage reduces the wavelength.

As electrons are accelerated through a potential difference of V volts, they have a wavelength (λ) equal to1.23nm/V. Therefore, increase in the accelerating voltage (V) leads to decrease in the wavelength (λ). As a result, the resolving power of the microscope increases. The resolving power also depends on various factors, such as the construction of electromagnetic lenses etc. However, higher voltages result in lower contrast due to greater beam penetration, so an optimal operative voltage is selected for better resolution as well as contrast.

Magnification: Magnification is the ratio of image distance versus object distance:

$$\mathbf{M = v/u} \quad \ldots\ldots \mathbf{(17)}$$

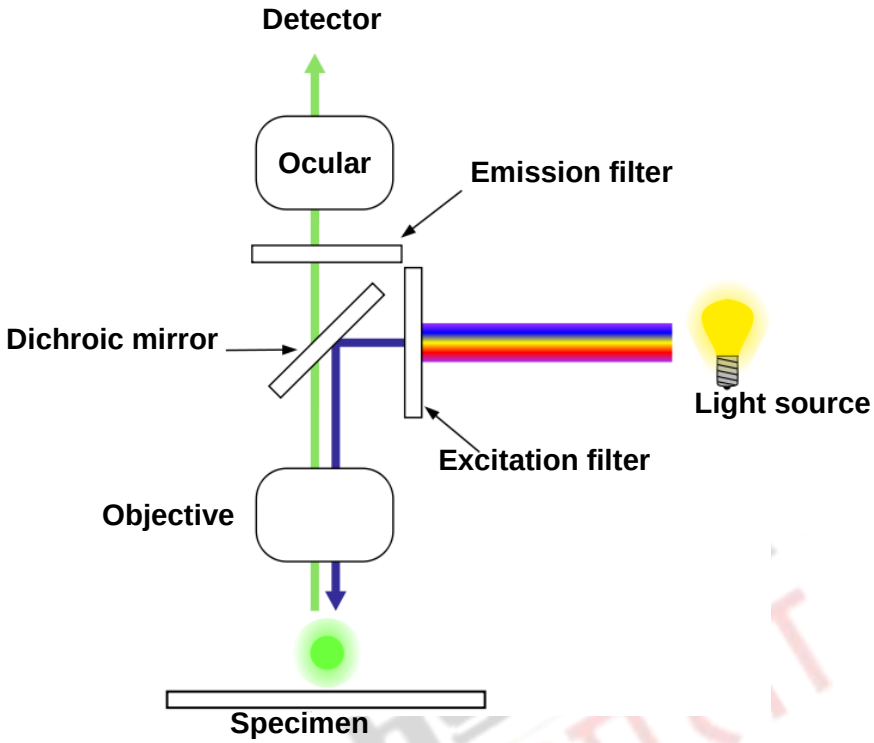
M is 'Magnification', **u** is the 'Object distance' and **v** is the 'Image distance'.

Magnification can also be defined as the ratio of the resolving power of the eye to the resolving power (δ) of the microscope. The resolution can not be less than half the wave length used. Magnification has no meaning without the simultaneous increase in resolution.

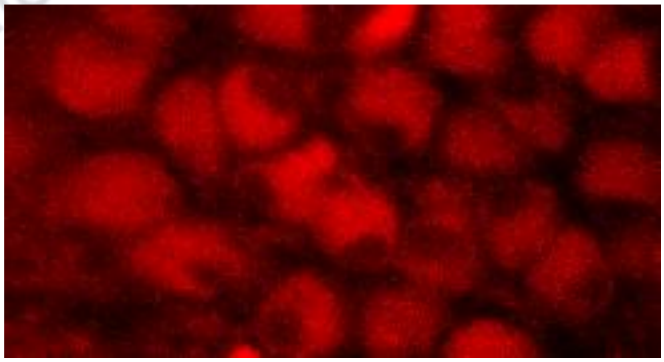
$$\mathbf{M = \delta\ eye\ /\ \delta\ microscope} \quad \ldots\ldots \mathbf{(18)}$$

The magnification obtained in an electron microscope is much higher than that of the light microscope. This is due to the illumination source used (electrons), which can provide even a theoretical atomic resolution.

2 Schematic representation of a Fluorescence microscope



Cytoplasmic localization of constitutively present calretinin (calcium binding protein) stained red (rhodamine labeled secondary antibody) in the neurons of the auditory nucleus - n. magnocellularis in the posthatch day 1 chick by immunofluorescence. Note the black unstained nuclei in the neurons. Scale bar = 20 μm. (**Courtesy Dr. Shashi wadhwa lab, Anatomy Dept. AIIMS**). See below



The complex interaction between the accelerated electrons and the specimen results in various physical products such as elastically scattered electrons, secondary electrons, X-rays, etc. Based upon these various physics products, different kinds of electron microscopes have been developed. Some of the commonly used electron microscopes are:

- Transmission electron microscope (TEM)
- Scanning electron microscope (SEM)

Electron microscopes were initially used in material science. It was only in 1960's, when specimen preparative techniques for biological material were developed. A newly evolved branch of science "Nanotechnology" is also based on the electronmicroscopic observations. **Table 7.4** shows the comparison of a typical Light microscope (LM) and Electron microscope (EM).

Factors	**LM**	**TEM**
Source of illumination	Light rays	Electrons
Wave-length	400-800nm	0.0037nm (100kv)
Medium	Air	Vacuum
Lenses	Glass	Electromagnetic
Image visibility	Direct	Fluorescent screen
Focusing/alignment	Mechanically	Electrically
Magnification	5-2000 X	Up to 500,00 or more
Resolving power	200 nm	0.1 nm