Shree H.N.Shukla College of Science

Subject: Microbiology

Paper-101 [Sem-1] Fundamental of Microbiology

Unit-2: Types of Microscopy

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Parts of a light microscope

The light is produced by a lamp source and focused on the specimen by the condenser. The light diffracted by the sample is then collected by the objective lens that generates a real magnified image as shown in Figure.This image is further magnified by the eyepiece.

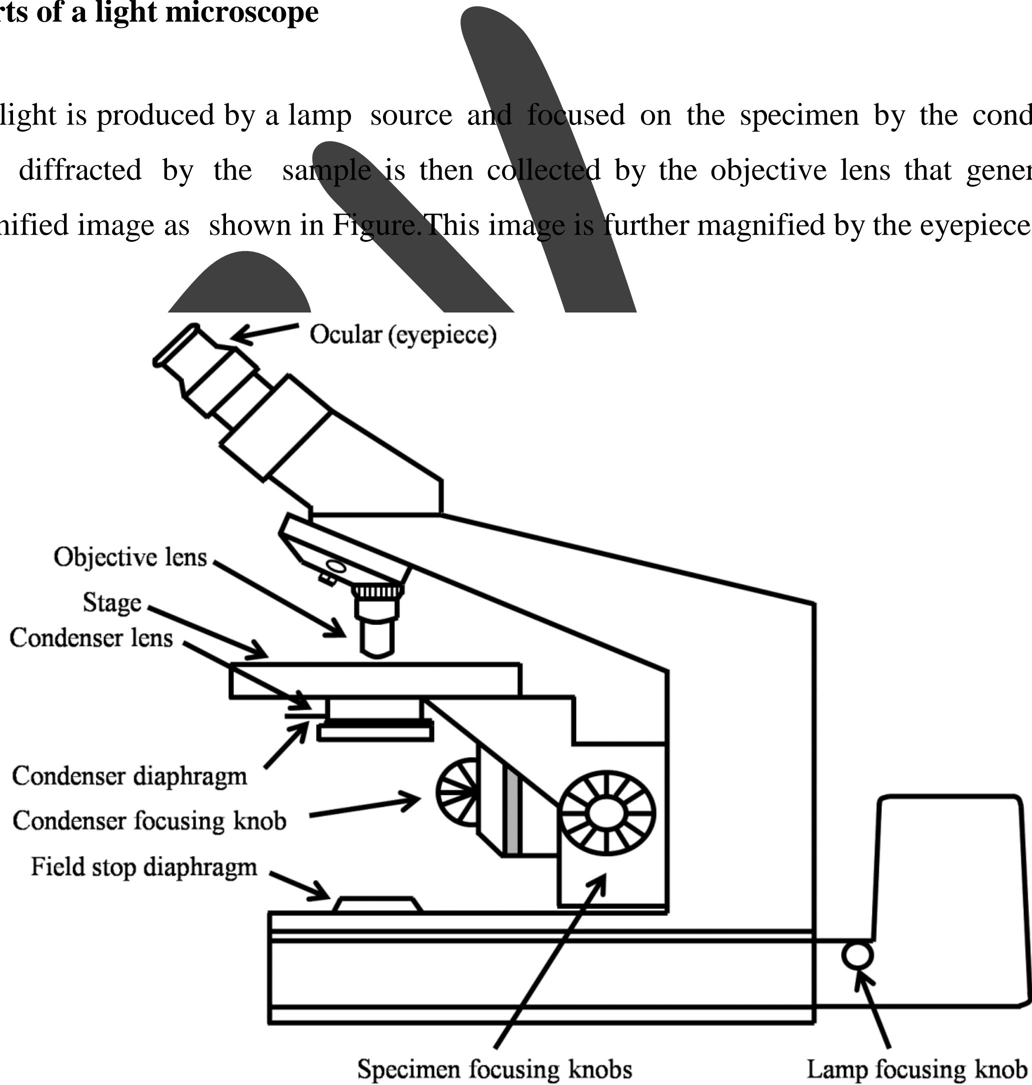

Figure Schematic diagram of a compound microscope showing its different components

Bright-field microscopy

In a bright-field microscope, both diffracted (diffracted by the specimen) and undiffracted (light that transmits through the sample undeviated) lights are collected by the objective lens (Figure). The image of the specimen is therefore generated against a bright background, hence the name bright-field microscopy. Most biological samples are intrinsically transparent to the light resulting in poor contrast. To increase the contrast of the image, the specimens are therefore generally stained with the dyes. However, intrinsically colored samples such as erythrocytes can directly be observed using bright-field microscopy.

Dark-field microscopy

Dark-field microscopy increases the contrast of the image by eliminating the undiffracted light. The specimen is illuminated by the light coming from a ring at an oblique angle (Figure). If there is no specimen in the optics path, no light is collected by the objective lens. Presence of specimen results in the diffraction of light; the objective lens collects the diffracted light generating a bright image against a dark background.

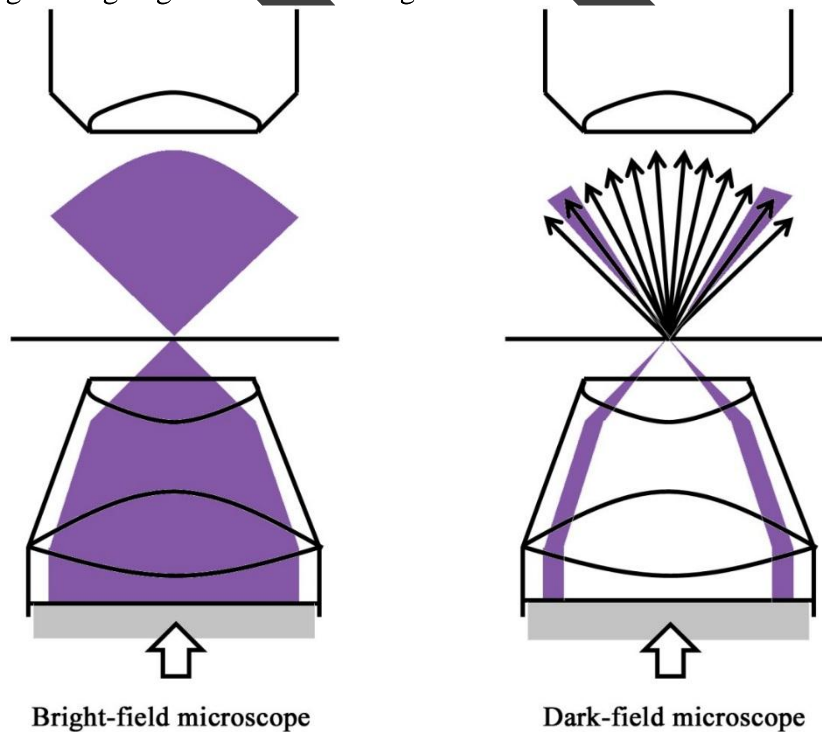

Figure Optical diagrams of bright-field and dark-field microscopes

Phase contrast microscopy

A phase contrast microscope provides very high contrast as compared to the bright- field and dark-field microscopic methods. The image in a phase contrast microscope is generated from both diffracted and undiffracted lights as shown in Figure. Like dark-field microscopy, the specimen is illuminated by the light coming from a ring, called a condenser annulus. The diffracted and the undiffracted lights are separated in space allowing selective manipulation of their phases and intensities. The diffracted as well as the undiffracted light is collected by the objective lens. A phas

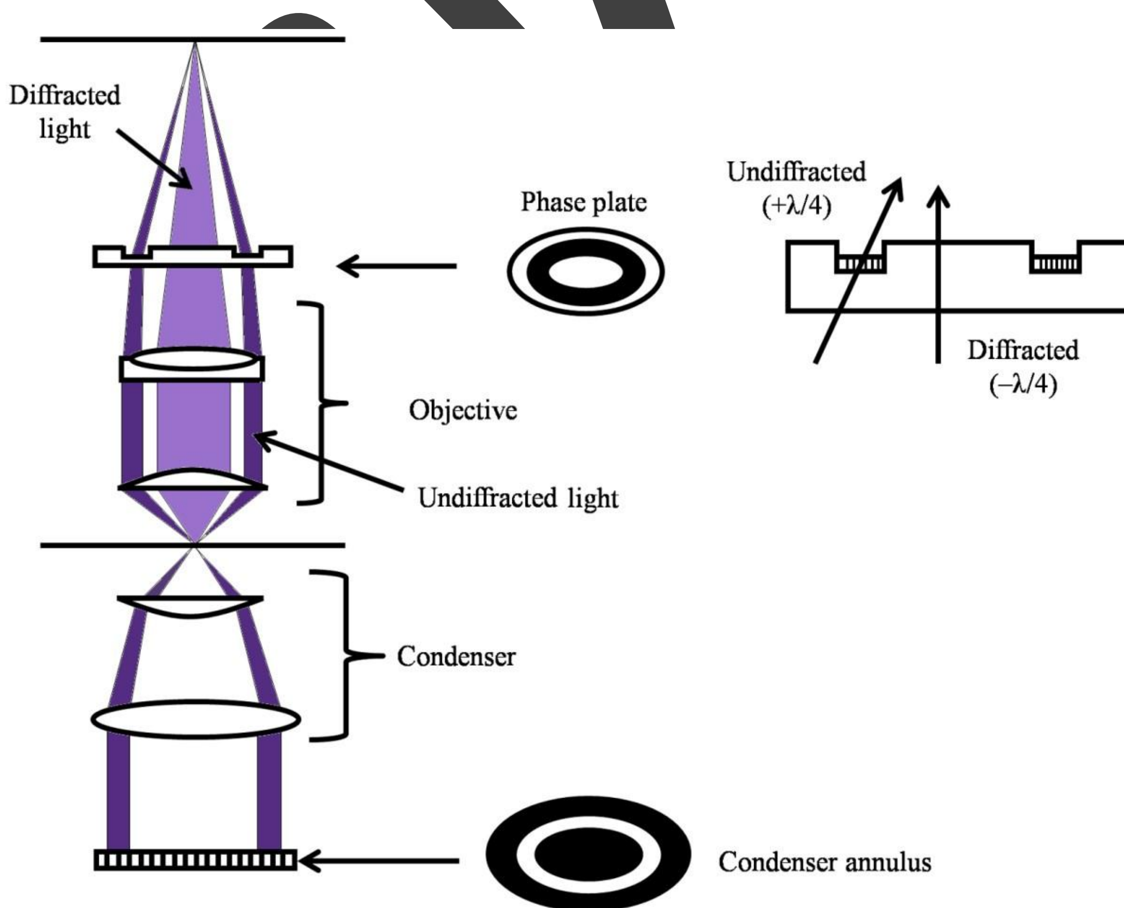

Figure: Optical diagram of a phase contrast microscope

Fluorescence microscope

fluorescence microscopes. In an epifluorescence microscope, the illumination of the specimen as well as the collection of the fluorescence light is achieved by a single lens. This has become possible due to the incorporation of dichroic mirror in the optics. A dichroic mirror is largely reflective for the light below a threshold wavelength and transmissive for the light above that wavelength.

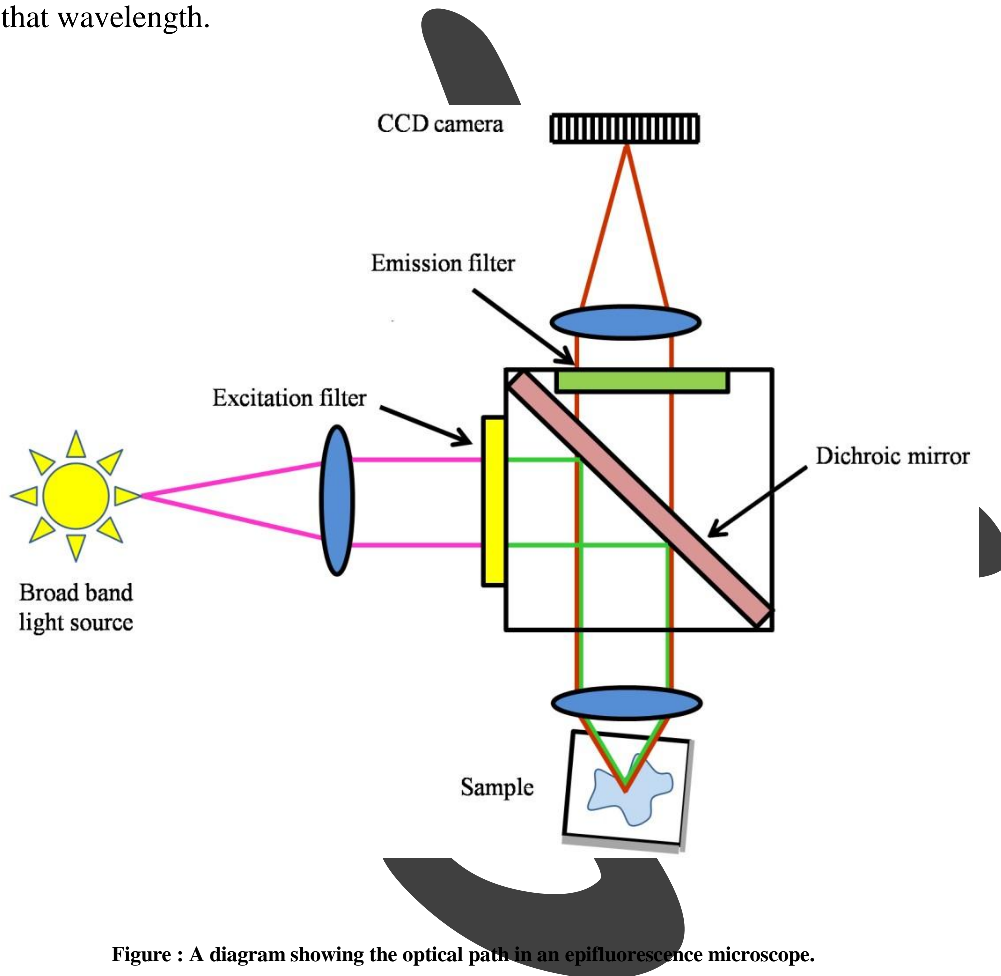

Figure : A diagram showing the optical path in an epifluorescence microscope.

The microscope has a high power lamp source, usually a mercury or xenon arc lamp. An excitation filter transmits the band of the excitation radiation. The excitation radiation is reflected by the dichroic mirror towards the condenser/objective lens that focuses the light on the specimen. Light emitted by the fluorescent molecules (higher wavelength due to Stokes shift) is collected by the same lens and is transmitted by the dichroic mirror towards the ocular lens. Figure 15.3 shows a comparison between a brightfield and a fluorescence image of the Cos-7 cells expressing GFP.

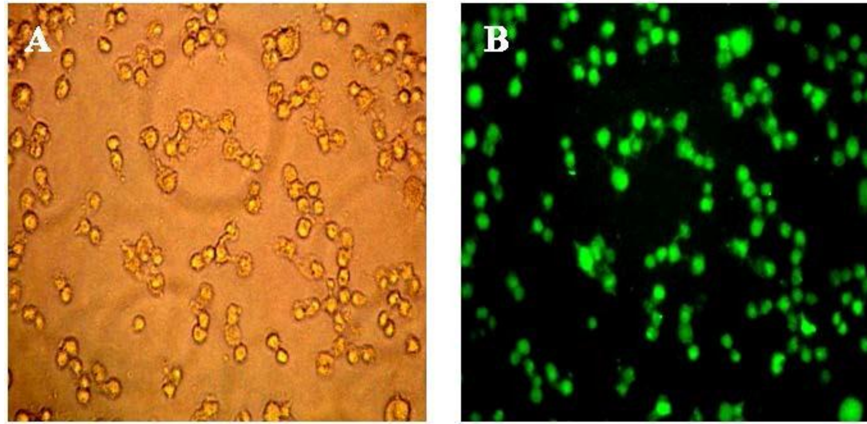

Figure Bright-field (A) and epifluorescence (B) images of Cos-7 cells expressing GFP.

Light microscopes come in two designs: upright and inverted (Figure 15.4).

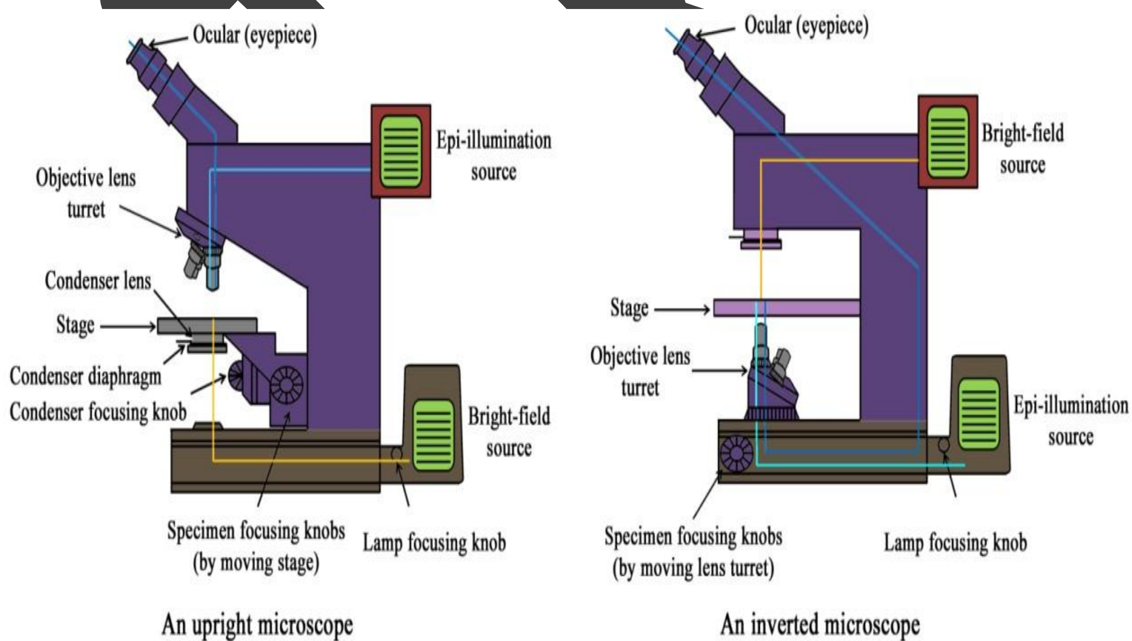

Figure: Designs of upright (A) and inverted (B) microscopes

In an upright microscope, the objective turret is usually fixed and the image is focused by moving the sample stage up and down. In an inverted microscope, the sample stage is fixed and objective turret is moved up and down to focus the final image. Inverted microscopes offer certain advantages over upright microscopes and are therefore becoming more popular:

i. As the objective turret is at the bottom of the stage, the sample stage is more accessible allowing manipulations of the sample.
ii. The specimen need not be covered at the top by a coverglass.
iii. The centre of mass is closer to the bench thereby providing more mechanical stability to the microscope.
iv. Inverted design provides an excellent platform for attaching the total internal reflection fluorescence accessories (discussed later in this lecture).

Electrons in microscopy

Louis de Broglie in 1924 theorized that particles have wave-like characteristics. Three years later, electron diffraction experiments carried out independently by 'Davisson and Germer' and 'Thomson and Reid' demonstrated the wave behavior of the electrons. Within next five years, the idea to use electrons for microscopy was realized when Knoll and Ruska published the images recorded using electrons

Electron sources and lenses

Of the various methods of generating electrons, two are more frequently used in the electron guns used for electron microscopy: *thermionic electron emission* and *field emission*. Most electron microscopes use thermionic emission of electrons from a heated filament. Being one of the cheapest and simplest thermionic sources, tungsten is most widely used in thermionic electron guns. Figure 17.1A shows a diagrammatic representation of a tungsten filament electron gun. The filament is placed in a cylindrical case called a Wehnelt cylinder or Wehnelt cap. Wehnelt cap has an aperture and the filament is situated immediately above the aperture. Below the Wehnelt cap lays an anode that causes the emitted electron to accelerate. A negative potential is applied to the Wehnelt cap that focuses the electrons emitted by the filament into a narrow beam. An electron gun therefore acts both as an electron source as well as a lens. The brightness of the electron beam is defined as the current density.

Further ten-fold increase in brightness can be achieved using lanthanum hexaboride (LaB_6) instead of tungsten filament.

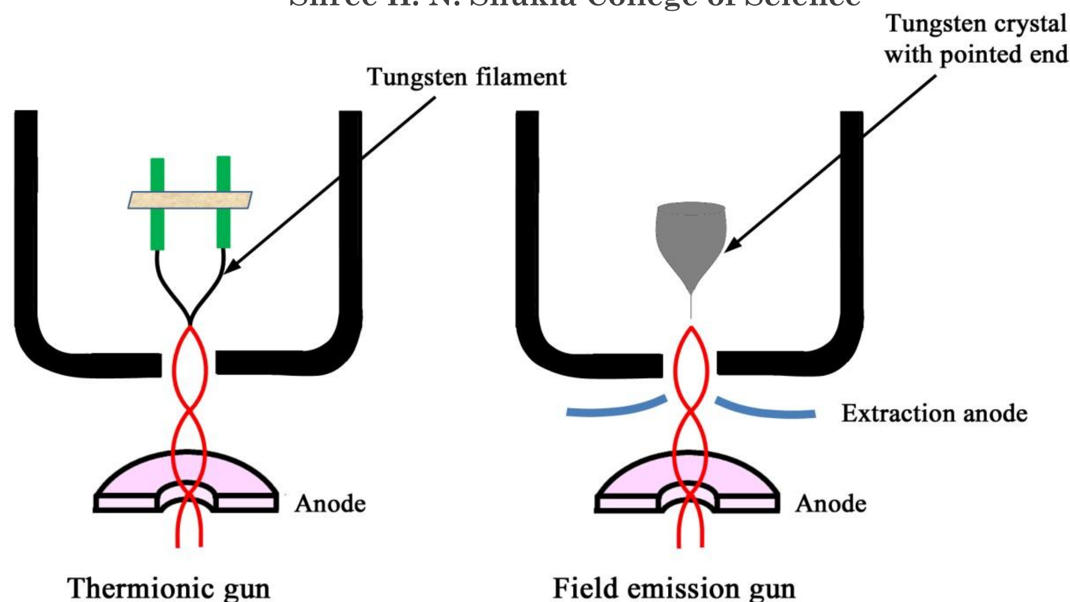

Figure Electron guns: A tungsten filament Wehnelt thermionic gun (A) and field emission gun (B)

For further higher brightness, another electron source called a field emission gun is used. A field emission gun typically uses a single crystal tungsten filament that has a very fine tip (Figure). The electrons are not ejected by heating the filament but by applying a very strong electric field called an extraction voltage. The field at the pointed tip is very large (>10^9 V/m) and results in electron emission through *tunneling*. As more number of electrons can be emitted compared to field thermionic emission, field emission guns have very high brightness (>10^{13} $A \cdot m^{-2} \cdot sr^{-1}$).

Scattering of electrons

We see various objects around us; but how exactly do we see them? How does a light microscope allow us to see a magnified image of a specimen? Why is milk white while water transparent? The answer to all these questions is same: the interaction of light with matter alters one or more properties of the light that it receives. We can see objects around us because they absorb, reflect, or scatter the visible light. A specimen becomes visible only if it brings about changes in the radiation used to visualize it. How do then we image samples using electrons? Electron microscopy is possible because interaction of electrons with matter

brings about changes in the electrons or generates new electrons with different energies. A specimen will be transparent to electrons if it does not scatter them and therefore be invisible when analyzed using an electron microscope. Figure shows the different processes that result through interaction of electrons with matter.

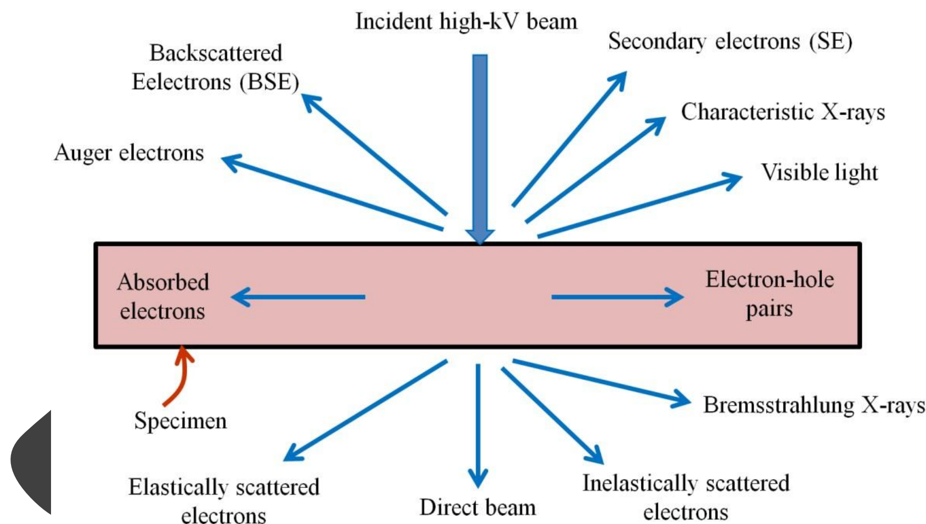

Figure Various phenomena that take place during electron interaction with a thin specimen

Elastic scattering: In elastic scattering, the scattered electrons do not lose their energy. The scattering only causes change in the electrons' trajectories. Elastic scattering gives a strong forward peak in a thin specimen.

Inelastic scattering: All scattering processes that result in the loss of energy of the primary electrons fall under inelastic scattering.

Secondary effects: Secondary effects include the phenomena that are brought about by the primary electron beam. The phenomena that we are concerned with here are:

- Secondary electrons: Secondary electrons are ejected from the atoms in the specimen. The term is usually used for the electrons that have energies below 50 eV. Such electrons can therefore include the primary electrons that lose their energies through successive scattering and reach the surface of the specimen. Secondary electrons are produced in abundance and form the basis of the scanning electron microscopy (discussed in the next lecture).
- Backscattered electrons: The primary electrons that do retain substantial energy before

escaping the specimen surface. Back-scattering is a function of the atomic number wherein samples with larger atomic number give brighter signals.

- Cathodoluminescence: An electron can knock off a valence electron from the colliding atom creating an electron-hole pair. An electron falls back into the hole releasing the excess energy as light
- X-rays: If an electron is knocked off from the inner shells of the atom, an electron in the higher energy shells can fill the vacancy in the lower energy state. The energy associated with inner electron transitions fall in the X-ray wavelength region.

We are now ready to see how electron microscopes work. Electron microscopes come in two basic designs: *scanning electron microscopes* and *transmission electron microscopes*. The two microscopes differ from each other in the electrons that are detected.

Scanning electron microscope

The electrons produced by the electron gun are guided and focused by the magnetic lenses on the specimen.

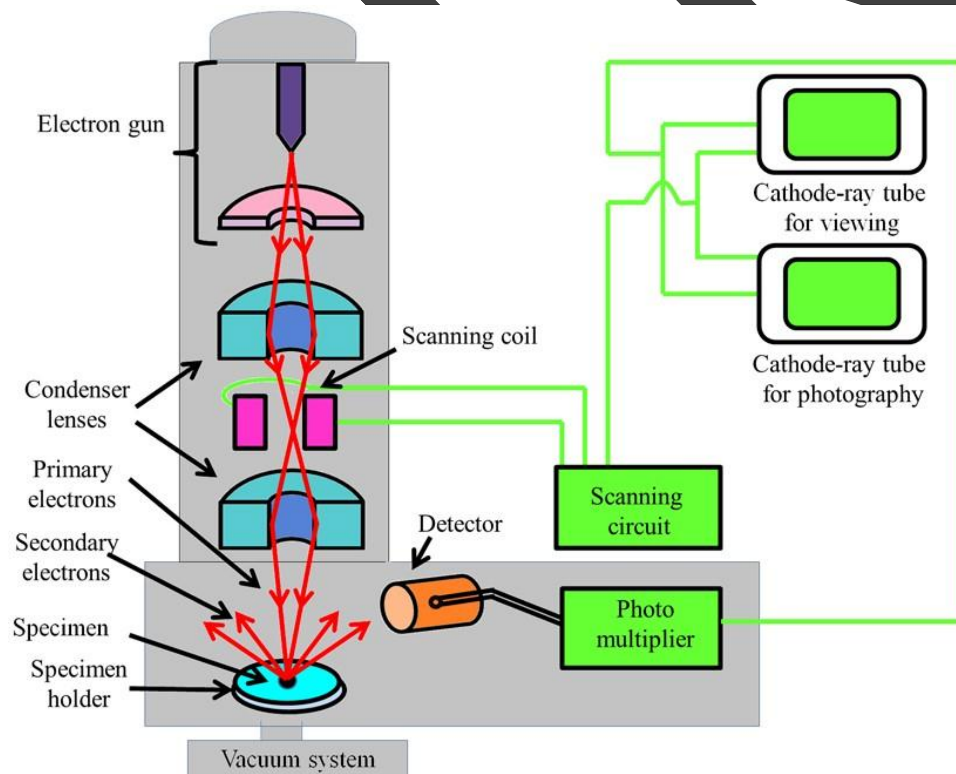

Figure : A simplified schematic diagram of a scanning electron microscope

The focused beam of electrons is then scanned across the surface in a raster fashion (Figure). This scanning is achieved by moving the electron beam across the specimen surface by using deflection/scanning coils. The number of secondary electrons produced by the specimen at each scanned point are plotted to give a two dimensional image.

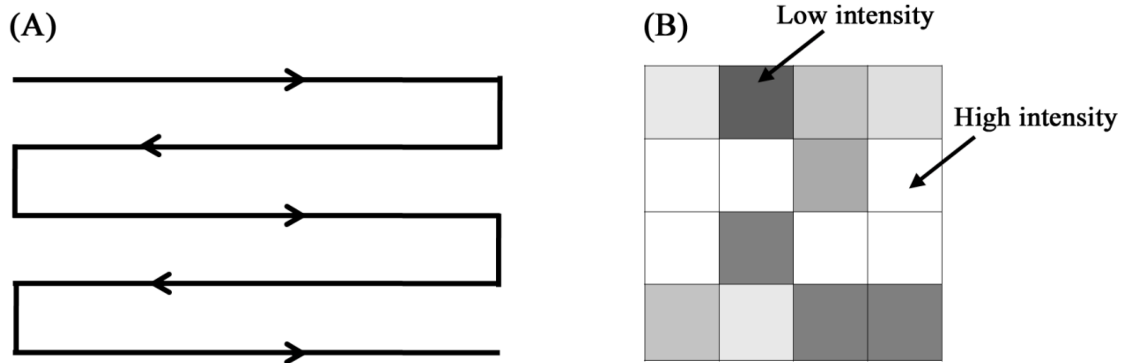

Figure A diagrammatic representation of the raster scanning (A) and the intensity plot for the scanned area (B).

In principle, any of the signals generated at the specimen surface can be detected. Most electron microscopes have the detectors for the secondary electrons and the backscattered electrons. Figure shows the interaction volume within the specimen showing the regions of secondary electrons (energy < 50 eV) and backscattered electrons.

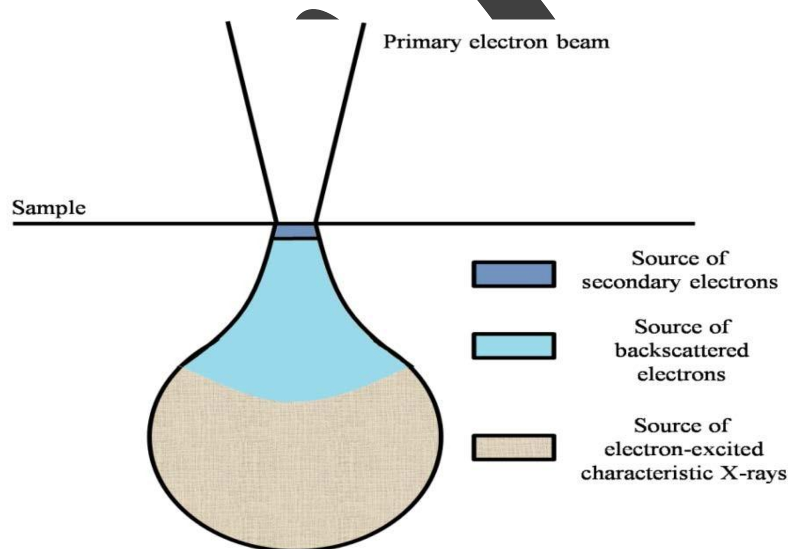

Figure: Specimen-electron interaction volume within the specimen. Notice the different regions where secondary electrons and backscattered electrons come from.

A secondary electron detector is biased with positive potential to attract the low energy secondary electrons. Detector for backscattered electrons is not biased; the high energy backscattered electrons strike the unbiased detector. As backscattered electrons come from a significant depth within the sample (Figure 18.3), they do not provide much information about the specimen topology. However, backscattered electrons can provide useful information about the composition of the sample; materials with higher atomic number produce brighter images.

Sample preparation for SEM: A specimen to be analyzed by electron microscopy has to be dry which most biological samples are not. As dehydration might lead to structural changes, the specimens are first fixed to preserve their structural features. Fixation is the first step and can be achieved using chemical methods such as fixation with glutaraldehyde or physical methods such as cryofixation in liquid nitrogen. The fixed specimens are then dehydrated usually by exposing them to an increasing gradient of ethanol (up to 100%). The specimens are then dried using critical point method. The dried specimens are then coated with a conducting material usually gold to make the surface conducting and cause it emit more secondary electrons. A SEM image of human erythrocytes coated with gold is shown in figure

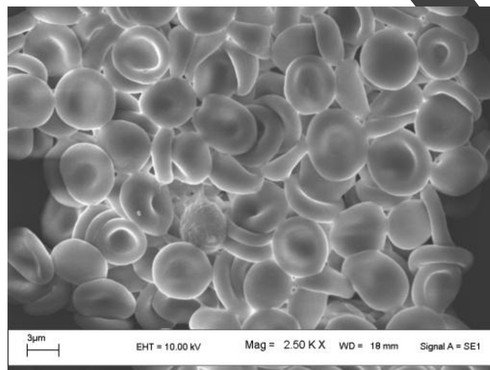

Figure A scanning electron micrograph of human erythrocytes.

Transmission electron microscope

The first electron microscope was developed by Knoll and Ruska in 1930s. It was a transmission electron microscope; the electrons were focused on a thin specimen and the electrons transmitted through the specimen were detected. Figure 18.5 shows a simplified optical diagram comparing a light microscope with a transmission electron microscope.

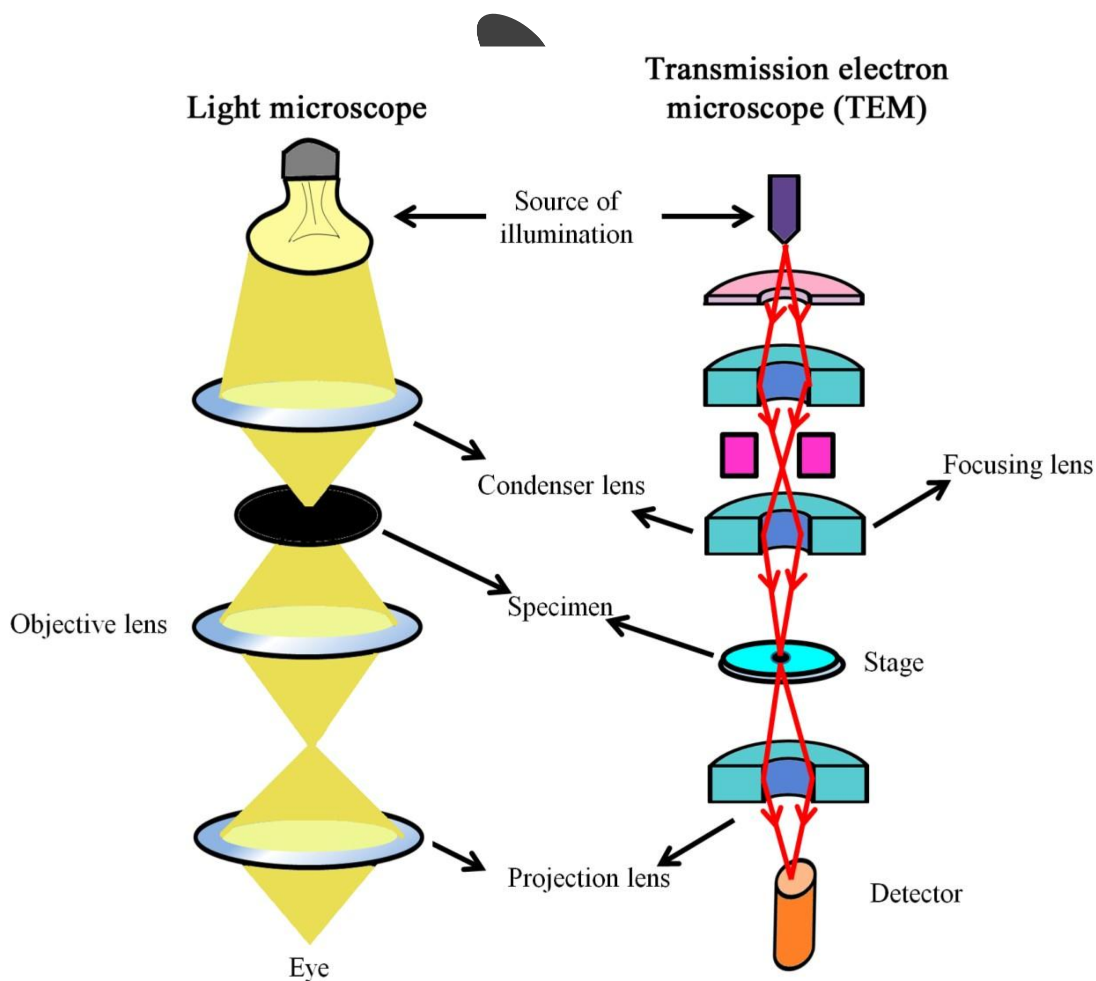

Figure 18.5 A simplified comparison of optics in a light microscope with that in a TEM.

Transmission electron microscopes usually have thermionic emission guns and electrons are accelerated anywhere between 40 – 200 kV potential. However, TEM with >1000 kV acceleration potentials have been developed for obtaining higher resolutions. Owing to their brightness and very fine electron beams, field emission guns are becoming more popular as the electron guns.