INVERTIS UNIVERSITY BAREILLY

LECTURE 9

ELECTRON MICROSCOPE

(TRANSMISSION AND SCANNING ELECTRON MICROSCOPE)

- Transmission electron microscopy utilizes the wave properties of moving electrons to generate highly resolved images of specimens.
- In 1986, the Nobel prize in physics was awarded ½ to Ernst Ruska (for work in electron optics and for design of first electron microscope) and ½ to Gerd Binnig and Heinrich Rohrer (for their design of the scanning tunneling microscope).

- Electrons are negatively charged subatomic particles. Their use in imaging can be explained in terms of their quantum ***mechanical wave-like property***, which allows a beam of electrons to be considered in exactly the same way as a beam of electromagnetic radiation such as light.
- Electrons possess two additional advantages for use in microscopy:
- **First:** by accelerating the electron between charged plates with a very high applied voltage, the wavelength of the 'radiation' used in the microscope could be made very small and this is associated with increase in resolution and one can increase magnification.
- **Second:** This actually allows the use of electrons for microscopy. That is, electromagnetic radiation of short wavelengths (X-rays & electrons) can be focussed by means of magnetic lenses.
- ***This is dependent on the principle in physics that a charged particle in a magnetic field is deflected in a direction perpendicular to its direction of motion.***

$$\text{Resolution } (r) = \frac{\lambda}{NA}$$

- The effect of the wavelength of light on resolution, at a fixed numerical aperture (0.95), is listed in Table, with longer wavelengths producing lowered degrees of resolution.

Wavelength (Nanometers)	Resolution (Micrometers)
360	.19
400	.21
450	.24
500	.26
550	.29
600	.32
650	.34
700	.37

- A magnetic lens is a device used for focusing or deflection of moving charged particles (electrons or ions), by use of the magnetic force. Its strength can often be varied by usage of electromagnets.

ELECTRON OPTICS:

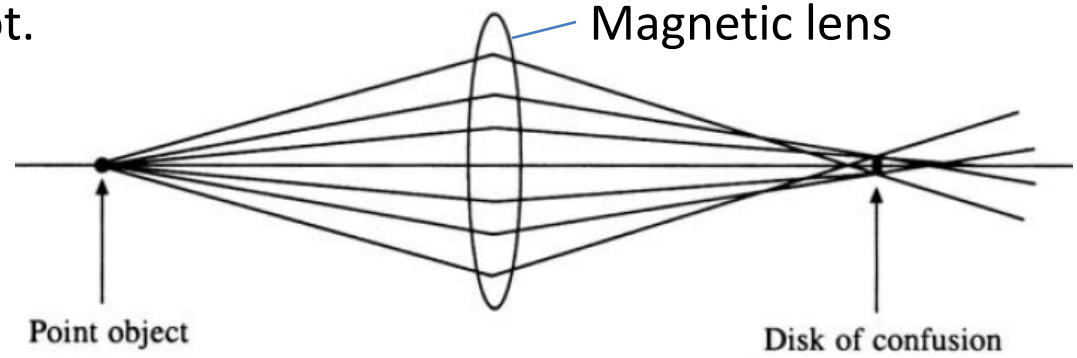
- The wavelength of the electron beam depends on its energy, which is in turn dependent on the voltage used to accelerate the electrons.
- Modern electron microscopes use accelerating voltages in the range 1000 volts to 1000 kilovolts. ***The most popular microscopes use about 100 kilovolts.***
- This voltage will correspond to wavelength of which gives the resolution about 0.03 Å.

$$E\,(keV) = {}^{12.4}/_{\lambda}$$

12.4 keV	1 Å
8 keV	1.54 Å
17.5 keV	0.71 Å

- However, several factors come in the way of reaching this high resolution i.e. Vibration, damage to the specimen and contamination are some of the problems encountered in microscope design.

- The most important factor limiting the resolution is the inability of the magnetic lenses to focus the beam accurately k/a aberration of the lens.
- Aberration is chiefly of two types:
- First is **spherical aberration**: Arises from the fact that all the electron beams scattered from the specimen which fall on the lens should be focussed on to the same spot but in practice are not.



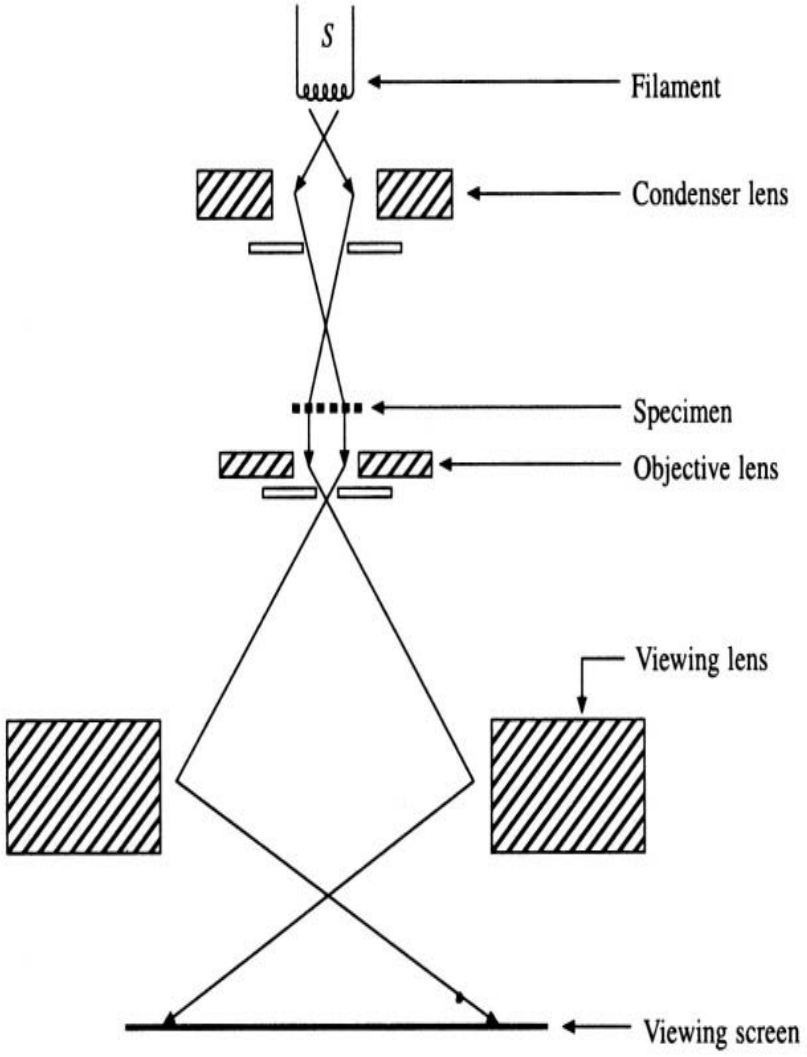
- Thus the image of the point will be seen as a disk, called the disk of confusion.
- Second is **chromatic aberration:** Arises from the fact that the focal length of the lens is different for different wavelengths.
- Electron source cannot produce a uniformly energetic electron beam, thus image point again spreads out into disk of confusion (caused by chromatic aberration).

- Final quality of image depends not only on resolution but also on contrast.
- Contrast is difference in intensities b/w object and background as a fraction of the background intensity (For visibility, this ratio should be larger than 0.2)
- Contrast can be enhanced if the object is dense as compared to the background.
- Usually, the support for the specimen is a carbon/copper film and biological materials (have about the same density).
- The use of EM in biology is thus especially problem prone (one can use staining methods to increase the contrast).
- It is also possible to increase contrast by increasing the intensity of the incident electron beam (background intensity varies as the square root of the incident intensity / while the image intensity will vary directly as the incident intensity).

- However, increasing the intensity of the incident beam leads to another severe problem in the study of biological materials, viz. radiation damage.
- This will destroy the sample leading to very poor quality images.
- Thus because of the above problems (not all), the actual practical resolution and magnification in biological electron microscopy is below what in principle is possible.
- The EM is far superior to the light microscope in its resolution and magnification.
- Objects such as cells, viruses, DNA strands, ribosomes, etc. can be imaged clearly at resolutions of a few tens of Angstroms ($A^0 = 10^{-10}m$), at magnification of 100000 X or more.

THE TRANSMISSION ELECTRON MICROSCOPE (TEM)

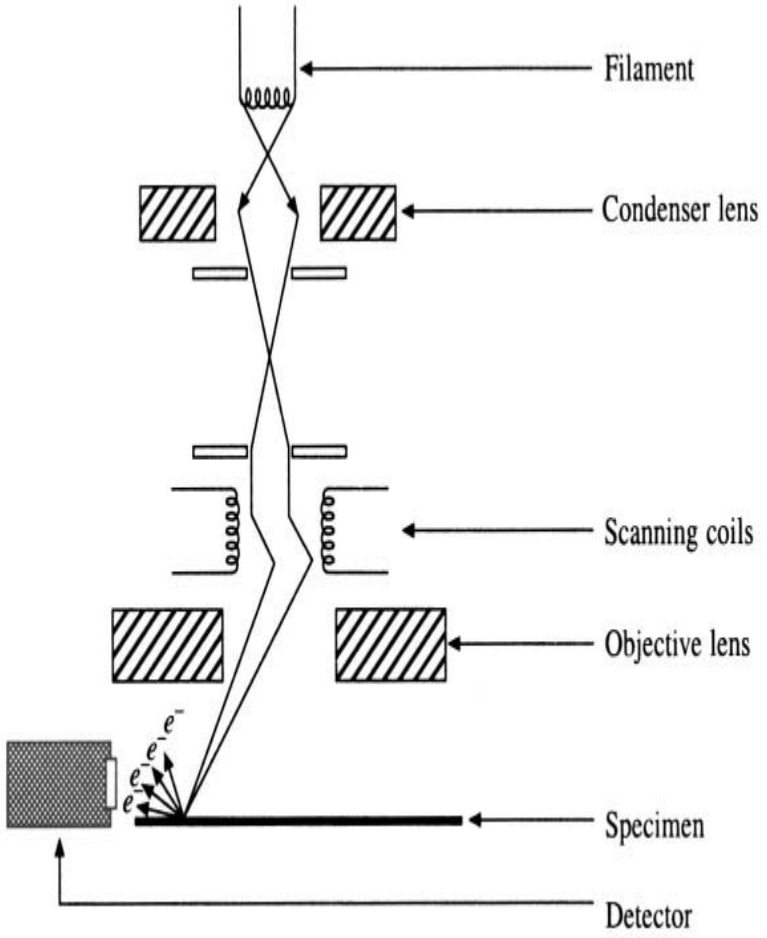
- This is one of the most commonly used instruments.
- The source *S* of the electron beam may be a tungsten filament, but other materials like lanthanum hexaboride are also used.
- The beam path, the placement of the lenses, the specimen, the aperture etc, follow the plan of the light microscope.
- The entire arrangement, including the specimen, has to be placed in high vacuum to avoid extraneous scattering and absorption of the electrons by air.



- The lenses are magnetic lenses in EM, thus image is not viewed directly through an eyepiece, but is projected on to a fluorescent screen on which electrons form the image.
- An extra aperture called the ***objective aperture*** is placed in EM (abscent in LM), this is because the image forming process in the TEM is different from LM.
- In a LM, light is transmitted or absorbed by the specimen and this creates a contrast b/w the different parts of it and hence the image.
- In TEMs, most of the incident beam passes through the specimen unimpeded and very little is absorbed, and only a small portion is actually scattered in the forward direction which is used to form the image.
- Contrast is created by differentiating between electrons scattered into wide angles from those scattered into small angles.
- The objective aperture cuts off the large angle electrons, and the amplitude of the electron beam from different portions of the specimen is different leading to amplitude contrast.
- Most biological materials do not have enough contrast to be viewed (unless stained).
- Dark field imaging is also possible by cutting out unscattered beam (as in phasecontrast).

The Scanning Electron Microscope (SEM)

- A SEM is very useful in obtaining images of the surface of thick specimens.
- In scanning transmission mode (STEM) of SEM, it can be used to study thin specimens and in this STEM has some advantages over the conventional TEM.
- The most obvious difference as compared to a TEM is that the electron beam, after passing through the condenser lens is deflected in a raster pattern over the specimen stage, similar to the pattern in a television picture tube.



- The objective lens is split into two parts:
- ***One part of objective*** is placed b/w condenser lens & specimen (in fact worked as additional condenser lens), which focuses the electron beam onto a small spot on specimen.
- Image signals can be collected by the detector, this detector is used in the surface-scanning mode and collects the secondary electrons of the primary electron beam ***scattered by the sample surface***.
- Image signals can also be collected in the STEM mode by the detector after passing through the second half of the objective.
- These are the ***forward scattered electrons***.

- Images obtained in the STEM mode are very similar to those obtained in the fixed beam TEM but the surface SEM image is quite different.
- The contrast in surface imaging arises from:
    a. Differences in the scattering powers of the different atoms in the specimen, and
    b. Differences in the surface structure of the specimen (variations in topography).
- **In both STEM & surface SEM modes images are generated in following way:**
- As the electron beam scans the specimen, the scattered electrons from each position is measured by the detector, one after the other in time.
- Measured intensities are displayed on imaging screen one after other.
- Thus, if scattering is high at particular point during the scan, then the corresponding point on the viewing screen is bright, and, if the scattering is low the corresponding point is dark. ***This creates an image of the object.***
- Resolution of image depends on size of the electron spot used to scan the specimen and this may be minimum 50 Å.
- Currently, both surface SEM and STEM are used in quantitative studies.

Sample Preparation for TEM (Tissue Sample):

- Collected tissue samples are washed with saline to remove blood content.
- Few pieces of approximately 5 mm cube of sample is stored in a mixture of 4% paraformaldehyde & 1% glutaraldehyde in phosphate buffer (FOR-GLU) for EM.
- **Grossing:** Tissue is placed on a piece of dental wax in few drops of buffer to avoid drying & cut into small pieces of 1 mm^3 with a razor blade (desired portion).
- **Fixation (to fix architecture):** After grossing, the tissue samples are transferred to vials containing fixative (FOR-GLU) and left for at least 1-3 hours at 4°C.
- Fixative is removed & sufficient amount of cacodylate buffer is immediately added into vials.
- Subsequently the specimens are washed thrice with cacodylate buffer.
- After buffer washing, cacodylate buffer is replaced with 1% Osmium tetroxide (OsO_4) for post fixation for 2 hours at RT.
- This is followed by rinsing with cacodylate buffer thrice (care was taken to prevent the drying of tissue).

- **Dehydration:** Fixed tissue samples are gradually dehydrated by passing through a series of solutions of ascending concentration of ethanol.
- Samples are finally dehydrated with two changes of 100% ethanol followed by two changes of propylene oxide.
- The dehydrated tissues are then gradually impregnated with resin, using ascending concentration of resin (resin + propylene oxide mixture) at RT.
- **Embedding :** Processed tissue samples are embedded in Beem capsules. For this few drops of resin mixture are placed in capsule & a piece of processed tissue is placed at bottom of resin mixture in capsule by forceps.
- Air-bubbles are removed from the face of capsule by using Stereo Microscope and then capsules are filled with resin mixture by syringe.
- **Block Preparation:** After embedding the capsules (in moulds) are kept for polymerization in an oven at 60-70°C for 20-24 hours.
- The polymerized blocks are used for sectioning.

- **Trimming:** Resin blocks containing the embedded specimens are trimmed under stereo microscope for the removal of excess resin.
- **Semithin Sectioning (for LM):** Semithin sections of 1-micron are cut by an Ultra microtome with glass knives (obtained from glass strips).
- Section folds are removed by warming the sections & are allowed to dry at 80°C.
- **Staining of Semithin Sections:** Sections are stained (after drying) by few drops of 1% Toluidine blue in 1% Borax for approximately 30 seconds at 60°C.
- Excess stain is removed by rinsing slide with DDW & dried on hotplate.
- **Examination of Semithin Sections:** Toluidine stained semithin sections are observed under LM (10X or 20X) to observer and/or decide presence of desired area in the block for EM studies.
- Tissue blocks in which the desired area is present is taken for ultrathin sectioning.

- **Ultrathin Sectioning (for EM):** Tissue blocks containing desired area is again trimmed for the removal of excess resin surrounding the specimen.
- After trimming, then ultrathin sections of 60-90nm are cut with the help of diamond knife (sections are appeared as silver ribbon), and picked on copper grids (as per the specimen).
- **Staining Procedure for EM:** The picked ultrathin sections on grid is stained with Uranyl acetate and Lead citrate and examined under TEM.
- Photographs of areas of interest are taken using Camera attached with EM. Photographs stored in computer are printed and analyzed for ultrastructural changes.

SAMPLE PREPARATION FOR SEM:

- All samples must be of an appropriate size to fit in the specimen chamber and are generally mounted tightly on a specimen holder called a specimen stub.
- Models of SEM with different specimen holder are available which can examine any specimen up to 6-inch (15 cm) and can tilt an object of this size to 45°.
- For SEM, specimens must be electrically conductive (at least surface) and must be electrically grounded to prevent accumulation of electrostatic charge at surface.

Metal objects require little special preparation for SEM:

- **Nonconductive specimens** tend to charge when scanned by the electron beam, and this causes scanning faults and other image artifacts.
- Thus, they are coated (ultrathin coating) with an electrically conducting material by specialized methods (low-vacuum sputter coating / high-vacuum evaporation).
- Gold, gold/palladium alloy, platinum, osmium, iridium, tungsten, chromium, and graphite etc. are used as conductive materials for coating.

- **For biological samples and materials specimens (where electron penetrates):** Embedding in resin with further polishing to mirror-like finish is required.
- For SEM of biological samples, a specimen must be completely dry because the specimen chamber is at high vacuum.
- Hard, dry materials such as wood, bone, feathers, dried insects, or shells can be examined with little further treatment.
- But living cells & tissues and whole soft-body organisms usually require chemical fixation to preserve and stabilize their structure.
- Fixation is usually performed by incubation in buffered fixative (glutaraldehyde in combination with formaldehyde) and followed by postfixation with osmium tetroxide. The fixed tissue is then dehydrated with organic solvents like ethanol or acetone, and replacement of these solvents in done with a transitional fluid such as liquid carbon dioxide and dried. The dry specimen is usually mounted on a specimen stub using an adhesive such as epoxy resin or electrically conductive double-sided adhesive tape, and sputter-coated with gold or gold/palladium alloy before examination in the microscope.

Temperature sensitive materials (ice & fats):

- For this SEM should be equipped with a cold stage for cryo-microscopy, cryofixation may be used and low-temperature scanning electron microscopy is performed on the cryogenically fixed specimens.
- Cryo-fixed specimens may be cryo-fractured under vacuum in a special apparatus to reveal internal structure.
- Specimens were then coated, and transferred onto the SEM cryo-stage for analysis and the samples are still in frozen state.
- **Cryo sectioning is also available in TEM.**

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