ORIGINAL RESEARCH PAPER

Anatomy

KEY WORDS:

Electron microscopy: a short review

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ABSTRACT

The basic aim of a microscope is to provide magnification, resolution and contrast to the specimen under observation. The limit of resolution is indirectly proportional to the wavelength of the illuminating light. The visible light lies in a wavelength range of 400-700nm, since the resolution cannot be less than half of the wavelength , the ultimate resolution attainable by using light microscope is 200nm. In the 1920s, it was found that electrons when accelerated in vacuum behave like light. The wavelength of electrons being 100,000 times smaller than that of light gave the scope that resolution of microscope can be increased if electrons are used as source of illumination. There are varieties of electron microscopes, each having a peculiar function. The most commonly used electron microscopes are transmission and scanning electron microscope. Electron microscopy has wide applications in physical, chemical and biological sciences. The future of electron microscopy rests on its ability to create a 3D view of the artefacts found in the specimen.

Introduction to microscopy

When provided ample amount of light, the unaided human eye can distinguish two points that are 0.2 mm apart. This distance being called as the Resolving power of the eye. When the distance between these two points is less than 0.2 mm they cease to be distinguished as two points by the unaided eye and only single point is seen.

The word microscope is derived from the Greek mikros(small) and skopeo (look at). The light microscope was developed from the Galilean telescope during the 17th century. In a light microscope, a beam of visible light is focused on a thin object and a combination of glass lenses gives an image , which can be viewed by our eyes through an eye piece. The image thus formed is real. The light microscope can magnify the image up to 1000 times as compared to the eye. The limit of resolution is indirectly proportional to the wavelength of the illuminating light. The visible light lies in a wavelength range of 400-700nm, since the resolution cannot be less than half of the wavelength , the ultimate resolution attainable by using light microscope is 200nm.

In the 1920s, it was found that electrons when accelerated in vacuum behave like light. The wavelength of electrons being 100,000 times smaller than that of light gave the scope that resolution of microscope can be increased if electrons are used as source of illumination 1. In addition to that it was observed that electric and magnetic fields have the same effect on electrons as glass lenses and mirrors have on visible light. These observations were used by Dr. Ernst Ruska (1931) at University of berlin to build the first Electron microscope , a transmission electron microscope with a resolution of 100nm using two magnetic lens 1,2. Today the resolving power of 0.2 nm has been achieved by using 5-7 magnetic lenses.

Electron the central player

This elementary particle called the corpuscle was discovered by J.J.Thompson in 1897. It carries a negative charge over it and is a constituent of an atom, the size of it being 1000 times smaller than hydrogen atom and mass being 1/1836 of that of a proton.

The complex interaction between the scattered electrons and the specimen results in elastic electron interactions and inelastic electron interactions . In elastic electron interactions no energy is transferred from electron to sample. These signals are mainly exploited in Transmission electron microscopy and Electron diffraction methods. On the other hand inelastic electron interactions the energy is transferred from electron to specimen and this results in variety of signals such as X rays, Auger electrons , secondary electrons, plasmons, phonons, UV quanta or cathodluminescence. These signals are used in analytical electron microscopy – scanning electron microscopy.

Electron microscopy and electron microscope

Electron microscopy is a diagnostic tool with diversified combination of techniques and gains insight to the structure, morphology, topology and composition of a material. The electron microscope is a special type of microscope having a high resolution (0.2nm) and magnification (100 to 250000 times the light microscopy) of images which are formed by controlled use of electrons in vacuum captured on a phosphorescent screen. It helps in study of objects less than 0.2 µm in size, analysis of subcellular structure, intracellular pathogens such as viruses, cell metabolism and other minute structures in nature.

Applications of electron microcope

it has wide applications in biological, physics and chemical sciences 3 . Biological and life sciences 4

- Cryobiology
- Cryoelectron microscopy
- Diagnostic electron microscopy
- Electron tomography
- Particle analysis
- Particle detection
- Drug research
- Protein localization
- Toxicology
- Virology
- Tissue imaging

Materials research

- Material qualification
- Medical research
- Nanometrology
- Device testing and characterization
- Industry
- Chemical/petrochemical
- Food science
- Forensics
- Mining
- Pharmaceutical quality control

Types of electron microscopy

1. Transmission electron microscopy

a. Bright field
b. Dark field
c. High resolution Transmission electron microscopy
d. Energy filtered transmission electron microscopy
e. Electron diffraction

2. Scanning Transmission Electron Microscopy

a. Bright field/ Dark field
b. High angle annular Dark field

3. Analytical electron microscopy
a. X ray spectroscopy
b. Electron energy loss spectroscopy(EELS)
c. Electron spectroscopic imaging (ESI)

4. Scanning Electron Microscopy
a. Secondary electron imaging (SE)
b. Back scattered electron imaging(BSE)

Commonly used electron microscope in biological sciences
Transmission and scanning electron microscope are used mainly for studying various life forms and microbes. On the other hand to visualize the molecules and individual atoms even in motion scanning tunneling and atomic force microscope can be used.

Transmission electron microscopy
The first transmission electron microscope was built by Max Knoll and Ernst Ruska in 19311. The first commercial TEM was built by them in 1939. It is a microscopy where a beam of electrons is transmitted through an ultra-thin specimen, interacting with specimens as it pass through. An image is formed from the interaction of the electrons transmitted through the specimen. This image is magnified and focused onto an imaging device such as fluorescent screen, on a layer of photographic film or to be detected by a sensor such as CCD camera5. It has a wide application in biological as well as chemical and physical sciences. It can be used in cancer research, virology, material sciences, nanotechnology and many more 6,7,8.

Components of TEM
1. Vacuum system: in which electron travel in a straight line
2. Electron emission source : for generation of the electron stream (tungsten filament or lanthanum hexaboride (LaB6)
3. Voltage source 100-300 kV ; by a series of electromagnetic lens and electrostatic plates to guide and manipulate the beam
4. Insertion device to allow insertion into, motion within and removal of specimens from the beam path
5. Imaging devices are subsequently used to create an image from the electrons that exit the system on phosphor screen having zinc sulphide.

Types of TEM
1. Bright field
2. Dark field
3. High resolution transmission electron microscopy
4. Energy filtered transmission electron microscopy
5. Electron diffraction

The most common mode of operation for a TEM is the bright field imaging mode. As the thickness of the sample increases, the contrast also increases. Thicker regions of the sample or the regions with high atomic number will appear dark and regions with no sample in beam path will appear bright 9.

Sample preparation
It is a crucial step in electron microscopy and any error in any step can lead to failure to obtain result. There are 3 main steps for sample preparation: Processing, embedding and Polymerization.

Processing
This includes : fixation, rinsing, post fixation, dehydration, clearing and infiltration.

1.Fixation : it prevents autolysis, change in volume and shape and preserves chemical constituents of cell. The aim of fixation is to preserve the structure of cells and tissues with minimal alteration from the living state, to protect them against alterations during embedding and sectioning and to prepare them for subsequent treatments such as staining and exposure to the electron beam 10 . The commonly used fixatives being glutaraldehyde, paraformaldehyde, acrolein, karnovsky's fixative (glutaraladehyde + paraformaldehyde) and osmium tetraoxide . glutaraldehyde and paraformal dehyde are the most commonly used in primary fixation (for proteins) followed by secondary fixation with OsO4 (for lipids)

2. Rinsing (washing) : specimen is thoroughly washed with buffer to wash off excess fixative. If traces are left in the specimen , they may react with OsO4 , producing precipitate of reduced osmium in the specimen

3. Post fixation (secondary fixation) : OsO4 is most frequently used for secondary fixation . It reacts mainly with lipids , converting unsaturated fatty acids to stable glycol osmate. It also acts as an electron dense stain. Thus the stability and contrast of the structure is increased. After fixation , specimens are rinsed thoroughly in buffer to wash off excess fixative 11.

4. Dehydration : the water content in the tissue sample should be replaced with an organic solvent since the epoxy resin used in infiltration and embedding step are not miscible with water. Tissue water is removed by passing the tissues through a series of ascending concentrations of the dehydrating agent. Ethanol or acetone act as a dehydrating agent. Acetone has an advantage over ethanol, as it is more readily miscible with clearing agents , viz. xylene and toluene.

5. Clearing : the dehydrating agent is replaced with a solvent that facilitates penetration of resin into tissues. Xylene , toluene and epoxy propane are some of the clearing agents used commonly.

6. Infiltration : epoxy resin is used to infiltrate the cells .It penetrates the cells and fills the space to give hard plastic material which will tolerate pressure of cutting 12.

Embedding
It can be done using flat moulds or plastic capsules. After filling the molds/capsules with the embedding medium, tissue is placed. When specific orientation is required, flat embedding mold is used.

Polymerization
It is the process by which resin is allowed to harden by heat treatment (50-60°C temperature)

Ultamicrotomy
1. Trimming of blocks : blocks are trimmed to produce a pyramidal shape at the tip of the block where the tissue is located.
2. Sectioning : diamond or glass knives are used for sectioning. Before one proceeds with ultrathin sectioning, thick sections (0.5-1.0 µm) are cut for viewing the tissue under a light microscopy. This enables to determine the quality of fixation, selection of the area for ultrathin sectioning and the size and position of the cutting face for final trimming. Ultrathin sections show interference colors while floating on the liquid of the knife boat. This allows determining the thickness of the sections. Silver colored sections

(60-90nm) are ideal for most purpose and gold colored sections (90-150nm) is useful for low magnification.

To obtain a good contrast of sections, a double staining method using uranyl acetate and alkaline lead citrate is routinely followed.

Limitations of TEM[13,14]

- Many materials require extensive sample preparation.
- It is difficult to produce a very thin sample
- Relatively time consuming process
- Structure of sample may change during the preparation process
- Small field view may not give conclusive result of whole sample.

Scanning Electron Microscope

In 1937, Manfred Von Ardenne who built the first scanning electron microscope. It is used for examining the surface details of microorganisms, cells and tissues. the specimen surface is scanned with a narrow electron beam and the generated signals are collected and amplified and then fed to the cathode ray tube for display. It can be used to study the topography, morphology, composition and crystallograp hic information of a material.15,16,17

Components of SEM

1. Electron optical column consists of
a. Electron source to produce electrons
b. Magnetic lens to demagnify the beam
c. magnetic coils to control and modify the beam
d. Apertures to define the beam and prevent

electron spray

2. Vacuum system consists of
a. Chamber which holds vacuum
b. Pumps to form vacuum
c. Valves to control vacuum
d. Gauges to monitor vacuum

3. Signal detection and display consists of
a. Detectors which collect the signal
b. Electronics which produce an image from the signal

As the electron beam hits the specimen, variety of signals are generated , different signals providing different information when collected by specific detectors. The secondary electrons provide information on surface morphology. X rays provide information for elemental detection and analysis. The backscattered giving knowledge about surface topography.

Sample preparation

The surface of an object to be studied by SEM should possess following characteristics

- It must be free from foreign particles
- ·It should remain stable when put under high vacuum
- It must be stable after exposure to electron beam
- It must emit sufficient quantity of secondary electrons

- Cleaning the surface of SEM : the proper cleaning of the surface of sample is essential because the surface might contain a variety of unwanted deposits, such as dust, silt , media components or other components depending on source of the biological material and the experiment that may have been conducted prior to SEM specimen preparation
- Stabilizing the specimen : apart from objects such as teeth, bone and wood , all other biological materials must be stabilized in order to prevent from undergoing structural changes , which is done by chemical or physical fixation (cryofixation). The fixative and fixation protocol remains same as that of TEM specimen preparation.
- Rinsing the specimen : the specimen is washed in buffer in order to remove excess fixative
- Dehydrating the specimen : the most commonly used dehydrating agents are ethanol and acetone. To avoid initial osmotic damage the specimens are dehydrated using increasing concentration of the dehydrating agent upto dry absolute acetone stage.
- Drying the specimen : it can be dried by various methods

a. Air drying : it is the simplest method but it creates maximum distortion due to compressive forces of surface tension of liquid gas interface leading to flattening of the sample.

b. Critical point drying : it is most commonly used technique to dry biological samples. The sample is transferred from an organic dehydration medium (acetone) to drying medium (liquid CO2 or Freon 13) in a chamber that is cooled and pressurized. When there is complete removal of dehydrating agent and the specimen is now impregnated with liquid CO2 , the chamber is now warmed up to the critical point where the density of drying medium is same in both liquid and gas phase. Carbon dioxide is removed after its transition from the liquid to the gas phase at the critical point , and the specimen is dried without critical damage.

c. Freeze drying : unfixed as well as chemically fixed samples can be freeze dried. Freeze drying consists of the sublimation of ice from cells and tissues under vacuum.

6. Specimen Mounting : after processing of sample it must be mounted on a holder that can be inserted into the scanning electron microscope. Samples are typically mounted on metallic (aluminium) stubs using a double sticky tape. It is important that investigators first decides on the best orientation of the specimen on the mounting stub before attaching it as a reorientation is difficult and can result in significant damage to the sample.

7. Metal coating : Biological specimens are poor electrical conductors. They develop electrical charge when scanned by high energy electron beam. This leads to charging artefacts , distorting the image. The conductivity of the specimen can be increased by if coated with a thin layer (15-30nm) of metal. The metal coating in addition to increasing conductivity of the specimen also increases the strength of fragile biological material and the number of secondary electrons emitted from specimen surface, leading to improvement in image formation. Metals commonly used for sputter coating are silver, gold, gold-palladium , platinum etc. Coating can be done in two ways : thermal evaporation and sputter coating. Sputter coating is the preferred method because it allows uniform coating on the specimen even on parts that are not directly facing the metal to be evaporated. On the other hand even under best conditions , the coating done by thermal evaporation may be relatively uneven and the chances of surface charge accumulation are higher.

After coating the specimen is ready to be viewed under SEM.

Limitations of SEM

- SEMs are costly, large and must be housed in an area free of any possible electric, magnetic or vibration interference.
- A steady voltage is required for maintenance of currents to electromagnetic coils and circulation of cool water.
- Special training is required to operate an SEM as well as prepare samples.
- Any fault in the specimen processing results in artefacts on imaging.
- Specimen size is small thus imaging gets limited
- SEMs carry a small risk of radiation exposure associated with the electrons that scatter from beneath the sample surface

Conclusion

The electron microscope uses beam of electrons as a source of illumination to create an image of the specimen. It allows us to study structures which cannot be visualiseed in a light microscope by providing higher magnification and resolution. They are huge, expensive pieces of equipment , generally standing alone in a small, specially designed room and requires special trained personnel for its operation. It has wide application in biological and material sciences as well as in industry for quality check.

Future of electron microscopy

The future oppurtinities wil be to achieve atomic or molecular resolution measurements in a short time scale and in real environment. Also enabling the materials to be viewed in multidimensional view i.e. the three spatial dimensional together with energy and momentum can be a strong scientific oppurtinity in development of electron microscopy.18,19,20

References

1. Ruska, Ernst (1986). "Ernst Ruska Autobiography". Nobel Foundation.
2. Rudenberg, H Gunther; Rudenberg, Paul G (2010). "Chapter 6 – Origin and Background of the Invention of the Electron Microscope: Commentary and Expanded Notes on Memoir of Reinhold Rüdenberg". Advances in Imaging and Electron Physics. 160. Elsevier.
3. Kruger DH; Schneck P; Gelderblom HR (May 2000). "Helmut Ruska the visualisation of viruses". Lancet. 355 (9216): 1713–7.
4. Steere, Russell L. (1957-01-25). "ELECTRON MICROSCOPY OF STRUCTURAL DETAIL IN FROZEN BIOLOGICAL SPECIMENS". The Journal of Biophysical and Biochemical Cytology. 3 (1): 45–60.
5. Kacher, J.; Cui, B.; Robertson, I.M. (2015). "In situ and tomographic characterization of damage and dislocation processes in irradiated metallic alloys by transmission electron microscopy". Journal of Materials Research. 30 (9): 1202–1213.
6. R.S.; Subramanian, S. (2009). "Role of transmission electron microscopy in the semiconductor industry for process development and failure analysis". Progress in crystal growth and characterization of materials. 55 (3–4): 63–97.
7. Florian, P.E.; Rouillé, Y.; Ruta, S.; Nichita, N.; Roseanu, A. (2016). "Recent advances in human viruses imaging studies". Journal of Basic Microbiology. 56 (6): 591–607.
8. Cushnie, T.P.; O'Driscoll, N.H.; Lamb, A.J. (2016). "Morphological and ultrastructural changes in bacterial cells as an indicator of antibacterial mechanism of action". Cellular and Molecular Life Sciences. 73 (23): 4471–4492.
9. Li, M.-H.; Yang, Y.-Q.; Huang, B.; Luo, X.; Zhang, W.; Han, M.; Ru, J.-G. (2014). "Development of advanced electron tomography in materials science based on TEM and STEM". Transactions of Nonferrous Metals Society of China. 24 (10): 3031–3050..
10. McDowell EM , Trump BF. Histologic Fixatives suitable for diagnostic light and electron microscopy ; Archives of pathology and laboratory medicine Aug 1976, 100(8);405-414
11. K Kato The osmium tetroxide procedure for light and ekectron microscopy of ABS plastics ;Polymer Engineering & science , 1967- Wiley Online Library
12. Arthur R. Spurr . A low viscosity epoxy resin embedding medium for electron microscopy : journal of Ultrastructure Research vol 26,Issues1-2, january 1969, Pages 31-43
13. Chen, X.D.; Ren, L.Q.; Zheng, B.; Liu, H. (2013). "Physics and engineering aspects of cell and tissue imaging systems: microscopic devices and computer assisted diagnosis". Biophotonics in Pathology: Pathology at the Crossroads. 185: 1–22..
14. Fagerland, J.A.; Wall, H.G.; Pandher, K.; LeRoy, B.E.; Gagne, G.D. (2012). "Ultrastructural analysis in preclinical safety evaluation". Toxicologic Pathology. 40 (2): 391–402.
15. Wilhelmi, O.; Roussel, L.; Faber, P.; Reyntjens, S.; Daniel, G. (2010). "Focussed ion beam fabrication of large and complex nanopatterns". Journal of Experimental Nanoscience. 5 (3): 244–250.
16. Bro ek-Mucha, Z. (2014). "Scanning electron microscopy and X-ray microanalysis for chemical and morphological characterisation of the inorganic component of gunshot residue: selected problems". Biomed Research International. 2014: 428038.
17. Wiiliam R. Herguth, president , Guy Nadeau. Applications of Scanning Electron Microscopy And Energy Dispersive Spectroscopy to practical Tribology problems
18. R.Gordon , R Bender , GT Herman . Algebraic reconstruction techniques (ART) for three dimensional electron microscopy and X ray photography ; journal of theoretical biology , 1970 – Elsevier
19. R.Strack . Microscopy : colorful electron microscopy : nature methods Vol 14; pages 8-9 (2017)
20. Rebecca G. Weiner et al . Electron Microscopy : Impact of membrane – induced particle Immobilization on seded growth monitored by in situ Liquid Scanning Electron Microscopy ; wiley online library vol 12, issue 20 pg 2267