12 Microscopy

Microscope *is an optical system designed for the study of objects not to be seen by the unaided eye.* It makes us see things which are very small and which are otherwise invisible (Mikros = ***small***; skopein = *to see, to **look***). It is an inevitable instrument for clinical and laboratory studies. The first microscope was designed by ***Jenssen*** and ***Hans*** (1590-1610).

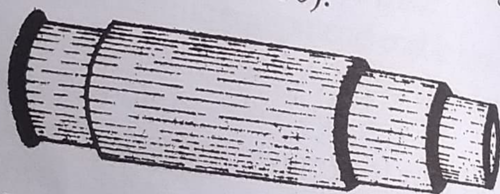


Fig.12.1: Jenssen's compound microscope.

Jenssen's microscope *is a compound microscope.It is the first microscope. It contains two tubes fitted one inside the other. Its magnification is **nine times.***

Magnification

The optical system of the microscope magnifies the objects to several folds of their original size. The ratio of this magnified image to that formed on retina of an unaided normal eye is termed the ***magnification***.

$$\text{Magnification} = \frac{\text{Size of retinal image seen with microscope}}{\text{Size of retinal image seen with unaided eye}}$$

Resolving Power

The ***resolving power*** *is the capacity of an optical system to show distinct images of points lying very close together.* The resolving power of unaided human eye is 0.1mm. Thus the human eye can distinguish objects upto the size of 0.1mm.

The resolving power depends on the ***wave length*** (l) of light and the ***numerical aperture*** (NA) of the objective lens.

The limit of resolution is the minimum distance between two points resolvable as separate points, when viewed through lenses.

$$\text{Limit of resolution } d = \frac{0.161}{NA}$$

where, NA = Numerical aperture

l = Wave length of light used.

The limit of resolution is inversely proportional to the resolving power. Numerical aperture is limited due to the stability of refractive index of the optical materials, which does not exceed 1.6. Thus it is evident that the resolving power can be increased by reducing the limit of resolution, i.e., by using smaller wave length.

Types of Microscopes

The microscopes are classified into three types based on the source of illumination. They are the following:

1. Light microscopes
2. Electron microscopes
3. X-ray microscopes.

1. Light Microscopes

In light microscopes, ***light*** is the source of illumination. They are sub-divided into the following types:

- *Simple microscope*
- *Compound microscope*
- *Binocular microscope*
- *Phase contrast microscope*
- *Interference microscope*
- *Polarizing microscope*
- *Dark field microscope*
- *Ultraviolet microscope*
- *Fluorescence microscope*
- *Cinematography*

Compound Microscope

The compound microscope is an *optical instrument* to ***magnify*** objects.

It is formed by the combination of two simple microscopes (a simple microscope is formed of only one lens).

It is a ***light microscope*** because light is the illuminating source.

Principle

The compound microscope works on the principle of ***optics***. The lenses ***magnify*** objects. By stacking lenses the magnification is increased.

The compound microscope has a ***light source***, a ***diaphragm***, an object an ***objective lens*** and ***an eye piece*** (eye lens).

The light passes through the ***diaphragm***. The diaphragm ***gathers*** the light on the object.

The objective lens produces a ***real, inverted magnified image*** of the object.

This magnified image acts as an ***object*** for the eye piece.

The eye piece produces a ***virtual inverted*** and ***magnified*** image of the object.

Magnifying Power

The magnifying power of a compound microscope is the ratio of the size of the final image to the size of the object.

$$M = \frac{\text{Size of the final image}}{\text{Size of the object}}$$

The magnification of the compound microscope can be obtained by multiplying the power of the objective lens and eyepiece.

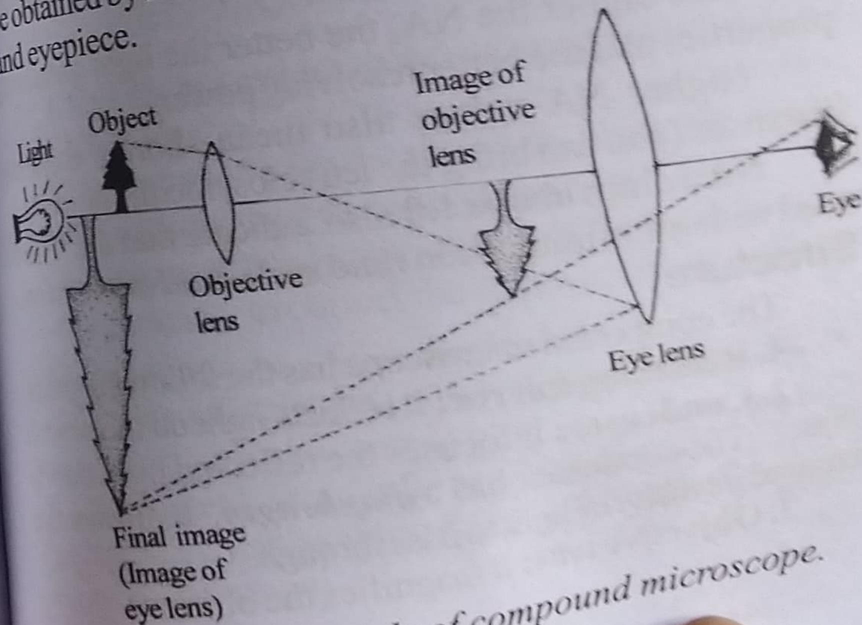


Fig.12.2: Principle of compound microscope.

If the power of the objective lens is 100x and that of the eyepiece is 10x, than the magnifying power of the compound microscope is 100x 10 = 1000 ie the compound microscope magnifies the object 1000 times.

Resolving Power

The ability of the microscope to distinguish two very small and closely spaced objects as separate entities is called 'Resolving power of the microscope'.

The resolving power of the microscope is improved by the following factors.

1. High numerical ***aperture.***
2. Fully opened condenser ***(diaphragm).***
3. Shorter wavelength of light (***blue light***).
4. Immersion fluid like ***oil.***

Numerical aperture (NA)

Numerical aperture is light gathering capacity of the lens. The NA of the lens is inscribed in the metal tube and it ranges from 0.25 to 1.4.

The higher the NA, the better the light gathering properties and the better resolving power.

Higher NA values also mean shorter working distances (you can bring the lens closer to the object).

NA values above 1.0 also indicate that the lens is used with some immersion fluid such as ***oil immersion.***

Structure

The compound microscope has the following parts:

1. Reflecting mirror: It reflects light on the object.

2. Condenser: It focuses the reflected light on the object. The condenser has a ***diaphragm***. It allows the required intensity of light to pass through.

3. Objective lens: It magnifies the object.

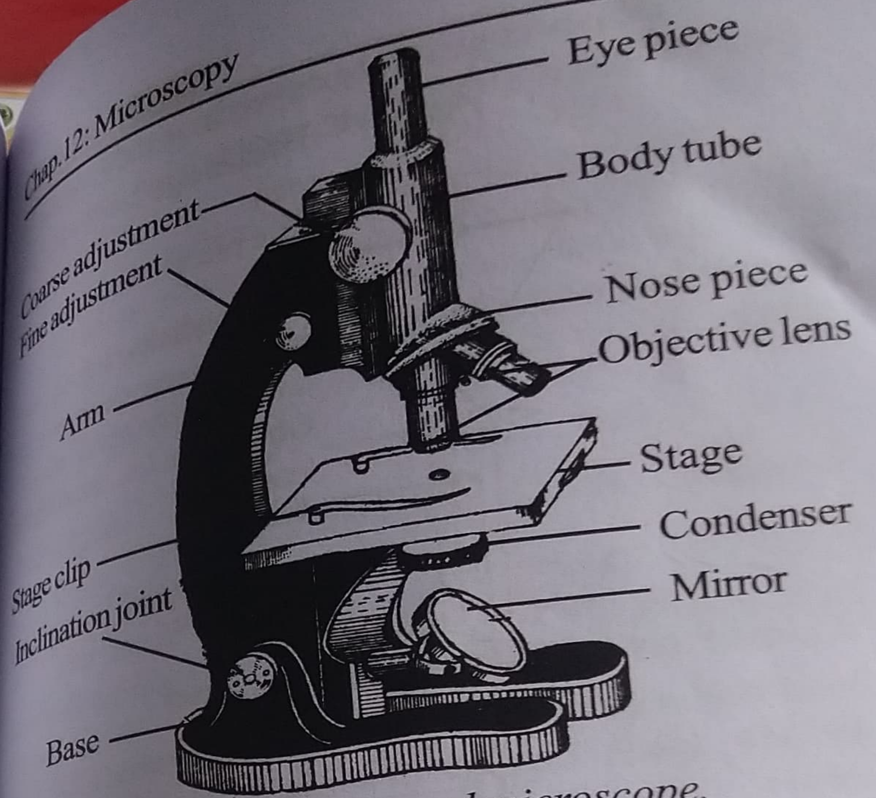


Fig.12.3: Compound microscope.

4. Eye piece : It magnifies the image produced by the objective lens.

5. Body tube: It is a tube with the objective lens at the lower end and the eye piece at the upper end.

6. Coarse adjustment: It moves the body tube up and down rapidly to correct the distance from the object to get focussing.

7. Fine adjustment: It moves the body tube up and down slowly to make exact focussing.

8. Stage: It is a platform with a hole in the centre. The light falls on the object through the hole. The slide is placed on the stage.

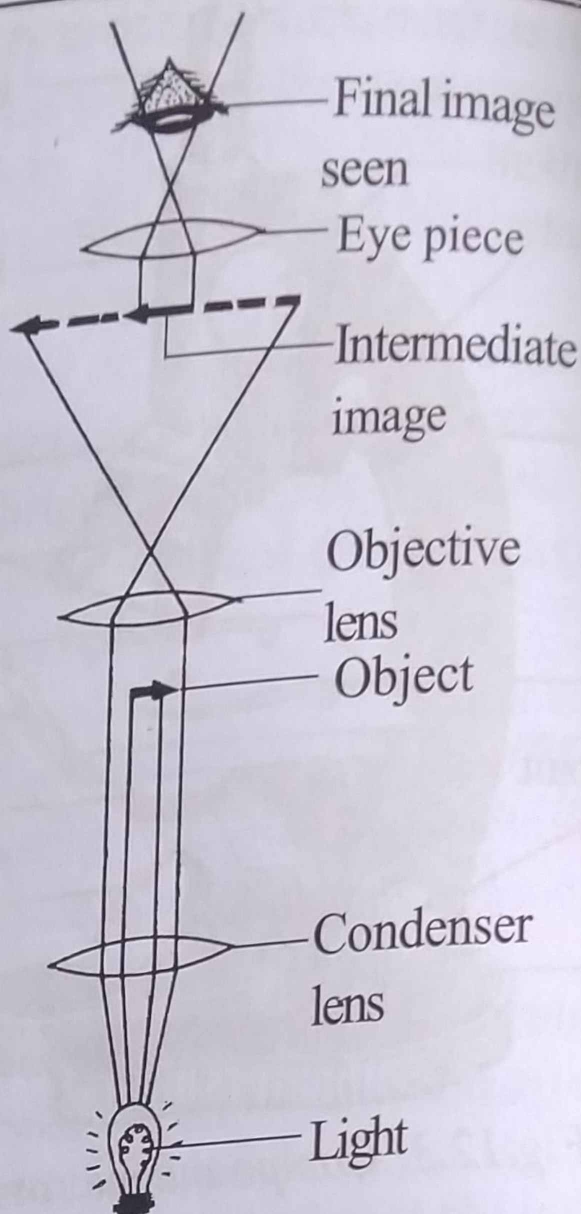


Fig.12.4: Components of a compound microscope.

9. Stage-clips: Stage-clips hold the slide firmly on the stage.

10. Nose-piece: It is in the form of a rotating disc having holes for fitting the objective lenses.

11. Arm: It holds body tube and coarse adjustment.

12. Inclination joint : It permits tilting of the upper part of the microscope to adjust to the level of eye.

13. Base or Foot: The f[illegible]t keeps the body in position.

3. It is used to examine water and other fluids for the presence of minute transparent microbes (bacteria and flagellates).

4. It is a must to study physiological processes such as ***phagocytosis, pinocytosis, cell divisions***, etc. in cells.

5. It is used to study the behaviour of chromosomes during ***mitosis*** and ***meiosis.***

6. It is best to study living cells without fixation and staining.

2. Electron Microscope

Electron microscope is a system of electromagnetic coils where electron beam is used as the source of illumination.

As the wave length of electron is very short (0.05A°), the magnification is very high. Electron microscope gives a magnification of 2000 times than that of light microscope.

It is much useful in studying virus, bacteria and the ultrastructure of cell organelles.

The first electron microscope was designed by ***Knoll*** and ***Ruska*** in 1932.

There are two types of electron microscopes, namely ***transmission electron microscope (TEM)*** and ***scanning electron microscope (SEM).***

In ***transmission electron microscope,*** the electron beam is transmitted through the ultra thin object.

In ***scanning electron microscope***, the electron beam scans the surface of the object and conveys its image to a TV screen. When the electron beam strikes the object, it penetrates a few micrometres into its surface and liberates low energy 2^0 electrons. A secondary electron detector traps the signals and converts them into a 3-D image on

the TV screen. SEM can also provide information about chemical composition of the specimens.

Principle

• Electron microscope uses ***electron beam*** as the illuminating source.

• The electron beam has ***very short wavelength*** ranging from 0.05 Å to 0.005 Å. So the ***resolving power*** is very high. It can magnify objects up to ***2 million times.***

• Electron microscope works in ***vacuum*** because the electrons move in a straight line in vacuum only.

• The electrons are produced by an ***electron gun*** containing a ***tungsten filament***. The filament is heated to release ***electrons.***

• The ***lenses*** used in electron microscope are called ***electromagnetic coils***. They work on ***electric current.***

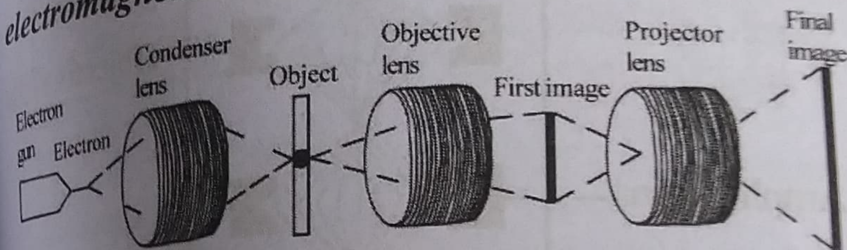


Fig.12.10:Principle of electron microscope.

• Two ***condenser lenses*** are kept on the path of the electrons. They make the electrons into a ***narrow beam*** and ***focus*** on the object.

• The brighter and finer the electron beam, the higher the level of observation of the object.

• The object ***absorbs, diffracts, reflects*** and ***transmits*** the electrons.

• The ***image*** results from the scattering of electrons by the atoms present in the specimen.

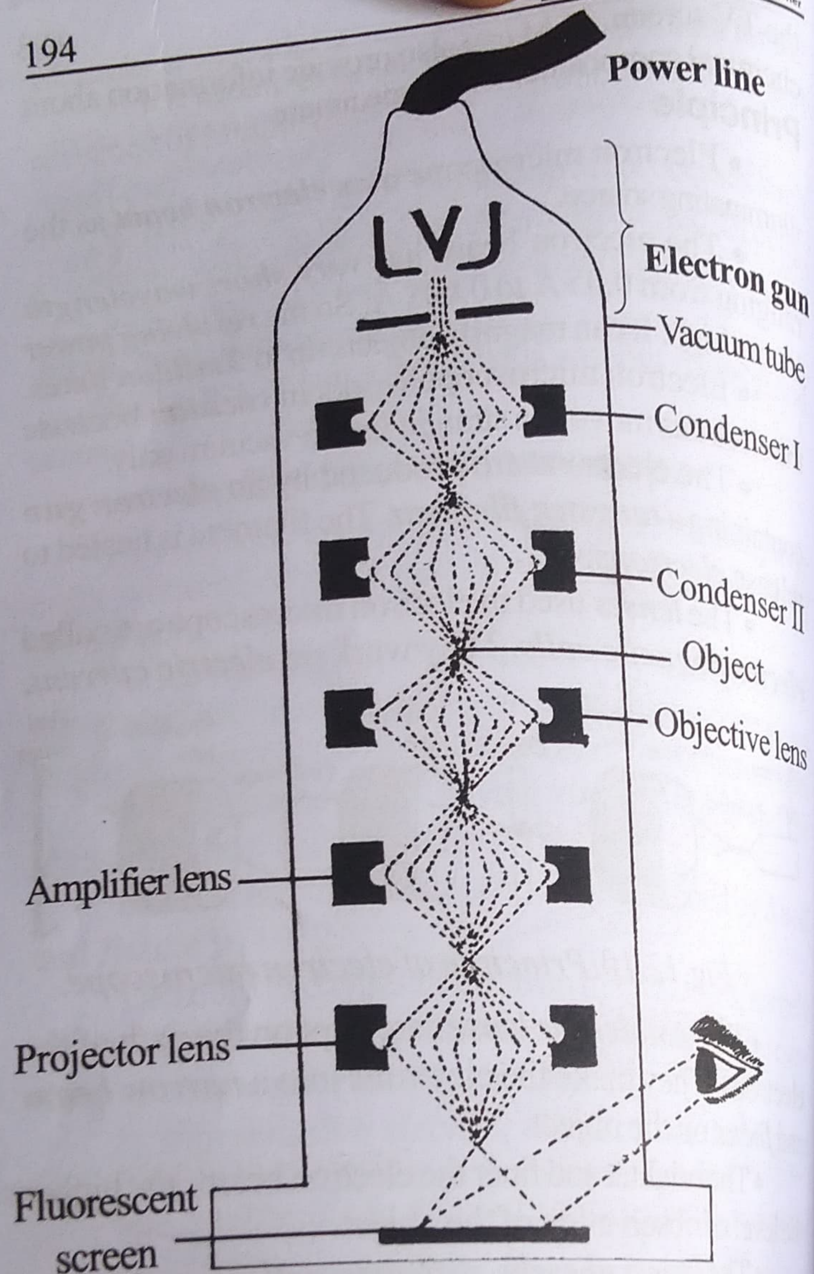


Fig.12.11: Transmission electron microscope.

• A heavy atom is more effective in scattering of electrons than one of low atomic number.

• The presence of ***heavy atoms*** will increase the image contrast. Hence the electron microscopists incorporate heavy atoms like **gold** into the specimen.

• *The part of the specimen which transmits electrons appears bright. The portions of specimen which absorb or scatter electrons appear dark.*

• The ***objective lens*** collects the image of the specimen and focuses it towards the ***amplifier lens***.

• The ***amplifier lens*** magnifies the image several 1000 times.

• A ***projector lens*** focuses the image on a ***photographic plate.***

Structure

The electron microscope consists of the following components:

1. *Electron gun*
2. *Condenser Lenses*
3. *Object*
4. *Objective coils*
5. *Amplifier coils*
6. *Projector coils*
7. *Fluorescent screen or photographic plate*
8. *Vacuum tube*
9. *Cooling system*

1. Electron Gun: Electron gun is the source of illumination. It consists of a V- shaped filament and two circular metal plates with holes in the centre. The two metalplates are named as ***cathode shield*** and ***anode plate***. A high voltage is applied between the filament and the anode plate. A current flows through the filament and heats it to incandescence, causing it to emit electrons. The electrons are attracted towards the anode plate and

they pass through the hole in its centre. The cathode shield focuses the electrons in the form of a beam through the hole present in the middle of the anode plate.

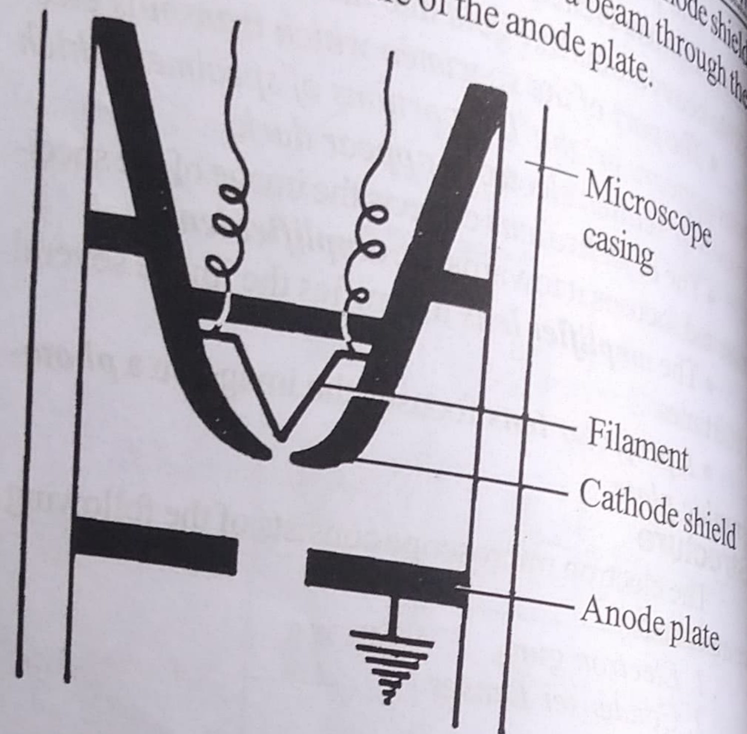


Fig.12.12: Electron gun.

2.Condenser Lenses: There are two condenser lenses set one below the other. Each lens is a coil of wire called ***electromagnetic coil*** or ***electromagnetic lens.*** They make the electron beam very narrower and focus the beam in the direction of object.

3. Object: The object is placed below the second condenser lens in the focal length of the beam. The object is placed on a ***supporting film*** mounted on a ***copper grid.***

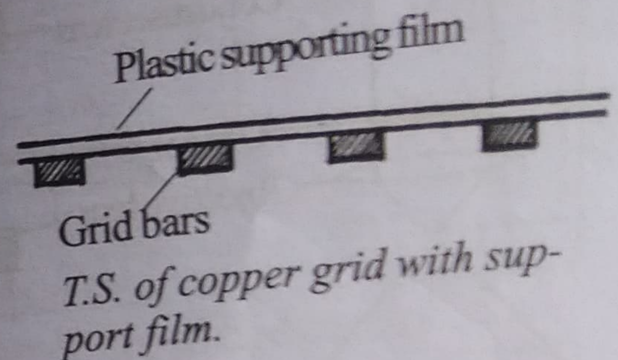
4. Objective Lens: It is another ***electromagnetic coil,*** situated below the object. It captures the transmitted

electrons from the object and magnifies the image.

5. Amplifier Lens: It amplifies the image produced by the objective coil.

6. Projector Lens: It projects the final image on a screen.

7. Fluorescent screen: It receives the final image of the object. If the image is received on a photographic film, it is developed to make a ***microphotograph***.



T.S. of copper grid with support film.

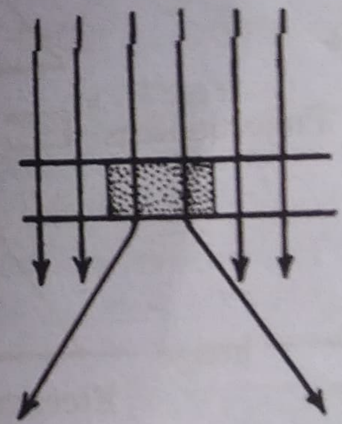


Fig.12.13: Scattering of electrons while passing through dense region of the specimen but not in less dense region.

8. Vacuum tube: The entire set-up is placed in a vacuum tube, because the electrons move in a straight line only in a vacuum.

9. Cooling system: Moderately large current is flowing through the electromagnetic coils. So the coils get heated. The coils are cooled by water circulating around the vacuum tube.

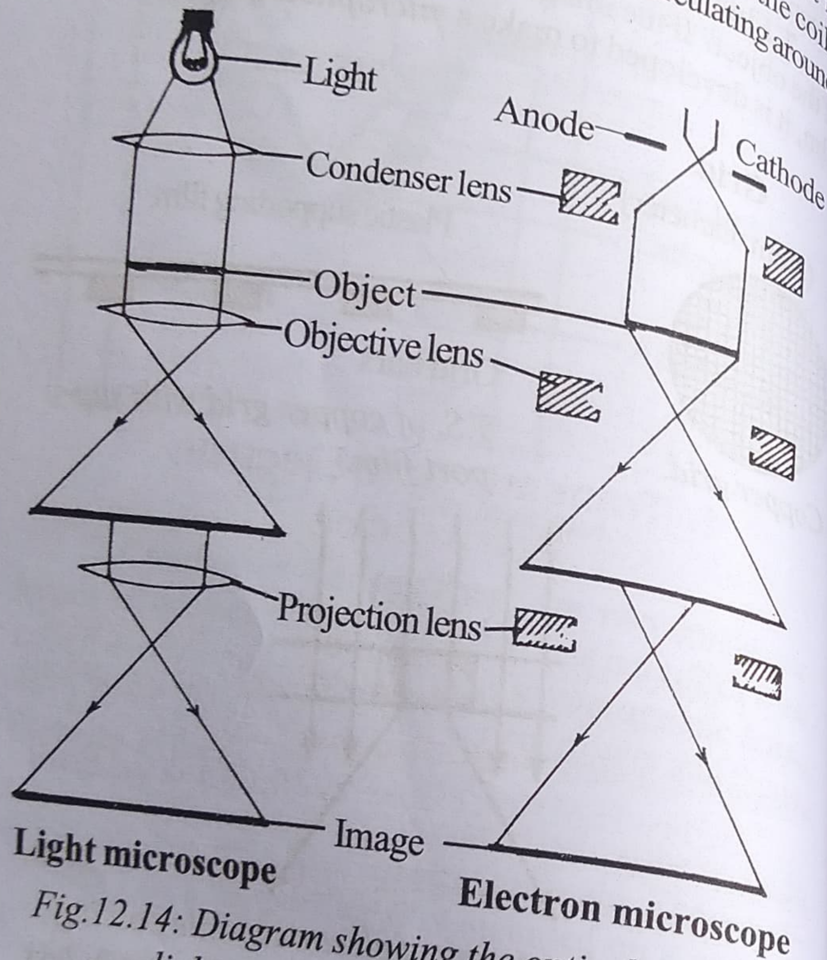


Fig.12.14: Diagram showing the optical parts of light and electron microscopes.

Table 12.1: Comparison of Electron and Light Microscopes.

	Electron Microscope	Light Microscope
Illumination	Electron beam from a high voltage system	Tungston lamp or sunlight
Lens	Electromagnetic coil	Glass or quartz lens
Object	Less than 1000 A° thick	More than 50000A° and 500000A° thick.
Projection	Image on fluorescent screen or film	Image on ground glass
Working site	Works in vacuum column	Works in air column
Starting time	Requires one hour	Viewed immediately
Type of samples	Dry samples only	Live materials also
Resolving power	High 1.5A°. 2000 times more powerful than light microscope	Low 2000A°
Wavelength	Short wavelength radiations	Long wavelength radiations
Layout	0.5 to .05A° inverted	5000A° upright.

1. Transmission Electron Microscope (TEM)

Electron microscope in which electron beam is passed through the specimen to produce its image is called ***transmission electron microscope*** (TEM).

The first TEM was designed by ***Max Knoll*** and ***Ernst Ruska*** in 1931.

The TEM was first made available in the market in 1939.

The TEM has wide applications in the research on ***virology, oncology, pollution studies, material science*** and ***semi-conductor research.***

Principle

The basic principle of electron microscopes is similar to the optical principle of ordinary compound microscope. Here, ***electron beam*** is substituted for ***light beam*** and ***electromagnetic coils*** are substituted for ***optical lenses***.

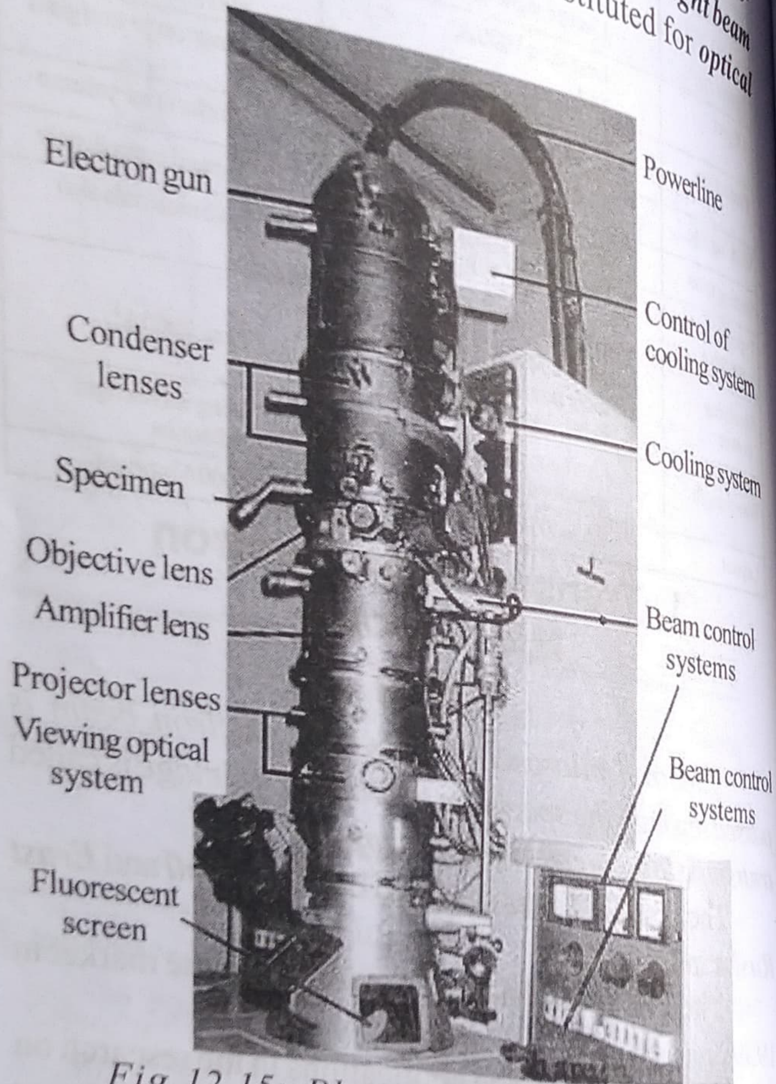


Fig.12.15: Photograph of a transmission electron microscope.

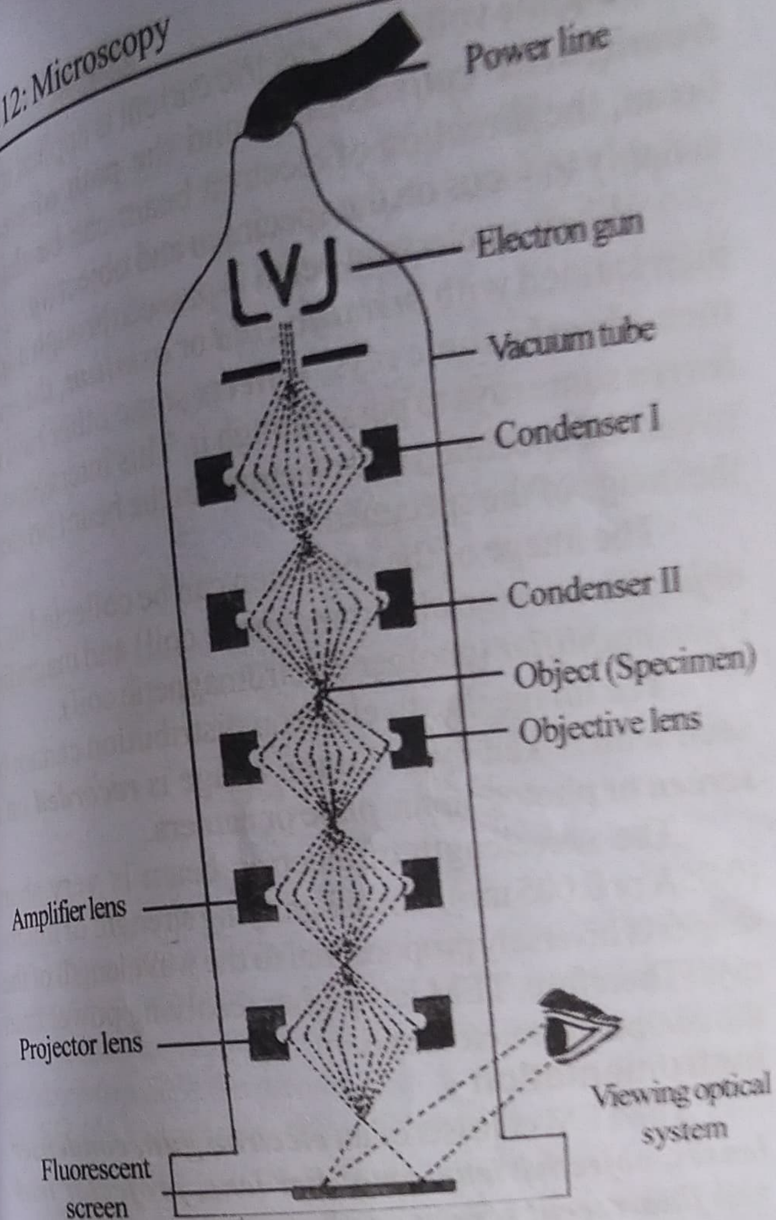


Fig.12.16: Simplified diagram showing the electron path of TEM.

When high voltage current is passed through a filament of cathode ray tube, electron beams are produced from the filament.

If some voltage of electric current is applied to ***electromagnetic coils*** kept around the path of electron beam, the direction of electron beam can be changed suitably to focus on the specimen and objective.

When an electron beam is passed through a specimen stained with ***metallic gold*** or ***osmium***, the specimen ***absorbs*** some rays, ***reflects*** some other rays and ***leaves*** some rays to pass through it. This interaction between the specimen and electrons in the beam produces the image of the specimen.

The image of the specimen can be collected by an ***objective lens*** (an electromagnetic coil) and magnified by an ***amplifier*** (another electromagnetic coil).

The image due to electron distribution cannot be seen with naked eyes. So the image is ***recorded*** on a ***screen*** or photographic plate or camera.

The wavelength of electron beam is very short (0.05A or 0.005 nm). The magnifying strength of microscopes is inversely proportional to the wavelength of the rays. Therefore, TEM has higher resolving power than the compound microscope.

Instrumentation

The TEM consists of an ***electron gun, condenser lenses, objective lens, amplifier lens, projector lens*** and ***fluorescent screen*** or ***photographic plate.***

Electron gun is the source of electron beam used in this microscope. It consists of a ***V-shaped filament, Whenelt cylinder*** and an ***anode plate*** with a hole at the centre. The Whenelt cylinder is a small ***cup-like structure*** with a ***hole*** at the bottom. The ***V-shaped filament*** is kept in the cup. The Whenelt cylinder acts as a ***cathode.***

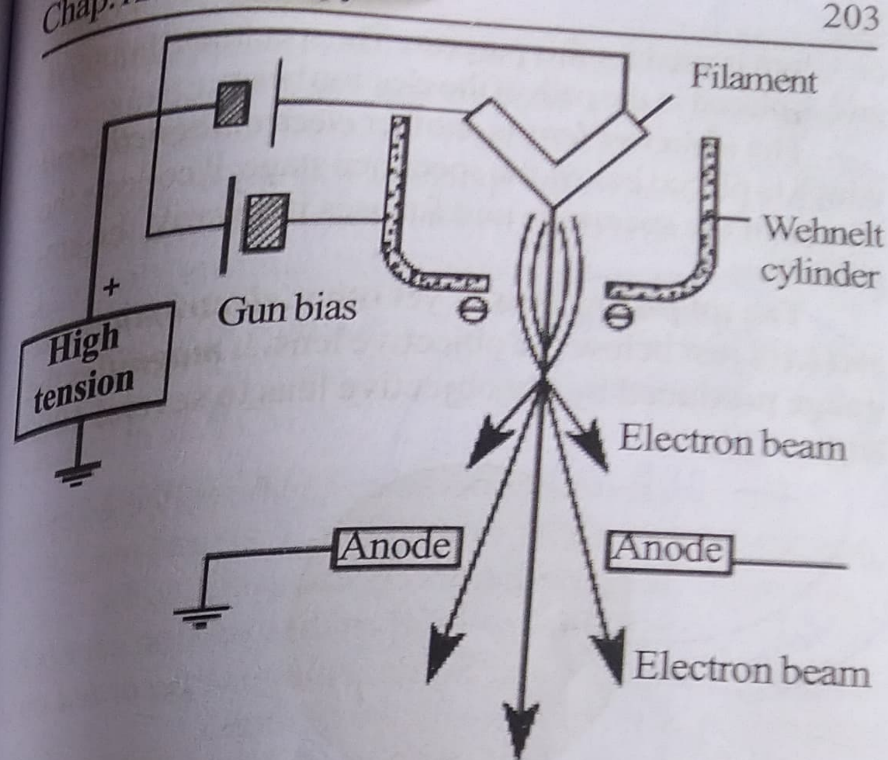


Fig.12.17: Electron gun.

When ***high voltage current*** is applied between the filament and anode plate, the filament is ***heated up*** to incandescence for emitting electrons. Since the electrons are attracted towards the ***anode***, they are forced out through the hole in the ***anode plate*** by the ***cathode shield***. The ***electron gun*** is placed at the ***top*** of the TEM.

There are two ***condenser lenses*** just below the electron gun. They are nothing but electromagnetic coils (coil of wires). They collect and concentrate the electrons into a strong electron beam before focusing it onto the specimen.

Just below the second condenser lens, there is a ***specimen stage.*** A thin section of specimen is placed on a thin plastic film mounted on a copper grid. The grid size of 2.2nm

or 3.2nm is used for this purpose. The specimen mounted grid is placed in the path of the electron beam.

The ***objective lens*** is another ***electromagnetic coil*** which is placed below the specimen stage. It collects the ***image*** of the specimen and focuses it towards the amplifier lens.

The ***amplifier lens*** is yet other ***electromagnetic coil*** kept just below the objective lens. It ***magnifies*** the image produced by the objective lens to several 1000 times.

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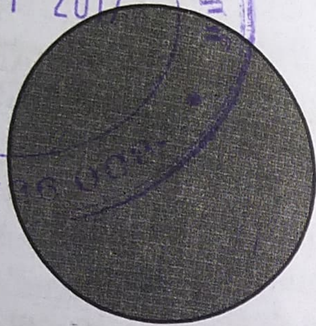

Fig.12.18: Copper grid (3mm diametre).

A ***projector lens*** collects the magnified image and focuses it onto a ***fluorescent screen*** or ***photographic plate***.

The entire setup is placed in a ***vacuum tube***, because electrons can move in a ***straight line*** only in a vacuum. Usually, vacuum pressure of 10^{-7} to 10^{-9} ***Pa*** is applied in the vacuum tube.

While TEM is working, a large amount of ***heat*** is flowing through the electromagnetic coils. As a consequence, the TEM is ***heated*** to a great extent. In order to keep the apparatus at a low temperature, ***cooling water*** is ***circulated*** through a ***cooling system*** around the TEM.

Sample Preparation

Biological specimens are formed of elements of relatively low atomic weight. The elements are ***carbon, hydrogen, oxygen*** and ***nitrogen***. They do not give high resolution images under TEM examinations. Therefore, the biological sample has to be loaded with heavy atoms such as ***gold*** or ***osmium*** via fixation. The ***heavy atoms*** protect the specimen from the ***destructive effects*** of vacuum in the TEM.

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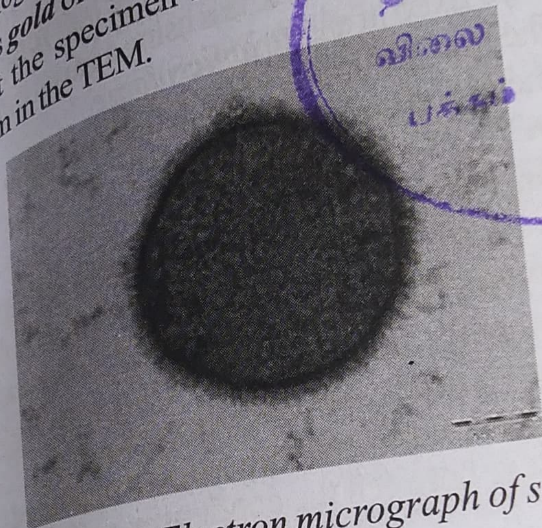

Fig.12.19: Electron micrograph of section of Bacillus subtilis taken by TEM.

The sample preparation and examination involve the following steps:

- The wet ***specimen*** is ***dehydrated*** (water removal) by keeping in increasing concentrations of ***ethanol*** or ***acetone***.
- Fixation is a chemical processing of biological specimens. It stabilizes the molecular organizations as such in them. It is done by immersing the specimen in chemical ***preservatives*** called ***fixatives*** for a considerable time. ***Osmium tetroxide***, ***glutaraldehyde***, ***potassium per-***

manganate, formalin, etc. are common fixatives for biological specimens. These fixatives form ***covalent bonds*** with biological molecules like proteins and lipids. These stabilize their structural organization in the specimen.

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- After fixation, the specimen is ***embedded*** in a hard embedding medium like ***araldite*** or ***vestopal-W*** or ***Epson-812*** or ***plastic*** medium.
- The embedded specimen is cut into ***thin sections*** of 50 – 100nm thickness using a ***glass*** or ***diamond knife*** fixed in an ***ultramicrotome***.
- The thin section is mounted on a ***copper grid*** of 3mm diameter and covered with ***parlodoan.***
- This section is then kept dipped in a solution containing heavy metal ions for ***metallic staining***. Solutions of ***phosphotunstate, lead acetate, lead hydroxide, osmium tetroxide,*** etc. are useful for this staining. These metallic stains give ***clear contrast*** to the specimen.
- The specimen-mounted grid is then placed on the ***specimen stage*** between the condenser coil and objective coil.
- Image of the specimens is viewed through the optical system to choose a suitable section.
- Image of the selected section is then viewed on the ***fluorescence screen*** or captured in ***photographic plate.***

Applications of TEM

1. TEM is an ideal tool for the study of ***ultrastructure of cells***.
2. It is used in the ***identification*** of plant and animal ***viruses*** based on their ***structural features***.
3. It is employed in the ***localization*** of nucleic acid, enzymes and proteins in cells and cell organelles.

4. It is used in cancer research for the ***cytological observation*** of cancer cells.

2. Scanning Electron Microscope (SEM)

Electron microscope that scans the surface of specimen by passing an electron beam is called ***scanning electron microscope*** (SEM). The first SEM was designed by ***Max Knoll*** in 1935.

It scans the surface of specimen using electron beam more like a xerox machine scanning the surface of paper by laser beam. The SEM is very useful to study the ***surface architecture*** of cells, membranes, organelles and others.

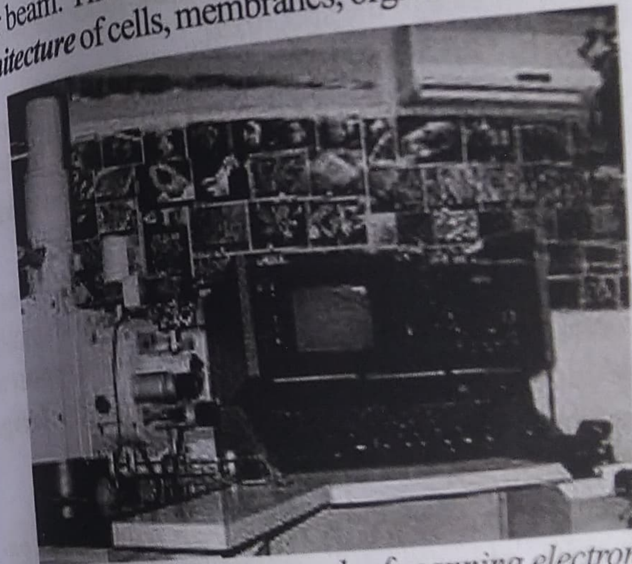

Fig.12.20: Photograph of scanning electron microscope.

Principle

The SEM also uses an ***electron beam*** as ***illumination*** and ***electromagnetic coils*** to direct the path of electron beam.

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When electron beam is focused on the specimen, it produces ***secondary electrons*** (SE), ***back-scattered electrons*** (BSE) and ***characteristic X-rays***.

The ***secondary electrons*** are those which are ***reflected*** due to ***interactions*** between the atoms at the surface of the specimen and electrons.

The electrons reflected by the sample due to its ***elastic scattering*** property are called ***back-scattered electrons (BSE)***. The BSE gives information about the ***distribution of different elements*** in the sample.

Characteristic X-rays are emitted by the sample when the electron beam removes electrons in the inner shells of the atoms at the surface of the specimen.

The SE, BSE and characteristic X-ray are detected accurately using separate specialized detectors. The beam current absorbed by the specimen is detected and used to create the ***current distribution*** in the specimen.

Electronic amplifiers of different kinds (collecter, scintillator and PMT) are used to measure the electric signals. The electric signals are converted into ***bright spots*** of varying density by a ***scanning circuit*** to give the ***image*** of the specimen on the screen.

Instrumentation

The SEM consists of an ***electron gun, two condenser coils, objective lens (coil), specimen stage, grid, scintillator, photomultiplier tube*** (PMT), ***scanning circuit*** and ***x-ray detector***.

Electron gun is the source of ***electron beam*** used in this microscope. It consists of a ***V-shaped filament, Whenelt cylinder*** and an [illegible] ***plate*** with a hole in the centre. The Whenelt cy[illegible]mall ***cup-like*** struc-

ture with a ***hole*** at the bottom. The V-shaped filament is kept in the cup.

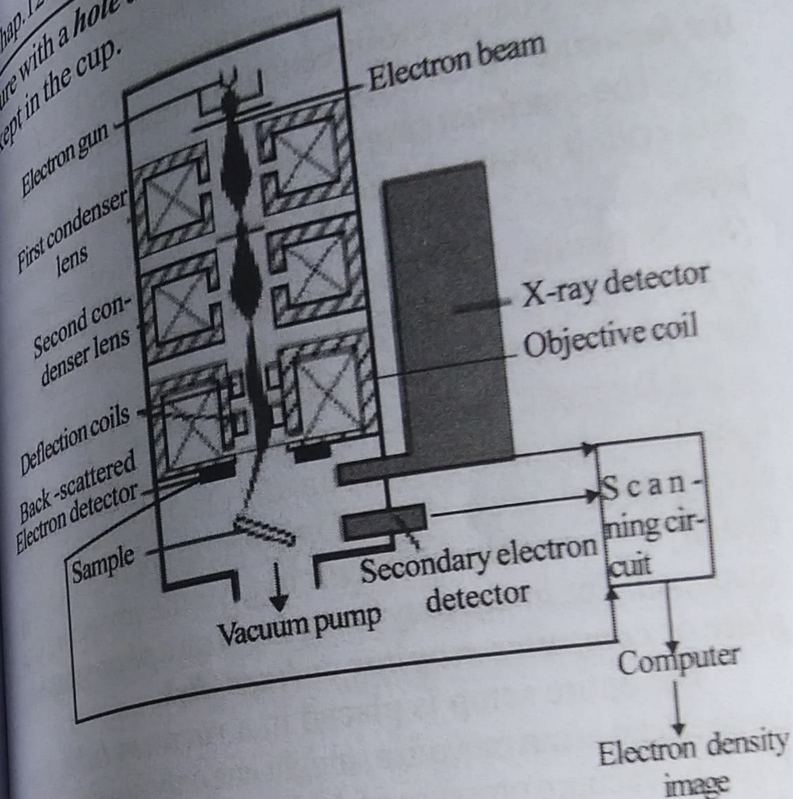

Fig.12.21: Schematic structure of scanning electron microscope.

The Whenelt cylinder acts as a ***cathode***. When high voltage current is applied between the filament and anode plate, the filament is ***heated up*** to incandescence for emitting electrons. Since the electrons are ***attracted*** towards the ***anode***, they are forced out through the hole in the anode plate by the cathode shield. The electron gun is placed at the ***top*** of the SEM.

There are two ***condenser lenses*** just below the electron gun. They are electromagnetic coils (coil of wires).

They collect and concentrate the electrons into a ***strong electron beam.***

Just below the second condenser, there is a ***deflection coil*** to change the direction of electron path. It helps for ***focusing*** the electron beam on the ***specimen stage.***

The ***specimen stage*** is placed just below the ***deflection coil***. It is in a ***slanting position*** (45°) in the electron path.

Separate electron detectors are attached to the vacuum tube of SEM. Each of these electron detector is formed of a ***collector,*** a ***scintillator*** and ***PMT.***

Electronic amplifiers are connected with the detectors. They are used to measure the electric signals. The electric signals are converted into ***bright spots*** of varying density by a ***scanning circuit*** to give the image of the specimen. The image may be captured on a ***photographic plate*** or ***computer monitor*** or ***hard disk.***

The entire setup is placed in a ***vacuum tube***, because electrons can move in a straight line only in a ***vacuum.*** Usually, vacuum pressure of ***10^{-7} to 10^{-9} Pa*** is applied to the vacuum tube.

While SEM is working, a large amount of heat is flowing through the electromagnetic coils. As a consequence, the SEM is heated to a great extent. In order to keep the apparatus at a low temperature, ***cooling water*** is circulated through ***cooling system*** around the SEM.

Specimen Preparation

Dry materials such as wood, bone, feathers, insect's wings and shells are coated with a thin film of electroconductive material. Generally, ***metallic gold, platinum, tungsten, iridium, osmium, chromium*** and ***graphite*** are used for coating.

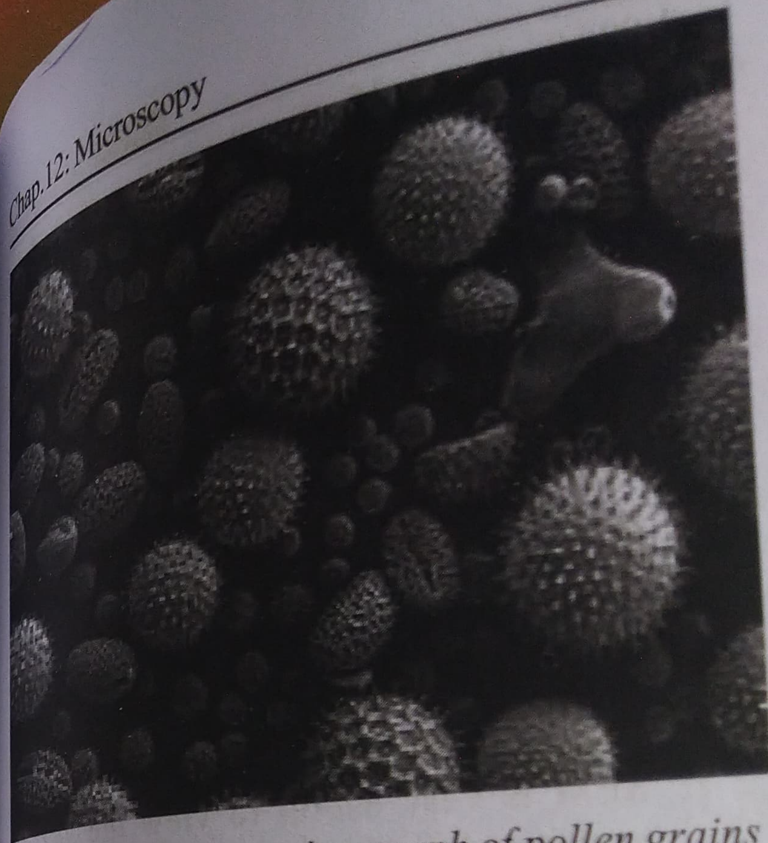

Fig.12.22: Electron micrograph of pollen grains taken by using SEM.

Then, the specimen is placed on a grid to place it on the ***specimen stub***.

The preparation of wet specimen for SEM examination involves the following steps:

- Wet specimen is fixed by immersing it in a solution of fixatives. The fixation stabilizes the molecular organizations as such in them. ***Osmium tetroxide, glutaraldehyde, potassium permanganate, formalin,*** etc. are common fixatives for biological specimens. These fixatives form ***covalent bonds*** with biological molecules such as proteins and lipids to stabilize their structural organization.

- After chemical fixation, the specimen is dehydrated (water removal) by keeping it is increasing concentrations of ***ethanol*** or ***acetone.***
- The specimen is then coated with an ultra ***thin layer*** of ***electroconductive alloy***. This metallic coating prevents the accumulation of electrostatic charges on the specimen.
- The specimen is placed in slanting position on the specimen holder called ***specimen stub***.
- When electron beam is focused on the specimen, the SEM detects the ***secondary electrons, back-scattered electrons, X-ray emission***.
- The image is formed on the ***computer*** monitor.

Applications of SEM

1. SEM is very useful to view the ***surface architecture*** of microscopic creatures like bacteria, diatoms, pollen grains, nematodes and others.
2. SEM gives the ***3-D structure of objects*** to reveal the structure of organism or organelles.
3. SEM is employed in the analysis of ***structural features*** of compound eyes of insects.
4. ***Hairs*** and ***scales*** on plant and animal surfaces are characterized with the SEM.
5. SEM is used to study the surface of ***small archeological specimens*** and ***fossils.***

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