

Chapter 4

Microscopy

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Abstract: Microscopy is one of the powerful tools of microbiology without which the study and examination of the bacteria and microorganisms is really impossible. Based on the source of examination microscopes are classified as light microscopes and electron microscopes. Electron microscopy is an advanced technique where we will study the fine details of bacterial organelles and virus structure.

Keywords: Electron Microscopy, Examination, Light microscopy, Source of Examination, Viruses

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1. Auxochrome:

Auxochrome is a group of atoms that can increase the intensity of the colour when grouped to chromophore. Auxochrome cannot induce colour by itself but can impart colour to the chromophore. Some of the examples of auxochrome are :

- Hydroxyl group (-OH)
- Amine group (-NH₂)
- Aldehyde group (-CHO)
- Methyl mercaptan group (SCH₃)

2. Chromophore:

Chromophore is a substance that can impart colour to the compound. It consists of atoms that contain molecular orbitals with energy differences that fall in visible spectrum. When chromophore hits the visible light it can absorb and the electrons excite from ground state to excited state. So chromophore does not absorb the light but appears in colour due to excitation.

3. Light Microscopy:

First simple microscope was made by two dutch scientists Zaccharias Janssen and his father Hans who made spectacles by combining lenses in to tubes and appearance of objects closer and larger. Later on Father of microbiology Anton von Leeuwenhoek made first simple microscope and observed the tiny creatures in the pond which observed as dots. Light microscope is one of the basic tool of microbiology lab and the microscope will consists of lenses for magnification of the specimen and formation of image. Image is carried by two or three lenses finally magnifying the image. Based on the number of lenses light microscope is classified as simple and compound microscope. Simple microscope is made up of single lens where as compound microscope is made up of two or more lenses required for the magnification of the image.

Lenses in the microscope can bend the light as per requirement and the transparency of the specimen makes the light to be easily focused on the specimen and passage through the lenses finally forming the image. The specimen is kept closer to the lenses and final image can be observed with the help of eye piece. Light microscopy works with the principle of bending the light by lenses which finally forms image. Normally when light travels between two phases it will bend which can be determined by refractive index. When the light travel from medium with low refractive index such as glass to air it will travel fast and move away and when the light travel from high refractive index medium such as air to low refractive index medium like glass it will slow down and focus at a point. The focus of the light will depend on difference between the two refractive indexes of the medium.

If we place prism in between the two phases the light will be bend at an angle and if we allow the light to pass through the convex lens it will focus at a particular point called focal point. The distance between center of the lens to focal point is known as focal length. The resolution of the image is defined as ability of the lens to distinguish two closer points as separate entities. The resolution of the microscope will depends on the numerical aperture defined as two different wavelengths after the light deviate from the touched object. Abbe's equation can be used to calculate the wavelengths required to distinguish two close entities as separate ones. Where $n \sin \Theta$ = Numerical aperture and λ = wavelength.

$$d=0.5 \lambda/n \sin \Theta$$

Examples of some modern light microscopes include:

- Bright field microscope
- Dark field microscope

- Phase contrast microscope
- Fluorescence microscope

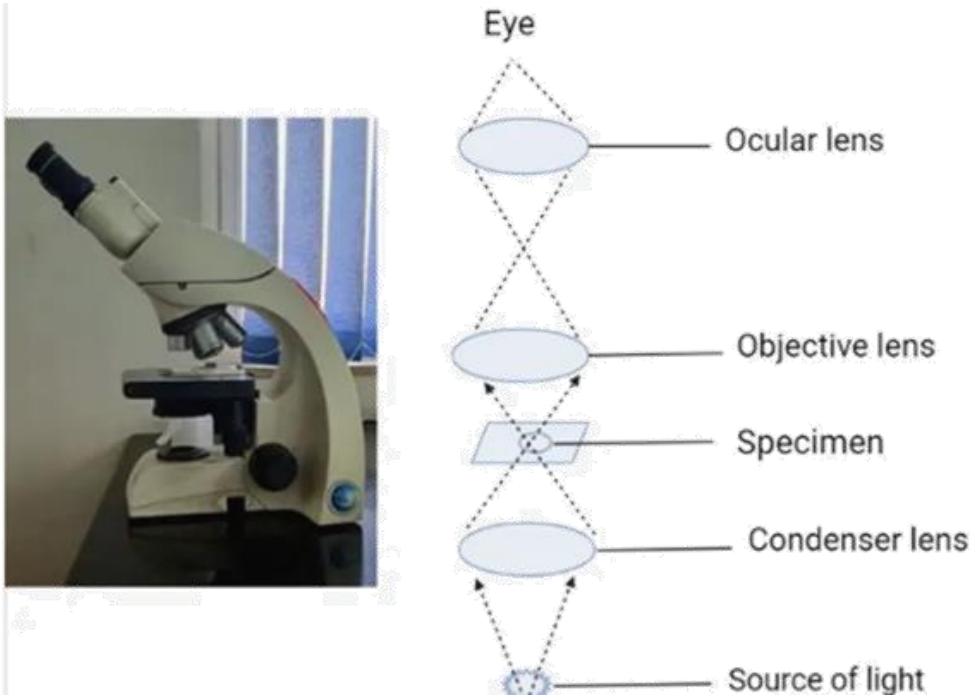


Figure:5 Compound Microscope and working principle of Light Microscope. [Taken from Madigan, M. T., Martinko, J. M., Stahl, D. A., & Clark, D. P. (2011). *BROCK Biology of Microorganisms* (13th edition). Benjamin Cumming].

Parts of light microscope includes arm which holds condenser lens, objective lenses and eyepiece or ocular lens. Condenser lens can be movable to adjust the intensity of the light from the light source. Two adjustable knobs coarse adjustment knob and fine adjustment knob. Coarse adjustment knob is used to move the metallic body up and down to adjust the image. Fine adjustment knob used to further focus the image formed by coarse adjustment.

4. Electron Microscope:

Electron microscopes utilizes high velocity electrons to study the fine details of the object. Using electron microscopy we can yield details of topology, morphology, size and shape of the molecules and crystallographic arrangement of the molecules in the substance. Types of electron microscope includes Scanning electron microscope used to study the surface of the object whereas other one Transmission electron microscope is used to study

inner surfaces of the object. Transmission electron microscope was made by Knoll and Ruska in 1931 and the first TEM was came in to the market in 1939. TEM works by transmission of electrons through the ultra thin specimen and the image developed is captured on the photographic film or fluorescent screen or sensor connected to CCD camera.

TEM consists of :

- Electron Gun
- Condenser lens
- Objective lens
- Amplifier lens
- Projector lens
- Vacuum tube
- Fluorescent screen

Electron Gun:

Electron gun is one of the source for electrons. It is made of V-shaped filament which acts a anode and with in it cylinder with central hole acts as cathode. Acceleration of filament at high voltage emits high velocity e^{-s} and focused towards anode plate. The e^{-s} are forced in to hole due to cathode shield.

Condenser lens: Condenser lens collects and focuses the e^{-s} on to the specimen.

Objective lenses: Objective lens collects the e^{-s} from the specimen and forms an image.

Amplifier lens: The image formed at objective lens is amplified up to 1000 times by the amplifier lens.

Projector lens: It magnifies the image and further focuses it on to the fluorescent screen.

Vacuum tube: The entire setup is carried out in the high vacuum conditions. A pressure of around 10^{-7} to 10^{-9} psa of vacuum is applied inside the vacuum tube.

Cooling system: During operation of electron microscope large amount of heat is generated inside the microscope so cooling system is used to reduce the temperature.

Preparation of specimen:

1. Dehydration: The specimen is dehydrated by a series of increasing concentrations of ethanol or acetone

2. Fixation: The specimen is subjected to fixation using chemical fixatives. Osmium tetroxide, Glutaraldehyde, Potassium permanganate and formalin are some of the common fixatives used in TEM.

3. Embedding: After fixation the specimen is embedded in a plastic film. Commonly used plastic embeddents are plastic medium, Araldite, Vestopal - W and Epon - 812 etc., After the process thin section of 100 nm is made through sectioning by ultramicrotome.

Staining: The thin specimen generated by using ultramicrotome is stained using heavy metal stains like osmium tetra oxide, Lead acetate and Lead hydroxide. The stained specimen is mounted on the copper grid and the final image can be visualised by viewing through optical system.

Scanning electron microscope:

SEM utilizes high velocity electrons to focus and form the image which can be viewed on the phosphorescent screen. SEM uses various sensors and detectors for the electron capture and final image is built on the phosphorescent screen.

Parts of SEM include:

Electron gun: It produces the e^- beam

Condenser lens: It will collect and concentrate the electrons before to focusing on to the specimen.

Specimen stage: Specimen grid is mounted at an angle of 45° to the e^- path and when e^- beam focuses on to specimen it will generate secondary electrons, back scattered electrons and X- rays.

Secondary e-s detector: SED detects and capture secondary e^- s scattered from the specimen.

Additional Sensors: These will capture back scattered electrons and X-rays.

Amplifiers: Amplifiers further capture and magnifies the signal and common amplifiers include Photo multipliers, grid or scintillators.

Phosphorescent screen: Final image will be captured and viewed on the phosphorescent screen.

Vacuum tube: The entire setup is carried out in the high vacuum conditions. A pressure of around 10^{-7} to 10^{-9} psa of vacuum is applied inside the vacuum tube.

Cooling system: During operation of electron microscope large amount of heat is generated inside the microscope so cooling system is used to reduce the temperature.

Staining:

Dry Specimen:

Metallic objects: Metallic objects are stained by coating with electrical conducting materials like gold, tungsten, osmium, Chromium and Graphite.

Wet specimen:

Wet specimens are stained with heavy metal dyes like osmium tetra oxide, Lead acetate and lead hydroxide after dehydration, fixation and embedding. After staining the specimen is mounted on to a specimen grid and examined for the details.

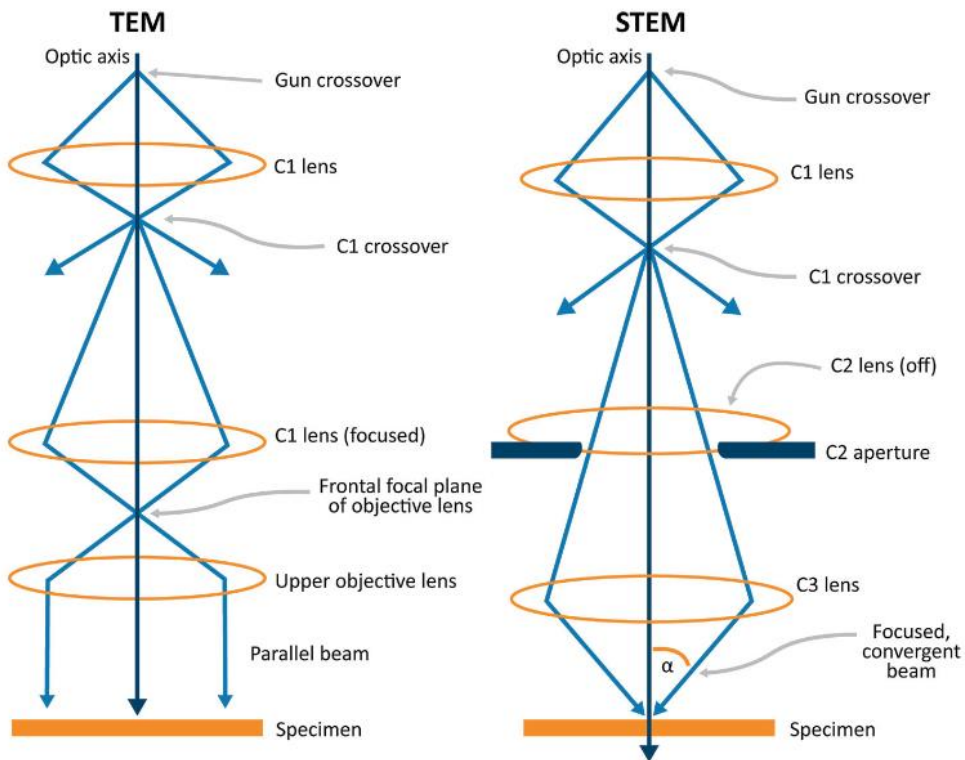


Figure:6 Working principle of TEM and SEM. [Taken from Pennycook and P. Nellist, Scanning Transmission Electron Microscopy, New York: Springer, 2011].

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