MICROSCOPY

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I. INTRODUCTION

The word microscope is originated from Latin words "Micro"-little and "Scopos:- to look at. It is an instrument that magnifies and resolves small details of an item using one or more lenses. A microscope is a vital tool for examining living organisms that are too small to see with the naked eye. Microscopy is the science of examining small objects and structures with such instruments.

II. HISTORY

Microscope is invented by **Zacharias Janssen in 1590.** The father of microscopy, In 1676, Antonie Von Leeuewenhoek discovered the microorganisms with the help of microscope. **Robert Hooke** used a **compound microscope** first time in 17th century. Fritz Zernike devised the phase-contrast microscope in 1932, allowing researchers to explore colourless and transparent biological materials. He was awarded the Nobel Prize in Physics in 1953 for this discovery.

III. PRINCIPLE

Microscopy is the study of tiny things and structures using a variety of microscopes. Microscopy is based on the use of lenses and/or electron beams to magnify objects so that they can be examined in detail. Here are the fundamental principles of several types of microscopes.

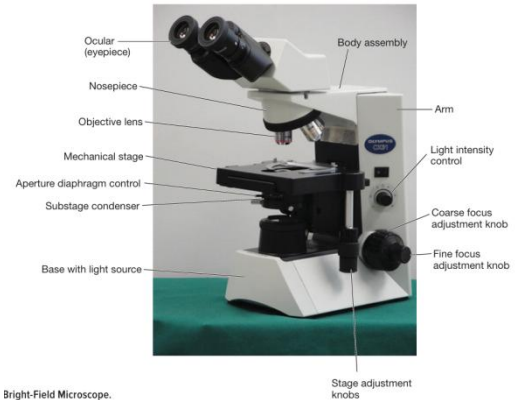

Figure 1: Bright Field Microscope (Prescott's Microbiology 11th Edition)

IV. TYPE OF MICROSCOPE

The light and electron microscopes are the two main categories of microscopes that differ from one another. When focusing light to create a picture, light microscopes utilise a sequence of glass lenses, whereas Electron microscopes (EM) employ lenses with electromagnetic fields to direct electron beams. There are many types of light microscopes based on the specimen and the observer's aim: Bright field, dark field, and confocal microscopes In contrast to phase contrast and fluorescence microscopes, electron microscopes are classified into two types: SEM (scanning electron microscope) and TEM (Transmission electron microscope).

V. BRIGHT FIELD MICROSCOPE

The specimen is dark (Dark Object), with a bright background. This can be produced with a minimum of optical equipment. In brightfield pictures, contrast is often provided by the specimen's colour. (Wilson and Walker). Bright-field imaging is frequently used to capture images of histological sections, cell culture, and pigmented tissues dyed with colourful dyes.

Advantage

- Very easy to use, with few changes required to see the specimen.
- Certain samples can be examined without staining, and the optics used in the bright-field technique do not alter the colour of the specimen.

Disadvantage

- Poor contrast.
- The majority of cells must be dyed to be seen.
- The practical limit of magnification is around **1300x.**

Darkfield: Lighting produces photos of brightly lit things against a black background. This technique is frequently used to observe the contour of objects in liquid medium, such as bacteria or cells growing in cultures of tissues, living spermatozoa, or to swiftly assess the status of a biochemical preparation.

Application: Dark field microscopy is very effective for studying live, unstained samples that would be harmed or altered by staining methods. It is widely used in microbiology to investigate microorganisms, as well as cell biology and materials science.

Limitation: Dark field microscopy necessitates precise lighting angle adjustments and is susceptible to vibrations and dust, which can degrade image quality. Furthermore, because it uses scattered light, it may not produce as much information as other approaches in some situations.

VI. CONFOCAL MICROSCOPE

A confocal microscope is a powerful optical imaging tool used in a variety of scientific disciplines, including biology, medicine, and materials research. Confocal microscopes, unlike ordinary microscopes, use a focussed beam of light to produce high-resolution images of specimens.

Figure 2: Confocal Microscope

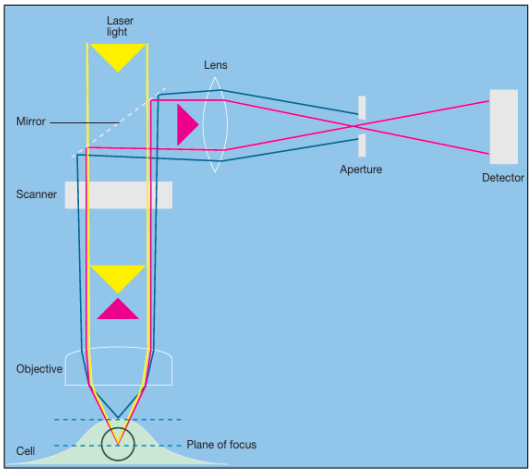

Figure 3: Confocal Microscope Ray Diagram **(**Prescott's Microbiology 11th edition**)**

VII. KEY FEATURES

1. **Point Scanning:** Confocal microscopes employ a point scanning approach in which a concentrated laser beam sweeps across the specimen. The scanning procedure is coordinated with the detecting system.

2. **Pinhole:** A pinhole opening is located on either side of the detector. This pinhole allows light coming from the focal point to pass through while rejecting out-of-focus light. This confocal aperture is crucial for doing optical sectioning.

3. **Optical Sectioning:** Confocal microscopy allows for optical sectioning by rejecting out-of-focus light. This means that researchers can reconstitute three-dimensional pictures by stacking these thin pieces.

4. **Resolution:** Confocal microscopes provide higher resolution than ordinary light microscopes due to its ability to reduce out-of-focus blur. This makes them suitable for analysing the tiny features of cells and tissues.

5. **Fluorescence Imaging:** Confocal microscopy is widely used with fluorescently tagged materials. It may use appropriate filters and lasers to selectively see different fluorophores (fluorescent molecules), enabling multi-color imaging and colocalization research.

Application: Confocal microscopy is used to research cell biology (such as organelle structure and dynamics), neuroscience (to image brain circuits),

developmental biology (monitoring embryo development), materials science (evaluating surface features), and many other fields.

VIII. PHASE CONTRAST MICROSCOPE

Phase contrast is used to detect uncoloured cells in tissue culture and to assess cell and an organelle composition for lysis. The method identifies changes in the refractive index (RF) of cell structures. Light is held up in thicker parts of the cell than in thinner cytoplasm.

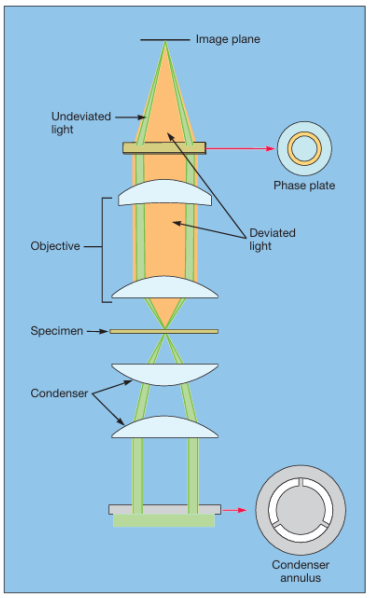

Figure 4: Phase-Contrast Microscope (These Picture is Adopted from Prescott's Microbiology 11th Edition)

IX. APPLICATION OF PHASE CONTRAST MICROSCOPE

- Create high-contrast photographs of translucent specimens, including cells that live and microbes.
- Fine tissue sections.
- Subcellular particles include nuclei and other cell organelles.
- The movement of mitotic chromosomes, mitochondria, and vacuoles.
- How cells multiply through division.

X. FLUORESCENCE MICROSCOPE

When certain chemicals are exposed to high intensity light, they produce light of a different, lower frequency. This effect is known as fluorescence. This

microscope is used to visualise specimens which fluorescence. Specimens are usually stained with **fluorochromes.** Stained sample or immunofluorescence microscope is a technique which uses antibodies conjugated to fluorescent dyes to evaluated cell in suspension, cultured cell or tissue.

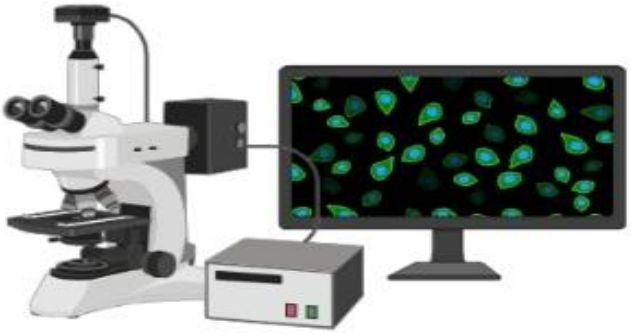

Figure 5: Fluorescence Microscope

Applications

- Localisation of specific proteins and other subcellular structures within cells.
- Identified which cell compartment a protein localises to and whether it colocalised with other protein.
- Analysis of signalling pathways in individual cell (Calcium imaging).
- Chromosomal abnormalities (FISH)

XI. MICROSCOPY OF LIVING CELLS

Microscopy is a significant technique in cell biology. It allows scientists to examine and study cells in their natural condition without the need of fixation or labelling, which might affect cellular architecture and activities. Here are some important points about the microscopy of living cells.

Advantage

Microscopy enables real-time observation of dynamic processes like cell division, migration, and interactions.

- It enables the investigation of cellular responses to stimuli or treatments.
- It provides details regarding cell shape, behaviour, and function under physiological settings.

Challenges

- Living cells are sensitive to external factors such as temperature, pH, and oxygen levels.
- Movement and focus changes might be difficult, particularly in thick or tightly packed samples.
- Managing cell viability and physiological significance during imaging may necessitate specialised equipment and approaches.

Applications

- Cell biology is the study of cellular processes like mitosis, death, and intracellular transport.
- Microbiology involves studying bacterial proliferation, movement, and interactions with host cells.
- Developmental biology is the study of how cells and tissues develop and differentiate.
- Drug Discovery: Evaluating the impact of medicines on cellular function and viability.
- Cancer research focuses on tumour cell behaviour and response to treatment.

XII. CONCLUSION

Finally, imaging of living cells is important in modern biological research because it allows scientists to monitor and analyse cellular dynamics and behaviours in settings that closely resemble their natural environment. This expertise is critical for improving our understanding of basic biological processes and developing new illness treatments.

1. **Electron Microscope:** Knoll and Ruska co-invented this in 1931. An electron microscope examines objects on a very tiny scale by focussing a beam of highly powerful electrons. Electrons possess a shorter wavelength than light hence electron microscopes have better resolution than light microscopes. When electron beam strike the specimen three phenomenon takes place:

    - Electron passes without any scattering.
    - Electrons undergoes elastic scattering.
    - Electron undergoes inelastic scattering.

Electromagnetic Lenses are used for the formation of final magnified image. Electron gun is used to produce electron beam. There are two types:

- The transmission electron microscope (TEM)
- The scanning electron microscope (SEM)

Transmission Electron Microscope (TEM)

Unscattered electrons along with **elastically** scattered electrons is used for image formation. It is used to visualize **internal structures.**

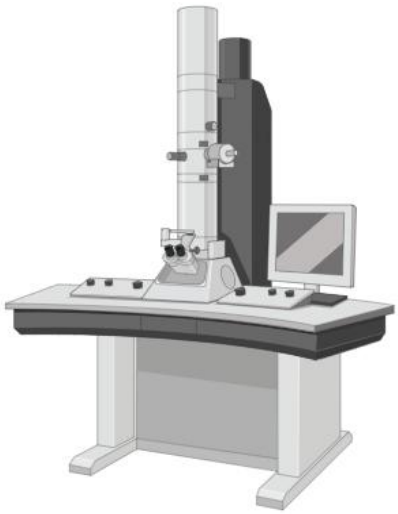

Figure 6: TEM

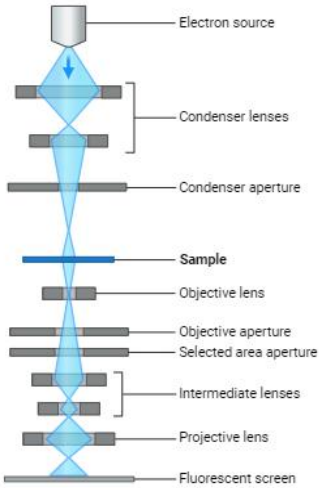

Figure 7: Ray Diagram (TEM)

XIII. SAMPLE PREPARATION

1. **Sample Collection and Fixation:** Collect an accurate representative of the material to analyse and Fixatives such as glutaraldehyde or formaldehyde can help to preserve the structure of the material. This procedure is necessary for biological specimens to retain their cellular architecture.

2. **Dehydration:** Gradually replace the water in the sample with progressively higher quantities of alcohol or acetone. This avoids ice development in later processes.

3. **Embedding Medium:** Place the dehydrated sample in an embedding medium, such as epoxy or acrylic resin. This adds support and structure to the sample.

4. **Polymerization:** Depending on the type of resin being used, heating or UV light are usually required to harden the resin.

5. **Sectioning**

    - **Cutting:** Cut the implanted sample into extremely thin slices, typically 50–100 nanometres thick, using an ultramicrotome. To make sure the portions are thin enough for electron transmission, this procedure demands precision.

    - **Gathering Sections:** Place the slender segments onto a TEM grid. To sustain the sections, these grids are usually covered with a thin layer of formvar or carbon.

6. **Staining:** Apply heavy metal stains to improve contrast in the electron microscope, such as lead citrate and uranyl acetate. These stains attach themselves to the constituents of cells, raising their electron density.

7. **Dring:** Prior to imaging, make sure the material is totally dry. By doing this, TEM image artefacts are avoided.

8. **Mounting on Grid:** Make sure the stained sections are clean and firmly affixed to a TEM grid by carefully positioning them on it.

9. **Imaging:** Insert the ready-made grid into the TEM to capture images. To view the sample at the desired resolution, change the parameters

Advantage

- TEM offer very powerful magnification and resolution.
- TEM provide information on element and compound structure.
- Image is high quality and detailed.

Disadvantage

- TEM are Large and very expensive.
- Laborious sample preparation.
- Operation and analysis require special training.
- TEM require special housing and maintenance.
- Image is black and white.

XIV. SCANNING ELECTRON MICROSCOPY (SEM)

Inelastic scattered electrons along with secondary electrons are used in the image formation in SEM. It is used to monitor surface structure of organism. These microscopes cannot be used to image living cells. Produces images of sample by scanning the surface.

Figure 8: SEM

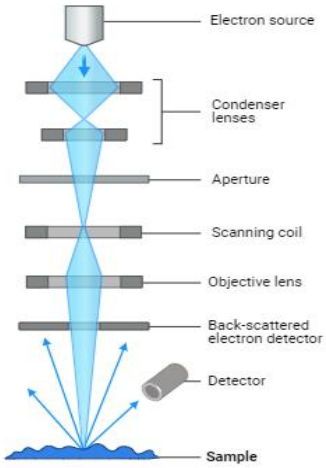

Figure 9: Ray Diagram (SEM)

XV. APPLICATIONS OF SEM

- For investigation of virus structure
- 3D tissue imaging
- Insect, spore, another microorganism or cellular component visualis

XVI. IMAGE PROCESSING METHODS

Image processing methods cover a wide range of approaches for manipulating and analysing images. Here are some popular methods categorised according to their aims.

Preprocessing

1. **Noise Reduction:** Uses techniques such as Gaussian blur, median filtering, and wavelet denoising to eliminate noise from images.

2. **Image Enhancement:** Methods for improving image quality and visual appeal include histogram equalisation, contrast stretching, and sharpening.

Segmentation

1. **Thresholding:** Divide a picture into foreground and background using pixel intensity thresholds.

2. **Edge Detection:** Identifying the borders of objects inside a picture, commonly utilising techniques such as Sobel, Canny edge detection, or Laplacian of Gaussian.

3. **Region Based Segmentation:** Pixels are clustered into clusters based on similarities in colour, intensity, or texture.

Feature Extraction

Corner Detection: Identifying key points in an image, such as Harris corner detection or FAST features.

Comparison between TEM and SEM

Sl. No.	TEM	SEM
1.	Used to study internal structures.	Used to study topology of the cell.
2.	Transmitted electrons are measured.	Secondary electrons are measured.
3.	Sectioning of specimen is required	No sectioning is done
4.	Magnification 10,000–100,000X	Magnification 1000–10,000X.

XVII. DIGITAL MICROSCOPE

Digital microscopy is the use of digital cameras and computer technologies to acquire, process, and analyse images of specimens. It has transformed microscopy by allowing for better visualisation, documentation, and sharing of microscopic pictures than classical optical microscopy can provide.

The Key Elements of Digital Microscopy are:

1. **Image Capture and Processing:** Digital microscopy enables real-time viewing and capture of pictures on a computer screen. This functionality allows for immediate image analysis and processing, such as changing brightness, contrast, and colour balance.

2. **Digital Camera:** High- quality digital cameras are used to photograph objects under a microscope. These cameras range from basic USB-connected devices to sophisticated digital cameras built into microscope systems.

3. **Storage and Documentation:** Digital microscopy allows you to store and organise massive volumes of picture data digitally. This makes it easy to keep track of observations, compare photographs over time, and share results with colleagues or for publication.

4. **Analysis and Measurement:** Digital microscopy software enables quantitative analysis and measurement of numerous properties in images, including cell size, count, and morphology. This quantitative data can be extremely useful in research and diagnostic applications.

5. **Sharing and Collaboration:** Digital photographs are easily shared over networks or the internet, promoting global collaboration among researchers and educators. This skill improves the transmission of scientific knowledge and speeds up research progress.

6. **Integration and Other Technologies:** Digital microscopy can be combined with other technologies such as fluorescence microscopy, confocal microscopy, and even artificial intelligence to enable enhanced picture processing and automated decision-making.

Difference between Light and Electron Microscope

Sl. No.	Light Microscope	Electron Microscope
Resolution	0.2	0.5
Magnification	1000-1500	Over 100000
Travel through	Air	High vacuum
Source of Radiation	Visible light	Electron Beam
Mount on specimen	Glass	Metal Grid (mainly Cu)
Contrast	Differential light Absorption	Scattering of electron

XVIII. FIXATION TECHNIQUE

Fixation processes of materials for microscopy are critical in preparing biological material for examination under a microscope. Fixation is the technique of maintaining biological tissues or cells in as lifelike a state as possible in order to maintain structure and prevent degradation. The following are some common fixation procedures used in microscopy.

Chemical Fixation

1. **Glutaraldehyde Fixation:** Glutaraldehyde is a more potent fixative than formaldehyde and is commonly used in electron microscopy to retain ultrastructural details.

2. **Formaldehyde Fixation:** Formaldehyde crosslinks proteins within cells, stabilising them. It is commonly utilised because it successfully preserves cellular structure.

XIX. CRYOFIXATION

Freezing: Samples are quickly frozen with liquid nitrogen or other cryogens. This approach keeps the sample in a near-native state by preventing the production of ice crystals, which could harm cellular structures. Cryofixation is widely employed in cryo-electron microscopy (cryo-EM).

Heat Fixation

Smear Technique: This technique is passing a sample (such as a bacterial smear) momentarily through a flame to kill the microbes and fix them to the slide. It is often used in bacterial microscopy.

Acetone Fixation: Acetone is used to stabilise frozen slices or air-dried specimens for immunofluorescence microscopy.

Osmium Tetroxide Fixation: Osmium tetroxide is a potent fixative used largely in electron microscopy to preserve lipid structures in cell membranes.

Methanol Fixation: Methanol, like acetone, is used to fix cells for immunofluorescence microscopy.

The fixation technique chosen is determined by a number of criteria, including the type of sample, the purpose of the study, and the microscopy technique utilised. Each fixation process has advantages and disadvantages, and researchers choose the one that best preserves cellular morphology and structure for precise observation and analysis under the microscope.

XX. STAINING TECHNIQUE

Microscopy staining procedures are vital for increasing the visibility of biological material under a microscope. Here are some typical staining techniques:

1. **Gram Staining:** Used to divide bacteria into two groups depending on their cell wall construction. Gram-positive bacteria stain purple-blue, whereas Gram-negative bacteria stain pinkish-red.

2. **Giemsa Staining:** Used to stain blood cells and other microbes such as bacteria. It stains cytoplasm, nuclei blue-purple, and other structures in varying colours of pink.

3. **Fluorescent Staining:** Uses dyes that are fluorescent or antibodies linked with fluorescent markers to identify specific molecules or architectures. It provides for more precise and sensitive detection than standard stains.

4. **Haematoxylin and Eosin Staining:** This is a commonly used colouring method in histology. Haematoxylin colours nuclei blue-purple, but eosin stains cytoplasm and extracellular structures in a variety of pink tints.

5. **Methylene blue Staining:** Used for staining animal cells and bacteria. It stains acidic substances blue.

Freez Fracture Technique

Freeze-fracture techniques are highly specialised methods used in electron microscopy to investigate the interior architecture of cells and tissues.

References

[1] Willey, J. M., Sandman, K. M., Wood, D. H., & Prescott, L. M. (2019). Prescott's microbiology (11th ed.). McGraw Hill.