

Observing Microorganisms through a Microscope

I Microscopy

Magnification = how much larger an object is made to appear compared to its real size.

Resolving power = The minimum separation distance at which two points can still be distinguished as two separate points.

Resolving power of a lens

$d = 0.5 \lambda / NA$ - resolution increases with decrease in λ

- d = minimal distance between two objects
- λ = wavelength of light used to illuminate the specimen
- NA = numerical aperture of lens (the ability of the lens to gather light)

SI prefixes

T	=	tera	=	10^{12}
G	=	giga	=	10^9
M	=	mega	=	10^6
k	=	kilo	=	10^3
m	=	milli	=	10^{-3}
μ	=	micro	=	10^{-6}
n	=	nano	=	10^{-9}
p	=	pico	=	10^{-12}
f	=	femto	=	10^{-15}
a	=	atto	=	10^{-18}

1 Angstrom (\AA) = 10^{-10} m

Types of Microscopy

A. Light Microscopy

- focus visible or UV light with glass lens
 - ⇒ 1000 – 2000X useful magnification
 - ⇒ Maximum resolution - $0.2 \mu\text{m}$ resolution
 - ⇒ larger subcellular organelles & bacterial cells

Types of Light Microscopes

1. Bright-field Microscopy

- Most common type of microscopy
- Forms a dark image against a light background
- Specimens must be stained to provide contrast

2. Phase Contrast Microscopy

- Converts slight differences in medium and cell refractive index into easily detected variations in the light intensity (contrast; Fig. 2.9).
- Allows one to observe living and unstained cells by changing how the specimen is illuminated
- Useful in studying microbial motility, determining the shape of living cells, and detailed examination of internal structures (e.g., bacterial components such as endospores and inclusion bodies)

3. Dark-field Microscopy

- Only light passing through the object will enter the objective lens. Light passing through the background will not enter the objective. Object is bright while background is dark (Fig 2.7)
- Like phase contrast microscopy, darkfield microscopy allows one to observe living and unstained cells by changing how the specimen is illuminated. May also be desirable when staining distorts the specimen or causes artifacts.
- Useful in observing internal structure of larger eukaryotic microorganisms

4. Differential Interference Contrast (DIC) Microscopy

- Also known as Nomarski Microscopy
- like phase contrast, DIC creates images by detecting differences in refractive index and thickness
- Uses polarized light to create interference patterns
- Live unstained specimens appear brightly colored and three dimensional
- Used to observe structure such as ornate cell walls, endospores, granules, vacuoles and nuclei of eukaryotic cells

5. Fluorescence Microscopy

- Specimen illuminated (excited) with one wavelength of light (UV, violet or blue light) and observed by the image formed from the lower energy (i.e., longer λ) emitted light (fluorescent light).
- The specimen is usually stained with special dye molecules (fluorochromes) that absorb the light energy from the excitation light and fluoresce brightly.

- Fluorescence microscopy is an essential tool in modern medical microbiology and microbial ecology. Specific fluorochromes, fluorescent antibodies and fluorochrome labeled nucleic acid probes can be used to visualize and identify particular microbes

Newer Techniques in Light Microscopy

6. Confocal Microscopy

- Confocal Scanning Laser Microscope (CSLM) overcomes image clarity problems resulting from light from all areas of the specimen, not just in the plane of focus, entering the microscope. This instrument uses a laser beam to illuminate only the parts of the specimen in the plane of focus. The light is collected through a pinhole aperture resulting in very sharp 2 dimensional images
- Computer collects data from each plane of the specimen and can assemble composite images as well as three dimensional images
- Can also be used in cellular physiology studies by measuring distributions and concentrations of substances such as ATP and Ca^{2+}

7. Two-Photon Microscopy

- Specimens are stained with fluorochromes. Uses long wave red light rather than the short wave blue light used with (CSLM). The fluorophore is excited by 2 photons rather than one so only parts of the specimen in the tight focus of the laser are excited.
- The longer wavelength infrared laser allows deeper tissue penetration (up to a depth of 1 mm compared to > 0.1 mm for CSLM), reduced phototoxicity and efficient light detection. It can be used to track cell activity in real time.

Preparation and Staining of Specimens for Light Microscopy

- While a number of the techniques described above can be used to observe live microorganisms without staining, these specimens are often stained to increase visibility, accentuate specific features and preserve them for future study

Fixation

- A process whereby the external and internal structures are of cells and microorganisms are preserved and fixed in position
- The process inactivates destructive enzymes and toughens cell structures so they do not change during staining and observation
- The microorganism is usually killed and attached to the microscope slide during fixation

Methods of fixation

- Cells are often applied to a slide as a thin film or smear
- Heat fixation, such as gently heating a film of cells (smear) by passing a slide through a flame, preserves overall structure but not structures within cell. This process is usually used with prokaryotic cells
- Chemical fixation may be used if one requires fine cellular substructure or is working with larger more delicate eukaryotic cells. Chemical fixatives (e.g., Methanol, ethanol, acetic acid, formaldehyde and glutaraldehyde) enter the cell and react with proteins and lipids rendering them inactive, insoluble and immobile.

Staining

- There are many dyes that can be used to stain cells. They all have two common features – they have chromophore groups (groups with conjugated double bonds that give the dye its color) and they can bind with cells by ionic, covalent or hydrophobic bonding
- Most dyes are used to stain the cell or object directly (Positive staining), but some dyes are used to stain the background (Negative staining)
- Dyes that bind by ionic interactions are the most commonly used stains. They can be divided into two groups based on the nature of their charged group

Basic dyes – Have positively charged chromophores (e.g., pentavalent nitrogen) and bind negatively charged molecules such as nucleic acids, many proteins and the surface of prokaryotic cells (bacteria tend to be slightly negatively charged at pH 7). Examples include: crystal violet, methylene blue, safranin, malachite green

Acidic dyes – Have negatively charged chromophores (carboxyl and phenolic hydroxyls) and bind positively charged cell structures or background. Examples include: eosin, Rose Bengal and acid fuchsin

i) Simple staining - single stain is used; stains all cells and structures the same color

ii) Differential staining - multiple stains are employed. Different types of microorganisms or cell structures exhibit different staining reactions resulting in color differences. The initial stain or primary stain must be fixed following staining so that unbound stain can be washed away and the specimen can be treated with an additional stain(s) or counterstain(s)

e.g., Gram Stain (developed by Christian Gram - 1884)

e.g., Endospore stain

B. Electron Microscopy

- focus a beam of electrons with electromagnetic lenses.
 - Practical resolution - 0.2 nm resolution and useful magnification > 100,000 x
 - Can observe cellular ultrastructure
 - chemical and physical preparation is lethal to cells and may lead to artifacts

1. Transmission electron microscopy (TEM)

- electron transmitted through the specimen
- Practical resolution - 0.2 nm resolution and magnification from 10,000 to 100,000X
- study internal cellular ultrastructure
- chemical fixation, physical preparation (thin sectioning) and staining is lethal to cells and may lead to artifacts
- Contrast is provided by positive staining, negative staining and shadowing (metal stain such as platinum is applied in a thin film at a 45° angle so that the metal strikes the specimen on only one side)
- Can use freeze etching to disclose the shape of internal organelles

2. Scanning electron microscopy (SEM)

- scanning beam excites secondary electrons on specimen surface. These are collected and focused onto a viewing screen.
- produces a 3-D image with great depth of field.
- used for studying the surface of a specimen.
- Resolution of 7 nm or less and magnification in the range of 1000 to 10,000X

C. Scanned-Probe Microscopy

- Measure surface features by moving a sharp probe over the surface
- Scanning tunnel microscope – Tungsten probe tip may have only a single atom on the tip. This instrument can achieve magnifications of 100,000,000X. Special preparation of the specimen is not needed.
- Atomic force microscope also uses a thin probe – made of metal and diamond. The specimen surface is scanned with probe. The probe movement is recorded and this information is used to generate a three dimensional image.