

Microscopy

Techniques that make it easy to see things this small.

What is a Microscope?

- An instrument for viewing objects that are too small to be seen easily by the naked eye.
- Galileo Galilei developed an “occholino” or compound microscope with a convex and a concave lens in 1609.
- Christian Huygens developed a simple 2-lens ocular system in the late 1600's that was achromatically corrected and therefore a huge step forward in microscope development.
- Anton van Leeuwenhoek is generally credited with bringing the microscope to the attention of biologists, even though simple magnifying lenses were already being produced in the 1500's, and the magnifying principle of water-filled glass bowls had been described by the Romans.
- Van Leeuwenhoek's home-made microscopes were actually very small simple instruments with a single very strong lens.
- It actually took about 150 years of optical development before the compound microscope was able to provide the same quality image as van Leeuwenhoek's simple microscopes.

Simple vs. Compound

- A simple microscope, as opposed to a standard compound microscope with multiple lenses, is a microscope that uses only one lens for magnification.
- Van Leeuwenhoek's microscopes consisted of a single, small, convex lens mounted on a plate with a mechanism to hold the material to be examined (the sample or specimen).
- This use of a single, convex lens to magnify objects for viewing is still found in the magnifying glass.
- In its simplest form - as used by Robert Hooke, for example - the compound microscope would have a single glass lens of short focal length for the objective, and another single glass lens for the eyepiece or ocular.
- Modern microscopes of this kind are usually more complex, with multiple lens components in both objective and eyepiece assemblies.
- With a few exceptions, they utilize light passing through the sample from below and special techniques are usually necessary to increase the contrast in the image to useful levels.
- Typically, on a standard compound optical microscope, there are three objective lenses: a scanning lens (4x), low power lens (10x), and high power lens (40x).
- Advanced microscopes often have a fourth objective lens, called an oil immersion lens.
- To use this lens, a drop of oil is placed on top of the cover slip, and the lens is moved into place where it is immersed in the oil.
- An oil immersion lens usually has a power of 100x. The actual power or magnification is the product of the powers of the ocular (eyepiece), usually about 10x, and the objective lens being used.

Types of Light Microscopy

- Compound Light Microscopy
- Darkfield Microscopy
- Phase-Contrast Microscopy
- Differential Interference Contrast Microscopy (DIC)
- Fluorescence Microscopy
- Confocal Microscopy

Compound Light Microscopy

- Illuminator-
- Condenser-
- Objective lens-
- Ocular Lens-
- Resolution- the ability of a microscope to distinguish fine detail.
- Refractive index- a measure of the light-bending ability of a particular medium.
- A compound microscope is typically used in teaching and research laboratories.
- A specimen is magnified as light passes through the objective and ocular lens.
- Resolution distinguishes magnified objects clearly.

Darkfield Microscopy

- This technique is used to examine unstained (live) microorganisms in liquid media.
- The light emitted by the lamp passes through an opaque disc, effectively eliminating all light in the center of the beam.
- The light comes in from an angle, therefore, light that is reflected by the specimen reaches the objective lens.
- The background is dark (black).

Phase-Contrast Microscopy

- This technique is also used to examine unstained (live) microorganisms.
- Specifically, this is used to examine the fine detail (internal structure) of a microorganism.
- A phase ring diaphragm allows light to pass through and hit the specimen and a diffraction plate in the objective lens.
- Direct and reflected light rays come together to form an image.
- The background should have a blue-gray background.

Differential Interference Contrast Microscopy (DIC)

- This technique can provide three-dimensional images of unstained organisms.
- It is similar to phase contrast in that differences in refractive indices are used to produce an image.
- The beam of light is split into two beams by a prism.
- Because of this prismatic effect, the image appears colored.

Fluorescence Microscopy

- This is used to rapidly detect and identify organisms.

- Some organisms may naturally fluoresce. If they do not fluoresce, a fluorochrome may be used to dye the cells.

Confocal Microscopy

- This is another technique where fluorochromes are used to dye organisms.
- A laser is used to illuminate the organism and each time it is illuminated, it is scanned.
- The result is a two-dimensional image with improved resolution over other types of microscopy.

Other Types of Microscopy

- Scanning electron microscopy (SEM)
- Transmission electron microscopy (TEM)
- Scanning tunneling microscopy (STM)
- Atomic force microscopy (AFM)

Scanning Electron Microscopy

- This type of microscopy is used to examine the surface structure of organisms.
- Instead of a light source, an electron gun “shoots” electrons at the prepared specimen.
- The electrons are bounced off of the specimen onto an electron collector, where it is amplified and transmitted to a viewing screen.
- Three-dimensional images are produced.

Transmission Electron Microscopy

- This type of microscopy is used to examine ultra-small objects (i.e. viruses, internal ultrastructure of cells).
- Here, the same principle is used as SEM, however the electrons pass through the sample and onto a photographic plate.
- This gives two-dimensional images.

Scanning Tunneling Microscopy

- A tungsten probe is used to scan a specimen.
- The scan will reveal bumps and depressions of atoms on the surface of the specimen.
- Voltage is applied between the probe and surface to be imaged, to detect a weak electric current flowing between the tip and the surface.
- It can resolve features 1/100 the size of an atom.
- The STM can obtain images of conductive surfaces at 0.2 nanometer and can be used to manipulate individual atoms, trigger chemical reactions, or reversibly produce ions by removing or adding individual electrons from atoms or molecules.

Atomic Force Microscopy

- A metal and diamond probe is pressed onto a specimen.
- As the probe moves along the specimen, the movements are recorded and a 3-D image is produced.

Preparing Specimens

The method of specimen preparation depends on:

- The condition of the specimen: living or preserved
- The aims of the observer:
 - Observation
 - Identification
 - Movement
- The type of microscopy available

Wet Mounts or Hanging Drop Mounts

- Cells are suspended in a suitable fluid: water, broth, or saline
- Fluid maintains viability and provides a medium for movement
- Provide a true assessment of size, shape, arrangement, color, and motility of cells

Wet Mounts

- Consist of a drop or two of culture placed on a slide overlaid with a coverslip
- Advantages: quick and easy to prepare
- Disadvantages:
 - Can damage larger cells
 - Susceptible to drying
 - Can contaminate the handler's fingers

Hanging Drop Mounts

- Prepared with concave (depression) slide, Vaseline adhesive or sealant, and a coverslip
- Overcomes the disadvantages of wet mounts

Fixed, Stained Smears

Smear technique:

- Spread a thin film made from a liquid suspension of cells on a slide
- Allow the slide to air dry

Fixing Smears

- Heat fixing:
 - Heat the slide gently after it has been air dried
- Important functions of heat fixing:
 - Kills the cells
 - Secures the specimen to the slide
 - Preserves cellular components in a natural state with minimal distortion
 - Alcohol and formalin can also be used for fixation

Staining Smears

- Provides contrast
- Makes inconspicuous features stand out
- Dyes impart colors to cells by becoming affixed to them through a chemical reaction
- Dyes used in microbial staining:
 - Basic (cationic): have a positive charge, attracted to acidic, negatively charged

- components on bacterial cell walls
- Acidic (anionic): have a negative charge, repelled by acidic, negatively charged components on bacterial cell walls

Negative vs. Positive Staining

Positive stain:

- Positively charged stain is attracted to negatively charged cell walls
- Stick to the cell and give it color

Negative stain:

- Negatively charged dye is repelled by negatively charged bacterial cell walls
- Produces a black background around the cells

Simple Stains

- Require a single dye
- Uncomplicated procedure

Differential Stains

- Use two differently colored stains to clearly contrast cell types or cell parts
- Complex staining technique
- Types:
 - Gram stain
 - Acid-fast stain
 - Endospore stain
 - Capsule stain

Gram Stain

- Developed a century ago, it remains the universal diagnostic staining technique for bacteria
- Permits ready differentiation of major categories based on the color reaction of cells:
 - Gram-positive bacteria stain purple
 - Gram-negative bacteria stain pink

Acid-Fast Stain

- Differentiates acid-fast bacteria from non acid-fast bacteria:
 - Acid-fast bacteria stain red
 - Non acid-fast bacteria stain blue
 - Detects the agents of tuberculosis and leprosy

Endospore Stain

- Used to distinguish **endospores** from **vegetative** cells
 - Detects endospore-forming members of the genera *Bacillus* and *Clostridium*
 - Endospore stain showing vegetative cells in pink and spores in green

Special Stains

- Some staining techniques (spore, capsule) which are differential are also in the “special” category as they pinpoint a particular characteristic, such as the presence of an endospore
- Used to emphasize certain cell parts that may not be revealed by conventional staining methods

The Capsule Stain

Capsule:

- An unstructured protective layer surrounding the cells of some bacteria and fungi
- Visualized through negative staining with India ink or special positive stains
- Useful in identifying pathogens such as *Cryptococcus*

Flagellar Staining

Flagella:

- Tiny, slender filaments used by bacteria for movement

Flagellar staining:

- Flagella must be enlarged in order to be seen
- A coating is deposited on the outside of the filament and then stained