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.
- Dutch spectacle-makers Hans Janssen and his son Zacharias Janssen are often said to have invented the first compound microscope in 1590.
- The date is certainly not likely, as it has been shown that Zacharias Janssen actually was just about born in 1590.

- Another favorite for the title of 'inventor of the microscope' was Galileo Galilei.
- He developed an "occhiolino" or compound microscope with a convex and a concave lens in 1609.
- Christian Huygens, another Dutchman, 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.

- They were awkward in use but enabled van Leeuwenhoek to see highly detailed images, mainly because a single lens does not suffer the lens faults that are doubled or even multiplied when using several lenses in combination as in a compound microscope.
- 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.

- These multi-component lenses are designed to reduce aberrations, particularly chromatic aberration and spherical aberration. In modern microscopes the mirror is replaced by a lamp unit providing stable, controllable illumination.
- Compound optical microscopes can magnify an image up to 1000x and are used to study thin specimens as they have a very limited depth of field.
- Typically they are used to examine a smear or a thinly sectioned slice of some material.

- 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.

- A lens magnifies by bending light (through the process of refraction).
- Optical microscopes are restricted in their ability to resolve features by a phenomenon called diffraction which, based on the numerical aperture (NA) of the optical system and the wavelengths of light used (λ), sets a definite limit (d) to the optical resolution.
- Assuming that optical aberrations are negligible, the resolution (d) is given by:

- Usually, a λ of 550 nm is assumed.
- With air as the medium, the highest practical NA is 0.95, and with oil, up to 1.25.
- Due to diffraction, even the best optical microscope is limited to a resolution of 0.2 micrometers.

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.

- Resolution can be increased by using immersion oil.

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 difraction plate in the objective lens.
- Direct and reflected light rays come together to form an image.
- The background should have a blue-gray background.

- Examples of a dark-field and phase-contrast microscope.

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.

- Example of differential interference.

Fluorescence Microscopy

- This is used to rapidly detect an identify organisms.
- Some organisms may naturally fluoresce. If they do not fluoresce, a fluorochrome may be used to dye the cells.

- Example of fluorescent microscopy

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.

- Example of a confocal microscope.

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.

- Example of Scanning Electron Microscopy (SEM)

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.

- Example of Transmission Electron Microscopy (TEM)

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.

Schematic of Scanning Tunneling Microscope

Salt

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.

- Examples of atomic force microscopy.

- Summary of optical and electron microscopes.

- Comparison of optical and electron microscopes.

Preparation and Staining of Specimens

- increases visibility of specimen
- accentuates specific morphological features
- preserves specimens

Fixation

- preserves internal and external structures and fixes them in position
- organisms usually killed and firmly attached to microscope slide
  - heat fixation – routine use with bacteria and archaea
    - preserves overall morphology but not internal structures
  - chemical fixation – used with larger, more delicate organisms
    - protects fine cellular substructure and morphology

Dyes and Simple Staining

- dyes
  - make internal and external structures of cell more visible by increasing contrast with background

- have two common features
  - chromophore groups
    - chemical groups with conjugated double bonds
    - give dye its color
  - ability to bind cells

- dyes
  - ionizable dyes have charged groups
    - basic dyes have positive charges
    - acid dyes have negative charges
  - simple stains
    - a single stain is used
    - use can determine size, shape, and arrangement of bacteria

Figure 2.17

Differential Staining

- divides microorganisms into groups based on their staining properties
  - e.g., Gram stain
  - e.g., acid-fast stain
- differential stain used to detect presence or absence of structures
  - endospores, flagella, capsules

Gram Staining

- most widely used differential staining procedure
- divides bacteria into two groups, Gram positive and Gram negative, based on differences in cell wall structure

Acid-Fast Staining

- particularly useful for staining members of the genus *Mycobacterium*
  e.g., *Mycobacterium tuberculosis* – causes tuberculosis
  e.g., *Mycobacterium leprae* – causes leprosy
  - high lipid content in cell walls (mycolic acid) is responsible for their staining characteristics

Staining Specific Structures

- endospore staining
  - heated, double staining technique
  - bacterial endospore is one color and vegetative cell is a different color
- capsule stain used to visualize capsules surrounding bacteria
  - negative stain - capsules may be colorless against a stained background
- flagella staining
  - mordant applied to increase thickness of flagella

Figure 2.19

Specimen Preparation for TEM

- analogous to procedures used for light microscopy
- for transmission electron microscopy, specimens must be cut very thin
- specimens are chemically fixed and stained with electron dense materials, such as heavy metals, that differentially scatter electrons

Other Preparation Methods

- negative stain
  - heavy metals do not penetrate the specimen but render dark background
  - used for study of viruses, bacterial gas vacuoles
- shadowing
  - coating specimen with a thin film of a heavy metal only on one side
  - useful for viral morphology, flagella, DNA

- freeze-etching
  - freeze specimen then fracture along lines of greatest weakness (e.g., membranes)
  - allows for 3-D observation of shapes of intracellular structures
  - reduces artifacts