

Microscopy

- Because microbiology deals with organisms too small they cannot be seen distinctly with the unaided eye, the microscope is essential.
- The ***light microscope*** is the ***single most important research tool*** that microbiologists have ever had.
- The ***light microscope*** is an optical instrument which operates on the principal that light energy will pass through and around a suitably thin object, and, with the aid of lenses form a magnified impression on the visual sensory layer of the eye.

In order to know how the microscope works we must first have a basic understanding about how lenses bend and focus light to form images.

- When light energy passes from one medium to another (i.e. AIR and GLASS) the light rays are bent at the point of interface. This process is called ***refraction.***
- The measure of how greatly a substance slows the velocity of light is called the ***refractive index***
- The direction and magnitude of bending is determined by the refractive indexes of the two media forming the interface.
- As parallel rays of light encounter a convex lens, they are slowed and bent towards the normal path.
- The point at which these rays converge is called the ***focal point.***
- The distance between the center of the lens and the focal point is called the ***focal length.***
- The strength of a lens is directly related to its focal length. A lens with a short focal length has a greater capacity for magnification than a lens with a longer focal length.
- Our eyes cannot focus on objects nearer than about 25 cm or 10 inches. We overcome this limitation by using a convex lens as a simple magnifier (or microscope) and holding it close to the object.

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The Light Microscope

There are a variety of *light microscopes* mostly employed in Microbiology:

- Bright-field
- Dark-field
- Phase-contrast
- Fluorescence

The modern scopes are all compound scopes, meaning the magnified image formed by the objective lens is further enlarged by one or more additional lenses.

The Bright-field Microscope

- Called the ordinary microscope because it forms a dark image against a brighter background.

See Transparency

- *Parafocal:* image should remain in focus when objectives are changed.

Path of Light:

- The objective lens forms an enlarged real image within the microscope and the eyepiece lens further magnifies this primarily image.
- Upon looking in the microscope, the enlarged specimen image – *Virtual image* appears to lie just beyond the stage about 25 cm away.
- Total magnification is calculated by multiplying the objective and eyepiece magnification together.

Three factors determine the quality of an optical image:

- a. Magnification
- b. Resolution
- c. Contrast.

Magnification.

Magnification is the apparent increase in size affected by a convex lens

- A compound microscope uses two sets of lenses, with differing focal lengths, to facilitate magnification

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- The total magnification achieved by the lens array is the product of each individual lens.

Magnification (total) = magnification (obj. lens) x magnification (ocu. lens)

- Example: $Mag_{(obj)} = 40X$ and $Mag_{(ocular)} = 10X$
- Then $Mag_{(total)} = (40X) (10X) = 400X$.

It is much easier to make two lenses with average magnifying powers and put them together in a compound microscope than to make a single lens with a very high magnifying power.

Compound microscopes are usually designed to give a highest possible magnification of only 1,000-1,500X.

Resolution

The most important part of the microscope is the objective, which must produce a clear image, not just a magnified one.

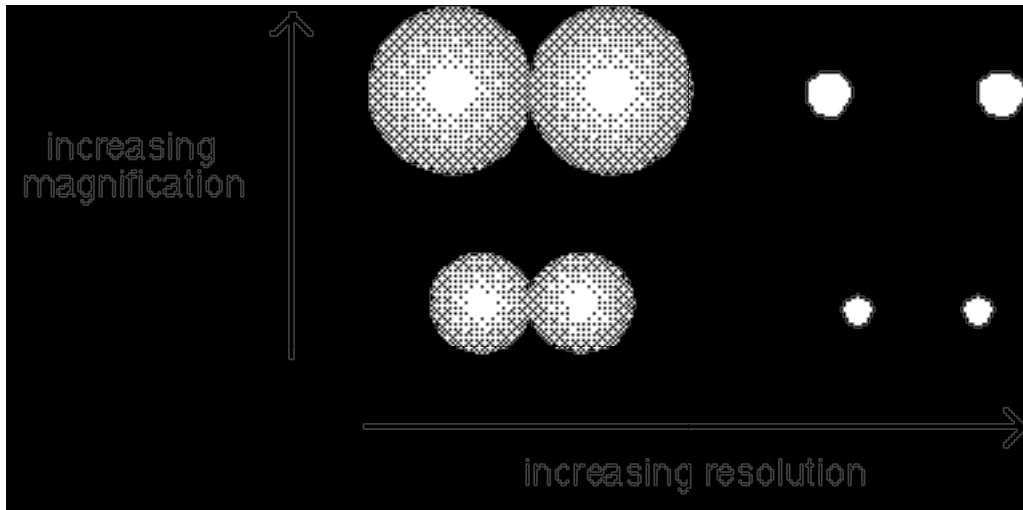
Resolution is the ability to separate points (in other words, to observe fine detail).

- the ability to of a lens to distinguish between small objects that are close together.

Example: car headlights.



Resolution is not the same thing as magnification.



- One way to increase the resolution of an image is to increase the amount of light that enters the objective lens by using *immersion oil*.

We have already discussed Refraction, that is the deflection of a light ray that occurs when it passes between substances that have different densities.

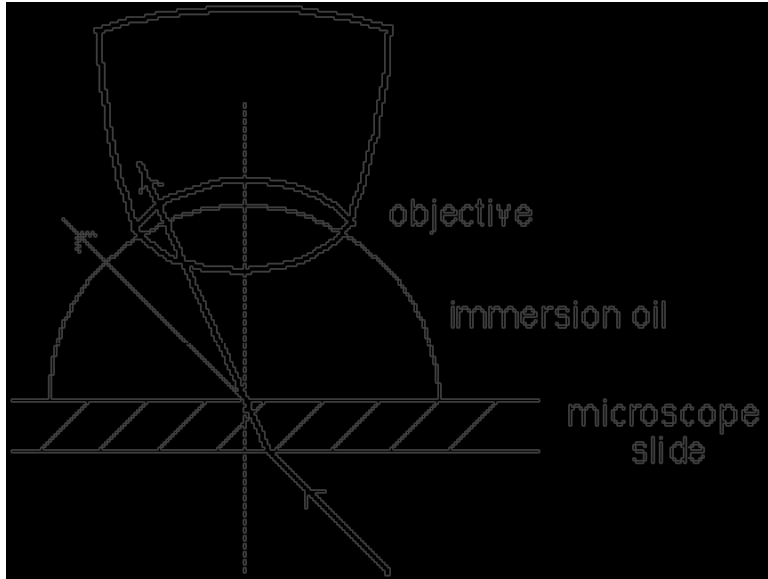
A convex lens exploits refraction to focus light at a specific point.

However not all refraction results in the convergence of light, in many cases, the rays of light diverge, which results in an overall decrease in the intensity of light.

This typically occurs as light passes through the air, in the space between the specimen and the objective lens.

Immersion oil (with a density closer to glass) can be used to reduce the refraction of light rays (compared to air) and allows more light to enter the objective. This improves resolution.

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Only the highest power (100X) objective on the microscope is designed for use with immersion oil. **DO NOT USE OIL WITH THE LOWER POWER (10X AND 40X) OBJECTIVES!**

Another way to increase resolution of an image is to decrease the wavelength of light that is used to illuminate the specimen (use blue light instead of white light).

Contrast

Microbes are composed of water, nucleic acids, proteins, and lipids. Most appear colorless against a colorless background when observed using bright field microscopy. Therefore in order to see them, we must ***devise a way to increase the contrast.***

Brightfield microscopy

- Direct staining of the microorganisms
- Indirect (negative) staining of the background

In order to stain a specimen, it must first be fixed to the slide and chemically altered. This results in the death of a specimen.

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Additional microscopic techniques have been developed to increase contrast of living microorganisms

Dark-field microscopy is one such technique that is often used to observe living, unstained cells and organisms.

- Dark field microscopes illuminate the sample in such a way that unreflected, and unrefracted light does not enter the objective, only light that has been reflected/refracted by the specimen passes through the objective and forms the image.
- This results in a specimen that is brilliantly illuminated on a dark field
- The field surrounding the specimen appears black.

Phase-contrast microscopes convert slight differences in refractive index and density into variations in light intensity.

Fluorescence microscopy. Specimens are treated with dye molecules called fluorochromes which brightly fluoresce when exposed to light of a specific wavelength.

Anatomy of a compound Microscope

1. lamp (light source)
2. Substage Condenser: Used to vary the intensity of light. Focuses the broad light beam into a cone on the slide.
Highest intensity is achieved when the focal point is at the surface of the slide. (Illustrate). Lower intensity occurs when the light is focused above or below the slide.
3. Iris Diaphragm. Modulates the amount of light that passes through the slide.
4. Stage and microscope slide with specimen
5. Objective lenses (usually 10X, 40X, and 100X) on revolving nosepiece
6. Body tube
7. Ocular lens (usually 10X)

ELECTRON MICROSCOPY

- A physical relationship exists between resolution and light: ***resolution of an image increases as the wavelength of light used to illuminate it decreases.***
- The wavelengths of light in the visible spectrum range between 800 nm (red) and 400 nm (Blue).
- With these wavelengths of light, the maximum resolution that can be achieved is about ***200 nm.***
- Since bacteria are generally ***1000 nm in diameter***, the most we can resolve using light microscopy is their ***general shape and some of the major morphological features (is spores, flagella, capsules)***
- ***Electron microscopes*** are used to achieve up to more than ***100,000 greater magnification and more than 1000 times greater resolution than the light microscope.*** At these limits, ***subcellular structures can be easily observed.***
- The technique of electron microscopy is ***based on the principal that electron beams behave like waves and can be focused much like light in a compound microscope.***
- Electron microscopes ***do not use lenses to focus the electron beams***, instead, they use ***magnetic fields.***

Wavelength of electrons that have been accelerated across a 100,000-volt electric field is about 0.04 nm. (compared to 400 nm blue light)

There are two types of electron microscopes SEM and TEM

1. Transmission Electron Microscope

- Transmission electron microscope (TEM) ***project a beam of electrons through a specimen.***
- Maximum resolution that can be achieved by TEM is ***about 0.5 nm.***
- A TEM uses a series of electromagnets to focus an intense electron beam onto/ and through the sample.
- Electrons that collide with a structure are absorbed or scattered and are not recorded by the phosphorescent detector
- Electrons passing through the specimen unscattered strike the phosphorescent detector screen to form the image.

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- Phosphorescent image is then recorded on photographic film

Limitations of TEM

- Electrons can only travel through a vacuum, so the specimen must be completely dehydrated.
- Electrons have poor penetrating ability. The specimen is usually imbedded in a plastic block and cut into thin sections (no more than 50 nm thick) for viewing.
- Use oil on HOH analogy, if it has a color it is too thick >50 nM=grey/gold
- The image contrast results when electrons are scattered by the specimen. Most biological materials scatter electrons poorly. Therefore specimens are usually **“stained” with a coat of heavy metal (uranium, osmium, and tungsten) to increase scattering ability.**

2. Scanning Electron Microscope

- In SEM, ***the electron beam is focused*** (via electron magnets) and ***projected onto (not through) the specimen in a raster pattern.***
- Image is formed as the scattered electrons collide with a phosphorescent detector screen.
- To improve contrast, the specimen is coated with an ***ultra thin layer of heavy metal (such as gold) to improve electron scattering***
- This technique can be used to observe specimens that are ***several millimeters thick***, and produces ***spectacular three dimensional images.***
- The SEM is able to achieve resolution of ***about 2.5 nm.***

Newer Techniques in Microscopy

1. Confocal Microscopy:

- *Confocal Scanning Laser Microscope or Confocal Microscope*
- Eliminates a murky, fuzzy, and crowded image.
 - Fluorescently stained specimen
 - A focused laser beam strikes a point in the specimen
 - Light from the illuminated spot is focused by an objective lens onto a plane above the objective.
 - An aperture above the objective lens blocks out any stray light from parts of the specimen that lie above and below the plane of focus.
 - The laser is scanned over a plane in the specimen (beam scanning) or the stage is moved (stage scanning) and a detector measures the illumination from each point to produce an image of the optical section. Many sections are scanned, a computer combines them to form a three-dimensional image from the digitized signals. Image can then be measured and analyzed quantitatively.
- *Improves images in two ways:*
 1. Illumination of one spot at a time reduces interference from light scattering by the rest of the specimen.
 2. The aperture above the objective lens blocks out stray light.
- Image has excellent contrast and resolution

2. Scanning Probe Microscopy

- *Scanning Probe Microscope* measure surface features by moving a sharp probe over the object's surface.
- *Scanning Tunneling Microscope*
 - Invented in 1980
 - Can achieve magnification of 100 million and allow the viewing of the atom on the surface of a solid.
- *Atomic Force Microscope*
 - Moves a sharp probe over the specimen surface while keeping the distance between the probe tip and the surface constant.
 - Accomplished by exerting a very small amount of force on the tip.

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Preparation and Staining of Specimens

- *Microorganisms must be fixed and stained to increase visibility, accentuate specific morphological features, and preserve them for future study.*

Fixation:

- Process by which internal and external structures of the cells and microorganisms are preserved and fixed in position.
- This process inactivates enzymes that might disrupt cell morphology and toughens cell structures so that they do not change during staining and observation. A microorganism usually is killed and attached firmly to the microscope slide during fixation.

Two types of fixation:

- **Heat-fixed** bacterial smears by gently flame heating an air-dried film of bacteria, preserving the overall morphology but not structures within cells.
- **Chemical Fixation** is used to protect fine cellular substructures and the morphology of larger, more delicate microorganisms.
 - chemical fixatives penetrate cells and react with cellular components, usually proteins and lipids, to render them inactive, insoluble and immobile.
- Common fixative mixtures:
 - Ethanol
 - Acetic Acid
 - Mercuric Chloride
 - Formaldehyde
 - Glutaraldehyde

Biological Stains

- One way to increase contrast of an image is to **stain** colorless microorganisms.
- Stains are very pure dyes which first came into common use in biology around 1850.
- Stains are typically salts, composed of positive and negative ions (e.g., Na⁺Cl⁻). Only one of the ions carries the **chromogen**, or *colored part of the molecule*.
- Many dyes used to stain M.O. have two common features:
 1. They have **chromophore groups, groups with conjugated double bonds that gives dyes its color.**

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2. They can bind with cells by ionic, covalent, or hydrophobic bonding.

If the positive ion (cationic) contains the chromogen, then the stain is referred to as a basic stain.

- *Example: methylene blue is a chloride salt (MB+Cl-)
Basic fuchsin, crystal violet, safranin, malachite green.*
- *Basic dyes bind to negatively charged molecules like nucleic acids, proteins, and cell wall components, and the surface of the cells themselves.*
- *Because the surfaces of bacterial cells are negatively charged, basic dyes are most often used.*

If the negative ion (anionic) contains the chromogen, the stain is an acidic stain.

- *Example: eosin is a sodium salt (Na+E-)*

Would you expect a basic stain or an acidic stain to react with the bacterial cell itself?

- *Acidic stains do not react with the negatively-charged bacterial cells and therefore become deposited in the background.*
- *This results in a preparation of colorless cells within a stained background (indirect or negative staining).*

Staining a bacterial smear

- *How to make a smear.*
 - Too thick vs. Too thin***
 - *Too thick can cause dye to accumulate between cells*
 - *Too thin, too hard to see*
 - *Ideal smear is one cell layer thick*

Simple stains make use of a single staining agent

- *Cover the fixed smear with stain*
- *Wash excess stain off with water*
- *Blot slide dry*
- *Frequently used basic dyes: CV, MB, CF are used to determined size, shape and arrangement of BACTERIA.*

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Differential Stains

*In contrast, we can have **Differential stains** make use of more than one stain to identify two or more types of bacteria.*

i.e. Different bacteria can be stained different colors (to help distinguish between them).

You will be learning how to do several differential stains in the laboratory.

The Gram stain

The most important differential stain is the Gram stain, which was devised by Christian Gram in 1884.

- Almost all bacteria can be divided into two groups (Gram-positive and Gram-negative) by this stain.
- Results of the Gram stain are correlated with cell wall composition.
- The Gram stain is usually the first step taken to identify an unknown bacterium.

Procedure

- a. The primary stain is crystal violet (purple color). 30 sec - HOH rinse
- b. The mordant is a solution of iodine. 1 min - HOH rinse
 - The mordant forms a complex with crystal violet and helps it adhere to the cell. (iodine increases the interaction btwn CV and cell).
- c. The decolorizing agent is alcohol (95% ethanol). 10-30 sec - HOH rinse
 - This is the Differential step.
 - Gram positive bacteria retain the CV, whereas Gram negative lose CV and become pink upon the addition of the counterstain.
- d. The secondary stain or counterstain is safranin (pink color). 30-60 sec - HOH rinse

Gram-positive cells retain the crystal violet-iodine complex and stain purple. Gram-negative cells lose this complex during the decolorizing step, react with safranin, and stain pink.

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Acid Fast Staining

Some bacteria have an *unusually high lipid content in their cell walls which will not react with simple stains.*

- Bacteria from the *genus Mycobacterium*
- *High concentrations of mycolic acids (branched chain hydroxy lipids) in their cell walls.* These fatty acids give the colonies an overall, waxy, appearance, and will not stain by conventional methods.
- To stain mycobacteria, you must use a combination of heat and phenol to drive the basic fuchsin stain into the cell wall.
- Once the stain has penetrated, it is not easily removed, or decolorized with acid alcohol.
- Thus the cells are said to be **ACID FAST**
- Bacteria without the waxy cell wall are easily decolorized with the acid alcohol, and must be counterstained with methylene blue before they can be visualized.
- *Therefore acid fast=red*
- *Non acid fast= blue*

Other examples of Differential stains

- Spore stain: used to identify endospores
Read in book
- Capsule stain, also an example of negative stain: used to identify capsules
Read in book

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Early taxonomists placed all living organisms into two kingdoms, Plants and Animals. In this classification scheme, bacteria were considered to be plants

Today living organisms are placed into 5 kingdoms

Plantae, Animalia, Fungi, Protista, and Monera

According to this classification scheme, bacteria are placed in the monera kingdom.

In the 1950's scientists began to use the electron microscope to study cellular structures, and they found that all living organisms could be placed into one of two categories (based on its cellular structure)

*The divisions were referred to as, Prokaryote (pre-nucleus) and Eukaryote (true-nucleus)
The electron microscope revealed very clear differences in structure which are summarized on the following table*