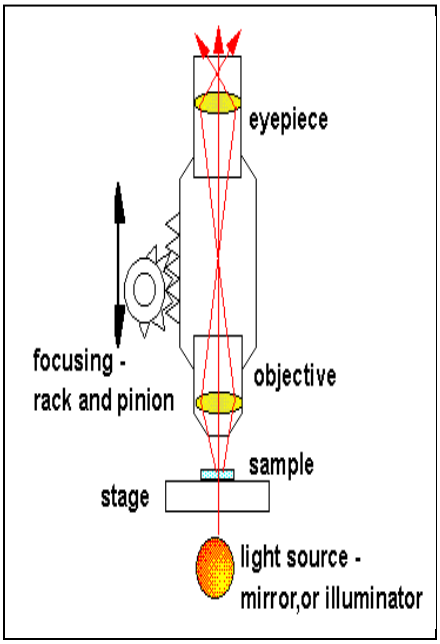
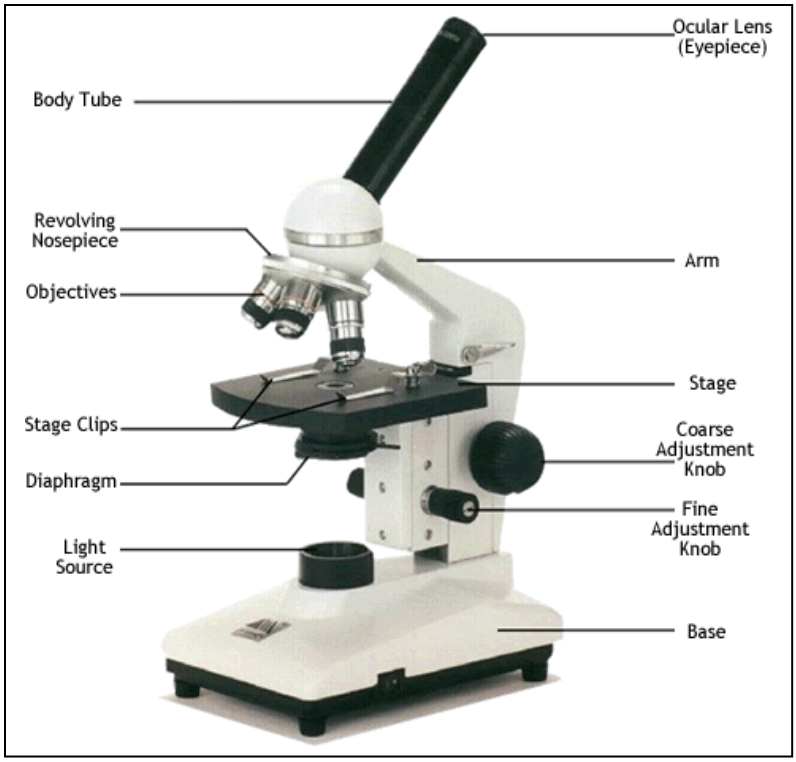
Light Microscopes

Light microscopy uses visible or ultraviolet light to illuminate an object. The light passes through several glass lenses that alter the path of the light and produce a magnified image of the object.

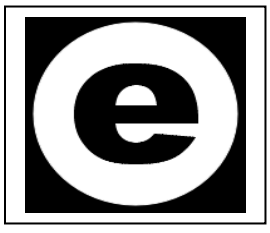




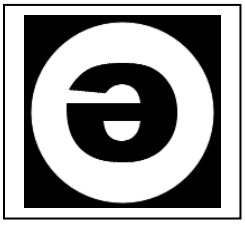
<u>Observing and Drawing Objects</u>:

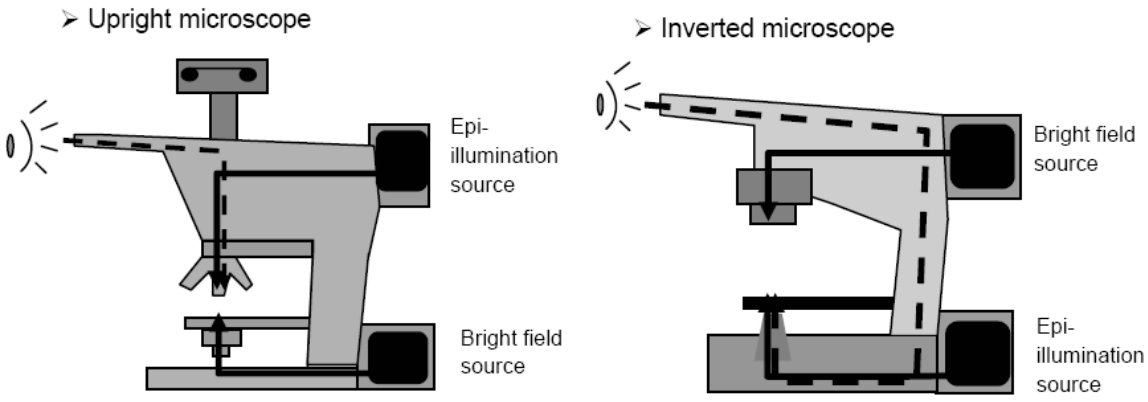
Because the light rays from an object cross before reaching your eye, the image you see through our light microscopes will be **inverted** and **upside down**.

Sitting on the stage



Viewed through the lens





Basic Parts of a Compound Light Microscope:

- **Eyepiece (Ocular):** Usually contains a 10X lens.
- **Arm:** contains the housing for the fine and coarse adjustments and connects the base of the microscope to the nosepiece and ocular.
- **Nosepiece:** A rotating head that has the objective lenses attached to it. The lens to be used should "click" into position when the wheel is gently turned so that it is directly over the speciman slide.
- **Objective:** Basically housing for a lens. Microscopes have three objective lenses - 4X, 10X, and 40X or 100X.
- **Stage:** The speciman slides rests on this part of the microscope.
- **Coarse adjustment knobs:** The larger of two sets of knobs located on either side of the arm, just above the base. This adjustment is used to make large adjustments in focusing by moving the lenses up and down. *Never use this adjustment when using the 40X objective.*
- **Fine adjustment knobs:** The smaller of two sets of knobs located on either side of the arm. This adjustment is used to make small adjustments in focusing. It has a limited amount of movement and is most efficiently used after focusing with the 4X objective and coarse focus, then increasing magnification and making final adjustments with the fine focus knob.
- **Light source:** Located directly under the stage.

- **Adjustable diaphragm:** This rotating wheel on the underside of the stage allows the user to adjust the amount of light that passes through the specimen. As a general rule, the lowest intensity of light that allows you to resolve the structure of the object you are viewing should be used.

Magnification: the increase of an object's apparent size.

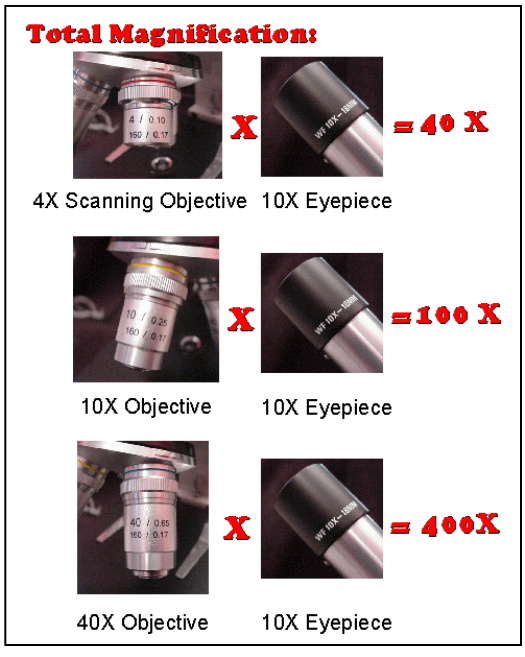
Total magnification is the product of the magnifying powers of the individual lenses. The magnifying capability of a microscope is the product of the individual magnifying powers of the two lenses;

1- Ocular lens (eyepiece) : The lens nearest the eye to magnify object 10 times (10X)

2- Objective lens: The lens nearest the specimen to magnify object 4, 10, 40, and 100 times (4X, 10X, 40X, 100X)

Total magnification = ocular x objective

If the ocular lens enlarges by a factor of 10 (10X) and the objective lens enlarges by a factor of 40 (40X), the total magnification is the product of the two - 400X.



Field of view: the area visible through the microscope lenses. Field of view decreases as magnification increases.

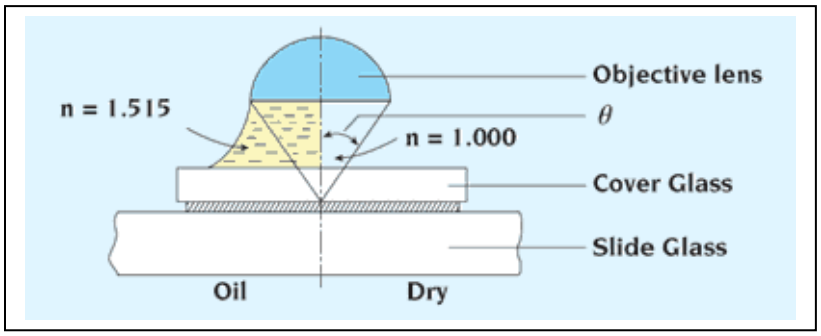
Resolution: is the degree to which the detail in the specimen is retained in the magnified image. The ability to see in detail is essential lest everything appears as an unresolved blur. Magnifying object by using microscope is useful only if detail can be accurately preserved and observed.

Resolving power (R): is the closest spacing between two points at which the points can still be seen clearly as separate entities. In reference to microorganisms, it is the distance between two structural entities of a specimen at which the entities still can be seen as individual structures in the magnified image. The smaller the value for resolving power, the smaller the object that can be seen distinctly. The best resolving power of a light microscope is approximately 200 nanometer (nm), or just below the size of many bacterial cells.

Resolving power (R) = 0.61λ/ NA

λ: Wavelength of light

NA (Numerical Aperture): A property of a lens that describes the amount of the light that can enter it.

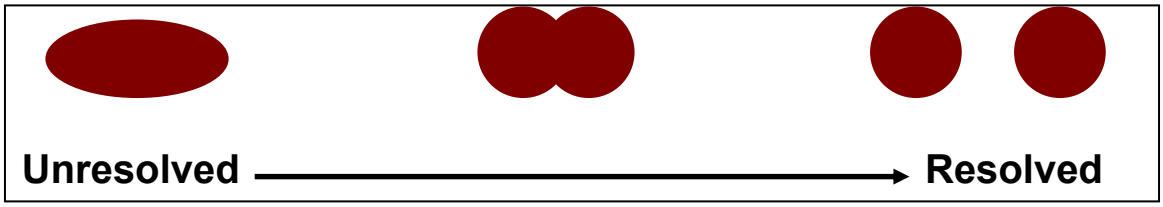


NA = n x sin θ

n: Refractive index of the medium filling the space between the specimen and the front of the objective lens

θ: Angle of the most oblique rays of light that can enter the objective lens

Resolution of Objects



MEASUREMENT EQUIVALENTS

1 centimeter (cm) = 10^{-2} meter (m) = 1/100 m = about 0.4 inch

1 millimeter (mm) = 10^{-3} m = 1/1,000 m = 1/10 cm

1 micrometer (μm) = 10^{-6} m = 1/1,000,000 m = 1/10,000 cm

1 nanometer (nm) = 10^{-9} m = 1/1,000,000,000 m
= 1/10,000,000 cm

1 meter = 10^{2} cm = 10^{3} mm = 10^{6} μm = 10^{9} nm

Inspection Before Use:

Before using your microscope on a laboratory exercise, thoroughly inspect the instrument as follows:

- Make sure it is clean.
- Oil and mounting medium not cleaned off can cause operating and visual distortion problems.
- Clean the ocular lenses with a piece of lens paper. Then clean the four objective lenses, 4X (scanning-lens), 10X (low-power lens), 40X (high-power lens) and 100X (oil-immersion lens).
- Make sure all the parts are present and functioning.
- Check each objective to see that it is clean and focusing properly.
- If you have a problem with your microscope reports it to the instructor immediately.

Care and Handling of the Microscope:

- A microscope is a **delicate** piece of equipment and should be treated with care.
- Use **two hands** when carrying the microscope. Place one hand around the arm of the microscope and the other under the base for support.
- Carry the microscope **upright** and close to the body.
- Place the microscope **flat on the table**, but not too near the edge where it might be knocked off.
- **DO NOT** slide the microscope back and forth on the lab table.
- If it becomes necessary to clean the lenses on the microscope, ask your facilitator for a piece of **lens paper**. Other materials, such as paper towel, can scratch the surface of the lens.

Making a wet-mount slide:

- Place a clean slide on the lab table. **Handle slides at the ends**, not the center, to avoid getting fingerprints in the viewing area of the slide.
- Add specimen to the slide. For liquid samples, place one small drop in the center of the slide. For solid samples, place the sample in the center of the slide and add one drop of **water or stain**.
- Hold the coverslip by the edges to avoid fingerprints. Set one edge against the slide and lower it until it contacts the liquid. The liquid should spread across the whole area of the coverslip.
- **Never view a slide without a coverslip.** The coverslip protects the objective lens from the liquid on the slide.

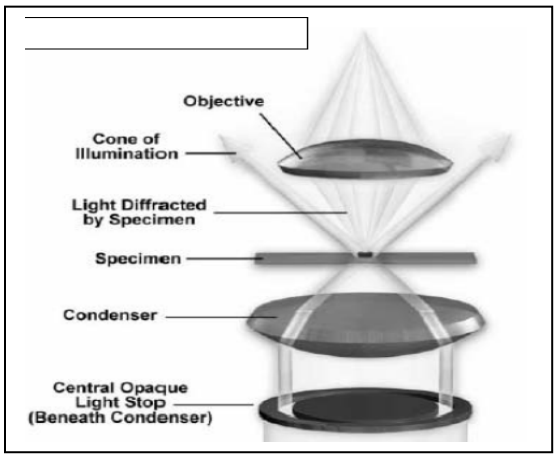
Cleanup:

- Unless otherwise instructed, wipe the sample and coverslip off the slide with a paper towel when finished.
- Throw the paper towel and its contents away.
- Return the microscope slide to its container.

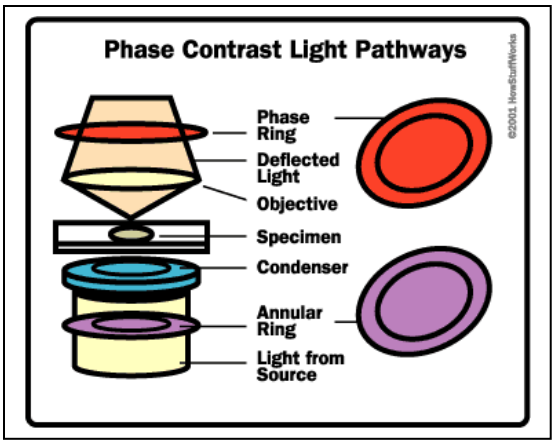
Types of Light Microscopes

Brightfield Microscope: The most common type of microscope in biology is the brightfield microscope, a light microscope in which visible light is transmitted through the specimen. This microscope usually requires staining of specimens and rarely is used to observe live microorganisms. It has a light source, a condenser lens that focuses the light on the specimen, and two sets of lenses that contribute to the magnification of the image. The specimen generally appears dark on a bright background.

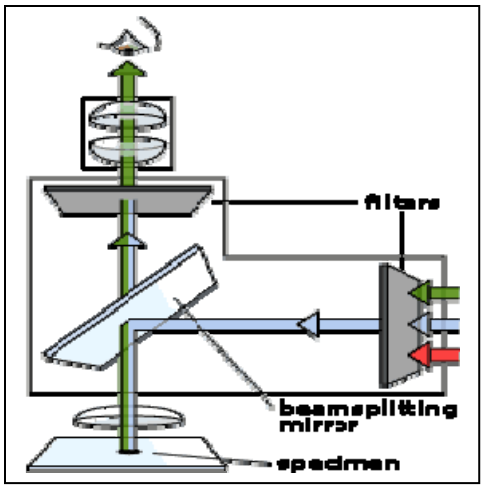
Darkfield Microscope: The darkfield microscope is designed to eliminate the need for staining to achieve contrast between the specimen and the background. The condenser lens of the darkfield microscope does not permit light to be transmitted directly through the specimen and into the objective lens. Condenser lens focuses light on the specimen at an oblique angle, such that, light that does not reflect off an object does not enter the objective lens. Therefore only the light that reflects off the specimen will be seen and the light simply passing through the slide will not enter the objective. The field will appear dark. Sample that is viewed with a darkfield microscope appear very bright on a dark background.



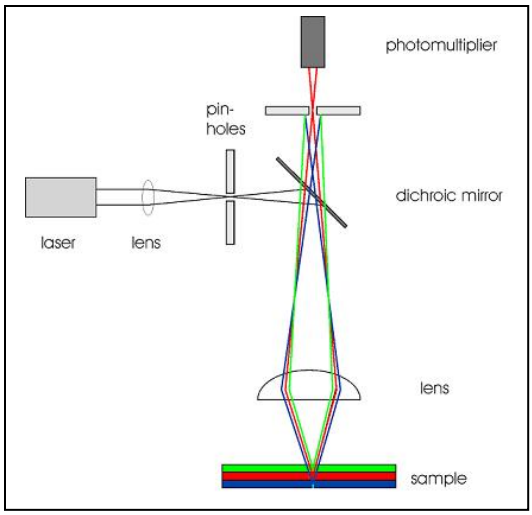
Phase Contrast Microscope: This microscope is designed so that staining is not required to view object because staining usually kills cells. Light that passes through a cell or a cell structure is slowed down relative to the light that passes directly through the less dense surrounding medium. The greater the refractive index of the cell, the greater the retardation of the light wave. Even difficult –to-stain structures often are conspicuous under a phase contrast microscope. The phase contrast microscope optically changes differences in intensity, thereby producing differences in contrast.



Fluorescence Microscope: The fluorescence microscope is based on the phenomenon that certain material emits energy detectable as visible light when irradiated with the light of a specific wavelength. The sample can either be fluorescing in its natural form like chlorophyll and some minerals, or treated with fluorescing chemicals. The basic task of the fluorescence microscope is to let excitation light radiate the specimen and then sort out the much weaker emitted light to make up the image. First, the microscope has a filter that only lets through radiation with the desired wavelength that matches your fluorescing material. The radiation collides with the atoms in your specimen and electrons are excited to a higher energy level. When they relax to a lower level, they emit light. To become visible, the emitted light is separated from the much brighter excitation light in a second filter. Here, the fact that the emitted light is of lower energy and has a longer wavelength is used. The fluorescing areas can be observed in the microscope and shine out against a dark background with high contrast.



Confocal Scanning Microscope: Confocal Laser Scanning Microscopy (CLSM) is an optical microscopy technique that is based on conventional wide-field fluorescence microscopy. The use of modern technology (laser, photomultiplying detectors) in CLSM gives this method a lot of advantages. The laser beam is focussed into the sample and by using electronic lenses and apertures only the fluorescence light that comes directly from the confocal plane is detected by a photomultiplier.



EXPERIMENT I

Use of Light Microscopy to Study Different Cell Types

Materials

- Binocular Microscope
- Microscope slides and coverslips
- Freshly cut onion
- Cotton swabs
- Forceps
- Water

Procedure

1. Pick up a microscope from the cabinet by placing one hand under the base and the other on the arm of the microscope. **Most microscope damage is due to careless transport.** It is important that you carry the microscope securely, with two hands, and in an upright position. Remember that you are handling over $1,000 of precision instrumentation.

2. Place the microscope in front of you, unwind the power cord and plug it in. The microscope is normally provided in its storage position, that is, with its eyepieces pointed back over the arm. This takes less room in a cabinet, but is not the position for which it was designed to be used. If your instructor approves, slightly loosen the screw holding the binocular head and rotate the entire binocular head 180°. Carefully (and gently) tighten the screw to prevent the head from falling off. You will notice that all parts of the microscope are now conveniently located for your use, with an uninterrupted view of the stage, and substage (Fig.1.3). The focus controls are conveniently at arms-length.

3. Note the magnification power and the numerical aperture of the lenses which are on your microscope's nose-piece. These values are stamped or painted onto the barrels of the objectives. Record the magnification power and numerical aperture of each lens in the space provided below.

Magnification (x#)	**Numerical Aperture (NA)**

Using the lowest NA value from above as the working numerical aperture, calculate the limit of resolution for your microscope, assuming violet light with a wavelength of 400 nm.

Resolving power (R) = 0.61λ/ NA

the calculated value for your microscope is:

Limit of resolution = _____ μ

Wet Mounts

1. Add a small drop of water to the center of a clean slide.
2. Cut a fresh onion in half and remove a layer.
3. Using pointed forceps, strip a small piece of epidermis from the concave surface of a layer and place it on the drop of water. Make sure that the onion layer does not fold over on itself!
4. Add a drop of water and a coverslip.
5. Examine the cells under different magnifications.

    a) Draw the image observed.
    b) How many cell layers are there?
    c) What cell parts, if any, are visible?

6. Add a small drop of water to the center of a clean slide.
7. Using a cotton swab, obtain epidermis cells from the inner side of your cheek.
8. Swipe it onto the glass slide.
9. Add a drop of water and a coverslip.
10. Examine the cells under different magnifications.

    a) Draw the image observed.
    b) How many cell layers are there?
    c) What cell parts, if any, are visible?

Phase-Contrast Microscopy

Materials

- Prepared, pre eosin-stained slide
- Phase Contrast Microscope

Procedure

1. Observe cells under bright-field microscopy at 10X. Draw their images.
2. Draw the image of cells viewed under phase contrast. Label organelles which are more clearly visible with phase contrast than with bright field microscopy.
3. Return the phase control on the condenser to the normal bright field setting, switch to a higher magnification (20X or 40X) and observe the cells at the higher magnifications with and without phase enhancement.

Viability Staining

Materials

- glass slides and coverslip
- Microscope
- A culture of fibroblasts
- PBS
- Trypan Blue
- Eppendorf tube
-

Procedure

1. Resuspend cells in 100 µl PBS.
2. In an Eppendorf tube, mix 50 µl PBS with 50 µl Trypan Blue
3. Put 50 µl Trypan Blue:PBS and 50 µl cell suspension on the glass slide.
4. Place a coverslip on them.
5. Examine the cells under various magnifications, and draw the images.