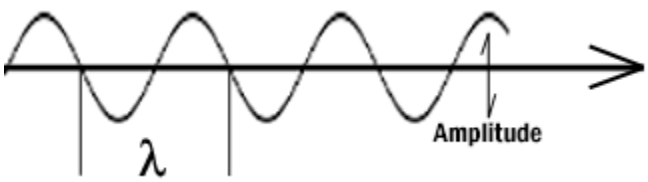
The physics of light microscope:

Light microscopes play an important role in many research laboratories, including electron microscopy facilities. They can be used as a primary visualization tool or in support of electron microscopy. Samples for light microscopy are prepared in an ever-increasing number of techniques, and can range from sliced biological organisms and tissue cultures to materials science and geological samples. Light and electron microscopes share many similarities in their optical principles. Understanding how a light microscope works is not only critical for obtaining optimum light images, but also for understanding electron microscopy.

Principles of Light, Electrons, & Microscopy

In microscopy we take advantage of waveform properties of light. These waves when produced at a particular source vibrate at right angles to the line of propagation. Each wave has a peak and trough. The distance traveled forward by the light ray is one wavelength (lambda). Wavelength varies with the color and intensity of the source.



How the images are formed:

The structures the light microscope is called upon to resolve exert only a small influence on the light they transmit. What is changed is the phase of momentary vibration. Conventional bright field illumination will lack contrast and the details of the sample remain invisible. When the emerging waves have acquired a larger phase difference due to changes in refractive index, greater contrast is produced. This manifests itself by an edge effect (diffraction, refraction, and reflection). Sample details may be resolved in a number of ways. When a light passes through stained structures intensity is reduced selectively depending on the color and density of the sample as the light is absorbed. Selective absorption of wavelengths of white light produces colored light. Refraction changes the direction of a light ray as it passes from one medium to another. The shorter the wavelength, the greater the refractive angle. Diffraction is the bending of light rays around objects with sharp edges. A new wave front is created at this edge. Diffraction can be useful, but can also reduce resolution. When light is dispersed it is separated into its constituent wavelengths as a result of refraction on entering a transparent medium. Contrast can be defined as a steep slope between bright and dark image points. Adequate contrast MUST be achieved before the specimen can be resolved.

Two terms that are often confused, but are central to microscopy are magnification and resolution. Magnification is the degree by which dimensions in an image are, or appear to be, enlarged with respect to the corresponding dimensions in the object. Resolution is the act or result of displaying fine detail in an image. Magnification without resolution would be meaningless.

The theoretical resolution of the light microscope was first defined by Abbe in the following equation.

Abbe's equation for theoretical resolution of the light microscope:

$$d = 1.22 \times \lambda o / n \sin \mu$$

d=distance of resolution
λ=wavelength of incident light
μ= 1/2 angle of incoming light

The actual resolution achievable with a light microscope is not as great. We will discuss the reasons for this later.

It is important to understand and to recognize the various components of the light microscope. The first and perhaps the most important element are the lenses.

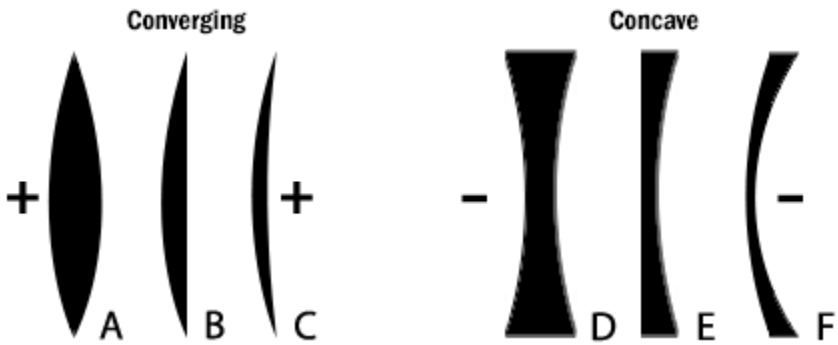


Figure 2: *The six simple lenses. A, B, & C are converging or positive lenses. D, E, & F are concave or negative lenses.*

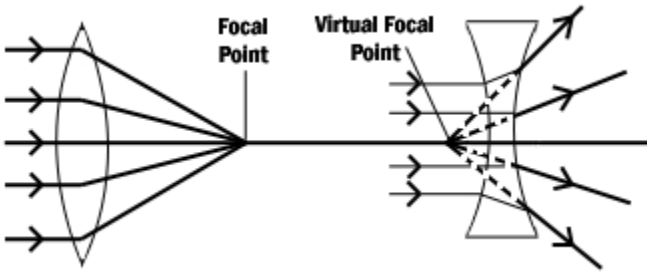


Figure 3: *Converging and diverging lenses. Parallel rays enter a convex lens converge and meet at the principle focus. The distance between the center of the lens and the principal focus is the focal length. Parallel rays entering a concave lens diverge and never meet.*

The Objective Lens is the first part of the imaging system; the objective lens forms a primary, enlarged image of the object. Very fine details are distinguished with the objective lens. The

eyepiece sometimes called the ocular lens is the second lens, which forms a secondary, further enlarged image. By multiplying the magnifying power of the objective lens and the magnifying power of the ocular the final magnification is found. A Substage Condenser lens is the third optical component. It is placed on a platform beneath the object. Light is directed through the substage condenser and converges to a point at the position of the specimen. The light rays diverge as they pass through the specimen and form an inverted cone, whose base is just large enough to fill the aperture of the objective. The size of the light beam is controlled by a diaphragm beneath the condenser called the aperture diaphragm.

The light source should contain both a lens to project an image of the lamp filament called a field condenser and a diaphragm to control the size of the illuminated field called a field diaphragm.

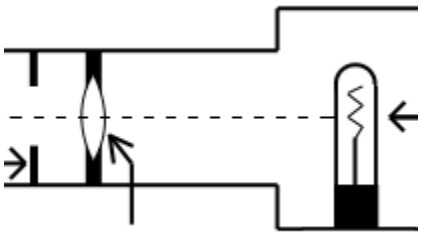
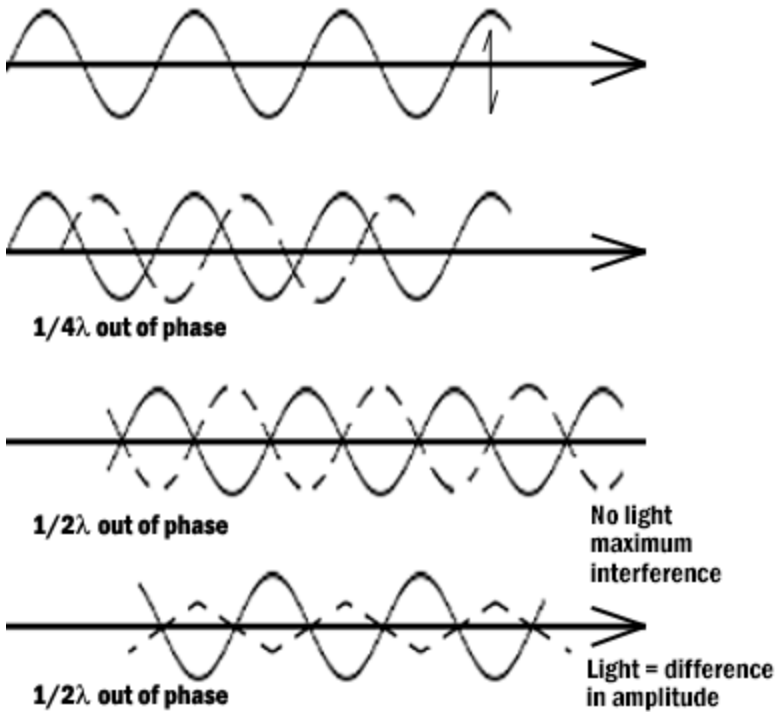


Figure 4: *Typical lamp for light microscope.*
Kohler Illumination is the most common method of illumination. In Kohler illumination the image of the source is projected by the field condenser onto the substage condenser, to the top of the plane of the object. This method assures even illumination.

Modern light microscopes use several different modes of operation depending on the needs of the investigator. The most common of these being bright field microscopy in which direct light passes through the objective aperture and illuminates the background against which the image is seen. Since the structural elements being resolved have little variance in refractive index, the image will lack contrast and the details remain invisible. Small structure detail can be revealed by changing the absorption of the object by means of staining.

Many microscopes have special lenses that allow an investigator to image using phase-contrast. This method reveals details in specimens possessing very slight differences in optical path or refractive index in the surrounding media. Phase contrast requires no staining and can be used with living tissue. Contrast is produced in this method by phase differences in the light leaving the object to amplitude differences in the image. A phase plate alters the optical path length traveled by diffracted light from that traveled by direct light.



lightwave before encountering the object. Wave B represents the wave phase after passage in bright field (unstained mode). C compares the wave phase of an object viewed with phase contrast.

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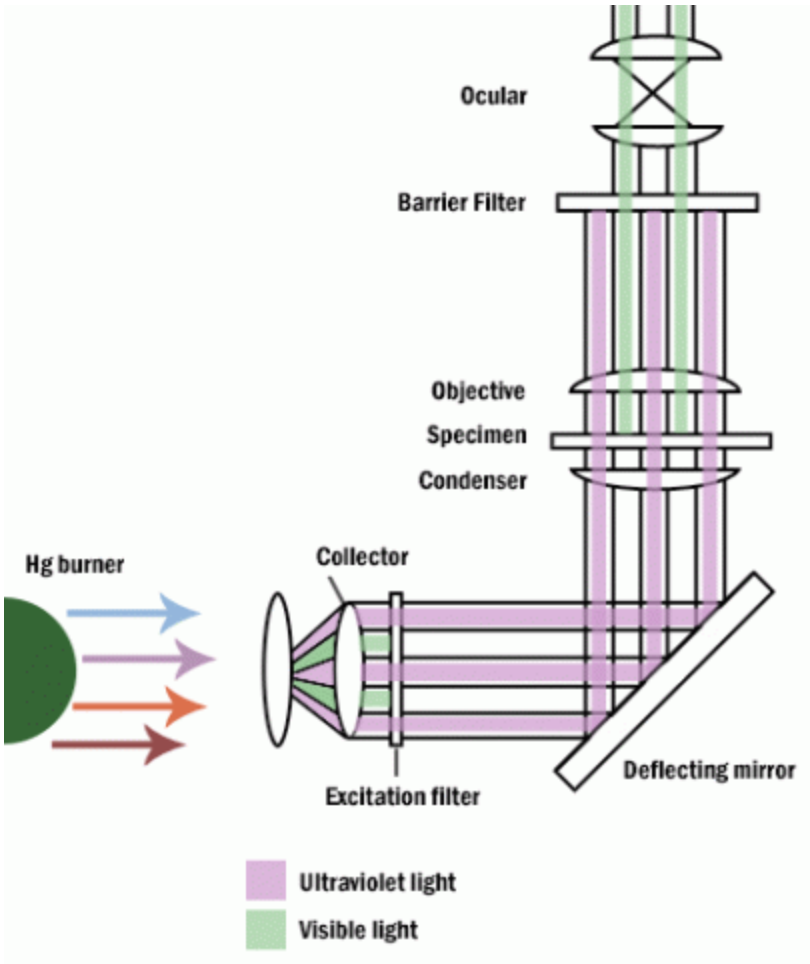
Figure 5: *Differential Interference Contrast Schematic.*

Dark field microscopy is a mode in which direct light is prevented from passing through the objective aperture, but a hollow cone of light forms an apex in the plane of the specimen. The image is formed by light scattering from features of the object. Details appear bright white against a dark background. Darkfield can also be used for living bacteria, algae and plankton.

Some materials produce light when excited by short wavelengths of radiation. This effect is called fluorescence or auto-fluorescence. Specimens that do not fluoresce by themselves may be treated with fluorochromes which produce a secondary florescence. By illuminating with a high intensity mercury or xenon source and filtering out all but the desired excitation wave length to contact the specimen, the resulting longer (less energetic) wavelengths of emission from the specimen its self viewed. Fluorescence microscopy can be used to enhance particular organelles, immunocytochemistry, in-situ hybridization, enzyme cytochemistry and elemental localization.



2.) **Principle of Centrifugation:**

- In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it floats to the top.
- The greater the difference in density, the faster they move. If there is no difference in density (isopycnic conditions), the particles stay steady.
- To take advantage of even tiny differences in density to separate various particles in a solution, gravity can be replaced with the much more powerful “centrifugal force” provided by a centrifuge.

- A centrifuge is a piece of equipment that puts an object in rotation around a fixed axis (spins it in a circle), applying a potentially strong force perpendicular to the axis of spin (outward).
- The centrifuge works using the sedimentation principle, where the centripetal acceleration causes denser substances and particles to move outward in the radial direction.
- At the same time, objects that are less dense are displaced and move to the center.
- In a laboratory centrifuge that uses sample tubes, the radial acceleration causes denser particles to settle to the bottom of the tube, while low- density substances rise to the top.

Brands of Centrifuge: High-Speed micro centrifuge
Bench top Centrifuge with swing buckets, 4000rpm
Allegra 64R Refigerated Bench top Centrifuges
Allegra X-30 Benchtop Centrifuge
Allegra X-30R Refigerated Bench top Centrifuge

Maintenance and care of centrifuge:

- Always place the centrifuge on a flat surface first.
- Always unplug the power cord before cleaning.
- Emergency phone numbers and procedures should be posted and kept up to date.
- Wear disposable gloves.
- Follow your facility's safety procedures when cleaning and disinfecting the centrifuge.
- Before moving the centrifuge to a new location, the exterior and interior surfaces should be cleaned and disinfected.
- Plug in centrifuge only when completely dry.

There should be schedule of regular preventive maintenance with a trained bio-medical engineer because this will increase the durability and functionability of the centrifuge. Regular preventive maintenance include: inspection of the physical condition, electrical condition, cleaning and testing of the centrifuge

b.) **Principle of Automatic tissue processor:**

Most ATPMs are easy-to-program interface. The Leica processor model has ten 1.8L (60.9oz.) reagent beakers and two 1.8L (60.9oz.) wax baths.
The tissue basket oscillates up and down in each station at three-second intervals to ensure thorough and even mixing of the reagents and optimum tissue infiltration. Infiltration time is separately programmable for each station. Up to nine programs may

be run with immediate or delayed starting times.
When it's time for tissue to be transferred to the next beaker or jar, the cover of the machine is raised up, and the lifting mechanism carefully removes the tissue basket and gently transfers it to the next beaker.

Brands of Automatic tissue processor: Automatic Sample preparation system(SLEE MEDICAL)

Automatic Sample preparation system(Bio optical)

Maintenance and Care of Automatic Tissue processor: Any spillage or overflow should be cleaned immediately

Accumulation of wax on any surface should be removed

The temperature of the paraffin wax bath should be set above the melting point of the wax

Timings should be checked be when placing the cassettes in the processor

Temperature of all paraffin wax dispensers

Flotation water baths and automated processors are carefully monitored.

c.) **Principle of Microtome:** is a sectioning instrument that allows the cutting of extremely thin slices of a material known as section . **Microtome** are used in microscopy, allowing for the preparation of sample for observation under transmitted light or electrons radiation

Brands of Microtome:

Maintenance and care of microtome: Keep the edge of your knife clean at all times

Store the knife in its case to prevent oxidation from occuring