Lesson: Principles of Microscopy: Light Microscopy and Phase Contrast Microscopy

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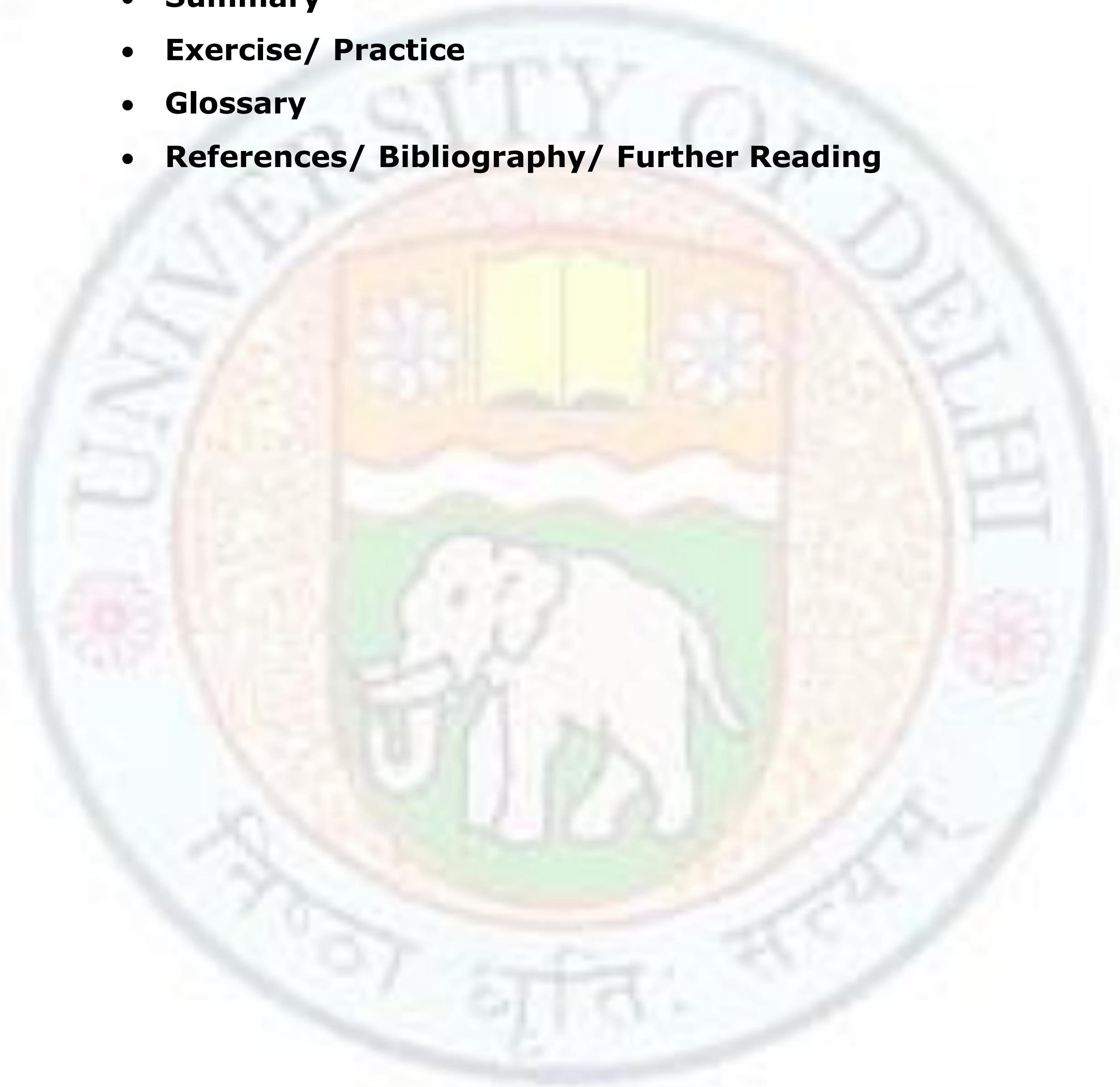


Introduction

Microscopes constitute the very basic requirement for cell biologists, thereby facilitating the deciphering the fine details of intracellular components. The microscope is an obligatory tool for this purpose because most cellular structures are too small to be seen by the unaided eye. The beginnings of cell biology can be traced to the invention of the light microscope, which made it possible for scientists to examine enlarged images of the cells and thus to analyze cellular structure and its dynamics. The first light microscope was developed in 1590 by Z. Janssen and H. Janssen. During the next century, many microscopic observations were reported, notably those of Robert Hooke (who observed the first cells) and Antonie van Leeuwenhoek (who provided first glimpse of internal cell structure through improved microscopes). Since then, the light microscopes have undergone numerous improvements and modifications till the present time. Light microscopy has experienced a renaissance in recent years through specialized technological improvements that allow researchers to explore aspects of cell structure and behaviour. Most images produced by microscopes are now recorded electronically using digital imaging techniques, like digital cameras, digital image acquisition software, digital printing and digital display methods. Additionally, vast improvements have been made in the biological aspects of specimen preparation. These advancements have fostered many more applications of the microscope in biomedical research. These advances have involved the merging of technologies from physics, engineering, chemistry and molecular biology, that have greatly expanded the ability to study cells using light microscope. Biochemical analyses is frequently accompanied by microscopic examination of tissue, cell or organelle preparations. Such examinations are used in many different applications, for example:

1. to evaluate the integrity of samples during an experiment;
2. to map the fine details of the spatial distribution of macromolecules within cells;
3. to directly measure biochemical events within the living tissues.

There are fundamentally two different types of microscopes:

- the light microscope and
- the electron microscope.

Light microscopes use a series of glass lenses to focus light in order to form an image whereas electron microscopes use electromagnetic lenses to focus a beam of electrons.

Light microscopes are able to magnify to a maximum of approximately 1500 times whereas electron microscopes to a maximum of approximately 200000 times. Standard light microscopes have a lateral resolution limit of about 0.5 micrometers for routine analysis. In contrast, electron microscopes have a lateral resolution of up to 1 nanometer. Both living and dead specimens are viewed with a light microscope, and often in real colour, whereas only dead ones are viewed with an electron microscope and never in real colour.

Applications of the microscope in biomedical research may be relatively simple and routine, for example, a quick check of the status of a preparation or the health of cells growing in a plastic dish in the tissue culture. The application may be more involved, for example, measuring the concentration of calcium in a living embryo over a millisecond timescale through a more advanced light microscope (often called an imaging system). Some microscopes are more suited to specific applications than others but there may be constraints imposed by the specimen. Images may be required from specimens of vastly different sizes and magnifications; for example, for imaging whole animals (metres), through tissues and embryos (micrometres) and down to cells, proteins and DNA (nm). The study of living cells may also require time resolution from days (like when imaging neuronal development or disease processes) to milliseconds (like when imaging cell signalling events).

Principles of microscopy

The principles of microscopy need to be examined through special emphasis on the factors that determine how small an object can be observed and analyzed with current technologies.

Illuminating wavelength

Three elements are always needed to form an image, regardless of the type of microscope being used:

- a source of illumination
- a specimen to be examined
- a system of lenses that focuses the illumination on the specimen and forms the image.

The source of illumination in a light microscope is visible light (in the wavelength range of 400-700 nm) and the lens system consists of a series of glass lenses. The image can either be viewed directly through the eye piece or focused on a detector, such as photographic film or an electronic camera. In an electron microscope, the illumination source is a beam of electrons emitted by a heated tungsten filament, and the lens system consists of a series of electromagnets. Despite these differences in illumination source and instrument design, both types of microscopes depend on the same principle of optics and form images in a similar manner. When a specimen is placed in the path of light or electron beam, physical characteristics of beam are changed in a way that creates an image that can be interpreted by the human eye or recorded on a photographic detector. To have a better understanding of illumination source and specimen, the concept of wavelength needs to be understood. The ability of an object to perturb a wave's motion depends significantly on the size of the object in relation to the wavelength of the motion. This principle is of great importance in microscopy because the wavelength of the illumination source sets a limit on how small an object be seen. When a beam of light (or electrons) encounters a specimen, the specimen alters the physical characteristics of illuminating beam. Since an object can be detected only by its effect on the wave, the wavelength must be comparable in size to the object that is to be detected. As we understand this relationship between wavelength and object size, one can readily appreciate that why small objects can be seen only by electron microscopy. The wavelength of electrons is very much shorter than those of photons and thus, objects such as viruses and ribosomes are too small to perturb the waveform of photons, but can readily interact with electrons. (Web link 1)

Resolution

The image formed when waves of light or electrons pass through a lens and are focused, results from a property of waves called interference. Thus, the image seen when you look at a specimen through a series of lenses, is just a pattern of either additive or cancelling interference of the waves that went through the lenses (a pattern called diffraction). A light microscope uses glass lenses to direct photons, whereas an electron microscope uses electromagnets as lenses to direct electrons. However, both kinds of microscopes have two fundamental properties in common:

- focal length
- angular aperture

The focal length is determined by

- The index of the refraction of the lens itself
- The medium in which it is immersed
- The geometry of the lens

The magnifying strength of a lens (measured in dioptres) is the inverse of focal length (measured in meters). The angular aperture refers to a measure of how much of the illumination that leaves the specimen actually passes through the lens. This, thus determines the sharpness of the interference pattern and, therefore, the ability of the lens to convey information about the specimen. The angular aperture is about 70° in the best light microscopes.

Resolution, and not magnification, is a more reliable estimate of the utility of a microscope. Three factors influence a microscope's resolution:

- The wavelength of light used to illuminate the specimen
- The angular aperture
- The refractive index of the medium surrounding the specimen

The effect of these variables on resolution is described quantitatively through Abbé equation:

$$r = (0.61\ \lambda) / (n \sin \alpha)$$

where r = resolution

λ = wavelength of light being used to illuminate the specimen

n = refractive index of the medium between the specimen and the objective lens of the microscope

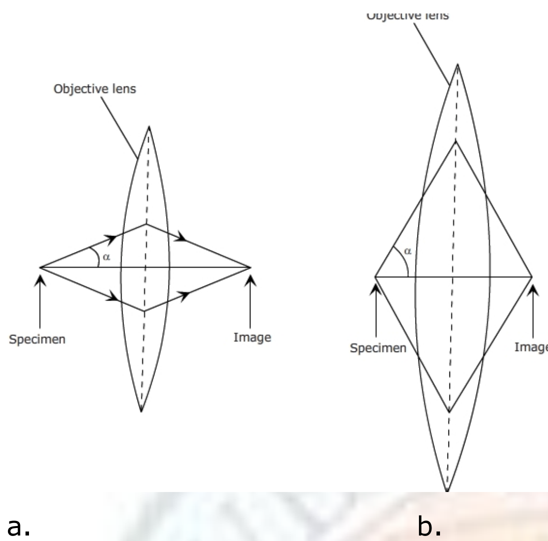
α = angular aperture

The constant 0.61 implies the degree to which image points can overlap and still be identified as separate points by an observer.

Alternatively, the above equation can also be written as:

$$r = (0.61\ \lambda) / NA$$

where NA refers to the quantity ($n \sin \alpha$) and is called as numerical aperture of the objective lens.



a. b.

Figure: The angular aperture of a lens is the half-angle ɑ of the cone of light entering the objective lens of the microscope from the specimen. A low aperture lens (a) and a high aperture lens (b) is shown.

Source: Author

NA is a measure of the ability of a lens to collect light from the specimen. It is usually better to choose the lens with higher NA if there is a choice between lenses of same magnification.

Practical limit of resolution is roughly 200 nm for light microscopy

An important goal in both light and electron microscopy is to maximize resolution. Resolution improves as r becomes smaller because r is a measure of how close two points can be and still be distinguished from each other. Therefore, for best resolution, the numerator in the above equation should be as small as possible and the denominator should be as large as possible. If we consider a glass lens that uses visible light as an illumination source, the minimum value for wavelength is set by the shortest wavelength in the range of visible light (i.e. 400-700 nm) that is practical to use for illumination that turns out to be blue light of approximately 450 nm. This would make the numerator as small as possible. In order to maximize the denominator, both the values of refractive index and sine of the angular aperture must be maximized to achieve optimal resolution. The maximum value for sine ɑ is about 0.94 (because the angular aperture for the best objective lenses is approximately 70°). The refractive index of air is 1. Thus, resolution of a sample illuminated in air with blue light of 450 nm can be calculated as follows:

$$r = (0.61\ \lambda)/\ NA$$
$$= (0.61)\ (450)/\ (1)\ (0.94)$$
$$= 292\ nm$$

Limit of resolution is thus, 300 nm for a glass lens in air.

In order to increase the numerical aperture, some microscopic lenses have been designed to be used with a layer of immersion oil between the lens and the specimen. Immersion oil (higher refractive index than air) allows the lens to receive more of the light transmitted through the specimen. Since the refractive index of immersion oil is about 1.5, the maximum NA for an oil immersion lens is about (1.5) X (0.94) = 1.4. Resolution of an oil immersion lens is approximately 200 nm. In actual practice, such limits of resolution (best possible resolution for a microscope) can rarely be reached because of aberrations in the lenses. The resolution can be further enhanced by using ultraviolet rays as an illumination source. Because the wavelength of UV rays is shorter (200-300 nm), the resolution can be enhanced to approximately 100 nm. However, when using ultraviolet light, special cameras (because UV rays are invisible to the human eye) and expensive quartz lenses (because ordinary glass is opaque to UV rays) must be used.

The limit of resolution sets an upper boundary on useful magnification that is possible with any given lens. Generally, the greatest useful magnification that can be achieved with a light microscope is approximately 1000 times the numerical aperture of the lens being used. Since NA ranges from approximately 1.0 to 1.4, the useful magnification of a light microscope is limited to roughly 1000X in air and 1400X with oil immersion lens. The magnification for each objective lens can be increased above a point where it is impossible to resolve any more detail in the subject. Any magnification above this point is called empty magnification. The best way is to use a higher magnification and higher NA objective lens.

In order to achieve better magnification in an effective way it is necessary to switch from visible light to electron beam as the illumination source. The theoretical limit of resolution of the electron microscope (0.002 nm) is orders of magnitude better than that of light microscope (200 nm) because the wavelength of an electron is approximately 100,000 times shorter than that of a visible light. However, practical problems in the design of electromagnetic lenses (to focus the electron beam) prevent the electron microscope from

achieving this theoretical potential. Electromagnets produce considerable distortion when the angular aperture is more than a few tenths of a degree. This angle is of several orders of magnitude less than that of a good glass lens (about 70°), providing a numerical aperture considerably smaller than that of a light microscope. The limit of resolution for an optimal electron microscope is therefore, only approximately 0.2 nm, far from the theoretical limit of 0.002 nm. Practical limit of resolution is often closer to 2 nm when viewing biological samples because of the problems with sample preparation and contrast. Resolution in an electron microscope is generally about 100 times better than that of light microscope. The useful magnification of an electron microscope is approximately 100 times than that of a light microscope or approximately 100,000X. (Web link 3)

Light microscopy

Light microscopy can be considered under the following aspects:

Magnifying lens

The simplest form of light microscope is a magnifying lens which consists of a glass lens mounted in a metal frame. The specimen hardly requires any sample preparation and is usually held close to the eye. Focusing of the region of interest is achieved by moving the lens and specimen relative to one another. The source of light is usually the sun or indoor light. The detector is human eye and the recording device is a hand drawing.

Bright field microscopy

The most basic mode of light microscope is called brightfield (bright background) which can be achieved with the minimum optical elements. All light microscopes include a system of three glass lenses:

- The condenser lens that focuses light from the source onto the specimen
- The objective lenses which magnify the specimen to different degrees
- The eyepiece lenses which further magnify the image.

Upon comparison with other microscopes, these are inexpensive and simple to align and use. Contrast in images obtained from such microscopes is usually produced by the colour

of the object itself or by exploiting some other property that affects the amount of light that can pass through. This kind of microscope is, therefore, used to collect images from pigmented tissues or histological sections, or from tissue culture cells that have been stained with dyes.

Major components of all modern light microscopes include a condenser lens, the objective lens and the eyepiece lens.

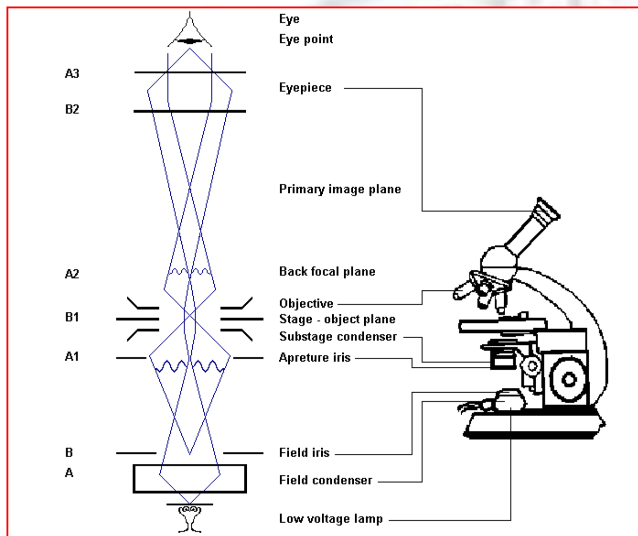


Figure: The path taken by light rays that form the image of the specimen and those that form the background light of the field. Light from a lamp is focussed at the specimen by a glass condenser lens. The image is magnified with an objective lens and projected onto a detector with an eyepiece lens. The detector can be the eye or a digital camera.

Source: Author

Each of these components is, in turn, made up of a combination of lenses. This is necessary to produce magnified images with reduced artefacts and aberrations. Problems, such as chromatic aberration, were encountered in the early microscopes of Antonie van Leeuwenhoek and Hooke. All modern lenses are now corrected to some extent to avoid such problems.
The main components of the compound light microscope include:

Light source

A proper illumination of the specimen is important for achieving high-quality images and photomicrographs and this is achieved using a light source. Light sources typically include mercury lamps, xenon lamps, lasers or light-emitting diodes (LEDs). Light from the light

source passes into the condenser lens (mounted beneath the microscopic stage in an upright microscope and above the stage in an inverted microscope) in a bracket that can be raised and lowered for focusing. The specimen is illuminated with parallel beams of light through the condenser focused light from the light source. Correct position of condenser lens produces illumination that is uniformly bright and free from glare across the viewing area of the specimen.

Light that passes through the specimen (transmitted light) or is reflected back from the specimen (reflected light) is focused by the objective lens into the eyepiece lens. The image is either directly viewed by the eye in the eyepiece or often projected onto a detector (like photographic film or more likely a digital camera). The images are stored in a digital format and reproduced using digital methods.

Condenser misalignment and an improperly adjusted condenser aperture diaphragm contribute to major sources of poor images in a light microscope.

Stand

This part of microscope holds all the components firmly in position. There are two basic types of microscope stand: an upright or an inverted one. The light source is below the condenser lens in the upright microscope and the objectives are above the specimen stage and this is the most commonly used format for viewing specimens. However, in an inverted microscope, the light source and the condenser lens are above the specimen stage and the objective lenses are beneath it. The condenser and light source can often be swung out of the light path and this also allows additional room for manipulating the specimen directly on the stage.

Specimen stage

A mechanical device that is finely engineered to hold the specimen firmly in place. It enables the specimen to be moved and positioned in fine and smooth increments, both horizontally and transversely, in the X and Y directions (for locating a region of interest). The stage is moved vertically in the Z direction for focusing the specimen unlike in inverted microscopes where the objectives are moved and the stage is fixed. There are usually fine and coarse focusing controls for high and low magnification viewing respectively. The fine focus control can be moved in increments of up to 1 micrometer in the best research microscopes. The specimen stage can either be moved by a hand or a stepper motor (attached to the fine focus control of the microscope) controlled by a computer.

Objective lens

This component is responsible for producing the magnified image and can be the most expensive component of the microscope. Objectives are available in many different varieties and with much information inscribed on each one. This information can include manufacturer, magnification (4X, 10X, 20X, 40X, 60X and 100 X), immersion requirements (air, water or oil), coverslip thickness and more specialized optical properties of the lens. In fact, lens correction for optical artefacts, such as chromatic aberration, may also be included in the lens description. Like the words Fluo st (for fluorite in cases of least correction) and plan or plan apo (for plan apochromat in case of most highly corrected) might also appear on the lens surface.

Objective lenses can either be dry (glass/air/coverslip) or immersion lenses (glass/oil or water/coverslip). Generally, most objectives below 40X are air (dry) and those of 40X and above are immersion (oil, glycerol or water) based. An objective designed to operate in oil will be labelled as oil or oel. Other immersion media include glycerol and water and the lens will be marked accordingly. Dipping lenses are specially designed to work without a coverslip and are dipped directly into water or tissue culture medium (like used in case of physiological experiments).

There is even a mark for numerical aperture on the lens (a number usually between 0.04 and 1.4). Lenses with a low NA collect less light than those with a high NA. High power objectives with a higher NA have a better resolution than the low power lenses.

The objective lens is most prone to be damaged by mishandling although many lenses are coated by a protective coating. Even a scratch in front of lens can lead to serious image degradation.

Eyepiece

It works in combination with the objective lens to further magnify the image and allows it to be detected by eye, or usually to project the image into a digital camera for recording purposes. Eyepieces usually magnify by 10X since an eyepiece of higher magnification merely enlarges the image without any improvement in resolution. There is an upper limit to the useful magnification of the collection of lenses in a microscope.

Stereomicroscopes

Another type of light microscope, referred to as stereomicroscope is used for the observation of surfaces of large specimens. Stereomicroscope is used when 3D information is required, like for screening through vials of fruit flies. These microscopes are useful for micromanipulation and dissection where the ability of wide field of view and ability to zoom in and out in magnification is insignificant. Quite a wide range of objectives and eyepieces are available for different applications. The light sources can be from above or from below the specimen, encircling the specimen using a ring light, or from the side, giving a darkfield effect. These different light angles help to add contrast or shadow relief to the images.

Many configurations of the light microscope have been added over the years, specifically to add contrast to the final image. Improved contrast with transparent cells can be gained by closing to an extent, the condenser diaphragm on a bright-field microscope, but excessive closure has the deleterious consequence of effectively reducing the NA of the lens system and thereby the resolution. To avoid all this, research microscopes can be converted to phase contrast optics. This allows the detection of different components of cells by virtue of slight variations in their refractive indices. This method is straightforward, and specimens mounted in their appropriate media can be observed directly. Invariably, this allows visualization of not only the outline of the cell, but also of many of its organelles and their arrangements. It is used for examining living, unstained cells because biological materials almost inevitably diffract light.

Darkfield illumination produces images of brightly illuminated objects on a dark (black) background. This technique has conventionally been used for viewing the object outlines in liquid media, such as for a quick check of the status of a biochemical preparation or for cells growing in a tissue culture medium. For lower magnifications, a simple darkfield setting on the condenser is sufficient. However, for higher magnifications (for more critical darkfield imaging), a darkfield condenser along with a darkfield objective lens is required.

Phase contrast microscopy

Principle

For examining cells through brightfield microscopy, cells are often killed, sliced into thin sections and stained. Although such procedures are useful for examining the details of cell's internal architecture, however, with an increased emphasis on the study of cell dynamics (especially in the fields of motility, division, and cell death), techniques that allow visualization of intact or still living cells have become increasingly important. This improves contrast (without sectioning and staining) by exploiting differences in the thickness and refractive index of various regions of the cells.

Phase contrast microscopy was introduced by the Nobel laureate Frits Zernike. It is a form of interferometry whereby contrast is derived from refractive elements within the sample, which cause phase shifts in light that are subsequently converted into amplitude changes which are registered as light or dark regions against a uniform reference. Cell organelles are made up of different proportions of various molecules like DNA, RNA, protein, lipid, carbohydrate, salts and water. Regions of different composition have different refractive indices. As the rays pass from the light source through the object, their velocity might be affected by the physical property of the specimen. Along its route, the velocity of the rays slows down to varying extent upon encountering different regions of the specimen. Light passing through the thicker parts of the cell is held up relative to the light that passes through thinner parts of the cytoplasm, resulting in a change in phase relative to light waves that have not passed through the specimen. Although such changes in phase are not detected by the human eye, phase contrast microscope overcomes this problem by converting differences in refractive indices into differences in intensity (relative brightness and darkness), which are visible to the eye. Phase contrast microscope accomplishes this result by: 1. separating the direct light that enters the objective lens from the diffracted light emanating from the specimen and 2. by causing these two types of light rays to interfere with one another, producing an image with highly contrasting bright and dark areas against a uniformly illuminated background. The relative brightness or darkness of each part of the image reflects the way in which light from that part of the specimen interferes with the direct light. On an average, light passing through the transparent specimens is retarded by approximately ¼ wavelength and the direct, undiffracted light passes through a portion of the phase plate that speeds it up by approximately ¼ wavelength.

Setting up a microscope for phase contrast

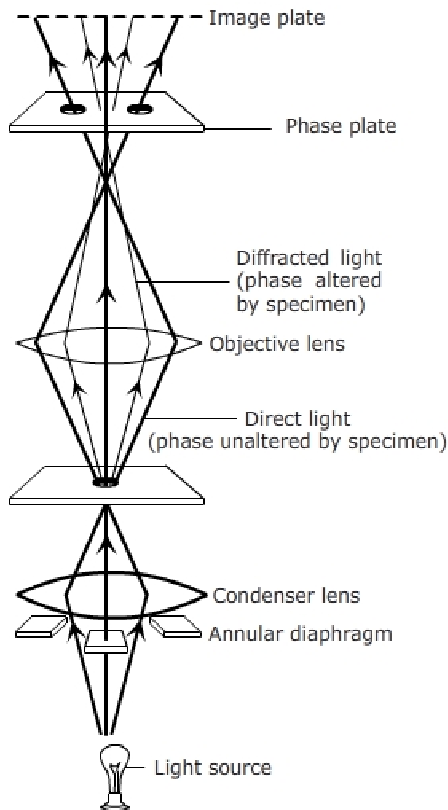


Figure: Configuration of the optical elements and the paths of light rays through the phase-contrast microscope.

Source: Author

Setting up a phase contrast microscope is straightforward, provided the microscope is equipped with phase lenses and a corresponding phase ring (annulus) in the condenser. This conversion is accomplished using a phase plate. The phase lenses contain a phase plate that works in tandem with the phase annulus to retard direct light (which does not pass through the specimen) by ¼ of a wavelength. Light that passes through the specimen will be diffracted by organelles, cell margins, etc., and this light is retarded in phase. Background light, and light that has passed through the specimen, interact at the image plane causing wave interference with objects appearing bright against a uniform background. This microscope consists of a specialized phase condenser and phase objective lenses (labelled as 'ph'). Each phase setting of the condenser lens is in line with the phase setting of the objective lens. These are usually numbered as phase 1, phase 2 and phase 3 and are visible on both the objective and condenser lens. This, thus, results in a better

visualization of the internal structure of cells through phase contrast microscopy than with bright field optics. (Web link 4)

Applications

Phase contrast microscope is most useful for examining intracellular components of living cells at relatively high resolution. It is widely used in microbiology and tissue culture research to detect bacteria, cellular organelles or for testing cell and organelle preparations for lysis. Also, the dynamic motility of mitochondria, mitotic chromosomes, and vacuoles can be followed and filmed using this optics. The greatest benefit derived from its invention has not been in the discovery of new structures, but rather its everyday use in research and teaching laboratories for observing cells in a more revealing way.

This microscope is only suitable for observing single cells or thin cell layers. Additionally, it has optical handicaps that result in loss of resolution, and the image suffers from interfering halos and shading where sharp changes in refractive index occur. Cells and their components appear to be surrounded by a white halo caused by interference fringes. This prevents one from observing distinct edges on the specimen. This restricts the ability to identify and distinguish between organelles within a typical flattened cell. Therefore, many researchers focus on other types of microscopes, like differential interference contrast (DIC) microscope that produces images with a shadow relief. (Web link 5).

Polarized Light Microscopy

Light and other forms of electromagnetic radiation normally vibrate in all directions. A polarizing microscope utilizes polarizing filters, which are able to select light that vibrates only in one plane. By using these filters, information is provided about the cellular structures that are composed of well-aligned elements, such as microtubules or microfilaments (normally below the limit of resolution of a light microscope). This is possible because of the optical property of such structures, referred to as birefringence. An object manifests birefringence by appearing bright when positioned between two polarizing filters oriented with their particular plane of transmission at right angle to one another. Light passing through the first filter becomes plane polarized (i.e. the light waves vibrate in only one direction) and in the absence of a birefringent specimen, none of the plane polarized light rays can pass through the second filter, causing the field to appear black. However, if

the specimen contains oriented elements that rotate plane polarized light, the object appears bright against a black background. Birefringent cell structures include myosin filaments, chloroplasts, plant cell walls and various types of crystalline inclusions

Other contrast enhancement methods, like Hoffman modulation contrast (named after its discoverer Robert Hoffman) increase contrast by detecting optical gradients across a transparent specimen using special filters and a rotating polarizer. This results in a shadow-casting effect similar to that in Differential Interference Contrast microscopy. (Web link 6 and 7)

Other most widely used contrast techniques include fluorescence microscopy since it gives superior signal-to-noise ratios for many applications. This would be discussed in the next chapter.

Sample preparation for Light microscopy

One striking feature of using light microscopy is how easily most specimens can be arranged for examination. Specimens to be observed with the light microscope are broadly divided into two categories: whole mounts and sections.

Whole mount

A whole mount is an intact object, either living or dead, and can consist of an entire microscopic organism or a dissected organ. To collect images from the microscope, the specimen must be in a form that is compatible with the microscope. As long as the sample is sufficiently transparent, it can be viewed with the transmitted light. Quite often, opaque objects can be made translucent by substituting water with alcohol, and immersing the object in solvents, such as toluene or xylene, in which they become clear. In some cases, sample preparation involves mounting a small piece of the specimen in a mounting medium (water, tissue culture medium or glycerol) on a glass slide and covering it with a glass coverslip. The slide is then positioned on the specimen stage of the microscope and examined through the ocular lens, or camera. To take maximum advantage of the resolving power of the light microscope, specimens are usually prepared in a way designed to enhance contrast (difference in the darkness or color of the structures being examined). A

regular means of enhancing contrast is to apply particular dyes that color or otherwise adjust the light transmitting properties of cell constituents.

Sections

Many specimens are too opaque for microscopic analysis unless examined as a very thin slice or section, using a device called microtome. Sample preparation involves:

Fixation

Tissues are first often treated with fixatives that kill the cells while maintaining the structural integrity of the cell. The most widely employed fixatives are acids and aldehydes such as acetic acid, picric acid, formaldehyde and gluteraldehyde. Aldehydes act by cross linking proteins while, alcohols act by precipitation. A good fixative rapidly penetrates the cell membrane and immobilizes all of its macromolecular structures in such a way that the structure of the cell is maintained as close as possible to that of the living state. One way of fixing tissues is to plunge them in the fixative solution. An alternative approach for animal tissues is a technique known as perfusion (i.e. to inject the fixative into the bloodstream of the animal before removing the organs). After fixation, the specimen is usually permeabilised to allow a stain to infiltrate the entire tissue. The amount of permeabilisation (time and severity) depends on several factors, like the size of the stain or the density of the tissue. The goal is to infiltrate the intact tissue with a consistent staining.

Embedding

After fixation, the tissue is dehydrated through a series of alcohols (because paraffin is insoluble in water, therefore, any water in the specimen must first be removed) and then embedded in a medium such as plastic or paraffin wax that provides mechanical support during sectioning. Paraffin is mostly used as an embedding medium because it is readily dissolved by organic solvents. Dehydration is less critical if the specimen is embedded in a water soluble medium instead of paraffin.

The processed tissue is usually placed in warm, liquefied paraffin and allowed to harden. The wax, which both surrounds the tissue and infiltrates it, hardens upon cooling, therby supporting the tissue externally and internally. The resulting solid paraffin block is then trimmed to the appropriate shape before being sectioned. Specimens may also be embedded in epoxy plastic resin or tissue can be simply frozen (an alternative way of providing support).

Sectioning

The specimen is then mounted in a block of wax and cut with the knife of a device called microtome into thin sections (thickness ranging from 100 μm – 500 μm) .The specimen is

thus sliced into sections that are thin enough to transmit light. The specimen is basically mounted on the arm of microtome, which advances the specimen by small increments toward a metal or glass blade that slices the tissue into thin sections. The microtome knives are usually constructed of a polished steel for light microscopy.

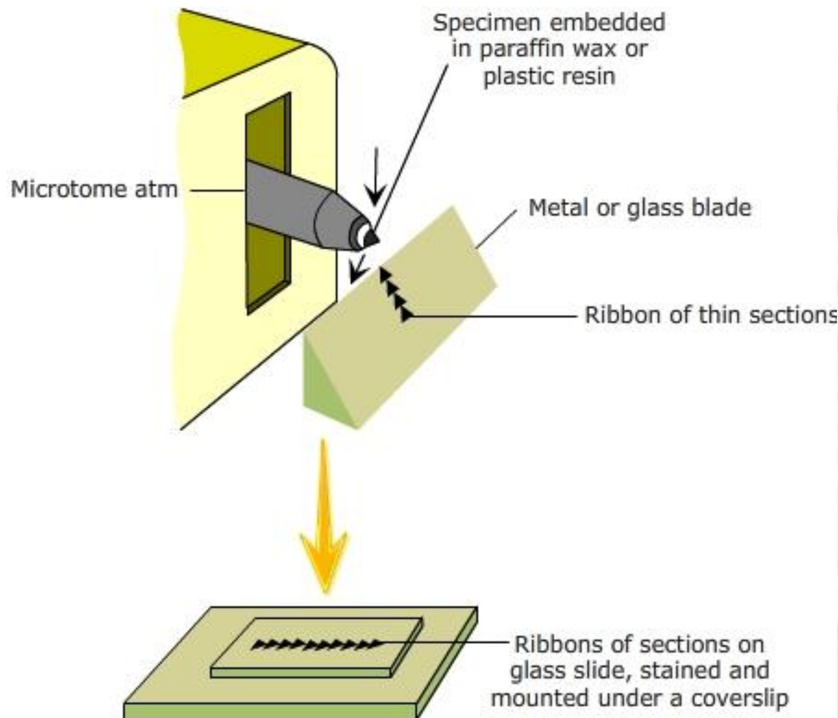


Figure: Sectioning through a microtome. The fixed specimen is embedded in paraffin wax or plastic resin and mounted on the arm of a microtome. The blade cuts successive sections as the arm moves up and down through a circular arc. A ribbon of thin sections (sections adhered to one another) is formed that can be mounted on a glass slide, stained and protected with a coverslip.
Source: Author

Some samples are frozen, and cut on a cryostat. Frozen sections are more suitable for immunolabelling. The sections are then mounted on a glass slide. Slides containing adherent paraffin sections are immersed in toluene, which dissolves the wax, leaving the thin slice of tissue attached to the slide.

Staining

The sections are then subjected to staining with any of the dyes or antibodies adapted for this purpose or enzymes. Quite often, a series of treatments are applied, each with an

affinity for a different kind of cellular component. After staining, a glass coverslip is mounted over the tissue using a mounting medium that has the same refractive index as the glass slide and coverslip.

Summary

- Transmitted light microscopy has undergone a renaissance during the later years of the 20th century and early stages of 21st century.
- Bright-field microscopy is the usual form utilizing the compound light microscope.
- Most living imaging subjects, however, do not provide inherent colour or contrast and, therefore, are not well defined by bright-field microscopy.
- Subsequently, procedures that improve the contrast of transmitted light images of living samples are fundamental for active study of cellular processes.
- Methods such as phase contrast or differential contrast can help in attaining contrast-enhanced transmitted light images to satisfy certain experimental objectives, while less commonly used techniques, such as dark-field or polarized microscopy, are useful for more specialized applications.

Exercises

1. What would be the effect on resolution of a microscope if the numerical aperture of a lens is (a) increased (b) decreased?
2. Highlight the significance of phase contrast microscope over light microscope.
3. What can be done to increase the numerical aperture of a lens?
4. Which microscope can be used to directly monitor live, unstained specimens?

Glossary

Angular aperture: Half angle α of cone of light entering the objective lens of microscope from the specimen.

Birefringence: The resolution or splitting of a light wave into two beams, each refracted at a different angle, and each polarized at a right angle to the other by an optically anisotropic medium such as calcite or quartz.

Chromatic aberration: Aberration caused due to failure of a lens to focus all colours of the spectrum to the same convergence point (i.e. due to the differences in refraction of the colored rays of the spectrum) resulting in rainbow of colours around the edges of the objects in the image.

Cryostat: A microtome used for cutting thin slices of biological specimens at constant low temperature.

Focal length: Distance between the midline of lens and the point at which rays passing through the lens converge to a focus.

Interference: It refers to the process by which two or more waves combine to reinforce or cancel one another, producing a wave equal to the sum of the two combining waves.

Magnification: Amount or degree to which the observed object is enlarged. It is measured by multiples such as 20X, 40X indicating that the object is enlarged 20 times, 40 times respectively.

Microtome: A special instrument for cutting a specimen (as of biological tissues) into thin sections for later examination by light microscope or electron microscope.

Phase plate: An optical material inserted into the light path above the objective lens in a phase contrast microscope.

Refractive index: Measure of the bending of a ray of light when passing from one medium to another. It is the ratio of sine of angle of incidence to the angle of refraction.

Resolution: The minimum distance that can separate two points that still remain identifiable as separate points when viewed through microscope.

Wavelength: The distance between successive crests of a wave, especially points in a sound wave or electromagnetic wave.

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Web Links

1 http://www.microscopy-uk.org.uk/index.html
2 http://www.microscopyu.com/articles/formulas/index.html
3 http://www.nature .com/milestones/light-microscopy
4 http://www.microscopyu.com/articles/phasecontrast/index.html
5 http://www.microscopyu.com/articles/dic/index.html
6 http://www.olympusmicro.com/primer/techniques/hoffman.html
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