Chapter 6

Microscopy

Microscopy is a technique for making very small things visible to the unaided eye. An instrument used to make the small things visible to the naked (unaided) eye is called a microscope. There are two fundamentally different types of microscopes: the light microscope and the electron microscope.

6.1 Light microscope

Light or optical microscope uses visible light as a source of illumination. Because the light travels through the specimen, this instrument can also be called a *transmission light microscope*. The light microscope creates a magnified image of specimen which is based on the principles of transmission, absorption, diffraction and refraction of light waves.

The simplest form of light microscope consists of a single lens, a magnifying glass. Microscope made up of more than one glass lens in combination is termed *compound microscope*. Compound microscope includes condenser lens, the objective lens and the eyepiece lens. Condenser lens focuses the light from the light source at the specimen. The one facing the object is called the *objective* and the one close to the eye is called the *eyepiece*. The objective has a smaller aperture and smaller focal length than those of the eyepiece (also referred to as the *ocular*).

The objective lens is responsible for producing the magnified image. It is available in different varieties (4x, 10x, 20x, 40x, 60x, 100x). The power of a lens is described with a number followed by the letter 'x'. For example, if through a microscope one can see something 25 times larger than actual size, its magnification power is 25x. The eyepiece works in combination with the objective lens to further magnify the image. A compound microscope with a single eyepiece is said to be *monocular* and one with two eyepieces is said to be *binocular*. Eyepieces usually magnify by 10x, since an eyepiece of higher magnification merely enlarges the image, with no improvement in resolution.

Both living and dead specimens are viewed with a light microscope. The visibility of the magnified specimen depends on contrast and resolution. In general, variation in the light intensity (contrast) within an image occurs because different parts of the specimen absorb light to differing degrees. Brightness contrast arises from different degrees of absorption at different points in the specimen. Color contrast can also arise from absorption when the degree of absorption depends on the wavelength and varies from point-to-point in the specimen.

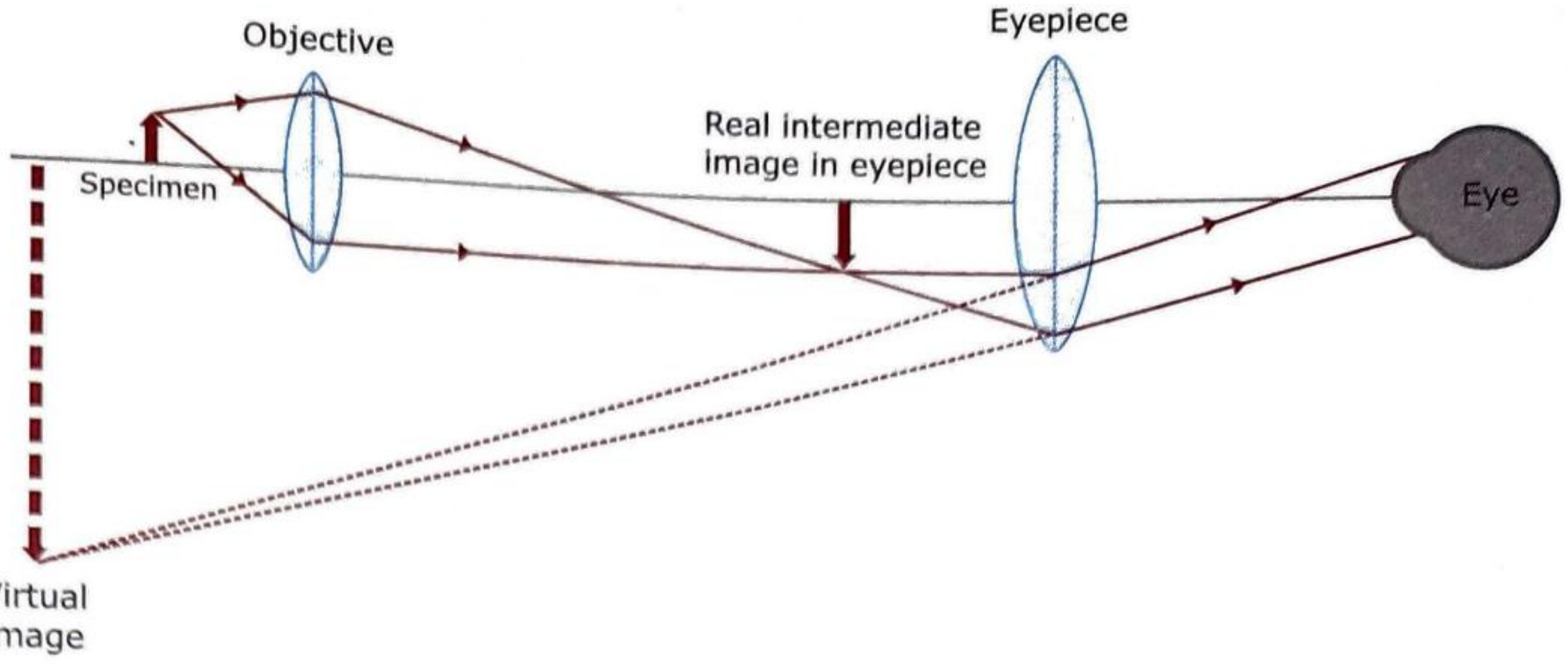


Figure 6.1 Compound microscope. The objective lens forms a real and inverted magnified image of the specimen or object (called the real intermediate image) in the focal plane of eyepiece. This image works as an object for the eyepiece. The final image is, then, formed at infinity. It is erect with respect to the first image and hence, inverted with respect to the object.

For colorless specimens, as is the case for most biological material, contrast is achieved in various ways. The specimen may be stained, thus reducing the amplitude of certain light waves passing through the stained areas. However, this usually requires the fixation and staining of specimens. But fixation and staining kills the specimens. For colorless living specimens, contrast can be achieved in different ways. A living biological specimen is almost uniformly transparent, and therefore the intensity variation in the image will be poor. However, the light does not go through the specimen unaffected. The light going through the specimen will be shifted in phase due to scattering and diffraction. Our eyes cannot detect this phase shift. However, using phase contrast microscopy, we can develop a contrast based on the phase shift.

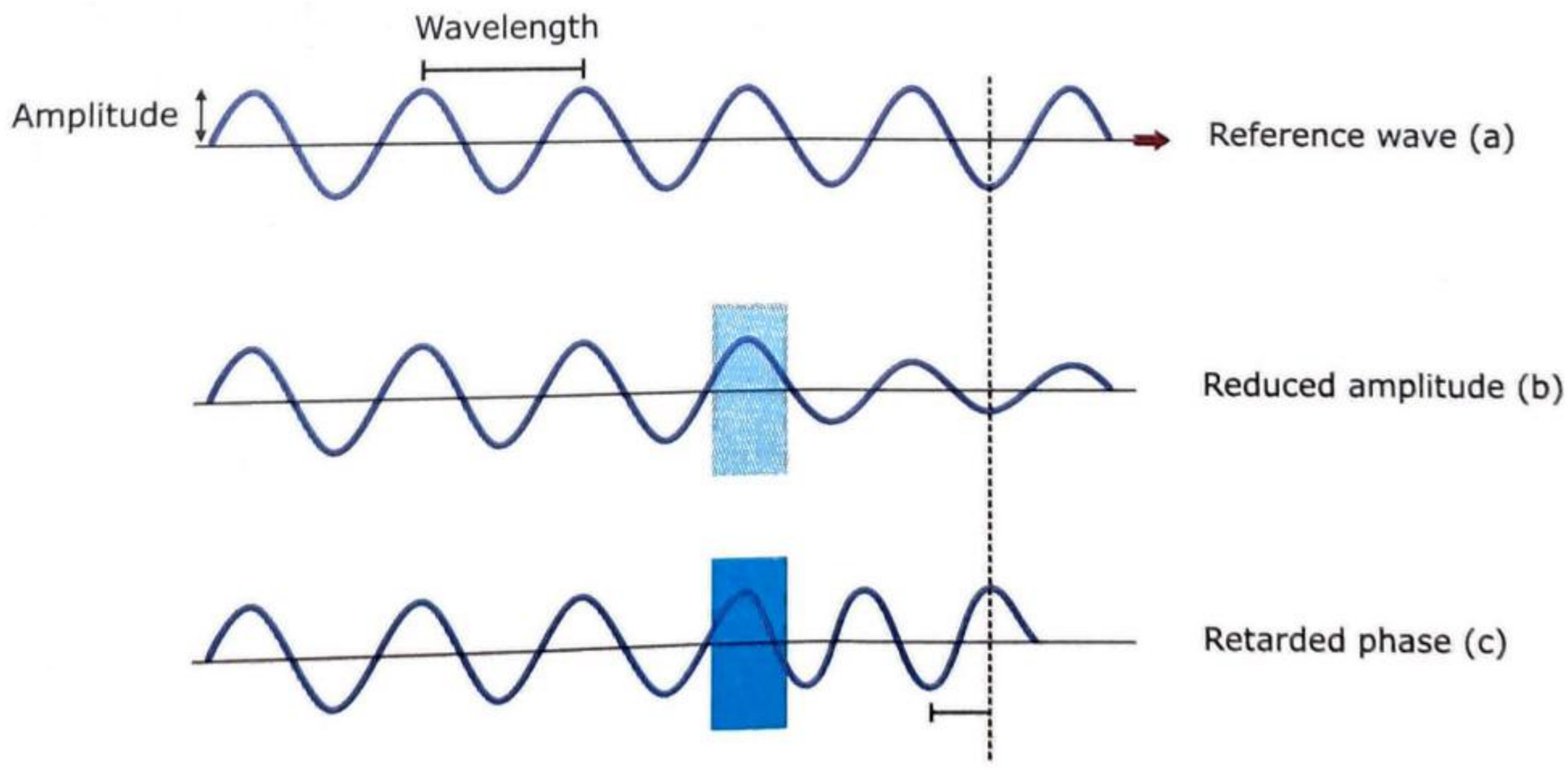


Figure 6.2 Changes in amplitude and phase of light. (a) Reference wave with characteristic amplitude, wavelength and phase. (b) An object absorbs energy and reduces the amplitude, but does not alter the phase of an emergent ray. (c) An object alters velocity and shifts the phase, but not the amplitude of an emergent ray.

Magnification

The magnification or linear magnification of a microscope is defined as the ratio of the image size to the object (specimen) size. If the image and object are in the same medium, then it is just the image distance divided by the object distance. There is a difference in the meaning of the two terms, *magnification* and *magnifying power*. Magnifying power or angular magnification is the ratio of the angle subtended by object and image.

The magnification of a compound microscope is the product of the magnification of the objective and the eyepiece. The magnification of the objective is called the linear magnification, because it is measured in linear dimensions. The magnification of the eyepiece is called the angular magnification. The overall magnification is the product of the linear magnification of the objective lens and the angular magnification of the eyepiece with the first image at the focal length.

Resolving power

Resolving power is the ability of magnifying instrument to distinguish two objects that are close together. The resolving power is inversely related to the *limit of resolution*. The limit of resolution is defined as the minimum distance between two points that allows for their discrimination as two separate points. Thus, the higher the resolving power, the smaller the limit of resolution. The limit of resolution of the light microscope depends upon the three factors: the *wavelength* (λ) of the light used to illuminate the specimen, the *angular aperture* (α) and the *refractive index* (n) of the medium surrounding the specimen. The effect of these three variables on the limit of resolution is described quantitatively by the following equation known as the *Abbe equation*:

$$\text{Limit of resolution} = \frac{0.61\lambda}{n \times \sin\alpha}$$

The quantity $n \times \sin\alpha$ is called the *numerical aperture* of the objective lens, abbreviated NA. The NA is a measure of the ability of a lens to collect light from the specimen. Lenses with a low NA collect less light than those with a high NA.

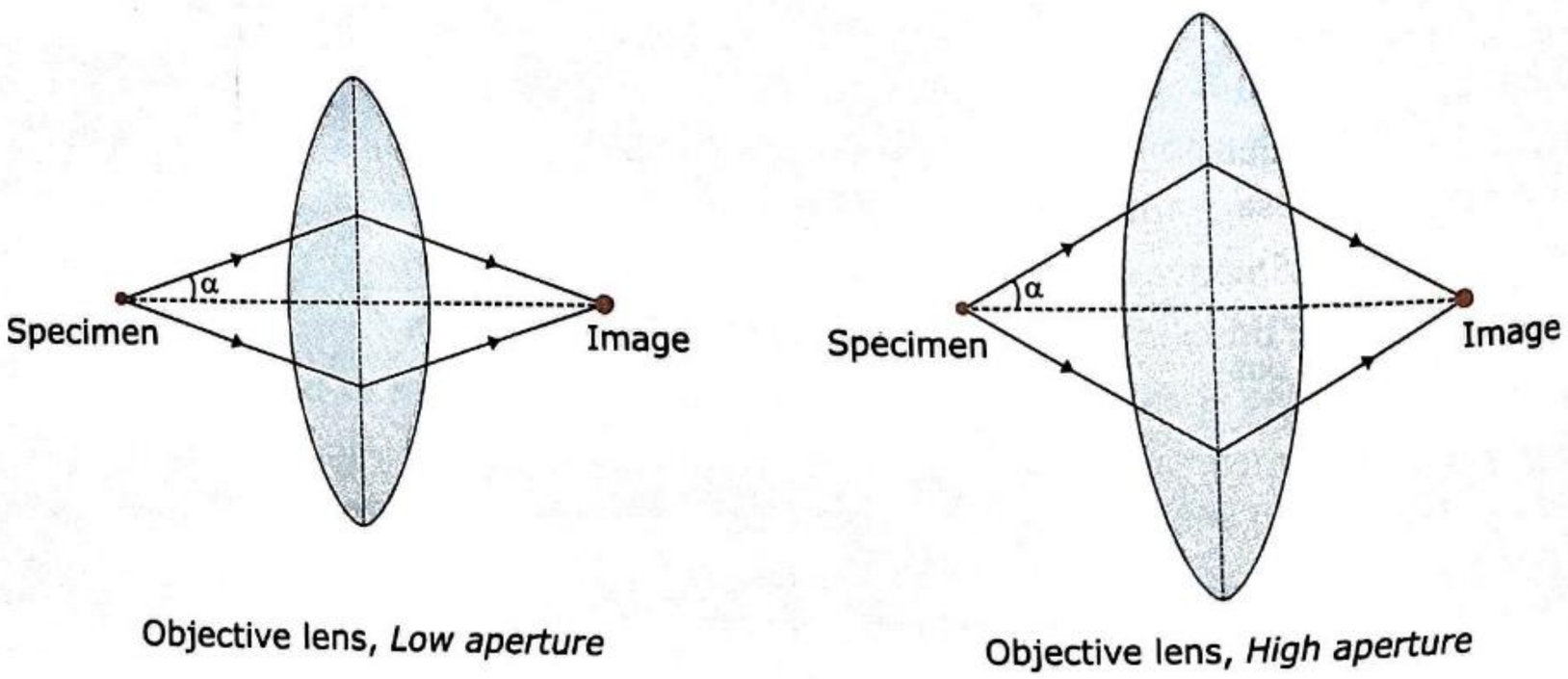
$$\text{Limit of resolution} = \frac{0.61\lambda}{NA}$$

For small value of limit of resolution, the numerator of the equation should be as small as possible and the denominator should be as large as possible. Equation shows that resolution can be improved by shortening the wavelength of the illuminating light, increasing the index of refraction on the objective lens, and increasing $\sin\alpha$. The angle α can be increased either by shortening the distance between the lens and the object or by increasing the diameter of the lens.

Magnification is how large the image is as compared to real specimen size, whereas resolution is the amount of information that can be seen in the image – defined as the smallest distance below which two discrete objects will be seen as one.

Magnification is a function of the number of lenses. Resolution is a function of the ability of a lens to gather light.

The maximum magnification of compound light microscopes is usually 1500x and has a limit of resolution of about 0.2 μm.



Objective lens, *Low aperture* — Objective lens, *High aperture*

Figure 6.3 The angular aperture of a lens. The angular aperture is the half-angle α of the cone of light entering the objective lens of the microscope from the specimen. It is, therefore, a measure of how much of the illumination that leaves the specimen actually passes through the lens. The larger the angular aperture, the more information the lens can transmit.

For minimum value of numerator, the wavelength should be small. Thus, for the best resolution, specimen is illuminated with blue light of 450 nm. The angular aperture for the best objective lenses is about 70°. Hence, the maximum value for sinα is about 0.94. The refractive index of air is about 1.0, so for a lens designed for use in air, the maximum numerical aperture is about 0.94. In this situation, the limit of resolution for a glass lens in air is roughly 300 nm. To increase the numerical aperture some microscope lenses are designed to be used with a layer of *immersion oil* between the lens and the specimen. Immersion oil has a higher refractive index than air and, therefore, 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 numerical aperture for an oil immersion lens is about 1.5 × 0.94 = 1.4.

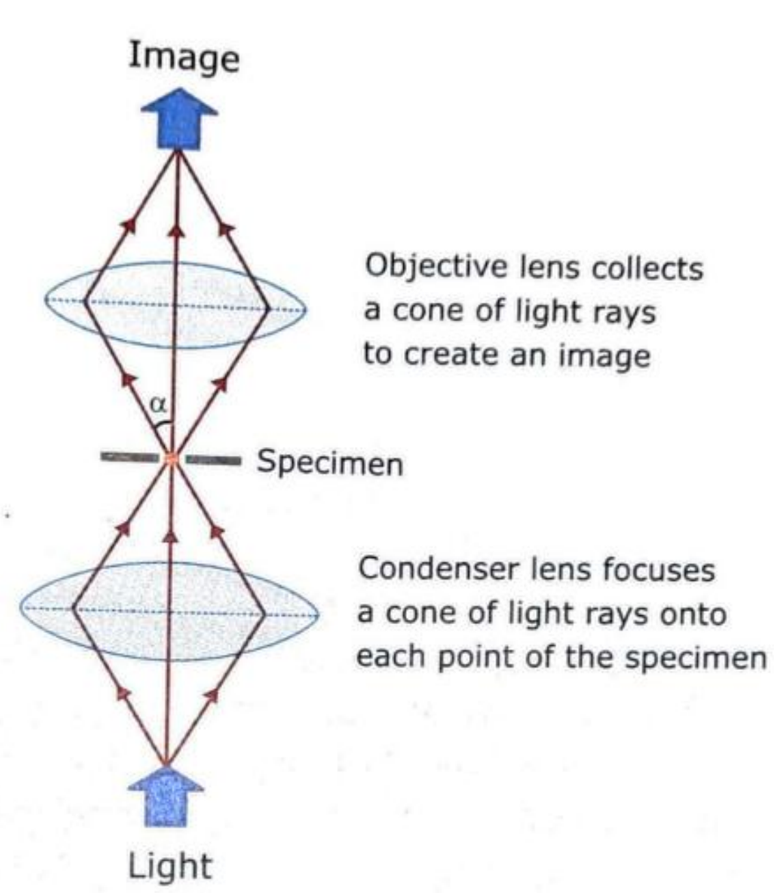


Figure 6.4 The angular aperture is the half-angle α of the cone of light entering the objective lens of the microscope from the specimen.

Thus, the limit of resolution for a microscope that uses visible light is roughly 300 nm in air and 200 nm with an oil immersion lens. The limit of resolution of the unaided human eye is 100 µm.

Oil immersion lens

In most microscopes, air is present as a medium through which light rays pass between the coverslip protecting the sample and front lens of the objective. Objectives of this type are referred to as *dry objectives*. Air has a refractive index of 1.0003, very close to that of a vacuum and considerably lower than most liquids, including water (n = 1.33). In light microscopy, *oil immersion lens* is used to increase the resolving power. An objective lens specially designed to be used in this way is termed as an *oil immersion objective.*

Refractive index
The refractive index of a medium measures the extent of interaction between electromagnetic radiation and the medium through which it passes. The refractive index of water at room temperature is 1.33, which means that light travels 1.33 times slower in water than it does in vacuum.

In this system, air is replaced by transparent oil (termed immersion oil) of high refractive index (very similar to refractive index of glass). Immersion oil such as paraffin oil, cedarwood oil has been placed at the interfaces between the objective lens and the cover slip protecting the specimen (also between the condenser lens and the underside of the specimen slide).

If the air is present between the cover slip and the objective lens, light is refracted, scattered and effectively lost. This happens because the refractive index of air is very different from that of glass and light passing through a glass-air interface is refracted (bent) to a large degree. By reducing the amount of refraction at this point, more of the light can be directed

to the narrow diameter lens of the high-power objective. The more the light, the clearer will be the image. Placing a material with a refractive index equal to that of glass in the airspace between cover slip and objective, more light can be directed through the objective which improves resolution. Immersion oils improve resolution by performing same function.

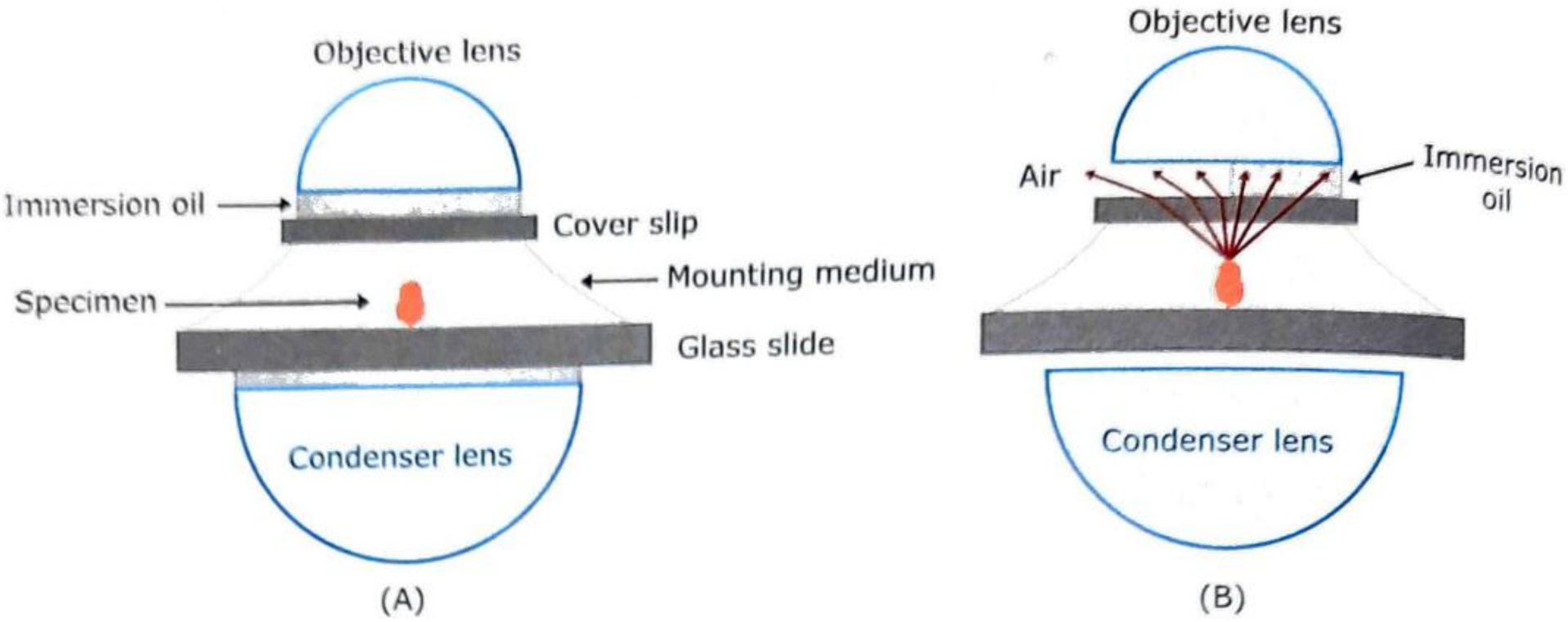


Figure 6.5 (A) An oil immersion lens where immersion oil has been placed at the interfaces between the objective front lens and the specimen covered with a cover slip and also between the condenser lens and the underside of the specimen slide. (B) In case of a dry objective, light rays pass through a specimen that is covered with a cover slip. These rays are refracted at the coverslip-air interface and only the light rays closest to the optical axis of the microscope have the appropriate angle to enter the objective lens. When air is replaced by oil of the same refractive index as glass, the light rays now pass straight through the glass-oil interface without any deviation due to refraction. In this case, the numerical aperture is, thus, increased by the factor of n, the refractive index of oil.

6.2 Types of light microscope

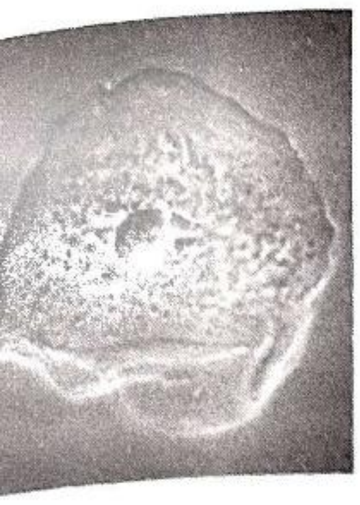
Brightfield microscopy

It is the original and most commonly used form of microscopy in which the specimen is viewed by transmitted light from a condenser lens. Light is aimed towards a condenser, through the specimen, through an objective lens, and to the eye through a second magnifying lens, the ocular or eyepiece. Specimens are visible in the light path because the natural pigmentation or stains absorb light differentially, or because they are thick enough to absorb a significant amount of light despite being colorless.

Darkfield microscopy

A dark-field microscope is a type of microscope in which objects are illuminated at a very low angle from the side so that the background appears dark and the objects show up against this dark background. It is a technique for improving the contrast of unstained, transparent specimens. Darkfield illumination uses a carefully aligned light source to minimize the quantity of directly-transmitted light and collecting only the light scattered by the sample. To view a specimen in a dark field, an opaque disc is placed underneath the condenser lens, that blocks light from entering the objective lens directly; light reflected by specimen enters the objective, and the specimen appears light against a black background.

Phase–contrast microscopy



ase contrast micrograph
a human cheek cell.

When light passes through a living cell, the phase of the light wave is changed according to the cell's refractive index: light passing through a relatively thick or dense part of the cell, such as the nucleus, is retarded; its phase, consequently, is shifted relative to light that has passed through an adjacent thinner region of the cytoplasm. The phase-contrast microscope (invented by Frits Zernike) exploits the interference effects produced when these two sets of waves recombine, thereby creating an image of the cell's structure. The specimen appears as different degrees of brightness and contrast. It is used for the study of live and unstained cells, which are, in general, transparent to light.

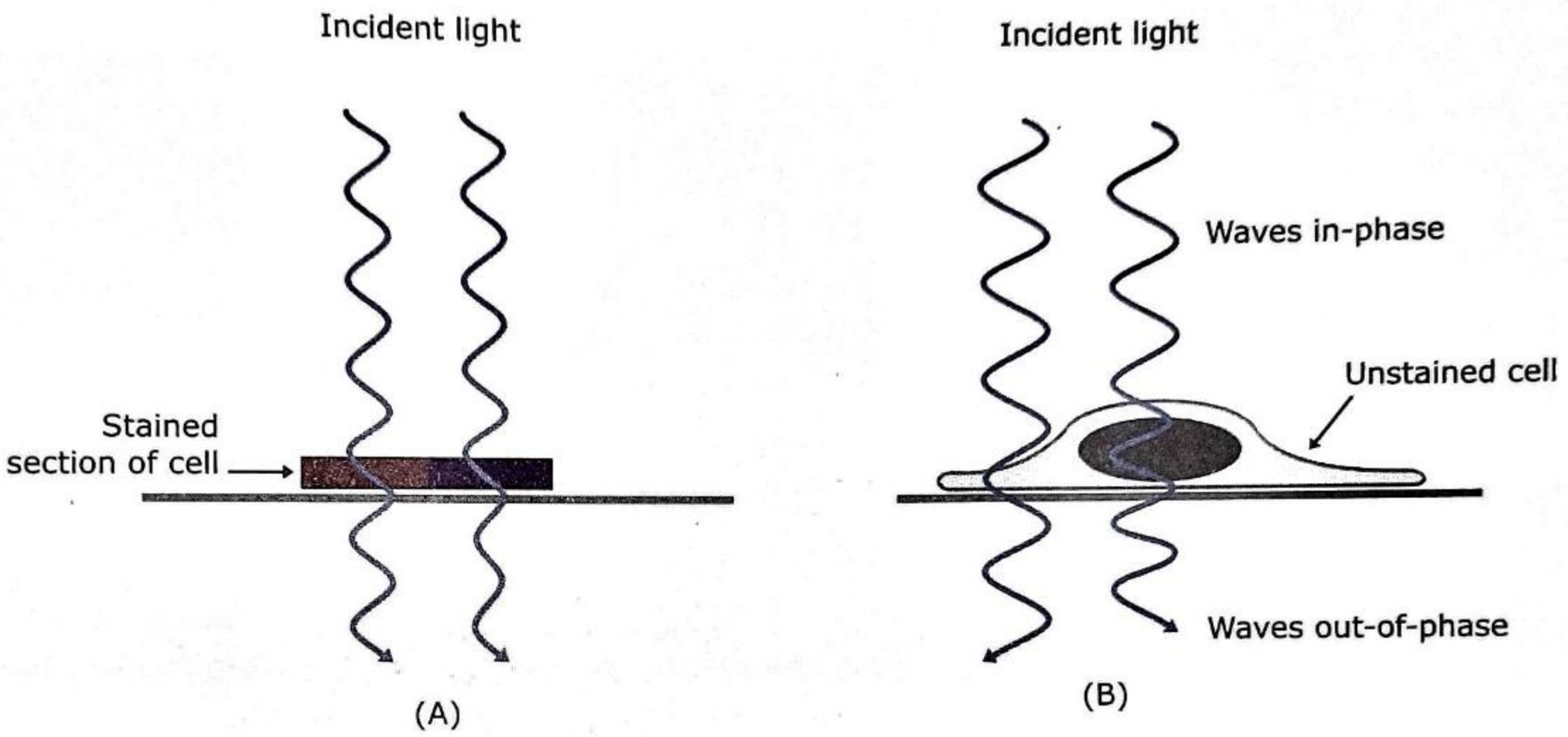


Figure 6.6 Two ways to obtain contrast in light microscopy. The stained portions of the cell in (A) reduce the amplitude of light waves of particular wavelengths passing through them. A colored image of the cell is, thereby, obtained that is visible in the ordinary way. Light passing through the unstained, living cell (B) undergoes very little change in amplitude, and the structural details cannot be seen even if the image is highly magnified. The phase of the light, however, is altered by its passage through the cell, and small phase differences can be made visible by exploiting interference effects using a phase-contrast or a differential-interference-contrast microscope.

Fluorescence microscopy

In fluorescence microscopy, the specimen itself acts as a light source. The specimens used to study are either fluorescent materials or stained with fluorescent dyes. A chemical is said to be fluorescent if it absorbs light at one wavelength and emits light (fluoresces) at a specific and longer wavelength. Most fluorescent dyes (or fluorochromes) emit visible light, but some emit infrared light. Fluorochromes exhibit distinct excitation and emission spectra that depend on their atomic structure and electron resonance properties. Two fluorescent dyes that are commonly used are fluorescein, which emits an intense green fluorescence when excited with blue light, and rhodamine, which emits a deep red fluorescence when excited with green-yellow light.

The fluorescence microscope is similar to an ordinary light microscope except that the illuminating light is passed through two sets of filters – one to filter the light before it reaches the specimen (*excitation filter*) and other to filter the light emitted from the specimen (*barrier* or *emission filter*). The excitation filter passes only the wavelength that excites the particular fluorescent dye, while the barrier filter blocks out this light and passes only those wavelengths emitted when the dye fluoresces. Only fluorescent light emitted by the fluorescently

stained specimen is used to form an image. The wavelength that excites the specimen and induces the fluorescence is not allowed to pass the filters placed between the objective lens and the eye.

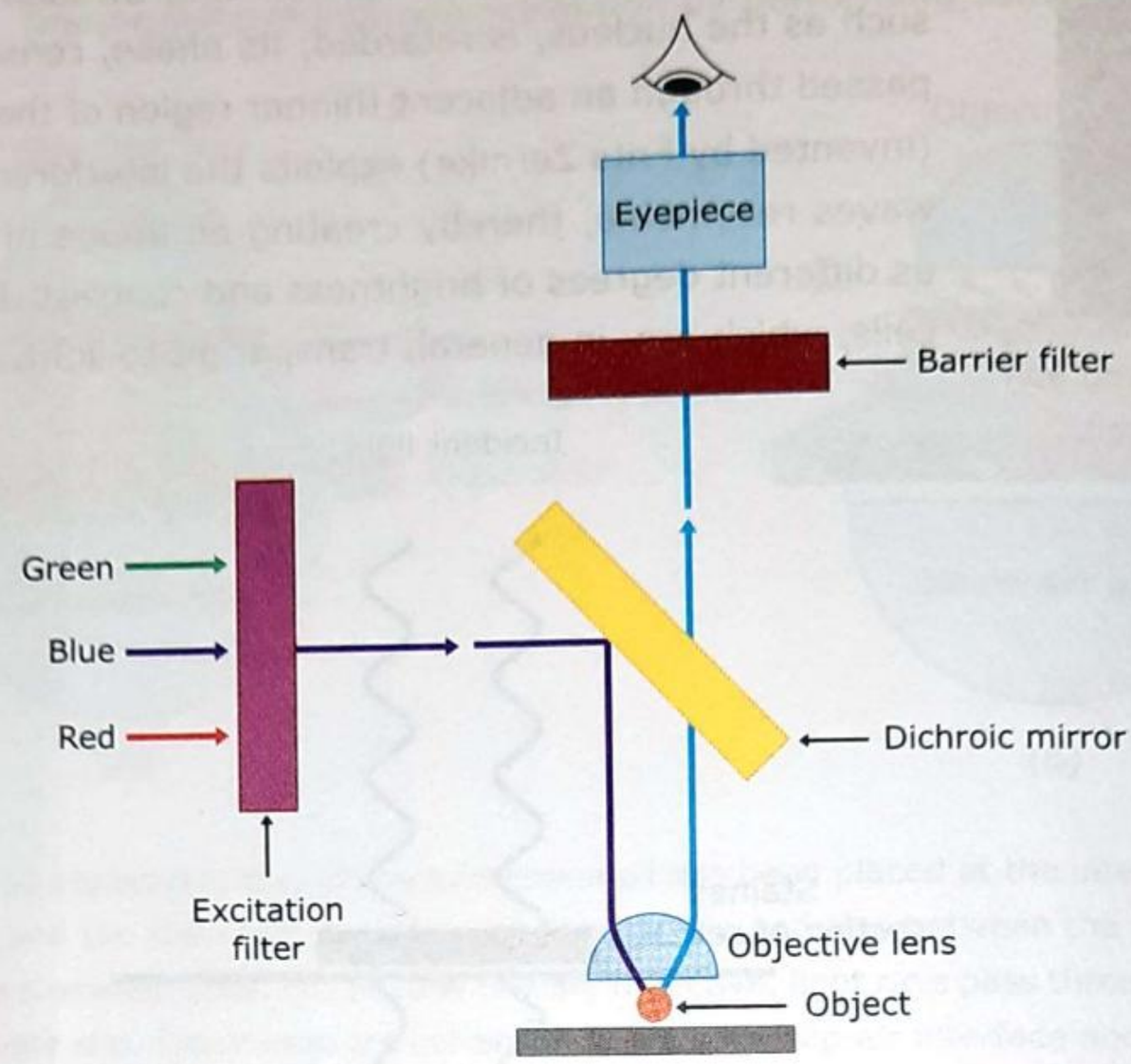


Figure 6.7 The optical system of a fluorescence microscope. Fluorescence microscopes contain special filters and employ a unique method of illumination to produce images of fluorescent light emitted from excited molecules in a specimen. It contains two essential filters – excitation filter and barrier or emission filter. The diagram shows the orientation of filters. The excitation beam (blue line) passes through the excitation filter and is reflected by the dichroic mirror and directed towards the specimen. The return beam of emitted fluorescence wavelengths (cyan line) passes through the dichroic mirror and the emission filter to the eye or camera. Excitation wavelengths that manage to pass through the dichroic mirror are blocked by the barrier (emission) filter.

Confocal microscopy

A confocal microscope creates sharp images of a specimen that would otherwise appear blurred when viewed with a conventional microscope. This is achieved by excluding most of the light from the specimen that is not from the microscope's focal plane. The image has less haze and better contrast than that of a conventional microscope and represents a thin cross-section of the specimen. Thus, apart from allowing better observation of fine details it is possible to build three-dimensional (3D) reconstructions of a volume of the specimen by assembling a series of thin slices taken along the vertical axis.

Confocal is defined as *having the same focus*. What this means in the microscope is that the final image has the same focus as or the focus corresponds to the point of focus in the object. The object and its image are *confocal*. The microscope is able to filter out the out-of-focus light from above and below the point of focus in the object. Normally when an object is imaged in the fluorescence microscope, the signal produced is from the full thickness of the specimen which does not allow most of it to be in focus to the observer. The confocal microscope eliminates this out-of-focus information by means of a confocal *pinhole* situated in front of the image plane which acts as a spatial filter and allows only the in-focus portion of the light

to be imaged. Light from above and below the plane of focus of the object is eliminated from the final image. The confocal microscope uses a laser beam to illuminate a specimen, usually one that has been fluorescently stained. A diagram of the confocal principle is shown below.

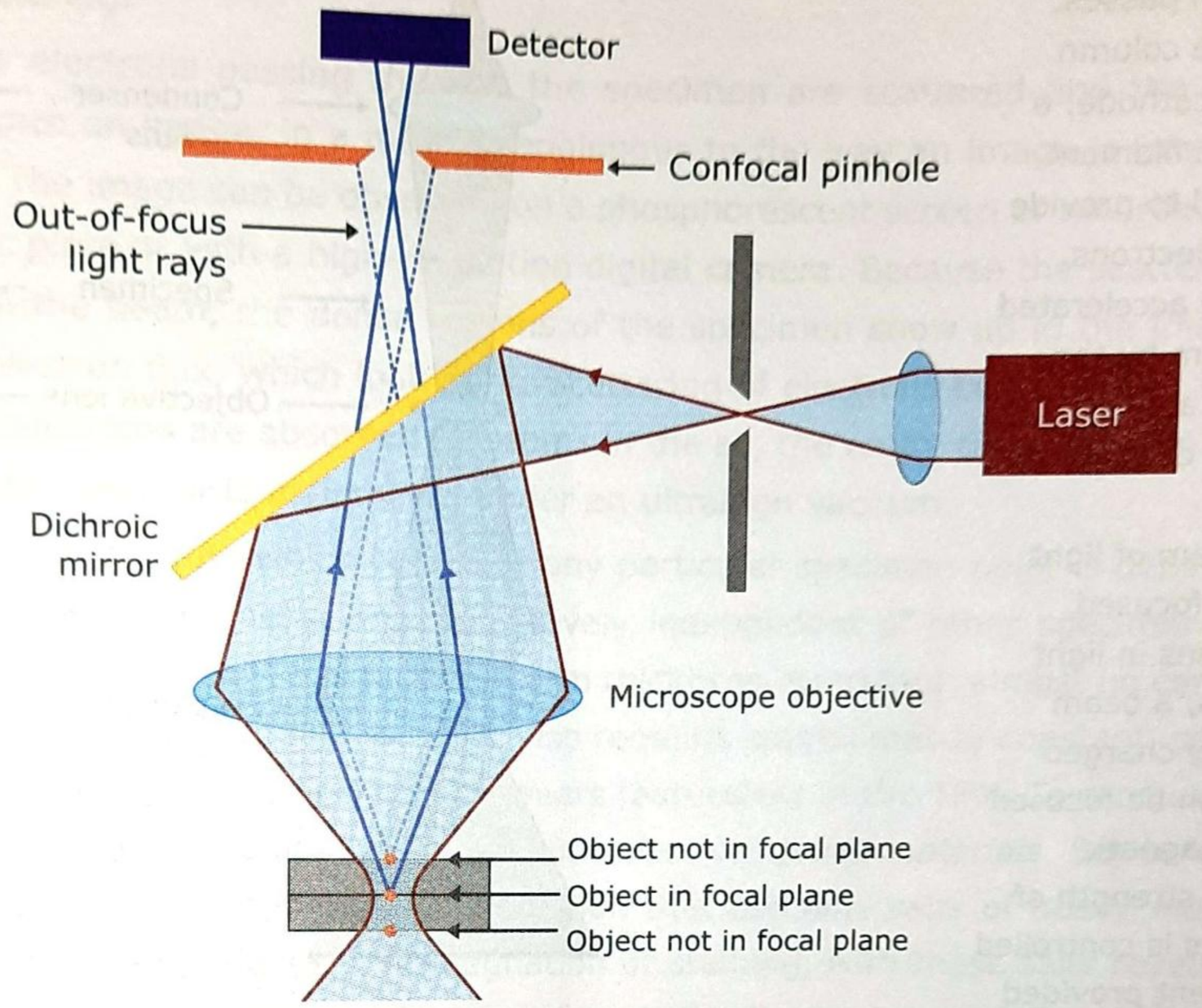


Figure 6.8 Ray path in a confocal microscope. A microscope objective is used to focus a laser beam onto the specimen, where it excites fluorescence. The fluorescent radiation is collected by the objective and efficiently directed onto the detector via a dichroic beam splitter. The wavelength range of the fluorescence spectrum is selected by an emission filter, which also acts as a barrier blocking the excitation laser line. The pinhole is arranged in front of the detector, on a plane conjugate to the focal plane of the objective. Light coming from planes above or below the focal plane is out-of-focus when it hits the pinhole, so most of it cannot pass the pinhole and, therefore, does not contribute to forming the image.