Design, Alignment, and Usage of Infinity-Corrected Microscope

2

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Contents

What You Will Learn in This Chapter

This chapter discusses how infinity-corrected microscopes work, as well as the principles of optics that are applied to their development. Proper designing and good alignment of an optical microscope are essential for accurate studies of cells, observation of cellular growth, identification, and counting of cells. To design an optical microscope the two important aspects are, namely, a better understanding of the function of each component and how their control influences the resulting images. The design of the infinity-corrected optics is routinely incorporated into multiple lenses, filters, polarizers, beam-splitters, sensors, and illumination sources. This chapter discusses the development of microscope with infinity optics and the design of infinity-corrected optics with optical ray diagrams. The microscope design parameters and aberrations are discussed to understand the necessity of multiple lens

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objective system. To get the best resolution and contrast, the condition for Köhler illumination should be maintained within a microscope. The unstained sample is unable to image in bright field microscopy. The chapter also discusses label-free techniques with infinity-corrected optics, such as dark field microscopy, Zernike phase contrast microscopy, differential interference contrast (DIC), and digital holographic microscopy and their applications to study the various type of specimens without dye or label.

2.1 Development of Compound Microscope with Infinity Optics

A microscope is an instrument that produces a magnified two-dimensional image of an object which is not normally resolvable by the human eye. The human eye cannot resolve the object smaller than 150 μm [1]. A decent optical microscope can resolve the object which is near 0.2 μm, so it has multiple times better resolution over independent eye [2, 3].

Antonie van Leeuwenhoek (1632–1723) was a Dutch scientist who was first to document microscopic observation of bacteria using a single-lens microscope which consisted of a small, single converging lens mounted on a brass plate, with a screw mechanism to hold the sample to be examined. In the single-lens system, the object is magnified with a convex lens that bends light rays by refraction. The rays from the object are converged behind the lens to form a focused image. The distance from the object to the lens divided by the distance of the image from the lens determines the magnification of this system.

The disadvantage of Leeuwenhoek single-lens microscope is that it has to be placed very near to the eye to get a magnified image. The *compound microscope*, built by Robert Hooke (1635–1702), overcame this problem by using two thin lenses: the *objective* and the *eyepiece*. The highest magnification achieved by Leeuwenhoek microscope was limited to only 300×, whereas in Hooke's compound microscope, magnification up to 1500× was possible. As the application of microscope in studying biological samples grew by the beginning of the twentieth century, there was an increasing need to add multiple optics between objectives and eyepiece to enhance the contrast. However, the introduction of extra optical elements in the Hooke's microscope deteriorated the quality of images. In the early 1930s, the German microscope manufacturer Reichert introduced an infinity-corrected optical configuration for microscope to overcome this drawback [3]. The infinity-corrected microscope is a three-lens system where *objective*, *tube lens*, and *eyepieces* are used to get an image. Most of modern optical microscopes today use the infinity-corrected optical configuration due to the improved functionality of the microscope.

In microscope a specimen under observation is uniformly illuminated through a lens by an incoherent light source. The compound microscope (Fig. 2.1) is a two-step imaging system where the first lens (objective lens) is placed close to the object and creates a real, inverted, and magnified image of the object at the focal plane of the second lens (eyepiece).This image is also known as an intermediate

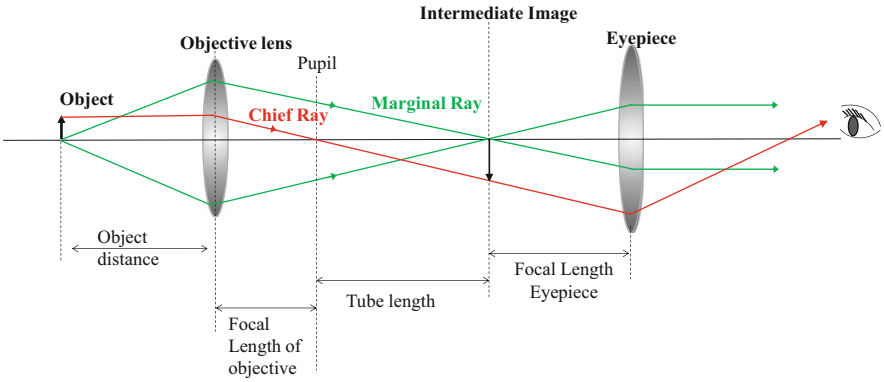

Fig. 2.1 Ray diagram for image formation in general compound microscope

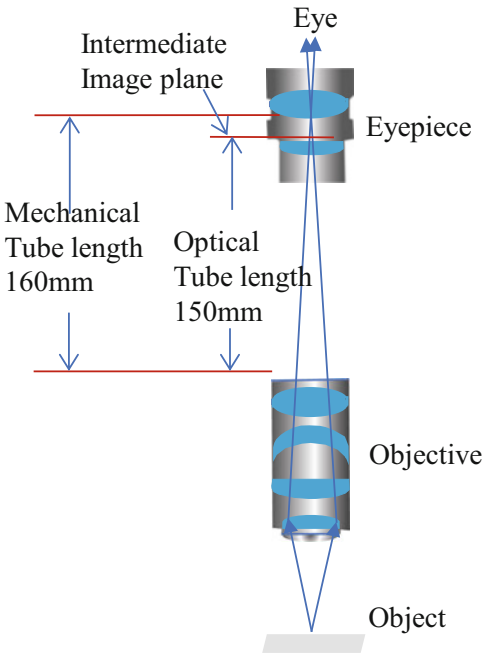

Fig. 2.2 Different tube lengths in compound microscope

image and the plane is the intermediate image plane. The eyepiece produces a virtual image that projects an image to infinity, and the human eye creates the final magnified image. In most of the compound microscope systems, the final image is located at the minimum focusing distance of the human eye, which is generally 250 mm from the eyepiece [3, 4].

The distance between the objective and eyepiece is known as mechanical tube length. As illustrated in Fig. 2.2, the distance from the rear focal plane of objective to the intermediate image plane is named as optical tube length. The value of finite

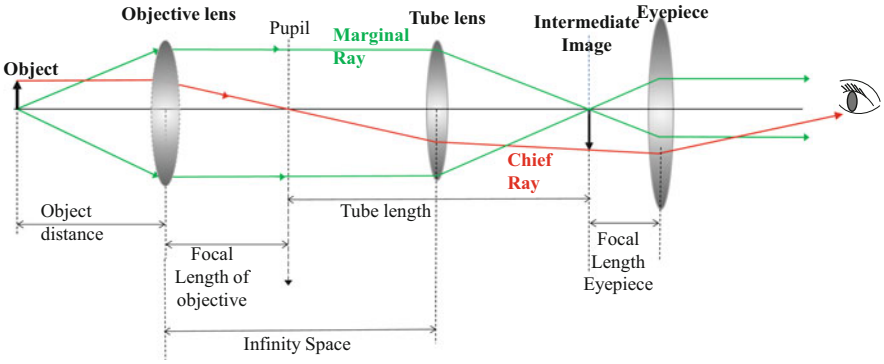

Fig. 2.3 Ray diagram for image formation in infinity-corrected compound microscope configuration

mechanical tube length is standardized to 160 mm. It is observed that adding auxiliary optical components such as beam-splitters, polarizers filters into the light path of a fixed tube length microscope increases the effective tube length and deteriorates the quality of images. But in the contrast-enhancing methods (DIC, fluorescence microscopy) these auxiliary optics are essential for a microscope. The introduction of infinity optics enables the use of auxiliary optics. Figure 2.3 show the schematic diagram of infinity optics where the objective collects the light transmitted through or reflected from the specimen and produces a parallel bundle of rays. The parallel light rays are then focused at the intermediate image plane by the tube lens, and subsequently magnified intermediate image is seen by the eyepiece. The area between the objective rear plane and the tube lens is called *infinity space*, where auxiliary components can be introduced without producing focus artefacts or optical aberrations. The term *infinity space* refers to the production of a bundle of parallel light rays between the objective and tube lens of a microscope.

The three main advantages of the infinity optical system are:

- The magnification and location of the intermediate image remains constant even when the distance between the objective lens and tube lens is altered.
- There is no image aberration even when prisms, filters are interposed between the objective lens and the tube lens.
- It allows users to switch between objectives with different magnification without needing to refocus the specimen (Parfocality), even when extra elements are added.

2.2 Design Parameters: Resolution Limit, Numerical Aperture, Magnification, Depth of Field, and Field of View

Resolution The resolution is one of the key parameters of the microscope. Resolution is defined by the minimum spacing between two specimen points which will be distinguished as separate entities. The resolution of an optical system is limited by diffraction and aberration [4]. The high resolution of an optical instrument means the ability to see a structure at a high level of detail.

Light propagates as a wave, when light passes through the objective it is diffracted and spread out in the focal region, forming a spot of light which is about a minimum 200 nm wide and minimum 500 nm along the optical axis [4, 5]. The diffraction pattern consists of a central maximum (zeroth order) surrounded by concentric first, second, third, etc., maxima with decreasing brightness, known as Airy disc or point spread function (Fig. 2.4). The size of the central maxima of the Airy disc is dependent on the numerical aperture of lens and wavelength of light. The limit of resolution of a microscope objective refers to its ability to distinguish between two closely spaced Airy discs in the image plane. The two principal ways of recognizing and calculating resolving power and the limit of resolution in the microscope are Rayleigh's resolution criterion and Abbe's resolution criterion. In Rayleigh's resolution criterion we consider how closely two finely detailed points can lie to one another and remain distinguishable as discrete entities, whereas Abbe's resolution criterion considers how many diffracted orders of light can be accepted by the objective to form an image.

Rayleigh Criterion Two-point sources are regarded as just resolved when the central zeroth order maximum of one image coincides with the first minimum of the other (Fig. 2.5). The minimum distance between the two points is

$$R = \frac{0.61\lambda}{n \cdot \sin\theta} = \frac{0.61\lambda}{\mathrm{NA}}, \tag{2.1}$$

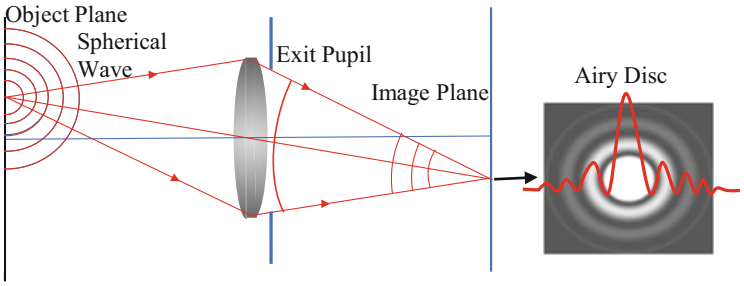

Fig. 2.4 Airy disc in a lens system as broadening the image of a single object point

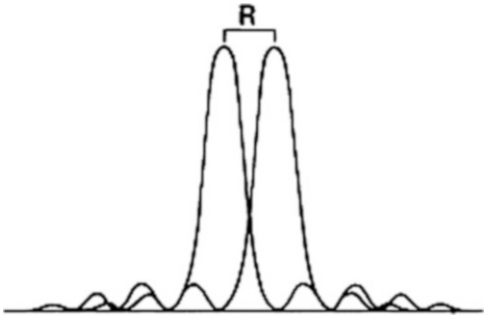

Fig. 2.5 Rayleigh Resolution criterion in diffraction-limited system

where λ is the average wavelength of illumination. The numerical aperture is defined by the refractive index of the immersion medium (the medium between objective and specimen, n; usually air, water, glycerine, or oil) multiplied by the sine of the half aperture angle ($\sin(\theta)$).

$$\mathrm{NA} = n \cdot \sin(\theta). \tag{2.2}$$

The numerical aperture is used to define the resolution and light gathering ability of the lens. The values of NA range from 0.1 for very low magnification objectives to 1.4 for high-performance objectives utilizing immersion oils in imaging medium.

Abbe's Resolution Criterion The Abbe diffraction limit depends on the number of diffraction order from object are accepted by objective and the NA of the lens involved for image formation. It assumes that if two adjacent diffraction orders of two points incident on the aperture of objective, these two points are resolved. Therefore, the resolution depends on both imaging (objective lens) and illumination apertures (condenser lens) and is

$$\text{Abbe Resolution}_{x,y} = \frac{\lambda}{\mathrm{NA}_{\text{condenser}} + \mathrm{NA}_{\text{objective}}}. \tag{2.3}$$

The above two equations indicate that the resolution of an optical system improves with an increase in NA and decreases with increasing wavelength λ.

Due to the large difference between the refractive indices of air and glass; the air scatters the light rays before they can be focused by the lens. To minimize this difference a drop of oil can be used to fill the space between the specimen and an immersive objective. The oil has a refractive index very similar to that of glass, it increases the maximum angle at which light leaving the specimen can strike the lens. This increases the light collected and, thus, the resolution of the image as shown Fig. 2.6. For example, the image spot size produced by a 100× magnification dry objective of NA 0.95 in green light (550 nm) is approximately 0.34 μm, whereas the spot size for 100× oil immersion objective of NA 1.4 is approximately 200 nm or 0.2 μm.

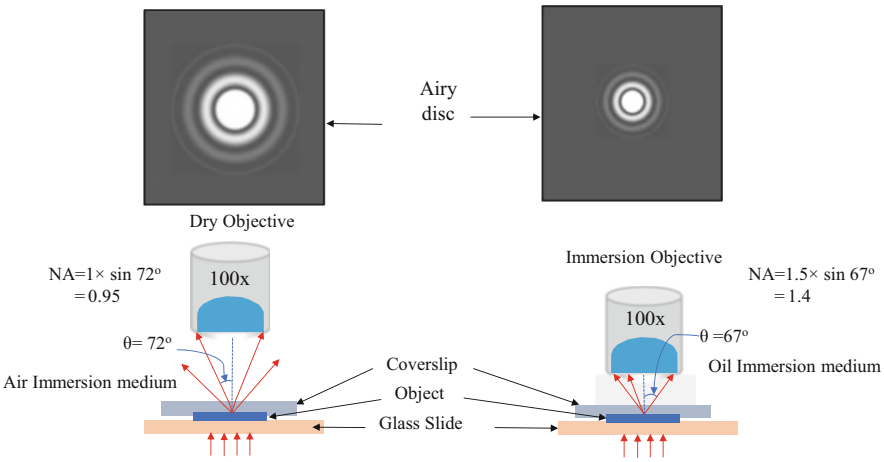

Fig. 2.6 Numerical aperture and resolution of dry objectives of NA 0.95 and oil immersion objective of NA 1.4

Magnification In compound microscopy, the magnification depends on the objective magnification (M_o) and the eyepiece magnification (M_e), where M_o is the ratio of tube length and object distance and M_e is the ratio of the virtual image distance and the focal length of eyepiece. So total magnification of the microscope (M_T) is

$$M_T = \frac{\text{Finite tube length}}{\text{Focal length}_{\text{objective}}} \times \frac{250\text{ mm}}{\text{Focal length}_{\text{Eyepiece}}} = M_o \times M_e. \tag{2.4}$$

So, if the objective magnification is 4× and the eyepiece magnification is 10×, $M_T = 40\times$

In a microscope with infinity-corrected optics, the magnification of intermediate image is defined by the ratio of focal length of tube lens and objective lens. The focal length of the tube lens varies from 160 to 250 millimetres depending upon the manufacturer and model.

$$\text{Magnification}_{\text{infinity-corrected objective}} = \frac{\text{Focal length}_{\text{tubelens}}}{\text{Focal length}_{\text{objective}}}. \tag{2.5}$$

Depth of Field and Depth of Focus Depth of field is the axial depth of the *object plane* within which the object plane is possible to shift without loss of sharpness in the image plane while the image plane position is fixed. Depth of focus is that the axial depth of the *image plane* within which the position of the image plane is allowed to move and the image appears acceptably sharp while the positions of the object plane and objective are maintained (Fig. 2.7).

Various authors [6, 7] have proposed different formulas for calculation of the depth of field. Suggested by Rudolf Oldenbourg and Michael Shribak [6] the total depth of field is

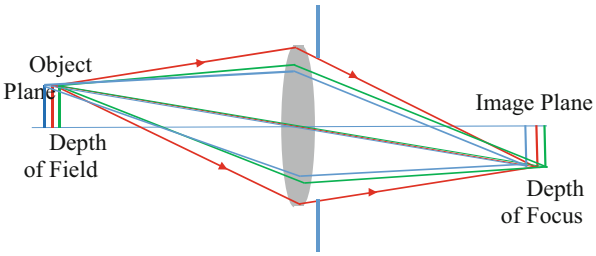

Fig. 2.7 Depth of field and depth of focus of an objective lens

$$d_z = \frac{\lambda \cdot n}{\mathrm{NA}^2} + \frac{n \cdot p_{\mathrm{sens}}}{M \cdot \mathrm{NA}}, \tag{2.6}$$

where d_z is the depth of field (or axial resolution), λ is the wavelength of the illuminating light, n is the refractive index of the medium between the specimen and the objective lens, NA is the objective lens numerical aperture, M is the objective lens magnification, and p_{sens} is the pixel size of the image sensor placed in the intermediate image plane.

Two important aspects of depth of field and depth of focus are:

1. High magnification objectives with large aperture have extremely limited depth of field at object or specimen's plane and relatively large depth of focus at eyepiece or camera plane. This is why at high magnification focusing of specimen is very sensitive and accurate.
2. Low magnification objectives with small aperture have a relatively large depth of field at object plane and extremely shallow depth of focus at the eyepiece or camera plane. That is why eyepiece setting is critical to being properly adjusted.

Field of View The field of view on a microscope determines the size of the imaged area. The size of the field of view depends on the objective magnification; the greater the magnification smaller the field of view. In an eyepiece-objective system, the field of view from the objective is magnified by the eyepiece for viewing. In a camera-objective system, that field of view is relayed onto a camera sensor. The maximum field of view of the microscope is affected by the objective lens, the tube-diameter of the microscope's internal optical system, the eyepieces, and the scientific camera sensor size. Equations (2.7) and (2.8) can be used to calculate the field of view in the aforementioned systems.

$$\mathrm{Field\ of\ View}_{\mathrm{Camera-Objective}} = \frac{\mathrm{Camera\ Sensor\ size}}{\mathrm{Magnification}_{\mathrm{objective}}}. \tag{2.7}$$

$$\mathrm{Field\ of\ View}_{\mathrm{eyepiece-Objective}} = \frac{\mathrm{field\ stop\ diameter}_{\mathrm{eyepiece}}}{\mathrm{Magnification}_{\mathrm{objective}}} \tag{2.8}$$

2.3 Optical Aberrations and Their Corrections

Aberrations in optical systems are often defined as the failure of getting a faithful image of an object. True diffraction-limited imaging is usually not achieved due to lens aberrations. Aberrations fall into two classes: monochromatic and chromatic. Monochromatic aberrations are caused by the geometry of the lens and the refraction of light through the lens. The monochromatic aberrations are: spherical aberration, coma, astigmatism, curvature of field, and distortion. Chromatic aberrations are caused by lens dispersion, the variation of a lens' refractive index with wavelength.

Spherical Aberration occurs when light waves passing through the periphery of a lens are not brought into identical focus with those passing near the centre. Light rays passing near the centre of the lens are refracted slightly, whereas rays passing close to the periphery are refracted to a greater degree resulting in different focal points along the optical axis (Fig. 2.8). The resolution of the lens system is degraded by this aberration because it affects the coincident imaging points along the optical axis, which will seriously affect specimen sharpness and clarity.

A simple way of reducing the spherical aberration is to place an aperture or lens stop over the entrance pupil of the lens to block out some of the peripheral rays. In biological imaging, the most common approach to correct spherical aberration is adjusting the objective correction collar which axially translates a movable lens group within the objective. The effect of spherical aberration in biological imaging has been corrected in several ways including altering the tube lens [8, 9], adjusting the rear pupil aperture of the objective lens [10], optimizing the immersion medium [11], and optical refocusing, which involves the use of multiple objectives lenses [12].

Chromatic Aberration is a result of the fact that white light consists of various wavelengths. When white light passes through a lens, the rays are refracted according to their wavelengths. Blue light rays are refracted with the greater angle followed by green and red light, this phenomenon commonly referred to as

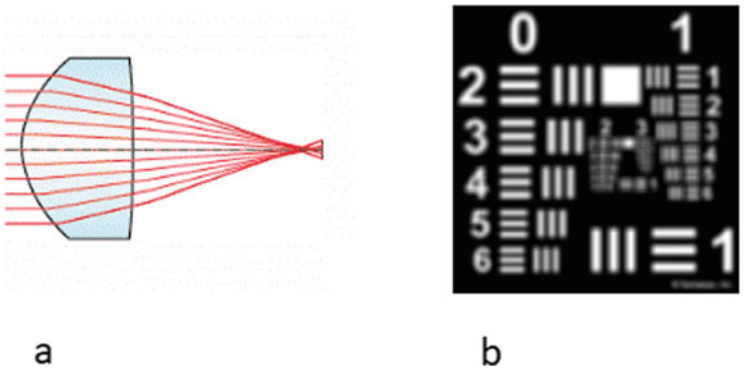

Fig. 2.8 (**a**) Representation of spherical aberration. (**b**) Simulation of spherical aberration by Zemax optical design software

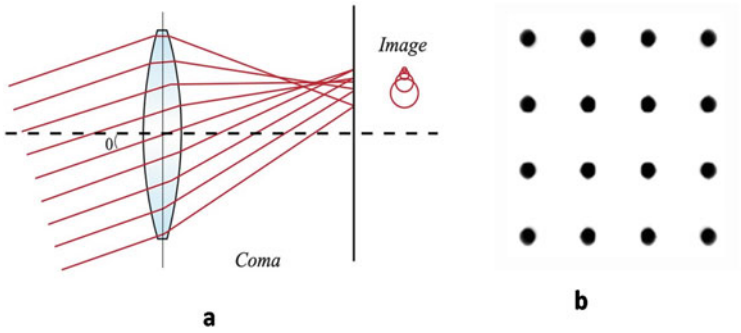

Fig. 2.9 (**a**) Representation of coma. (**b**) Simulation of coma by Zemax optical design software

dispersion. The inability of the lens system to bring all of the colours into a common focus results in the increase of image size and colour fringes surrounded the image.

By combining a special type of glass, crown glass and flint glass (each type has a different dispersion of refractive index) in the lens system it is possible to bring the blue rays (486 nm) and the red rays (656 nm) to a common focus, near but not identical with the green rays (550 nm). This combination is known as achromat doublet where each lens has a different refractive index and dispersive properties. This is the most widely used lens system in microscopes. The chromatic aberration is reduced in the doublet system by properly adjusting the lens thickness, curvature, and refractive index of glass.

Coma is an "off-axis aberration" that causes point objects to look like comets with a tail extending towards the periphery of the image plane (Fig. 2.9). Usually, coma affects the points located near the periphery of the image, resulting in a sharp image in the centre of the field and blurred towards the edges. When a bundle of oblique rays is incident on a lens, the rays passing through the edge of the lens may be focused at a different height than those passing through the centre. Coma is greater for lenses with wider apertures. Correction of this aberration is done by accommodating the object field diameter for a given objective.

Curvature of Field is another serious off-axis aberration. Field curvature indicates that the shape of the image plane is a concave spherical surface as seen from the objective (Fig. 2.10). In the infinity-corrected system, the field curvature is corrected by accommodating different lens systems (doublet, triplet) in the objective and tube lens.

Distortion is a monochromatic aberration produced mainly by the eyepiece in the microscope. Distortion changes the shape of the image while the sharpness maintained. If the image of an off-axis point is formed farther from the axis or closer to the axis than the actual image height given by the paraxial expressions, then the image is said to be distorted. The distortion provides a nonlinear magnification in the image from the centre to the edge of the field. Depending on whether the gradient in magnification is increasing or decreasing, the aberration is termed as pincushion or barrel distortion (Fig. 2.11). Corrections are made as described for field curvature.

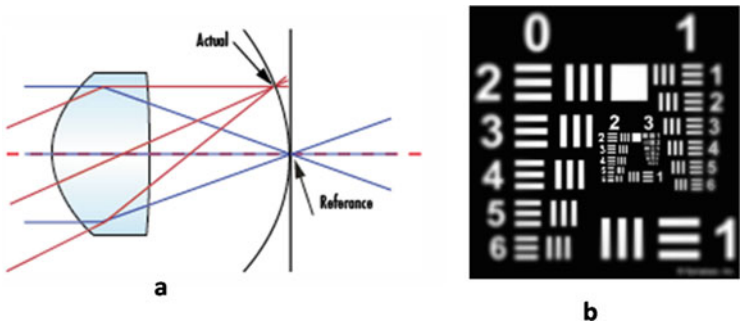

Fig. 2.10 (**a**) Representation of field of curvature. (**b**) Simulation of field of curvature

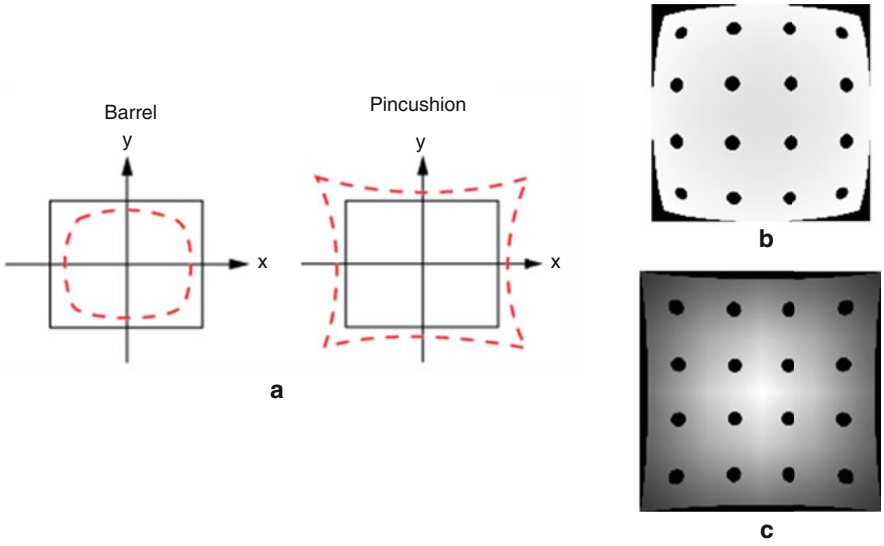

Fig. 2.11 (**a**) Representation of distortion. (**b**) Simulation of barrel distortion aberration. (**c**) Simulation of pincushion distortion aberration

Astigmatism Light rays lying in the tangential (T1) (planes contain chief ray and optic axis) and sagittal plane (S1) (planes contain only chief ray) are refracted differently. Therefore, both sets of rays intersect the chief ray at different image points, resulting in different focal lengths for each plane. This discrepancy in focal length is a measure of the astigmatism and will depend on the inclination angle of the light rays and the lens. The off-axis rays enter the optical system at increasingly oblique angles, resulting in larger focal length differences (Fig. 2.12).

These rays fail to produce a focused image point, but rather produce a series of elongated images ranging from linear to elliptical, depending upon the position within the optical train. Astigmatism errors are usually corrected in objectives through the precise spacing of individual lens elements with the appropriate choice

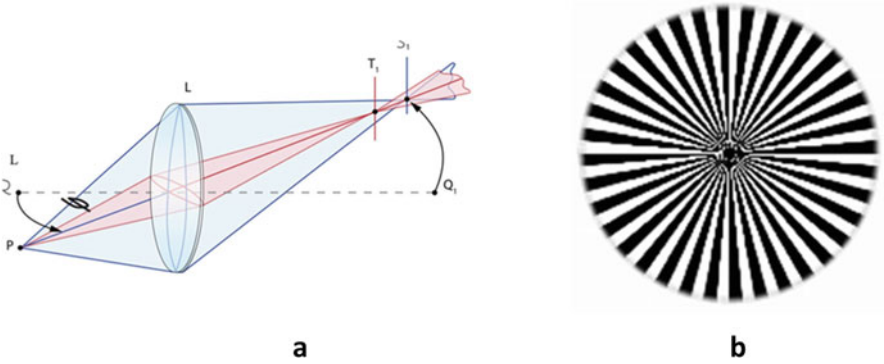

Fig. 2.12 (**a**) Representation of astigmatism. (**b**) Simulation of astigmatism

of lens shapes, aperture sizes, and indices of refraction. The correction of astigmatism is often accomplished in conjunction with the correction of field curvature aberrations.

2.4 Design Specifications of the Infinity-Corrected Microscope Objective

The objective is the eye of the microscope. Modern objective lenses are infinity-corrected, i.e. the object is placed in the front focal plane and its image is formed at infinity. Most compound microscopes have four or five objectives usually of 4×, 10×, 40×, and 100× (or 5×, 10×, 20×, 50×, 100×) which revolve on a nosepiece (turret) to allow different magnifying powers. The 4×, 10×, and 40× are generally "dry" objectives which operate with air imaging medium between the objective and the specimen. The 100× may be a "wet" objective which operates with immersion oil between the lens and the specimen. The three design specifications of the objective set the ultimate resolution limit of the microscope. These include the wavelength of light, the numerical aperture, and the refractive index of the imaging medium. Higher magnifications yield higher numerical apertures, but shorter working distances with smaller fields of view. Lower magnifications yield lower numerical apertures, but longer working distances with larger fields of view. In modern microscopes, both objectives and eyepieces are formed by many different groups of lenses; by assembling lenses in the right way, very high magnification values may be obtained [13]. Nikon CF (Chrome Free) and Zeiss Jena objectives are fully corrected so as not to require additional chromatic correction by tube lens or eyepiece.

The most common types of objectives are: plan achromats, plan apochromats, and plan fluorite. "Plan" designates that these objectives produce a flat image plan across the field of view. "Achromat" refers to the correction for chromatic aberration featured in the objective design. The achromats are colour corrected for two wavelengths red (656 nm) and blue (486 nm) and are corrected for spherical

aberration in the green wavelength (546 nm). Plan achromats are particularly used for monochromatic applications.

Plan apochromats are corrected for three or four wavelengths (red, green, blue, and violet), and the chromatic aberration is comparatively well corrected for other wavelengths. These objectives are corrected for spherical aberration for three or four wavelengths and have a high degree of flat field correction. They contain more lens elements than achromats. It is also possible to get very large NAs (up to 1.49) with this objectives design for high-resolution and low light applications. With the most effective colour correction and highest numerical apertures, plan apochromat objectives deliver brilliant images in bright field, DIC, and fluorescence techniques.

Fluorite or semi-apochromat objectives are made by glass materials, i.e. fluorite or fluorspar (CaF2) or synthetic lanthanum fluorite, giving high transmission and low colour dispersion. These objectives are corrected for chromatic aberrations at two wavelengths (red and blue) and spherical aberrations at two to three wavelengths.

The objective illustrated in Fig. 2.13a is 250× long working distance (LWD) infinity-corrected plan-apochromat objective, which contains 14 optical elements that are cemented together into three groups of lens doublets, a lens triplet group, and three individual internal single-element lenses. The objective has a hemispherical front lens and a meniscus second lens which allows to capture the light rays at high numerical aperture with minimum spherical aberration. The internal lens elements are carefully designed and properly oriented into a tubular brass housing that is encapsulated by the objective barrel. For infinity corrected objectives, an infinity symbol will be written on the body. The other parameters such as NA, magnification, optical tube length, refractive index, coverslip thickness, etc., are engraved on the external portion of the barrel (Fig. 2.13b).

The working distance is the distance between the surface of the front lens element of the objective and the top surface of the coverslip nearest to the objective. Working distance also depends on focal length and NA of the objective. Long working distance (LWD) objectives allow focusing over an extended range of up to several mm. For example, 40×/1.3 NA oil immersion objectives have a short working distance of just 0.2 mm, whereas 40×/1.0 LWD water immersion objective has a working distance of over 2 mm.

It is necessary to use a coverslip to protect the sample like bacteria, cell cultures, blood, etc., and microscope components from contamination. The light path from sample to objective depends on coverslip thickness and immersion medium. A cover slip, or glass microscope slide, affects the refraction angle of the rays from the sample. As a result, the objective needs proper optical corrections for coverslip thickness to provide the best quality image. Objective denotes a range of cover slip thicknesses for which they are optimized and it is imprinted after the infinity symbol (infinity-corrected design) on the objective barrel. The coverslip thickness ranges from zero (no coverslip correction) to 0.17 mm. The thickness of coverslip is different for upright and inverted microscope configuration. The upright microscope objective images the specimen between the coverslip and slide glass. The standardized value of the cover slip thickness for upright configuration is 0.17 mm

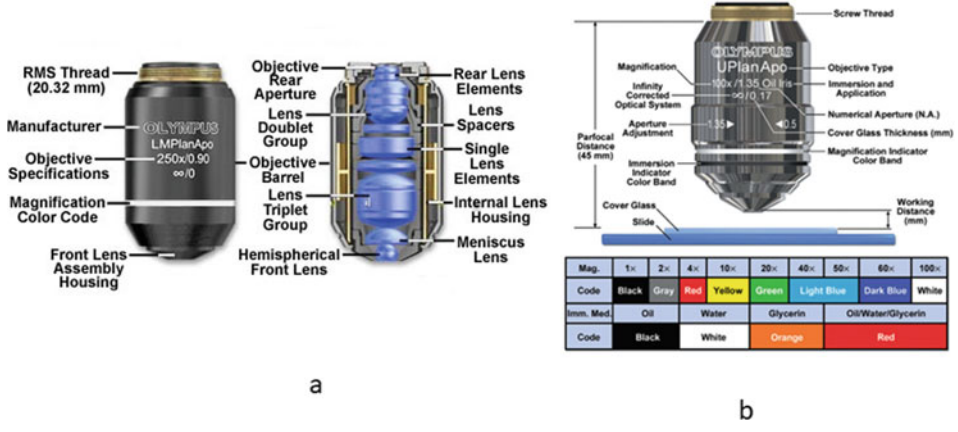

Fig. 2.13 (**a**) LWD infinity-corrected apochromat objective and its internal configuration (Source: Olympus Microscopy Resource Center). (**b**) NA, magnification, optical tube length, refractive index, and coverslip thickness are inscribed on the barrel of objective. The colour-coded ring, farthest from the thread, denotes the type of immersion medium (Source: D. B. Murphy and M. W. Davidson, Fundamentals of Light Microscopy and Electronic Imaging, Wiley-Blackwell 2012)

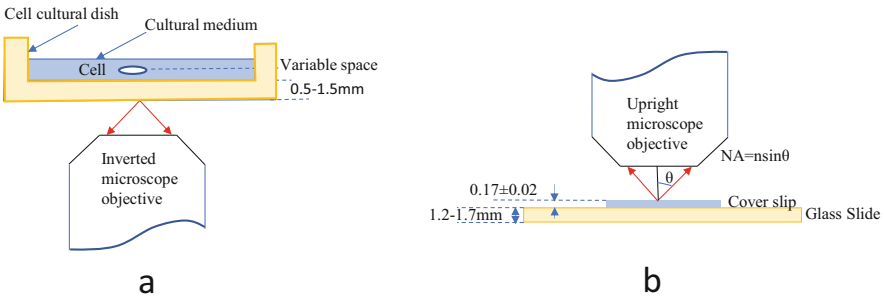

Fig. 2.14 (**a**) Inverted microscope observation. (**b**) Upright microscope observation

in biological applications (Fig. 2.14). The thickness of slide glass usually varies between 1.2 and 1.7 mm for different applications. The cover glass thickness typically has a tolerance of ±0.02 mm because the performance of the objective with NA of 0.95 is reduced by 71% for 0.02 mm thickness error [14]. Therefore, the objectives are designed in such a way so that it can also compensate for the induced coverslip aberration in the high NA system. If the objective has "–" (dash) inscribed on the barrel instead of 0.17 which indicates that the coverslip does not require to examine the sample. For example, the coverslip is not used for water glass dipping objectives.

The inverted microscope objective images the cell floating in the culture medium through the bottom of the cell culture dish. Therefore, the distance between the cell and the bottom surface of the dish is not fixed. The bottom thickness of the dish is varying between 0.5 and 1.5 mm. Furthermore, the inverted objectives often work with slide glass or without substrate. Consequently, the conventional inverted microscope objective with NA $>$ 0.4 must be designed with correction collar for large range (0–2 mm) of cover glass (CG) correction. So, the objective must be flexible for a large scale of working distance for the large correction range. Thus, the conventional inverted objectives were mostly designed with relatively longer working distance. The LWD objectives always bring with more difficult correction of chromatic aberration, spherical aberration, and coma.

Immersion objectives generally have higher NA greater than 1.0 and less coverslip aberration. Oil immersion objectives require the use of a drop of immersion oil between and in contact with the front lens of the objective and the cover glass of the sample. These are very common on both upright and inverted configuration. They need to be treated with care, in order that immersion oil does not drip down into the objective. Sometimes small plastic protective covers will be placed around them to catch excess oil. Water immersion objectives are designed to work best with a drop of water between the objective and specimen, while water-dipping objectives are designed to interface directly with the specimen and it has long working distance. These objectives are not suitable for inverted microscopes; they are usually used in upright microscopes, where they can dip directly into the culture dish. Immersion

media should never be used on dry objectives. This means that if immersion oil is on a sample, we cannot use a dry objective of more than 10× magnification on that sample without cleaning it, or the objective lens will touch the oil. Dry objectives have no protection shields against oil penetration and are easily destroyed. Note that if an immersion or dipping objective is used without the immersion medium, the image resolution becomes poor.

Another characteristic of the objective is Brightness. The ratio of NA to lateral magnification (M) determines the light gathering power (F) of an objective. The light gathering power determines the image *brightness* (*B*). *F* is defined as

$$\begin{aligned} F &= 10^4 \times \left(\frac{\mathrm{NA}}{M}\right)^2 (\text{Transmission mode}) \\ \text{and, } F &= 10^4 \times \frac{\mathrm{NA}^4}{M^2} (\text{Epi-illumination or reflection mode}). \end{aligned} \tag{2.9}$$

The 60×/1.4 NA apochromatic objective gives the brightest images because its image is well chromatic corrected across the entire visual spectrum and substantially free from other aberrations (flat field and spherical aberration), it is popular in fluorescence microscopy. The 40×/1.3 NA fluorite objective is significantly brighter, but is less well corrected. The brightness of the image does not only depend on the geometry of the lens, but also on the number of reflecting surfaces and the material of the optical glasses used to construct the lens elements in objective.

The last most significant parameter that influences the objective structure is the parfocal length, which is the distance between the object and the objective shoulder. The parfocal length basically determines the amount of space for integrating different lens elements. By designing microscope objectives with identical parfocal length, the focus position is fixed when changing the objectives with different magnifications. A system with smaller parfocal length typically has a smaller number of elements but more critical sensitivity. Utilizing longer parfocal length, although more elements are used, better tolerance and reduced cost could be achieved. 60×/1.48 45 mm parfocal objective from Olympus used two cemented triplets in the middle group, whereas the 60×/1.45 60 mm parfocal objective utilized four doublets. These two objectives have similar functionality in spherical and chromatic aberration correction. But the triplet setup could relatively save space, resulting in the overall length of microscope is reduced.

2.5 Critical and Köhler Illuminations

The first stage of the light microscope is the illumination unit. Illumination is a critical determinant of optical performance in the microscopy. Two different types of illumination systems are commonly employed in a standard microscope for the illumination: (1) *Critical* illumination, (2) *Köhler* illumination. With critical illumination, an incoherent source such as a filament lamp is used to illuminate the object through a condenser lens. As illustrated in Fig. 2.15 the image point S′ of the light

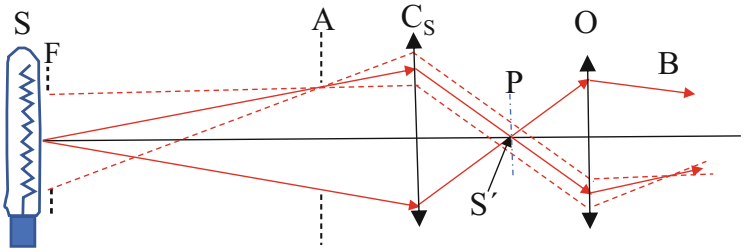

Fig. 2.15 Principal of critical illumination

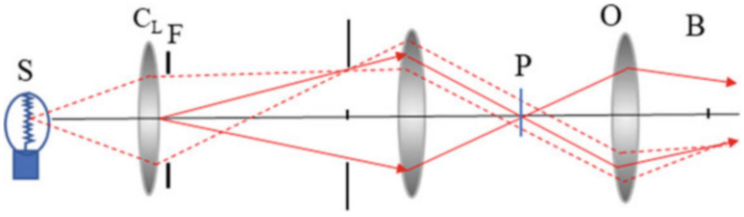

Fig. 2.16 Principal of Köhler illumination

source S is formed in the object plane P by the condenser lens (Cs). A field diaphragm F, placed close to the light source, controls the area of the illumination field, so it acts as a field stop. Simultaneously the iris diaphragm *A* in the front focal plane of the condenser controls the lights illuminating the object and entering the objective, i.e. it acts as an aperture stop. So, an image of the filament lamp is focused directly onto the sample in critical illumination system. A disadvantage of the critical illumination is that the source filament lamp is imaged onto the object plane and this type of source generates significantly highly non-uniform illumination.

In *Köhler illumination system* (Fig. 2.16) the light source and collector lens (C_L), and condenser lens (Cs) are responsible for establishing the primary illumination conditions for the microscope. In Köhler illumination, light from a source *S* is focused by a collector lens C_L onto the aperture diaphragm (A) that lies in the front focal plane of the condenser lens *Cs*. The light from this diaphragm passes through the object plane P as parallel rays inclined to the optic axis. These rays enter the microscope objective O and are brought to focus in its back focal plane B. Simultaneously the condenser forms an image of the field diaphragm F, which lies at the back focal plane of collector lens, in the object plane P. The field diaphragm controls the diameter of the light beam emitted by the illumination system before it enters the condenser aperture. This system allows to optimize light quality and resolution in the image plane by aligning and adjusting each component of this optical system. It minimizes internal stray light, and allows for control of contrast and depth of an Image.

The advantages of Köhler illumination are listed below:

- Only the specimen area viewed by a given objective/eyepiece combination is illuminated; no stray light or "noise" is generated inside the microscope.
- Even, uniform illumination of the specimen area is achieved by distributing the energy of each source point over the full field.
- Full control of the illumination aperture (condenser field diaphragm) provides for best resolution, best contrast, and optimal depth of field.

2.6 Components of Infinity-Corrected Microscope System

The infinity-corrected microscope typically consists of an illuminator (including the light source and collector lens), a substage condenser, specimen, objective, tube lens, eyepiece, and detector, which is either some form of camera or the observer's eye (Fig. 2.17). There are two configurations of compound infinity-corrected system, based on the positions of the light source and the objective. With an inverted microscope, the source for transmitted light and the condenser are placed on the top of the sample stage, pointing down towards the stage. The objectives are located

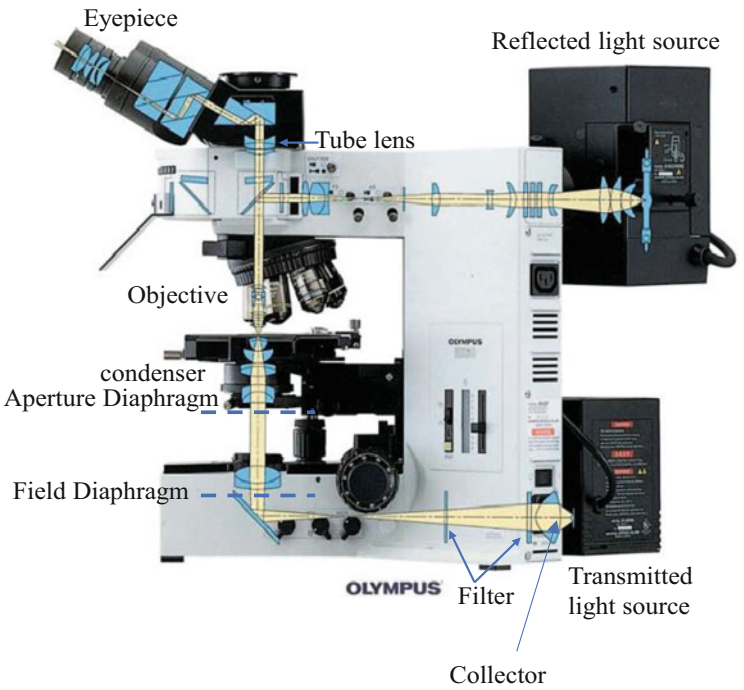

Fig. 2.17 Olympus upright microscope (Source: D. B. Murphy and M. W. Davidson, Fundamentals of Light Microscopy and Electronic Imaging, Wiley-Blackwell 2012)

below the sample stage pointing up. The cells are observed through the bottom of the cell culture vessel. With an upright microscope, the source of transmitted light and the condenser are located below the sample stage, pointing up and the objectives are placed on top of the stage, pointing down. The optical microscope design depends on two diaphragms to control the passage of light through the microscope. A diaphragm or *stop* is an opaque gate with a circular opening (often adjustable) that controls light flow through the microscope. Mainly, two diaphragms are utilized in the microscope: the *aperture diaphragm*, which adjusts the aperture angles within the microscope, and the *field diaphragm* that controls the dimension of the field imaged by the microscope. The primary role of diaphragms is to prevent light rays with aberration and stray light from reaching the image planes, and to balance the resolution against the contrast and depth of field of the image.

Light Source Most common light sources for optical microscopy are incandescent lamps, such as tungsten-argon and tungsten (e.g. quartz halogen) lamps. A tungsten-argon lamp is popular for bright field, phase contrast, and some polarization imaging. Halogen lamps are less costly and a convenient choice for a variety of applications that require a continuous and bright spectrum.

Xenon (XBO) and mercury (HBO) arc lamps are usually brighter than incandescent lamps but these are difficult to align and more expensive. Arc lamps are appropriate for high-quality monochromatic illumination when it is combined with the appropriate filter. Their spectral range starts in the UV range and continuously extends through visible to the infrared. Another popular light source is the gas-arc discharge lamp, which includes mercury, xenon, and halide lamps. About 50% of the spectral range of mercury arc lamp is located in the UV range. For imaging of biological samples using mercury arc lamp, the proper selection of filters is important to protect living cell samples and micro-organisms from the UV rays. (e.g., UV-blocking filters/cold mirrors). The xenon arc lamp can provide an output power greater than 100 W, and is often used for fluorescence imaging. However, over 50% of its power falls into the IR; therefore, IR-blocking filters (hot mirrors) are necessary to prevent the overheating of samples. Metal halide lamps were recently introduced for high-power sources (over 150 W). Light-emitting diodes (LEDs) are a new, alternative light source for microscopy applications. The characteristic features of LEDs include a long lifetime, a compact design, high efficiency, and easy to align.

Filter Microscopy filter is mainly used to increase the contrast of the image by allowing or blocking selective wavelengths of light. Two common types of filters are absorption filter and interference filter. Absorption filters normally consist of coloured glass which selectively absorbs wavelengths of light and transfers the energy into heat. The interference filters selectively transmit the wavelengths based on the interference effect. The other filters are neutral density (ND) filters that reduce the light intensity without changing wavelength and heat filters that absorb the infrared radiation to prevent the specimen from heating.

Collector Lens This lens is used to create an image of the filament onto the front focal plane of the condenser lens (Kohler illumination) or onto the specimen itself (critical or confocal illumination). The diameter of the field illuminated by the light source is controlled by the field diaphragm which is placed just behind the collector lens.

Condenser Imaging performance by a microscope depends not only on the objective lens but also on the light delivery system, which includes the illuminator, collector lens, and condenser lens. There are three types of condenser lens: (1) Abbe condenser, (2) Aplanatic condenser, and (3) Achromatic aplanatic condenser. The Abbe condensers have two lenses, and they are usually uncorrected for spherical and chromatic aberrations. The three-lens aplanatic condenser is superior to Abbe condenser. This type of condenser is corrected for spherical aberration and field curvature but still exhibits chromatic aberration. The highly corrected achromatic aplanatic condenser has five lenses including two achromatic doublet lenses, provides NAs up to 1.4, and is essential for imaging fine details using immersion-type objectives. These condensers are corrected for chromatic aberration at red and blue wavelengths, spherical aberration at green wavelength, and field curvature. Achromatic aplanatic condenser is suitable for both types of objectives (dry and oil immersion). Note, however, that for maximal resolution, the NA of the condenser must be equal to the NA of the objective, which requires that both the condenser and the objective should be oiled.

Tube Lens To create an image with an infinity-corrected objective, a tube lens must be used to focus the image. A typical infinity-corrected microscope employs a doublet pair as a tube lens (Fig. 2.18). The first doublet is Plano convex. It provides three features: optical power, correction of spherical aberration, and correction of axial colour. The second doublet is a meniscus lens with little optical power. It provides correction of coma and lateral colour. The distance between the objective and the tube lens (L) can be varied, but this will affect the image field diameter. In infinity-corrected system the tube lengths between 200 and 250 mm are considered optimal, because longer focal lengths will produce a smaller off-axis angle for

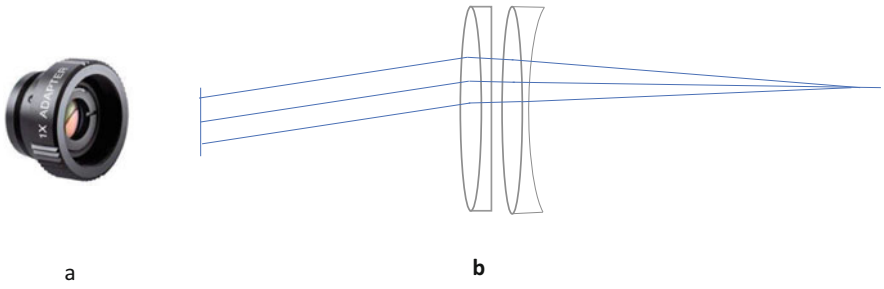

Fig. 2.18 (**a**) Standard 1× tube lens. (**b**) Doublet-pair tube lens layout

Fig. 2.19 Ultra-wide 10× eyepiece with its internal lens system

diagonal light rays, reducing system artefacts. Longer tube lengths also increase the flexibility of the system with regard to the design of accessory components.

Eyepieces The eyepieces are the multi-lens system at the top of the microscope that the viewer looks through; they are usually 10× or 15×. The magnification of an eyepiece is defined as 25 cm divided by the focal length of eyepiece. The eyepiece illustrated in Fig. 2.19 is marked with UW, which suggests it is an ultra-wide view field. Often eyepieces will have an H designation, which indicates a high eyepoint focal plane that permits microscopists to wear glasses to look at samples. Other inscriptions often found on eyepieces include WF for Wide-Field; UWF for Ultra-Wide-Field; SW and SWF for Super Wide-Field; HE for High Eye point; and CF for Chrome Free. The eyepiece magnification in Fig. 2.19 is 10× and also the inscription A/24 indicates the field number is 24, which refers to the diameter (in millimetres) of the fixed diaphragm in the eyepiece. Good eyepiece should also have the widest possible field of view. This is often helpful in estimating the actual size of objects. The field-of-view numbers vary from 6.3 to 26.5.

Digital Camera Nowadays a charged coupled device (CCD) camera is used to capture and store the images in microscopy. The CCD consists of a large matrix of photosensitive elements (referred to as "pixels") that capture an image over the entire detector surface. The incident light-intensity information on each pixel is stored as an electronic charge and is converted to an analogue voltage by a readout amplifier within CCD. This analogue voltage is subsequently converted to a numerical value by a digitizer in CCD chip resulting in the visualization of the digital image in the computer. The spatial and brightness resolution of digital image gives the information of fine details that were present in the original image. The spatial resolution depends on the number of pixels in the digital image. By increasing the number of pixels within the same physical dimensions, the spatial resolution becomes higher. The digital spatial resolution should be equal or higher than the optical resolution,

i.e. the resolving power of the microscope. To capture the smallest degree of detail, two pixels or three pixels are collected for each feature. This criterion is called as Nyquist criterion, is expressed by this equation: $R * M = 2 *$ pixel size (ref), where R is the optical resolution of the objective; M is the resulting magnification at the camera sensor and it is calculated by the objective magnification multiplied by the magnification of the camera adapter. Consider a 10× Plan Apochromat objective having NA 0.4 and the wavelength of the illuminating light is 550 nm, so the optical resolution of the objective is $R = 0.61 * \lambda/\text{NA} = 0.839$ μm. Assuming further that the camera adapter magnification is 1×, so the resulting camera magnification $M = 10\times$. Now, the resolution of the objective has to be multiplied by a factor of 10 to calculate the resolution at the camera, i.e. $R \times M = 0.839$ μm * 10 = 8.39 μm. Thus, in this setup, we have a minimum distance of 8.39 μm at which the line pairs can still be resolved, this is equivalent to 1/8.39 = 119-line pairs per millimetre. The pixel size is calculated by the size of the CCD chip or CMOS sensor divided by the number of pixels. If 0.5-inch chip has a dimension of 6.4 mm * 4.8 mm, the total number of pixels for this chip needs to meet the Nyquist criterion with 2 pixels per feature is $(1/(R \times M)) \times$ chip size × 2 = 119 line pairs/mm × 6.4 mm × 2 = 1526 pixels in horizontal direction and 1145 pixels in vertical direction. If we take 3 pixels per line pair, the result is 2289 pixels in horizontal direction. The system with a higher magnification objective has a small field of view so the number of pixels is reduced.

2.7 Alignment for Designing the Infinity-Corrected Bright Field Microscopy

Two basic types of microscopic optical illumination are possible: those using reflected light (episcopic or epi-illumination) and those using transmitted light (diascopic). In transmitted illumination system light allows to pass through the specimen, whereas in reflection illumination method light reflects from the specimens. Reflected light microscopy is used for imaging the opaque specimens, e.g. metals, minerals, silicon wafers, wood, polymers, and so on and transmitted light microscopy is for transparent samples such as bacteria, cell, etc. Today, many microscope manufacturers offer advanced models that permit the user to alternate or simultaneously conduct investigations using both reflected and transmitted illumination.

Figure 2.20 shows the two optical ray paths of imaging and illuminating for bright field transmissive infinity-corrected configuration. The design of a microscope must ensure that the light rays are precisely guided through the microscope. The knowledge of optical ray paths under Köhler illumination is important for proper designing and aligning the optical microscope [15]. Generally, the microscope contains two groups of optical planes which belong together. Within a group, the planes are always imaged one on the other, so they are known as conjugate planes. The first group of conjugate planes in the path of illuminating light rays includes the lamp filament, aperture diaphragm, the rear focal plane of the objective, and pupil of

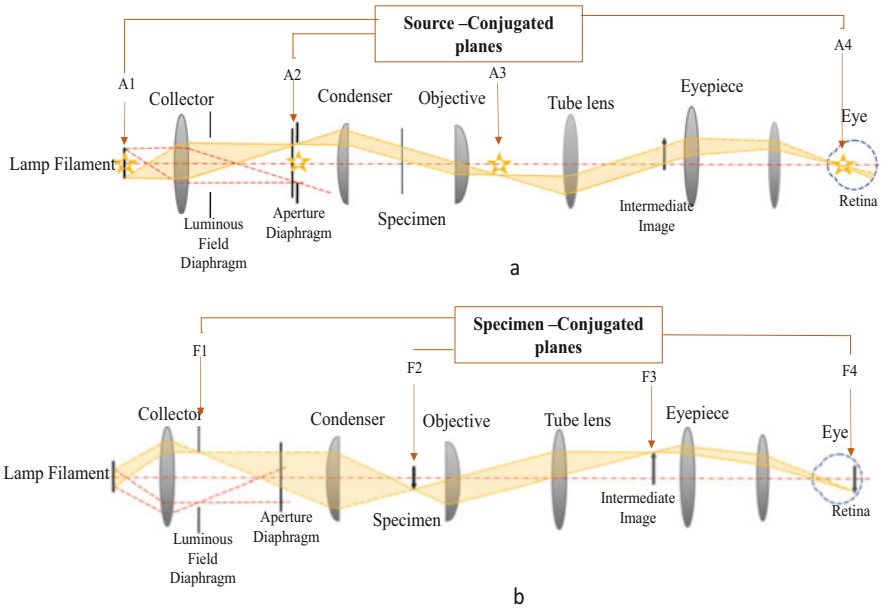

Fig. 2.20 (**a**) Illuminating light path consists of four conjugate planes A1, A2, A3, A4, known as source-conjugated planes. (**b**) Image-forming light path has four conjugate planes F1, F2, F3, F4, known as specimen-cojugated planes

observer's eye. As illustrated in Fig. 2.20, in the illuminating light ray path, from the light source to eyepoint, there are four images of the light source. These are known as source-conjugated planes, i.e. A1, A2, A3, A4. The final source image in the exit pupil of the eyepiece is located in the same plane as the entrance pupil of the observer's eye. The second group of conjugate planes in the image-forming light path includes the luminous field diaphragm, the specimen, the intermediate image plane, and the retina of the eye. Thus, from field stop to final image, there are again four specimen-conjugated planes, i.e. F1, F2, F3, F4, respectively.

Generally, these two conjugate groups occur simultaneously in microscopy. Suggested by Hammond [16], the complete symmetrical ray diagram, combing two conjugate groups, is shown in Fig. 2.21. Under the Köhler illumination condition the lamp collector and auxiliary lenses focused the illuminating rays to form an image of the filament (A1) in front of the condenser. The focused illuminating rays, from the conjugate plane A2, incident on the object as a series of parallel bundles of light and converged at the back focal plane of the objective (conjugate plane (A3)). The final image of filament is produced at the exit pupil of the eyepiece (conjugate plane (A4)). In the specimen conjugate optical path, the light rays from the filament (as shown in fig the green, red, and blue rays) is focused at different points in the field diaphragm plane (F1) which is front focal plane of the auxiliary lens. The parallel bundle of rays generated from the auxiliary lens is focused on the specimen plane (F2) in front of the objective. The infinity-corrected optics, the objective and

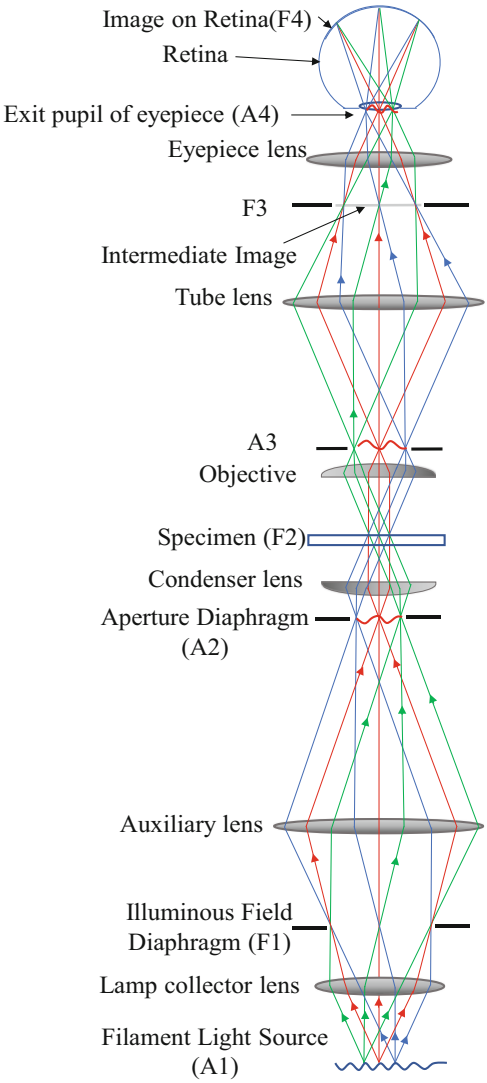

Fig. 2.21 Optical ray diagram of transmissive configuration in bright field mode (Source: C. Hammond, "A symmetrical representation of the geometrical optics of the light microscope," Journal of Microscopy)

the tube lens, generate the intermediate image (F3) at front focal plane of the eyepiece and the final image of specimen is formed on observer's retina (F4). Under this alignment of microscopy if we decrease the size of the field diaphragm, a narrow bundle of rays will illuminate a smaller region of the specimen. If we decrease the size of the aperture diaphragm, then a smaller area of the filament contributes to the illumination at the object and the angles of aperture of the condenser will be smaller which causes a decrease in resolution and increase in contrast. Figure 2.22 shows the ray diagram in epi-illumination or reflection mode of microscopy. In reflection configuration, the positions of the field and aperture

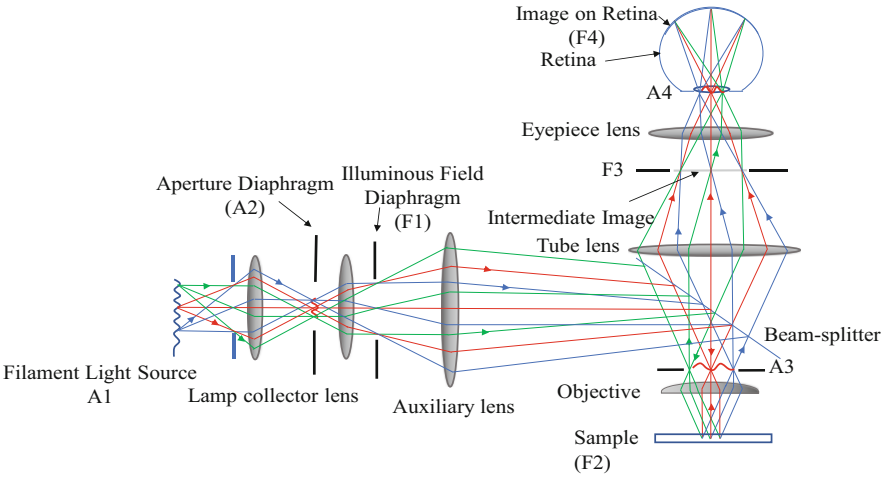

Fig. 2.22 Optical ray diagram for reflection configuration in bright field mode (Source: C. Hammond, "A symmetrical representation of the geometrical optics of the light microscope," Journal of Microscopy)

diaphragms are reversed and the objective performs the dual operations which are focusing the illuminating lights onto the specimen and collecting the imaging lights from the specimen. Fluorescence microscopy is usually performed using reflected light, even on microscopes where the bright field examination is done using transmitted light.

The fundamental step to get a good image in the microscope is to align the illuminating system correctly. The alignment of the illumination system depends on three factors: (1) Proper adjustment of field diaphragm, (2) Focusing and centre of condenser, and (3) Adjusting aperture diaphragm. The Steps for alignment in the illumination system in bright field (BF) observation mode are:

1. Open the aperture diaphragm and field diaphragm.
2. Place the 5 to 10× objective and focus the specimen.
3. Set intensity to a comfortable level (varying lamp intensity setting and/or neutral density filters).
4. Adjust the interpupillary distance of the eyepiece.
5. Close down the field diaphragm until its image is just visible in the field of view.
6. Rotate the condenser height adjustment knob to bring the field iris diaphragm image into focus, along with the specimen.
7. Centre the condenser to align the image of the field diaphragm concentric with the circular field of view.
8. Open the field diaphragm lever until its image inscribes the field of view. If using a camera, the field diaphragm should be adjusted to disappear just beyond the field of view of the camera image.

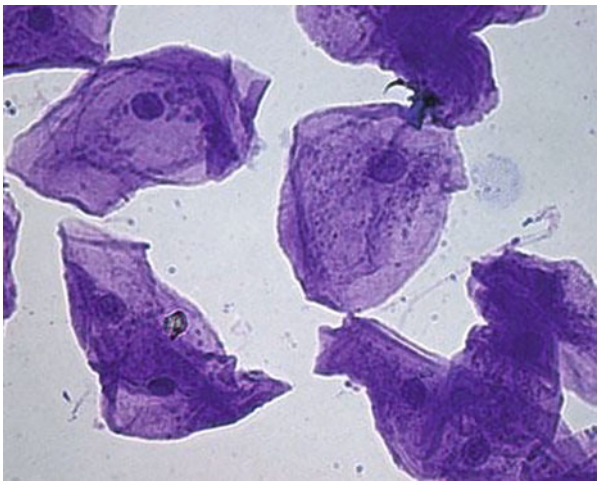

Fig. 2.23 Bright field microscopy image of stained cheek cell (Source: M.K. Kim, Digital Holographic Microscopy: Principles, Techniques, and Applications, Springer Series in Optical Sciences)

9. For optimal imaging the adjustment of aperture diaphragm is the last step of alignment. The aperture diaphragm is not able to be imaged directly through the microscope eyepieces or to a camera as it is in an intermediate plane in the optical path. By using phase telescope or Bertrand lens or removing the eyepiece the aperture diaphragm is possible to image. Normally, if the aperture diaphragm is closed to 70–80% of the numerical aperture of the objective, then a clear image with reasonable contrast will be obtained. The scale on the condenser aperture diaphragm ring shows numerical aperture, so adjust the condenser aperture diaphragm ring in accordance with the scale. Once we are able to see the aperture stop adjust it to the proper size and return the eyepiece or remove the Bertrand lens for normal imaging (Fig. 2.23).

2.8 Label-Free and Quantitative Phase Microscopy

Unstained biological samples, such as bacteria or cells, are phase objects/samples. This type of object does not absorb incident light, it only alters the phase of light. Conventional bright field microscopy gives only the information about the intensity changes or amplitude changes not the phase changes introduced by the object. The human eye also relies on changes in amplitude of a light wave, cells can be difficult to visualize using a light microscope without dyes/labels which enhances cell contrast. Therefore, phase sample or unstained (without dyes/labels) biological samples are problematic for analysing in conventional bright field microscopy. *S*uch samples may be either transmissive or reflective in nature [17]. Rather than using contrast-enhancing dyes/labels, label-free solutions rely on components of the optical setup that use cells' inherent contrast characteristics (thickness and refractive index (RI)) to create image contrast.

Here some of the popular label-free imaging techniques such as dark field illumination, phase contrast, differential interference contrast, digital holographic microscope are discussed.

2.8.1 Dark Field Microscopy

Dark field illumination requires blocking out the central zone of light rays and allowing only oblique rays to illuminate the specimen. This is a simple and popular method for imaging unstained specimens, which appear as brightly illuminated objects on a dark background.

Dark field conditions are created when bright field light from the source is blocked by an opaque dark field stop (annular stop) in the condenser. This stop must be of sufficient diameter to block the direct light (zeroth order illumination) passing through the condenser, but it must also be open around the edges, letting light pass by the outside of the stop. So, a hollow cone of light from the condenser lens illuminates the specimen. With the direct light blocked from entering the objective, the central zone of light fails to reach at image plane causing the background field of view becomes black instead of white. Only the interference of scattered light from the specimen contributes to image formation. When, objects, e.g., small particles of bacteria, are in the object plane, light is laterally diffracted away. Provided that this diffracted lights within the aperture cone of the objective, it is gathered by the objective and forms an image. The object becomes brightly visible in front of a dark background. If there is no sample, the image seen in the eyepieces remains completely dark. For dark field microscopy, it is necessary for the objective aperture to be smaller than the inner aperture of the condenser.

To design a dark field microscope, we need standard light source (halogen lamp or LED), condenser with dark field stop, infinity-corrected objective, tube lens, eyepiece, and CCD camera. The turret condenser is the best condenser option because the dark field stop is placed in exactly the same location as the condenser aperture as illustrated in Fig. 2.24.

While specimens may look washed out and lack detail in bright field, protists, metazoans, cell suspensions, algae, and other microscopic organisms are clearly

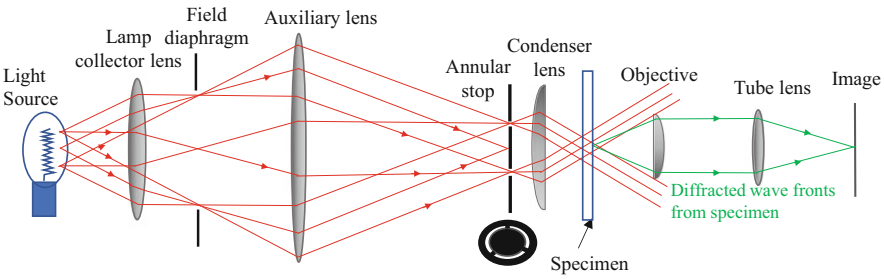

Fig. 2.24 Principal of dark field microcopy

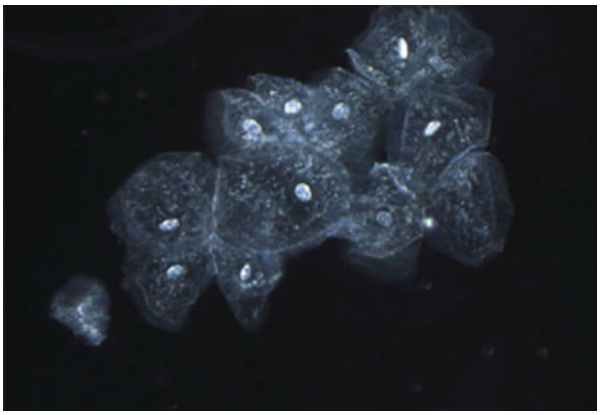

Fig. 2.25 Dark field microscopy image of cheek cells (Source: M.K. Kim, Digital Holographic Microscopy: Principles, Techniques, and Applications, Springer Series in Optical Science)

distinguished in dark field mode. Using 100× objective in darkfield mode we can see bacteria and some structures (rods, curved rods, spirals, or cocci) and their movement.

Alignment steps in transmitted dark field condenser:

1. Engage the 10× objective and bring the specimen into focus.
2. While looking through the eyepieces and using the condenser height adjustment knob, carefully adjust the height of the condenser until a dark circular spot becomes visible.
3. Turn the condenser centring screws to move the dark spot to the centre of field of view. This completes the centration.
4. Engage the desired objective. Using the condenser height adjustment knob, adjust until the dark field spot is eliminated and a good dark field image is obtained (Fig. 2.25).

2.8.2 Zernike Phase Contrast Microscopy

According to Ernst Abbe, a microscope objective can form the image of an object, by superposing all the diffracted object beams in the image plane. Basically, the resultant image is an interference pattern generated by the diffracted beams. Frits Zernike (Nobel prize in Physics, 1953) invented phase contrast microscopy using Abbe's image formation theory (Fig. 2.26).

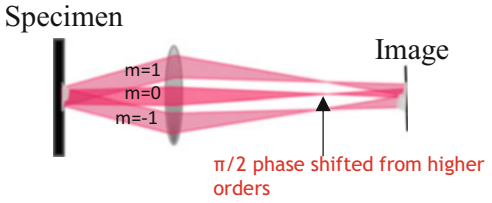

Fig. 2.26 Abbe principle for image formation in Coherent illumination (m=diffraction order)

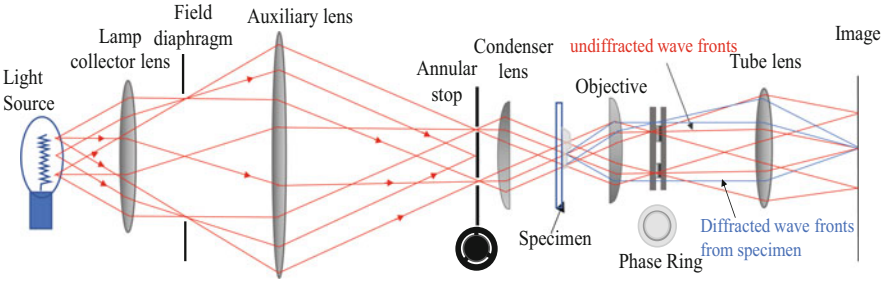

Fig. 2.27 Schemetic diagram of Zernike phase contrast microscopy

For phase contrast microscopy two elements are needed. One is an annular aperture insert for the condenser, the other is special objectives that contain a phase plate. Light incident on a specimen emerges as two components: an un-diffracted wave and a diffracted wave that pass through the specimen. The diffracted wave is phase shifted by an amount δ that depends on the RI of the medium (n_1), and the specimen (n_2) along with specimen thickness t. The optical path difference $(\Delta) = (n_1 - n_2) \cdot t$, the phase shift δ is

$$\delta = \frac{2\pi\Delta}{\lambda}. \tag{2.10}$$

The refractive index of a cell is usually ~1.36. The phase shift is introduced by the cell is nearly equal to $\lambda/4$. The spatially separated diffracted and un-diffracted wave from the object traverse through the objective. A phase plate introduced in the back focal plane of objective is used to modify the relative phase and amplitude of these two waves. The phase plate then changes the un-diffracted light's speed by $\lambda/4$, so that this wave is advanced or retarded by $\lambda/4$ with respect to the higher order diffracted waves. The total $\lambda/2$ phase difference introduced between the two waves [3]. Thus, when the two waves come to focus together on the image plane, they interfere destructively or constructively (Fig. 2.27).

There are two forms of phase contrast: positive and negative phase contrast (Fig. 2.29). They mainly differ by the phase plates used for illumination. In positive phase contrast the narrow area of the phase plate is optically thinner than the rest of the plate. The un-diffracted light passing through the narrow area of phase ring travels a shorter distance resulting its phase is advanced compared to diffracted light. This causes the details of the specimen to appear dark against a lighter background, and so is called positive or dark phase contrast. In negative phase contrast the ring phase shifter is thicker than the rest of the plate, the un-diffracted wave is retarded in phase. The image appears bright on a darker background for negative or bright contrast. This is much less frequently used.

The central component of a phase contrast microscope is the phase ring. Usually it is composed of a neutral density filter and a phase retardation plate. The portion of light that passed the specimen without experiencing diffraction passes the phase ring

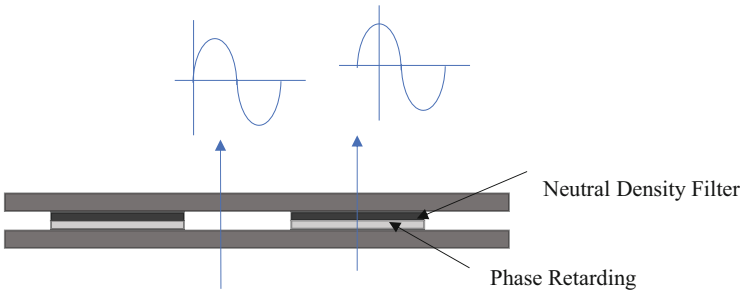

Fig. 2.28 Structure of phase ring

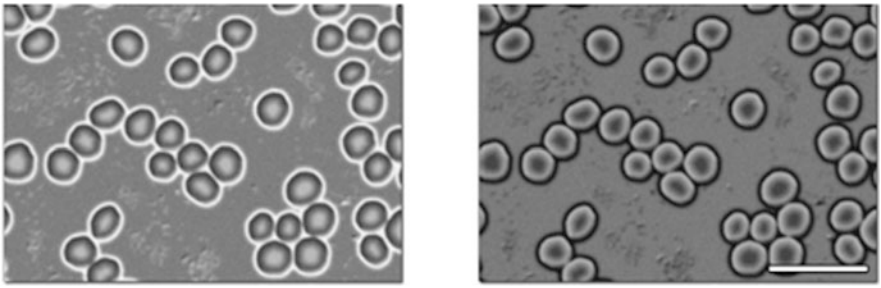

Fig. 2.29 Images of erythrocytes in positive and negative contrast optics (Source: D. B. Murphy and M. W. Davidson, Fundamentals of Light Microscopy and Electronic Imaging, 2 edition. Wiley-Blackwell, 2012)

(right arrow). The neutral density filter reduces the light intensity to avoid irradiation. The phase retardation plate retards the phase of the non-diffracted light to allow interference with the light waves that experienced phase shift and diffraction by passing the specimen (left arrow) (Fig. 2.28).

Limitations of Phase Contrast System

1. The phase contrast microscope, however, has some problems with its image quality. One is the so-called halo effect. This effect causes spurious bright areas around phase objects or reverse contrast images. Halos form because the low spatial frequency wave fronts, diffracted by the specimen traverse the phase ring as well. The absence of destructive interference between these diffracted wave fronts and un-diffracted light waves produces a localized contrast reversal (manifested by the halo) surrounding the specimen. These halos are optical artefacts and can make it hard to see the boundaries of details.
2. Another problem in phase contrast microscopy can be contrast inversion. If the objects are thick with very high refractive index, they will appear brighter instead of darker (for positive phase contrast). In such regions the phase shift is not the usual shift of $\lambda/4$ for biological specimens, and instead of destructive interference, constructive interference occurs (opposite for negative phase contrast).

Alignment of Phase Contrast Microscopy

1. Set up the Köhler illumination in microscope.
2. Install the phase ring in the condenser.
3. Remove one of the eyepieces and replace these with the phase contrast cantering telescope.
4. Put the phase contrast telescope into focus, so that the phase plate of objective and phase ring are in focus. Observe a sharp image of the phase ring in the back focal plane of objective.
5. Put the lowest magnification phase objective and corresponding phase annulus in place. For example, a 10× Ph1 objective with a Ph1 phase annulus (low magnification objectives have large diameter phase annuli (normally inscribed "Ph1" for "phase 1" and suitable for 5× or 10× objectives); intermediate magnification objectives have Ph2 annuli (e.g. 20× and 40× objectives) and the 60× or 100× objectives have the smallest diameter annuli, generally inscribed "Ph3").
6. Look at the phase plate and phase ring through the phase telescope.
7. Use the centering screws for the condenser inserts to centre the phase contrast ring, so that the bright ring overlaps the dark ring within the field of view (Fig. 2.30). If the phase ring and annulus are slightly misaligned (rotate the turret slightly), the background light intensity increases, and the quality of the phase contrast image falls.
8. Repeat the steps 5, 6, 7 for each phase and contrast ring set.
9. Once the centering operation is complete, remove the centering telescope and replace it with the eyepiece.

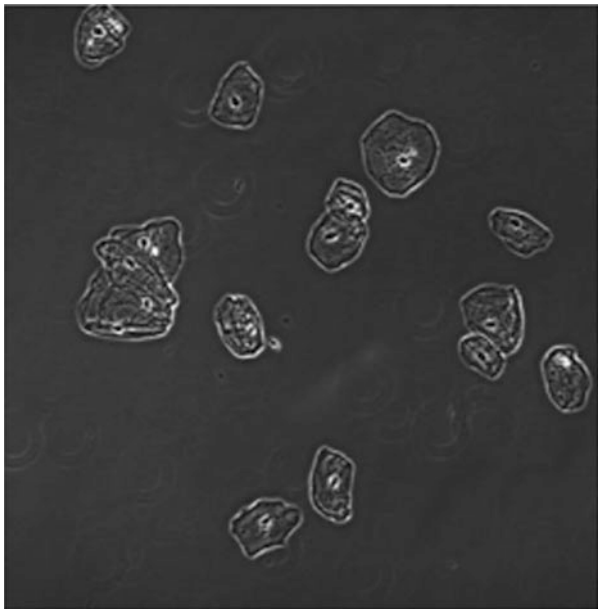

Fig. 2.30 Cheek cell image using phase contrast microscopy (Source: M.K. Kim, Digital Holographic Microscopy: Principles, Techniques, and Applications, Springer Series in Optical Sciences)

10. Focus the image with the fine focus of the microscope.
11. Widen the field iris diaphragm opening until the diaphragm image circumscribes the field of view.

2.8.3 Differential Interference Contrast (DIC) Microscope

Differential interference contrast (DIC) microscope uses the dual beam interference mode where the light beam from sample is replicated and sheared by the passage through specially designed Wollaston prism (or Nomarski prism). When these two identical and mutually coherent sample beams are made to overlap in the image plane with small shear between them, minute phase variations are visualized in white light illumination as graded and colourful intensity variations. The difference between DIC and phase contrast microscopy is discussed in Table 2.1.

The major advantage of DIC over phase contrast is that the full aperture of the microscope is used. In phase contrast the condenser's annular stop restricts the aperture, and therefore the resolution of the system. Compared with phase contrast images, differential interference contrast: (1) produces high-resolution images, (2) has better contrast, (3) can be used with thick specimens, and (4) lacks the distracting halo of phase contrast.

The main element in DIC microscope is Wollaston prism. Wollaston prism is a polarizing beam-splitter made of quartz or calcite (which are birefringent, or doubly-refracting materials). This device splits the light ray into two linearly polarized rays, and the resulting rays vibrate perpendicular to each other. One of the waves is designated the *ordinary* (*O*) wave and vibrates in a direction perpendicular to the optical axis of the prism, while the other is termed the *extraordinary* (*E*) wave with a vibration direction parallel to the prism optical axis.

Table 2.1 Difference between DIC and phase contrast technique

Study	DIC observation	Phase contrast observation
How contrast is added	Contrast added by gradients in sample thickness	Contrast added at sample borders or points
Image features	Bright/dark or colour contrast added, conveying a three-dimensional appearance Shadows added depending on orientation	Bright/dark contrast added Pronounced halo around thick samples
Contrast adjustment and selection	Fine adjustment of three-dimensional contrast possible	Choice of negative or positive contrast
Suitable sample	Capable of observing structures with sizes ranging from minimum to large. Sample thickness up to several 100 μm	Useful for observing minute structures Sample thickness up to 10 μm
Resolution	High	Poor compared to DIC

This is briefly how Nomarski DIC images are produced:

1. Light passes through a standard polarizer before entering the condenser, producing plane-polarized light at a 45-degree angle with respect to the optical axes.
2. This light enters a Wollaston prism situated in the front focal plane of the condenser. The two wavefronts, ordinary and extraordinary, are separated by a very small difference (less than the resolution of the system). A separation like this is called shearing and is one of the most important features of the system.
3. The two wavefronts pass through the specimen and are retarded to varying extents in doing so.
4. The light now enters a second Wollaston prism which recombines the wave fronts. If there has been a phase shift between the two rays as they pass through areas of different refractive index, then elliptically polarized light is the result.
5. Finally, the light enters a second polarizing filter, termed an analyser. The initial polarizer and this analyser form a crossed polarized light. The analyser will permit the passage of some of the elliptically polarized light to form the final image (Figs. 2.31 and 2.32).

Basic Components of DIC

Condenser: The condensers designed for DIC usually have a built-in polarizer. This can be slid out of the light path for bright field illumination. The polarizer can fully be rotated, but is marked to permit correct east-west orientation and a locking screw is provided. The main body of the condenser is the rotating, phase contrast type.

Wollaston Prism: In DIC each Wollaston prism consists of two precision made wedges of quartz, cemented together so that their axes of birefringence are at right angles to each other. The prism itself is mounted in a circular cell. These prisms are specific for the objectives to be used, so if DIC observation at 10×, 40×, and 100× is required, then three matching prisms need to be installed.

Objectives: Theoretically any objectives can be used, but in practice higher grade objectives (fluorite and apochromatic types) are generally specified to benefit from the high-resolution potential. In many cases phase contrast fluorite objectives are chosen, permitting bright field, DIC, phase contrast, and fluorescence observation with a single set of objectives.

DIC slider: The second Wollaston prism arrangement is a slider fitted above the objectives but below the tube lens. In this case only one prism is required, and it is provided with a means of sliding it across the light path. The DIC slider is orientated northwest–southeast, i.e. diagonally in the light path.

Analyser: The output polarizer in the system, termed the analyser, is installed above the DIC slider. The polarizer and analyser need to be aligned so that their transmission axis is orthogonal to each other.

Alignment of DIC

1. In place of the condenser used for bright field observation, a “universal condenser” fitted with a built-in polarizer and a DIC prism are required.
2. A “DIC prism (DIC slider)” and an “analyser” are required below the objective.

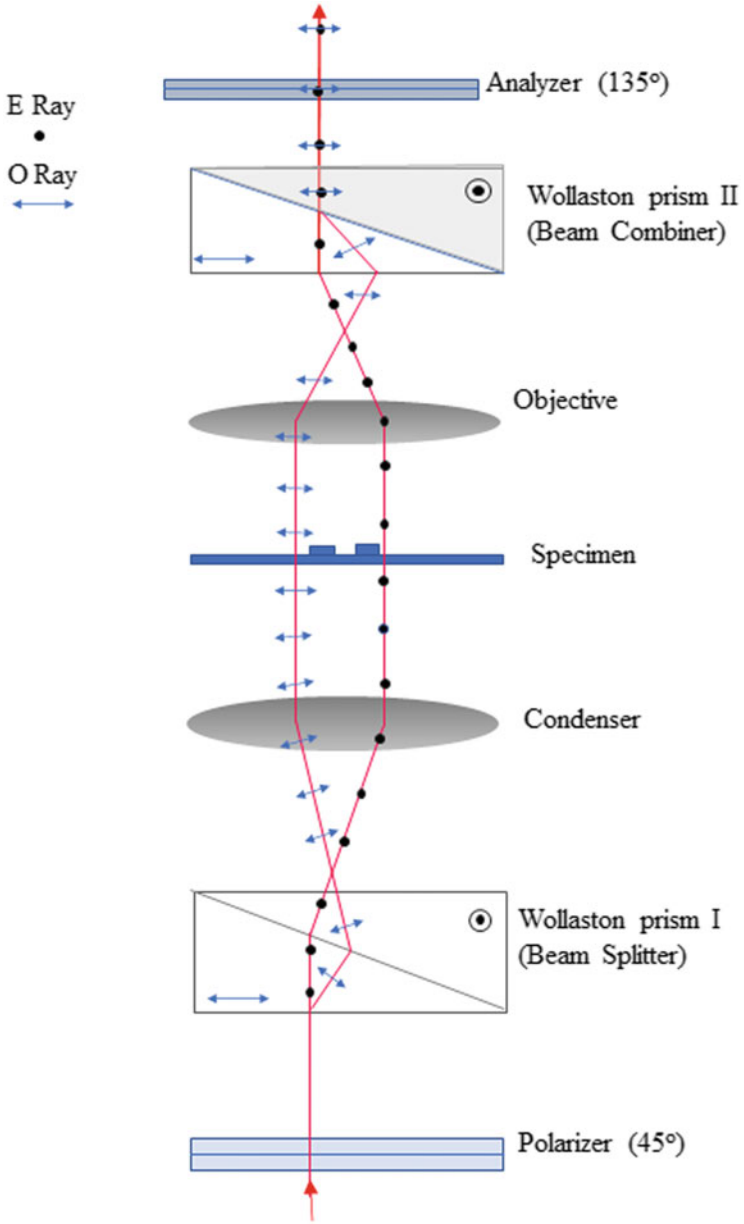

Fig. 2.31 Schemetic diagram of DIC

3. Focus on a blank sample plate using either a 4× or 10× objective in bright field mode.
4. Move the DIC slider with the analyser into the light path.

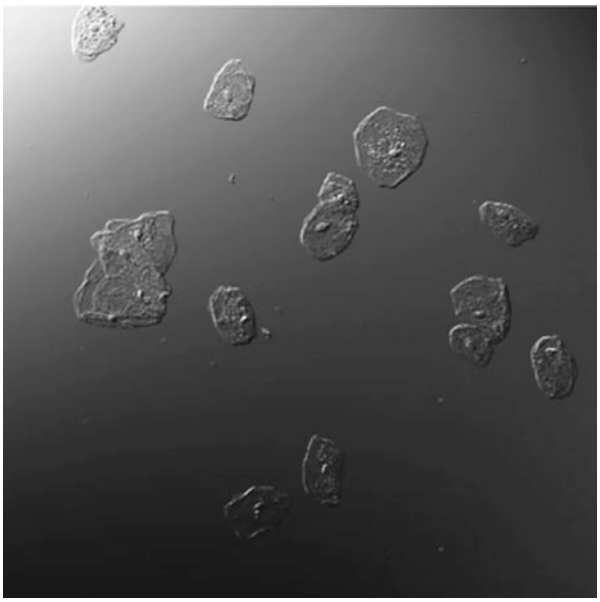

Fig. 2.32 Cheek cell image using DIC (Source: M.K. Kim, Digital Holographic Microscopy: Principles, Techniques, and Applications, Springer Series in Optical Sciences

5. If using a trinocular head, remove one eyepiece and view the sample directly down the trinocular head.
6. (a) When using eyepieces: rotate the polarizer until there is a dark stripe through the centre of the field of view. This indicates that the transmission axis of polarizer and analyser is aligned at 90 degrees to each other. (b) When using a camera: rotate polarizer until the image is at its darkest.
7. If the condenser and objectives were removed, put them back in their position and also check the illumination condition.
8. Place the specimen on the stage and bring the specimen into focus by moving the objective up or down.
9. Adjust the field iris diaphragm so that its image circumscribes the field of view.
10. Adjust the aperture iris diaphragm to enhance the contrast.
    Move the prism movement knob of the DIC slider to select the interference colour that can provide the optimum contrast in accordance with the specimen.

2.8.4 Digital Holographic Microscopy for Quantitative Phase Measurement

The phase contrast microscope, DIC allowed only qualitative evaluation of phase which was sufficient to visualize the internal structure of living cells without the need of dyes. The alternative approach to phase imaging is through the use of interferometry where small phase variations of the light emerging from the specimen are rendered in intensity as shifts of the interference fringes. Interferometry provides

the measurement of the defects of samples with resolutions of fractions of the wavelength of light. Based on interference phenomenon, digital holographic microscopy (DHM) is developed for quantitative phase imaging (QPI) [17, 18]. The knowledge of this microscopic technique is important because it permits true three-dimension (3D) visualization and 3D phase display of any unstained specimen. A comparison study of different microscopes can be found in Table 2.2.

The basic DHM setup consists of an illumination source, an interferometer with microscopic imaging optics, a digitizing camera (CCD), and a computer to run the algorithms. A laser is used for illumination with the necessary coherence to produce interference. The common interferometer for DHM is Mach–Zehnder configuration as depicted in Fig. 2.33. The spatially filtered and collimated laser beam is amplitude divided by the cube beam-splitter (CBS1) into an object (O) and reference beam (R). The specimen or object is placed at the working distance of the microscope objective (MO1) and this MO1 collects the object wave transmitted through the transparent sample. After passing through CBS2 these beams interfere and hologram (interference pattern) is recorded by the CCD. The image is numerically reconstructed from the hologram. The reconstruction algorithm consists of two steps: (1) Multiplication of a reference wave with the hologram and (2) Convolution of the propagation transfer function with the digital hologram. The propagation transfer function is calculated by diffraction integral using Fresnel transform method or angular spectrum method. Two images, real and virtual image, are formed from this digitally recorded hologram. The hologram (E_h), recorded by CCD, can be expressed as

$$E_h = |R + O|^2 = |R|^2 + |O|^2 + O^* R + O \cdot R^*. \tag{2.11}$$

In the above equation, the first term $|R|^2 + |O|^2$ is known as the dc term. Real and virtual image of the object are, respectively, given by the terms OR* and RO*. There is no significant difference between real and virtual image besides 180° rotation; both are known as the twin images.

The image reconstruction algorithm is depended on well-developed fast Fourier transforms (FFT). Reconstruction of image is described by the following equation:

$$E_I = \mathcal{F}^{-1}[\mathcal{F}(E_h \cdot R)\mathcal{F}(h)], \tag{2.12}$$

where R is the reference wave and h is the propagation transfer function which is calculated by Fresnel diffraction integral.

The Fourier transform of first two terms $|R|^2 + |O|^2$ in Eq. (2.10) being real, and in frequency plane their transform is centred at origin. The object can only be reconstructed from the last two terms. To improve the reconstruction quality, the dc and twin-image terms have to be eliminated. One of the methods to achieve this is to apply the spatial filtering operation. The spatial filtering method is used not only to suppress the DC term, but also to select one of the twin terms as well as to eliminate spurious spectral components due to parasitic reflections and interference.

Table 2.2 Main features and usage of different infinity-corrected microscopic techniques

Name of method	Features	Main area of use
Bright field microscopy	Commonest observation method Entire field of view illuminated by light source	Observation of stained specimen
Dark field microscopy	Zeroth-order un-diffracted light is rejected at the objective back focal plane and does not contribute to image formation. Only interference of higher order diffracted light contributes to image formation. Transparent specimens appear bright against a dark field of view	Suitable to the examination of minute refractile structures, which scatter light well. Observation of phase objects, such as the silica of the frustules (i.e. shells) of diatoms, bacteria, aquatic organisms, small inclusions in cells, and polymer materials.
Phase contrast microscopy	By using annular stop into the condenser, the zeroth-order un-diffracted lights will appear as a ring at the objective back focal plane, whereas the specimen diffracted light will be inside or outside this ring. Introduction of an annular quarter-wave ($\lambda/4$) plate at the objective back focal plane results in a total $\pm\lambda/2$ (90°) phase shift of diffracted light relative to un-diffracted light, as well as specific attenuation of the undiffracted light. At the image plane, interference of this "modified" diffracted and un-diffracted light leads to good image contrast without sacrificing resolution.	Observation of phase objects, such as bacteria, living cells. Does not work well with thick specimens
Differential interference microscopy	Wollaston prisms (one at the condenser aperture plane and the other very close to the objective back focal plane) are used to create two parallel and orthogonally polarized beams (O-rays and E-rays) out of every beam that would be incident upon the sample. Any phase difference between O-rays and E-rays is converted into elliptically polarized light when the rays are recombined. Specimen appears three dimensional.	Observation of phase objects, such as bacteria, living cells
Digital holographic microscopy	Two-step imaging process: Recording the hologram and numerical reconstruction of image Interferometric technique where object and reference wave interfere to generate the hologram. Reconstruction algorithm depends on optical configuration	Observation of phase objects, such as protozoa, bacteria, and plant cells, mammalian cells such as nerve cells, stem cells, tumour cells, bacterial-cell interactions, red blood cells or erythrocytes, etc. Quantitative depth measurement is possible.

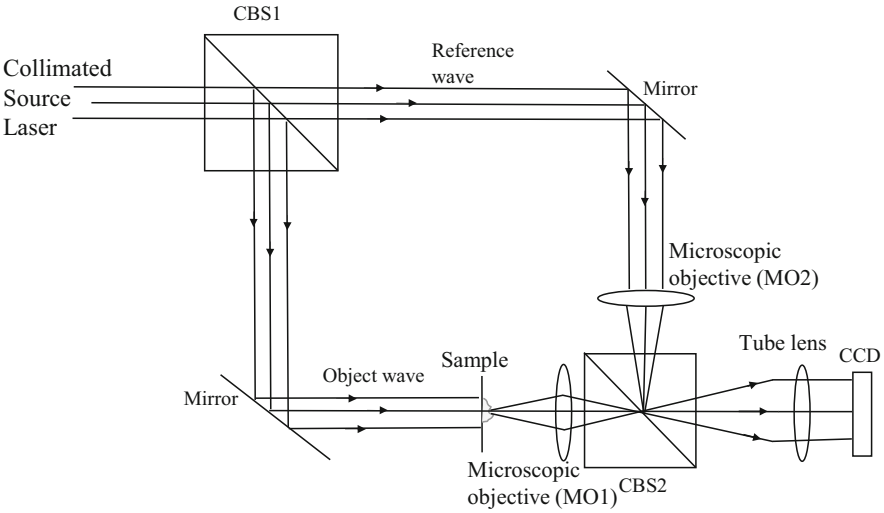

Fig. 2.33 Schematic diagram of a digital holographic in microscopic configuration

The intensity image is calculated from the complex amplitude distribution E_I and it is represented by following equation:

$$I = \mathrm{Re}\,[E_I]^2 + \mathrm{Im}[E_I]^2. \tag{2.13}$$

If n is the refractive index of the sample, then the sample thickness t is possible to calculate from the reconstructed phase information of the sample. The sample phase reconstruction is given by

$$[\delta(x,y)]_{\text{Sample phase}} = \tan^{-1}\left[\frac{\mathrm{Im}(E_I)}{\mathrm{Re}\,(E_I)}\right]. \tag{2.13}$$

$$\text{So}, t = \frac{\lambda[\delta(x,y)]_{\text{sample\_phase}}}{2n\pi}. \tag{2.14}$$

In DHM, the phase image is a quantitative representation of the object profile with subnanometre precision [18–20] (Fig. 2.34).

A well-known distinctive feature of holography is the reconstruction of image from the single hologram at various distances. Spatial resolution of DHM is limited by the wavelength of source, NA of objective, and pixel size of CCD. The interferometers may also include various apertures, attenuators, and polarization optics to control the reference and object intensity ratio. The polarization optics may also be used for the specific purpose of birefringence imaging. There are also low-coherence sources (LED) used in DHM for reducing speckle and spurious interference noise, or generating contour or tomographic images.

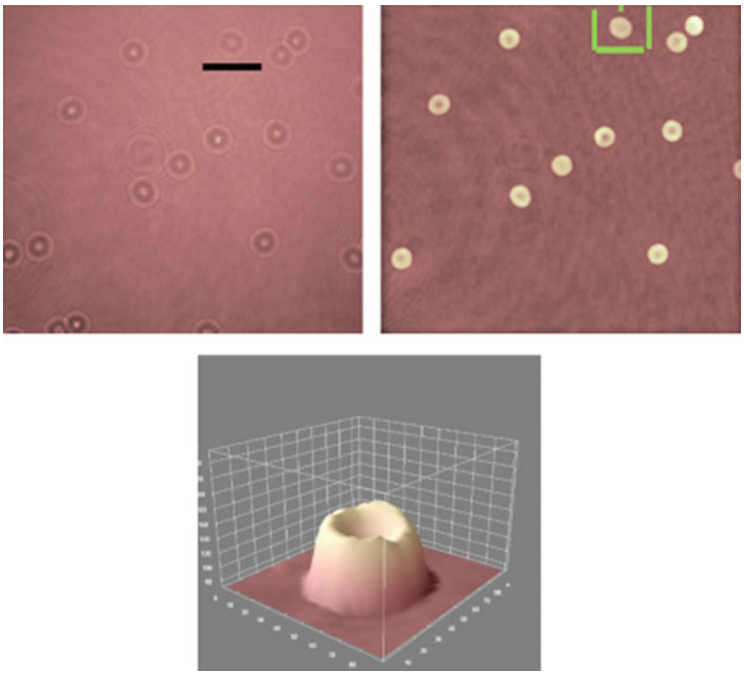

Fig. 2.34 (**a**) Recorded hologram of red blood cells (black bar is 500 μm), and (**b**) reconstructed phase image (green bar is 10 μm). (**c**) Phase reconstruction of single RBC cell (Source: [20])

Take Home Messages

The limiting resolution of all microscopes depends on the wavelength *(λ)* of the light used and the NA of the objective. Dirty or misaligned optics or vibration, or both, can reduce the achieved resolution. To reduce the aberration different types of infinity-corrected objectives are designed. Test resolution regularly, and especially pay attention to the iris setting and full illumination of the condenser aperture, to assure optimal performance of the microscope.

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