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A Primer on the Fundamental Principles of Light Microscopy: Optimizing Magnification, Resolution, and Contrast

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SUMMARY

The light microscope is an indispensable tool in the study of living organisms. Most biologists are familiar with microscopes, perhaps being first introduced to the wonders of the world of small things at a very early age. Yet, few fully comprehend the nature of microscopy and the basis of its utility. This review (re)-introduces the concepts of magnification, resolution, and contrast, and explores how they are intimately related and necessary for effective microscopy.

Magnification is simple and easy to produce, but must be accompanied by both resolution and contrast for it to be of value.

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INTRODUCTION

The Romans first discovered glass and realized that it could be formed into various shapes, and that certain shapes allowed them to observe small objects. The first compound microscope wasn't invented until two Dutch spectacle makers, Hans and Zaccharias Jenssen, started to experiment by placing a series of lenses into a cylinder, circa 1590. The astronomer Galileo Galilei was the first to describe how these lenses magnified objects. Yet, ''microscopy'' did not emerge as a useful tool to describe biological phenomenon until Antonie van Leeuwenhoek (1632–1723) made considerable improvements in the lenses, allowing him to systematically describe biology.

This mini-review provides an overview of the fundamentals of microscopy. It is intended to lay a foundation of the principles, components, and terminology underlying how a microscope works—and provides a primer for more specialized applications and advances in microscopy that exploit various aspects of these principles to achieve technological advances. For a microscope to be of use, it must achieve three fundamental goals: First it must create *magnification*; ostensibly, this is the motivation of microscopy and is simple and easy to produce. Magnification, however, must be accompanied by both *resolution* and *contrast* to be useful.

MAGNIFICATION

Magnification is the enlargement of the appearance of an object. Consider the appearance of an object as

Abbreviations: λ, wavelength [of light]; Θ, sin of the half-angle [of a lens or objective]; D, resolution; M, magnification; n, refractive index [of a lens or objective]; N.A., numerical aperature.

observed by the human eye: The apparent size of that object, as projected onto the human eye, is considered a magnification of 1-times (or 1×). The breadth and height of the scene captured by the eye is the field of view. Using a simple lens configuration, one can map a smaller portion of the field of view onto the same eye, resulting in the object appearing larger; we refer to this as magnification. In contrast, a larger field of view mapped onto the eye is negative magnification or de-magnification. For example, the left image in Figure 1A represents an object as seen by the uncorrected eye and has a magnification of 1×. In the right image, one-fifth of the width and one-fifth of the height of the original object are projected onto the eye. The magnification (M) is 5× and the field of view is reduced by $1/M^2$ or 1/25 of the original field of view. For a simple lens, this 5× magnification may be accomplished by creating a lens whose distance from the object to the lens is 1/5th as long as the distance from the lens to the focus (Fig. 1B).

In older microscopes, the primary lens of the microscope (the objective lens) had a back-focal distance that focused directly onto an image plane at a finite distance. In modern microscopes, objective lenses are said to be infinity-corrected because the objective lens focuses at an infinite distance while a second lens (the tube lens) focuses the light from the objective lens to a finite distance (Fig. 1C). Nonetheless, the net effective distance from the focal to the tube lens (f_1) divided by the distance from the objective lens to the object (f_0) is the magnification of the system (i.e. magnification $= f_0/f_1$) (Fig. 2).

It is important to note that one consequence of magnification is a decrease in image intensity or brightness. The area of the scene is proportional to the magnification squared (M^2). If a smaller scene is projected onto the same detector (the eye, in our example), then the density of light also decreases by M^2. As we will explore later, a consequence of higher magnification is that more light must be provided at the specimen to overcome this loss in light intensity.

OPTICAL RESOLUTION

Resolution is a term that describes our ability to distinguish discrete objects. Classically one can imagine two small objects brought closer and closer together until they no longer appear to be two separate objects, but rather appear as one. The distance at which the objects barely appear to be two is described as the resolution (D) of the optical system. The resolution of a microscope is determined by both the wavelength of light (λ) and the maximum angle of light that can be collected by the objective lens.

The wavelength of light describes its energy, and we perceive a portion of the light spectrum (the ''visible'' spectrum) as color. Light that appears blue is of shorter wavelength and contains more energy than red light. For a given wavelength of light, the resolution is determined by the angle of light that can be collected by the optical system (the angular aperature); the greater the angular aperature of light, the more detail is possible in the collected image.

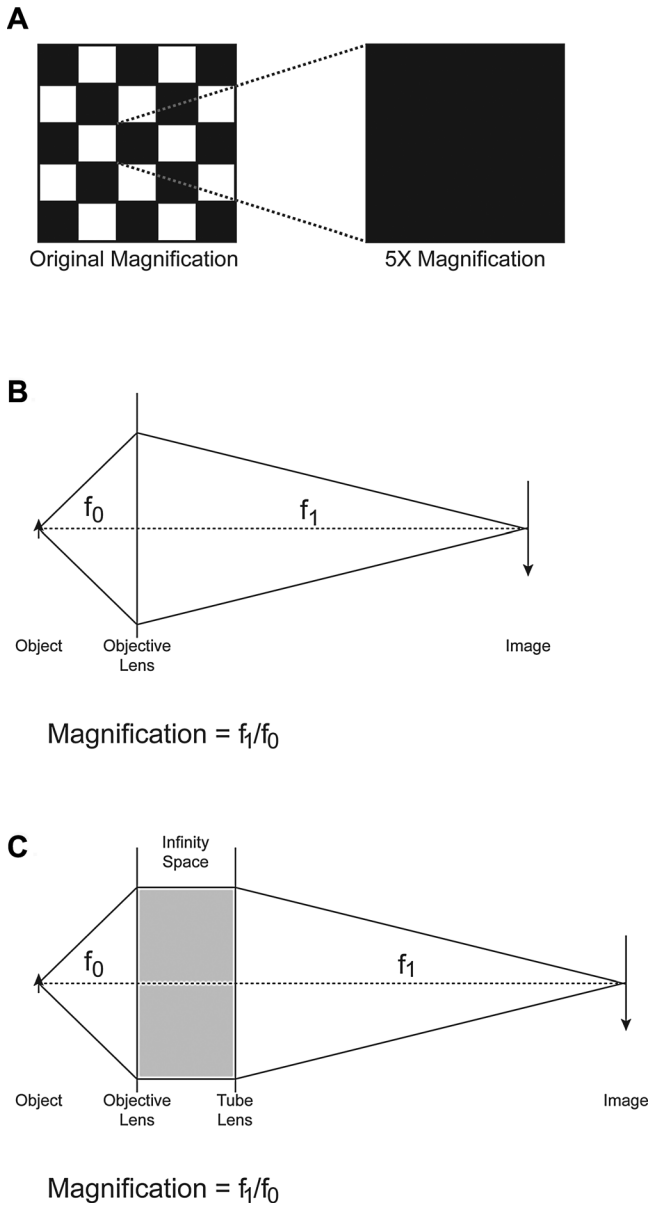

Figure 1. Magnification can be represented by a reduction in the size of the field of view projected onto an image plane. **A**: In a system with 5-times (5×) magnification, one fifth of the width or breadth of the original object is projected to create an image. The area projected onto the image is thus 1/25th or $1/M^2$. **B**: For a simple lens, positive magnification is generated by a lens whose focal length from the object to the lens (f_0) is shorter than the focal length from the lens to the image plane (f_1). For a simple microscope lens, the magnification of the lens is the distance from the object to the objective lens (f_0) divided into the distance from the objective lens to the primary focal plane (f_1). Prior to the 1990s, most microscopes were designed such that all objective lenses focused to the same distance (160 mm). **C**: By the end of the decade, however, all of the major microscope manufacturers switched to ''infinity-corrected optics'' in which the objective lens focused at infinity and a second lens, the tube lens, focuses light from the objective onto the image plane. In this case, the magnification is the distance from the object to the objective lens (f_0) divided into the tube-lens focal length to the image plane (f_1) (Sluder and Wolf, 2007).

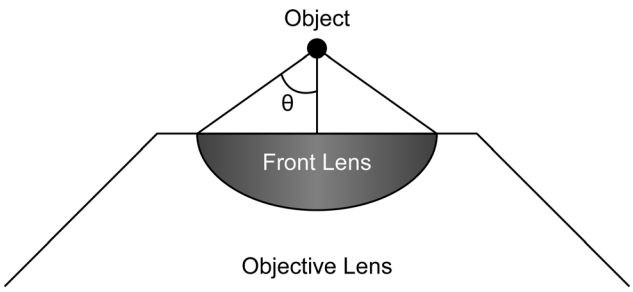

Figure 2. Angular Aperture of Objective Lens. The front lens of an objective lens gathers light from the field of view. The amount of light gathered by the lens is described by the angular aperture of the lens, which is defined by the half-angle of light (Θ).

This is analogous to attempting to view around a solid object. If the observation angle is fixed (i.e., a small angular aperature), the object appears to extend to infinity. As the angular aperature is increased, the detail of the object increases. If an observer is able to collect views from all angles around the object, they could assemble a complete image of the observed object; in practice, the practical angle from which the image is collected is limited. The *practical angle* of light that can be collected by a lens is described by the numerical aperture (N.A.), which is the product of the lowest refractive index between the sample and the front lens of the objective lens (n) and the sine of the half-angle (Θ) that describes the cone of light between the focal plane and the front lens of the objective lens:

$$\text{Numerical Aperture}(\text{N.A.}) = n\sin(\Theta)$$

For a sample mounted on a slide and viewed with an objective lens designed to observe a specimen through an air medium ($n = 1.0$), the N.A. is simply $\sin(\Theta)$. To achieve higher resolution and more efficient light collection, a higher N.A. can be obtained with objective lenses intended to view the sample through an immersion medium, which has a higher refractive index, such as water, glycerol, or oil. The manufacturer of an objective lens labels the barrel with the magnification and N.A. of the lens (e.g., $60\times/1.40$), and specifies the immersion media through which the sample should be viewed. Disregarding the manufacturer's recommended immersion media leads to substantial chromatic aberration, which is a distortion due to the inability of lens to properly focus all the wavelengths of incoming light.

Many objective lenses, especially those with high N.A.s, are intended for viewing the specimen through cover glass with the specimen sitting directly on the cover glass. When a specimen is on the cover glass, the lowest refractive index will be that of the immersion medium. When the sample is off of the cover glass, however, the mounting media surrounding the specimen may have a lower refractive index; this difference will lower the resolution of the optical system.

When an image is formed by passing light through a specimen (transmitted light), the overall resolution of the microscope depends on the N.A. of the illumination system (provided by the condenser lens) as well as the objective lens. The lateral resolution (in the focal plane) of the microscope is described in by the Rayleigh resolution limit (Inoué and Spring, 1997; Sluder and Wolf, 2007; Mertz, 2010):

$$D = \frac{1.22\lambda}{(\text{N.A.}_{\text{Objective}} + \text{N.A.})}$$

In transmitted light, then, the optimal resolution is only obtained when the N.A. of the condenser and the objective lens are optimized. In the case of fluorescence and chemiluminescence microscopy, however, the detected light originates within the sample itself so the illumination does not affect the resolution. In such cases, the lateral resolution becomes:

$$D = \frac{0.61\lambda}{\text{N.A.}}$$

Systems that obtain this level of optical resolution are limited by diffraction of the light, and are said to be ''diffraction-limited''. Until 1994, the diffraction limit was believed to be a hard boundary; now we know that the resolution limit can be exceeded with specialized techniques (Hell and Wichmann, 1994; Betzig, 1995; Gustafsson, 2000; Betzig et al., 2006; Rust et al., 2006; review in Schermelleh et al., 2010). Such systems are deemed as ''super-resolution'' since they can surpass the diffraction limit.

Axial resolution (i.e., the resolution of objects along the z-axis) is much more difficult to define, and a number of formulae have been offered (Shillaber, 1944; Françon, 1961; Martin, 1966; Piller, 1977). Here, we use the formula for depth of field from Inoué and Spring (1997):

$$\text{Depth of field} = \frac{\lambda_0 n}{\text{N.A.}^2} + \frac{n}{M \times \text{N.A.}}e$$

where λ_0 is the wavelength of light (emission wavelength in the case of fluorescence), n is the lowest refractive index, M is the lens magnification, and e is the resolution of the detector (for example, the pixel spacing of the camera). A list of the resolution limits for some objective lenses is given in Table 1.

The formulae for lateral and axial resolution assume an infinitely small object of very high contrast in a thin optical section. In practice, however, resolution is limited by contrast within the specimen.

CONTRAST

Contrast describes our ability to detect an object against a background. Depending on the specimen and the optical conditions, object detection is limited by either noise or

TABLE 1. Resolution Limits for Different Lens Types

Magnification	N.A.	λ_0	n	D (μm)	Depth of field (μm)
60	1.4	525	1.516	0.188	0.406
60	1.35	525	1.33	0.194	0.383
60	0.75	525	1.0	0.350	0.933
40	0.75	525	1.0	0.350	0.934

background. *Noise* is the uncertainty in the measured signal. Some sources of noise are statistical in nature while others are the consequence of instrumentation. The process of capturing and recording individual events (such as the arrival of photons at a camera) is subject to the uncertainty commonly referred to as ''shot noise'' or ''Poisson noise''. Such noise is stochastic, and follows a Poisson distribution:

$$\text{Poisson Noise} = \sqrt{\text{Events}}$$

Poisson noise is the result of uncertainty in measurement, and is the statistical consequence of the number of measurements that are made. As the number of events increases, the absolute Poisson noise is greater even though the relative Poisson noise (defined as the Poisson noise divided by the number of events) is reduced. Specifically, the relative Poisson noise falls off as the inverse of the square root of the number of events. For example, if the actual number of events is 100, the Poisson noise is ten and the relative uncertainty is 10%. For 10,000 events, the Poisson noise is 100 and the relative uncertainty is 1%.

Additional forms of noise also affect our ability to detect objects. Each type of detector has noise associated with it. Some examples include camera-read noise, electron-gain noise, photo-multiplier noise, etc. Object detection that is limited by the noise of an image is referred to as ''noise-limited'' or ''photon-limited''. Noise-limited contrast is typical of weak signals where the total number of photons is low and the background is negligible, and is often referred to by the signal-to-noise ratio.

Object detection can also be limited by high signals that are not specific to the parameter being detected. These non-specific signals are often referred to as ''background''. In many biological assays, background is more limiting than noise. This is due to an inability to tune detection specifically to the signals of interest. This lack of specificity can occur optically, electronically, and even chemically within the sample itself. For example, when observing biological samples using fluorescence, the inherent fluorescence in many biological structures and macromolecules confounds the detection of specific fluorescence signals. While systems can be made more specific, the trade off is lower overall signal detection. This balance between specificity and detection underlies the technology of instrumentation.

On a microscopic level, most biological materials have little inherent contrast; with the exception of pigments and granules that may exist within cells, cells are nearly transparent and absorb light poorly. Put another way, if visible light is passed through a thin biological specimen (<50 μm) and the intensity of light that is detected is compared with the intensity that was used to illuminate the sample, the intensities are nearly identical unless the sample is pigmented or stained. Thus, visualization of cells, tissues, and structures requires creating or enhancing contrast.

Light has a number of properties that can be exploited to generate contrast. The intensity of the light exiting a sample can be compared to the intensity of light that entered the sample, where the difference represents light absorbed and scattered by the material. The amount of light that is absorbed by a material is wavelength-dependent; those wavelengths that are not absorbed are detected as particular colors. For example, when light passes through the leaf of a green plant, much of the violet, blue, and red light is absorbed by the chlorophyll in the chloroplasts whereas green light is poorly absorbed, or is reflected, by these organelles, so the plant appears green. In contrast, most of the wavelengths that we can detect with our eyes almost entirely pass through a squamous epithelial cell, making the cell difficult to detect. This property of minimal light absorbance can be enhanced by staining the cell with dyes or chromophores that absorb light at specific wavelengths, and is used widely in histology—e.g., hematoxylin and eosin, which respectively bind to nuclei and proteins. Additionally, specific molecules can be identified by generating light absorbing deposits in association with antibodies in immunohistochemistry (Nakane and Pierce, 1966; Nakane, 1968).

Light has additional properties besides wavelength and intensity that are useful for optimizing the contrast of unstained specimens. As light passes through materials with differing refractive indices, it can either slow down or speed up. Light passing through a vacuum travels at maximum speed ($n = 1.0$); light that travels through water travels about 30% slower ($n = 1.33$); and light traveling through lipids and fats is slowed by about 40% ($n = 1.4$). The phase of the light that passes only through water can be made to cancel out, while the light that passes additionally through lipids can then be accentuated, which allows for the optimal detection of boundaries, especially of cellular membranes. This property is exploited in phase-contrast microscopy (Inoué and Spring, 1997; Sluder and Wolf, 2007). Light also has polarity, that is, an axis of oscillation. Most materials do not rotate the polarity of light, but some materials and structures do. Regularity in structure can be used be generate contrast using polarized light microscopy (Inoué and Spring, 1997; Sluder and Wolf, 2007). Differential Interference Contrast (DIC) microscopy is a common mode of contrast enhancement that uses both polarization and phase information to render a three-dimensional image of the sample (Inoué and Spring, 1997; Sluder and Wolf, 2007).

A powerful tool for generating contrast is fluorescence. Fluorescent molecules absorb light at one wavelength (the excitation wavelength) and then generate new photons at a second, longer wavelength (the emission wavelength). Contrast is created by illuminating a sample with an excitation wavelength, and then observing it at the specific emission wavelength. Most biological molecules fluoresce

weakly, but adding fluorescent molecules to a sample can be used to generate tremendous molecular specificity and sensitivity. Using this strategy, families of synthetic molecules have been synthesized with tremendous molecular specificity that also acquire fluorescence properties when associated with their target. For example, 4', 6-diamidino-2-phenylindol (DAPI) is only weakly fluorescent until it binds to the A-T rich regions of DNA, where its fluorescence increases by several orders of magnitude. Other fluorescent molecules are sensitive to their environments: Fura-2, an aminopolycarboxylic acid, undergoes a change in excitation spectrum that is proportional to calcium ion binding, and is thus readily used for measuring free Ca^{+2} in living cells and tissues. One of the most powerful tools in cell and developmental biology is the green fluorescent protein (GFP) (Shimomura, 1979; Chalfie et al., 1994) and its derivatives. GFP and other intrinsically fluorescent proteins can be genetically encoded, allowing them to help identify and track specific proteins in cells, tissues, and whole organisms.

Cells and tissues exist in three-dimensional space, and thus represent a number of challenges for microscopists. As seen previously, the depth of field of a microscope is inversely proportional to the square of the N.A. At first glance, this appears to be beneficial since the axial resolution improves faster than the lateral resolution (inversely proportional to the N.A.). Unfortunately, contrast in the microscope is much worse axially than it is laterally. Since the N.A. of an objective lens is limited by the sample, the observation angle is limited. This relationship consequently limits one's ability to distinguish objects in the axial direction; that is, the axial contrast is poor relative to the lateral contrast. This is problematic since the amount of information that is in focus (depth of field) decreases with increased N.A. with no improvement in the contrast. Thus, more of the image is out of focus when a higher N.A. objective is used. Prior to the 1980s, the only reasonable solution was to mechanically section specimens to physically remove any out-of-focus material. This severely limited the ability to study living specimens in three dimensions.

One of the important features of fluorescence is that the observed photons are generated in situ. Light generated by the fluorochrome at a single point spreads radially in all directions. This feature of fluorescence makes it more amenable to a number of contrast-enhancing techniques not available to transmitted light. The most significant contrast-enhancing techniques (deconvolution, confocal, and multiphoton microscopy) are able to exploit the in situ generation of fluorescence to create axial contrast, allowing investigators to render three-dimensional reconstructions of their images without mechanically sectioning their samples.

ANATOMY OF A LIGHT MICROSCOPE

As described previously, the magnification of a microscope necessarily reduces the intensity of the signal. The higher the magnification, the more light is lost purely as a consequence of the reduction in field-of-view. To overcome this, microscopes provide a means of illuminating the sample. Depending on the application, the *source* can be an incandescent bulb, a plasma arc lamp, a light-emitting diode (LED), or a laser. Each of these methods has costs and benefits. The issue is not just more photons (brighter light) but the photon density of the light source.

Light is constrained by physical laws that do not apply to matter. Importantly, light is not easily concentrated. A large light source is difficult to focus to a small spot; however, a small spot is easily defocused to a large spot. This explains the recent rise in popularity of LED and laser illumination versus the fall in popularity in incandescent and arc-lamp illumination sources. In most light microscopes (see Fig. 3), the illumination source is expanded to fill the back aperture of the condenser using a *collector lens*. The illuminated area of a sample is controlled by the *field aperture*, which is projected at the focal plane by the *condenser*. Like the objective lens, the condenser produces a cone of light that illuminates the sample. The angle of this cone is controlled by the *aperture diaphragm*. The condenser focuses light onto the sample, whose lateral position is controlled by the specimen stage. Light from the sample is then collected by the *objective lens*, with the position of the focal plane being determined by the focus of the microscope.

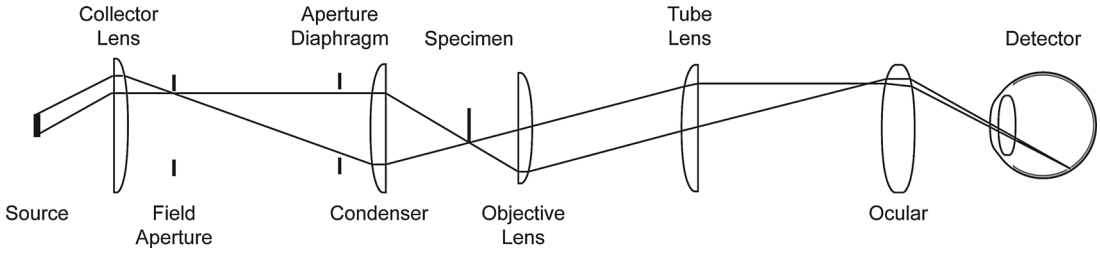

Figure 3. Anatomy of a light microscope. Conventional microscopes have a common set of components. Light from the *source* is gathered by a *collector lens*. Light from the collector lens passes through the *field aperture* en route to the *aperture diaphragm*, where it is collected by the *condenser* lens and then on to the *sample*. Light from the sample is collected by the *objective lens*. Light rays from the objective lens are focused onto the primary image plane by the *tube lens*. Light from the primary image plane is re-projected through the *oculars* onto the retina of the observer or on to a *detector*, such as a camera.

TABLE 2. Objective Lens Types

Objective type	Color correction	Transmission	Price
Achromat	+	+	+
Fluor	+	+++	++
NeoFluor	++	+++	+++
Apochromat	+++	++	++++

Depending on design of a microscope, the position of the focal plane can be fixed and the sample moved through the focal plane, or the focal plane can be positioned by moving the objective lens closer or further from the sample. Light from a modern objective lens only focuses at infinity, so a secondary lens (*tube lens*) is required to refocus the light onto an intermediate focal plane. Light from the intermediate focal plane can ultimately be projected onto the eyes of the observer (*detector*) by the *oculars* (see Fig. 3).

OBJECTIVE LENSES

The objective lens plays a substantial role in defining the resolution of the microscope (along with the condenser for transmitted-light applications). Objectives typically have the strongest lenses in the microscope, and are largely responsible for the quality of the image formed by the microscope. They therefore represent some of the more expensive components of a conventional microscope.

The curvature of lenses refracts light. The magnitude of this refraction is wavelength-dependent, causing light of different wavelengths to focus at different points in space, introducing a color-dependent aberration. Manufacturers provide a variety of types of lenses whereby color correction, light transmission, and price are balanced to meet the needs of the user (see Table 2).

An objective is made by combining a series of lenses. These individual lenses are generally made based on spherical surfaces, so they naturally focus not to a plane but to a sphere. Manufacturers can largely correct this effect by adding additional lens elements with the opposite curvature. These corrected lenses are referred to as PLAN, and can be applied to most objectives; however, since PLAN correction normally requires additional lens elements, adding PLAN correction usually reduces light transmission and adds to the cost.

SUMMARY

This mini-review has briefly touched on the basic principles of the light microscope. Much more comprehensive reviews are available, and the interested reader is encouraged to examine works by Inoué and Spring (1997), Sluder and Wolf (2007), and Mertz (2010).

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