Light Microscopy

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The light microscope has the function of revealing fine detail in transparent (usually) or opaque specimens. Although much depends on the preparation of the specimen, an understanding of the operation of the microscope itself is a requirement to help distinguish a good image from an inferior one.

Introductory article

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Introduction

The images produced by microscopes have fascinated observers for several centuries. Today, the instrument is often used as the definitive symbol of scientific investigation. It is unfortunate, then, that the understanding of what the microscope really does is poorly taught, and poorly understood. To start with, a microscope will only produce an image of the specimen (crystal, bacterium or whatever else) that is being observed; we see an image, not the specimen itself. The importance of this distinction is that different instruments could well produce different images of the same specimen. An understanding of microscope performance is therefore a requirement to help distinguish a good image from an inferior one.

What Makes a Good Image?

The most recognizable judgement of microscope performance is its ability to faithfully reproduce in the image the fine detail that is in the specimen. The term resolving power is used for the smallest distance – a linear measurement – that a lens system can separate or resolve. The smaller this distance, the closer together are the linear elements that a lens is capable of resolving, and the 'better' we think of the lens. There is an unfortunate confusion in language that defines high resolution performance with a small numerical value for resolving power. The term 'resolving power' is not a particularly good one; more recently the phrase minimum resolved distance (MRD) has been introduced, which is an accurate description of the concept.

The lens MRD is easy to understand and is reasonably easy to measure. But possibly of more practical importance in the study of microscope performance is the ability of a lens system to reproduce in an image the weak intensity variations that are present in the specimen, especially living biological specimens. The emphasis that is placed in introductory texts (and this account) on MRD should not override the very great significance of contrast transfer; but the subject is more difficult to explain in a simple way and is therefore confined to more theoretical dissertations.

The Basic Microscope

The specimen is normally placed on a 75 mm × 25 mm glass slide, that is 1 mm or less in thickness. The specimen should be very thin, less than 10 μm in thickness, in a very small quantity of mountant; the mountant is sometimes water but the specimen is more often permanently embedded in a polymerized plastic. A coverslip protects the specimen and prevents it from drying out. The slide preparation is supported on a large flat stage, usually rectangular in shape. It is helpful for higher-magnification studies to have a mechanical stage to facilitate holding and manoeuvring of the specimen in the horizontal x and y directions. One disadvantage of a mechanical stage is a lazy tendency of the observer to avoid examining the whole specimen. Specimen movement in the vertical or z direction is with the focusing system, with a coarse focus and a fine focus, although these may be on the one axis (coaxial). The focusing movement may operate on the microscope tube that holds the objective and eyepiece, or on the microscope stage (a feature of later instrumentation).

Microscopes for the observation of thin specimens normally use a transmitted light illumination system in which light is directed through the transparent specimen. Better microscopes will have a transformer or rheostat to permit variation of the intensity of illumination. This is the microscope illustrated in **Figure 1**. Opaque specimens are much less frequently encountered in the biological laboratory. For such specimens, an epi-illumination system is necessary ('epi' here meaning 'above'), in which the light source is placed on the same side of the specimen as the observation optics. Although epi-illumination is essential to fluorescence microscopy, a very important technique in the life sciences, this account is confined to transmitted light systems.

The heart of the microscope is the objective lens. It consists of four (or many more) glass elements housed in a protective metal mount that is screwed into the microscope nosepiece. Although the screw threads are identical for almost all objectives, it should not be assumed that they are interchangeable from microscope to microscope. There are several objectives on each microscope, a common combi-

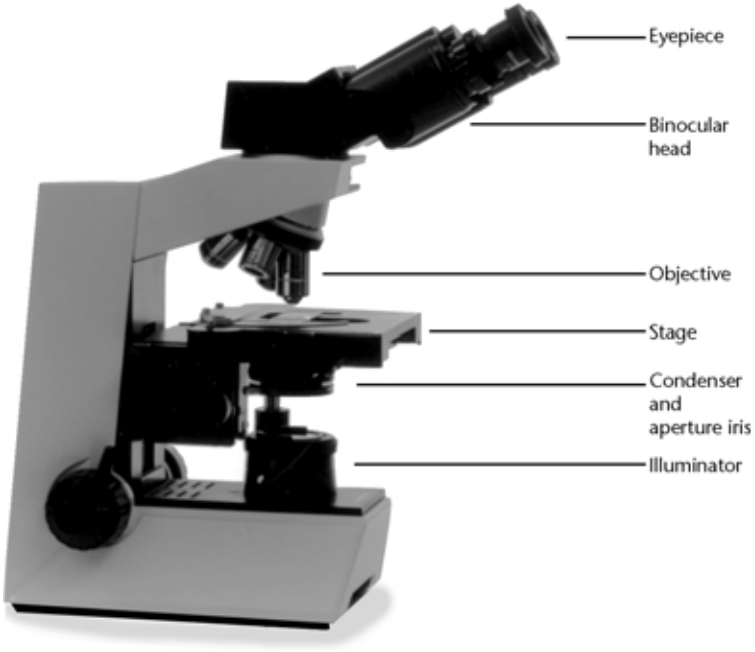

Figure 1 A modern classroom microscope with mechanical stage and binocular head.

nation being $\times 4$ (ultra-low power), $\times 10$ (low power), $\times 40$ (high power), and $\times 100$ (oil immersion). For many purposes it is sufficient to have only two or three of this range. We shall see later that this is a very inadequate, even improper, way of describing these lenses, but it is the common jargon of microscope users.

The objective forms an enlarged primary image of the microscope. An eyepiece (ocular) further enlarges the primary image. The objective and eyepiece together form an image that is enlarged, inverted, and laterally reversed and at infinity – that is, parallel rays emerge from the eyepiece. The lens of the observer's eye brings these parallel rays to a focus in the plane of the retina, for visual observation. The actual position of the primary image formed by the objective is one of the more recent changes introduced by microscope manufacturers – at 160 mm above the nosepiece in earlier instruments, at infinity in the most recent ones. This is one reason why compatibility between modern objectives cannot be assumed. For prolonged microscope observation, a binocular system with two eyepieces is preferable, but exactly the same field is presented to each eye. If stereoscopic viewing is required, as for dissection, two separate optical paths must be employed in the one instrument (a 'stereomicroscope'), in which the images are designed to be erect and laterally unreversed.

Beneath the specimen is another lens system, the condenser. Its function and purpose, often misunderstood, are discussed below.

Microscope Theory

Unless there is an understanding of very basic lens theory, it is not possible to appreciate the complexities of image control provided by the condenser. **Figure 2** shows a lens bringing parallel rays to a focus, but the arrows in the diagram have been reversed as a legitimate optical design practice. To look at the performance of an objective we should consider light coming from a specimen at the point of focus of the lens. According to Abbe (a German physicist, 1840–1905) the resolving power (MRD) of the lens is given by eqn [1].

$$d = \frac{\lambda}{2(n \sin \alpha)} = \frac{\lambda}{2\mathrm{NA}} \qquad [1]$$

where d = MRD, λ = wavelength of illumination, n = refractive index of the medium between the lens and the

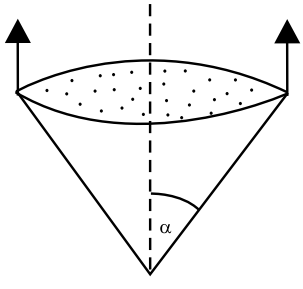

Figure 2 Defining numerical aperture (NA).

specimen (usually air, $n = 1.0$), α = half the angular intake of the lens, and ($n \sin \alpha$) is defined as the numerical aperture (NA) of the lens.

For improved resolution, the MRD value should be small, and therefore the wavelength should be as small as possible. In a biological laboratory, the wavelength cannot be varied much but, on theoretical grounds, blue light would give better resolution than red light. However, the equation predicted the MRD capability of very low-wavelength sources (ultraviolet light, X-rays, electrons, ions), and microscopes – the instrumental name persisted – were ultimately designed to utilize these sources.

The Abbe equation suggests that the lens NA should be maximized to keep the MRD as small as possible. The NA is a physical property of the lens, a function of the lens intake angle. This is determined by the lens designer and would seem to be beyond the control of the user, but incorrect use of the microscope invariably leads to a decrease in the effective NA of the objective.

Numerical aperture control with the condenser

To completely fill the objective NA with the required cone of light, another lens system, the condenser, must be used to provide that cone. The reason for having a condenser at all is to provide that cone of light. Two identical NAs are required, the condenser NA exactly matching the objective NA. If the condenser NA exceeds that of the objective, contrast will be lost by uncontrolled reflections in the objective. If the condenser NA is less than that of the objective, the objective NA will not be filled, and the objective will have its resolution performance compromised with an effective NA lower than its true NA.

This implies the need for a separate condenser for each objective; when the objective is changed, the condenser should also be changed. No-one does this. Instead, a condenser of high NA is chosen that has its NA reduced by an iris diaphragm – the aperture iris diaphragm – whenever a lower NA objective is used. The correct position of the aperture diaphragm is recognized by looking at the back (focal plane) of the objective. Remove an eyepiece and look down the tube of the microscope. Inside the objective there is an image of the aperture iris diaphragm, which should be adjusted so that some 7/8 of the diameter of the objective is filled with light. It is an adjustment to be made with each change of objective. Theoretically, exactly matching NAs of the condenser/objective would have the objective 8/8 filled with light by the aperture iris, but the imperfections of objectives require some small closure (1/8) of the aperture iris to maintain contrast. The aperture iris controls the effective NA of the condenser; it is used to match the NA of the condenser to the NA of the specific objective that is in use. See **Figure 3**.

The effects of closing the aperture iris diaphragm are to

- Reduce the effective NA of the condenser (appropriate only for low-NA objectives)
- Increase contrast (not required with well-stained material)
- Increase depth of field (not required with very thin preparations)
- Reduce light intensity (not the best way of doing this — use the transformer)

The normal position for the condenser is fully raised, to within 1 mm of the microscope slide. Some would say that it is a sure sign of lack of microscope training if ever the condenser is lowered.

The Objective

Lens correction

The MRD of the microscope is established by the front lens of the objective. Other lens elements that are a part of the objective are there to correct for deficiencies in image formation resulting from aberrations of the system. Objectives are categorized by the efficiency with which they minimize chromatic aberration (mainly) and curvature of the image plane. The very large majority of objectives are achromatic, in which blue and red rays, but not the green, are brought to the same focus. The images of sharp boundaries are characterized by green/magenta fringes as the focus is slightly changed (magenta = red plus blue). Apochromatic objectives have the blue, green and red rays focused together. A separate correction made by lens designers is for field curvature. The very best and most expensive objectives are called planapochromats.

Magnification

Since it is the NA of the objective that determines its MRD, it is unfortunate that we identify objectives by their magnification. Magnification serves only to enlarge the image resolved by the objective to a size in which all the image detail is resolved by the eye. The limit of useful magnification is about 1000 times the objective NA. Further magnification introduces artefact, not specimen detail.

Immersion objectives

The highest NAs are achieved by increasing the refractive index of the medium between the specimen and the objective; this is achieved in immersion objectives. They are always marked in some way to identify their special function, and to specify the proper immersion fluid, usually immersion oil but occasionally glycerine or water. These

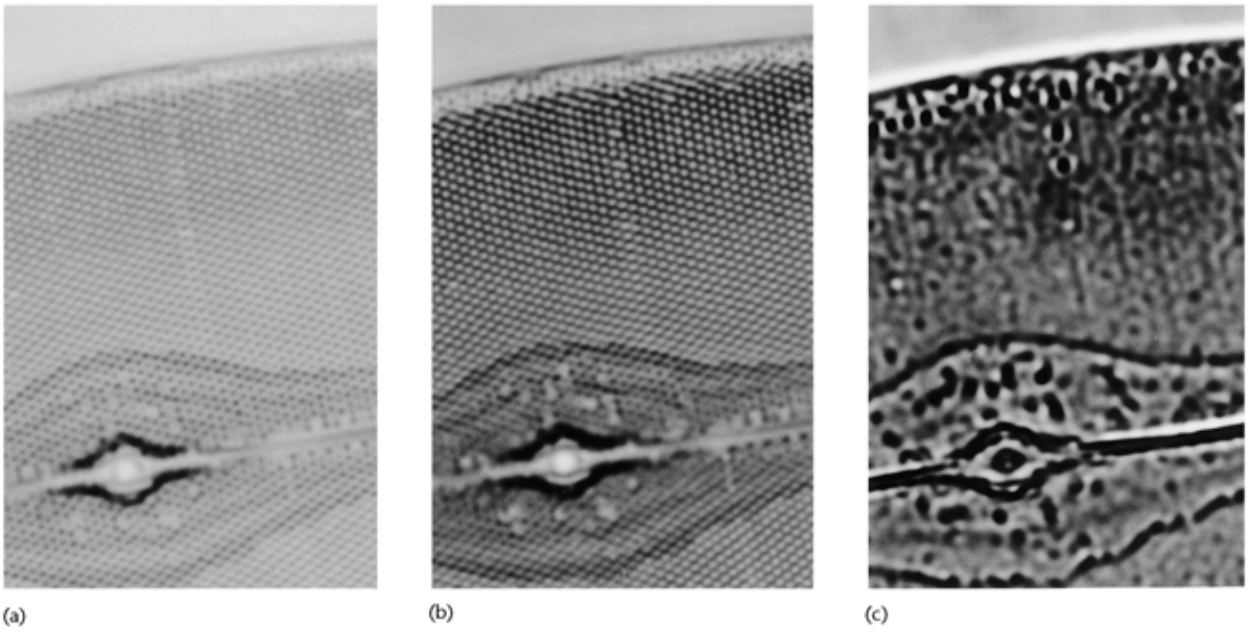

Figure 3 Images of the diatom *Pleurosigma angulatum*, to illustrate the function of the condenser aperture iris diaphragm. The objective used was a $\times$ 40, 0.65 NA achromat. The field size is 60 μm $\times$ 80 μm. In (b) aperture iris set to 7/8 of the objective NA as seen in the objective back focal plane. The image has optimal compromise between MRD (the ability to resolve the line pattern) and contrast. In (a) aperture iris opened too far. Flare has resulted in loss of image contrast, although the pattern can still be resolved. In (c) the aperture iris closed too far. Contrast and depth of field have been increased, but the effective objective NA has been reduced, the MRD adversely affected and the pattern is no longer resolved.

objectives have their lens mounts especially sealed to prevent seepage of the immersion fluids into the system. 'Dry' (nonimmersion) objectives should be carefully protected from contact with oil or similar fluids.

The coverslip

Objectives of NA higher than 0.4, that is those with magnifications in excess of about $\times$ 20, are sensitive to variation in the thickness of the coverslip (coverglass); the higher the NA, the more critical is the problem. By 'coverslip thickness' is meant the distance between the actual point of focus in a specimen and the front surface of the objective. When entire cells are being examined, this poses a major difficulty, for the cell itself may have considerable thickness. Most objectives for the life sciences are designed to have a coverslip thickness of 0.17 mm, but objectives are also made for use without a coverslip, a requirement of metallurgical specimens. One important advantage of oil immersion objectives is that the oil is chosen to have optical properties homogeneous with those of the coverslip, so that variations in relative thickness of oil/coverslip, even the absence of a coverslip, are consistent with high-quality imaging. A No. 1 coverslip, about 0.15 mm thick, is the normal compromise for coverslip choice in a biological laboratory.

Advances in Light Microscopy

Living biological material suffers from a lack of contrast, for there is little variation in light absorption by the cellular components that we wish to distinguish. One of the functions of specimen staining is to provide intensity and colour contrasts, but chemical (histochemical) information about the specimen is also a significant result. The emphasis with more advanced microscopical techniques is to introduce contrast optically, as in darkfield, phase contrast and differential interference contrast microscopy. Digital image capture techniques, image enhancement and computer reconstruction of images, especially as applied to the confocal microscope are transforming the expectations of the light microscope as a modern research tool.

Further Reading

Bradbury S and Bracegirdle B (1998) *Introduction to Light Microscopy*. Oxford: Bios Scientific Publishers.

Kapitza HG (1994) *Microscopy from the Very Beginning*. Oberkochen, Germany: Carl Zeiss.

Oldfield R (1994) *Light Microscopy; an Illustrated Guide*. London: Wolfe Publications.

Schade K-H (1995) *Light Microscopy, Technology and Application*, 2nd edn. Landsberg, Germany: Verlag Moderne Industrie.