Botanical MICROTECHNIQUE CYTOCHEMISTRY

/ JEROME P. MIKSCHE

PREFACE

BOTH of the authors had the privilege of doing their doctoral work in the laboratory of Dr. John Eugene Sass at Iowa State University. The present book has its origins in Sass's *Botanical Microtechnique*, but it has been greatly revised and extended at the personal request of Dr. Sass prior to his death in 1971.

We embarked on this task out of a deep sense of responsibility to a man we profoundly respected. In the intervening years we have come to share his conviction that a work of this nature is essential. Structure and function are the fundamentals of plant science, and the key to understanding them lies in the study of plant cells and tissues. It is, therefore, imperative that scientists—new and old—have at their command an effective set of techniques for investigating cell structure and chemistry.

The goal of the present book is to serve as a training manual and *not* as a reference work. It is designed to introduce future teachers and researchers in plant science to the basic principles of microtechnique and cytochemistry. We also recognize that more experienced researchers may also wish to consult this manual, as they have its predecessor. Therefore, each topic begins at an elementary level and proceeds to more advanced considerations. The first chapter is intended as an elementary introduction to one of the central themes of the book—microscopy; later chapters expand and enlarge on this elementary coverage. As in the previous book emphasis is placed on understanding the aim of the procedures and the reason behind each operation. After mastering the basic principles the reader is prepared to delve in the advanced and/or specialized literature. Thus no attempt has been made to compile an encyclopedia of techniques; such manuals are available elsewhere.

It is pertinent to ask what we mean by microtechnique. Sass defined it as consisting of three overlapping activities: (1) preparation of plant tissues for microscopic study, (2) skillful use of the microscope and related equipment for critical study and interpretation of the material, and (3) recording and illustrating the results by numerical and graphic arts.

This broad definition does not permit a purely physical separation between microtechnique and cytochemistry, and thus cytochemistry becomes

an activity that uses microtechnical methods to gain an understanding of cellular chemistry and thereby cellular function.

In this book we have introduced many more quantitative topics than were included in its predecessor. Not only is the mathematical preparation of plant scientists today greater than it was twenty years ago, but also botanists are now much more interested in and willing to deal with quantitative concepts. However, the eminent mathematician and philosopher A. N. Whitehead has aptly stated: "Geometry and mechanics, followed by workshop practice, gain that reality without which mathematics is verbiage." The primary reason for the mathematics introduced in this text is that its use provides vastly greater insights. It gains reality by its use at the desk and in the laboratory.

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/ JEROME P. MIKSCHE

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CHAPTER ONE

Microscope Construction, Use, and Care

THE MICROSCOPE is probably the most indispensable instrument used in the biological sciences. Intelligent purchase and effective utilization of a microscope require an understanding of at least the elements of its optical and mechanical construction. It is an expensive instrument, built to the highest standards of precision and having possibilities of performance that are not fully utilized by many users. Although some structural features are delicate and even fragile, the microscope has adequate durability to give many years of useful service.

The function of a microscope is to produce an enlarged image of an object. This is accomplished by a system of lenses. A lens may be defined as a transparent body having at least one curved surface. A simple lens, consisting of one piece of glass, may be used to illustrate how a lens produces an enlarged image by bending or refracting light. A ray of light coming from the object enters the upper portion of the curved face of a lens and is bent downward. Similarly, a ray entering the lower portion of the lens is bent upward. The rays that pass through the lens converge and then continue as a diverging cone. If a sheet of paper or ground glass is placed to intercept the rays that pass through the lens, an enlarged image of the object is produced on the screen. A photographic plate can be placed in the cone of light and a photographic image obtained. A hand lens or the lens on a simple dissecting microscope produces an image on a screen in this manner (Fig. 1.1A). The objective or lower lens of a microscope consists of a number of lenses which act as a unit to produce an image as described above. There are certain limitations on the magnification and quality of image obtainable with the objective alone. The primary image produced by the objective is intercepted and magnified and improved in quality by an eyepiece. The eyepiece or ocular consists of two or more lenses working as a unit and having a fixed magnification. If a ground-glass screen, a sheet of paper, or a photographic plate is placed at any plane above the eyepoint of the ocular, an image is produced (Fig. 1.1B). Note that the primary image is inverted and the projected image is erect.

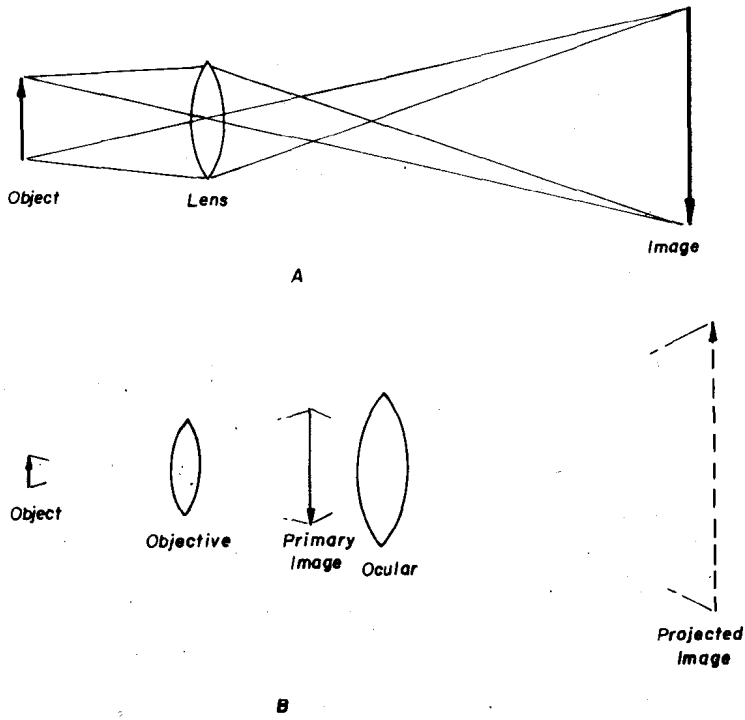

FIG. 1.1. Formation of projected images by the microscope: A, simple microscope; B, compound microscope.

With a given objective and ocular, the size of the image varies with the distance of the screen from the ocular. If the screen is placed 254 mm from the eyepoint, the size of the image will be approximately equal to the product of the designated magnifications of the objective and ocular. Thus an objective having a designated magnification of 10X used with a 10X ocular gives a total magnification of approximately 100X. Exact values must be determined by micrometry.

The foregoing discussion does not take into account the operation of the human eye working in conjunction with the microscope. However, most microscopic work is done by direct visual observation with the eye held at the eyepoint of the ocular. Let us turn for a moment to a consideration of the eye as an optical instrument. The lens of the eye operates as a simple lens, and the curved retina is the receptive surface on which the image is formed. If an object is held at a given distance in front of the eye, an inverted image of definite size is produced on the retina. If a larger object is substituted at the same distance, or if the original object is moved closer to the eye, the visual angle (the angle of the cone of rays between the object and the eye) is

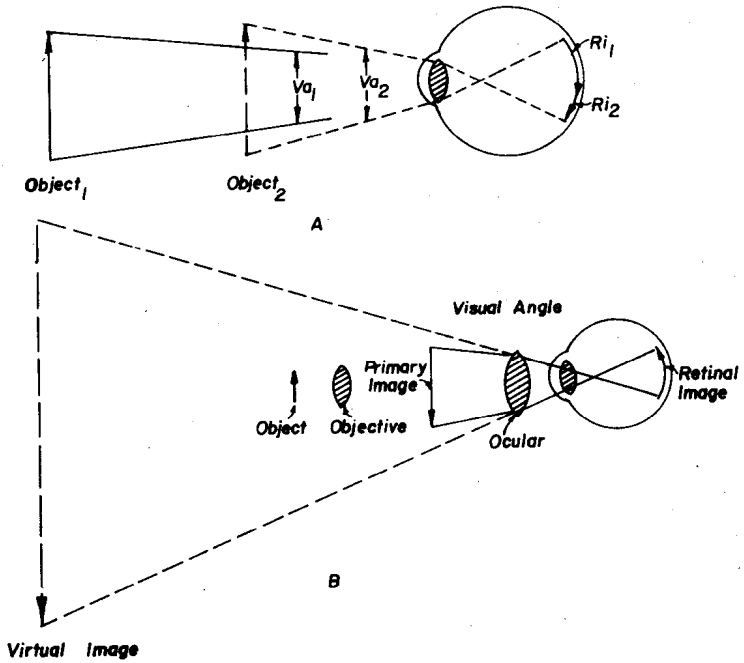

FIG. 1.2. A, Formation of images by the eye showing relative size of retinal image in relation to visual angle; B, retinal and virtual images obtained with a compound microscope.

increased, the size of the retinal image is increased, and the object appears to be larger. In Figure 1.2A compare the two objects shown in solid and dotted lines, their respective visual angles Va_1 and Va_2, and the retinal images Ri_1 and Ri_2.

When the eye is held at the eyepoint of the microscope, it intercepts the image-forming cone which has a definite angle established by the microscope, and a retinal image of definite size is produced (Fig. 1.2B). The observer sees a magnified virtual image, which appears to be near the level of the microscope stage, approximately 254 mm from the eye. The retinal image is erect, the virtual image is inverted, and the direction of motion of the object is reversed.

The apparent size of the virtual image is the same as if the observer viewed the projected image on a screen 254 mm from the ocular. As a specific case of magnification, let us view an object 0.1 mm long with a 100X microscope. This produces a retinal image of the same size and impression of magnitude as if we looked at the 10 mm image projected by the same microscope on a screen 254 mm from the eyepiece.

PROPERTIES OF OBJECTIVES

Magnification

The most obvious property of objectives is magnification, which is a fixed value under the conditions outlined above. The objective magnifications used most commonly on standard monoobjective microscopes range from 3.2X to 100X. Magnifications below this range are normally used on paired-objective stereoscopic prism binocular dissecting microscopes. Objectives above 100X have rather limited uses. The conventional low-power objective is 10X. The lower powers, from 1.0X to 6X, are not fully appreciated and deserve more serious consideration.

Working Distance

Free working distance is the distance between the objective and the cover glass, using a cover glass 0.16–0.19 mm thick. The catalogues of the manufacturers give the working distances of their objectives. A few selected illustrations show the relation between magnification and working distance: 10X, 7.0 mm; 43X, 0.6 mm; 45X, 0.3 mm; 95X, 0.13 mm. For an elementary class the most desirable high-power objective has a magnification in the forties and the longest available working distance. Objectives of higher magnification and short working distance must be used with care to avoid damaging the front lens and the slides.

Focal Length

If a beam of parallel rays is projected through a simple lens, the rays converge at a point. The distance from this point to the optical center of the lens is the focal length. In an objective consisting of several components, the situation is somewhat more complex, and a different value is used. The manufacturers engrave on the mountings and list in their catalogues a value known as the equivalent focus (EF). At the standard projection distance of 254 mm, an objective that has several components and an EF of 16 mm will give an image of the same size as a simple lens of 16 mm focal length. The EF should not be confused with working distance; the EF decreases as the magnification increases. The experienced worker is in the habit of speaking of an objective as a 4 mm objective, for instance. For class use it is much better to speak in terms of magnification, which is 43X in a certain 4 mm objective. In the past the manufacturers have paid undue attention to computing their objectives so that the EF is an even number; a series of objectives will have the EF in the orderly progression 16, 8, 4, 2 mm. A magnification may turn out to be some awkward fractional number like 3.2 or 5.1. A more practical series would be a sequence of magnifications such as 3, 5, 10, 20, 40, 60. There is a trend toward the use of the latter system.

Depth of Field (Depth of Focus)

Even a minute body or a very thin section has some thickness or depth. If a deep cell is being viewed with a 10X objective and the lens is focused on the upper wall of the cell, the bottom wall may also be in focus. If a 45X objective is focused on the top wall, the bottom wall may be completely out of focus and practically invisible. If the lens is focused on the bottom wall, the top wall becomes obscured. The vertical extent of the zone of sharp focus is the depth of field. The term "depth of focus" is also in common use. For instance, some camera lens mounts have depth of focus scales. Depth decreases as the magnification increases. There are mathematical limits to the depth encompassed by a given objective. Magnification and other factors being equal, objectives of the several manufacturers have the same depth of field.

Resolving Power

Resolving power is that property of a lens which makes possible the recognition, as distinctly separated bodies, of objects that are exceedingly close together or subtended by a small visual angle. The simplest illustration of resolving power is the visibility of double stars. Although the two stars may be separated by a vast distance, the visual angle reaching the eye is very small, and the stars appear to be close together. Many individuals can see only one star; other persons, whose eyes have better resolving power, can see the two stars distinctly. Applying this principle to the microscope, a lens of poor resolving power will show a slender chromosome as a single thread, whereas a lens of better resolving power may show the chromosome as two interwound threads. The important question concerning an objective is not how small a thing you can see but what is the minimal separation between two objects that the lens can resolve.

The mathematical derivation of the formula for determining resolving power will be discussed in Chapter 8. The formula contains the following factors:

λ = wavelength
n = the lowest index of refraction in the path of the rays—i.e., the index of refraction of water, glass, air, cedar oil, balsam.
u = half of the angle made by the effective cone of rays entering the objective. This value can be obtained from a table in Gage (1941) or from the manufacturers.
NA = numerical aperture, a number that is indicative of relative resolving power. $NA = n \cdot \sin u$

The value of the numerical aperture is engraved on most modern objectives and is given in the catalogues of the manufacturers. This number may be 0.25 in a certain 10X objective, for example, and it increases through pro-

gressively higher magnification, attaining the value 1.4 in an expensive 100X objective.

Knowing the numerical aperture, we can make a simple computation and arrive at a tangible value of resolution (h). Assume that we are using an objective of NA 1.0 and light having a wavelength of 0.0005 mm. The formula is $h = 0.61\lambda/NA = 0.61(0.0005)/1.0 = .00030$ mm $= 0.30$ μm. This means that if two bacteria or two chromomeres on a chromosome are separated by a space of 0.00030 mm, they can be seen as two distinct bodies. As the numerical aperture increases, the resolving power increases. Resolution (h) may be thought of as the minimal distance by which two objects can be separated and still be perceived as separate entities. Resolving power can be defined as the reciprocal of h, i.e., $1/h$. The formula is empirically accurate and stems from the Rayleigh-Airy theory of resolution. This will be discussed with more detail in Chapter 8.

The practicable upper limit of NA 0.95 is obtainable with dry lenses, used with an air space between the objective and the cover glass. In accordance with the foregoing formula, the NA can be increased by increasing the value of n or sin u or both. If the angle of the ray that passes from the glass slide to air is greater than 41°, the light is totally reflected back into the glass. This phenomenon limits the angle that determines n. If a drop of cedar oil or synthetic silicone immersion fluid is used to connect the immersion objective with the slide, the values of n and sin u are both increased; consequently the resolving power is increased. An NA of 1.10 can be obtained with a water immersion lens, 1.25 with glycerin, and 1.40 with oil.

OPTICAL CORRECTIONS

The foregoing discussion of the properties of objectives does not take into account the quality of the image produced. Spherical surfaces do not form perfect images; although the use of electronic computers has resulted in greatly improved optical design, there are still defects in images formed in the microscope. Perfection is approached by judicious choices of lens shapes, lens dimensions, and lens materials (crown glass, flint glass, fluorite). The goal is to counteract defects caused by one surface with equal but opposite defects in other surfaces so that the whole objective acts as a single lens to produce a high-quality image.

There are seven principal defects in the lens system of a microscope.

1. *Spherical aberration*—The objective has a hemispherical front lens, and those rays which pass through the thicker (more central) portions of the lens are brought to focus at a point farther from the lens than those rays passing the thinner (more peripheral) portions of the lens. Spherical aberration is evidenced by a loss of contrast in the image, especially the off-axis image, since the central (paraxial) rays are not bent as they pass through the lens. This defect is aggravated by a cover glass that is not within the thickness range of 0.15-0.20 mm. Every cover glass should be calipered before use; thickness No. 1½ generally gives the greatest yield of usable cover glasses.

The defect is particularly noticeable with the high dry powers and can be partially alleviated by going to oil immersion and/or using a 589 nm or 546 nm interference filter. Departures from the standard tube length (160 or 170 mm or equivalent, depending on the manufacturer) also aggravate this defect.

The point along the image axis where the least blurring occurs due to spherical aberration is the locus of the so-called circle of least confusion. Closing down the substage diaphragm reduces the size of the circle of confusion (includes a greater proportion of paraxial rays) but also reduces resolution (by decreasing u of $n \sin u$). Very often a compromise must be made in this regard.

2. *Chromatic aberration*–This defect is due to the dispersive property of the lens that causes light of short wavelength (e.g., blue light) to be brought to focus closer to the lens than light of longer wavelength (e.g., red light). Consequently the colors of the object are not reproduced accurately, and a color fringe or "rainbow" is visible along the boundaries of objects in the microscopic image. The use of monochromatic light will eliminate this error.

3. *Lateral color*–This results when light of one color is imaged at greater magnification than light of another color (closely related to chromatic aberration). The effect is a rainbow at the margin of the field of view. This problem is most prevalent at higher magnification; the use of a compensated eyepiece will usually correct the situation.

4. *Distortion*–This defect is caused by the lens giving rise to different

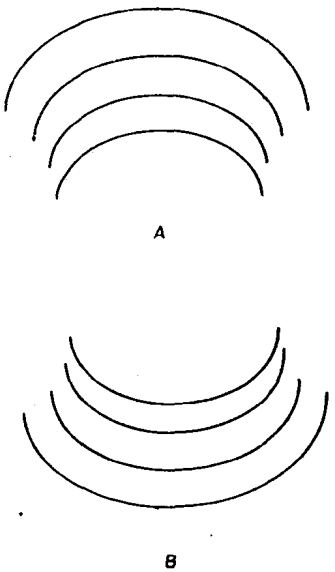

FIG. 1.3. Image of a series of parallel lines in the presence of (A) barrel distortion; (B) cushion distortion.

magnifications at the marginal and central portions of the image. There are two types of distortion–barrel and cushion (Fig. 1.3). It is the authors' experience that even high-quality instruments may have this defect. It can be checked by imaging an ocular disk containing a set of parallel lines. The defect is often encountered in microprojection and is serious when linear measurements are to be made. Good alignment of the instrument minimizes the defect, but it is not otherwise correctable by the operator.

5. *Astigmatism*–This error is related to distortion and occurs when the lens has unequal magnification at various azimuths. The off-axis image is badly deteriorated and cannot be brought to focus.

6. *Coma*–This is due to different lens-to-image distance for various parts of the lens (related to astigmatism). It derives its name from the fact that the Airy disk resembles a comet. Again the off-axis image is badly deteriorated.

7. *Curvature of field*–This results in a curved image of a flat field due to marginal portions of the microscopic field coming to focus at a different plane than the central portion of the field. This defect is present in the finest of high-power apochromatic objectives and minimized by the use of compensated eyepieces. Some compensated eyepieces have better correction than others of the same manufacturer but of a different power.

PARFOCALIZATION

Two or more objectives are parfocal with each other when it is possible to focus one objective on an object, turn to the next objective without focusing, and see the object in more or less sharp focus. This feature is especially important with respect to student (and in some cases even professor) use. If the 10X objective (low power) is not parfocal with the high dry power (40–47.5X) objective, the operator must refocus with the latter lens, which has a short working distance (ca. 0.3 mm), small field, and shallow depth of field. This often results in excessive breakage of slides and scratching of objectives, to say nothing of the loss of time involved.

TYPES OF OBJECTIVES

Achromatic–These are the lowest priced and are used on classroom microscopes, for routine work in research, and for maximum magnification fluorescence microscopy of weakly fluorescent objects. Chromatic aberration is corrected for two colors and spherical aberration for one color. Flat-field (planachromats) objectives of this design are now offered by several manufacturers.

Fluorite–The mineral fluorite is used in conjunction with special optical glasses. The corrections are for two colors in chromatic aberration and two colors in spherical aberration. This objective has been particularly useful in phase-contrast work.

Apochromatic–Chromatic aberration is corrected for three colors and spherical correction for two colors, affording brilliant images presented in

true colors and without distortion in shape. The highly actinic near ultraviolet rays are brought to the same focus as the longer visual rays, making these objectives highly desirable for serious work in photomicrography. Several manufacturers (Zeiss, Leitz) offer a series of planapochromatic objectives of complex design. These objectives are especially good for photomicrography of very thin specimens but may not be so suitable for thick specimens or where light transmittance is critical, as in high-resolution fluorescence (1000X) microscopy.

Ultrafluar—These objectives are manufactured by Zeiss and are primarily for microspectrophotometry. They are corrected for all wavelengths of light including the ultraviolet. Other manufacturers make mirror objectives which also transmit ultraviolet light, but these are somewhat less desirable.

OCULARS (EYEPIECES)

Oculars have distinctive optical characteristics that must be understood in order to use the correct ocular and the correct combination of ocular and objective under specific conditions. An ocular has a definite equivalent focal length which may be obtained from the catalogues. A more useful designation—engraved on modern oculars—is the magnification value, which ranges from 4X to 30X. For routine work and for classwork 10X is the most satisfactory magnification. The lower magnifications are likely to have marked curvature of the image; higher magnifications cause increasingly greater eyestrain, which is very pronounced with the 30X. Furthermore, there is an upper limit, beyond which the ocular produces only empty magnification with no gain in the resolution.

The maximum effective ocular magnification that may be used with a given objective can be easily computed. Assume that a 43X objective of NA 0.65 is being used; the formula is (1000) (NA of objective)/magnification of objective = (1000) (0.65)/43 = 15X, the approximate maximum ocular magnification. With a microscope on which the 43X objective is the highest power used, an ocular magnification of over 15X is of no value with respect to resolving power but of possible value for counting or drawing by projection. This simple calculation will enable a purchaser to specify the most useful lens combinations. Modern oculars are parfocal, making it possible to interchange oculars of different magnification without requiring much change of focus.

Optical Categories

Huygenian oculars are of comparatively simple two-lens construction. They are designed for use with achromatic objectives and yield inferior images with apochromatic objectives.

Compensating oculars are designed to correct certain residual aberrations inherent in apochromatic objectives. Therefore, it is imperative to use compensating oculars with apochromatic objectives, and oculars and objectives

should if possible be of the same make. These oculars may also be used with achromatic and fluorite objectives having magnification over 40X.

Most flat-field oculars are of the noncompensating type and yield images in which curvature has been considerably reduced. These oculars have various trade names, Hyperplane and Planascopic being the best known. A serious objection to some oculars of this type is that the eye must be held rigidly at a restricted position. The slightest lateral motion of the head cuts off part of the field, and prolonged use produces marked fatigue. Wide-field oculars have an exceptionally wide field and good correction for curvature but may have a restricted eye position as in the flat-field type. This objection may also be raised concerning high-eyepoint oculars, which are designed to permit the use of spectacles by the observer.

Workers who must or like to use spectacles with low-eyepoint oculars find that the lenses of the spectacles and oculars become scratched after some use. Some manufacturers supply rubber rings that fit around the periphery of the ocular. Alternatively one can paste a narrow ring of felt around the perimeter of each ocular. These devices permit the user to press his glasses against the ocular and so utilize the full field, without damage to the glasses or the ocular.

ILLUMINATION

There are two essential forms of illumination—transmitted and incident.

Incident or reflected light is discussed under fluorescence microscopy in Chapter 9. By far the most common method of illuminating a slide or other transparent object is by transmitted light. The light comes up from the base of the microscope, is projected through the hole in the stage, and passes through the preparation and into the objective. The simplest device for projecting light through the specimen is a concave mirror under the stage, designed to focus a converging cone of rays at the specimen plane. Such a device will fill the back lens of an objective of NA 0.25. Regardless of the character of the light source, whether daylight or artificial light, the curved mirror should be used only if the microscope has no condensing lens under the stage. If the microscope has a substage condenser, the flat (plano surface) mirror or prism should be used. Many microscopes, even elementary instruments, have internal illumination. These microscopes have a flat mirror or prism incorporated in the illumination system and thus require a condenser for transmitted light microscopy. Condensers provide for NAs of 0.25 and greater, which are necessary because the resolving power inherent in an objective can be fully utilized only if the illuminating system has a numerical aperture essentially equal to that of the objective.

A condenser is a system of two or more lenses under the stage, designed to receive a beam of parallel rays projected from a plano surface of a mirror or prism. The simplest type of condenser, known as the Abbe condenser, consists of two lenses. Although Abbe condensers are not corrected for color or curvature, they are suitable for most classroom work. The NA is 1.20 or 1.25. The upper lens may be unscrewed (not in an elementary laboratory!); the lower lens then serves as a long focus condenser of NA 0.30, suitable for

use with objectives of 10X (NA 0.25) or less. On some condensers the upper element is on a swinging yoke, whereby the upper lens can be swung aside, leaving the lower lens as a long focus condenser that fills the field of the lower-power objectives. A three-lens Abbe condenser with NA 1.40 is available for use with high-aperture objectives. One or both upper lenses are removable, giving NA 0.70 and 0.40 respectively. The Abbe condenser does not accurately focus the light in the object plane, and this results in considerable glare and flooding. It is impossible or extremely difficult to maintain oil-slide contact, and thus the Abbe condenser is not especially suitable for work requiring high resolution or high contrast. The best condensers for this purpose are the achromatic-aplanatic condensers that have a high degree of correction for image defects. Such condensers are usually provided with an oil-immersion cap giving an NA of 1.30 or 1.40. A maximum NA of 1.00 can be obtained with a condenser if the condenser lens and the slide are separated by a layer of air. Obviously an oil-immersion objective of NA 1.40 does not yield maximum performance unless the condenser, as well as the objective, is connected to the slide with immersion oil. For maximum resolution the condenser is immersed, but this practice is not recommended for routine work because (1) it is messy and time consuming; and (2) the condenser top lens must be carefully cleaned (with a good lens cleaner like Mir-o-len) after immersion, which greatly increases the wear on the top lens.

Kohler Illumination

It is not sufficient for the illumination system to potentially or actually fill the back lens of the objective; the light source must be focused properly if maximum visibility is to be attained.

The desirable source is a flat, luminous, grainless surface of sufficient size to cover the field of the lowest-power objective. This source, or more precisely the radiant field diaphragm of the source, is focused in the object plane. The mechanical procedure by which this is correctly accomplished will be explained in the next section. A rather minimal requirement for a microscope lamp is that it have a field diaphragm; for research use it should have its own condenser lens as well.

MECHANICAL OPERATION

A microscope usually has a set of two to five objectives installed on a revolving nosepiece. The objectives are normally precentered and parfocalized, each screwed into its designated opening in the nosepiece. Some advanced instruments have provision for centration of each of the objectives; in other cases the objectives are mounted singly in a centerable mount on the body tube of the microscopes. Precentered objective carriers, although not perfectly centered, are much preferred for classroom use because centerable objectives must be precisely centered before each use.

Some microscopes have a body tube that moves up and down for focus,