THE USE OF HOLLOW-CONE ILLUMINATION FOR INCREASING IMAGE CONTRAST IN MICROSCOPY

WILLIS W. MATHEWS
Biology Department, Wayne University, Detroit

Several optical methods for increasing the image contrast of transparent objects are available. Ordinarily, contrast is increased by reducing the numerical aperture (N. A.) of the illuminating cone (narrow-pencil illumination) or by using an objective of lower N.A. Either method necessarily reduces the resolution obtainable. Contrast may also be increased by the use of dark field illumination or specially constructed phase optics. We have found that another method, hollow-cone or annular bright field illumination, can give contrast and resolution superior to that obtainable with narrow-pencil illumination and under favorable conditions comparable to that obtained with the phase optics. The method has some advantages over dark field illumination and ordinary phase optics in that the contrast is variable and consists of both color and amplitude variations. Only standard optics are required and those made by the Bausch & Lomb Optical Co. were used.

To obtain maximum contrast with hollow-cone illumination, the N.A. of the cone is critical and may vary with the object, the mounting medium, the N.A. of the objective, and thickness of the cover glass. Hence, it is desirable to use an illumination method which will produce a hollow cone of continuously variable N.A. Such a method could also be used with phase contrast objectives and for dark field illumination.

Variable hollow-cone illumination was produced with an ordinary Abbe condenser (N.A. 1.25) and microscope lamp with a diaphragm. The top lens of the condenser was removed and the lower lens taken out of its mount, inverted, and returned to the mounting ring. The condenser diaphragm should be open. The lamp should be adjusted for Köhler illumination with the lamp diaphragm closed to a diameter of about 8 mm when the lamp is 35 cm from the microscope. A diffusing ground glass may be used on the lamp, if needed. The 4 mm achromatic objective (N.A. 0.65) gave the best results with hollow-cone illumination and it may be adjusted in the following manner. The objective should be focused on an object of low contrast. Epithelial cells from the mouth mounted in saliva are suitable. A cover glass 0.14 to 0.18 mm thick should be used. If the back lens of the objective is now observed, it will be seen that as the condenser is raised from a low position the disk of light will break up into a central spot and a ring. The spot will become smaller and the ring larger. This effect is caused by the high spherical aberration of the inverted condenser lens.

The diameter of the lamp diaphragm controls the thickness of the ring and serves as the field diaphragm. If an ordinary dark field stop, 16 mm in diameter, is now placed under the condenser, the central spot of light will disappear and the ring of variable diameter remains. The ring indicates that the objective is being illuminated by a hollow-cone of light of variable N.A.

The mirror should be carefully adjusted so that the ring is centered in the back lens of the objective and is of uniform brightness. The examination of the object as the condenser is raised and lowered will locate the con-

denser position for maximum visibility. Maximum contrast is usually produced as the ring of light reaches the edge of the objective aperture; that is, when the N.A. of the hollow-cone approaches the N.A. of the objective. Under these conditions a beautiful color and amplitude contrast image may be produced. Objects of higher refractive index are dark blue on a background of lighter blue-green. A light red halo surrounds the blue image. Objects of lower refractive index than the surrounding medium are yellow or red. Since raising the condenser increases contrast a wide range of contrasts may be secured by regulating the position of the condenser. If the condenser is raised so that the N.A. of the illuminating cone exceeds the N.A. of the objective, a gradual transition to dark field occurs. This characteristic of the method increases its usefulness. The condenser should be continually raised and lowered so that each detail of the image is illuminated for optimum visibility.

To obtain maximum contrast with hollow-cone illumination, glare should be reduced to a minimum. This may be done by: (1) closing the lamp diaphragm until only the field of view is illuminated; (2) excluding stray light from the direction of the lamp by using a large dark field stop 25 mm in diameter; (3) keeping the level of illumination low with neutral filters or voltage control on the light source. Contrast also increases with the wavelength of light used. Hence, a green, yellow or red filter progressively increases contrast and blue filters decrease it.

To use annular illumination with the 8 mm achromatic objective, the N.A. of the illuminating cone must be reduced to slightly less than 0.50 by racking the condenser downward. With the 16 mm objective the cone must be further reduced and the dark field stop can be no larger than 10 mm in diameter. These two objectives can be properly illuminated without inverting the lower condenser lens; but for the 4 mm objective, the greater spherical aberration produced by inverting the lens is required.

Cover glass thickness is critical when using the 4 mm objective with hollow-cone illumination. The best results were obtained with covers 0.14 to 0.18 mm thick. Most number 1 covers are within this range. Also, the cover glass should be pressed as close to the object as possible so that the optical path is not increased by the mounting medium.

In order to produce a hollow-cone of light with sufficiently high N.A. to use with immersion objectives, the method usually employed for dark field illumination is suitable. An assembled Abbe condenser or achromatic condenser may be used. A dark field stop should be in place with the condenser in immersion contact with the slide. The condenser diaphragm should be closed until it can just be seen at the edge of the back lens of the objective. The dark field stop should be large enough to obscure all but a narrow marginal zone of the back objective lens. Its size will be determined by the N.A. of the objective and of the condenser. With immersion objectives all adjustments are critical and they are best made while observing the back lens of the objective with a telescope such as is used with phase optics.

The method described of producing annular illumination with the the lower lens of a Abbe condenser was successfully used with all phase objectives available to us. These included the Bausch & Lomb 4 mm and 1.8 mm objectives and the Wild objectives of 0.45, 0.65 and 1.25 N.A. All were of the dark contrast type. A telescope for viewing the phase ring of the objective is needed. The ring of light is produced as first

described and centered on the back lens of the phase objective by adjusting the microscope mirror. The dark field stop should be 10 mm in diameter. The condenser is then raised or lowered until the annulus of light is coincident with the phase ring of the objective. If the annulus of light is thicker than the phase ring, it may be reduced by partially closing the condenser diaphragm. Little loss of quality in the phase image was noticeable with this method as compared with images produced using the phase condenser supplied by the manufacturers (fig. 3, 4). It then appears possible to utilize phase optics by merely purchasing the phase objective needed. This permits a cost saving of about 60% if only one phase objective is required.

Good results using hollow-cone illumination were obtained with dry objectives of N.A. 0.66 or less on a variety of objects. These included living protozoa, bacteria (fig. 7), moulds, algae, epithelial cells from human mouth (fig. 1, 2), from frog mouth (fig. 5), frog epidermis, nematode larva (fig. 8) and a culture of mammary carcinoma. Fixed, unstained verebrate tissues mounted in glycerin or mineral oil were well differentiated (fig. 6). The method is often useful on both stained and unstained tissue sections mounted in balsam or synthetic resin mounting media. For example, nerve fibers in the frog retina which had been fixed in Bouin's fluid and mounted in H.R.S. medium were well differentiated. Dry objectives with N.A. greater than 0.66 gave poor results.

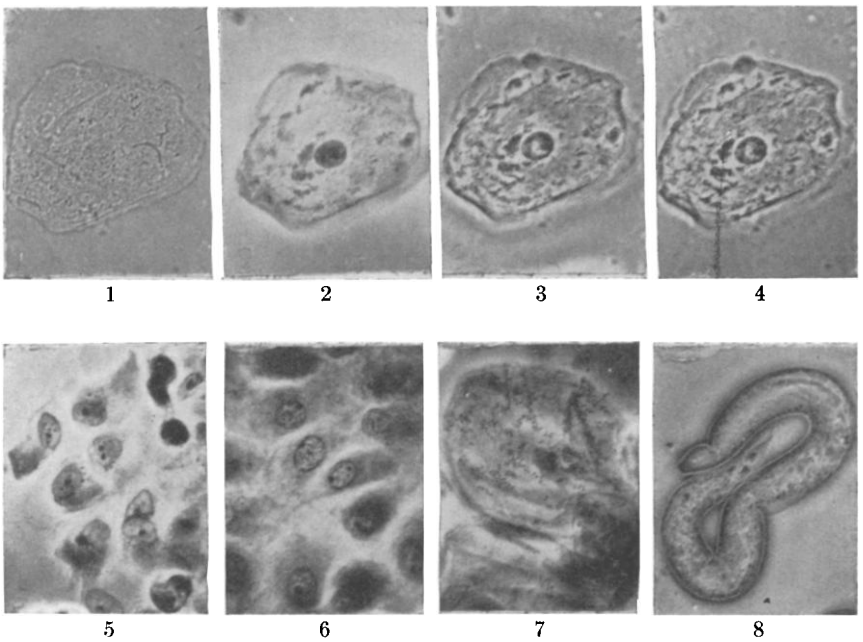
With a given object, the refractive index of the mounting medium is important and for good results it should be slightly lower than the refractive index of the object. These conditions are usually approximated when a tissue is fixed and mounted in the ordinary way. Apparently, the refractive indices which give good visibility with phase optics (Crossmon, 1949b) are also best for the hollow-cone illumination method.

Fixed cytological material is best when sectioned very thin and mounted in a medium of lower refractive index. For example, pancreas fixed in Flemming's fluid, sectioned 2 μ thick and mounted in mineral oil was well differentiated. Nucleoplasm was yellow and chromatin, secretion granules and mitochondria were blue when viewed with hollow-cone illumination using the 4 mm achromatic or the 4.3 mm, 1.00 N.A. fluorite immersion objective.

The performance of most immersion objectives with annular illimination was disappointing when compared to the good results obtained with dry objectives. However, the 4.3 mm fluorite immersion objective (1.00 N.A.) did give good dark contrast with annular illumination. With this objective the illuminting cone should be slightly less than maximum N.A. The 1.8 mm fluorite, the 1.8 mm achromatic, and 2 mm apochromatic objectives were less satisfactory.

The failure of most objectives of high N.A. to give good contrast with hollow-cone illumination appears to be related to the poorer correction of these objectives for spherical aberration. Objectives of higher N.A. than about 0.65 can not usually be corrected within the Rayleigh limit of $\frac{1}{4}$ wavelength phase shift (Foster, 1950). Apparently, the excessive phase distortion produced by the aberrations of these objectives prevents proper phase relations at the image plane for good contrast. The only objective of high N.A. which did give good contrast with hollow-cone illumination, the 4.3 mm fluorite objective, is corrected within the Rayleigh limit (Benford, J.R., personal communication).

Annular bright field illumination has occasionally been used in the past. Hogg (1911, p.194) suggested it for the resolution of diatoms Zernike (1942) investigated annular illumination in connection with his studies of phase microscopy and showed that as the radius of the annulus



FIGS. 1–4. Photomicrographs of the same unstained epithelial cell from the mouth mounted in saliva. In each, the focus was made sharp on the edge of the nucleus.

FIG. 1. 4 mm achromatic objective, N.A. 0.65, solid illuminating cone of light reduced to about one-half the N.A. of the objective.

FIG. 2. The same objective as in Fig. 1 but with the hollow-cone illumination. The nucleus is differentiated as if it were stained. The absence of some cytoplasmic granules which are visible in Figs. 1, 3, and 4 is due mainly to the reduced depth of field with hollow-cone illumination.

FIG. 3. 4 mm phase objective, N.A. 0.65, illumination with lower lens of an Abbe condenser as described in the text.

FIG. 4. Same objective as in Fig. 3, illumination with phase condenser supplied by manufacturer. Note the nearly identical appearance of Figs. 3 and 4.

FIGS. 5–8. Unstained objects illuminated with a hollow cone of light.

FIG. 5. Freshly isolated cells from frog's oral mucosa in Ringer's solution, 4 mm achromatic objective.

FIG. 6. Pig amnion cells fixed in Bouin's fluid and mounted in glycerin, 4 mm achromatic objective.

FIG. 7. Living bacteria on surface of epithelial cell from mouth mounted in saliva. Note surface markings on cells. 4 mm, achromatic objective.

FIG. 8. Living young nematode larva from frog. Both surface contours and internal details are visible, 8 mm achromatic objective.

All photographs were made with Wratten G and B filters on the light source. Ansco Supreme film was used and developed in DK50 for 7 min. at 70 F with continuous agitation. All were printed on Velox F-4.

is increased, the contrast of the image of the diatom markings is altered and even reversed. Oettlé (1950) has shown a similar effect with erthrocytes mounted in glycerin. He was able to increase the contrast further by partially absorbing the undeviated rays at the back focal plane of the objective and he referred to the effect as "amplitude contrast". This was accomplished by the use of a variable phase and amplitude microscope. Hallimond (1947) advocated annular illumination combined with opaque annuli which intercepted some of the deviated rays. Bennett, et al, (1951, p. 130) showed that the diameter of the annulus in phase objectives affects image contrast. However, the effectiveness of simple, annular bright field illumination for increasing contrast has not been fully appreciated. This may have been due, in part, to the lack of flexible control over the N.A. of the illuminating cone with the usually employed method of inserting annular stops under the condenser.

The contrast produced by hollow-cone illumination of high N.A. may result in part, from the oblique direction of the illumination. This causes deviated rays outside of the illuminating cone to be lost to the objective, thus darkening the image in respect to the background (Saylor, 1935). The colors of the image produced with oblique illumination arise from the dispersion effects of the object according to Wright (1913). However, tissue sections mounted in Crossmon's (1949a) high dispersion medium did not show increased color. In fact, when the refractive index of the medium approximated that of the object, the color and contrast were at a minimum. These conditions should give maximum color effects if the contrast with hollow-cone illumination were "dispersion staining" (Crossmon, 1949b).

The obliquity of the illumination also is responsible for the excellent resolution found which was sufficient with the 8 mm, 0.50 N.A. and the 4mm, 0.65 N.A. objectives to reveal the dot structure of *Pleurosigma angulatum*. The high N.A. of all rays of the illuminating cone also reduces the depth of field.

Because hollow-cone illumination is symmetrical, it does not produce the strong relief found with ordinary oblique illumination (Zernike, 1942). However, if such relief is desired, it may easily be produced in controlled amounts by blocking out from ⅓ to ⅔ of the illuminating cone. Unsymmetrical hollow-cone illumination produced in the manner emphasizes boundaries in objects of very low contrast.

It seems probable that much of the contrast found with hollow-cone illumination was true phase contrast in which the phase differences between the deviated and undeviated rays were introduced by slightly defocusing the image (Zernike, 1942). However, the quality of the image does not suffer by such defocusing with properly regulated hollow-cone illumination as it does with narrow-pencil illumination. With either type of illumination defocusing upward produces a dark-contrast image and defocusing downward a bright-contrast image. The former effect is usually preferable if the medium has the lower refractive index. The bright-contrast image is best if the medium has a higher refractive index than the object.

As the outer surface of the illuminating cone is made to advance beyond the aperture of the objective by raising the condenser, some of the undeviated rays are lost to the objective while the deviated rays inside the cone are all captured by the objective. This weakens the undeviated rays

relative to the deviated rays thus increasing the destructive interference at the image plane and therefore increasing image contrast. The partial absorption of undeviated rays in phase objectives and in amplitued contrast (Oettlé, 1950) increases contrast in the same manner.

It is possible that some of the phase change produced in hollow-cone illumination may be introduced by spherical aberration of the objective (Bennett, et al, 1951, p. 78). Since the undeviated rays of the cone pass through the marginal zone of the objective where the spherical aberration is great and the deviated rays pass through the more axial zones, a phase shift between the two paths appears possible. This effect might also contribute to the color in the image, since the phase shift introduced by spherical aberration is different for different wave lengths (Bennett, et al., 1951). Such a variation in phase shift would explain the high contrast produced with red light and the low contrast with blue light. Also, on this basis one would expect that the 8 mm, 0.65 N.A. apochromatic objective with its superior correction for spherical aberration would produce an image nearly free of color contrast with hollow-cone illumination. This is actually what is found.

The performance of hollow-cone illumination suggests that it is a system of variable color phase contrast microscopy using standard optics and that it is often as effective as special phase optics.

References

Bennett, A. H., H. Osterberg, H. Jupnik, and O. W. Richards. 1951. Phase microscopy, principles and applications. John Wiley & Sons, Inc., New York. Chapman & Hall, Limited, London.

Crossmon, G. C. 1949a. The "dispersion staining" method for the selective coloration of tissue. Stain Technology, 24: 61–65.

1949b. Mounting media for phase microscope specimens. Stain Technology, 24: 241–247.

Foster, L. V. 1950. Microscope optics. Jour. Optical Soc. Amer., 40: 275–282.

Hallimond, A. F. 1947. Production of contrast in the microscope image by means of opaque diaphragms. Nature, 159: 851–852.

Hogg, J. 1911. The microscope. 15th ed. George Routledge & Sons, Limited. London and New York.

Oettlé, A. G. 1950. IX. Experiments with a variable amplitude and phase microscope. Jour. Roy. Micr. Soc., 70: 232–254.

Saylor, C. P. 1935. Accuracy of microscopical methods for determining refractive index by immersion. Bur. Stand. Jour. Res. Wash., 15: 277–294.

Wright, F. E. 1913. Oblique illumination in petrographic microscopic work. Amer. Jour. of Sci., 35: 63–82.

Zernike, F. 1942. Phase contrast, a new method for the microscopic observation of transparent objects. Physica, The Hague, 9: 686–693, 974–986.