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# Diffracted-Light Contrast Enhancement: A Re-Examination of Oblique Illumination

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*KEY WORDS* contrast enhancement; light microscopy; oblique illumination; schlieren image; shadowcast

*ABSTRACT* A re-examination and modification of the very old oblique illumination technique has resulted in a method for contrast enhancement in microscopes, diffracted-light contrast (DLC), which provides high-contrast, high-resolution images of unstained biological material. The technique, which utilizes the diffracted light from the edge of a small, opaque plate, provides shadowcast images similar to those obtained by Nomarski DIC, anaxial illumination, modulation contrast, or single-sideband microscopy; however, it requires only a single additional component, which can be added to any bright field microscope. The contrast and three-dimensionality of the final image can be controlled by inserting differently shaped edges. Any bright field condenser will work with the technique and, consequently, it is a technique that may be especially useful with relatively basic, inexpensive laboratory and teaching microscopes although the image produced on a research grade microscope is of very high quality, comparable to that obtained with DIC. *Microsc. Res. Tech. 46:334–337, 1999.* 

## INTRODUCTION

Prior to the development of phase-contrast microscopy, contrast enhancement of unstained biological material was commonly accomplished by modifying the condenser so that the specimen was illuminated with an off-axis (oblique) beam of light (Inoué and Spring, 1997). A de-centering condenser aperture was developed by Abbé and, although this device is not present on today's microscope, its function may be duplicated by reducing the condenser iris diaphragm and rotating it laterally. This very basic anaxial technique results in a shadowcast image with improved contrast but with some degradation in resolution and increased depth of field due to the reduced numerical aperture of the condenser. Oblique illumination could also be accomplished by introducing large, opaque beam stops into the illuminating system of a microscope. This approach (reviewed by Bloss, 1967) was used primarily as a means of optical refractometry and its imaging capabilities were limited (Saylor, 1935). Regardless of the technique used, the object is to create and illuminate the sample with an oblique cone of light (Smith, 1994).

Oblique illumination was replaced by a host of more sophisticated contrast-enhancing techniques, e.g., phase-contrast (Zernike, 1935), differential interference contrast (Nomarski, 1955), modulation contrast (Hoffman and Gross, 1975), and single-sideband microscopy (Ellis, 1978). All of these techniques involve modifying the illuminating beam, generally by altering the condenser by the inclusion of special apertures, polarizers and prisms, or half-masks. The resulting image is then filtered or modulated at the image plane of the objective lens. Oblique illumination was largely abandoned until the 1980s when Kachar (1985) resurrected the technique by moving the light source slightly off-axis. Because Kachar did not need to alter the condenser, the resulting images exhibited excellent resolution but the contrast was not sufficient for viewing through the oculars. Therefore, to be of value, this method of oblique illumination must be harnessed to the contrast-enhancing capabilities of the video camera.

## MATERIALS AND METHODS

The high-contrast images presented in this report were formed simply by inserting a small, opaque plate (termed the edge-plate, Fig. 1) into the illuminating system (i.e., anywhere from the lamp to that region near the field stop, Fig. 2). Three differently shaped edges were used: convex and straight (shown in Fig. 1), as well as concave. Curved edges had a radius of 32 mm. If the plate is inserted just at the level of the field stop diaphragm, then the condenser must be slightly defocused from its usual Koehler position. When properly inserted and defocused, the blurred image of the edge-plate lies just within the field of view. Shadowcast images lie just within the darkened area found close to the edge of the edge-plate. By adjusting the extent of condenser defocus and the position of the edge-plate, the darkened area containing the best images may be broadened to fill the entire field of view (Fig. 3a–c). The light-dark background gradient, apparent in Figure 3b

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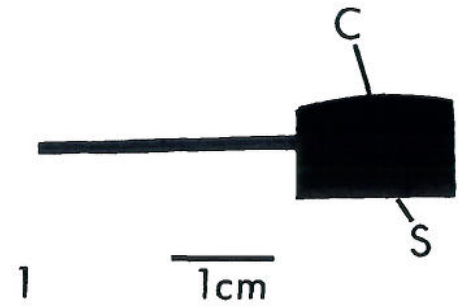



Fig. 1. Edge-plate used to generate the DLC image. The plate shown has two diffracting edges: straight (S) and convex (C), either of which can be used to form an image.

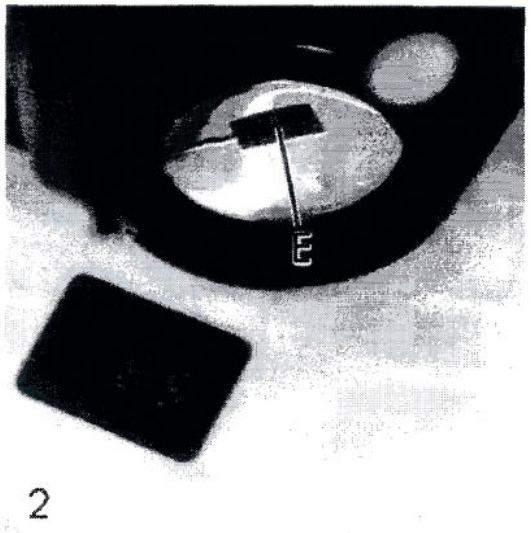



Fig. 2. Insertion of an edge-plate (E) just above the field stop of a Leitz Diaplan. The field stop and condenser iris diaphragms are left wide open.

and c, may also be minimized by continuing to defocus and reposition the edge-plate. This gradient is not apparent when using higher magnification objectives that have a smaller field of view. Although the technique can work if the edge-plate is inserted at the level of the condenser (just above, below, or within), the resulting image will suffer from excessive depth of field and can be quite astigmatic. Best results were always obtained with the edge-plate positioned in the illuminating system and with both the field stop and condenser iris diaphragms left wide open.

## RESULTS AND DISCUSSION

The high-resolution micrographs shown in Figure 4 demonstrates the effect of edge-shape on the final image. The convex edge provides the best shadowing, highest contrast, and most three-dimensional appearance while the concave edge gives a lower-contrast, more two-dimensional image. The relationship between image quality and edge-shape suggests that the edge itself is modifying the illuminating beam by diffraction and it is this light, not the oblique illumination that is primarily responsible for the images obtained with this method. Differently shaped edges produce different patterns of diffracted light. The various beams interact with a refractive sample in different ways and generate images that also vary.

The use of diffracted light as an illuminating beam is the basis of schlieren imaging. All the components for a type of schlieren imaging are found within DLC. The edge-plate serves as a source of diffracted light and the unstained cells serve as a refractive sample. As shown in Figure 1, more than one diffracting edge may be found on a single edge-plate and it is only necessary that the edges be sufficiently separated so that light from only one edge is being used to form the image. Defocusing the edge-plate with the condenser provides the necessary darkened background with which to view the sample and this appears to be functionally analogous to introducing a second knife. Focusing the condenser above or below the edge-plate will cause a reversal of the shadowing.

With the discovery that high-contrast, high-resolution images can be formed by simply placing a small edge-plate in the illuminating system of a microscope, many of the disadvantages inherent in earlier techniques have been overcome. Practitioners of the old oblique illumination technique were trying to create an oblique beam of illumination through the use of large beam stops. However, the beam stops used also provided a diffracting edge and it may be that, in some instances, the success achieved with that technique should have been attributed to that edge rather than the oblique illumination. In what is perhaps one of the most detailed discussions of oblique illumination, Wright (1913) reveals seven different procedures for creating oblique illumination (one of which includes sliding a finger across the lower focal plane of the condenser and adjusting the condenser focus). He shows a hand-drawn image and clearly that image is close to the shadow of his beam stop. Bloss (1967), while attributing successful results to the use of oblique illumination, states that best results are obtained when the image is formed close to the geometric shadow of the beam stop edge. These investigators failed to recognize the importance of the diffracted light coming from edges, and the subsequent importance of edge shape, in forming the image.

Saylor (1935, p. 282) shows a "double-diaphragm method of oblique illumination," which looks virtually identical to a classic schlieren apparatus. An adjustable beam stop is placed within the condenser and another fixed beam stop is located just behind the objective lens. This basic design is repeated in the modulation contrast microscope (Hoffman and Gross,

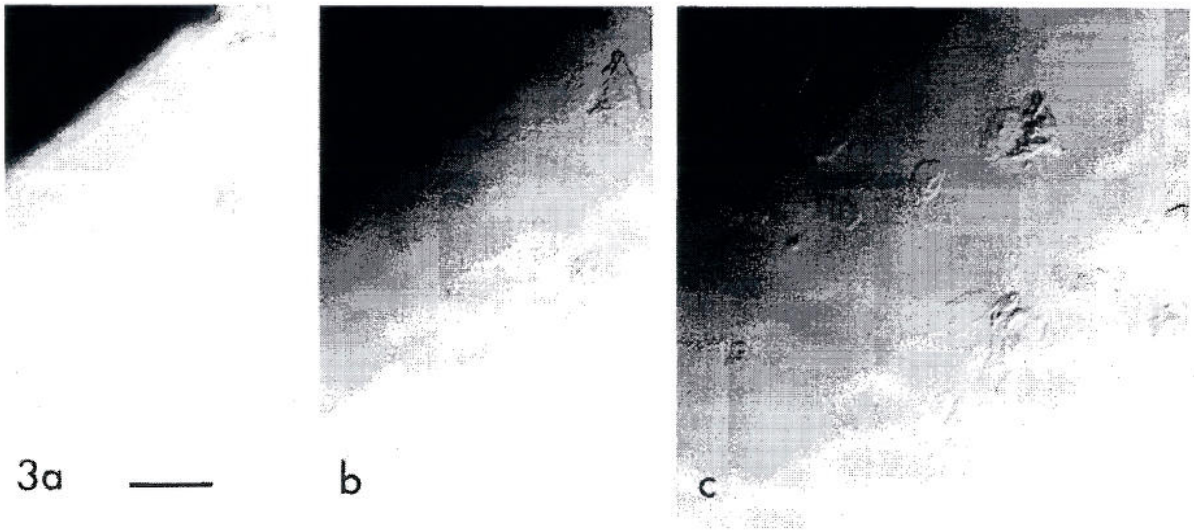



Fig. 3. a–c: Generation of contrast as the image of the edge-plate is increasingly defocused with the condenser. With the edge-plate focused (**a**), little contrast is seen and the image is basically what one would see when viewing unstained cells under ordinary bright field illumination. As the margin of the edge-plate is defocused (**b,c**) good contrast is generated over an expanding area. By continuing to adjust the condenser focus and the position of the edge-plate, it is possible to get good contrast over the entire field, with a minimal light-dark background gradient, even when using low power lenses. Leitz Fluotar 10/0.10 with white light. Scale bar = 200 µm.

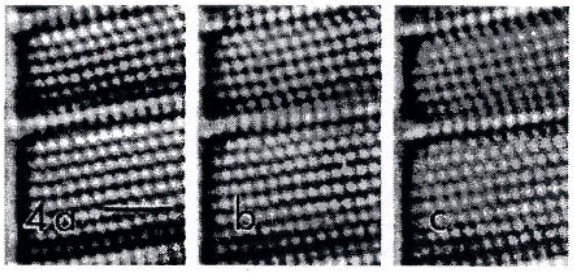
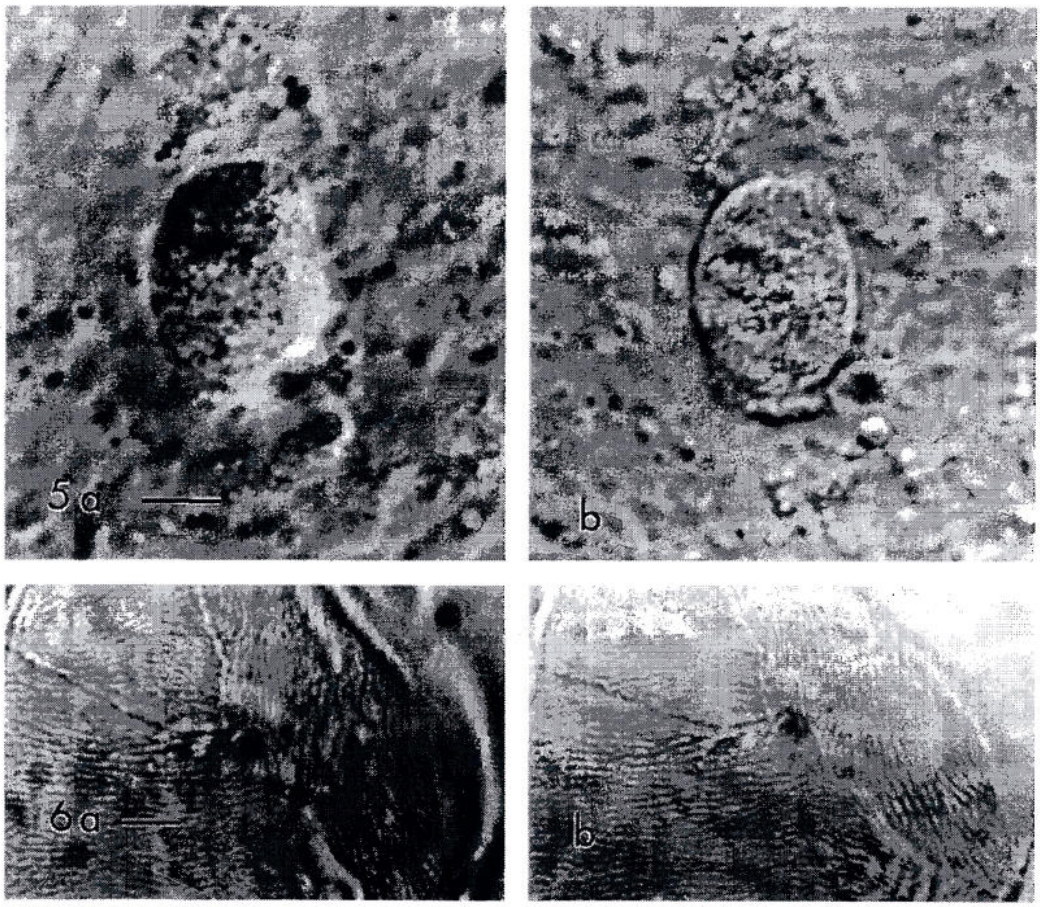



Fig. 4. a–c: Resolution and the effect of edge shape. The resolution obtainable with DLC is comparable to or better than that obtained with other contrasting techniques. Shown are identical areas on the frustule of the diatom *Surirella gema* (surface lattice of 0.41 µm). The lattice is easily visible, which suggests that the minimum resolution attainable with DLC is actually much better than the 0.41 µm reported here. The shape of the edge has a profound effect on the image with a convex edge (**a**) giving rise to the best contrasted, most three-dimensional image. Contrast and three-dimensionality are less when a straight edge (**b**) is inserted and are quite poor when concave (**c**) edges are used. Leitz Fluotar 100/1.32 oil with green filter. Scale bar = 2 µm.

1975), which has a slit aperture beneath the condenser and an adjustable modulator in the Fourier plane behind the objective. Axelrod (1981) duplicates this arrangement with the simple application of black masking tape to his bright field condenser and to the back focal plane of his objective lens. Once again, no mention is made of the importance of edge shape in image formation.

The small edge-plates used in the present study are unlike the earlier beam stops and serve primarily as a means of introducing a diffracting edge into the optical path. The large beam stop thought necessary to achieve oblique illumination, and which could occlude nearly 80% of the light, has been replaced by a relatively small edge-plate that blocks only a small fraction of the light, allowing for a brighter image. The edges are shaped so as to give those patterns of diffracted light which produce the best images and enable the user to vary the contrast and three-dimensionality. The image produced has excellent contrast and is sufficiently bright so as to be easily visible through the oculars. Also, the technique can be used with relatively simple microscopes that lack a movable light source or adjustable, multiple-aperture condenser. The insertion of the edge-plates below the condenser means that the full numerical aperture of the condenser can be used. This results in an image with a resolution and depth of field superior to that obtained by placing the plates near the condenser. Certainly the technique produces excellent images on research grade microscopes (comparable to those obtained with Nomarski DIC, Figs. 5 and 6); however, the ease and flexibility with which the technique may be performed make it suitable for use on any microscope, no matter how simple. Therefore, it may be especially useful with the more basic optics found on teaching/laboratory microscopes (the technique has been successfully used on an Olympus CH microscope), making it possible to routinely (and inexpensively) obtain high-quality images of living, unstained specimens in the classroom.




Figs. 5, 6. Micrographs comparing DLC imaging with Nomarski DIC imaging. The nucleus of a buccal epithelial cell shows better contrast and more detail when imaged with DLC (**5a**) than when imaged with Nomarski DIC (**5b**). Ridges on the cell surface also show better contrast when imaged with DLC (**6a**) vs. Nomarski (**6b**) illumination. Micrographs were printed on the same grade photographic papers and with the same enlarger settings. Zeiss Photomikroscop III, 100/1.32 oil (with phase ring), white light. Scale bars = 4 μm (5), 5 μm (6).

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