Adv Physiol Educ 31: 232–235, 2007;
doi:10.1152/advan.00028.2006.

Phase-contrast versus off-axis illumination: is a more complex microscope always more powerful?

Zdeněk Hostounský[1] and Radek Pelc[1,2]

[1]*The Stentor Institute, Praha-Západ; and* [2]*Institute of Microbiology, Academy of Sciences of the Czech Republic, Praha, Czechoslovakia*

Hostounský Z, Pelc R. Phase-contrast versus off-axis illumination: is a more complex microscope always more powerful? *Adv Physiol Educ* 31: 232–235, 2007; doi:10.1152/advan.00028.2006.—In this article, a practical demonstration suitable for any biology college classroom is presented. With the examples of a complex biological specimen (slug's radula) and a simple reference specimen (electron microscopical grid imprint in gelatin), both of which can be easily prepared, the capabilities of two imaging modes commonly used in optical microscopy are demonstrated. The results obtained under phase contrast (a rather sophisticated method, 1953 Nobel Prize to Zernike) and off-axis illumination (a very simple method) are compared. The off-axis illumination setup is capable of delivering noticeably better microscopic images of these two particular specimens, yet it can be easily assembled in a laboratory classroom. The outcome of such a demonstration is expected to be the realization on the part of the students that one needs to carefully choose the apparatus to address a given biological problem, with the "bottom line" being that a more complex one may not necessarily yield better results. An attempt to explain this "paradox" is presented, in the particular case presented here, partly from the physiology of vision perspective (the shape-from-shading problem). The overall aim of the present article is to induce in students critical thinking about the capabilities of a laboratory equipment in general and about data interpretation.

three-dimensional impression and differential imaging; depth perception; relief diaphragm; shape-from-shading; visual cues

IT IS GENERALLY BELIEVED by many students that considerable technical complexity is needed to achieve good science. The fact that there are numerous exceptions to this "rule" needs to be not only mentioned but also demonstrated in a laboratory classroom.

In the biology practical class, the optical microscope represents the most common "interface" between the macroscopic and little-known microscopic world. Optical artifacts stemming from an incorrectly adjusted microscope illumination are well known, e.g., the diffraction circles when the condenser aperture is closed too much. However, various artifacts still persist in the image even at the optimal (Köhler) setting (6, 11). These include, e.g., the disturbing "halo" artifact in the images obtained under the phase-contrast microscope (1, 6, 13, 19). A refined version, apodized phase contrast (9), introduced for the first time by Nikon (e.g., United States Patent No. 6317261), can partially eliminate the halo.

Here, we demonstrate that some of these issues can be easily addressed in a laboratory classroom by using, as an example, a simple biological specimen and a simple reference specimen, both of which can be easily prepared and examined under two different illumination modes commonly used in optical microscopy, namely, phase-contrast and off-axis illumination. The aim is to expand the scope of approaches that can be used to demonstrate various optical illusions (6–8, 14) and to relate the outcome of observation to the complexity of both the specimen and experimental apparatus.

Address for reprint requests and other correspondence: R. Pelc, Institute of Microbiology, Academy of Sciences of the Czech Republic, K Výzkumným ústavům 1083, CZ-14220 Praha 4-krc, Czechoslovakia (e-mail: rpelc@biomed.cas.cz and radekopelc@seh.oxon.org).

EXPERIMENTAL PROCEDURES

Apparatus

An upright laboratory microscope [model DN45–BH51, Lambda Praha (formerly Meopta Czechoslovakia)] was used. The (negative) phase-contrast images were taken with Plan 10Ph/0.25 (radula) or Plan 20Ph/0.40 [electron microscopical (EM) grid imprint] objectives (planachromatic); a condenser normally supplied with the model DN45-BH47Ph microscope was used (Lambda Praha, numerical aperture 1.25). All other images were acquired with 10/0.25 (radula) or 20/0.45 (EM grid imprint) objectives (achromatic) and a shifting relief diaphragm condenser (Abbé type, numerical aperture 1.2, model RCH-0128), with the relief diaphragm either engaged (off–axis illumination) or disengaged (axial, i.e., ordinary bright-field illumination). The condenser itself is shown in Fig. 1. All photomicrographs were taken with Minolta X300s or Praktica VLC3 camera on Fuji Superia 100 color print film; a photographic eyepiece (FU ×4, Lambda Praha) and a green filter were used.

Although a commercially available condenser with a built–in relief diaphragm (4, 5) and an accessory lens (5) was used, almost any laboratory microscope equipped with a classical condenser (i.e., not the long-working-distance type found on inverted microscopes) can be easily converted to provide the off–axis (or oblique, inclined) illumination of reasonably good quality.

INSTRUCTIONS. Cut a "D"–shaped piece from a black cardboard ("relief diaphragm") and place it as close as possible to condenser's iris diaphragm (front focal plane). Most condensers in upright microscopes are fitted with a filter holder placed just under the iris diaphragm. The relief diaphragm can be placed into this holder, which can be usually swung out of the optical axis. When viewing the specimen, try to experiment with the position and orientation of the relief diaphragm until the most satisfactory image is obtained. The most natural orientation of the diaphragm is the one providing an apparent illumination angle from the "north" (or northwest/northeast), which is reminiscent of overhead (sun) illumination (see RESULTS AND DISCUSSION for details).

Various other shapes of the relief diaphragm can be used as well, e.g., with its edge slightly concave, as in the present study or elsewhere (4, 5), or convex. A positive lens (instead of the relief diaphragm) placed in the above-mentioned filter holder slightly swung out from the condenser's optical axis represents a less efficient but usable way of producing off–axis illumination (18).

The relief diaphragm does not necessarily have to be inserted into (or close to) the front focal plane of the condenser. Other optical planes that are conjugate with it may be modified instead (6) or in addition (15). One of these options is the objective back focal plane, which is usually accessible in low-magnification objectives (1). In this way, a pseudorelief [quasi-three-dimensional (3-D)] effect is achieved in a manner similar to the more complex Hoffman modulation

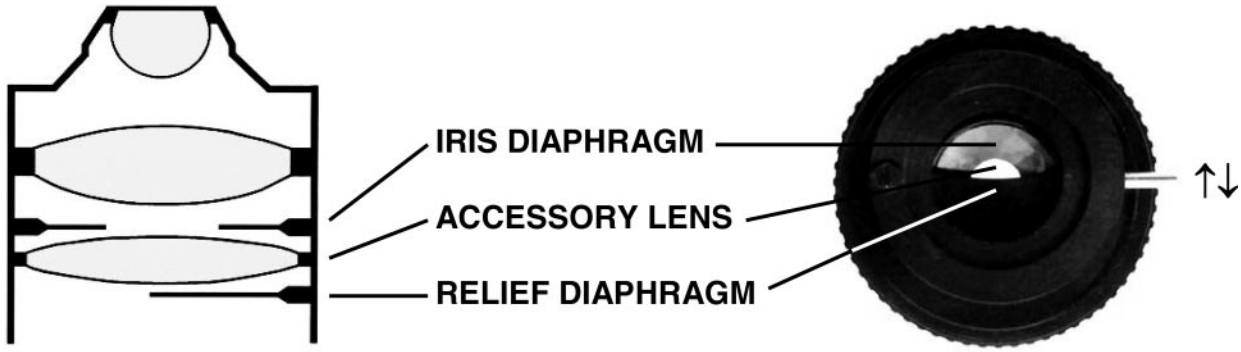

Fig. 1. A simple microscope condenser capable of delivering off-axis illumination. In addition to the original design (4), the present condenser (5) features a built-in positive accessory lens between the relief and iris diaphragms. Apart from collimating the light beam, the lens brings the two diaphragms optically closer together, thus enhancing the quasi-three-dimensional (pseudorelief) effect in the images and improving viewing field homogeneity. See the text for details. *Left*: side view (a simplified diagram, not to scale). *Right*: bottom view (i.e., in the direction of microscope illumination), with the outer casing removed. Arrows indicate the movement of the sleeved relief diaphragm, which can be either engaged (↑) or disengaged (↓). The fully engaged position is shown.

contrast (3). These various possibilities have been described in detail elsewhere (1, 5, 6, 15).

Note that it is critical to bring the relief diaphragm as close as possible to the condenser's front focal plane or a plane conjugate with it (6). This guarantees not only a strong relief (3-D) impression in the images but a uniform illumination of the viewing field as well. Failure to comply with this basic requirement can easily lead to rather disappointing results in that only a small portion of the image is of satisfactory quality (10), a factor that is also of importance in macroscopic schlieren imaging (15). In some condensers, mechanical constraints may not permit the relief diaphragm to be placed close enough to the iris diaphragm. In that case, an accessory lens, which is, in some condensers, already built in immediately under the iris diaphragm, may be utilized to bring the two diaphragms optically closer together, as in the present design (5) (Fig. 1).

Specimen Preparation

The biological specimen. The biological specimen (radula, a translucent chitinous ribbon in the mouth of certain mollusks) was isolated from the Spanish slug [*Arion lusitanicus* Mabille 1868 (*Stylomatophora, Arionidae*)], which was obtained locally.

INSTRUCTIONS. Let the slug stretch out. Cut off the front part (~1/4 of the slug) with a scalpel or scissors, and submerge it overnight into a concentrated solution of NaOH [~10% (wt/vol)].[1] Expose the radula by removing the remnants of the soft tissues, and then rinse the specimen in water, dry it, place it on a microslide, and cover it with a coverslip. It is recommended to attach the coverslip's edges to the microslide, e.g., with wax or Canada balsam, to keep the radula in place. In the adult slug we used (~10 cm long), the radula was a yellowish flat object ("scale") ~4 mm long and ~2 mm wide. Other slug or snail species would also be suitable. However, the animal should be at least ~2 in. (5 cm) long. Otherwise, the radula may be too small and difficult to locate and handle, and its relief structure may not be prominent enough to enable proper visualization under off-axis illumination.

The reference specimen. The reference specimen was prepared from gelatin.

INSTRUCTIONS. Make a few milliliters of an aqueous gelatin solution [~5–10% (wt/vol), ~50°C] in either a beaker placed in a water bath or a test tube kept warm on a laboratory burner (gelatin does not dissolve in cold water). Place a small gelatin drop on a microslide and let it spread out. Place an EM grid on the (still liquid) gelatin layer. A copper grid was used here of mesh 300 (meaning 300 "windows"/in.), a window width of ~54 μm, and a thickness of ~20 μm. Place the microslide into a refrigerator (for ~10 min) for the gelatin to harden. Remove the EM grid from the gelatin with a sharp pair of tweezers or a needle. At an appropriate angle, an imprint in the gelatin of the EM grid can be seen with a naked eye.

Examination of the Specimens

In both the radula and EM grid imprint specimens, the axial and off–axis illumination images were taken on exactly the same portion of the specimen while the phase-contrast images were acquired very close to that area (Fig. 2). In the radula specimen, all images were taken within 450 μm from its longitudinal axis and on the same side from that axis. Further details can be found in the text below and in Fig. 2.

RESULTS AND DISCUSSION

The complex microstructure of the biological specimen used here (the slug's radula) is apparently difficult to interpret as it appears very different under each of the three illumination modes used in the present study (Fig. 2, *top* and *middle*). The structure (and hence the function) of the radula can be assessed more reliably upon comparison with the images from the EM grid imprint serving as a reference specimen with a simple and predictable structure (Fig. 2, *bottom*). It is obvious that the off–axis (oblique, anaxial) illumination provides an image that closely matches the reference specimen's architecture (EM grid's bars imprinted into the gelatin).

The halo artifact in the phase-contrast images is profound in both preparations and is particularly disturbing in the radula specimen, as the density of prominent edges is very high. Indeed, the radula is designed for rasping food and has to be equipped with many indentations (teeth or denticles). The radula examined in the present study contained ~85 longitudinal and 125 transversal rows of teeth. The biggest ones are in radula's center, with their spacing being ~40 μm along the radula's longitudinal axis (=longitudinal *row 0*) by ~25 μm. At the same time, the performance of the phase contrast is poor

[1] For those who dislike the idea of killing a slug or snail to examine its radula, there is an alternative option, so far proved to work with various marine gastropods: a thin sheet (0.25 mm) of dental wax is placed on a microslide, which is then gently warmed up to make the wax adhere to it. The wax-coated microslide is placed into the slugs' dwelling place (aquarium, etc). As the animals try to sample various substrates in their territory, they leave radula graze marks in the wax within a few days or even hours. These specimens, essentially replicas of the radula, can then be examined under the microscope (17).

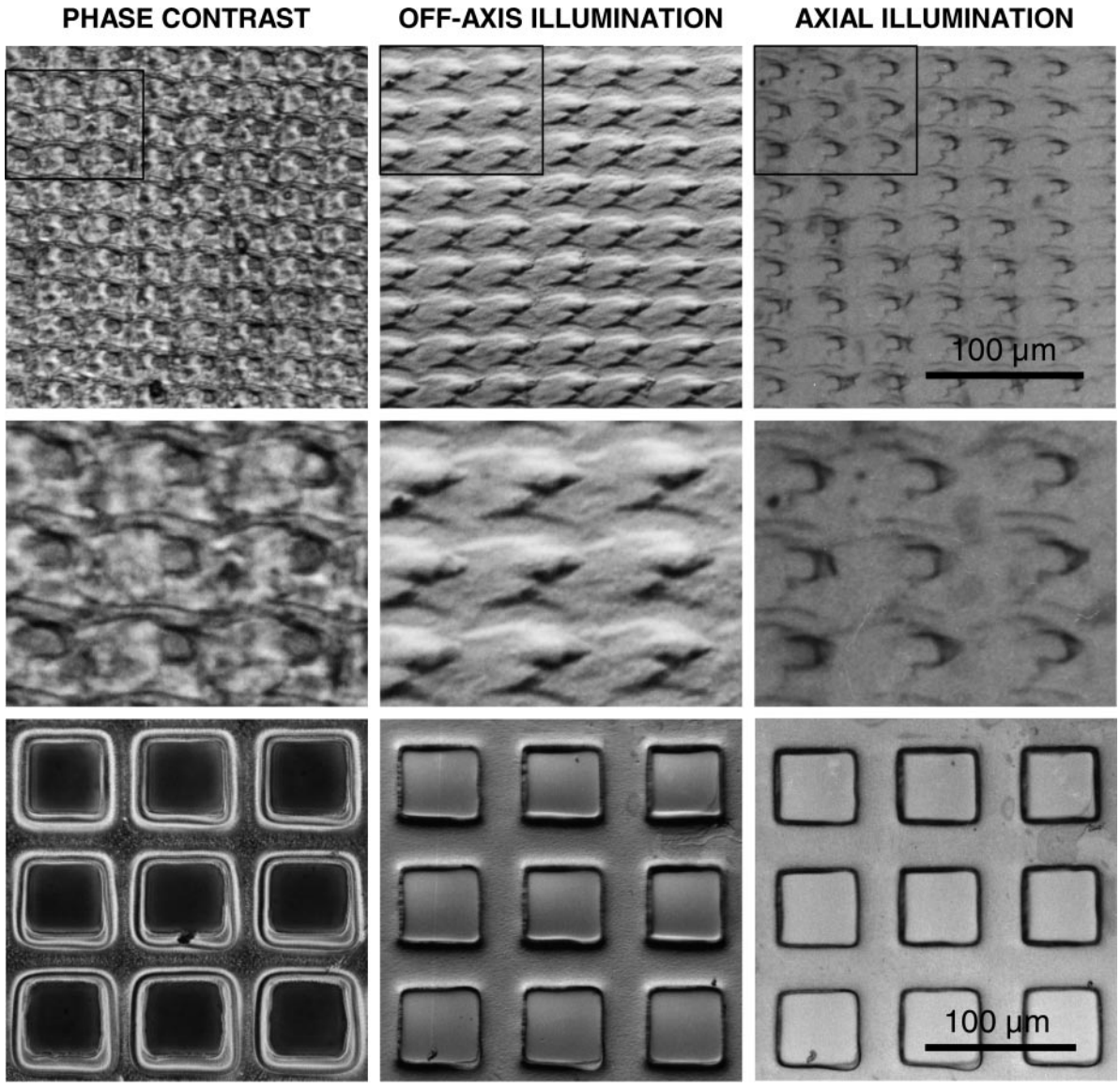

Fig. 2. Comparison between phase-contrast and off-axis (oblique, inclined) microscope illumination, as demonstrated on a biological specimen of unknown structure (*top* and *middle*) and a reference specimen whose architecture is predictable (*bottom*). Corresponding axial illumination (ordinary bright field) images featuring minimum contrast are also shown. In the off-axis illumination images, the apparent direction of illumination is from the "north." The contrast in the images has not been electronically manipulated, with the exception of the axial illumination images, where it has been enhanced. *Top*: radula of the Spanish slug (*Arion lusitanicus*) mounted in air; the radula's longitudinal axis (longitudinal *row 0*) is horizontal. Longitudinal *rows 3–13* and *7–15* are shown in the *left* and *middle*/*right* images, respectively. *Middle*: expanded scale details representing the boxed areas shown in the *top* images. The expansion factors (from *left* to *right*) were as follows: ×2.63, ×2.22, and ×2.22. *Bottom*: imprint in gelatin of an electron microscopical grid (reference specimen).

on too-thick specimens. This is partly due to the depth of field, which is smaller in the phase-contrast illumination mode compared with the off-axis illumination one, i.e., not due to the halo artifact alone. For this reason, the phase-contrast image of the radula was deliberately taken on a somewhat thinner portion of the specimen; note that the teeth are slightly smaller (Fig. 2).

The halo artifact, accompanied by the so-called shade-off (or shading-off) artifact, is brought about by sufficiently large optical thickness gradients (13). The halo is absent, e.g., on the edges of very thin cell extensions (6, 13). One way of reducing it is to place the object of interest into a solution of matching refractive index (13), but this approach cannot be applied to every object (e.g., highly refractive granules inside cells).

In this context, it is important to note that the human brain treats the visual scene (the microscopic image) as if it was acquired under the incident overhead (sun) illumination common in the macroscopic world, where most objects of interest are nontranslucent (with the rare exceptions being, e.g., a translucent tree leaf, a spider web, or a stained glass window in a church), with darker and brighter areas (shading) on their surface, depending on the direction of illumination and the object's shape. The shading patterns are then interpreted as projections and depressions of the object's surface (12). In optical microscopy, this effect can be used, e.g., when choosing the most convenient apparent direction of illumination (20).

These factors alone speak in favor of the off–axis, or oblique, illumination mode over the phase-contrast mode even

if the halo artifact is absent in phase-contrast images. The reason is that, of these two imaging modes, only the former is capable of mimicking the above-mentioned shading patterns encountered on the surfaces of macroscopic, mostly nontranslucent objects, by producing so-called schlieren images (15). The human visual system is thus presented with a more comprehensible type of microscopic image in that the images are enhanced with so-called visual cues to depth structure (6). This essentially enables "on-line" 3-D reconstruction, directly in the experimentalist's brain.

It is true that the shapes interpreted from the schlieren images do not necessarily represent a real physical profile; the luminance distribution (shading profile) in the image approximately translates to the distribution of the refractive index gradients (slopes' angles) (15). These gradients correspond to local variations in optical rather than physical thickness. However, optical and physical thickness profiles do coincide in an object with a uniform refractive index distribution, with this condition being more or less satisfied in the radula or gelatin specimens.

The shading profiles are particularly strongly modified at the object's edges (contours) by the off-axis (or generally schlieren) illumination. Overall image interpretation has been shown to be affected by their shape (12) and completeness (16). Involuntary saccadic (jumping) eye movements (6) may play a role in these phenomena by highlighting the edges (2).

In conclusion, the authors believe that this type of practical demonstration, suitable for any biology college classroom, would enable the students to *1*) prepare both a biological and reference specimen capable of unmasking some weak points of a technique that is now a commonplace in the biological laboratory, the phase-contrast microscopy [1953 Nobel Prize to Frits Zernike (19)]; *2*) convert a laboratory microscope to the off–axis illumination mode capable of producing, in this particular case, better results than phase contrast itself; *3*) compare visual impressions from the two image types and understand some of the physiological aspects of depth perception; and *4*) realize the need for a careful choice of an apparatus, with the "bottom line" being that more complex methods may not necessarily yield better results. The overall aim of the present article is to induce in students critical thinking about the capabilities of laboratory equipment in general and about data interpretation.

ACKNOWLEDGMENTS

The radula and the EM grid imprint specimens were prepared by Z. Hostounský and R. Pelc, respectively.

GRANTS

Partial support from public funds is hereby gratefully acknowledged from Academy of Sciences' Framework Grant AV0Z50200510. R. Pelc is a former Foreign and Commonwealth Office (Chevening)/George Soros scholar at St. Edmund Hall, Oxford, UK.

REFERENCES

1. **Axelrod D.** Zero–cost modification of bright field microscopes for imaging phase gradient on cells: schlieren optics. *Cell Biophys* 3: 167–173, 1981.
2. **Gaarder K.** Transmission of edge information in the human visual system. *Nature* 212: 321–323, 1966.
3. **Hoffman R.** The modulation contrast microscope: principles and performance. *J Microsc (Oxford)* 110: 205–222, 1977.
4. **Hostounský Z.** *Osvětlovací soustava pro mikroskopické pozorování průhledných neabsorbujících objektů.* Czech patent CZ4925 (utility model), 1996.
5. **Hostounský Z, Pelc R.** An efficient way of high-contrast, quasi–3D cellular imaging: off–axis illumination. *J Biochem Biophys Meth* 68: 23–30, 2006.
6. **Inoué S.** *Video microscopy.* New York: Plenum, 1986.
7. **Mojon D, Zhang W, Oetliker M, Oetliker H.** Psychophysiological determination of visual processing time by comparing depth seen in Pulfrich and Mach-Dvorak illusions. *Adv Physiol Educ* 12: 54–64, 1994.
8. **O'Drobinak DM, Woods CB.** Compelling classroom demonstrations that link visual system anatomy, physiology, and behavior. *Adv Physiol Educ* 26: 204–209, 2002.
9. **Otaki T.** Artifact halo reduction in phase contrast microscopy using apodization. *Optical Rev* 7: 119–122, 2000.
10. **Piekos WB.** Diffracted-light contrast enhancement: a re–examination of oblique illumination. *Microsc Res Tech* 46: 334–337, 1999.
11. **Plášek J, Reischig J.** Transmitted-light microscopy for biology: a physicist's point of view. Parts I and II. *Proc Roy Microsc Soc* 33: 121–127 and 196–205, 1998.
12. **Ramachandran VS.** Perception of shape from shading. *Nature* 331: 163–166, 1988.
13. **Ross KF.** *Phase Contrast and Interference Microscopy for Cell Biologists*. London: Arnold, 1967.
14. **Schaal S, Bogner FX.** Human visual perception–learning at workstations. *J Biol Educ* 40: 32–37, 2005.
15. **Settles GS.** *Schlieren and Shadowgraph Techniques*. Berlin: Springer-Verlag, 2001.
16. **Von der Heydt R, Peterhans E, Baumgartner G.** Neuronal processing of contours. *News Physiol Sci* 5: 152–155, 1990.
17. **Wai TC, Williams GA.** Monitoring spatio-temporal variation in molluscan grazing pressure in seasonal, tropical rock pools. *Mar Biol (Berl)* 149: 1139–1147, 2006.
18. **Wessenberg H, Reed MK.** The use of oblique illumination in microscopic observation of living protozoa. *Trans Am Microsc Soc* 90: 449–457, 1971.
19. **Zernike F.** How I discovered phase contrast. *Science* 121: 345–349, 1955.
20. **Žižka Z, Hostounský Z.** RCH–a ARC-mikroskopie–nové kontrastní metody. *Jemná Mechanika a Optika* 7–8: 199–201, 2004.

Adv Physiol Educ 31: 281, 2007;
doi:10.1152/advan.50001.2007.

Corrigendum

Volume 31, June 2007

Hostounský Z, Pelc R. Phase-contrast versus off-axis illumination: is a more complex microscope always more powerful? *Adv Physiol Educ* 31: 232–235, 2007; http://advan.physiology.org/cgi/content/abstract/31/2/232.

The correct e-mail address of Radek Pelc is as follows: radek.pelc@seh.oxon.org.