Relief Phase Contrast: A New Technique for Phase-Contrast Light Microscopy

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BIOGRAPHY

Jörg Piper obtained his medical degree from the University of Bonn in Germany. One field of his scientific activities is directed at light microscopical applications, especially reflection contrast. He has created several mathematical models for the three-dimensional quantitative analyses of opaque cellular specimens. Since 1998 he has also collaborated with the University of Oradea in Romania as an associate and honorary professor. In Germany, he currently works as senior consultant for internal medicine, angiology and diabetology.

ABSTRACT

Relief phase contrast is a new modification of conventional phase contrast which leads to visible improvements of image quality in light microscopy. In particular, the following parameters can be improved: contrast, focal depth, sharpness, three dimensionality, planeness, and halo artifacts. These effects can be achieved when the ring-shaped masks in the condenser are replaced by crescent- or punctate-shaped masks. Several solutions are described which are suitable to create this modification. The achievable improvements of image quality are relevant for all quality levels of objectives. The new technique can be used for phase contrast objectives from different manufacturers, so that the usual limitations of compatibility are eliminated.

KEYWORDS

light microscopy, phase-contrast microscopy, relief phase contrast, phase, interference, focal depth, cell relief, halo artifacts, spherical aberration, image quality

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INTRODUCTION

Phase contrast is a mode of light microscopy that is widely used for the examination of transparent and colourless specimens such as unstained cells and micro-organisms which typically have very low contrast. These objects do not absorb light, so the amplitude of the light waves passing through them remains nearly constant. However, they do modify the phase of transmitted light by around one quarter wavelength (λ/4) hence we call them phase objects. Such differences in phase cannot be perceived by the eye or by a photography. The Dutch physicist Frits Zernike developed phase contrast as a new illumination mode to convert phase differences into visible amplitude differences [10,11] for which he was awarded the 1953 Nobel prize.

To achieve phase contrast, two components of brightfield microscopes have to be modified: 1. The condenser has to be equipped with a ring-shaped aperture or mask (the condenser annulus), which is placed near the condenser aperture diaphragm. 2. A conjugate phase plate (or ring) is placed in the back focal plane of the objective. The condenser annulus and the phase ring in the objective have to be optically aligned so that they are conjugate. With this arrangement the specimen is illuminated by the apex of a cone of light. The light beams which are diffracted by the specimen pass through the objective lens at various angles which are dependent on the relative refractive index and the thickness of the specimen. The other light components, corresponding to the background, pass through the phase ring in the objective which produces an additional phase difference. Thus the phase differences between the specimen, its details and the background are amplified in the final image, so that minimal differences in refractive index are visible even in colourless specimens with a low contrast and thickness.

Depending on the configuration and properties of the phase ring in the objective, phase contrast microscopy can be positive or negative. In positive phase contrast the specimen is visible with medium or dark grey features, surrounded by a bright halo; the background is of higher intensity than the specimen. In negative phase contrast the background is darker and the specimen appears brighter, surrounded by a dark halo. The bright and dark halos are artifacts which are one of the major disadvantages of phase contrast; they are especially prevalent in specimens inducing large phase shifts. Recently, advances in the design of objective phase-ring configurations have led to a new technique which reduces halo-effects called apodized phase-contrast microscopy.

All these modifications used in phase contrast do not create three-dimensional images which could be compared with the 3D effects of interference contrast microscopy. Compared with brightfield, in phase contrast the depth of focus is smaller, because the condenser aperture iris diaphragm is fully open. In phase contrast, the intensity of contrast is dependent on the differences of refractive indices of the specimen and the surrounding medium, and the thickness and native contrast of the specimen.

The quality of phase contrast images is strongly determined by the quality of the lenses. Existing chromatic and spherical aberrations reduce the quality of the resulting images more intensively than in brightfield microscopy.

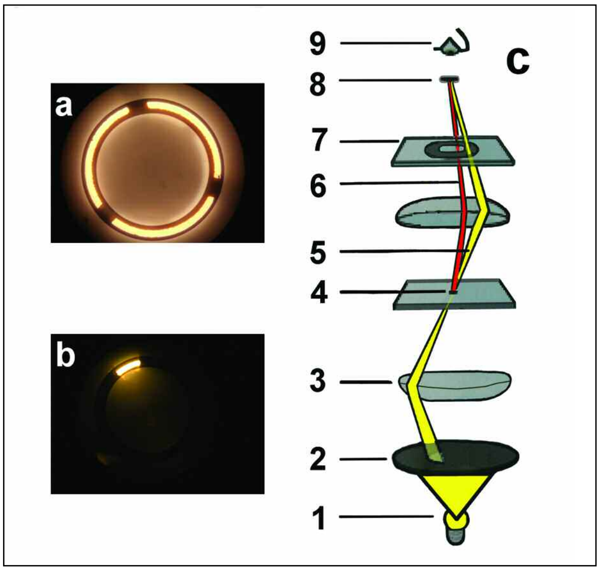

Figure 1:
(a, b) Optical alignments in conventional phase contrast and relief phase contrast microscopy.
(a) Conventional phase contrast. Condenser annulus (bright) and phase-ring (dark) are properly aligned, concentric and conjugate.
(b) Relief phase-contrast. The annulus is replaced by a modified arc-shaped mask (bright), which when properly aligned overlaps with the phase ring (dark).
(c) Simplified optical pathway for relief-phase contrast microscopy (modified from [5]).
Key: 1 = light source; 2 = modified mask; 3 = condenser; 4 = specimen; 5 = background light; 6 = light bent by the specimen; 7 = phase ring; 8 = eyepiece with intermediate image; 9 = eye.

Furthermore, phase contrast can only be achieved when the phase rings in the objectives and the condenser annuli are specifically adjusted for each other. Normally, a particular ring-shaped mask can be used for one or two special objectives within a well-defined range of magnification. For example, one condenser annulus could used with objectives for 10× or 16× magnification, a second mask for 25× and 40×, and a third mask for 100× magnifiing oil-immersion objectives. When objectives and condensers are used from different manufacturers, a misalignment can result. Therefore, the objectives and condenser should preferably be from the same manufacturer.

The principles of conventional phase contrast and its beam path were discussed in a recent article in Microscopy and Analysis [2]. Moreover, several very instructive internet-based interactive tutorials are available which demonstrate the effects of positive, negative and apodized phase contrast based on realistic animations [1,3]. The advantages of phase contrast in comparison with other illumination modes have been described [4,5,8,9].

In this article a new modification of phase contrast is presented which can improve the quality of the conventional phase contrast images by higher contrast, enlarged focal depth, reduced haloing and less-visible spherical aberration. This method, called relief-phase contrast, can also be used when existing phase-contrast objectives are built by different manufacturers. Moreover, in most cases this technique requires only one light-modulating element in the condenser, which is suitable for all existing phase-contrast objectives.

PRINCIPLES OF RELIEF PHASE CONTRAST

In conventional phase contrast, the condenser annulus is completely transparent, so that the passing light beams create a light cone which illuminates the specimen in a concentric circular manner (360°). When the condenser annulus and the objective phase ring are correctly aligned, both illuminating elements are exactly conjugate; the alignment is usually controlled using a centering telescope (phase telescope) as shown in Figure 1a.

To achieve relief phase contrast, the transparent annulus has to be replaced by a smaller transparent sector, which can be crescent-shaped or a small round aperture. This light sector has to be aligned with the phase ring in the objective in the usual manner, so that it is conjugate (Figure 1b). With this modification, the specimen is only illuminated from one defined direction by oblique light beams (Figure 1c). Typically, the resulting images have more contrast, focal depth and three dimensionality and less visible spherical aberration compared with conventional phase contrast.

Crescent- or circular-shaped sectoral masks can be achieved in several ways, controlled using a phase telescope: 1. Sliding components with an annular ring can be moved into the path of light and the condenser aperture diaphragm can be partially closed so that just a small part of the annular ring remains transparent for light. 2. Condenser turrets with a kit of several annular rings, usually existing in Zernike phase condensers, can be used for relief phase contrast, if the turret is rotated into an abnormal position, so that annular ring and phase ring overlap slightly. The condenser aperture diaphragm is closed as described above.

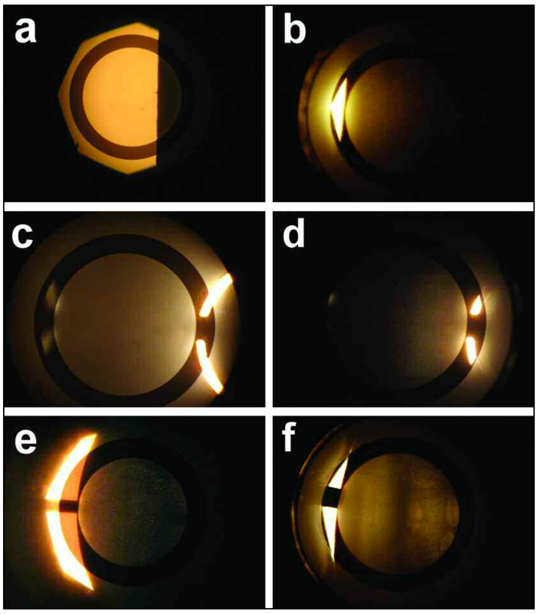

Figure 2:
Implementation of relief phase contrast using non-transparent slides with ring-shaped apertures. Images of the condenser aperture taken with a phase telescope.
(a) The aperture diaphragm is partially closed and then the margin of the slide is moved in from the right (shadowed area of aperture). The darker ring is the phase ring of the objective.
(b) Final alignment of the margin of the slide and the aperture diaphragm.
(c) An annular ring for phase contrast (small diameter, bright) is shifted from the right, overlapping the phase-ring (dark).
(d) Final alignment with partially closed aperture diaphragm, visible on the right (dark).
(e) An annular ring for darkfield (large diameter, bright) is overlapping the phase ring (dark) on the left side. The aperture diaphragm (dark) is partially closed, its edges visible on the left.
(f) Final alignment with partially closed aperture diaphragm.

The individual position of the phase ring in the objectives is not important for realising relief phase contrast, because the position of the illuminating light sector can be adjusted to the phase ring with a high degree of variability. Thus, objectives made by different manufacturers can be used simultaneously. Moreover, the condenser annuli can be directly modified as when they are covered by an opaque plate with a small aperture for the transmitted light. In this case it is not necessary to close the condenser aperture diaphragm. Alternatively, the annulus could also be replaced by other constructions, suitable to achieve circumscribed small light beams, which can be adjusted to the phase rings in the objective (see Discussion).

As in normal phase contrast, the quality of the resulting images can be optimized also in relief phase contrast by closing the field diaphragm so that it is just seen at the edges of the field of view (as in Köhler illumination).

MATERIAL AND METHODS:

Relief phase contrast was developed on two Leica (Leitz) microscopes, a Dialux and an HM-Lux III. These microscopes are both able to achieve positive phase contrast. The Dialux was equipped with a Zernike phase contrast universal condenser, the HM-Lux III with a kit of separate slides for phase contrast and dark field which can be shifted into an existing brightfield condenser. In this way, the several varieties of technical realisation described above could be evaluated.

The images of the phase ring and annular ring constellations, controlled by a phase telescope, were taken using Canon Powershot A 620 and Casio Exilim EX-Z 110 cameras. The microscopical images were taken using Olympus Camedia C 7070 and Canon EOS 350D/20D cameras operated with remote switches.

When slides for phase contrast are used (Figure 3a), the margin of the illuminating sector can be built by the edge of the slide and the aperture diaphragm (Figure 2 a,b) or by the annular ring itself and the aperture diaphragm (Figure 2 c,d). Annular rings for darkfield microscopy (Figure 3c), which are characterized by much higher diameters, can also be used (Figure 2 e,f). Moreover, a slide with an annular ring can directly be prepared

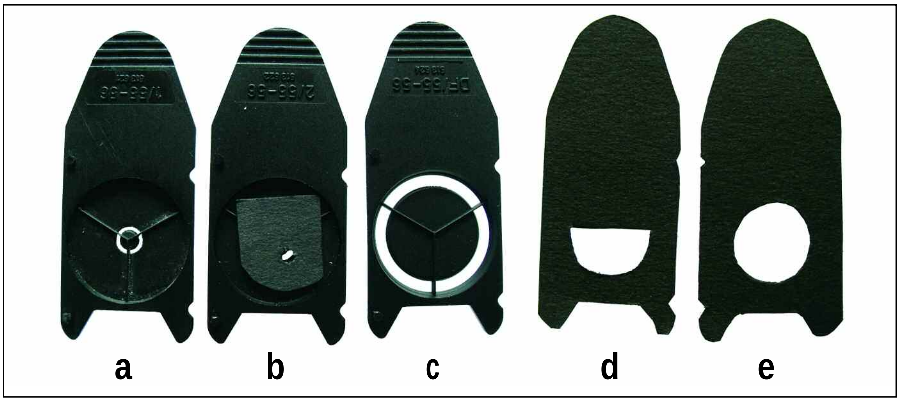

Figure 3:
(a-c) Standard condenser sliders for conventional phase contrast (a), relief phase contrast with a special mask (b), and darkfield (c) microscopy.
(d,e) Prototype condenser sliders with semicircular (d) and circular (e) shaped masks.

for relief phase contrast when it is covered by an opaque black mask (Figure 3b); in this case, the condenser aperture diaphragm has to be wide open. The resulting control image, which is visible with the phase telescope, is illustrated in Figure 1b.

The turret of a Zernike condensor can be rotated into several positions to achieve marginal overlappings of annulus and phase ring. The condenser aperture diaphragm has to be used for additional limitation of the illuminating field. Annular rings with various diameters can be used so that the configuration of the resulting illuminating field can be variable, corresponding to Figure 2 d,f.

A binocular viewing tube should preferably be used to align the illuminating elements. In this way, one eyepiece can be removed and the phase-telescope can be inserted in its place. Thus, the effects of manipulations can be controlled simultaneously by the remaining eyepiece. When an optimal alignment is found, the centering telescope can be replaced by the second eyepiece, so that normal binocular examination can then be made.

RESULTS

When phase-contrast objectives that were not highly corrected were used, the quality of the images could be dramatically improved. Relief phase contrast achieved a higher contrast with enlarged focal depth and often improved sharpness. The relief of the specimen mostly appeared more three dimensional, similar to interference contrast images. The planarity of the microscopical image was improved, because the effects of spherical aberration were lower. Halo artifacts were often reduced (Figure 4 a,b).

Even when highly corrected phase-contrast objectives were used (e.g. planachromatic or planapochromatic lenses), the images resulting from relief phase contrast had more contrast, enlarged focal depth and more apparent three-dimensional aspects (Figure 4 c,d).

Compared with interference contrast, relief phase contrast often produced images with higher or complementary information of specimen details (Figure 5 a,b, Figure 6 a-c).

The brightness of the microscopical image was lower than that using conventional phase contrast, because the area of the illuminating light beams was more reduced (about -2.0 or -3.0 EV). Therefore, higher light intensities have been necessary.

As specimens were illuminated from one direction by oblique light beams, the background of relief phase-contrast images sometimes appeared with variable brightness, especially when objectives with low magnifications were used. Similar effects are also known from interference contrast.

All the relevant findings are presented in Table 1 which compares the major similarities and differences of conventional phase contrast with relief phase contrast.

DISCUSSION:

When a conventional phase-contrast microscope is available, relief phase contrast can be achieved quite simply using a Zernike condenser; alternatively a brightfield condenser can be modified for phase contrast by placing special slides into the light path. In these cases, the condenser aperture diaphragm has to be closed to achieve correct alignment of the illuminating mask in the condenser and the phase ring in the objective. Closing the aperture diaphragm can improve the focal depth and sharpness in the same manner as is usual in brightfield. In conventional phase contrast, the quality of the microscopical images is not influenced by the aperture diaphragm. The resulting three-dimensional appearance can contribute to the gobal quality of relief phase contrast images as well as the diminution of existing spherical aberrations. It can be regarded as an additional advantage of these modifications that objectives made by different manufacturers can be used simultaneously also in cases of misalignments in conventional phase contrast caused by non-compatibility.

The resolving power is reduced when the aperture diaphragm is closed. The lower the diameter of the phase ring in relation to the diameter of the lenses, the lower the width of the aperture diaphragm and the remaining resolving power, when relief-phase-contrast is achieved. When ring-shaped masks are directly modified to crescent- or punctate-shaped masks, the condenser aperture iris diaphragm can be fully open. The specific features of the specimen and the individual relative diameter of the existing phase ring determines which option (large or small aperture diaphragm) leads to better optical results.

Relief phase contrast can be regarded as a complementary method which can improve the quality of conventional phase contrast images.

Manufacturers of microscopes could implement relief phase contrast if existing condenser annuli in conventional phase contrast systems were replaced by crescent- or punctate-shaped masks. In this case, relief phase contrast could be achieved without closing the aperture diaphragm. Moreover, manufacturers could create modified turrets equipped with unconventionally pre-aligned annular

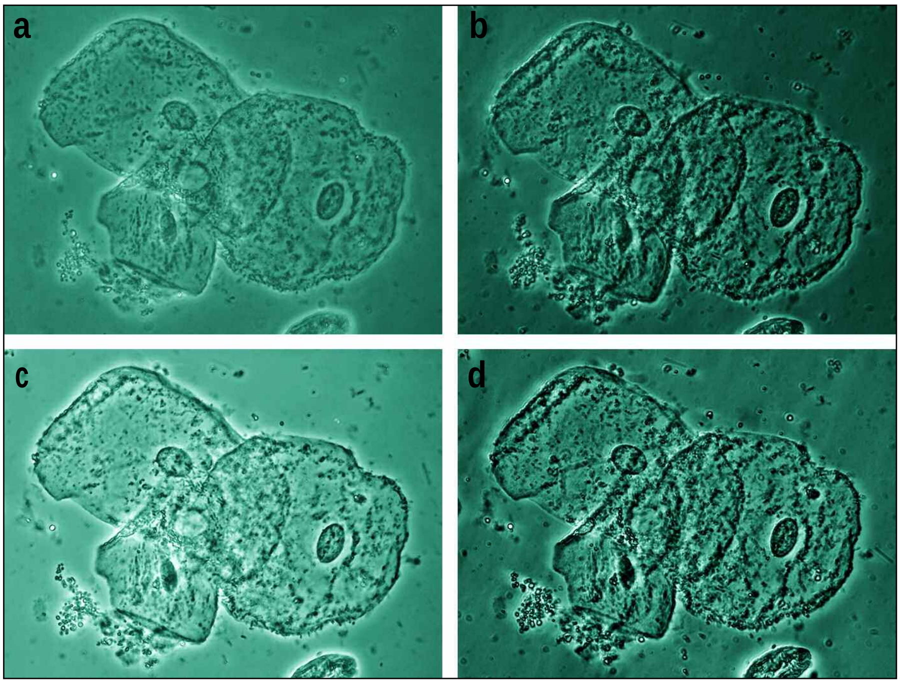

Figure 4:
Buccal epithelial cells, 40x objectives HFW = 100 µm. (a) Conventional phase contrast with a basic corrected objective (Olympus A 40x Plan 0.65NA). (b) Relief phase contrast using the same objective. (c) Conventional phase contrast with a more highly corrected objective (Leica Phaco Plan 40x 0.65NA). (d) Relief phase contrast with the Leica objective.

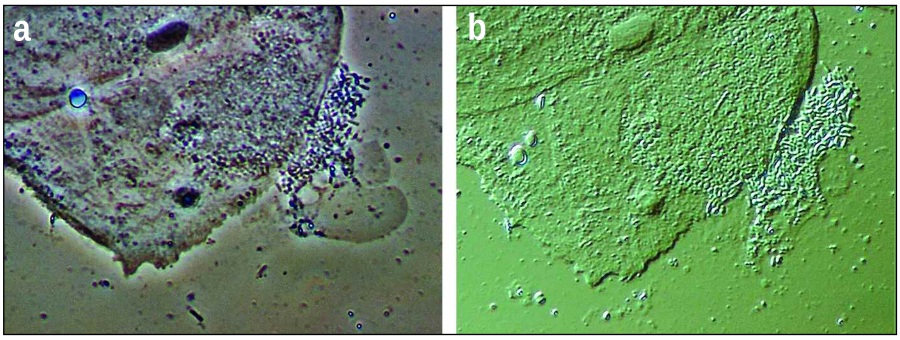

Figure 5:
Buccal epithelial cells in a very thin layer of saliva with Newton's rings. 40x objectives. HFW = 70 µm. Exposure by electronic flash. (a) Relief phase contrast using Leica Phaco Plan 40x 0.65NA objective. (b) Interference contrast using Leica NPL Fluotar 40x 0.65 ICT.

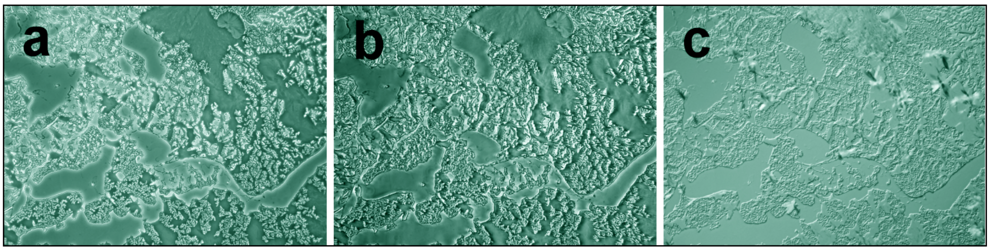

Figure 6: Thin-layer crystallization of a water soluble pigment. Coverslip preparation with Newton's rings. HFW = 240 µm. (a) Conventional phase contrast using Leica Phaco Plan 40x 0.65NA. (b) Relief phase contrast using same lens as (a). (c) Interference contrast using Leica NPL Fluotar 40x 0.65NA ICT.

rings which could overlap the phase rings tangentially when they are rotated into their fixed position. Thus, the aperture diaphragm could be closed to improve focal depth and sharpness.

Alternatively, circular or semicircular shaped masks could be built (Figure 3 d,e) which could be shifted excentrically into the light path to overlap phase rings tangentially. Thus, brightfield mode could be used when the aperture diaphragm is opened, and relief phase contrast could result when the aperture diaphragm is much smaller. Universal condensers could be equipped with several removable turrets to achieve all varieties of relief phase conrast as well as conventional phase contrast.

Moreover, special condensers for relief phase contrast could be created, equipped with various technical modifications to achieve small, circumscribed illuminating light beams with a variable position, length, width and shape. Thus, two separate iris diaphragms could be superimposed on each other, eccentrically aligned, which could then be shifted and turned eccentrically. Alternatively, two excentrically rotating disks could be created, one disk-shaped as a transparent light mask, the other disk as a non-transparent overlapping element, eccentrically superimposed on the light mask. Two non-transparent slides could also be used, overlapping marginally and suitably shaped, so that a small light beam could result. In all cases, small sectoral illuminating light beams could be achieved by variable transparent gaps, resulting from the position of the double iris diaphragm, the double-disk or double-slide system.

For high-end motorized microscopes, motorized condensers for relief phase contrast could be built, equipped with several freely programmable function buttons. In this way, illuminating light beams with optimized alignment could be achieved and reproduced with a high precision according to the existing kit of phase-contrast objectives.

When the microscope is equipped with a rotary stage, the position of the specimens can be changed according to their individual three-dimensional texture and the direction of the illuminating light beams. Thus, in special cases, the effects of 3D imaging could be optimized or intensified. Alternatively, this optimizing effect could also be achieved when the condenser is pivoted, so that it can rotate around the optical axis.

More intensified three-dimensional images could also be achievable when an inverted microscope is used for relief phase contrast, combined with phase-contrast objectives for long distances and thick glass slides. In this case, the illuminating beams will first pass through the coverslip instead of the object slide; the surface of the specimen will be illuminated from the top instead of from the bottom and shadow figures might occur on the surface of the object slide comparable with the shadow effects obtained by reflexion contrast with oblique illuminating beams [6,7].

When expensive high-end lenses are not available, relief phase contrast can still lead to excellent image quality when only less well corrected objectives are used. These improvements in quality are visible as well in live microscopy, photographic images or movies.

Key Features	Conventional Phase Contrast	Relief Phase Contrast
use objectives from all manufacturers	no	yes
illuminating light beams	concentric	eccentric
condenser aperture diaphragm	open	open or smaller (various modifications)
3D images / relief effects	no	yes
contrast	good	higher
sharpness	good	potentially higher (depending on the specimen)
depth of focus	narrow	higher
resolving power	high	sometimes lower (when aperture diaphragm is smaller)
halo artifacts	high	sometimes lower (depending on the specimen)
influence of spherical aberration	high	lower
influence of chromatic aberration	high	sometimes lower
brightness of the microscopical image	high	lower
homogeneity of background	high	sometimes lower (when objectives with low magnification are used)

Table 1: A comparison of the major characteristics of conventional phase-contrast and relief phase-contrast microscopy.

CONCLUSIONS

The quality of the images produced by conventional phase contrast microscopy can be significantly improved when the usual annular masks in the phase-contrast condenser are excentrically aligned with the phase rings in the objectives or replaced by other elements.

Several ways for the technical implementation of relief phase contrast exist; these are dependent or independent of the position of the condenser aperture diaphragm. Thus, the various parameters which are influenced by the illuminating aperture can be adapted to the individual features of the specimen. In contrast to conventional phase contrast, objectives from different manufacturers can be used simultaneously, so that their compatibility is improved.

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