Variable Phase- and Bright-Darkfield Contrast – new Illumination
Techniques for Advanced Imaging in Light Microscopy

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Variable phase-darkfield-contrast (VPDC) and variable bright-darkfield contrast (VBDC) are variant techniques in light microscopy promising significant improvements and a higher variability in imaging of many specimens, especially those consisting of high and low density components, affected with a high regional thickness or characterized by a complex three-dimensional architecture. Also fine reliefs and textures at the specimen’s surface can often be perceived in superior detail. In both methods, a darkfield-like partial image based on secondary and higher order maxima is optically superimposed on partial images which are based on the principal zeroth order maximum (phase contrast or brightfield). Moreover, the condenser aperture diaphragm can be used for modulations of the image’s appearance and optimizations of the final image quality in all variant techniques described.

In VPDC, phase contrast and darkfield illumination are simultaneously generated, and both partial images interfere with each other. The background brightness and character of the resulting image can be continuously modulated from a phase contrast-dominated to a darkfield-dominated character. An additional brightfield-like partial image can be added, if necessary. Thus, the visual information being apparent in each standard technique is summarized in the resulting final image. Low density and colorless phase structures can be perceived as well as high density colorized absorption structures.

In VBDC, two brightfield- and darkfield-like partial images are simultaneously superimposed and interfere with each other. In this variant, the background brightness can be continuously modulated from a brightfield-dominated to a darkfield-dominated appearance. When the weighting of the dark- and brightfield components is balanced, medium background brightness will result showing the specimen in a phase- or interference contrast-like manner. Specimens can either be illuminated axially /concentrically or obliquely / eccentrically. In oblique illumination, the angle of incidence and grade of eccentricity can be continuously changed, and so, the illumination can be optimally adjusted to the specific properties of the specimen. Even so-called “problem specimens” which cannot be adequately visualized in the standard techniques can be optimally contrasted.

In contrast with normal darkfield illumination, blooming and scattering are lower and when compared with phase contrast, any haloing is significantly reduced or absent. Axial resolution and depth of field are much higher than in current standard techniques. Nevertheless, lateral resolution is not visibly reduced. The specimen’s three-dimensionality is accentuated with improved clarity because the illuminating light beams associated with the respective partial images meet the specimen at different angles of incidence.

According to our practical experience up till now, the techniques described can be regarded as useful complimentary tools especially in biological specimens and crystallizations showing a complex morphology.

Keywords Phase contrast; darkfield, brightfield; variable phase-darkfield-contrast; VPDC; bright-darkfield-contrast; VBDC; illumination; focal depth; haloing; blooming; scattering; resolution; light microscopy

1. Introduction

Brightfield, phase contrast and darkfield are widely established as standard techniques in light microscopy – each affected with characteristic limitations. In brightfield illumination, only so-called absorption specimens which are characterized by a medium optical density can be properly visualized when an appropriate part of the incoming light transmitted through the specimen is absorbed. Especially in brightfield, the condenser aperture diaphragm can be used for optimization of the image quality. When the aperture diaphragm is moderately closed so that the aperture of the illuminating apparatus is reduced, not only can contrast and contour sharpness be enhanced but also the depth of field (focal depth) is increased [1]. On the other hand, in high density specimens, the incoming light can be totally or nearly totally absorbed so that such specimens appear as dark silhouettes situated in a bright background and fine details inside them cannot be seen. Moreover, low density specimens can be invisible or nearly invisible in brightfield when the proportion of absorbed light components is too low.

In darkfield illumination, even very high dense specimens can be visualized in their natural colors and the marginal contours of low density specimens can also be well demonstrated. On the other hand, marginal contours and high density details often appear highly irradiated because of blooming and scattering; fine internal structures can be invisible in darkfield when they are not hit by the incoming illuminating light beams running obliquely from the condenser’s periphery to the specimen. In darkfield, the principal zeroth order maximum does not contribute to the
image so that darkfield images are only based on reflected and scattered light components represented by secondary and higher maxima [2].

Phase contrast is preferably used for observation of so-called phase specimens, i.e. thin and low density colorless specimens. The advantages and limitations of this method have been compiled by several authors [3-6]. Halo artifacts can be regarded as a well known disadvantage of phase contrast. Moreover, the quality of phase contrast images is regularly reduced when the specimens or preparations are too thick or the cover slip is not situated parallel to the specimen slide.

Under normal circumstances, the condenser aperture diaphragm can be used for enhancements of contrast, sharpness and focal depth only in brightfield. For observations in phase contrast and darkfield illumination, the aperture diaphragm - if available in the condenser used - has to be wide open.

Taking into consideration the limitations described, variable phase-darkfield-contrast (VPDC) and variable bright-darkfield contrast (VBDC) have been developed by the primary author as new illumination techniques promising several improvements in examination of many specimens. In these methods, the characteristic advantages of the respective standard techniques remain present whereas particular disadvantages can be avoided.

### 2. General principles of variable phase- and bright-darkfield contrast

In the methods reported, a darkfield-like image is simultaneously superimposed on a phase contrast or brightfield-like image. The partial images generated interfere with each other so that additional contrast effects result. Brightfield and phase contrast images are both based on the zeroth principal order maximum, darkfield images on the secondary order maxima so that the zeroth order maximum does not contribute to the image. Thus, partial images based on secondary order maxima (darkfield) are superimposed on complementary partial images based on their principal order maximum (phase contrast or brightfield). The intensities of the respective partial images can be continuously adjusted by the user so that the resulting final image may be dominated by the darkfield or the brightfield / phase contrast component. In all technical variants, the alignment of the light modulating components can be controlled with a phase telescope – in the same way as usual in normal phase contrast examinations.

### 3. Achievement of variable phase-darkfield contrast (VPDC)

In order to achieve VPDC, the condenser’s light mask has to be modified into a twin annuli system as follows: In addition to the standard light annulus corresponding with the objective’s phase ring, the light mask has to be fitted with a larger sized light annulus which is projected outside the objective’s cross section area. The illuminating light components which pass the smaller sized internal condenser annulus are capable of producing a phase contrast image, and additional light components running through the external light annulus generate a darkfield image which is superimposed on the phase contrast image. The schematic light pathway of this arrangement is shown in Fig. 1.

![Fig. 1: Illuminating light pathway in VPDC. 1 = light source, 2 = modified light mask fitted with separate concentric light annuli for phase contrast and darkfield, 3 = condenser lens, 4 = specimen 5 = illuminating light for phase contrast, 6 = illuminating light for darkfield, 7 = objective lens, 8 = phase plate with phase ring, 9 = eyepiece, 10 = eye.](image)

The intensity of the darkfield partial image can be regulated with the condenser aperture diaphragm in tiny steps. When the breadth of the external light annulus is moderately reduced by the aperture diaphragm, blooming and scattering associated with darkfield can be mitigated, the background is moderately brightened, ranges of brightness and contrast can be equalized and the depth of field (focal depth) can be enhanced. When the external light annulus is completely covered by the aperture diaphragm, the illuminating light producing the darkfield is totally blocked so that a singular phase contrast image will remain. An additional brightfield image can be added to the phase contrast and
darkfield partial images when the internal light annulus is slightly turned into a moderate off-centered position so that a small part of the illuminating light transmitted through the specimen runs beside the phase ring within the objective. The total area of the internal light annulus corresponding with phase contrast has to be much smaller than that of the external darkfield producing annulus. Otherwise, the darkfield image will be superseded by the phase contrast image. To achieve a well balanced proportion of these different partial images, special light masks should be created fitted with a few small internal perforations instead of the circular phase contrast producing light annulus normally used. Thus, the intensity of the illuminating light associated with phase contrast is significantly reduced when compared with standard light annuli consisting of a circular gap. A hand-made prototype of a suitable light mask leading to well balanced partial images in darkfield and phase contrast is shown in Fig. 2a, the correct alignment of this light mask and the phase ring of a compatible phase contrast objective controlled with a phase telescope is demonstrated in Fig. 2b.

Fig. 2: Hand-made prototype of a condenser light mask for VPDC, mounted on a slide (a), correct alignment of the internal perforations and the objective’s phase ring, image taken with a phase telescope (b).

4. Achievement of variable bright-darkfield contrast (VBDC)

VBDC can be generated in two different ways, condenser- and lightstop-based. In both variants, the specimen can be illuminated by axial / concentric or oblique / eccentric light. Illuminating light pathways for condenser-based variants are shown in Fig. 3. Examples of some typical alignments are given in Fig. 4 and 5, the light stop used in Fig. 6.

Fig. 3: Illuminating light pathways for condenser-based concentric (a) and eccentric (b) and light stop-based axial (c) and oblique (d) VBDC. 1 = brightfield generating light, 2 = darkfield generating light, 3 = illuminating light meeting the light stop, LA = light annulus, LS = light stop, ID = iris diaphragm, SP = specimen, condenser lenses are not drawn in the figure.

Fig. 4: Examples of optical alignments for concentric VBDC (a, b), dominance of brightfield (a) and darkfield (b), alignment for condenser-based eccentric VBDC (c), images taken with a phase telescope.
Light stop with twin diaphragm can be generated. In reflected by the specimen is not drawn in the figure; it can pass both holes of the light stopping slide so that an intermediate image can be generated. In particular, the breadth of the internal illuminating zone producing the concentric brightfield image can be modulated by shifting the condenser as described. The breadth of the external zone associated with darkfield can be regulated with the condenser aperture diaphragm – in the same way as already explained for VPDC. The illuminating light pathway of this variant is schematically demonstrated in Fig. 3a. Some typical alignments controlled with a phase telescope are shown in Fig. 4a and b. When the condenser is shifted down, a higher proportion of the light annulus is projected into the objective’s cross section area so that the brightfield partial image will dominate (Fig. 4a). The projection size of the light annulus increases when the condenser is shifted up in tiny steps so that a higher proportion of the light annulus is projected outside the objective (Fig. 4b); the resulting image is now successively dominated by the darkfield component.

4.1 Condenser-based concentric VBDC

This variant can be achieved when the condenser is fitted with an appropriately sized light annulus in centered position. The inner diameter of this light annulus has to be somewhat smaller and its external diameter somewhat larger than the diameter of the objective’s internal cross section area. The internal part of the light annulus generates concentric brightfield, the external part leads to an additional darkfield image. The projection of the light annulus and the run of the illuminating light components can be continuously modified and adjusted when the condenser is slightly shifted up and down in a vertical direction. In particular, the breadth of the internal illuminating zone producing the concentric brightfield image can be modulated by shifting the condenser as described. The breadth of the external zone associated with darkfield can be regulated with the condenser aperture diaphragm – in the same way as already explained for VPDC. The illuminating light pathway of this variant is schematically demonstrated in Fig. 3a. Some typical alignments controlled with a phase telescope are shown in Fig. 4a and b. When the condenser is shifted down, a higher proportion of the light annulus is projected into the objective’s cross section area so that the brightfield partial image will dominate (Fig. 4a). The projection size of the light annulus increases when the condenser is shifted up in tiny steps so that a higher proportion of the light annulus is projected outside the objective (Fig. 4b); the resulting image is now successively dominated by the darkfield component.

4.2 Condenser-based eccentric VBDC

Instead of a concentric alignment, the condenser annulus can also be moved to an off-centered position so that the periphery of the objective’s cross section area and a small segment of the condenser annulus overlap with each other. Also in this arrangement, the internal zone of the illuminating light segment leads to a brightfield-like partial image, whereas the external light components which run outside the objective’s cross section area generate an additional darkfield-like image. The pathway of the illuminating light is shown in Fig. 3b, an example of a typical alignment is given in Fig. 4c. The proportion of the bright- and darkfield partial images can be modulated when the illuminating light segment is horizontally shifted and it can be influenced further by the aperture diaphragm.

4.3 Light stop-based axial VBDC

In this technical variant, a small light stop is inserted into the light path, centrically situated in the back focal plane of the objective or near its back focal plane so that the optical axis runs through this light stop. The condenser has to be fitted with an appropriately shaped light mask or light outlet which is congruent with the light stop. In this arrangement, the specimen is illuminated by rather small and collimated light beams running in axial and perpendicular direction. When the illuminating light beams are completely blocked by the light stop, the specimen is illuminated in axial (central) darkfield. When light stop or light mask are slightly shifted out of their centered position, a small part of the illuminating light can pass the light stop so that a brightfield partial image is additionally generated.

In our prototype, the light stop was built as a black slide fitted with two circular holes acting as a twin diaphragm (Fig. 6). This light stop was inserted into the light path a short distance above the objective’s back focal plane. In axial illumination, the black area between both holes was shifted into the optical axis so that it could act as a light stop. Moreover, a large sized condenser annulus was turned into an equatorial and axial position and appropriately collimated by the condenser aperture diaphragm. Condenser annulus and light stop could be turned into a complete congruent median position for axial darkfield illumination or slightly turned into paramedian and partially congruent positions when a brightfield-like image had to be added. The illuminating light path resulting from the arrangement described is shown in Fig. 3c. The small axial light beam is collimated by the condenser’s iris diaphragm and it is totally blocked by the light stop, so that the specimen is illuminated in axial (central) darkfield. The imaging light which is bent and reflected by the specimen is not drawn in the figure; it can pass both holes of the light stopping slide so that an intermediate image can be generated. In Fig. 5a-c, the alignment of all light modulating components is demonstrated for axial darkfield illumination as described above.
4.4 Light stop-based oblique VBDC

When light stop and condenser light mask are both shifted into a paramedian or paraequatorial positions, the specimen can be illuminated in oblique VBDC. In this way, the grade of eccentricity and the angle of incidence can be continuously varied. A maximum eccentricity can be achieved when the condenser annulus is turned into the marginal periphery of the objective’s cross section area and covered by one of the black areas beside the slide’s transparent holes. As an example for light stop-based oblique VBDC, Fig. 3d shows a schematic illuminating light pathway for oblique axial darkfield illumination achieved with the twin diaphragm from Fig. 6. Also in this arrangement, the illuminating light is completely blocked by the light stop so that oblique darkfield illumination results and the imaging light (not drawn in) can pass the lightstop’s holes as described. An appropriate alignment of the illuminating light segment and the light stop leading to maximum oblique darkfield illumination controlled with a phase telescope is shown in Fig. 5d-e.

5. Materials and methods

VPDC was achieved with a standard laboratory microscope from Leitz / Leica, equipped with a brightfield condenser and several slides acting as light masks for darkfield and phase contrast. These slides could be shifted into the condenser. The proper alignment of light annuli and phase rings was controlled with a phase telescope in the usual manner. Slides containing light annuli for phase contrast were modified by hand for VPDC as shown in Fig. 2a. In analogy with normal phase contrast, these modified slides were also shifted into the condenser so that their inner perforations were congruent with the objective’s phase ring, whereas their external circular perforations were projected outside the objective’s cross section area. The condenser aperture diaphragm was situated directly beneath the slide inserted so that the intensity of the darkfield partial image could be continuously regulated with this diaphragm.

In order to achieve VBDC, a separate microscope from Leitz / Leica was used, this being equipped with a universal condenser according to the construction type inaugurated by F. Zernike. For concentric VBDC, suitable objectives were selected so that their cross section areas were somewhat larger than the inner diameters of the corresponding condenser’s light annuli.

Condenser-based eccentric and light stop-based VBDC could be achieved with any type of objective when the universal condenser was equipped with a large-sized light annulus. In the light stop-based variants, the twin diaphragm shown in Fig. 6 was placed directly above the objective barrel and the revolving nosepiece (objective turret).

Photomicrographs were taken with a digital camera Olympus Camedia C-7070 and a CMOS camera manufactured by TheImagingSource, each mounted with Leitz/Leica vario photo oculars.

6. Results

In the new techniques presented, several “problem specimens” could be visualized in a high definition quality even when corresponding standard methods did not lead to adequate results. Fig. 7 gives an example for this.

Fig. 7: Scale of a Silver Angelfish (Pterophyllum scalare), permanent slide, cover slip preparation, horizontal field width (HFW): 0.9 mm, objective 10x, brightfield (a), phase contrast (b), darkfield (c), condenser-based eccentric VBDC, dominated by brightfield (d), equalized (e), dominated by darkfield (f).
The fish scale shown here is just barely visible in brightfield because its optical density is very low and only minimally different from the refractive index of the embedding medium (Fig. 7a). In normal darkfield, only artificial precipitations (some detritus, dust and other particles) are highly contrasted whereas the structure of the scale is nearly invisible (Fig. 7b). Normal phase contrast leads to poor images because the thickness of the preparation is beyond the critical limit and the cover slip of this permanent slide is somewhat inclined (Fig. 7c). In VBDC (condenser-based eccentric mode), the scale appears in excellent clarity, in bright-field dominated illumination (Fig. 7d) as well as in equalized (Fig. 7e) and darkfield-dominated VBDC (Fig. 7f). As the bright- and darkfield-like partial images interfere with each other, the final image resulting from superimposition can be compared with phase contrast when phase specimens are examined.

Fig. 8 shows a diatom frustule taken in brightfield (Fig. 8a), normal phase contrast (Fig. 8b) and eccentric condenser-based VBDC (Fig. 8c). In VBDC, some fine phase structures appear clearer than in phase contrast, although there is no phase ring within the objective, because halo artifacts are reduced or absent and the focal depth is enhanced.

Fig. 9 gives an example for the improvements achievable with concentric VBDC. The varnish cast of a snow flake prepared without cover slip and examined at low magnification appears like a linear drawing when examined in bright- or darkfield; only the snow flake`s marginal contours are clearly visible in these standard techniques (Fig. 9a and b). In VBDC however, additional impressions can be clearly perceived which correspond with the regional thickness and the three-dimensional profile of the crystal arms regardless of whether they are illuminated in brightfield - or darkfield-dominated variants (Fig. 9c and d).

A complementary example of concentric VBDC carried out at maximum magnification is shown in Fig. 10. In the very small diatom frustule presented (thickness: 8 µm), the fine linear pattern cannot be well perceived in brightfield, darkfield and phase contrast (Fig. 10a-c), but it appears in optimum clarity, maximum distinctness and highest vertical resolution when examined in concentric VBDC (Fig. 10d).
Also in light stop-based VBDC, the vertical resolution is enhanced and the three-dimensional architecture can be optimally visualized in complex structured specimens. In the pyramidal crystallization shown in Fig. 11, only the peripheral marginal contour can be well perceived in bright- and darkfield (Fig. 11a and b), whereas the pyramid’s three dimensional morphology can only be recognized in VBDC (Fig. 11c and d).

Some examples for VPDC are presented in the Figures 12 and 13. The specimens shown here consist of low density colorless phase structures and additional high density light absorbing components which are pigmented in brown (Fig. 12) or red (Fig. 13). In these circumstances, VPDC leads to maximum visual information because all relevant details can be perceived in the phase structures as well as in the high density components; the natural colors of the high density pigmented material are only visible in darkfield and VPDC, whereas these structures appear like dark silhouettes in phase contrast and brightfield. The low density phase structures, however, can only be well visualized in phase contrast and VPDC. When compared with phase contrast, halo artifacts are mitigated in most cases when VPDC is carried out.
The variant methods presented can be regarded as interesting tools promising high definition images especially in many “problem specimens”. Even in particular preparations which cannot be well perceived in bright-, darkfield or phase contrast, the techniques reported can lead to high fidelity images. In contrast to darkfield illumination, blooming and scattering are reduced and when compared with normal phase contrast, halo artifacts are mitigated or absent. In all technical variants, the appearance of final image can be modulated with the condenser aperture diaphragm. In particular, the background brightness can be modified, the vertical resolution and planarity of field can be enhanced, irradiations can be reduced and the contour sharpness can be intensified. Also in rather thick specimens, existing structures situated in different planes or heights are mostly imaged sharp and distinct. Three dimensional reliefs and fine internal structures can often be visualized in superior clarity, especially in complex structured specimens. In VBDC, several phase specimens can be imaged in a phase contrast-like manner without using phase contrast lenses, and in VPDC, phase structures can be perceived together with surrounding light absorbing details.

It can be regarded as additional advantage of VBDC that specimens can be examined in circular (concentric) as well as in oblique (eccentric) illumination whereby the angle of incidence can be varied by the user.
All in all, the techniques presented will lead to improved results in several fields of light microscopy, especially in preparations affected with a high regional thickness, high regional ranges in density and a complex morphology. The methods described could be easily integrated in standard laboratory microscopes by manufacturers. Thus, for instance, universal condensers for phase contrast could be fitted with modified light masks for VPDC so that this technique could be carried out with the phase contrast equipment available. Blooming and scattering could probably be reduced further when objectives for phase contrast were additionally fitted with an iris diaphragm inserted into or near their back focal plane; alternatively, existing special objectives for darkfield examinations could be equipped with a phase ring so that they could be also used for VPDC. Moreover, special universal condensers could be created for concentric VBDC containing a set of different sized light annuli which were all larger than those used for phase contrast. These light annuli should match the objective’s cross section area so that different weightings of bright- and darkfield could be achieved simply by changing the annulus used. Of course, universal condensers could also be constructed consisting of special light masks for concentric VBDC and normal phase contrast so that these different methods could be carried out by use of existing phase contrast lenses. In principle, VPDC and VBDC could also be carried out in epi-illumination when an illuminator for incident light was modified for these techniques. In the latter case, these methods could also give new impulses for material sciences.

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