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Oblique illumination in microscopy: A quantitative evaluation

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ABSTRACT

Many biological objects are barely distinguished with the brightfield microscope because they appear transparent, translucent and colourless. One simple way to make such specimens visible without compromising contrast and resolution is by controlling the amount and the directionality of the illumination light. Oblique illumination is an old technique described by many scientists and microscopists that however has been largely neglected in favour of other alternative methods. Oblique lighting (OL) is created by illuminating the sample by only a portion of the light coming from the condenser. If properly used it can improve the resolution and contrast of transparent specimens such as diatoms. In this paper a quantitative evaluation of OL in brigthfield microscopy is presented. Several feature descriptors were selected for characterising contrast and sharpness showing that in general OL provides better performance for distinguishing minute details compared to other lighting modalities. Oblique lighting is capable to produce directionally shadowed differential contrast images allowing to observe phase details in a similar way to differential contrast images (DIC) but at lower cost. The main advantage of OL is that the resolution of the light microscope can be increased by effectively doubling the angular aperture. OL appears as a cost-effective technique both for the amateur and professional scientist that can be used as a replacement of DIC or phase contrast when resources are scarce.

1. Introduction

In photography, oblique light (OL) is a technique that allows to show detail by creating shadows on the surface of the object. This is one of the reasons most of the photographers prefer to capture images at sunset or dawn. Side photography allows to emphasise texture, defining depth and bringing out pattern and detail. An old debate in microscopy was to elucidate why different illumination techniques will produce different results for different specimens. In general, it can be shown that oblique illumination improves the contrast of transparent specimens by introducing a pseudo-relief effect. Oblique illumination is an old technique that has been described by many scientists and microscopists (Stephanides, 1947; Olliver, 1947; Schleiden, 1849; Schacht, 1853; Spitta, 1920; Francon, 1961; Allen, 1944). Oblique lighting can be interpreted in the context of the Abbe's theory of diffraction that establishes that the finest details of the specimen are obtained from diffracted beams that are apart of the axial light (Stephanides, 1947). According with the Abbe's theory of diffraction, at least two orders of diffraction are needed to recover the details of the object. With oblique light the objective lens capture positive diffraction orders from one side of the specimen and negative from the other side (Wayne, 2014). In practice, oblique illumination is obtained by blocking part of the condenser's light through the use of particular patch stops with different shapes. The main advantage of OL is that the resolution of the light microscope can be increased by effectively doubling the angular aperture. One of the inconveniences of OL is that it appears to improve resolution in a directional way depending on the direction of the oblique mask. A simple variation of OL is produced by inserting an opaque stop with one or several small apertures or notches on the border of the stop. This procedure generates few narrow pencils of illumination in a very oblique way (Olliver, 1947). Both kind of unsymmetrical OL introduce similar strong pseudo-relief effect improving the edges of the objects. Another way to improve resolution and contrast is to use an inverted oblique “hollow-cone illumination” or circular oblique lighting (COL) (Mathews, 1953), and because COL is symmetrical it does not produce the strong pseudo-relief found with OL. In fact, as R. Wayne mentioned in his recent book “*there is no reason why all microscopes should not be equipped with annular illumination as a standard feature to*

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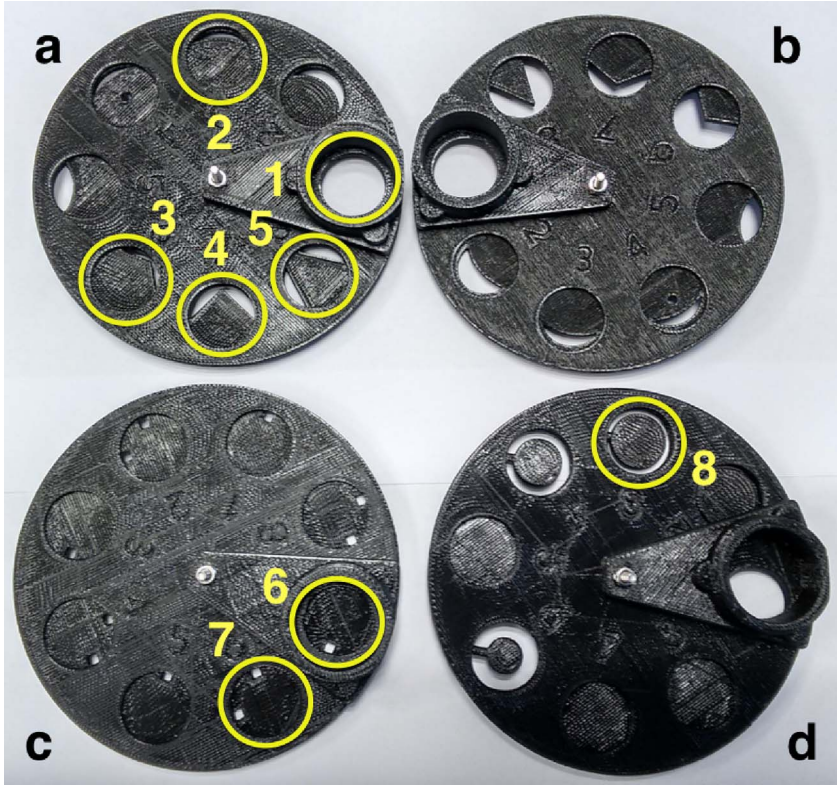

Fig. 1. Oblique filter wheels generated with a 3D printer using different shapes. In wheel (a), filter 1 corresponds to brighfield illumination and filters 2–5 provide oblique illumination with the best performance both in terms of sharpness and texture contents. In wheel (c), by inserting small aperture or notch-shaped the object will be illuminated with a very oblique pencil of light. In wheel (d) circular apertures are used producing an inverted oblique "hollow-cone illumination", i.e. circular oblique lighthing (COL).

maximise the resolving power" (Wayne, 2014). It is important not to confuse COL with dark field (DF) illumination which is again a very old technique. In the latter case (not discussed in this paper), the specimen is illuminated with a hollow cone of light which is too wide to enter the objective lens. Once the object is placed on the stage only the first and higher-order diffracted light will be able to enter the objective lens (Wayne, 2014). DF can be interpreted as a process to remove the low-frequency components of the Fourier spectrum and therefore will become more effective for improving the edges or boundaries of the objects and less useful for the inner texture (except for patterns with high transparent detail).

The main purpose of this paper is to provide a quantitative account of OL as a simple and efficient variation in brightfield microscopy. Improvements in contrast and enhancing details have been evaluated through the use of texture descriptors and the quality of the images has been assessed by considering an ideal observer model. Different oblique filter sets have been designed and printed out using a 3D printer. Such designs are provided and they can be easily adapted to different microscope configurations (Sanchez and Cristobal, 2017). This paper is structured as follows. The details about the materials and the equipment used are described in Section 2. Section 3 reviews the oblique illumination properties. In Section 4 the feature descriptors selected for characterising contrast and sharpness of the different diatom preparations analyzed are shown. Section 5 presents a quantitative evaluation study of the oblique illumination technique for different patch stop filters with different shapes and sizes. Finally, a discussion an few conclusions are drawn in the last section.

2. Materials and methods

Microscopic identification and counting of diatoms requires obtaining frustule suspensions free of organic matter. The frustules are the siliceous covering of the diatoms, their shape and ornamentation is the basis of the taxonomy of these algae. To empty the cell contents of the diatoms and eliminate other non-silicified organisms, it is necessary to process the sample to an intense oxidation by hydrogen peroxide. For the observation of diatoms under optical microscopy, it is necessary to mount permanent preparations using a synthetic resin (Naphrax®) as a medium, with a refraction index of 1.7. Sample images were captured using a low cost Brunel SP30 monocular microscope with standard Brunel DIN parfocal objectives of 60× (0.85 NA) and 100× (1.25 NA) using LED with different wavelengths from visible to UV. For white light (λ = 442 nm) a Brunel Digicam LCMOS 5 Mpixel camera was used for image acquisition (cell size 2.2 μm × 2.2 μm). The camera is connected to the computer through an USB2.0 connection, providing an image size of 2592 × 1944 pixels and the exposure time was 0.25 s. For UV light (λ = 365 nm), the Imaging Source camera DMK 72 with USB 2.0 was used. The DMK 72 is a monochrome 5Mpixel camera that has good sensitivity for 365 nm. Both cameras meet the resolution limit of the corresponding objectives for the wavelengths used. Concerning the appropriateness of the microscope optics with UV light, the fewer glass components the better. Flat field objectives are not suitable for UV due to the high absorption. Glass slides and coverslips produce minor losses (Hobel and Sterrenburg, 2011). A centering telescope (CT) eyepiece (Bertrand lens) was used for visualising the different diffraction spectra at the back focal plane of the objective lens. Results reported in Figs. 6 and 7 were obtained with a cooled Hamamatsu C9100-02 camera (CCD chip 1000 × 1000 pixel with 8 μm × 8 μm cell size) attached to a Leica AF6000LX multidimensional microscope with an objective lens PL APO 40 × 0.75 DRY. The radius of the Airy disk produced by such objective considering the Rayleigh criterion is about 14 μm what does not meet the Nyquist sampling theorem that requires two pixels per unit resolution distance (i.e. 16 μm). However such camera provides excellent sampling for 100 × 1.3 NA which turns to be very suitable for fluorescence applications. The Leica microscope was used because it was the only available equipment that provides all microscopic modalities described in Section 5.

3. Oblique illumination in the context of diffraction theory

When a transparent biological specimen is observed under a standard microscope brightfield illumination, light is retarded $\lambda/4$

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