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Contrast Methods in Microscopy: Rheinberg Illumination

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ABSTRACT

In 1896, Julius Rheinberg introduced a color contrast method to the microscopy world. This method uses colored discs relatively near the back focal planes of the objective, or more commonly, near the aperture focal plane of the condenser to provide a colored specimen on a colored background. Almost any microscope can be modified to generate this style of imaging, which can be accomplished using a few practical options. The Rheinberg technique is a modification of the darkfield (or darkground) method of illumination and the color contrast enhances the visibility of specimens and their texture. It also provides an esthetically pleasing view of specimens worthy of photomicrography awards.

Keywords: Rheinberg illumination, double illumination microscopy, darkfield microscopy, color contrast microscopy, photomicrography, numerical aperture (N.A.), near back focal plane (NBFP), condenser aperture focal plane (CAFP), Mikropolychromar microscope, color-phase contrast microscopy (PCM), variable phase darkfield contrast (VPDFC)

INTRODUCTION

On May 20, 1896, Julius Rheinberg introduced a color contrast method for enhancing features of a specimen using an optical staining method, instead of the more common physical staining techniques (1). Color, of course, is an important addition in obtaining optical contrast (2), and the human eye is capable of detecting differences in wavelength of 1–5 nm (3). Rheinberg continued his promotion of the method with similar updates (4–6). His original colored drawings illustrating the concept are shown in Figures 1–3. As noted by G.W. White, Rheinberg described three methods of differential color illumination: low power or refraction method, high power or diffraction method, and the composition method (7).

THE MECHANICS OF RHEINBERG DIFFERENTIAL COLOR ILLUMINATION

Following Rheinberg's three-type designation set forth by White, one can examine each method in more detail.

Type 1: Low Power or Refraction Method

A two-color disc is arranged in the substage at or close to the aperture focal plane. Some microscopes will have a filter holder at this location. The central disc is sized to allow the cone of light transmitted by the central color to just fill the whole of the objective aperture. The other colored disc matching the condenser cone is much wider than the objective cone and captures light from the far annular space. Therefore, the condenser numerical aperture (N.A.) needs to be greater than the objective N.A. The object can be identified by color contrast in the annulus color on a background of the central color (see below for a more formal description). This is a form of darkfield microscopy where the opaque central stop is replaced with a colored stop and the refracted light is further filtered

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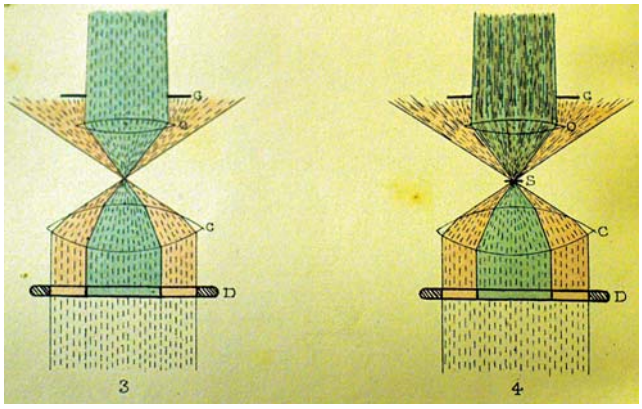

Figure 1. Original Rheinberg colored illustrations of the Type 1 method. No. 3 shows how the central stop color encompasses the full angle of capture (numerical aperture) of the objective without a specimen, whereas No. 4 shows the same arrangement but with a specimen (S) that allows the direct or zero-order blue-green. It also allows the indirect rays of the reddish-orange screen from interaction with the specimen to be captured by the objective.

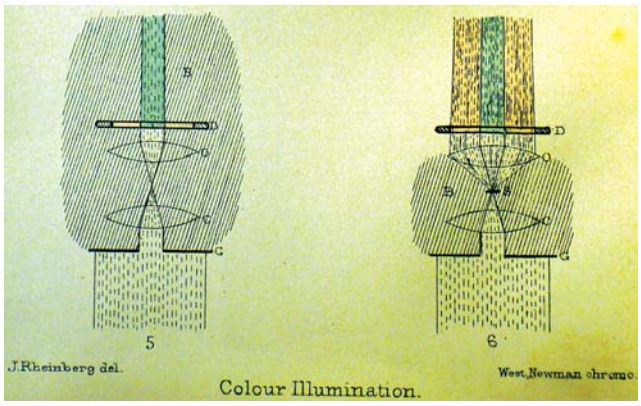

Figure 2. Original Rheinberg colored illustrations of the Type 2 method. No. 5 shows how the central stop color encompasses the restricted (zero-order blue-green) portion of the near back focal plane (NBFP) of the objective without a specimen, whereas No. 6 shows the same arrangement but with a specimen (S) that also allows the indirect rays from interaction with the specimen to be captured by the reddish-orange screen in the NBFP of the objective.

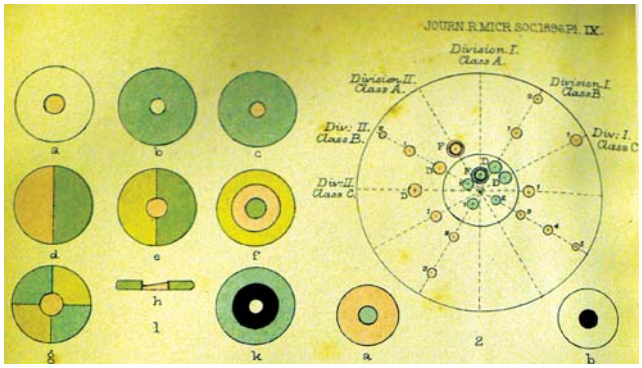

Figure 3. Original Rheinberg colored illustrations. No. 1 demonstrates nine discs of colored glass used in the technique (see text for general usage). The stop of type *h* is unusual in that a cross-section is drawn to demonstrate that a section is prismatic (not perpendicular to the axis of the scope). This creates a double image: one image in the red center and another projected to the side, thus overlapping the light transmitted directly through the green. (Although not tried by the author, it is likely that this type *h* disc will create sufficient displacement to create a pseudo 3-D image in red-green). No. 2 is a diagram representing various types of screens versus responses. Those in Division I are affected with less refraction and fall within the central stop, whereas Division II are those with strong refraction that fall outside the central stop. Within each numbered set, there are classes of fineness of structure causing "diffraction," with diffraction increasing from A to C and described as being correlated with the orders of diffraction as a parameter, from >2 orders for A, to first and second orders for B, and then one order for C.

with a color (Figure 1).

Type 2: High Power or Diffraction Method

A disc of two colors is placed above the objective lens in the near back focal plane (NBFP). The light transmitted through the substage condenser is reduced to a narrow cone using the substage condenser aperture so that it only fills the central color of the disc. This leaves the surrounding portion without direct illumination. The color of the center portion of the disc determines the background color, while the outer annular color (through which passes the diffracted rays) primarily sets the corresponding object (specimen) color (Figure 2). The reduction in the aperture will increase the color contrast at the expense of resolution.

Type 3: Composition Method

This method is usable for both lower and higher magnification. A dual (complimentary) color disc with a small central color filter is placed in the substage filter holder (or an equivalent location). The cone of light transmitted by the central portion is now smaller than that necessary to fill the objective aperture. The annular cone of light transmitted by the outer, complementary color, is limited by reducing the substage condenser aperture so that the two filters combine to provide a white or neutral background. When the specimen is placed in the field, it diffracts the two colors in unequal proportions and, therefore, appears colored on a white/neutral background. Again, a reduction in

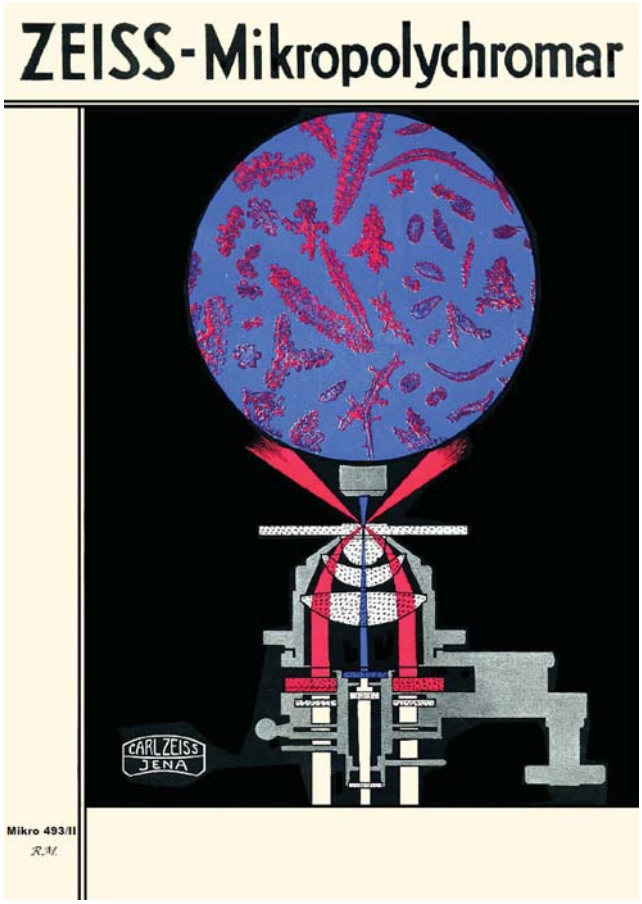

Figure 4. The cover of the Zeiss Mikropolychromar manual indicates the direct and indirect coloration effects generated by the microscope.

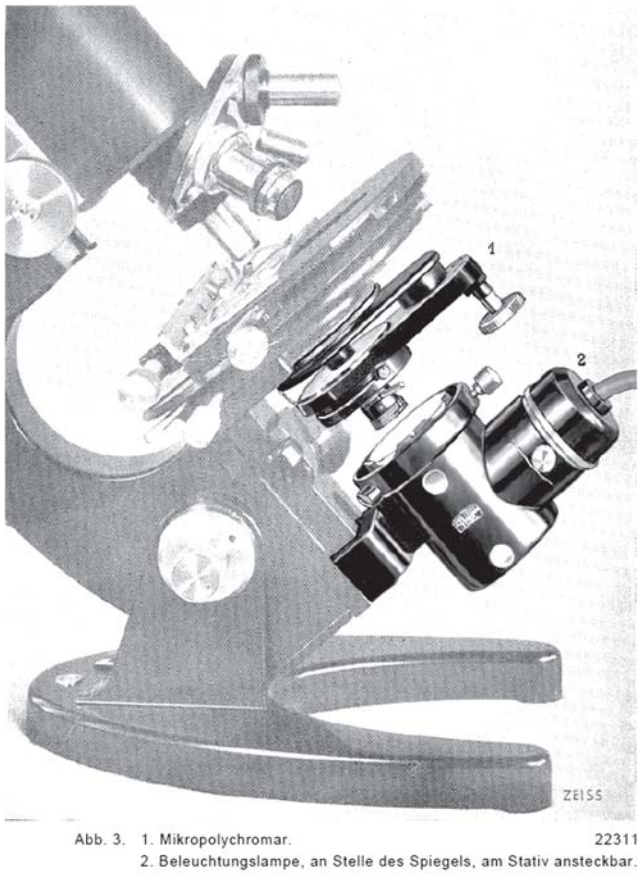

Figure 5. Black and white image of the Mikropolychromar microscope from the Zeiss manual.

the aperture will increase the color contrast at the expense of resolution.

PROMOTION AND VARIATIONS

Since their introduction, Rheinberg's techniques continue to be used on a variety of specimens: diatoms, foraminifera, polycistina, rotifers, other small aquatic organisms, frog embryos, blood, minerals, crystals, fibers, hairs, skin cells, textiles, pigments, polymers and liquid crystals as documented in various works over the years (7–20). Early on, and even into the 1950s, Kodak reportedly offered a kit of filters for this purpose (16, 21). A complete microscope based on this concept was created and sold by Zeiss beginning in 1933. It was called the Mikropolychromar (16, 22–24) (Figures 4 and 5). L. Leitz also patented a condenser system of this sort in 1956 (25). Although the Mikropolychromar was constructed for ease of use with regard to Rheinberg illumination, it apparently was found to have a quality no better than putting a patch in a simple Abbe condenser (26).

Although diatoms were the first subject described by Rheinberg, one interesting use of the method has been on flagella, such as sperm (27). The Rheinberg illumination technique was even repackaged under the trademarked term Light Staining Microscopes for use in sperm analysis (28–30), where it was referred to in the ASTM Method E2124, Standard Practice for the Specification for Equipment and Supplies in Sexual Assault Investigations.

A modified version of Rheinberg illumination was created by W.G. Hartley using overlaid polarizers in the condenser, a first-order red plate, and a rotatable analyzer (15), which he termed a variable Rheinberg system. S.E. Brolin prepared an ultraviolet version of Rheinberg that was later mimicked by P. Manigault (31, 32). In other modifications, D.J. Schuitema-Meijer

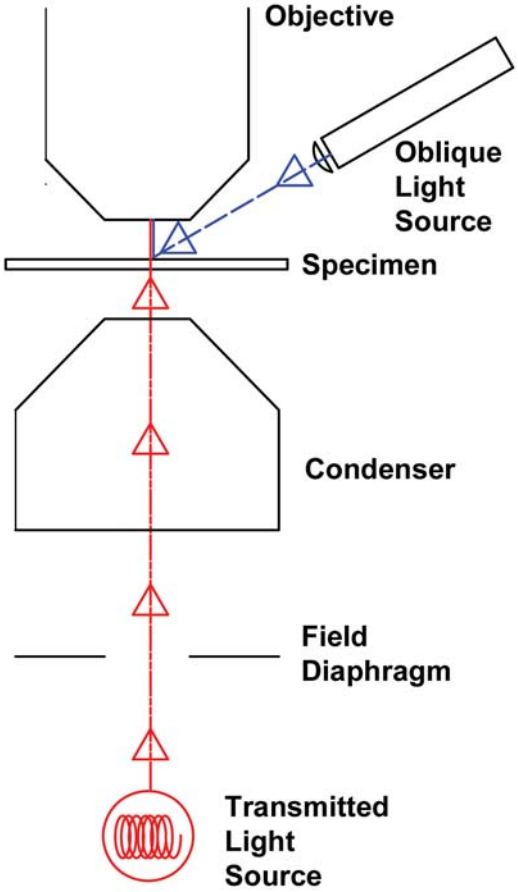

Figure 6. Diagram (after G.W. White) of the double illumination technique first described by William Carpenter in 1891 (it was not present in 1856 or 1883 editions).

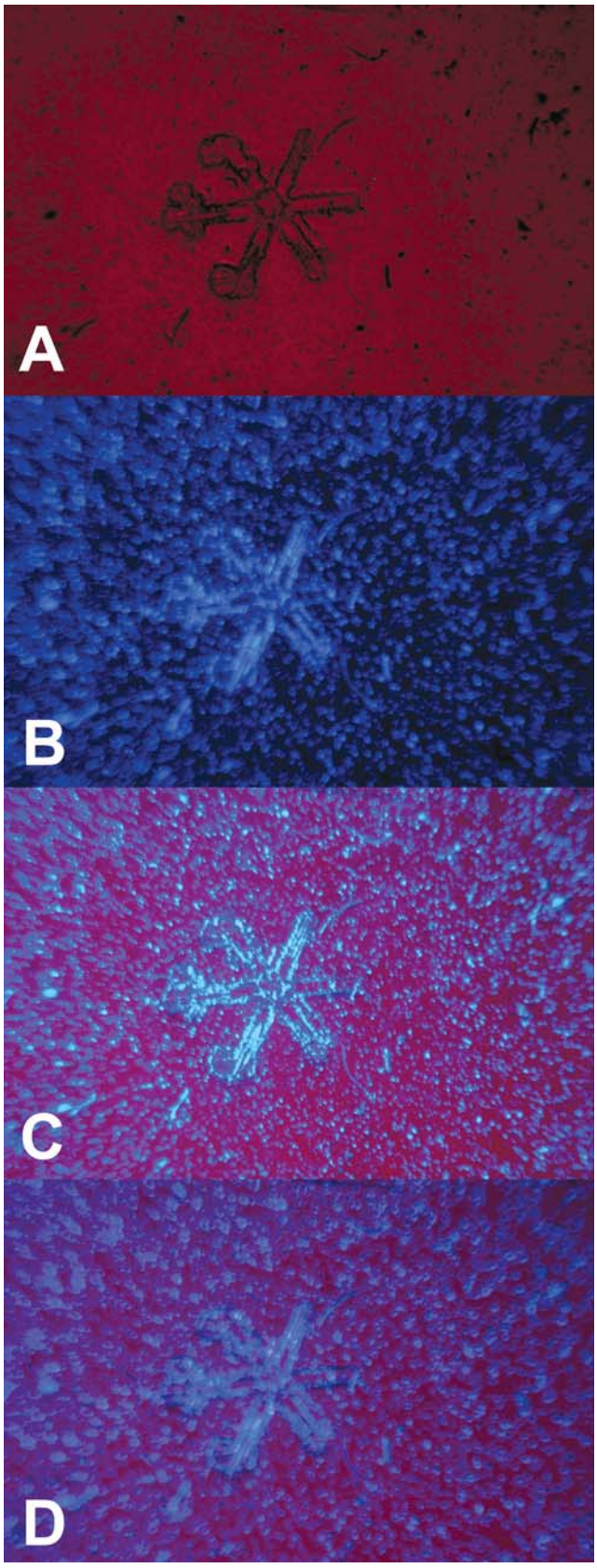

Figure 7 (right). A replica of a snowflake imaged by the Carpenter double illumination technique. The four images show, A) red transmitted light, B) blue light from the oblique Nightsea source, C) combined double illumination from both sources at the same time, and D) images A and B combined by using the Addition command in Image J. The shift to the magenta zero-order background in C is primarily a product of the combined wavelengths along with the camera (Pentax KD20) spectral response and the exposure period.

used two lamps and a prism for the central ray illumination (33), whereas K.F. Webb conducted Rheinberg without a condenser (34). To this effect, Martin (**35**) manufactured an annular LED illuminator for the outside of the condenser just below the stage level to provide an outer annular color while using a different color in the standard transmitted light source.

Usually thought of as a solely transmitted illumination technique, White details a dual transmitted/reflected illumination technique to generate a similar effect (**7**). This technique was reported by William Carpenter in 1891 (36) before Rheinberg. A diagram of the setup is shown in Figure 6, and an example of this compound Trans-Reflected method is provided in Figure 7. The example setup in Figure 7 consists of using a colored disc in the condenser and oblique top lighting from a Nightsea royal blue light for UV excitation (37, 38). The colored disc is one from a set of decorative confetti chips that make interesting filters (Figure 8). Figure 7 shows four images: one of the snowflake replica with transmitted light through the red filter; one with reflected (oblique) light of blue (broad band response from a 460–480 nm light); a combined view with both light sources, transmitted and reflected; and a combined image using the Addition command in Image J software (39). This approach with objectives of >10X magnification uses long working-distance objectives to permit the oblique top lighting to enter. This approach was also applied by Piper (40), who referred to it as "sandwich illumination," using transmitted darkfield and epi-illumination to beautifully display radiolarians. In addition, one manufacturer has marketed a combination device for ferrography (41), referring to the main component as a bichromatic microscope. It used a red reflected light source and a green transmitted light source, permitting (in general) metallic particles to be observed in red and non-metallic particles in green.

Color-phase contrast microscopy techniques have also been derived from the Rheinberg technique (42, 43). Because of this, it is understandable that Steven Ruzin describes a colored phase contrast-like effect if the central colored disc is thicker than the others (17). Ruzin also provides a wonderfully simple step-by-step modern version of creating Rheinberg illumination. Marcel Locquin refers to an interesting variation in which two techniques are used, e.g., darkfield and brightfield, or brightfield and negative phase contrast, each with one color applied to it (44). This would have required a dual exposure for 35 mm film at the time he described this. In the present day, this process may be accomplished digitally by the addition or averaging

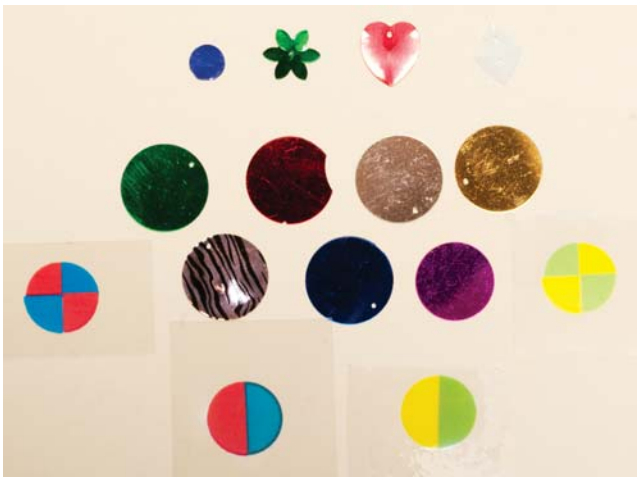

Figure 8. Various stops used for Rheinberg. Four of these — the left-most, right-most and two bottom-most — were printed from a color laser printer. The other discs and the top punched design pieces were gathered from party events.

of these two images (perhaps in varying proportions), as seen in Figure 7D. Alternatively, one can follow Piper's lead with a modified/manufactured arrangement of a modern scope to do this in real time, calling it variable-phase darkfield contrast (VPDFC) (45).

Classical darkfield technique tends to require a reasonably strong light source, whereas Rheinberg is a little more forgiving. Despite this, E.C. Samson provided a high lumen source by applying an LCD projector as a light source (46). Although this article is about direct viewing of observing static images, there are two papers utilizing Rheinberg illumination with video microscopy (47, 48).

PRACTICAL ASPECTS AND EXAMPLES

The sizing of the stops is the critical aspect, mostly for 20X and higher objectives. This can be aided by one of a few methods of estimating the diameter of the NBFP or condenser aperture focal plane (CAFP) location. The author has used the following to aid in estimating this field of view distance: a clear overhead transparency with hand-drawn grids, an Autocad-generated and laser printer- or copier-produced grid on transparency, a clear ruler, gelatin film with notches cut into it and a commercially available square 20 x 20 mm adhesive tape slide grid (Figure 9). A telescoping lens, Bertrand lens or pinhole cap (Figures 10 and 11) can assist in viewing the NBFP or CAFP for measurement. The telescoping lens or centering telescope provides the most versatility, as it

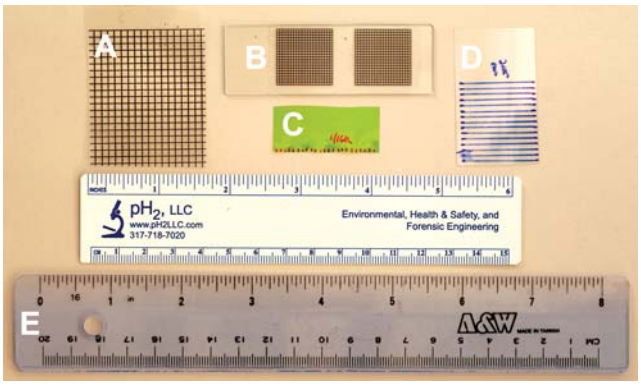

Figure 9. Measuring devices used for estimating the NBFP or CAFP diameter: A) 1/10 x 1/10-inch stepped grid created in Autocad, printed on a black and white laser printer, then photocopied onto a transparency sheet; B) 20 x 20 mm adhesive slide grids in steps of 1 mm purchased from Electron Microscopy Sciences; C) Kodak Wratten filter piece with 1/16-inch marks and cut notches; D) write-on transparency sheet with hand drawn lines approximately 2 mm apart; and E) see-through ruler.

Figure 10. Olympus telescoping lenses (centering telescopes) and pinhole eye caps. From left to right: side view of a telescoping lens, side view of a telescoping lens extended for viewing NBFP or CAFP, a top view of eye cap and a bottom view of the eye cap.

can focus through a large range of focal planes compared to other means. One can even take an eyepiece out and gaze down the eye tube to view the grid, ruler, etc. A diffuser to mute the light source (if not present already) is recommended for this step.

Once an estimated diameter is found (see Figure 12 for views using a Bertrand lens), it becomes trial and error to hone in on the best size, because the central stop is usually intended to absorb the complete direct field of view. Colored acetate sheets work well for creation of stops (49), but other transparent materials, including ink jet or laser-printed stops on transparencies (Figure 9) can be used. Color laser and ink jet printers use CMYK color overlays (cyan, magenta, yellow, and key or black) that allow voids on a microscale and, therefore, are generally not uniform or absorbing enough and must be supplemented by coloring over with a marker or stacking multiples. Rheinberg used glass coated with collodion or gelatin, with the required color in the coating (1), whereas Almroth Wright used dye dropped on glass or dye-stained filter paper (50). Cutting can be accomplished with punches, scissors, drilling a filter stop sandwiched between plastic, a compass cutter (51), a cork borer, commercial disc punches, etc. (52). One can attach magnets to a piece of cardstock to hold the Rheinberg filters to a condenser or place them in a pre-manufactured filter holder (which was common years ago [9]), lay the colored filters on the topside of the bottom lens in an Abbe condenser, put them on a glass slide and lay the slide on the condenser, etc.

Figure 11. An Olympus Bertrand lens location (focusable) at the "IN" position above the analyzer (graduated) and Red I waveplate. Below the analyzer and waveplate is the turret for the objectives.

For Type 2 Rheinberg illumination with a low power objective (≤10X), one can modify the back focal plane in the same manner as creating a dispersion staining objective (53, 54). If available, one can use the empty slot in a rotating turret-style dispersion staining objective (Figure 13) (55–57). Because the amount

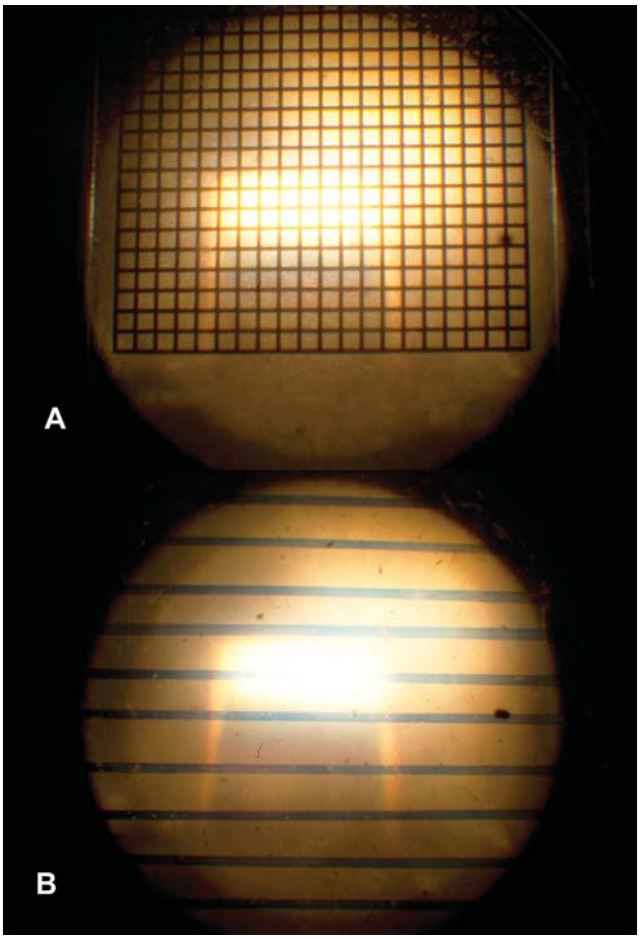

Figure 12. Photomicrographs of observation through a Bertrand lens at the CAFP. A) 20 x 20 mm (in 1 mm steps) adhesive slide grid mounted to a glass slide (diameter approximately 23 mm), and B) write-on transparency sheet with hand-drawn lines approximately 2 mm apart (diameter approximately 23 mm). Light filament is off-centered (therefore, not properly aligned Köhler illumination) and has been muted with a diffuser for imaging purposes.

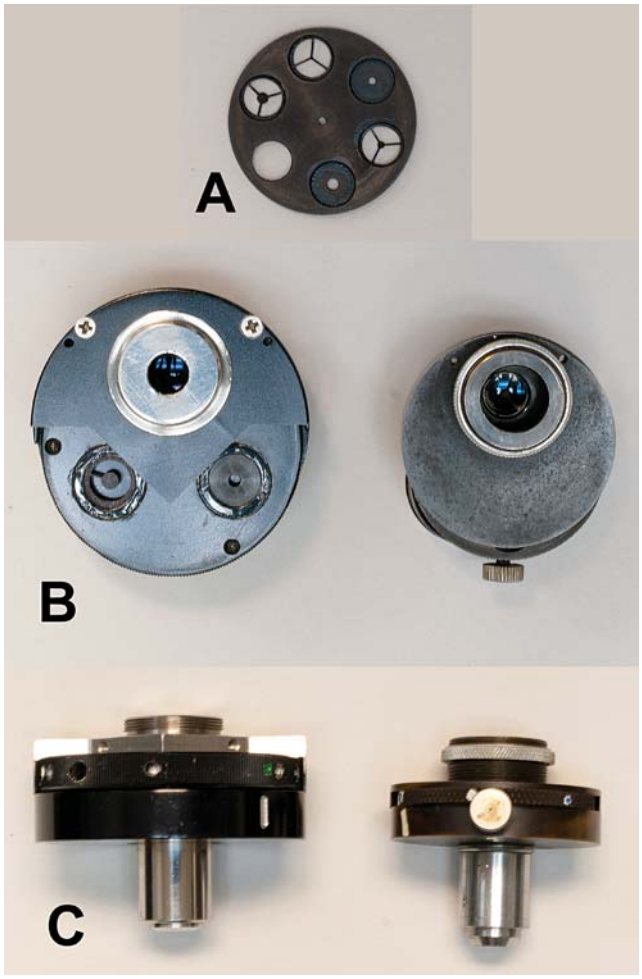

Figure 13. Variable stop turret-style 10X dispersion staining objectives for Olympus 160 mm tubelength scope. A) The plate removed from an old model showing six openings with three dark stops, two annular stops and one open slot. Colored discs may be inserted in these openings and the objective mechanics allows for centering the disc in the NBFP. B) Top view of a newer (left) and older (right) style dispersion staining objectives; note access on the newer (left) model to the central stop, annular and open slot (at the objective position and not viewable in this image). C) Side views of newer (left) and older (right) dispersion staining objectives.

of light transmitted to the eye from the central compared to the annular portion is much higher (Figure 14), it is advantageous to us a lighter color in the central portion. Alternatively, John Delly recommends using a neutral density filter to match the central portion, thereby reducing the illumination from the center color and simultaneously allowing the annular portion to increase relative to the central stop (58). Ruzin follows this suggestion in describing steps for preparing discs (17). When using concentric colors with a brightfield condenser, Locquin suggests using the shortest wavelength color in the periphery; however, when using a darkfield condenser, he uses the shortest wavelength color in the center (44). Two other possibilities are available. The first is placing an additional central black stop to reduce the central lighting; a piece of electrical tape punched or cut out works well. The second is using a central stop made from a polarizing sheet. This allows one to vary greatly the relative amount of center-to-outer annulus proportions by rotating a standard polarizer below or at the base of the condenser (Figure 15 is an example of an

Figure 14. An X-sectional display of a Rheinberg cone of light with a two-color set-up (inner center of blue and outer ring of red), using Robert E. Smith's technique (see References Cited, No. 72). Note that the inner cone has a higher intensity (more brightness).

image created using such a polarizer). When using a two-color approach, the primary color can almost be completely switched from one to the other using this system.

An example of the technique used on a diatom test slide is shown in Figure 16, where annular quadrants of red, green, red, green with a black central stop were used. Figure 17 is an example of a low-quality, 3-D image of a snowflake replica using double annular stops of red and blue with a black central stop. The red and blue sheets were cut from 3-D glasses. Viewing the image with a pair of red and white 3-D glasses will help create a pseudo 3-D image.

ARTISTIC LICENSE

The beauty of Rheinberg images is particularly demonstrated in instructional works on photomicrography such as Roger Loveland's two-volume set on the subject (59) and Delly's Photography Through the Microscope (58), where five of the nine images on the cover were taken using Rheinberg illumination. These pleasing aesthetics are also seen in photomicrography competitions. For instance, the 2012 Nikon Small World Competition Image of Distinction shows a butterfly tongue at 5X magnification using Rheinberg illumination. The 1995 first-place winner was Christian Gautier's image of the Larva of Pleuronectidae, which combined Rheinberg with polarized light (60). It is notable that Rheinberg illumination is listed as one of the Top 10 techniques for the Olympus Bioscapes

Figure 15. Navicula lyra diatom using a two-color (blue-magenta) system with the addition of a polarizer in the central area to control relative contributions of each color.

photomicrography competition (61), with a good example being the 2008 first-place winner, the fairy fly wasp (62). The author employed the method for a holiday postcard (Figure 18), using a simple hand-colored piece of tape on a universal condenser insert (Figure 19).

THE ORIGINATOR: RHEINBERG OR GORHAM — OR SOMEONE ELSE?

Although Rheinberg was given the credit for the concept of this technique, and he certainly attempted to propagate its use, it was John Gorham who likely introduced the concept but in a more dynamic way (63). He purposed a tri-colored plate with a rotating overlay to create a kaleidoscope effect (Figure 20). Gorham even noted in his article where the colored discs could be acquired: Smith, Beck and Beck. Similar to Rheinberg, Wright, a medical doctor in London, pro-

duced a book entitled *Principles of Microscopy* in 1906. In it he clearly describes Rheinberg's technique, but with a lot more detail and more examples, and with darkfield and colored darkfield diagrams to fully support his assertions (Figure 21) — but with no mention of Rheinberg. He used the colored stops in his frontispiece (Figure 22, images D and E) and described the use for examining textiles and glass beads (Figures 23 and 24).

No matter the origins — Rheinberg, Gorham, Carpenter or Wright — this color-contrast method will continue to find application as a technically valid contrast technique to aid in visualization and as a soul-inspiring method for producing exquisite photomicrographs.

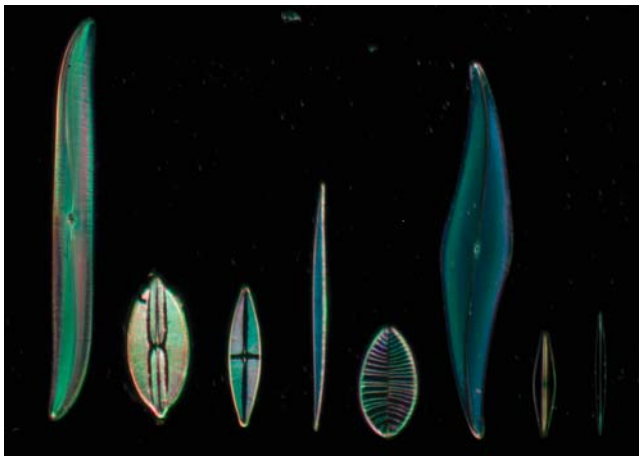

Figure 16. A red, green, red and green four-quadrant, disc-based Rheinberg illumination on a Klaus Kemp 8 Form Diatom Test Plate. Diatoms shown from right to left are: *Amphipleura pellucida*, *Frustulia rhomboides*, *Pleurosigma angulatum*, *Surirella gemma*, *Nitzschia sigma*, *Stauroneis phoenicentron*, *Navicula lyra* and *Gyrosigma balticum*. The image was taken on an Olympus BH2, with a 20X Long Working Distance objective with a low N.A., an opaque darkfield stop plus a four-quadrant disc. The intent was to show general directional spatial placement.

MATHEMATICAL APPROACH

Harold Hopkins (64) showed that by starting with a phase plate approach for phase contrast microscopy, the intensity of an image can be represented by:

Equation 1:

$$I = A^2 + [2(A\ SinP)\ Sin\varphi] + [2(1 - A\ CosP)\ (1 - Cos\varphi)]$$

Where P is a pupil angle and φ is the phase change in the object. In darkfield this can be reduced to the function:

Equation 2: $I = 2\ (1 - Cos\varphi)$

when one assumes that the phase plate/ring is completely absorbing (Amplitude A = 0 throughout that region). Others have independently arrived at this representation as well (65–67). If A = 0 (complete absorption) for all but a small range of wavelengths (λ_{a-b}), e.g., the transmitted central stop color, then a broad fairly uniform background of color wavelengths λ_{a-b} will form a base or zero-order image. Similarly, if the indirect (refracted and diffracted) wavelengths are restricted by an annular color of λ_{x-y}, these will reveal phase changes in the color range of λ_{x-y} from the annular portion. Assuming small phase changes (68), one can see that:

$$I \propto \varphi^2$$

This is a non-linear response to phase and is best observed clearly at changes in response to a strong phase gradient, i.e., edges. It is amazing to see that Conrad Beck (69) clearly recognized that the gain in visibility by this darkfield effect, particularly in diatoms, was due to a gain in contrast, not a gain in resolution. The author independently came to this conclusion a number of years ago as did H. Olivier more recently (70). Hartley (26) further recognized that Rheinberg effectively reduced glare by using one wavelength for the direct image and another wavelength for the dark-ground image, thereby eliminat-

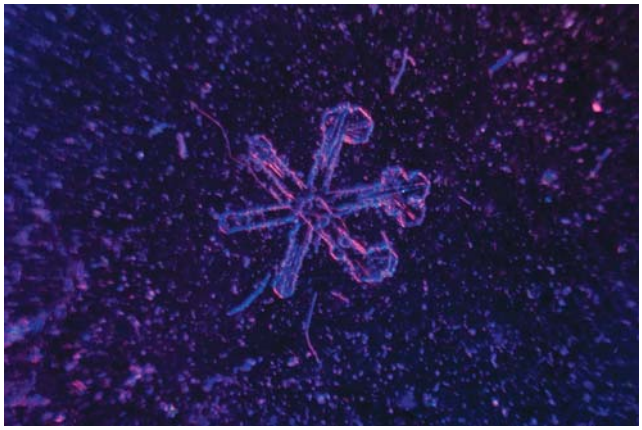

Figure 17. Snowflake replica by Rheinberg with a darkfield stop and two annular stops of red and blue derived from the lens material of a set of 3-D glasses. Use of 3-D glasses allows the observation of a pseudo 3-D image.

Figure 18. Fusion preparation of the polynuclear aromatic hydrocarbon (PAH) anthracene, often found in fossil fuels. It has a crystal habit of forming scimitar-like blades and lathes; in this case, grouped crystals that look much like a Christmas tree. The central stop portion is red and the outer annulus is green. Actual stop is shown in Figure 19 at insert plate 4.

ing mutual interference at the same wavelength. As Fourier planes can be considered additive on one another, this concept in conjunction with Equation 1 validates Hartley's comment with more than just a geometrical approach. Furthermore, it is recognized that visual distortion, or noise, in a dark image is more annoying than noise in a light image (meaning the average illuminance of an image) (71). By controlling the noise by limiting wavelengths, one would expect a reduction in annoyance that is often observed with white-light-based darkfield microscopy.

ACKNOWLEDGMENTS

Many thanks to the McCrone Research Institute for access to original color versions of the Royal Microscopical Society proceedings and journals. The University of Toronto provided a digital copy of Wright's 1906 book, *Principles of Microscopy*, including the plate images used here.

Figure 19. Top view of uncovered Olympus Universal condenser with a five-position turret. Plates in positions are as follows: 1) 40X phase contrast; 2) 40X Nomarski DIC; 3) 40X darkfield; 4) central-red, annular green Rheinberg disc from colored masking tape; and 5) open slot (not observable under top 0.8 N.A. lens).

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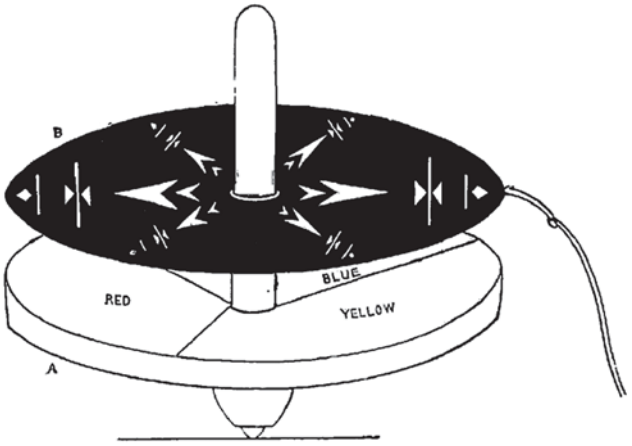

Figure 20. Original figure from John Gorham's 1859 article on a Kaleidoscope-like Rheinberg illumination system.

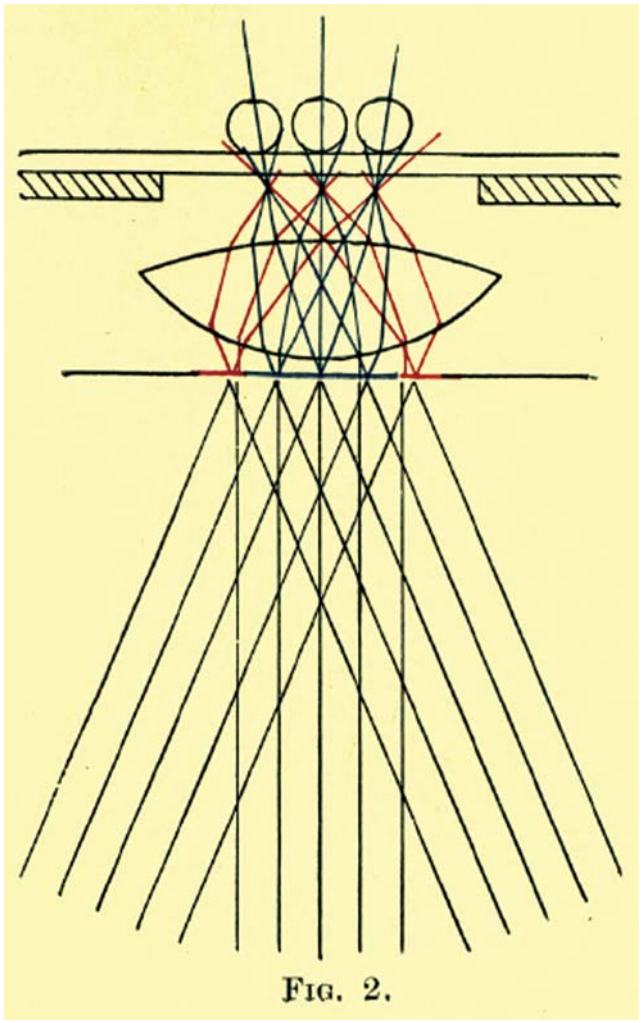

Figure 21. Original colored figure from Almroth Wright's 1906 book, *Principles of Microscopy*, illustrating the Rheinberg darkfield coloration effect: red central color for direct rays, blue annular color for indirect rays.

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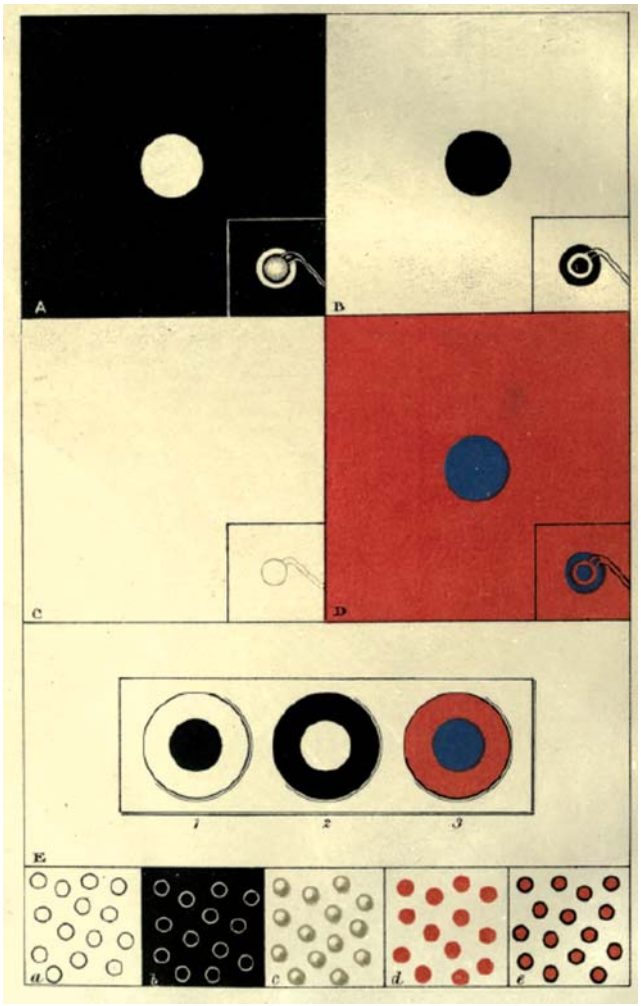

Figure 22. Frontispiece Plate I from Wright's 1906 book, *Principles of Microscopy*. Note the colored discs in D and E.

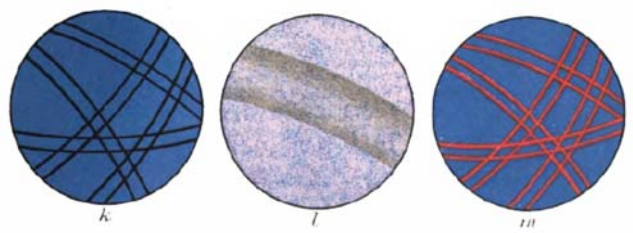

Figure 23. A portion of Plate II from Wright's *Principles of Microscopy*. Images of a glass wool fibers are: k) blue-colored stop with narrow aperture; l) Type 3 Rheinberg with both blue and red, using a stopped-down aperture; m) same view as k with a blue central and red annular stop with opened aperture. Overlapping adjacent original images have been digitally removed for clarity.

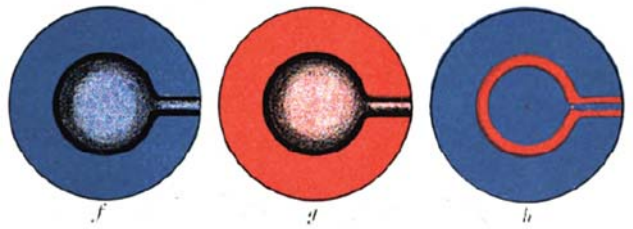

Figure 24. A portion of Plate II from Wright's *Principles of Microscopy*. Glass beads were viewed with a blue stop, red stop, then blue central and red annular stop. Overlapping adjacent original images have been digitally removed for clarity.

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