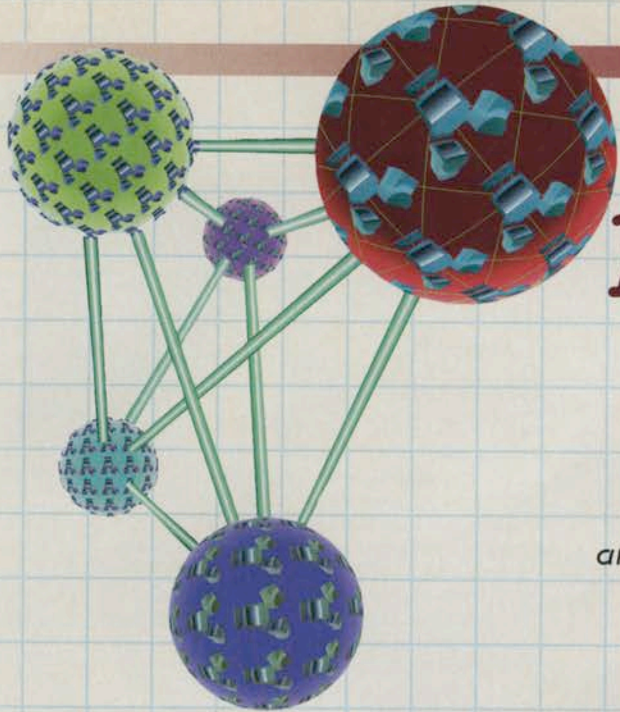


Bring Images to Life

Image-editing software, an ink-jet printer and transparency film can create microscope filters for a dark-field imaging technique.

by Michael R. Peres and Joseph Cowan

Making photographs at the microscope can be difficult. For example, magnification produces problems associated with the depth of field, and diffraction creates problems with image resolution.

One of the most difficult aspects of photographing through a microscope is creating image contrast, especially with thin, nearly transparent subjects. An early solution was the implementation of dark-field illumination, which involves using oblique-angle lighting from outside the lens's field of view. In this method, a white subject appears in front of a black background because light refracts to the lens from the subject.

In 1896, Julius Rheinberg published a paper in the *Journal of the Royal Microscopical Society* that described a method for optically coloring a subject's features using a microscope. The technique, now called Rheinberg differential color illumination, used concepts inherent to darkfield illumination methods and some of Ernst Abbe's optical theories.

The premise of the technique was simple (Figure 2): The disc stop that typically is used in dark-field techniques to subtract the background illumination would be replaced by a dense, colored disc. The usually clear region that surrounds the illumination system (the annulus) would be replaced with a lightly colored annulus. The effects that Rheinberg illumination can produce are very attractive and create interest in subjects that are otherwise monochromatic and bland (Figure 3).

Early methods of producing the required filters were slow and labor-intensive. As technology has improved, new ways to create the Rheinberg filters have evolved. The intent of our project was to explore more efficient and accurate methods of producing these filters using digital imaging technology.

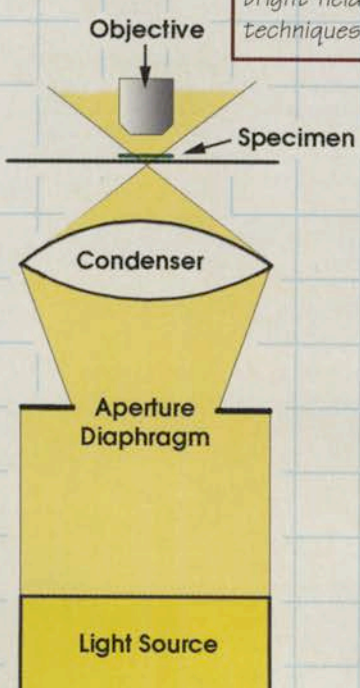
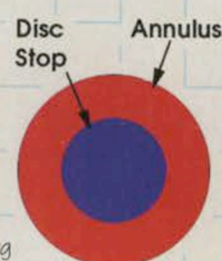


***Figure 1.** Microscopists have long struggled with producing high-contrast images, especially of thin, nearly transparent subjects, using traditional bright-field illumination techniques.*



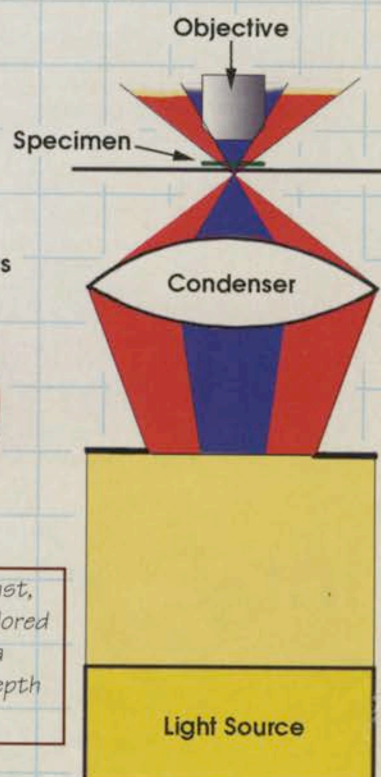


***Figure 2.** Dark-field imaging improves contrast, but replacing the disc stop with a dense, colored filter and the annulus (normally clear) with a lightly colored filter can create additional depth and color contrasts.*

We have found that using a standard microscope, a 10X objective and an Abbe or swing-out condenser, a person with access to image-editing software and a desktop printer can easily create many variations of Rheinberg filters and illumination.

Determining their size

Before creating the filters, the user must precisely measure various aspects of the microscope. The numerical aperture (NA) of the substage condenser must be determined for each objective that Rheinberg illumination will be used with. The higher the NA of the objective, the greater the disc stop that will be required. Abbe condensers provide the best access to the aperture diaphragm, which is where the filters must be placed. The following steps worked well:

1. Establish Köhler illumination with a slide (the sample is insignificant) at the subject stage.
2. With the objective in place, close the aperture diaphragm.
3. Remove one eyepiece.
4. Looking down the body tube of the microscope, open the aperture diaphragm until it leaves just the field of view.
5. Without disturbing the aperture diaphragm, remove the substage condenser.
6. Turn the condenser over and use a vernier caliper to measure the diameter of the aperture diaphragm's opening blades. Record this number, which is the diameter of the disc stop for that objective.
7. Replace the condenser and repeat the entire process for any objectives where Rheinberg illumination would be desirable.
8. To find the full diameter of a filter, open the blades of the aperture diaphragm all the way and measure the total diameter of the opening.

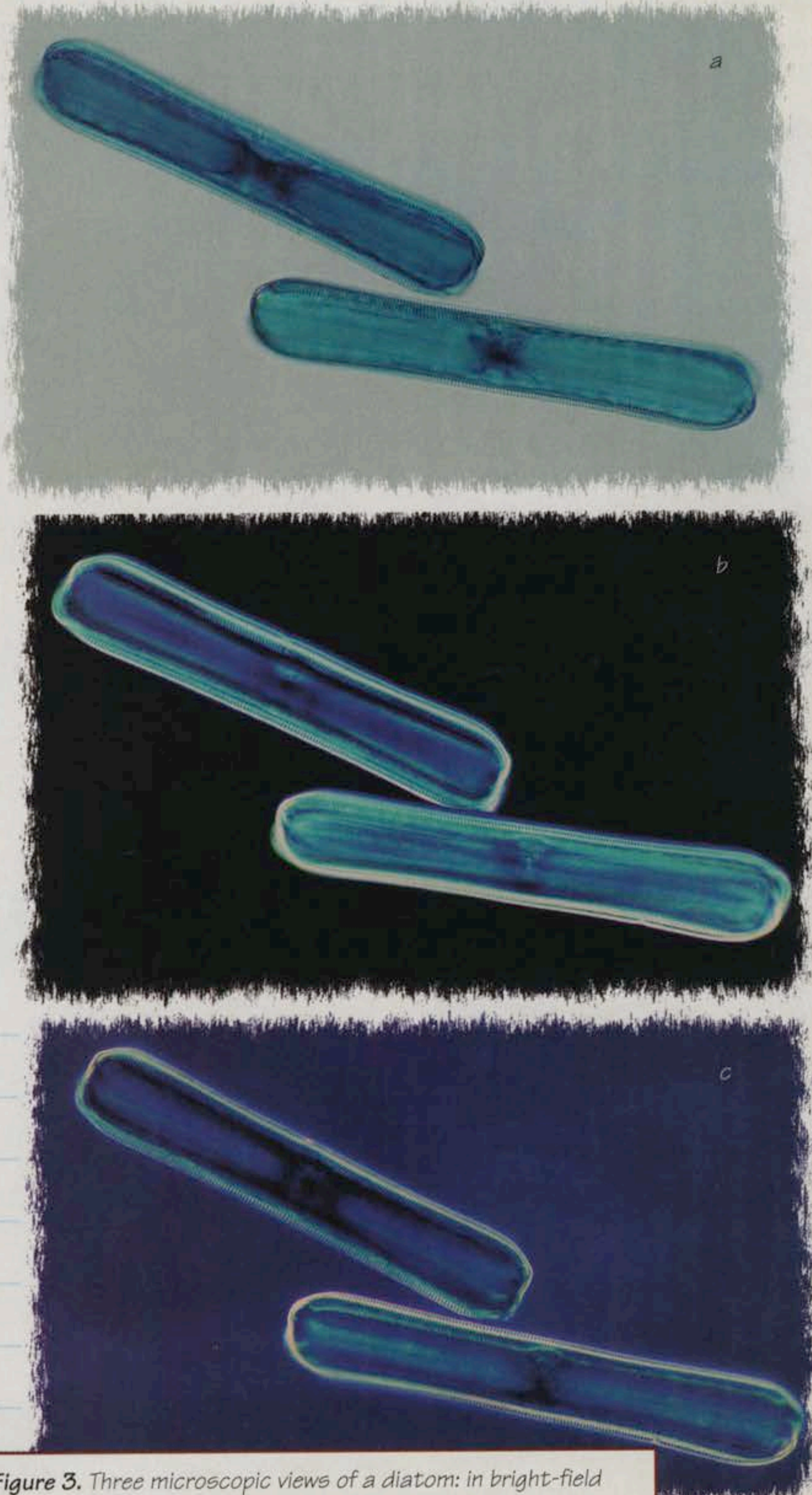


Figure 3. *Three microscopic views of a diatom: in bright-field illumination (a), in dark-field illumination (b) and using Rheinberg filters, a dark blue disc stop and a light red annulus (c).*

Making the files

To create your filters, open your favorite photo editing or illustration software; e.g., Adobe Photoshop or Illustrator, or Macromedia Freehand. Create a square image file with dimensions equal to the total diameter of the filter from Step 8 above. Using a black 1-pixel line, draw a circle that exactly fits, centered, inside that square. Still using a black 1-pixel line, draw another circle whose diameter equals the diameter of the disc stop from Step 6 above. Save this image as

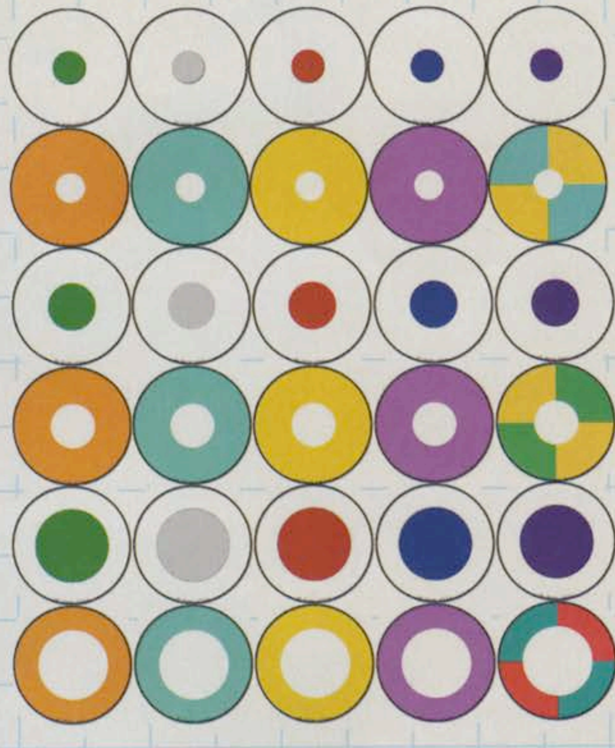


Figure 4. *After making one template, you can produce a sheet of Rheinberg filters that match your objective lenses. If you produce individual filters for the disc stop and annulus (as shown here), you decrease illumination efficiency slightly because you must double up filters; however, it increases flexibility. Quartering the annulus increases the contrast for linear samples such as crystals, more clearly revealing their internal structure.*

a template that you can use whenever you want a new filter for the measured objective.

The template will help you make a sheet of several filters. There are two basic ways to design the filters. The first is to create one filter with different colors on the disc stop and the annulus. The advantages to this are ease of use at the microscope and improved optical quality because the light passes through only one filter. The other way is to produce one filter for the disc stop and one for the annulus. The advantage to this approach is a higher degree of variability because they can be mixed and matched for different experiments.

Create a blank image that matches the size of the transparency film sheet that you will print on and the output resolution that matches your printer. Copy your uncolored template onto the blank image and make as many copies as you wish to make filters. Choose your colors and fill the center circle (disc stop) with one color and/or the surrounding ring (the annulus) with another.

In determining the colors for your filters, the only requirement is that the disc stop be denser than the annulus. When choosing colors, we seek the maximum contrast; e.g., red disc stop and yellow annulus or blue disc stop and yellow annulus. Also, if samples have inherent color, then filter colors should be based on their influence. This technique creates color contrast, so color selection is subjective and personal.

The most economical method to produce the filters is to use a color ink-jet printer or a color laser printer that can print onto overhead transparency film. Depending on the printer's characteristics, you may have to print the filters twice or use doubled filter sets to achieve enough color density.

We use an Epson Stylus Photo 1200 and have found that printing twice helps immensely because a single pass of ink is too transparent. We have not had problems with this overprinting; exactly aligning the page for the second pass is not that critical in this application because the goal is to produce large areas of color

A better way to have these filters printed is to have a local service bureau print them onto photographic transparency film. This process is considerably more expensive, but it provides the best color density.

To place the filters in the correct spot in the substage condenser, the ideal tool is a custom-made metal jig designed for the particular condenser. These jigs are difficult to make and may be expensive. As an alternative, we made small cylinders by rolling a piece of plastic cut from a milk carton. We then taped our filters to the end of the cylinder and inserted them into the condenser. We also found that plastic photographic film canisters will work just as well if their diameter size is appropriate for the substage condenser. □

Figure 5. *We use a custom-machined metal jig to place our filters in the substage condenser, but homemade jigs from plastic will work as well.*

Meet the authors

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