MODERN MICROSCOPY JOURNAL
www.modernmicroscopy.com, article 106

"How To" Tutorial Series : Enhancing Detail and Contrast in Light Microscopy Using a Combination of Circular Oblique (COL) and Oblique Illumination, Part II

by William A. Mark, Ph.D.

Published Friday, March 15, 2013

ABSTRACT

Work presented in this article is a continuation of a previously published document (Mark 2012). In the present article, enhancing detail and contrast in transparent specimens is accomplished using a deviation of the previously developed circular oblique lighting (COL) and oblique masks. The new filter arrangements incorporate a quarter-wave plate step-wedge, and an auxiliary field lens; in addition to a COL central stop, these modifications allow for the use of objectives ranging from 16X to 100X and provide an incremental enhancement to image contrast over the previously discussed designs. While the concept is still the same (combining COL and oblique illumination) the execution is slightly different, with enhanced flexibility.

What is presented in this article is a unique combination and application of COL, oblique illumination, quarter-wave step-wedge, and polarized light for the enhancement of detail in transparent specimens such as diatoms.

INTRODUCTION

This article is a continuation of previously published work dealing with simple combinations of COL, oblique illumination, and polarized light to improve resolution in transparent specimens such as diatoms. While the previous work focused on traditional oblique masks, elliptical and round COL stops, and polarized light with simple wave plates, the goal of this work is threefold:

1. To simplify some aspects of the previously established technique (such as the oblique mask).
2. Develop a system that is amenable to a wide range of magnifications.
3. Improve flexibility and resolving power over the previous setup.

While the concept is similar to the previously published work (combining COL and oblique illumination) the execution is different in three key ways:

1. The inclusion of a step-wedge increases the diversity of retarded light striking the specimen; this has been found to enhance resolution and improve contrast.
2. The oblique illumination can be varied and is provided by crossed Nichols rather than a static mask.
3. The central COL stop is smaller in size (2.7 mm vs. 7 mm) allowing for greater light throughput.
4. Inclusion of an additional field lens makes it possible to use the setup with lower power objectives
   such as 16X.

This study was undertaken to further develop an optical system that would allow for maximal resolving power using a standard halogen light source based on the previously discussed COL and oblique combinations.

MATERIALS

Experiments were conducted using a Zeiss Universal Photomicroscope II equipped with a Zeiss 1.4 NA Achr/Apl condenser. Two standard Zeiss biconvex field lenses having a focal distance of approximately 100 mm (as determined experimentally). Light was supplied by a 12V 100W halogen bulb and Zeiss illuminator. Objectives were a Zeiss 16X 0.35 NA Plan Achromat, Zeiss 40X 0.65 NA Plan Achromat, Zeiss Neofuar Pol 63X 0.90 NA dry, and Zeiss 100X 1.25 NA oil Plan Achromat.

Cameras used were one of the following: a Canon EOS 60D mated to a Zeiss intermediate basic unit 47 60 10 and Zeiss 4X projection eyepiece; Panasonic DMC GF1 mirrorless camera with Zeiss intermediate basic unit 47 60 10 and Leitz 4X projection lens; Moticam 2300 equipped with a Zeiss KPL 10X eyepiece and a Moticam 16 mm intermediate lens; or a Nikon Coolpix 4500 attached to a Leitz Periplan eyepiece. The specimens are from an eight- or ten-form diatom test slide.

METHOD

The circular polarizing films were all cut from commercially available film into 32 mm diameter filters using a commercial gasket cutter, and arranged so that they produce a black background when crossed (crossed Nicols). The COL stop was made from self-adhesive foil tape cut to a diameter of 7/64 inches (or 2.7mm). The additional lens is a standard Zeiss auxiliary biconvex lens, focal length approximately 100 mm, and was placed just above the step-wedge. The step-wedge was constructed using a 1” x 3” glass microscope slide, on which 7/8” x 1.25” sheets of polymer ¼ wave retardation film (WF-OG4-WE, from Aflash Photonics OPD 135 nm +/-10 nm Center wavelength 560 nm) was applied and held in place. The leading edge of the first sheet was placed in the center of the glass slide and each subsequent ¼ wave sheet was placed 1 mm from the previous leading edge. All sheets were arranged with the fast axis parallel with the length of the slide. Six sheets were stacked to form the step-wedge (Figure 1).

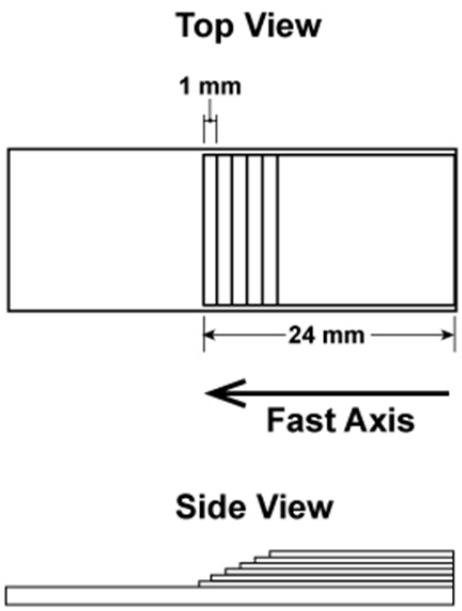


Figure 1: Schematic representation of step-wedge.

This type of wedge is known as a Fox Wedge (Clay 1914) and is usually constructed of mica sheets of a given retardation. Since difficulties were encountered when trying to source a mica step-wedge, a wedge was constructed using ¼ wave film instead. The ¼ wave film wedge gives ¼ wave, ½ wave, ¾ wave, 1 wave, 1-¼ wave, and 1-1/2 wave distinct bands of retardation in crossed polarized light.

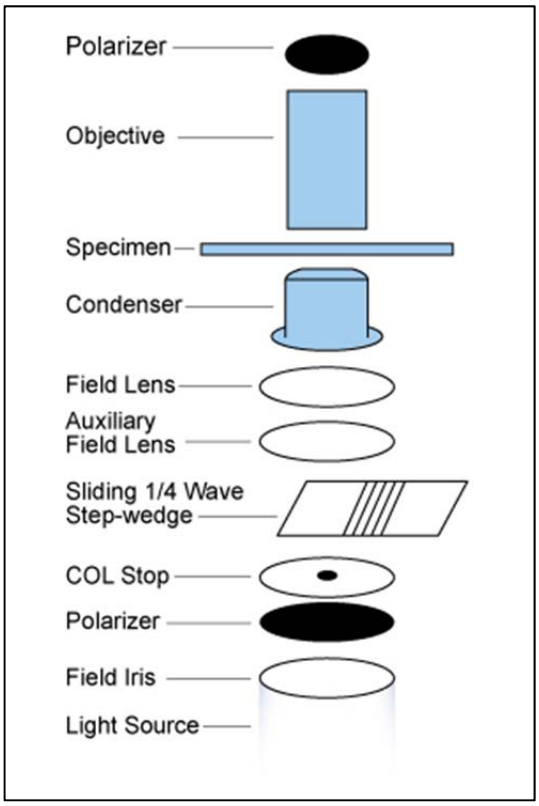
Images converted to black-and-white were done via desaturation based on luminescence using the shareware program GIMPshop.

Optical setup sequence

1. Focus on specimen.
2. Field and condenser iris fully opened.
3. Cross polarizers so that the background is black. Remove the analyzer from the light path.
4. Insert COL stop filter and center it in the objective back focal plane using a phase telescope or equivalent. Insert the analyzer into the light path. The background should be black.
5. Slide in the step-wedge until the desired objective back focal plane image is achieved. It has been my observation that the best image resolution is seen when the step wedge leading edge is near the outermost edge of the COL field stop as projected onto the objective back focal plane.
6. Focus substage condenser up or down until the observed image of the specimen is just free from the appearance of a central “dark” spot. The condenser can be kept at a lower position (slightly lower than the position where the dark spot is observed), or at a higher position (just above the position where the dark spot is observed). There may be some slight darkness at the edge of the field of view.
7. The step-wedge may be rotated to control light throughput, and slid in or out to enhance or reduce the oblique effect.
8. For a 16X objective, the lower auxiliary field lens is left in place, while the standard field lens is removed from the light path (Figure 2). For 40X the auxiliary field lens should be removed. For greater than 40X both the auxiliary and standard field lenses should be in place.
9. For images utilizing the 100X objective, both the objective and condenser should be oiled using a good quality immersion oil.

DEVELOPMENT AND RESULTS

A photo of the actual setup is shown in Figure 2, and a simplified schematic of the general setup and optical elements utilized is illustrated in Figure 3.



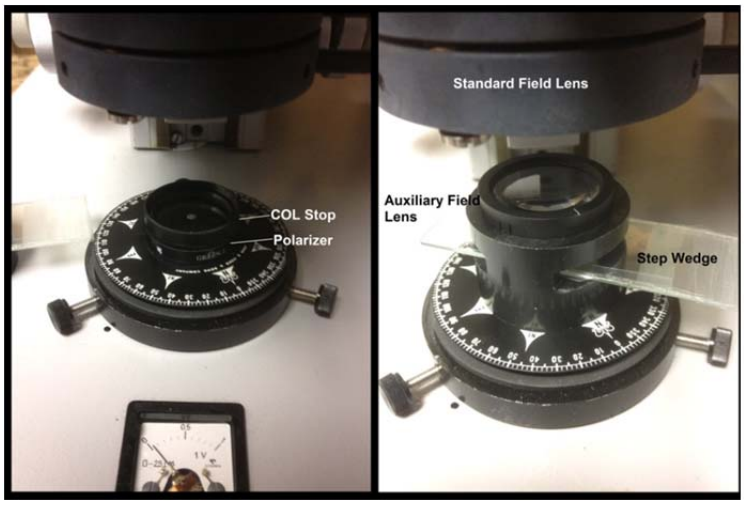


Figure 2, left. The general optical setup for experiments.

Figure 3, above. Actual setup of filters on a Zeiss Photomicroscope II. The field iris and light opening of the scope is just under the centerable unit.

As mentioned earlier, the stop and wedge are adjusted by viewing the objective back focal plane, as shown in Figures 4 and 5. It is important to center the COL stop, but likewise important to center the condenser and light-source filament. A phase telescope or equivalent device can be used to facilitate.

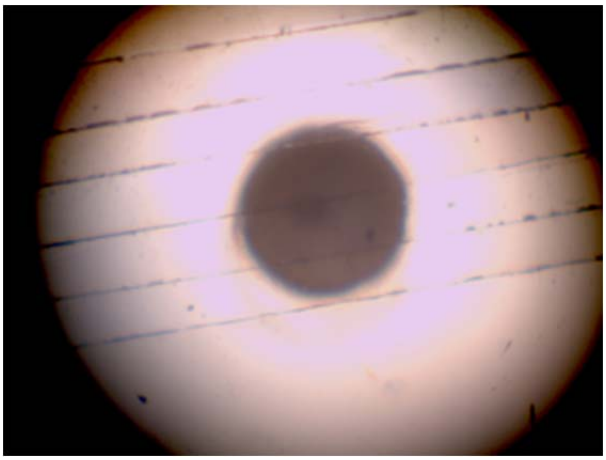


Figure 4. Objective back focal plane image of COL stop and step wedge without analyzer in place.

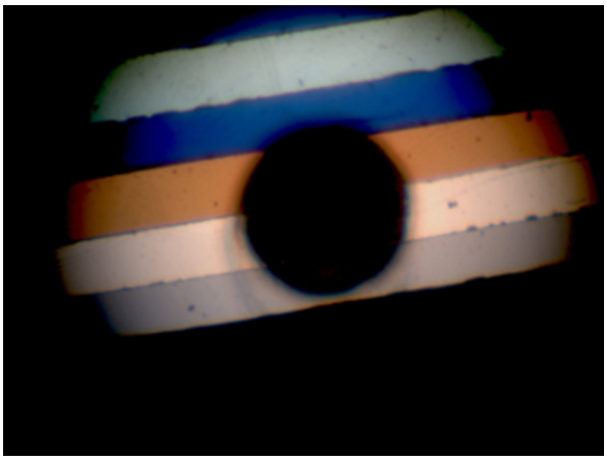


Figure 5. Objective back focal plane image of COL stop and step-wedge with analyzer in place.

Eight-form and ten-form diatom test slides were selected as the test subject. Striae spacing for test diatoms are given in the table below:

Table 1. Diatoms imaged with critical parameters.

Diatom	Length (mm)	Striae Spacing
Amphiplura pellucida	80	0.27[3] , 0.21 (0.22-0.25) [4]
Amphipleura lindheimeri	100	0.36[5]
Surirella gemma	60-100	0.33 (0.40-0.50) [4]
Nitzschia sigma	150	0.435[3]
Stauroneis phoenocenteron	170	0.714[3]

RESULTS AND DISCUSSION

Photographic results of the setup are shown in following figures. Figures 6 through 12 were taken of a professionally mounted diatom test slide. This represents a best case scenario. Figures 13 through 15 were images taken from fresh mounted samples. Figures 13 through 15 represent samples in less than ideal mounting. As can be seen in the photographs, good resolution is obtained with all the diatoms examined. In fact, even the most challenging striae, spacing is resolved (*Amphiplura pellucida* at ~210 nm), with moderate optics (Plan Achromat). In addition, overall color is kept natural. Good detail and image quality is also maintained in specimens that have not been prepared in an “ideal” mount, regardless of camera, as can be seen by an examination of Figures 13 through 17. Application of this technique with a professionally mounted stained sample is shown in Figure 18.

It is believed that the enhanced contrast seen in these samples is a result of the combined effect of annular lighting (provide by the COL stop), the polarization state of the light, and the interference colors; the latter effects being supplied by the crossed Nicols and Fox wedge. Each wave plate in the wedge not only changes the polarization state of the transmitted light, but also contributes an interference color. All of these combined factors result in the images as shown. Removal of any one of the three components results in an inferior image.

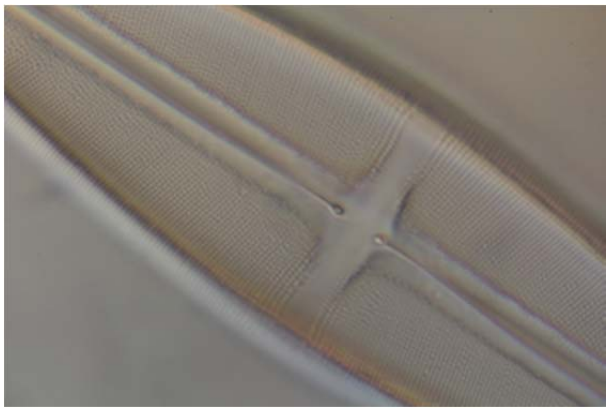


Figure 6.

Image of diatom *Stauroneis phoenocenteron* (striae spacing ~714 nm) taken with Zeiss 100X Plan Achromat Oil objective and Canon EOS 60D camera.

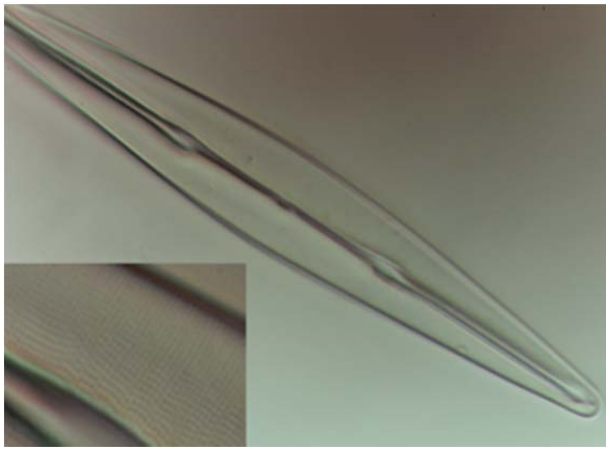


Figure 7.

Image of diatom *Amphipleura lindheimeri* (striae spacing ~360 nm) taken with Zeiss 100X Plan Achromat Oil objective and Canon EOS 60D camera.

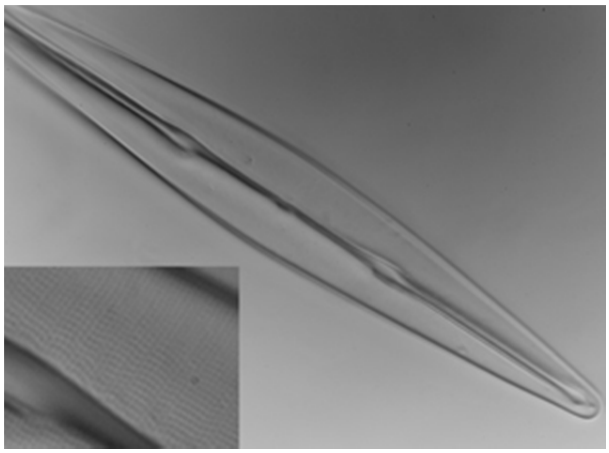


Figure 8.

Image of diatom *Amphipleura lindheimeri* (striae spacing ~360 nm) taken with Zeiss 100X Plan Achromat Oil objective and Canon EOS 60D (black and white conversion via Gimpshop).

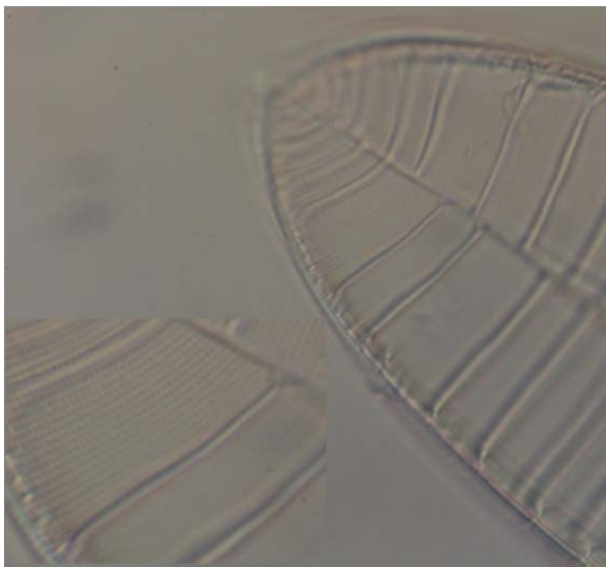


Figure 9.

Image of diatom *Surirella gemma* (striae spacing ~330 nm) taken with Zeiss 100X Plan Achromat Oil objective and Canon EOS 60D camera.

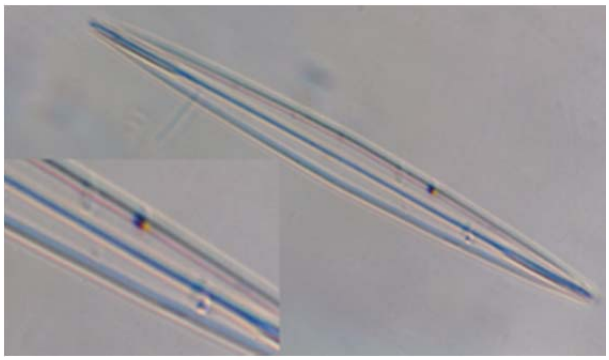


Figure 10.

Image of diatom *Amphiplura pellucida* (striae spacing ~210 nm) taken with Zeiss 100X Plan Achromat Oil objective and Canon EOS 60D camera.

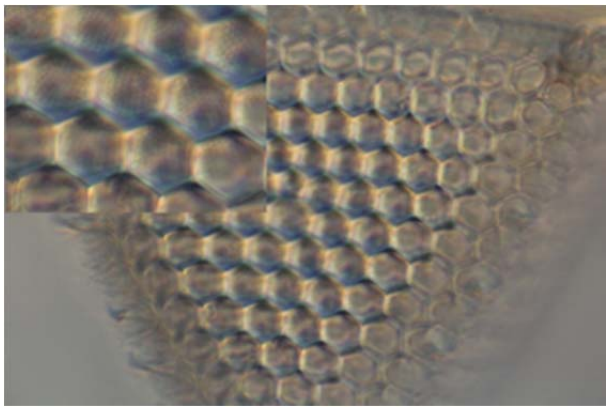


Figure 11.

Image of diatom *Triceratium favus* taken with Zeiss 100X Plan Achromat Oil objective and Canon EOS 60D camera.

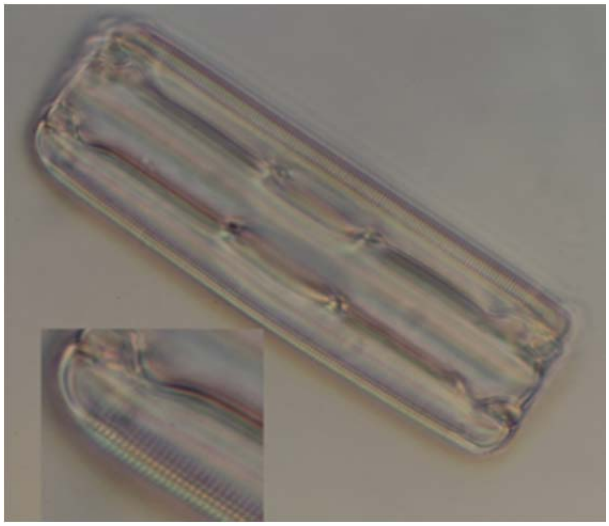


Figure 12.

Image of diatom taken with Zeiss 100X Plan Achromat Oil objective and Canon EOS 60D camera.

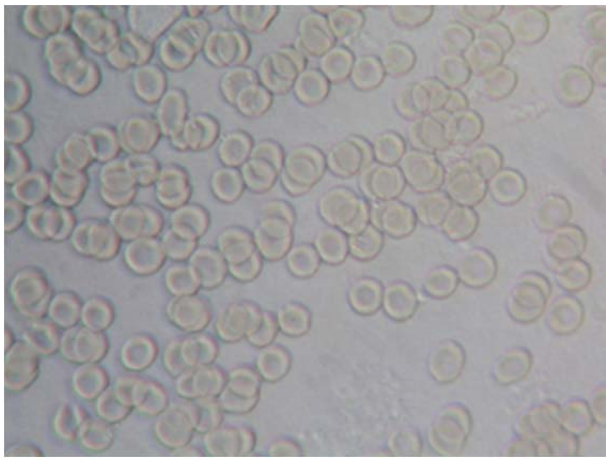


Figure 13.

Unfixed fresh blood smear unstained (in serum) with Zeiss Neofluar Pol 63X objective and Nikon Coolpix 4500 camera.

Figure 14.

Desmid water mounted taken with Zeiss Plan Acromat 40X objective and Canon EOS 60D camera.

Figure 15.

Desmid water mounted taken with Zeiss Plan Achromat 40X objective and Panasonic DMC-GF1 camera.

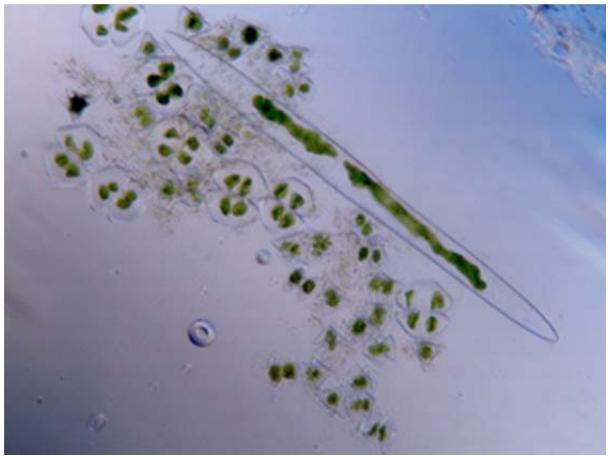


Figure 16.

Desmids water mounted taken with Zeiss Plan Achromat 16X objective and Moticam 2300 camera.

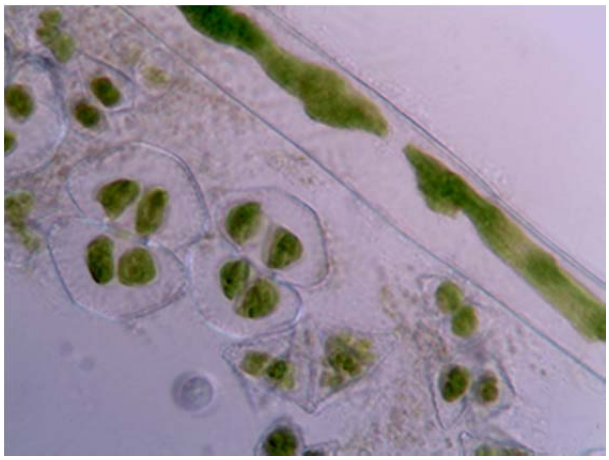


Figure 17.

Desmids water mounted taken with Zeiss Plan Achromat 40X objective and Moticam 2300 camera (same specimen as in Figure 16).

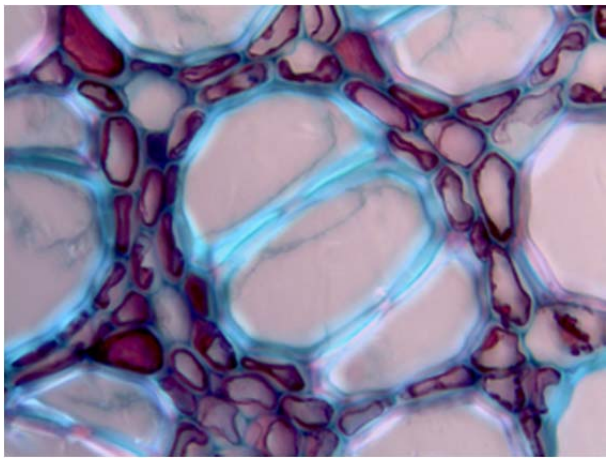


Figure 18.

Commercially prepared slide of fern root cross section taken with Zeiss Plan Achromat 40X objective and Moticam 2300 camera.

CONCLUSIONS

The system discussed in this article has shown the potential for very good resolution enhancement as well as contrast adjustment. These image enhancements can be achieved from a standard halogen light source and Plan Achromat objectives, and can be applied to a wide range of samples and sample preparations. Because a standard light source is used, the samples retain their natural color, which is of great advantage when examining live wet mounts.

Cited References:

Mark, William, Ph.D. (2012) *Combining Circular Oblique and Oblique Illumination to Enhance Detail and Contrast in Light Microscopy, Part I.* ModernMicroscopy.com http://www.modernmicroscopy.com/main.asp?article=100

Clay, Reginald S. (1914) *Practical Exercises in Light, Being a Laboratory Course for Schools of Science and Colleges*. Page 171, Macmillan and Co., Limited, London, 1914

University of Texas (2004) *Bio 205 lab PDFs Diatoms.* Retrieved September 28, 2011, from University of Texas EDU: http://www.zo.utexas.edu/courses/bio206/diatoms.htm

Piper, Jörg and Chmela, Gunther (2011) Advanced Techniques for Observation and Photomicrography of Subcellular Structures in Diatom Shells. *Microscopy Today* 19 (1) pg. 10

Journal of the Quekett Microscopical Club, Ser. 2, Vol. XII., No. 77, November 1915 pg 575http://www.archive.org/stream/journalofquekett0212quek/journalofquekett0212quek_djvu.txt