Articles

Test Slides: Diatoms to Divisions - What Are You Looking At? Part 1

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This article examines the history, types and uses of test slides, the problems that can be encountered, and a novel new test slide solution.

Are you confident that the image you see with the help of a microscope is a true representation of the object being viewed?

How do we know that the image we see is an accurate reproduction of the original object being viewed? A simple question, perhaps. The answer would seem to be to look at something well known and well understood, then judge the image against the 'reality' of past experience. This approach works well with cameras, binoculars, magnifying glasses or video systems, but what happens when one uses an optical instrument, or any imaging system, that brings images from the realm of the unknown? Telescopes and microscopes can both supply images of the unknown. Telescopes can be tested by looking at real things at a great distance: the dartboard in the common room of the next-door building, or the bolts in the television tower on a nearby hill. Testing microscopes presents a much greater challenge since there are few objects with which one has primary experience at microscopic scales. The creation of a set of known test objects which could be used in various microscope imaging systems seemed to be the logical solution to the discussion.

For as many years as there have been microscopes and microscope designers there have been debates over how well each new microscope, objective, eyepiece or illumination system performed. These debates were not trivial or pedantic matters driven by ego or scientific intellectualism, nor were they driven solely by commercial interests. The debates revolved around the questions of: How small an object could a given system resolve? How good was the contrast? How flat was the field of view? Were there distortions or aberrations in the final image? The central and perhaps most controversial question was, Just how small an object could a light microscope hope to resolve?

Delving into the journals of the late 1800s and early 1900s provides a wonderful insight into the discussions and arguments between theoreticians and modellers on one hand, and practical scientists and engineers on the other. The former group argued from the logic of theory and mathematics, while the latter group demonstrated their latest instrument successes as 'proof' of the achievement of their most recent advance. The real drive behind the debate came from the need to understand whether the state of the art was advanced by the latest effort, and if further advancement seemed possible. The design and production of successful instruments required an integration of theoretical and practical skills. One might argue that the giants of past instrument development were those who could successfully bridge the usually isolated realms of theory and practice.

Discussions concerning the theoretical limits of light microscopy have disappeared in the past several decades. There was general agreement that the limits of light microscopy had been reached. Most manufacturers offered optics with performance at or near the theoretical limits. Has microscopy really reached the limits of resolution, contrast and flatness of field, and minimized aberrations? Do all manufacturers' products deliver the same or substantially equal performance based on mature designs made possible by today's understanding of microscope theory? Is one manufacturer's system better than another's? If so, then in what way? Is one plan apochromat objective equal in performance to another manufacturer's plan apochromat? Is each objective of a given type equal in performance to every other objective of that type made by the same manufacturer? In many other technical fields there are clearly defined benchmark standards against which any product can be compared, but microscopy has not yet established such benchmarks.

Lately, the fundamental questions of imaging resolution have surfaced again with the technical advances in confocal and near-field scanning optical microscopy, and other new techniques. These techniques raise questions about what information an imaging system provides about an object. Is the information accurate and significant?

The best way to develop an understanding for the true performance of a microscope or imaging system would appear to be to use it to look at known, well-characterized, objects. So far so good; well, where does one go to get a library of (or even one) suitably characterized objects? First a definition of a 'well-characterized object' is needed. Secondly, it must be of a suitable size to substantially fill the field of view at the magnification or magnifications that are commonly used by the microscope being tested.

darkfield, phase contrast, DIC, confocal and near-field scanning optical microscopy. It should also be suitable for coated and uncoated use in scanning electron microscopes with standard or backscatter detectors.

In an attempt to satisfy the need for a reliable microscope test slide, work began in 1997 to define a suitable pattern of objects for a universally applicable standard test slide. The result of the research and design effort was the Richardson Test Slide pattern one. Subsequently Bio-Microtech Inc. produced a series of reproducible standard test slides, based on the Richardson pattern. These test slides are suitable for use in light microscopy and in scanning electron microscopy.

The overall pattern of the Richardson Test Slide is shown in Figure 1. The outer ring of arrows, which has an outer diameter of only 270μm, is provided as an aid to locate the inner pattern while searching at low powers. It was found that without the outer ring of arrows it was very difficult to find the test pattern. The outer ring also allows the pattern to be just visible to the naked eye so that one can maintain a sense of continuity between the scales of real world objects and the scale of objects on the test slide.

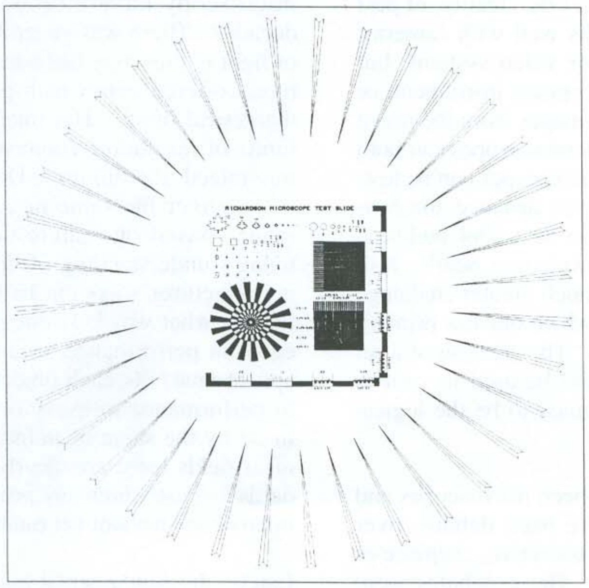

Fig. 1. Overall view of the Richardson Test Slide pattern one. The outside diameter of the ring of arrows is 270μm.

The geometrical features that were chosen for the test slide are arranged within the boundaries of a square inner test area shown in Figure 2. The most obvious choice of shape for a test slide is the circle, so often called upon in the past in discussions of resolution. The circles, noted as B in Figure 2, range from 4μm to 100nm in diameter. Since the true diameters of the circles on this test slide have been established, these circles can be used to study the formation of diffraction patterns such as Airy discs and rings. Squares offered an interesting alternative to circles as a resolution test since it would seem to be a harder test to resolve a sharp-edged square than a smooth-edged circle. The squares, C in Figure 2, range from 4.0μm to 100nm per side. While considering the impact of unusual shapes on resolution it seemed to be advantageous to include a range of random shaped 'amorphous' objects more typical of biological structures. Two ranges of such shapes were included on the test slide. A set of pointed objects shown as D and a set of rounded objects shown as E range from 2.0μm to 100nm. Maple leaves seem to be endemic to Canada, and they managed to find their way on to the test slide with the argument that they provide a well-known and demanding geometry whose abundant angles and sharp points provide a severe test of resolving power. The maple leaves, shown as A in Figure 2, range from 10μm to 100nm.

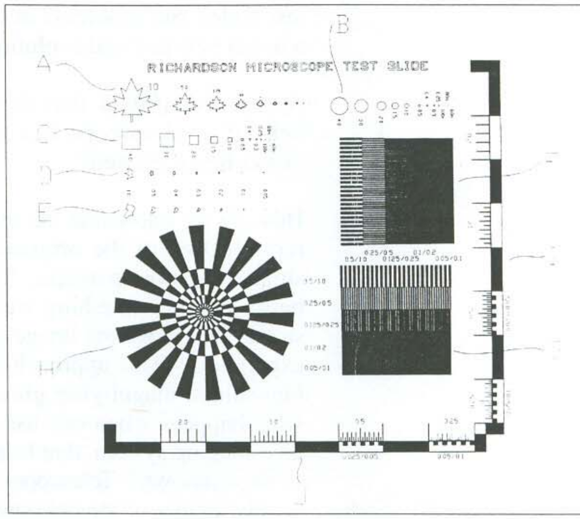

Fig. 2. Detailed view of the inner pattern area of the Richardson Test Slide pattern one. The black and white alternating bars in the horizontal and vertical scales are all 10μm in length. The overall dimension of each of the *X* and *Y* outer scale bars is 80μm (not including the centre and end features).

Grating patterns are very useful on a test slide since they provide a series of lines of known width at a known centre spacing. The gratings on the Richardson Test Slide range from 500nm lines on 1.0μm centre spacings to 100nm lines on 200nm spacings. There are two sets of gratings arranged at right angles to each other. The grating shown as F on Figure 2 has lines running horizontally, while the grating shown as G has lines running vertically.

The Siemens star pattern has been used in the past as a resolution test target. One of its disadvantages was its lack of scale detail. If the star fills the field of view completely so that no edge is visible, the ability to judge scale is lost completely. The Richardson Test Slide uses a new type of star similar to a dart-board design where alternating segments interrupt the 18 pie-shaped star segments. This alternating star is shown as H in Figure 2. The alternating segments are coded with triple segments of one colour which serve to demonstrate graphically which rings are being viewed in the image.

The question of scale needed to be addressed carefully. A set of scale bars was created for use with fields of view from hundreds of micrometres to fractions of a micrometre. The scale bars consist of eight alternating black-and-white segments where each segment is 10μm long. Each white segment has a set of scale markings adjacent to its inner edge. The scale markings range from 2.0μm centre spacings to 250nm centre spacings. The 250nm interval markings use 100nm lines on 250nm centre spacings. There are two such scale bars arranged at 90° to each other. The scale bars are shown in Figure 2 as I and J. The scale bars and the header information serve to contain the rest of the features in the inner part of the test slide. The 90° angle and the length of the scale bars form a powerful tool for studying image distortions, aberrations and flatness of field.

In all cases the individual patterns are identified with a scale marking directly adjacent to them so that one can be sure which size pattern one is looking at or imaging. The Richardson Test Slide is referenced to a set of scaled master pattern reference drawings which provide dimensions for all the features. Images of the test slide can then be tied back to the master pattern identification drawings so that the microscopist can be sure of the features that were imaged. Over a short period of use the microscopist can rapidly develop a sense of scale in images produced by each objective employed with the test slide. In the process a lot can be learned about resolving power, the effect of objective NA, the means of adjusting the illumination for optimum resolution and the imaging capabilities of the particular system in use.

Once the pattern described above was finalized, attention turned to the means of production. In producing the test slide the choice of substrate was the first consideration. It needed to have good optical qualities, long-term environmental stability and broad wavelength transmission capability. High grade, highly polished fused silica was chosen as the initial substrate for production. The pattern was then produced by etching into the surface of the fused silica. Using this method the pattern is formed by removing portions of the surface of the substrate material. The portions removed are of the order of 180nm deep. This method of pattern production is most suitable for darkfield, phase contrast and scanning electron microscopy with standard electron detectors.

A second set of patterns was produced by depositing a multilayer metallic pattern on a fused silica substrate. This metallic layer is designed to be opaque to light. Such a method of pattern production lends itself to brightfield and darkfield imaging for reflected and transmitted light applications, and to scanning electron microscopy with backscatter detectors.

The test slide substrates were mounted in suitable plates for use in light microscopes and in SEMs. It was now possible to examine the degree to which the physical test slides captured the design features.

It was important to learn whether the production process had accurately reproduced the features in the design drawings. What was the ultimate resolution achieved in the physical test slide objects? In order to determine the degree of success a number of scanning electron micrographs of the initial test slides were produced. The test slides used for these micrographs were metallic patterns on fused silica substrates. No conductive coating was used so charging of the sample was a consideration. Low accelerating voltages were used to produce the initial micrographs to minimize sample charging. Figure 4 shows results of using the standard detector at low voltage. To produce Figures 6 and 8 the voltage was increased to provide better resolution. Figures 3, 5, 7 and 9 show the results of using of a backscatter detector to allow the use of higher voltages in order to obtain higher resolutions.

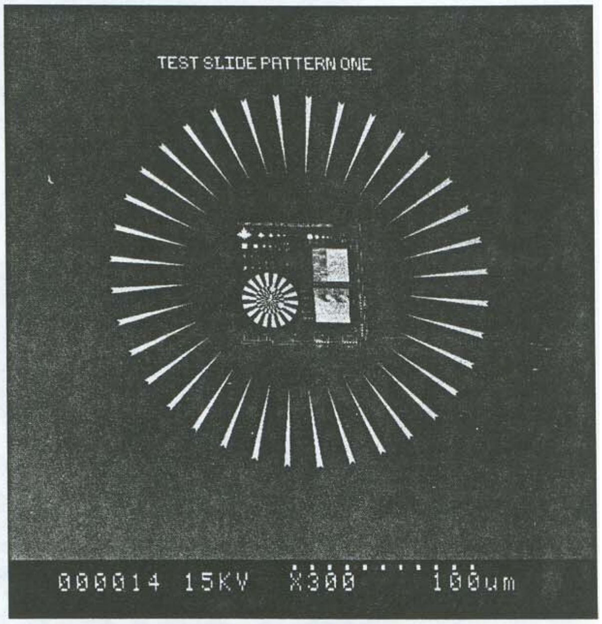

Fig. 3. Scanning electron micrograph of the entire Richardson Test Slide pattern one. The outside diameter of the ring of arrows is 270μm.

Figure 3 shows an overall image of the whole pattern on the test slide. The inner square area with the detailed shapes and scales is shown in Figure 4.

Figure 5 shows a view of the alternating star pattern. The locating marks formed by three adjacent sets of white segments can be seen in the second and fourth rings from the outside. The set of two locating marks in the second ring are separated from each other by seven intervening segments. The set of locating marks in the fourth ring is separated by five segments.

Even if the whole field of view is taken up with the star pattern one can still maintain an understanding of scale through the position of the locating marks. The egg-shaped distortion of the image from the expected circular image appears to be a function of the SEM employed for these micrographs, and is not present in the real object, as was demonstrated in later micrographs.

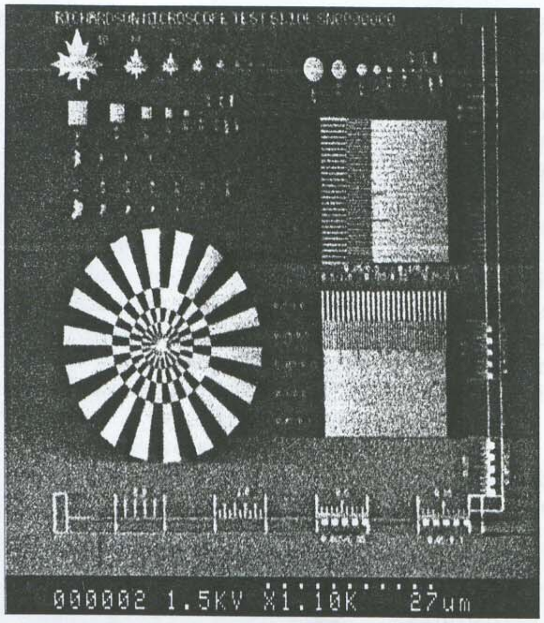

Fig. 4. Scanning electron micrograph of the inner pattern of the Richardson Test Slide pattern one. The horizontal and vertical scale bars on the bottom and right side of the pattern are 80µm long and the major scale divisions are 10µm.

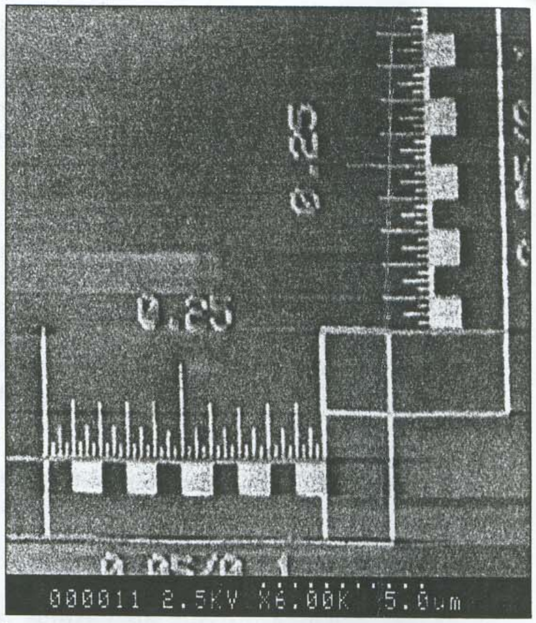

Fig. 6. Scanning electron micrograph of the finest scales of the horizontal scale bars at the point where they meet in the lower left corner of the inner pattern. The fine scale is 100nm lines on 250nm centre spacings. This image was made using the standard secondary electron detector.

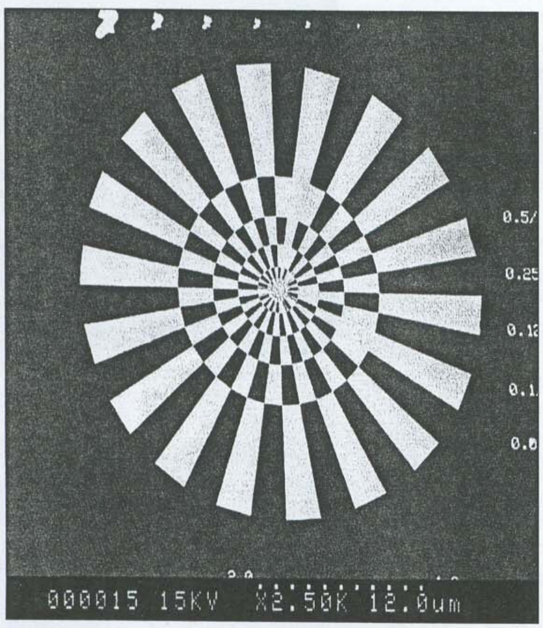

Fig. 5. Scanning electron micrograph of the alternating pie star. The location marking segments can be seen in the second and fourth rings from the outside edge. The overall diameter of the star pattern is 40µm. The internal rings are 20, 13, 8, 4 and 2µm in diameter.

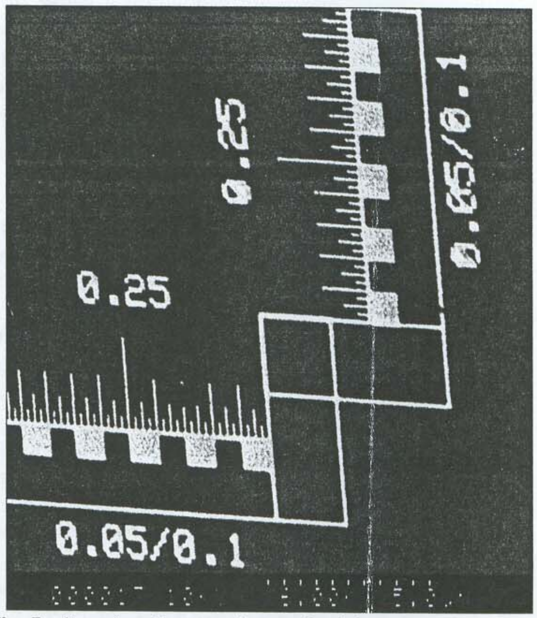

Fig. 7. Scanning electron micrograph of the same area as Fig. 6 except that it was made using a Robinson backscatter detector at a much higher voltage. The details of the fine scale divisions can be clearly seen in this micrograph; however, the use of this detector causes a distortion of the image which was not in the previous images.

Figure 6 shows a detail of the finest scale divisions at the intersection of the *X* direction and *Y* direction scale bars in the lower right-hand corner of the test pattern. Here the 100nm lines, on 250nm centre spacing, which form the finest reference markings, can be seen plainly. In order to obtain finer detail the same area was imaged at a higher voltage with the backscatter detector, shown in Figure 7. The image quality improves in terms of resolution but suffers in terms of angular distortion. Once again the image distortion appears to be due to the characteristics of the particular SEM used and is not present on the test slide. The scale bars seem to become slanted and the included angle appears to change from 90° to less than 90°. It is interesting to relate these scale markings to the wavelength of light. Two divisions of this scale are equal to the wavelength of aqua (green-blue, 500nm) monochromatic light.

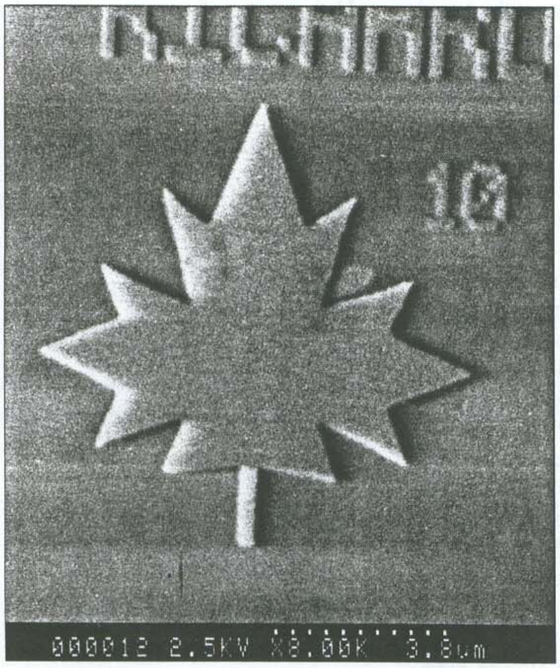

Fig. 8. Scanning electron micrograph of the maple leaf in the upper left corner of the inner pattern. The distance from the leftmost tip of the maple leaf to the rightmost tip of the maple leaf is 10μm.

Figure 8 shows a detailed view of the 10μm maple leaf located in the top left corner of the test pattern. The sharp edges and sharp points of the maple leaf provide a good test of the fabrication method and of the SEM imaging system. Figure 9 shows an image of the maple leaf using the backscatter detector. The resolution is better but the distortion of the image is considerably greater than in the previous figure.

Fig. 9. Scanning electron micrograph of the same area as Fig. 8 except that it was made using the Robinson backscatter detector at the higher voltage. The details of the points, outer edges and internal angles of the maple leaf are clearly shown although this mode of operation introduces some distortion to the image.

Electron microscopes usually provide scale bars in, or adjacent to, their images. All the SEM images included in this article contain the scale bars supplied by the manufacturer. These scale bars can conveniently be used to check the size of an image. How accurate are the manufacturers' scale bars? The test slide can be used to test the scale bars of the SEM and to check the focus, astigmatism, and many other parameters. In the process of producing these SEM images of the test slide the microscopist operating the SEM had a chance to study several factors affecting the image fidelity of the particular SEM being used. The operator discovered that the test slide provided a powerful way for him to adjust the operating parameters of the SEM to provide greatly improved imaging performance. The test slide also allowed the production of reference images, which could be used in later discussions with factory service personnel to illustrate required or desired adjustments or improvements to aspects of the SEM that were not available to the operator. The operator had many suggestions for the use of the test slide, both for routine calibration and characterization of instruments and for educating and testing students.

Light microscopes do not routinely provide scale bars in the field of view against which to judge the size of an image. One of the most important uses of the test slide is to provide accurate scale information for measuring objects with a light microscope.

The test slide was produced for many reasons. The first reason was simply to see how far the state of the art could be extended to produce an extremely high-resolution test slide at a reasonable cost. The more practical reason for producing the test slide was to provide an easily used and interpreted test target with a wide assortment of test objects for use in microscopy and imaging education, research, calibration and characterization of instruments. It was important to provide features which challenge the limits of current optical instrument design so that there is a readily available test object which today's best designers can use to test their ideas and instruments. It is hoped that designers and manufacturers will use the test slide to communicate the performance of their instruments to their clients and other researchers so that all can understand the exact capabilities and performance of any imaging system.

For the purchaser of microscopes the test slide provides an invaluable tool to assess the image quality of the vendor's offering. This can help to ensure that one is purchasing the system which provides the grade of images required for the task at hand. The test slide can also be used as a powerful tool in intercomparing one offering against another. For routine test of an instrument, system, or a microscopist, the test slide provides a standard and repeatable way to produce routine test images. These test images can be used to prove and record the performance of instrument, optics, hardware, software and operator skill.

In education the test slide provides an unmatched tool for rapidly conveying the basic concepts of microscopy to a student. At the same time the test slide provides a very well-understood object for testing the skills acquired by students.

These test slides provide a standard benchmark for intercomparisons of every optical and imaging component in a microscopy system, from the light source to the final monitor. The performance of objectives, CCD cameras, motion control systems and image processing algorithms can be fully understood and communicated to others at distant locations. The Richardson Test Slide can be used to address recent requirements for documentation, quality control, quality assurance and standardization such as the ISO standards for manufacturing. Richardson Test Slides provide the key to routine qualification of imaging systems, methods and personnel in medical research and diagnostic environments as well as industry.

The goal is to provide a tool to assist skilled microscopists so they can confidently judge image size, image geometry, limits of resolution, limits of detectability, aberration and distortion with the same ease as when looking through a pair of binoculars.

Acknowledgements

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Note

In order to encourage serious microscopists to explore the limits of their instruments, optics and imaging systems, Bio-Microtech will make available 100 test slides at a special price of £250.00 each to members of the Royal Microscopical Society and the Quekett Microscopical Club. Enquiries about ordering Richardson Test Slides can be directed to: Bio-Microtech Inc., Unit 4, 670 Hardwick Road, Bolton, Ontario, L7E 5T1, Canada. Phone: 905-951-9996; Fax: 905-951-7052; email: bmtinfo@ibm.net

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The Microscope User and Microscopy Education
An Inverse Relationship?

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Summary

Light microscopy has become a sophisticated field of expertise with a corresponding explosion in technological complexity. This in turn has led to a lag in user education where many professionals, who use the microscope on a routine basis, are either self-taught or have had only a brief exposure to the subject's theoretical and practical aspects. This study examined a portion of the Middle Eastern scientific community that routinely uses the light microscope, to establish the baseline of theoretical knowledge, practical skills and working environment of this population. A questionnaire established theoretical knowledge and information regarding operational parameters. The working environment and practical skill of the participants were assessed by observation. For the majority of subjects, work experience had the greatest impact on their understanding of microscopy as opposed to other educational resources with which they had been in contact. Although most microscopes used by the participants were capable of Köhler illumination they were not optimally adjusted. It appeared that most people had poor postures when using the microscope but yet they reported little major discomfort. Light microscopy courses held within the workplace are likely to have the greatest impact on user education and should cover not only basic theory but also practical and ergonomic issues.