Tricks and Treats in Photomicrography

H. Wolfgang Zieler

Wolfgang Zieler was a salesman all of his life. He sold light microscopy and microscopes. He was a most unusual salesman of microscopes in that he used microscopes himself and thoroughly understood how they were produced and how they should be used. He was also a great teacher and lecturer. His two books on Microscope Optics and Illumination in The Microscope Series of books from the McCrone Research Institute are thorough and lucid. Once head of the Leitz Organization in the U.S., he went on to an independent sales outlet with Werner Kessel in Chicago and then to Atlantex-Zieler in Massachusetts. He was a life member and post President of the New York Microscopical Society and a Fellow of the Royal Microscopical Society. He has been teaching and lecturing on microscopy behind the Pearly Gates since February 9, 1973. I wish we could get him back here to teach some of our courses

Walter C. McCrone, McCrone Research Institute

Success in photomicrography depends, not only upon the acquisition of a technique, but also upon the selection of equipment. Unless the equipment is provided with certain facilities and controls, it is impossible to create optimum final image quality in the object plane.

We shall direct our attention to that part of the equipment which - next to the performance of objective and eyepiece - has the greatest influence upon the quality of the image, viz., the illumination system. For photomicrography, the following facilities and control are essential:

1) A light source of suitable shape, dimensions, and spectral composition. It should be provided with centering and focusing capabilities.

2) A lens system (lamp condenser) at the correct distance from the light source, collecting light within a large angular aperture. This system must be able to focus the lamp filament in the front focal plane of the substage condenser.

3) An iris diaphram near the lamp condenser lens to be focused by the substage condenser into the object plane. This iris serves as a "field diaphram" of variable diameter at high (and medium) magnifications. To illuminate the full field of view evenly at low magnifications may require removal of the condenser top lens and refocusing of the field diaphram image in the field of view.

4) A substage condenser system must be so designed that correct illumination conditions are created in the object plane from lowest to highest magnifications. The intensity of the light in the object plane must be uniform, not only throughout the small field prevailing at high magnifications, but also the large field at low magnifications. Moreover, the numerical aperture of the illumination must be variable from a maximum, when it is equal to the selected objective, to a minimum which depends upon the optical character of the object and other factors. These requirements can be fulfilled only if the condenser system has several components which can be used individually as well as in combination with each other.

5) An iris diaphram in the front focal plane of the substage condenser to serve as an "aperture diaphram" of variable diameter to vary the angular aperture of the condenser. The iris diaphram in the lower portion of the substage fulfills this purpose. Here to, it may be necessary to remove the top lens of the condenser to achieve proper image contrast at low magnifications.

6) Centering facilities with which the optical axis of the substage condenser can be brought into coincidence with that of the microscope. This requirement is especially important at higher magnifications.

For visual observation, light sources of low intrinsic intensity are adequate, because the image formed on the retina of the human eye is quite small and the sensitivity of the eye is high. However, the images formed on the film in photomicrography are much larger and require light sources of considerably higher intrinsic intensity. That is why special halogen lamps with concentrated filaments are used for photomicrography.

In former years, the owner of a microscope for visual observations who wanted to take photomicrographs had to purchase either a separate camera and an independent light source, or an optical bench to which all of the components, including those of the illumination system, were mounted on separate "riders." The particular technique for photomicrography that offered the greatest difficulty was the alignment of the illumination system, because it was not always easy to correlate a specific criterion of unsatisfactory performance to misalignment of a specific component of the entire optical system. The difficulties increased at low magnifications, because the condenser of the microscope, when used in its entirety, performed correctly only at highest magnifications. In order to illuminate the larger fields at low magnification with reduced numerical aperture it became necessary to swing out the top lens of the condenser. When that was done, however, the field diaphram must be refocused in the field of view by lowering the condenser.

In recent years, microscopes have been produced in which all of the essential facilities and controls for photomicrography, including a suitable light source have been built into the microscope base. With this modification, the manipulation of the illumination system has been simplified somewhat so that even the beginner can more easily master the technique of photomicrography. Simple directions outline a systematic procedure in which unmistakable criteria indicate correct optical conditions in the object plane from lowest to highest magnification and consistent success becomes a matter of routine.

There is only one "trick" which must be learned - quite simple, but of utmost importance - if every photomicrograph is to be a "treat" even to the critical expert.

Before describing this trick, I want to give a brief description of the procedure of aligning the illumination system of the built-in type. Most of the component parts of the illumination system have been prealigned by the manufacturer to such an extent that 1) centering of a sharp image of the field diaphram in the field of view, 2) centering and focusing of the filament (when possible), and 3) centering of the rotating stage (of a polarized light microscope) indicates that all components have the same optical axis as the objective and that they are at the correct distances from each other.

For alignment of the built-in illumination system, a "high dry" objective (40-60X) should be used. All of the components of the microscope substage should be swung into the light path. After having obtained a well-focused image of the object, the aperture iris diaphram should be adjusted for optimum contrast, the field

Continued on Page 18

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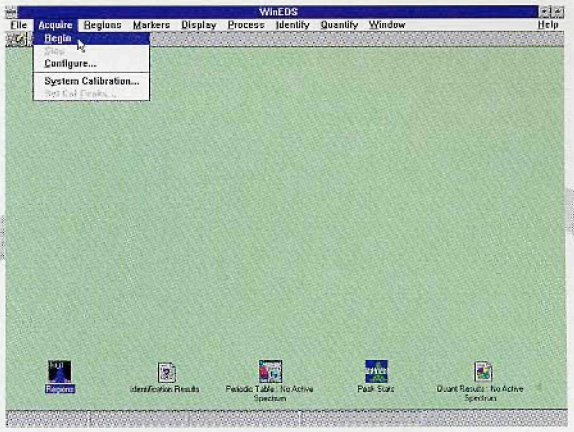

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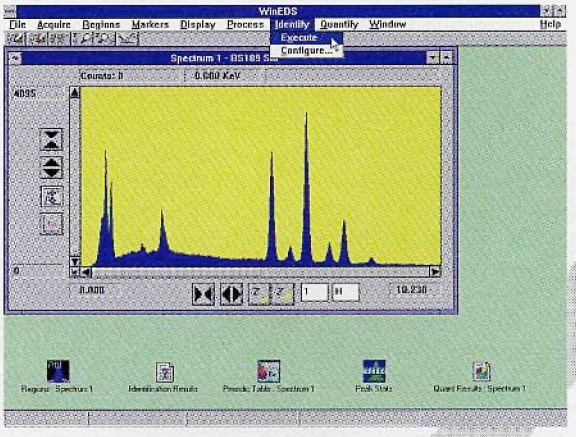

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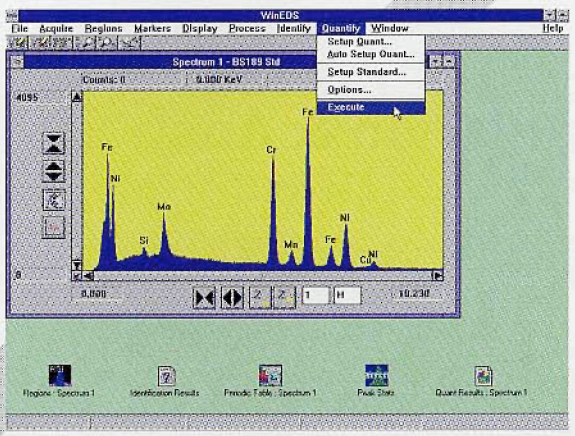

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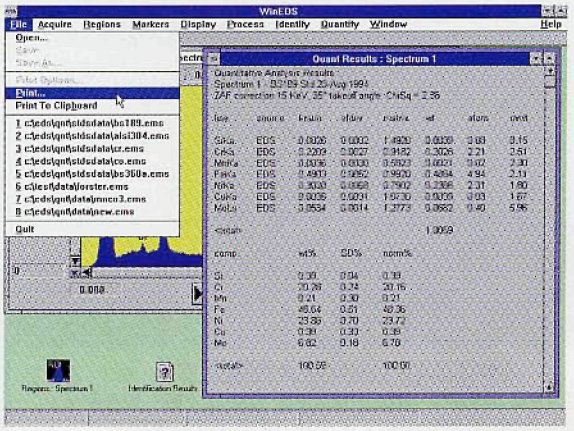

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Tricks And Treats - Continued from page 16

diaphram should be closed and the rack and pinion motion of the substage should be operated until the outlines of the iris leaves appear in focus simultaneously with the image of the object. The centering screws of the substage should be used to center this field diaphragm image with respect to the circumference of the field of view. Thereupon, it should be opened sufficiently, to illuminate the area to be photographed, *but not farther.* If an objective of lower magnification, i.e., 4-5X, is to be used, the top lens of the condenser will likely have to be moved out of the light path, usually by swinging out of the light path, and the field diaphram will then have to be refocused into the plane of the preparation.

This simple procedure completes the alignment of the built-in illumination system for photomicrography, except for the "trick" we will describe later on. The user of a polarized light microscope follows the same procedure but, in addition, the rotating stage must be centered so that any object at the center of the field of view stays at the center during rotation of the stage and the filament of the lamp must be focused and centered in the objective back focal plane. This is best done with a phase telescope or a Bertrand lens.

The reduction of the illuminated area of the object plane to the size of the area to be photographed has a special purpose, and becomes necessary only at high magnifications and correspondingly high numerical apertures of illumination. The purpose is: reduction of "glare" throughout the image area. It must be realized that every time light passes obliquely from glass to air or air to glass, there is partial reflection. The intensities of these reflections are higher, the greater the angle of obliquity of the light. In the light path through condenser, slide, and objective, there are the plane surfaces of upper condenser lens, lower and upper surface of the microscope slide and the lower plane surface of the front lens of the objective. All of these surfaces are parallel to each other. Between them, a certain part of the light "bounces back and forth" repeatedly, and each time, a small portion passes on through the objective and causes glare. It is evident that through such repeated partial reflections, detectable portions of light pass into the objective even if they have traversed the object plane in an area surrounding the field revealed by the eyepiece. Moreover, they are increasingly detrimental at higher numerical apertures of illumination. That is why manipulation of the field diaphram is an effective step for glare reduction at *high magnifications*, whereas at low magnifications and correspondingly lower numerical apertures of illumination, there are no highly oblique rays traversing the object plane and the field stop is not so important.

The "trick" that is so very important for the production of photomicrographs of highest quality involves the correct use of the variable aperture diaphram in the substage. I have had many occasions to observe that the correct use of this diaphram is misunderstood by many microscopists because quite frequently, I have been asked:

"What is the correct procedure for controlling and varying the light intensity in the object plane?" Should we open or close the iris diaphram of the substage or should we raise or lower the condenser with the rack and pinion?"

My answer, that if only variation of intensity is to be achieved neither the first nor the second procedure is correct, was generally received with surprise and skepticism. Yet, the following considerations will show that my answer is correct.

The only correct position of the condenser is that at which a real image of the field diaphram is formed in the object plane. By racking the substage assembly up or down away from that position, the image of the field diaphram is not formed in the object plane. This affects the uniformity of the illumination throughout the field of view. furthermore, control of glare at high magnifications becomes impossible. The photomicrographs may show the unpleasant effect of a fading of the intensity towards the periphery of the field, or traces of non-uniformity of the intensity become noticeable, for instance in the form of blurred images of the filament, when the light source is a concentrated filament lamp.

The other procedure of opening or closing the aperture iris diaphram causes, not only variations in intensity, but also changes of resolving power, depth of field, flatness of field and, last but not least, the entire optical character of the image.

It is this possibility of changing the optical character of the image by changing the ratio of the numerical apertures of objective and illumination, which is the "essence" of the "trick" to which I referred. The nature of this change of optical character can be observed by anyone who has a gift of keen observation (an essential requirement for every microscopist, especially if he knows what to look for).

To observe this change of image character, proceed as follows: select as an object a stained thin section or natural fibers in a mounting medium and covered with a coverglass. Use an objective of 4 mm focal length (preferably a fluorite system or apochromat), adjust the illumination system as described and open the aperture iris diaphram as far as possible. The numerical aperture of the illumination should then be equal to that of the objective and the back focal plane of the latter should be fully illuminated.

Keep one hand on the fine adjustment to maintain optimum sharpness of the image and with the other hand, close the aperture diaphram *gradually* while keenly observing the image for any possible changes.

The keen observer will notice that the iris diaphram can be closed to about 3/4 or even 2/3 of its maximum diameter, before any major change of image occurs. Such change occurs rather abruptly. At a certain position of the aperture diaphram, there is a sudden increase of contrast, but this enhancement of contrast is *restricted to the smallest observable object detail.* The general character of the image, as far as the coarser detail is concerned, remains almost unchanged. Whatever additional change becomes noticeable can be described as an enhancement of the colors of the stained object, a slight increase in the depth of field which also slightly improves the flatness of the field and, altogether a slight improvement of the overall sharpness of the image.

I have drawn the attention of many microscopists to these changes which I have described most unscientifically (but I believe effectively) by saying that at a rather critically defined position of the substage iris diaphram, the entire image seems to have more "OOMPH." The consistent improvement of the photomicrographs taken by those who had been made conscious of the change in image character, proved to me that I had conveyed the right thought to them.

The "OOMPH" position of the substage aperture diaphram is that at which the image quality is optimum. If this iris diaphram is closed further, there is a decrease in resolving power and an undesirable increase of contrast, also of the coarser object detail. If it is opened further, there is an undesirable decrease of contrast of

Continued on Page 20

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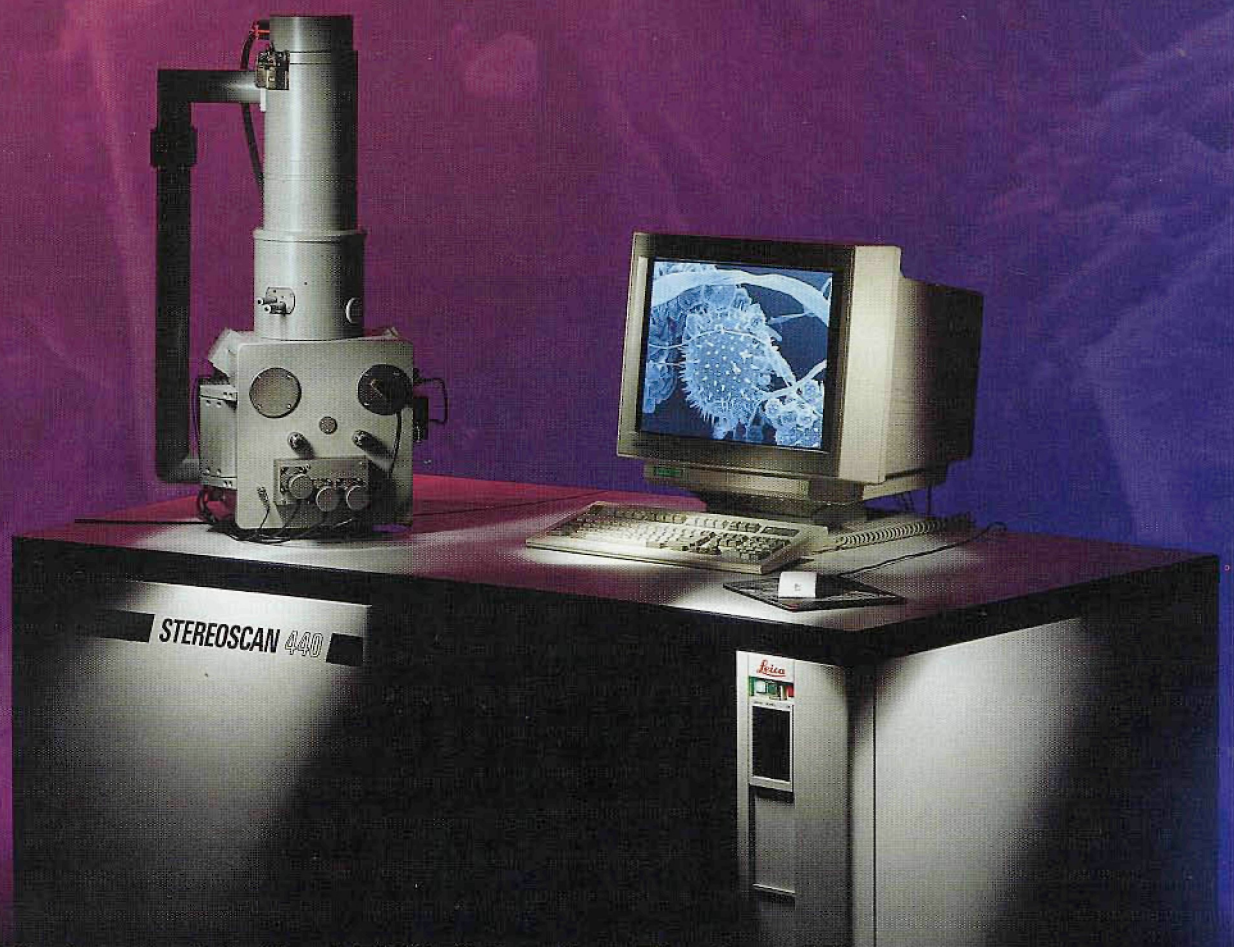

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the finest object detail with no gain of image quality in other respects.

If the intensity of the image is to be varied, *without* change of numerical aperture or spectral distribution of the illumination and without undesirable change of condenser position, the only correct method to accomplish this is by use of neutral density filters. Even the method of varying intensity with a rheostat (varying voltage) is not entirely correct because by such procedure not only the intensity, but also the spectral composition of the light is changed. This is not objectionable in black and white photography, but must be careful avoided when color film is used. Only very small deviations from the correct aperture of the iris diaphram are permissible, for instance, to adjust the illumination to a value indicated by a photoelectric exposure meter, to correspond to an available shutter speed. Let us assume that at the "OOMPH" position the exposure meter reading is 64, and experience has shown that for an exposure time of 1/25 second, a deflection to 75 is required. The slight increase in diameter of the substage iris diaphram necessary to obtain this intensity value is permissible.

The only factor which influences, not only resolving power, but also the entire optical character of the image, is the ratio of the aperture of the objective to that of the illumination.

To summarize the practical aspects of the "OOMPH" position of the aperture iris diaphram; optimum image quality prevails when the field of view is illuminated by light of uniform intensity; when the illuminated area is not larger than the field of view; and when the numerical aperture of the condenser comes closest to matching the numerical aperture of the objective considering the inherent contrast of the specimen.

The microscopist who guides himself by these suggestions will consistently produce photomicrographs which are a real "treat" to everybody who knows how to evaluate them. ■

The *Why* and *What* of Flow Cytometry

The following is the introduction in the Royal Microscopical Society Handbook No. 29 "Flow Cytometry," which is available from Microscophy Today ($27.00 plus S&H).

Why Flow Cytomertry?

In the last 10 years, the applications of flow cytometry have spread through all branches of biological sciences. Most research institutes now have several machines and even quite small research groups expect to have access to an instrument. People have used flow cytometers to measure the properties of, and to sort, mammalian and plant cells, yeast and bacteria and isolated nuclei, chromosomes and mitochondria. The biggest growth has been in the clinical field. Flow cytometers are now being used for routine measurements in immunology, haematology and, to a lesser extend, pathology departments.

In cells, a variety of properties may be measured, for example the DNA content of a nucleus, the expression of a surface antigen, the activity of an intracellular enzyme or the pH. Several properties might be measured simultaneously. Indeed, the scope of the technique is only limited by the fluorescent dyes available and the investigator's imagination - the latter probably being the most important.

What is Flow Cytometry?

As its name implies, flow cytometry is the measurement of cells in a flow system which has been designed to deliver particles in single file past a point of measurement. Although, in theory, many types of measurement could be made, in practice the term is applied to instruments which focus light on to cells and record their fluorescence and the light scattered by them. Electronic cell volume and absorbed light additionally may be measured.

The power of flow cytometry lies in the ability to measure several parameters on tens of thousands of individual cells within a few minutes. The method can therefore be used to define and to enumerate accurately sub-populations. Once identified, such sub-populations can be sorted physically for further study.

Typically, five parameters might be measured on 20,000 cells. Using blue light for excitation, one might record green, orange and red fluorescence and blue light scattered in a forward direction and at right angles to the laser beam. The large amount of data generated cannot be processed adequately without a powerful, well-programmed computer. The computer is not an optional extra but an essential part of the instrument.

The major disadvantage of flow cytometry is that a preparation of single particles (cells, nuclei, chromosomes) is required. Of necessity, tissue architecture is destroyed, so that spatial information about the relationships of cells to each other is lost. Also, no information is acquired about the distribution of entities within a cell and little, if any, information about a cell's shape. Flow cytometry can be contrasted to conventional light microscopy. The eye makes qualitative estimates of large numbers of parameters on a few cells, recording detail within each cell; the cytometer quantifies an average parameter for each cell but measures thousands of cells.

A comparison may also be made with a biochemical measurement in which an average value of, for example, an enzyme activity, is made for all the cells in a sample. Flow cytometry makes a measurement on each cell individually so that, if a small sub-set of cells has a high value, this feature will be recorded. Such a sub-set would not be detected biochemically.

Several recent books have given overviews of flow cytometry (Bauer *et al.*, 1993; Givan, 1992; Melamed *et al.*, 1990; Shapiro, 1988; Watson, 1991) and others have given detailed descriptions of many of the methods in common use (Darzynkiewicz and Crissman, 1990; Macey, 1994; Ormerod, 1994; Radbruch, 1992; Robinson, 1993). ■

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