50 Most Frequently Asked Questions About Optical Microscopy

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A significant percentage of technical experts who employ optical microscopes have had little or no formal training in optical microscope basics. Some, typically, were required to use microscopes during their technical education but, in general, microscope terminology and technology was a sideline to their major training. As a result, many useful basic microscope technical details were not learned because they were not necessary to accomplish what was needed in order to survive their major class work. At Florida State University, we try to make the learning of microscope technology an inherent part of the students training. An important part of this training is this compendium of 50 of the most frequently asked questions about Optical Microscopy.

The following are answered by Mortimer Abramowitz, consultant, technical information at Olympus America, Inc.

Question 1. What does the inscription "0.17" on the objective signify?
Answer: "0.17" refers to the thickness, in millimeters, of the cover glass that was assumed by the lens designer in computing the corrections for the objective. For objectives with a numerical aperture higher than 0.45, a departure in this assumed thickness (or no cover glass at all) may result in deterioration of the image.

Question 2. What is the significance of the N.A. number inscribed on the outer barrel of an objective?
Answer: This number is the numerical aperture of the objective, a measure of the light-gathering capacity of the objective. The higher the numerical aperture, other things being equal, the better the objective is able to separate the details of the specimen in forming an image; also the brighter the image. Higher N.A. objectives are usually more expensive. The higher the numerical aperture of the objective, the shallower the depth of field (see definition of depth of field).

Question 3. What does the objective inscription "160" mean?
Answer: This number identifies a finite tube length objective. 160 millimeters is the distance from the nosepiece opening (where the objective is screwed in) to the top of the observation tube (where the eyepiece is inserted). If this distance is lengthened, *e.g.,* by insertion of accessories in the light path above the nosepiece, spherical aberration will result; unless optically corrected lenses are included in the accessory.

Question 4. What does the objective inscription signify?
Answer: This inscription identifies the objective as an infinity-corrected objective. Light rays emerging from such an objective are in parallel bundles projected toward infinity. Such an objective, with its many advantages, requires a tube lens in the light path to converge the parallel rays so that they come to focus at the plane of the eyepiece diaphragm.

Question 5. Some objectives have the inscription "Plan". What does that mean?
Answer: "Plan" designates an objective which projects, at the eyepiece diaphragm plane, an image which is flat from edge to edge of the field of view. Some objectives, *e.g.*, the new planapochromats and new planfluorites, will give flat images even with eyepieces of field numbers (F.N. see below) up to 26.5; others up to F.N. 22.

Question 6. If an objective carries the inscription "Planapo", what does that mean?
Answer: The term "Planapo" signifies a planapochromat, an objective of the highest correction, corrected for four colors chromatically and spherically. Such an objective, for its magnification, will have a higher numerical aperture than objectives of lesser correction. Planapochromats are the best objectives for critical resolution and color photomicrography. Other things being equal, they usually have shallower depth of field. They are also more expensive.

Question 7. What does the inscription "PlanFl" mean?
Answer: "PlanFl" denotes an objective, which is a planfluorite, also called a plan-semi-apochromat. (Some manufacturers call such objectives "Fluars" or "Neofluars") These objectives are also corrected for four wavelengths, but not quite as completely as planapochromats. Planfluorites are also well-suited for color photomicrography and are less expensive than planapochromats.

Question 8. Some objectives have no inscription about their corrections or only the inscription "Plan". What kinds are these?
Answer: Such objectives are achromats or planachromats. They are now corrected for 3 wavelengths chromatically, and 1 or 2 wavelengths spherically. They give their best images in green light. However, in white light, the planachromats will yield satisfactory images for color photomicrography but not as good as objectives of better correction.

Question 9. Objectives usually have a color ring inscribed. What do these colors signify?
Answer: The 4X or 5X has a red ring; the 10X yellow; the 20X green; the 40X or 50X or 60X blue; the 100X white. These rings make it easier to visually identify the magnification of the objective; the colors are standard for most manufacturers. In addition, Olympus phase objectives are further identified by having a red ring nearer to the front lens of the objective (LB series) or all inscriptions in green for the infinity series of phase objectives.

Question 10. What is a No-cover glass or NCG or NC objective?
Answer: Such an objective has been designed to look at a specimen that is not covered by a cover glass; *e.g.*, a smear. At numerical apertures above 0.45, such an objective will yield images free of spherical aberration when smears or other uncovered specimens are examined. Metallurgical objectives are almost always designed for looking at uncovered objects; e.g., polished metals, wafers, etc.

Question 11. Why would one use an objective marked "LWD" or "ULWD"?
Answer: The letters identify a long working or ultra-long working distance objective. The vertical distance from the front lens of the objective to the focused specimen (working distance) is much longer than that for a similar magnification standard objective. Such objectives are invaluable for looking up through a culture vessel or Petri dish in inverted biological microscopy; or examining IC wafers to prevent inadvertent contact with the wafer; or inspecting solder connections of mounted chips.

Question 12. Some objectives are marked "NIC" or "DIC". Why?
Answer: This designates an objective that is preferred for use in Nomarski (NIC) or differential interference microscopy (DIC).

Continued on page 14

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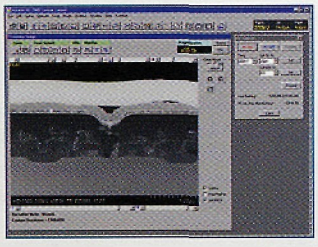

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50 Most Frequently Asked Questions About Optical Microscopy

Continued from page 12

Question 13. What does the inscription "oil" or "oel" or "WI" on the objective mean?
Answer: Such an objective is an immersion objective, requiring a drop of immersion oil (or water) between the front lens of the objective in contact with the cover glass or top of a smear. Unless there is oil contact with such an objective, the image will be very poor. Some manufacturers inscribe a black ring on the lower part of the barrel to enable the user to instantly recognize the need for oil contact. Similarly, the letters WI refer to an objective that requires water, rather than oil, as the immersion contact medium. To achieve a numerical aperture of 1.0 or above requires an immersion objective. WI objectives are especially useful in the observation of living biological specimens.

Question 14. Why are some objectives marked "UV"?
Answer: Ordinary glass is relatively opaque to ultra-violet light wavelengths below 400 nm. UV objectives contain specially formulated glass elements and coatings to transmit a relatively high percentage of light of such wave lengths. These objectives are very useful for near ultra-violet excitation in reflected light fluorescence work. The new Olympus infinity-corrected U-planapochromats and U-planfluorites have such improved transmission in the near ultra-violet.

Question 15. What do the letters "PL" or "NH" mean on a phase objective?
Answer: The letters "PL" stand for positive low, a phase contrast in which the specimen appears darker than the background of the field of view. Less commonly used, the letters "NH" stand for negative high, a type of phase contrast in which the specimen appears lighter than the background.

Question 16. Why are some objectives marked "P" or "POL" or "SF"?
Answer: These letters designate the objective as being relatively strain-free (that is, the objective itself has little or no effect on polarized light). Such objectives are required for high quality polarized light qualitative and/or quantitative investigations.

Question 17. Eyepieces, in addition to having the magnification inscribed, are marked 20, or 22, or 26.5. What do these numbers mean?
Answer: The designation is the field number of the eyepiece. The higher the field number of the eyepiece being used with a particular objective, the more specimen area will appear in the field of view. The diameter of the field of view, in millimeters, is calculated by dividing the field number of the eyepiece by the magnification of the objective. (For example, with a 10X objective and an eyepiece with a field number of 22, the diameter of the field of view would be 2.2 millimeters) For many microscopists, *e.g.*, haemotologists, it saves time to be able to see more of the specimen at a given time. Eyepieces with a field number of 26.5 are called super-wide eyepieces.

Question 18. What do the inscriptions "C" or "K" or "WF" or "H" on the eyepiece mean?
Answer: "C" or "K" identifies a compensating eyepiece. Some microscope objectives do not include correction for lateral chromatic aberration. For such objectives (Olympus LB series), the compensating eyepiece completes the correction. "WF" means widefield; more of the specimen to be seen at a given time. "H" signifies high eyepoint which means that the user's eyes do not have to be placed very close to the top lens of the eyepiece during observation; a particular boon to eyeglass wearers.

Question 19. Why do some observation tubes have notches cut into them?
Answer: The notch is meant for placement of the projecting "locator" pin on eyepieces which have a reticle installed. Such an eyepiece can be focused by rotating its diopter adjust upper lens, while the "locator" pin keeps the reticle (*e.g.*, crosshairs or micrometer scale) properly oriented.

Question 20. What are the outer dimensions of the most common observation tubes?
Answer: These are usually 25 millimeters or 30 millimeters (as on the latest Olympus B-max microscopes and on all Olympus superwide observation tubes).

Question 21. What is a photoeyepiece?
Answer: This is an eyepiece meant for photomicrography, not to be used for observation. the eyepiece "picks up" the image projected by the objective and projects that image onto film plane inside a camera. Photoeyepieces (also called projection lenses) usually come with low magnification power because the images they project onto film are often subsequently further enlarged (to lessen chances of "empty" magnification).

Question 22. Some objectives, 20X magnification or higher, have a spring-loaded or retractable front lens assembly. Why?
Answer: Such objectives have a very short free working distance; hence the danger of inadvertently crashing the front of the objective into the cover glass or specimen. A spring-loaded front lens assembly allows this part to retract upon gentle pressure contact with a specimen. It will NOT protect against rough, continuous pressure contact.

Question 23. Some objectives come with a built-in iris diaphragm. What is the use of this diaphragm?
Answer: The diaphragm is partially closed down during darkfield microscopy in order to reduce the numerical aperture of the objective below the lower of the two numerical apertures inscribed on a darkfield condenser. This action preserves the darkness of the background in darkfield observation. The iris diaphragm is absolutely necessity for high numerical aperture oil immersion objectives (above N.A. 1.2) when using an oil immersion darkfield condenser. For ordinary brightfield observation, this iris diaphragm is left wide open.

Question 24. What is the purpose of the correction collar found on some "dry" 20X, 40X, or 60X objectives?
Answer: The correction collar, when rotated, separates or brings together some of the internal glass elements of the objective. This action can correct for incorrect cover glass thickness. In upright microscopes, the range of correction of the collar is usually from 0.11-0.22 millimeters. For inverted microscopes, the range of correction capability is from 0 (uncovered) to 2 millimeters to correct for thick culture vessels. The purpose is to eliminate spherical aberration.

Question 25. What is meant by the "free working distance of an objective?"
Answer: This is the vertical distance in millimeters, or a decimal fraction of a millimeter, from the front of the objective to the coverglass or uncovered specimen, when the specimen is in focus. High magnification objectives customarily have very short working

Continued on page 16

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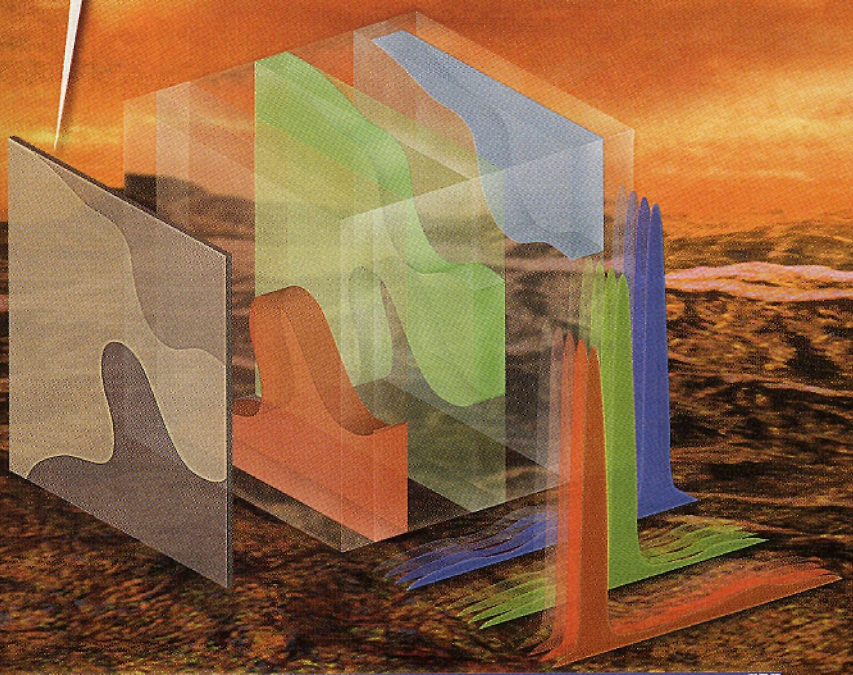

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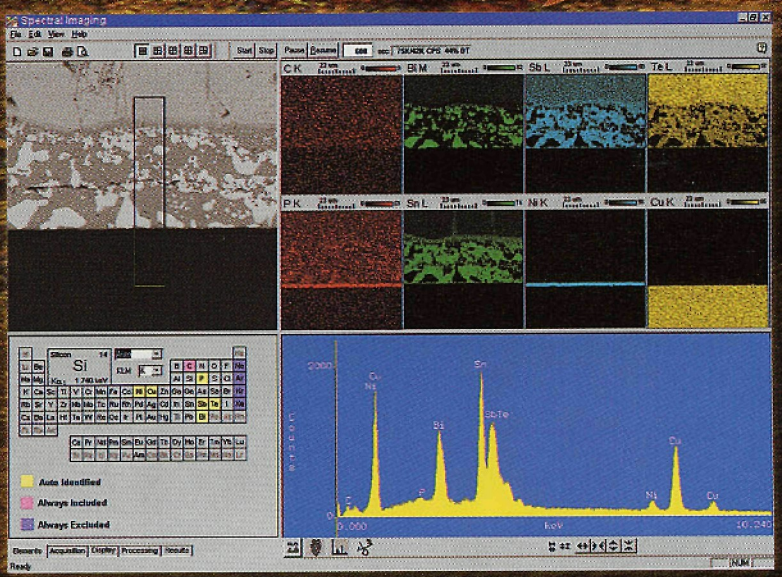

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50 Most Frequently Asked Questions About Optical Microscopy

Continued from page 14

distances. The manufacturer should be able to supply you with working distance data for each objective.

Question 26. What is meant by the adjustment distance or parfocalizing distance of a microscope?
Answer: In most modern microscopes, this distance (45 millimeters) is the chosen distance from the opening of the nosepiece to the focused specimen. By having a standard distance for all objectives, it is easy to rotate each objective, in turn, into the light path with a minimum of refocusing. Such a battery of objectives, despite various lengths of the objectives themselves, is described as being parfocal. If, in addition, a focused feature, which has been centered in the field of view, remains centered as objectives are changed, the objectives are described as parcentric.

Question 27. What is meant by resolving power of an objective and how is that distinguished from resolution?
Answer: Resolving power of an objective refers to the ABILITY of that objective to yield an image which clearly separates points or lines lying close together in the specimen. The shorter the distance (between points or lines) the better the resolving power of the objective. Resolving power is related to the numerical aperture of the objective--the higher the numerical aperture, the better the resolving power. The formula is expressed as $d = \lambda / 2N.A.$ or $0.61\lambda / N.A.$; the former according to Abbe, the latter according to Rayleigh. In the formula d is the distance between two closely lying points; λ is the wavelength of light being used; N.A. is numerical aperture. Resolution is the ACTUAL separation achieved by the microscope system. For example, in Köhler illumination, the condenser diaphragm is usually closed down somewhat in order to effect a compromise between resolving power and contrast—to enhance visibility.

Question 28. What is the highest numerical aperture for a "dry" objective (which requires air between its front lens and the specimen?)
Answer: The highest N.A. for a "dry" objective is 0.95. If such an objective is meant to look through a cover glass, it must have a correction collar. If such an objective is designed for uncovered objects, *e.g.*, smears or metallurgical, it will not require a correction collar.

Question 29. What is the relationship between numerical aperture and brightness of an image and between magnification and brightness of an image?
Answer: Other things being equal, the brightness of an image varies directly as the square of the N.A.; as the fourth power in reflected light fluorescence. Thus, higher N.A. yields brighter images. Conversely, brightness varies INVERSELY as the square of the magnification. Thus, other things being equal, higher magnification will reduce the brightness of the image.

Question 30. Can I use an infinity corrected objective on a finite tube length microscope?
Answer: No, because the finite system does not include a tube lens to bring the parallel rays to focus.

Question 31. Can I use a finite tube length objective on an infinity-corrected system?
Answer: Although you may be able to screw the objective into the nosepiece, the presence of a tube lens in the light path will result in a deteriorated image.

Question 32. Can I use an infinity-corrected objective from another microscope manufacturer on an Olympus infinity-corrected system?
Answer: No, that would not be advisable. The focal length of Olympus' tube lens (180 mm) is not the same as that of other manufacturers. As a result, the magnification of the objective would not be accurate. Also, aberrations would be introduced because other companies correct for lateral chromatic aberration in the tube lens; the new Olympus objectives for the B-Max series achieve this correction in the objectives themselves. Also, the objective probably would not be parfocal with Olympus objectives.

Question 33. Can the new series Olympus infinity-corrected metallurgical objectives be useful on a biological B-Max microscope?
Answer: Yes. For covered or uncovered specimens, metallurgical objectives of N.A. 0.40 or less should give satisfactory images. For an N.A. above 0.40, the specimen should not be covered by a cover glass, since the metallurgical objectives are corrected for uncovered specimens. The working distance may be very short, and the parfocality with biological objectives may not be as accurate.

Question 34. Can a planachromat objective be useable in reflected light fluorescence?
Answer: The answer is a provisional yes. Planachromats may serve satisfactorily for blue or green excitation waves lengths. However, the planachromat's glass elements may themselves fluoresce when excited by near ultra-violet. Also, planachromats, for their respective magnifications, have lower N.A.'s than do planfluorites or planapochromats-thus the images may be not as bright.

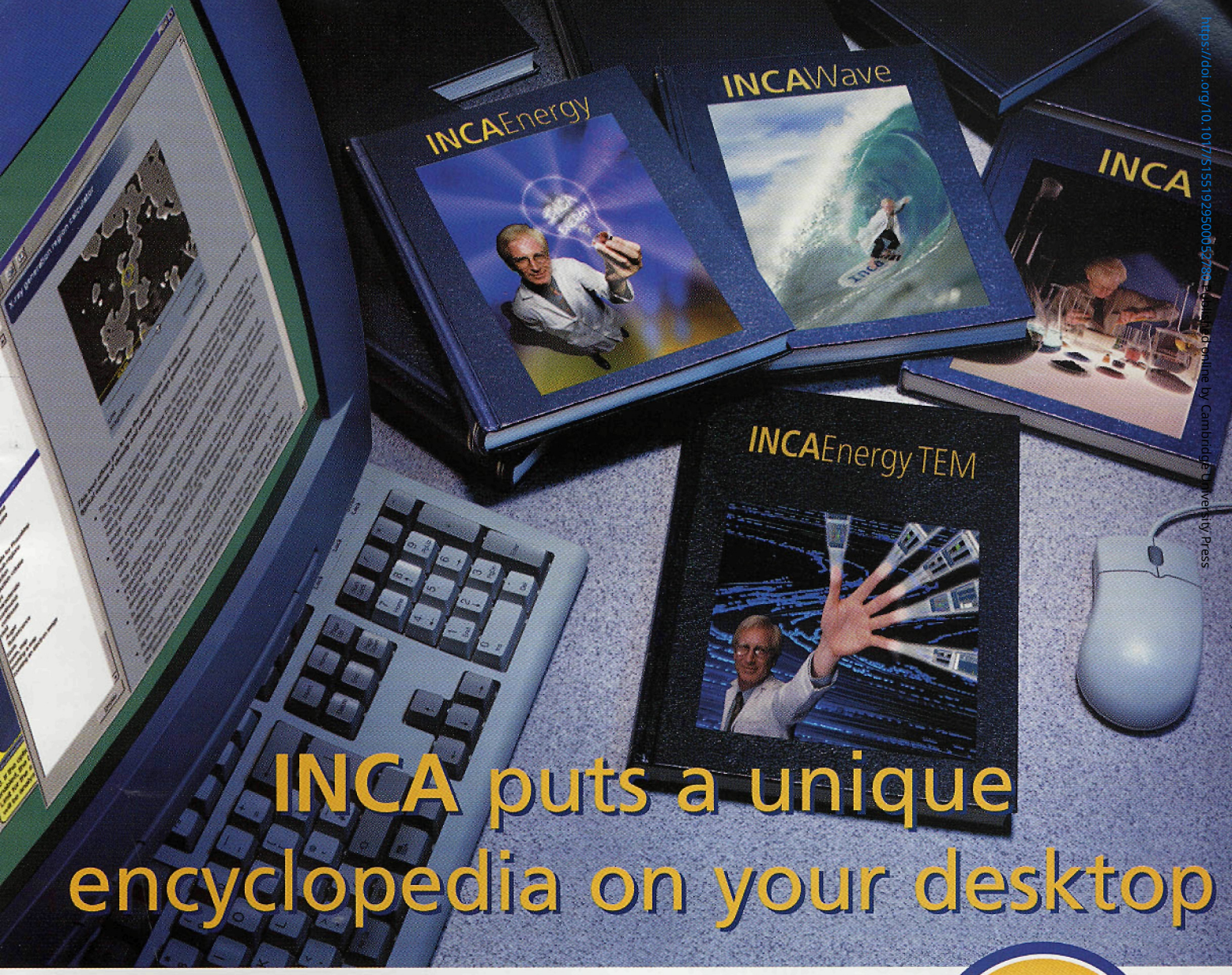
Question 35. Can phase contrast objectives be used for regular brightfield observation?
Answer: Yes. The phase condenser should be moved to the O position and standard Koehler illumination procedure should be employed. The brightfield images will be nearly as good as if a regular brightfield objective were being used.

Question 36. What objectives are generally chosen for clinical laboratory microscopes?
Answer: Such microscopes can function satisfactorily with ordinary achromats or planachromats. For research, or for best color photomicrography, planfluorites or planapochromats are preferable (and more expensive).

Question 37. When a 40X objective is used, the image may appear worse than with a 20X objective. Why?
Answer: The specimen may be covered with a cover glass much thicker than the standard 0.17 millimeters or may be mounted in a thick mounting medium under the cover glass. The solution could be to use a "dry" objective with a correction collar; or to substitute a 40X or 50X oil immersion objective for the 40X "dry" lens since the immersion objective lens will be less sensitive to variations in cover glass thickness.

Question 38. With an objective of a given magnification, why should not one use increasingly higher magnification eyepieces to achieve higher total magnification?
Answer: To maintain useful magnification, that is, magnification yielding satisfactory clarity and resolution, one must avoid making the specimen appear bigger but not clearer ("empty" magnification). The general rule of thumb in microscopy is that the TOTAL

Continued on page 18



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Continued from page 16

magnification should probably not exceed 750X-1000X the numerical aperture. For example, with a 40X, N.A. 0.65 objective, the total magnification (The multiple of the eyepiece and the objective magnifications) should be between 480-650X.

Question 39. What is meant by depth of field?
Answer: This is the vertical distance in the specimen, usually in microns, measured from above and below the exact plane of focus which still yields an acceptable image. The higher N.A. the shallower this distance will be.

Question 40. What is the depth of focus?
Answer: This is the vertical distance in the IMAGE space (at film plane), from above and below the exact image plane, that appears satisfactorily sharp. Contrary to what might be expected, the depth of focus is shallower for low magnification objectives. This makes it more difficult to get sharp images, in photomicrography, with low power objectives.

Question 41. What is the value of a green filter placed in the light path?
Answer: Since achromats and planachromats are best corrected spherically for green light, and since the use of monochromatic light eliminates chromatic aberration, the performance of achromats is markedly improved with the use of a green filter. Also, phase contrast objectives are computed to give best phase images in green light.

Question 42. Some microscopes come equipped with a so-called daylight blue filter. What is this used (or misused) for?
Answer: The "daylight blue" filter is a filter for observation only. It furnishes a pleasant pale blue-gray background to the field of view. It is NOT meant for photomicrography with daylight color film. Such film requires a blue CONVERSION filter such as the Olympus LBD or Kodak 80A filter. The conversion filter boosts the color temperature of the light source, thus simulating light of daylight color temperature quality (5500 degrees Kelvin) required for daylight balanced color film.

Question 43. How does a substage condenser function in helping to provide excellent images?
Answer: The working numerical aperture of a microscope is the sum of the N.A. of the objective plus the N.A. of the condenser divided by 2. In order to retain more good contrast with good resolution, the condenser iris (aperture diaphragm) is usually opened to about 3/4 of the N.A. of the objective. Condensers also vary in their color and spherical correction-from the relatively modest correction of an Abbe condenser to the highest correction aplanat-achromat condenser. The aplanat-achromat condenser, with its high N.A., is the best choice for color photomicrography. Some condensers have a swing-out upper element. With this element out of the light path, the condenser is able to fill the field of view, without vignetting, of a 4X or a 2X objective.

Question 44. Some nosepieces have wider diameter openings into which the objectives are screwed. Why?
Answer: In metallurgical microscopy, objectives are available for reflected light brightfield and/or darkfield observation. These objectives have a wide diameter barrel for use in darkfield reflected light; hence need for wider openings of the nosepiece. Such objectives may be labeled Neo or B/D or BF/DF.

Question 45. How can you minimize the likelihood of immersion oil getting on the 40X "dry" objective?
Answer: One expedient often used in clinical laboratories is to mount the "dry" 40X on the opposite side of the nosepiece from the 100X oil immersion objective. This mounting arrangement reduces the likelihood of inadvertent dipping of the 40X "dry" objective into immersion oil as you rotate the nosepiece between the 100X oil objective and the 40X "dry" objective.

Question 46. Is there a good alternative choice for the 40X "dry" objective to avoid the oiling "hazard" described?
Answer: If the 100X oil objective is used frequently, it might be advisable to substitute a 50X OIL immersion objective in place of the 40X "dry" objective. The 50X (N.A. 0.90) oil planachromat will yield much brighter images, with better resolution than the standard 40X (N.A. 0.65) dry planachromat or achromat.

Question 47. Are there disadvantages to choosing a 50X oil objective as suggested?
Answer: The 50X oil planachromat is approximately twice as expensive as the 40X "dry" planachromat. Also, oil objectives are difficult to use with haemocytometers because the oil may adhere and inadvertently lift off the coverslip.

Question 48. Is it usually advisable to buy the "best" (highest correction) objectives that your budget will allow?
Answer: Yes, but with a few caveats: If you are doing mostly observations of specimens thicker than several microns, planachromats or planfluorites may serve quite well because they have greater depth of field than comparable magnification planapochromats. For color photomicrography, planfluorites are capable of rendering better color images than can planachromats. For the finest color photomicrography (and observation) of minute details, planapochromats are the best choice but several times more expensive than the planfluorites.

Question 49. Is it important to choose the highest available N.A. objective for video microscopy of minute specimen details?
Answer: Yes. Although the image observed through the eyepiece, with the condenser aperture 100% open, may have so much glare that details become invisible, the "information" probably is there. Video enhancement techniques, controlling brightness and contrast, can process this "information" and render and excellent, high resolution, visible video image of the details.

Question 50. Are the NEW Olympus infinity-corrected metallurgical objectives parfocal with the NEW infinity-corrected biological objectives?
A: Yes, they are. ■

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