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Tutorial on Practical Confocal Microscopy and Use of the Confocal Test Specimen

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INTRODUCTION

The other chapters in this book give the reader an in-depth description of every important aspect of biological confocal microscopy that we could think of. This chapter is to provide the novice user of this instrument with a basic understanding of the practical information needed to use it effectively. Because the computer interfaces of the various commercial instruments vary greatly, this chapter will stress the important features of microscopical optics and the basics of sampling that are common to all instruments. The underlying agenda of these suggestions is based on two principles:

- Don't waste photons.
- Get all of the optical performance that you have paid for.

Getting Started

In order to be able to operate a confocal microscope properly, one must first be thoroughly familiar with the basic principles of nonconfocal microscopy. The user must understand the concept of conjugate image planes, Köhler illumination, and how to set up a microscope to produce it [i.e., the field diaphragm must be in focus in the image plane and the condenser aperture set so that the illumination almost fills the back-focal plane (BFP) of the objective lens. See the Appendix to this chapter or Chapter 6, *this volume*, for a refresher]. The user must also understand how the phenomenon of diffraction acts to place **the** fundamental limitation on the resolution that any optical system can attain (see Chapters 1, 8, and 11, *this volume*, or an introductory text such as Bradbury, 1984, Inoué 1986, Pawley and Centonze, 1997).

The next step is to get an image of some specimen that you understand. Without going into the details of the many available commercial confocal systems, we note that most confocal microscopes permit the user to set up the microscope for normal, nonconfocal use, and then switch over to confocal operation by changing the position of a single control. The user should follow the instructions from the manufacturer to produce such a confocal image using a live mode (i.e., producing an image that is continuous and not frame-averaged or Kalman filtered).

In most cases, the fluorescent specimen chosen for this first attempt at using the confocal microscope should be similar or identical to one that has just been viewed with success on a conventional instrument. Almost invariably, new users will close the detection pinhole in order to get the highest resolution and increase the zoom magnification to increase the size of the image on the display. They will then try to adjust the focus, but after a few seconds, the image will begin to fade and become indistinct. Reducing the zoom magnification will reveal the terrible truth: the observations so far have severely bleached the dye in the rectangular area that was being scanned at the higher zoom magnification. This is the moment at which would-be users either decide right then and there that the confocal microscope clearly produces far more bleaching than they are used to and leave the room, never to return, or they decide that there may be a bit more to operating this instrument than they had bargained for and set out to do better. This chapter is written for those who fall into the latter category.

Bleaching — The Only Thing That Really Matters

There are three reasons that the bleaching rate of a confocal microscope often **seems** to be much higher than that of a conventional epi-fluorescent microscope:

1. The instrument **can** be used in such a way that the **supposition is correct**! A laser is a far more intense light source than a Hg arc. Although the total power striking the entire specimen may be less with the laser, the area of the specimen over which it is absorbed can be reduced arbitrarily by increasing the setting of the zoom magnification control, thus increasing the power/unit area. The crucial difference between the two microscopical methods is that in normal epi-fluorescence, the power/area on the specimen is fixed by the type of arc and the illumination optics, while in confocal microscopy, the power/area increases with the square of the zoom magnification. In normal epi-fluorescence, one does not expect to see an image that is **both large enough** to see the finest details and **bright enough** to view by eye because it is not possible. In the confocal microscope, the extreme intensity of the laser does make it possible, though only for the moment until bleaching occurs!
2. If one is accustomed to viewing a widefield (WF) image in which fluorescence from a large number of focal planes is added together into a single image, a confocal image may look somewhat anemic because, ideally, it records only the fluorescence features present in a single optical section. When presented with an image of the few features that happen to be in the focus plane, one may be tempted to use a much longer exposure than is necessary in a vain attempt to record features that are not really there.
3. A final factor contributing to the perception that confocal bleaches faster is that, in normal epi-fluorescence, one expects to make one exposure that may require 30 to 60 s, while in the confocal microscope one often wants an image that is bright enough to look at after collecting for only 1 to 2 s and then sets out to

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record 20 to 30 similar images of the adjacent planes to form a three-dimensional (3D) stack. Each of these 20 to 30 images will cause additional bleaching.

In actual fact, what one should be interested in is not so much the bleaching rate but some sort of efficiency ratio of information recorded to illuminating dose. This ratio is set by the numerical aperture (NA) and transmission (T) of the optics and the quantum efficiency (QE) of the photodetector. Therefore, assuming equal optics, the confocal microscope is 10× to 100× more efficient than photography, because at these low light levels, the photomultiplier tube (PMT) photodetector that it uses is 10× to 100× more efficient than film (Chapter 12, *this volume*). The only photodetector more efficient than the PMT is the cooled charge-coupled device (CCD), and this detector can be used in disk-scanning confocal microscopes (Chapters 10, 12, 21, 25, and Appendix 3, *this volume*).

While on the subject of bleaching, there are a few additional items that should be mentioned. It is generally assumed (1) that bleaching is proportional to the total dose of light where dose is the illumination level (in photons/μm^{2s}) × time, and (2) that, as absorption is usually low, the total number of photons passing through any horizontal plane is a constant. From these facts, some jump to the conclusion that bleaching occurs at the same rate in the focus plane as it does outside this plane. While this may be true for short distances above and below the focus plane and when using lenses of low NA, it is not true once the height of a stack of images becomes comparable with the width of the scanned area. Figure 28.3(B) in Chapter 28 shows an *xz*-image through such a bleach pattern made with an NA 1.4 in a specimen of fluorescent plastic. It confirms that there is a pyramid of bleached dye expanding above and below the plane of focus. As the illumination intensity at any level is proportional to the cross-sectional area of the light cone at that level, one can see that, with a large NA lens, the illumination level and, therefore, the bleach rate drops off quite quickly with distance away from the focus plane. The illumination level at these planes is not constant within the cone but is less strong near the edges.

Figure 35.1 is a diagram to help visualize this process. We assume that the BFP of the objective is uniformly illuminated and, therefore, light is focused into the focused spot from all angles equally. As a result, the total number of photons passing through any particular level within the cone of the beam is a constant. The intensity itself decreases roughly with the square of the distance away from the focus plane but, as the beam scans over the raster, this effect is counterbalanced by the fact that most points in non-focus planes will be illuminated for a longer time. Although points that are only illuminated by the outer rays of the cone when the beam is scanning near the edge of the raster will only be illuminated once per scan, those near the center of the raster will be illuminated for a time that is longer in direct proportion to the reduction in the illumination intensity at any instant. All of the points for which this is true for a particular focus position will lie inside a bleaching octahedron having the scanned area as its base and a defining angle equal to the acceptance half-angle of the objective (α). Inside this octahedron the bleach rate is constant, while outside it the bleach rate decreases slowly until it becomes zero in the region where even the outer rays of a beam scanning the edge of the raster never reach.

As the focus plane moves up or down in the specimen during a *z*-series, the total bleaching will be proportional to the superposition of the octahedra of the individual planes. The result is that points inside the octahedron associated with the central focus plane will receive a maximum dose while those farther from the center will receive less. The reason for mentioning these simple consequences of geometrical optics here is to emphasize the difficulty of devising a computer program to correct for bleaching artifacts even if the bleach rate is assumed to be directly proportional to the energy deposited and the dye stays in one place. The best plan is to be careful not to waste photons and thereby minimize the amount of bleaching that occurs.

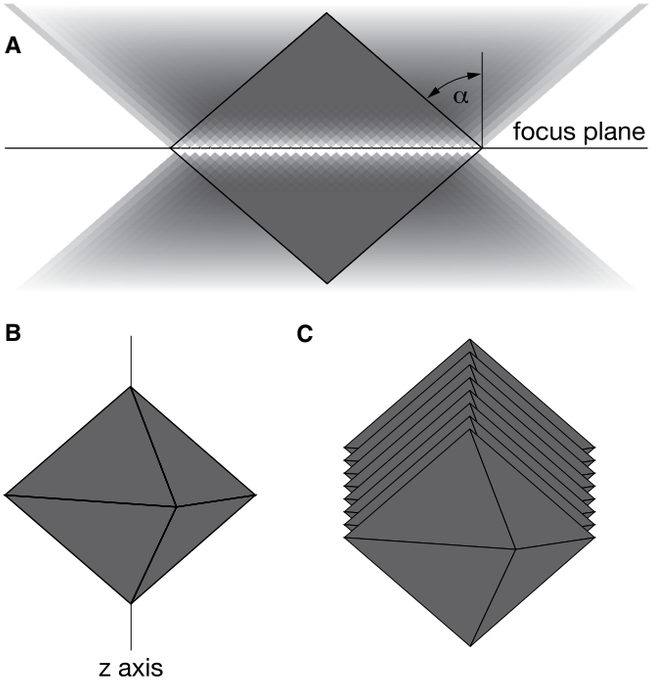

FIGURE 35.1. Pattern of bleaching in the confocal microscope. A stationary, focused light beam will bleach a conical volume in a uniform fluorescent specimen. The severity of the bleaching at any one level in the cone is inversely proportional to the area of the cone at that level and is most severe at the focus plane. However, as the beam is scanned over the focus plane, points in this plane are only illuminated a few times while points in adjacent planes will be illuminated for a longer time. As the beam is scanned over a line in the focus plane, the convergence angle of the beam (α) defines a triangle inside which reside points at which the lower intensity of the flux associated with not being in the focus plane is exactly compensated for by the increased amount of time they are illuminated [*black diamond* (A)]. As this line scans to create a 2D image, the triangle of equal total illumination becomes an octahedron (B). As the 2D focus plane is scanned in *z* to create a 3D image, the octahedrons add up to produce more damage near the center of the raster, where the planar constant bleach pattern is thickest (C). It should be noted that, because of the rather slow response of the mechanical scanning mirrors, a considerably higher level (10×–20×) of bleaching will occur to either side of the imaged area of the focus plane where the horizontal motion of the mirrors slows down and reverses. In some confocal instruments, the beam is blanked during this retrace period.

Although the bleaching problem may be no worse in confocal microscopy than in normal fluorescence microscopy, it is still a very serious problem. In fact, it is not unlikely that, particularly when viewing living cells, one's success at using either method will depend on how successful one is at collecting and recording as great a fraction as possible of the fluorescent photons produced and at recording only the information that one really needs while using no more than the minimum necessary exposure. We explain how to do this in the sections that follow.

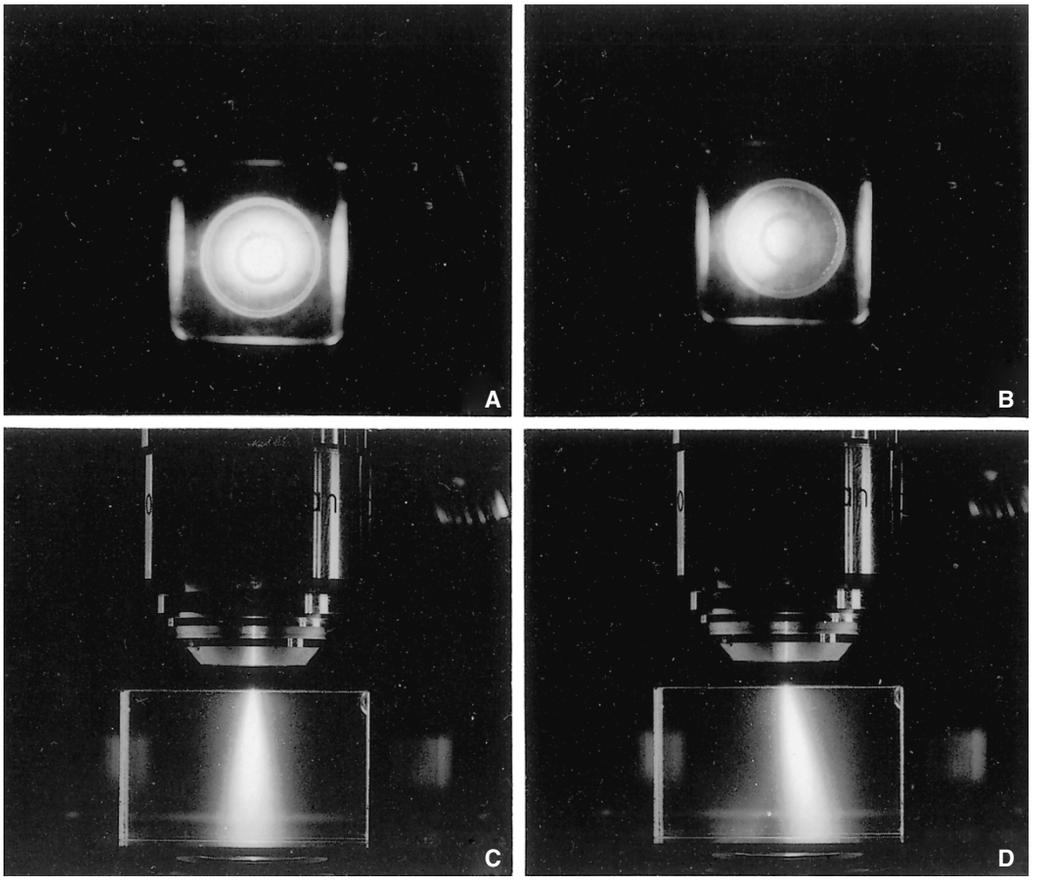

FIGURE 35.2. Alignment of the laser in the back-focal plane (BFP) of the objective as seen using a 45° prism that allows one to view a circular target situated in the BFP. (A) Properly centered, (C) misaligned. The two lower images show the bundle of light rays projected into the specimen under each alignment condition. These images were made by photographing the fluorescent light emanating from a cube of uranium-doped glass placed in the specimen plane of a 10×/0.45 objective. The angles involved are somewhat smaller than expected because the glass has a very high index of refraction.

GETTING A GOOD CONFOCAL IMAGE

To obtain an optimal confocal image, a fluorescent specimen must first be in focus under the right type of objective: that is, one that is designed to focus into a medium having the refractive index of your specimen, thereby avoiding problems with spherical and chromatic aberration (see Chapters 7 and 20, *this volume*). Because of the optical sectioning property on the confocal microscope, specimens that are far out of focus will produce **no signal**! If you are having trouble finding the specimen, open the pinhole all the way, focus on the specimen, then close it down again.

Next, the scanhead must be properly aligned. When the beam scans over the center of the raster pattern, the light from the laser should be both on axis and fill the BFP of the objective, and the light passing up from the focused spot to the PMTs must be aligned to pass through the pinhole. Once alignment has been achieved, the pinhole size should be set for the best compromise between signal strength and *xy*- and *z*-resolution, (for details see Chapter 22, *this volume*) the PMTs should be adjusted to produce an appropriate signal level and the monitors should be adjusted so that the full range of signal can be viewed.

Proper alignment[1] of the scanhead first requires adjustment of the mirror(s) that direct(s) the laser light down the optical path of the microscope. A 45° angle prism (or even just a small piece of lens-cleaning tissue) can be positioned in place of an objective so that the laser light path can be viewed with respect to the BFP (Fig. 35.2). Although the **angle** at which it passes the BFP is proportional to the instantaneous position of the beam in the imaged plane, the laser beam should **always** pass through the center of the BFP and then be focused by the objective to form a symmetrical spot at the specimen plane [Fig. 35.2(A,B)]. Misalignment at the BFP may mean that the light does not fill the pupil of the objective, reducing its effective resolution and causing it to pass through the specimen at an angle [Fig. 35.2(C,D)].

Next, a standard fluorescent or reflective test specimen must be used to align the scanhead so that the central maximum of the

[1] Many newer confocal microscopes come pre-aligned and there are no user controls for adjusting the alignment as described in this section. However, some of the tests used to check performance can still be used to see if you need a service visit.

diffraction-limited spot (Airy disk) in the specimen is focused through the pinhole where it can be detected by the PMT. When aligning for peak signal, it may be helpful to change the display to have a colorized lookup table (LUT). A colorized LUT emphasizes small changes in signal intensity because the eye is more sensitive to absolute hue than to absolute gray level. While scanning, adjust the scanhead mirrors or pinhole location to maximize the signal received by the PMT. For the time being, you need only be concerned with obtaining the brightest image, not one of maximum quality. It is advisable to make the mirror adjustments using the objective you plan to use for your final high-quality image. Because wedge errors in the dichroic beam-splitter can displace the apparent position of the pinhole (Chapter 9, *this volume*), older instruments will require re-alignment of the scanhead whenever the dichroic is changed.

Once one has a reasonable image, it is probably worthwhile to make a note of the settings of all the user controls. These can later be used to monitor instrument (and operator!) performance and to serve as starting conditions after one has implemented major service or development changes.

With basic alignment complete, you may find it interesting to view the Airy disk in the pinhole plane. On older scopes this can be accomplished by placing a small CCD camera in the intermediate image plane, but if your instrument uses a larger, iris-diaphragm aperture rather than a real pinhole, you may be able to see the light directly by viewing it scattered by a piece of paper placed in front of the pinhole. Figure 35.3 shows the sort of image you can expect to see if you do this by placing a piece of paper in the second filter well of a Bio-Rad MRC series scope that has been set up to measure BSL from a plane mirror surface that is either in focus [Fig. 35.3(A)] or just out of focus [Fig. 35.3(B)]. The in-focus image shows some astigmatism, probably caused by imperfections in the beam-splitter. It is informative to see how the image of the Airy disk degrades if one reduces the zoom magnification, a process that reveals the deleterious effect of off-axis aberrations such as astigmatism, curvature of field, and coma (see also Chapter 11, *this volume*). One can also use this setup to view the asymmetrical increase in Airy disk size (and decrease in resolution) caused by misaligning the illumination in the objective BFP (as in Fig. 35.2).

Actually, viewing the Airy disk helps to make clear the idea that each objective has an optimum pinhole size because it becomes evident that the light at the pinhole plane is simply a magnified view of the light in the image plane and that, therefore, at a fixed NA, the size of the image of the spot will be proportional to the magnification of the objective. A pinhole aperture equal to the diameter of the first minimum in the Airy disk (i.e., 1 Airy unit) will pass about 80% of the in-focus signal and still have a bit better *xy*-resolution than a widefield microscope. Once this benchmark detector aperture is known for a given optical setup (wavelength, NA, magnification), it is possible to make informed choices about the most appropriate aperture for any other objective.

Specimen preparation, objective lens, pinhole size and alignment, and focus plane all affect the amount of light collected from the specimen. Adjustment of the display monitor and the PMT affect the image viewed. The black level of the PMT amplifier must be set so the full range of signal can be detected. This can be done by scanning while light is blocked from entering the PMT and adjusting the black level until the rastered area becomes **just visible** compared to the unscanned part of the monitor screen.[2]

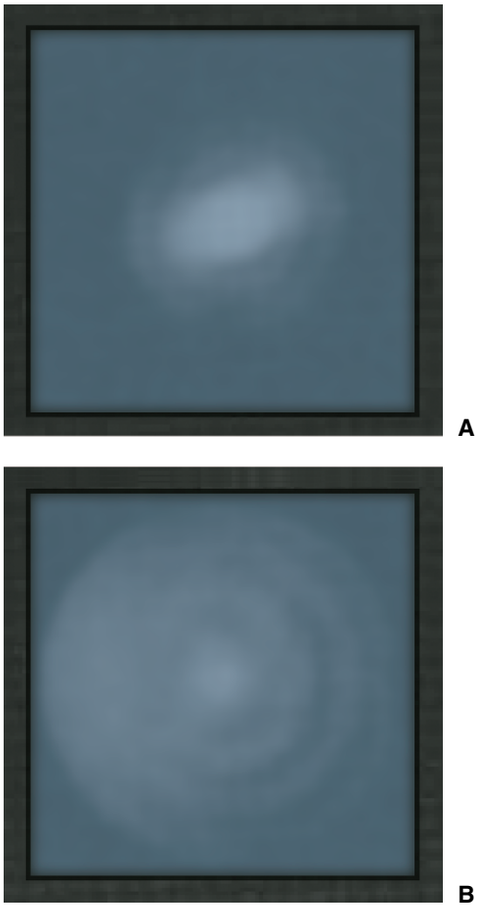

FIGURE 35.3. The Airy disk reflected back from a mirror test specimen can be viewed directly at the pinhole plane of a Bio-Rad MRC confocal microscope by removing the second filter block and placing a piece of paper into the bottom of the well. (Turn the PMT all the way down before doing this!) (A) In focus, but showing slight astigmatism, probably because of distortion by the dichroic; (B) slightly out of focus. Random points of light well away from the optical axis defined by the Airy disk represent stray light. This is less evident in (A) because of the higher relative brightness of the central spot and because the beam dump in first filter block had been improved by the addition of a piece of black velvet.

Note that if the black level is set too high, some intensity values may fall below the voltage corresponding to the zero value of the analog-to-digital converter (ADC) and so not be recorded. If, after setting the black level in this way, the image on the display has insufficient brightness, this can usually be best adjusted by modifying the display LUT in the computer.

The gain of the detector circuitry (i.e., how much it amplifies) is changed by adjusting the accelerating voltage of the PMT. In round figures, a 50 V increase in the accelerating voltage doubles the gain. Practically speaking, the gain controls both the brightness and the contrast of the image. When it is properly adjusted, the pixels in the brightest areas of the image should be bright white in order to utilize the full gray-scale range of the data handling system. However, one must also be careful not to **saturate** the ADC and this may be more difficult to avoid than expected because, in fluorescence confocal microscopy, the brightest signal may represent only <16 counts/pixel. Because of statistical factors discussed later in this chapter, an average value of 16 counts/pixel really implies 16 ± 4 counts/pixel. Therefore, if one is to avoid

[2] When the black-level control is set correctly, black areas will register as 2 to 3 ADU levels out of 255.

truncating data representing such small numbers of quantum events, one must be careful that the highest value of the ADC output (usually 255) represents **not the average** value of the brightest pixel but rather an unusually high measurement of this bright value (at least 16 + 4 = 20 in this example). In other words, if the gain is set properly, the value of the brightest pixel of a fluorescent image resulting from the average of several frames[3] should probably be no more than 200 to 220 and not 255. One can check the gain setting by plotting a histogram of pixel intensities or by using a colorized LUT to verify that the brightest pixels in a live (not averaged) image are below the saturation level of the digitizer.

It should now be possible to obtain a reasonably good image of the specimen. If, however, the images are not satisfactory, one should check the following parameters:

No Signal

- Can you see laser light of the appropriate color **emerging** from the objective? Is a shutter or a prism obscuring the beam?
- Is the specimen near the focus plane?
- Is the computer system adjusted to display the signal from the PMT that is actually receiving the light signal?
- **Never panic** and turn up the PMT all the way in a vain search for a signal that does not exist only to find that you have damaged the PMT by overloading it when you finally reset the control that had been obscuring the signal!

Low Signal

- Do **small** adjustments of the alignment knobs **reduce** the signal level as they should if the beam is properly aligned?
- Is the laser power sufficient? Are neutral density filters attenuating the light too much? Does the laser cavity need retuning? Are the optics used to launch the laser light into the fiber correctly aligned?
- Is the laser line appropriate for exciting your dye and are the correct filters in place?
- Is the specimen stained properly and has an anti-fade agent been included in the mounting media? Check by viewing the same specimen in the non-confocal fluorescence mode if available.
- Is the sample too opaque for the laser light to reach the focus plane and for the emitted light to exit the specimen without excessive scattering losses?
- Are the settings of pinhole size, PMT gain, and computer display system correct?

Simultaneous Detection of Backscattered Light and Fluorescence

The exercises that will be described in the following sections are easier to perform if one can arrange the system so that it can detect both backscattered light (BSL or reflected light) and fluorescent light simultaneously (Pawley *et al.*, 1993). Such a system is diagrammed in Figure 2.7 and provides the optimal removal of specular reflection artifacts from optical surfaces above the objective. A 1/2-wave plate in front of the laser is used to rotate the polarization plane of the beam so that a suitable fraction of it (0.8%–5.0%) is reflected down the microscope axis by an uncoated, clear glass beam-splitter in the first filter block. The light then passes through a 1/4-wave plate between the ocular and the objective before reaching the specimen. Light either reflected and/or emitted from the specimen returns via this same pathway, through the 1/4-wave plate and the dichroic beam-splitter to the second filter set. This system is constructed to pass the fluorescence signal to PMT 1 and the reflectance signal to PMT 2 via a rotatable 1/4-wave plate, analyzer, and laser-line filter. During setup, the rotatable elements of the second filter block are adjusted to eliminate axial specular reflections and the lower 1/4-wave plate is rotated to obtain a maximum signal from a biological specimen (Fig. 35.12 shows a stereo, BSL image obtained with this system). The operation of a similar system is described in more detail in Chapter 17. Because this system uses a different beam-splitter, the mirrors of the scanhead will need to be re-adjusted as described above.

NEW CONTROLS

Getting the best out of a confocal fluorescence microscope requires consideration of several matters that do not affect normal light microscopy. In order to extract as much information as possible from every photon emitted by the specimen, the user must set two unfamiliar parameters properly: pinhole size and zoom magnification.

Photon Efficiency

If either the pinhole size or the zoom setting are set incorrectly, the specimen may be subjected to much greater levels of exciting light than should be necessary to produce an image of a given quality. This will increase bleaching unnecessarily.

This is particularly important when examining living specimens containing fluorescent substances because, in this case, the exciting illumination inevitably produces both bleaching and cytotoxicity (MacIntosh *et al.*, 1990; Chapter 39, *this volume*). The bleaching itself will be more severe than with fixed specimens because on living specimens, one generally cannot use anti-fade agents (except, perhaps, oxygen deprivation or Trolox). In general, the **biological reliability** of the data is likely to be inversely proportional to the laser power used. In other words, even though using more light may make the **image appear more distinct**, it may also damage the object that the image is supposed to represent so seriously that the data is merely a better image of a worse specimen (see Chapters 38 and 39, *this volume*).

Pinhole Size

The advantage of the confocal method of image formation is that light emitted or scattered into the objective from planes other than the focus plane will be out of focus when it reaches the pinhole plane [Fig. 35.4(A)]. As a result, most of it will **not** pass through the pinhole and, to the extent that this happens, any image produced from the detected signal will be characteristic of only a single plane. For this reason, the confocal microscope is said to make optical sections.

The diameter of the aperture in front of the photodetector is usually adjustable so that it can be matched to the size of the image of the Airy disk (r_{Airy}) that the objective lens projects from the excited spot in the specimen to the pinhole plane [Fig. 35.4(B)]. The diameter of this disk is directly proportional to the total magnification between the object and the pinhole plane (see Chapter 2, *this volume*) and through the Abbe equation,

[3] Averaging several frames reduces statistical noise and means that the number recorded is closer to the real intensity value for each pixel.

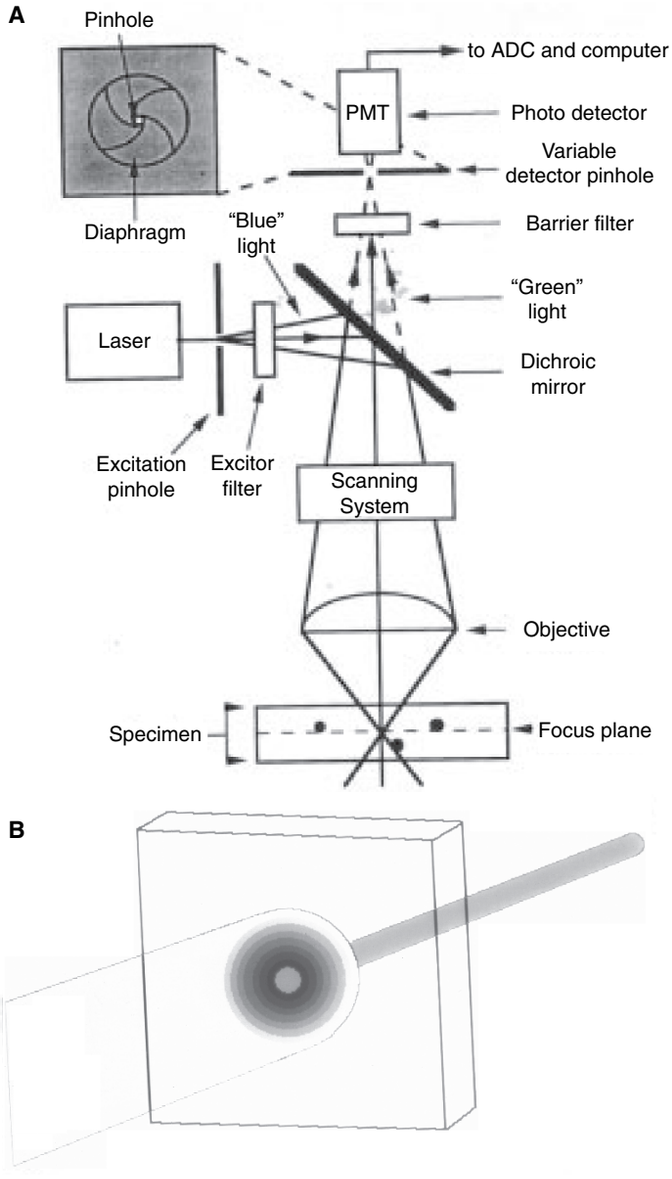

FIGURE 35.4. Confocal schematic. (A) Block diagram of the optical components of a fluorescent, laser-scanning confocal microscope: Short wavelength blue light from the laser passes through an exciter pinhole and filter and is then reflected by the dichroic mirror into the objective lens. The lens focuses it at a spot within the specimen. Some of the green fluorescent light from the specimen returns through the objective. Because of its longer wavelength, it can pass through the dichroic and barrier filters, and it comes to a focus at the plane of the detector pinhole. That fluorescent light which originated from the plane-of-focus passes through the pinhole to the detector while that from other planes is out-of-focus at the pinhole plane and so is selectively excluded from the detector. (B) The relationship between the hole in the pinhole and the Airy disk focused at this plane.

$$r_{\text{Abbe}} = 0.61\lambda/NA \quad \textbf{(1)}$$

it is proportional to the wavelength λ and inversely proportional to the NA of the objective. The **area** of the central spot of the Airy disk at the pinhole plane increases by a factor of over 12 if, for example, a 10× NA 0.5 lens is replaced by a 100× NA 1.3 lens (assuming that both are used in such a way that their BFPs are fully illuminated). Because the signal that passes through the aperture from a non-planar specimen is generally proportional to the pinhole area, an incorrect adjustment of the pinhole size can significantly reduce the effective sensitivity of the microscope.

However, there is a second constraint on the choice of pinhole size. Because almost all of the light originating from the plane of focus will pass through a properly-aligned pinhole, 1 Airy unit in size, one might expect that there could be no reason for ever wanting to use any other aperture size. This might be the case if the diameter of the pinhole did not also affect the spatial resolution of the microscope in both the *xy*-plane and, to a lesser extent, in *z*. If the pinhole is made very small (<0.1 Airy units), the *xy*-resolution of the instrument is improved by ~40% over that set by the Abbe limit, but only at the cost of reducing the signal level by 95%. As the pinhole is made larger, it begins to accept more light while the *xy*-resolution is reduced. When it equals 1 Airy unit, 80% of the light originating from the focus plane is accepted, while a 10% resolution gain is still being realized. On the other hand, when the pinhole is opened still more, any extra light that it accepts **must** be that originating from either above or below the focus plane, and this reduces the optical sectioning effect as well as providing more photons.

Therefore, depending on the particulars of the experiment, it may be preferable to either close the pinhole down to improve *xy* spatial resolution or open it up in order to collect more signal at somewhat reduced *z*-resolution. The latter is particularly true when viewing sensitive living specimens because, as these will usually not tolerate the multiple exposures needed to record many, closely-spaced optical sections in any case, the *z*-resolution is bound to be compromised by inadequate sampling (see below) **unless** the optical section is broadened. In this case, lower *z*-resolution will actually **improve** the image.

For most specimens protected by anti-fade agents (Chapters 18 and 39, *this volume*), the best compromise between sensitivity and spatial resolution is found by setting the aperture to be equal to 0.5 Airy units, the diameter at which the Airy disk reaches 50% of its peak intensity. At this setting, about 60% of the light from the plane of focus will reach the detector, and the *xy*-resolution will still be a bit better than that produced by the same optics when used in a non-confocal manner.

It should be emphasized that, as the presence of epi-fluorescence and DIC attachments on an older non-infinity microscope may increase the effective magnification of the objective lens, these figures should only be used as a guide.

Stray Light

Stray light can reach the pinhole plane in many ways but, when imaging in BSL mode, the most common is **laser light** reflected back by the beam dump and then up the optical axis by the upper surface of the beam-splitter or scattered in all directions by dust on optical and mirror surfaces. Most of the diffuse off-axis scattering visible in Figure 35.3 is from this source, but one must also be wary of **room light** entering through the objective or through holes in the light-tight box surrounding the detection optics. Stray light can sometimes be reduced by improving the efficiency of the beam dump (see Appendix 1, *this volume*) but, in general, one can only control for it by measuring the PMT output for different pinhole sizes **without** a reflective specimen in the focus plane and then subtracting these values from the corresponding readings made with the reflecting surface in focus.

Is the Back-Focal Plane Filled?

Some early confocal microscopes applied the laser output directly to the BFP without first expanding it (Chapter 9, *this volume*). Though the exact size of the beam depends on the details of the design of the laser, it is usually ~1 mm. While this is a fairly good match to a 100× NA 0.8 objective, it is too small by a factor of 10 for a 10× NA 0.45 objective. If such a beam is used with such a lens, the effective NA on the illumination side will be only 10% of that marked on the lens, and the *xy*-resolution will be 10× larger. On air lenses, one can check the filling of the BFP by turning on the microscope with no specimen present and either parking the beam on axis or going to the highest possible zoom and then observing the pattern that the light leaving the objective makes when it falls on a screen (a piece of lens tissue will do) held a few centimeters away from the front of the lens. If the BFP is overfilled, the boundary of the bright ring of light will have a sharp edge to it. (For a listing of the dimensions of the BFP for representative objectives see Table 9.2, *this volume*.)

Pinhole Summary

- Make the pinhole a little smaller than the first dark ring of the Airy disk (~0.8 Airy unit).
- On **a non-planar (bulk) specimen**, the signal level is generally proportional to the area of the detector pinhole.
- At a fixed NA, the optimal pinhole diameter is proportional to the magnification of the objective, that is, the diameter appropriate for 40× NA 1.3 is only 40% as large as that proper for 100×, NA 1.3.
- Not adjusting for this fact can reduce signal 6.25×.

Circumstances may often require deviations from the benchmark settings of the pinhole size given above, but this should only be done after considering the potential cost in terms of biological reliability (smaller pinholes give better *z*-resolution but less signal, meaning the specimen must endure more excitation light).

Statistical Considerations in Confocal Microscopy

The visibility of small objects viewed in a confocal microscope depends not only on the optics of the beam-forming system but also on detecting a sufficient number of photons to permit the signal from the feature to be statistically discriminated from that of the background. The matter of visibility is complicated by the fact that the images of small features are transmitted through the optical system with less contrast than are the images of large features. In other words, one must detect and record an image with more statistically-defined gray levels if one expects to make small features visible.

A confocal image is recorded by detecting the light signal from each point in the scanned raster and storing a number proportional to this light intensity in a computer. The image can be seen by displaying these numbers as distinct gray levels at the appropriate location on a monitor (see Chapter 4, *this volume*).

The PMT detector used in most laser confocal microscopes is extremely sensitive. It is capable of producing one recordable event (a photoelectron, PE) for every 5 to 10 photons striking it, and as long as it does not get too warm and is shielded from all sources of extraneous light, it has almost no dark current. Using the fast photon-counting mode found on some instruments, useful images can be recorded in which the brightest pixel may represent only 10 PE (~100 photons) while the dimmest pixel may average only 0.1 PE (1 photon).[4]

Even though this performance is impressive, it is important to remember that the visibility of features in any image is limited by a relationship between three factors: the contrast intrinsic to the signal from the feature, the transfer of this contrast by the optics to the detector, and the statistical uncertainty associated with the number of photons detected from it.

When one records an image photographically, the number of photons involved is so much greater and the chance of detecting an individual photon is so much less, that the topic of photon statistics is seldom explicitly considered. In the discussion that follows, contrast refers to a measure of the variation of the signal intensity within the image.

The term "photon statistics" recognizes the fact that a beam of light is actually composed of photons, which, as elementary particles, are governed only by statistical laws. If, after many measurements, the mean brightness of a given pixel is found to be 25 PE, random statistical variations ensure that the actual number counted on any given measurement will be somewhere in the range of ±1 standard deviation (SD) from this mean value only 67% of the time. As photons obey Poisson statistics, the standard deviation is simply the square root of the mean, so 67% of the measurements will be in the range of 20 to 30 counts/pixel, and 33% of the measurements will be outside this range!

As a practical matter, a feature that is one pixel in size will only be recognizable or visible above the statistical noise present in the surrounding pixels if it differs from the mean of the background by 5 SD (Rose, 1948). In the case noted above, the 25-count single-pixel feature would only be visible if the average background signal level was about 1 count/pixel. In other words, in order to produce a visible feature in the image, the staining of an isolated bright pixel must be sufficiently intense that, even after its contrast has been reduced by the optical system, it can still produce a detectable signal that is ~25× larger than that of its surroundings. The effects of this statistical limitation can be seen in Figure 35.5, in which part of the test specimen has been imaged with an average of 2, 4, 8, and 16 counts/pixel in the bright features. We must emphasis that the improved visibility *per se* of the bars in the middle two patches is not caused by improved optical resolution, but merely by counting enough particles so that the features of smaller size can be recognized.

[4] Novice confocal users, having made an intensity histogram of one of their images and having found that at least a few pixels have every one of the 256 intensities possible in an 8-bit image, may jump to the conclusion that the stored number is equal to the number of detected photons. Although not quite impossible, this situation is extremely unlikely to be true. The misunderstanding is related to the role of the multiplicative noise that characterizes the performance of all PMTs. Once a photon is absorbed and a free photoelectron (PE) produced, the PE is attracted to the first dynode where it collides producing, say, four secondary electrons (SE), which then proceed to the second dynode. As Poisson statistics controls this process, on many repetitions, 4 ± 2 SE will be produced. Because a PE that produces 6 SE that strike the next dynode is likely to produce a larger final pulse at the far end of the dynode chain than one that produces only 2 SE, it becomes clear that all PE are not amplified equally. As a result, given suitable signal levels and contrast settings, even an image that actually consists of only two signal intensities (0 and 1 ± 1) **can** yield an image histogram showing all possible stored values from 1 to 255. Multiplicative noise masks the fact that fluorescent confocal images usually represent the detection of very few photons/pixel. If it did not, and if the system gain was scaled so that 1 PE = 1 unit in the memory, the posterized nature of the image would warn the viewer against having great confidence in the values actually recorded.

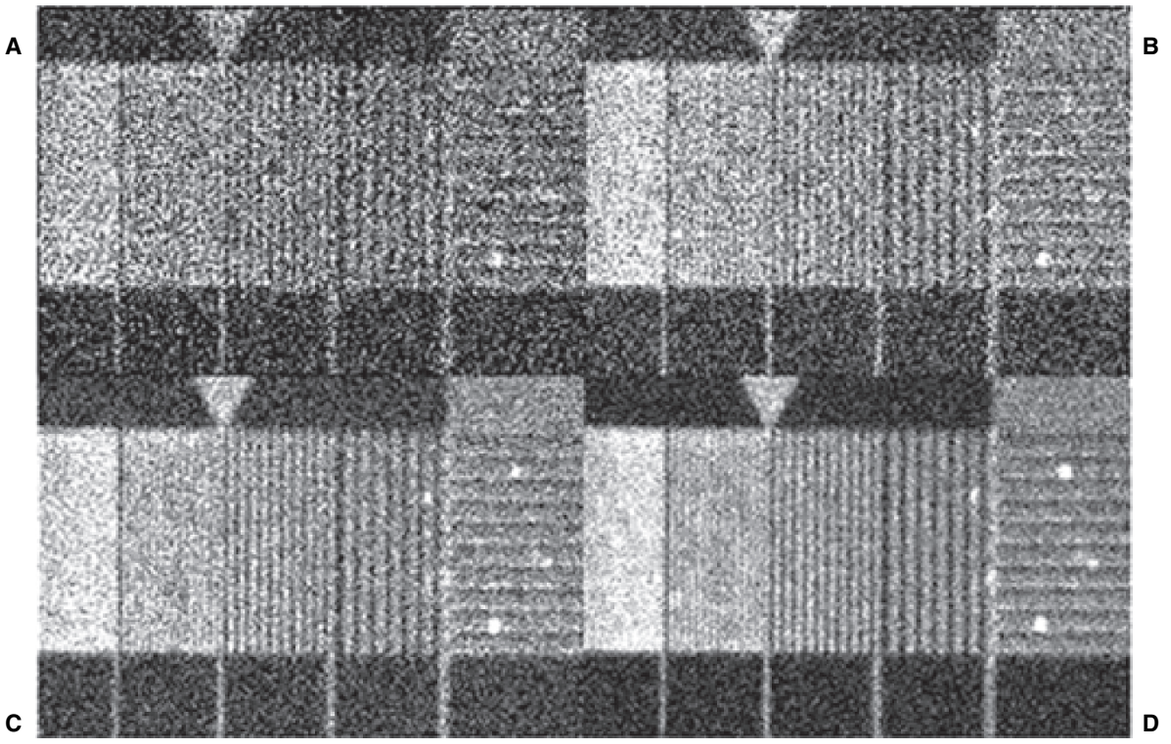

FIGURE 35.5. Confocal images made with few photons. Part of the test specimen has been imaged with an average of (A) 2, (B) 4, (C) 8, and (D) 16 counts/pixel. The improved visibility of the bars in the smaller patches is not caused by improved optical resolution but merely by counting enough particles so that these low-contrast features can be recognized.

Fortunately, as is shown in Chapter 4, with Nyquist sampling (see also next section) all real features cover at least 16 to 25 pixels in a two-dimensional (2D) image. Under these circumstances, it is the total number of counts recorded for the whole feature that is relevant to its visibility. Images containing six gray levels (corresponding to 0, 1, 4, 9, 16, and 25 events) can be usefully recorded with ~25 counts in the brightest pixel. The number of photons needed to see the bars in Figure 35.5 is less than the 25 PE used in the example above because the features are much more than one pixel in size.

The limitations imposed by counting statistics on feature visibility are immutable and are not affected by linear image processing (i.e., contrast and brightness settings). Therefore, when imaging specimens that have a stain contrast that is lower than 25 : 1, it is often necessary to average a number of scans to produce a useful image. This process does not literally "remove the noise," it merely permits more photons to be utilized, and this reduces the statistical variations in each of the thousands of the intensity measurements that make up the image. Because this process obeys Poisson statistics and the ratio of signal-to-noise (S/N), $S/N = n/\sqrt{n} = \sqrt{n}$, and counting 4× as many photons, will double the S/N making it possible to detect features in the image having only half as much contrast.

The Importance of Pixel Size

A pixel is that part of a digital image whose intensity can be accurately represented by a single number. Normally, each horizontal line of the rectangular scanning raster is divided into either 512, 768, or 1024 subdivisions, and each one of these subunits represents one pixel. The area of the specimen that can be properly represented by a single pixel varies with the magnification and NA of the objective and on the adjustment of the zoom magnification. The zoom setting controls the magnitude of the current waveforms supplied to the scanning mirrors and hence the size of the raster scanned on the specimen. Smaller currents make the light beam scan over smaller areas of the specimen, producing a higher magnification in the final image as displayed on the computer screen; larger scan currents produce lower magnification as the raster scanned on the object is larger in area.

Although the ability to arbitrarily change the magnification in this way is usually seen as a great convenience in terms of being able to fit the scanned area to the size of the object of interest, there can be hidden dangers associated with the incautious use of this control. As there is usually a fixed ratio between the size of the raster and the size of an individual pixel, adjusting the zoom control changes the area of the specimen represented by a single intensity value. However, all possible pixel sizes are not equally suitable for recording a set of digital data that retains all of the spatial information present in the original analog image.

The Nyquist Sampling Theorem states that, when a continuous, analog image is digitized, the information content of the signal will be retained only if the diameter of the area represented by each pixel (referred to the specimen) is at least 2.3× smaller in linear dimension than the resolution limit of the optical system (in x, y, and z!).[5]

[5] In various chapters in this book, the optimal number of samples/resolution-element varies between 2.3 and 4.0. Although reasonable arguments can be made for all these numbers, it seems safe to say that the larger ones are appropriate when the noise level in the image is low and when viewing small periodic objects. The smaller numbers are suitable for most confocal fluorescence images, because in these images, the high noise level prevents one from seeing small, low-contrast features. Consequently, the actual resolution may be considerably less than that predicted by the Abbe equation.

The optical resolution limit in the *xy*-plane is set by the Abbe equation (Eq. 1). This implies that, for each λ and objective lens used, there is an optimal setting for the zoom control. In the same way, the interplane sampling interval in a 3D data set should ideally be a bit less than half the *z*-resolution. Taking 200 nm as a typical value for r_{Abbe} for a high-resolution, NA 1.4 objective, a pixel width of 200/2.3 = 80 nm is needed to properly sample the data in the *xy*-plane, while the interplane spacing for the same optical conditions should be about 3× larger, or 240 nm. This means that the blob in the image that represents a point object will always be at least 5 pixels wide and 5 planes in height. It also means that the diameter of the probing beam is ~5 times larger than the interline spacing.

Measuring Pixel Size

Increasingly, the control software of the confocal microscope calculates and displays the pixel size currently in effect. If this is not so on your instrument, it is easy to calculate the pixel size as long as you know the dimensions of your raster. Just image a stage micrometer (available from major microscope manufacturers — a hemacytometer makes an inexpensive substitute for magnification calibrations), determine the actual width of the field of view in micrometers by matching it to the calibrated lines on the test specimen, and divide this number by the number of pixels in a line.

Alternatively, assuming that the service engineer calibrated the *x*- and *y*-magnification when the instrument was installed and that you have entered into the computer the NA and magnification of the objective in use, you can display a scale bar, and by measuring both it and the entire screen with a ruler, you can again find the field width and make the same calculation.

Finally, if your system allows you to measure the length of the scale bar in pixels, you can use this information to calculate the dimensions of the pixel. To meet the requirements set by the Nyquist criterion for a NA 1.4 objective, a 5 µm bar should be 5 µm/0.08 µm = 62 pixels long.

Adjust the zoom setting until this criterion is met. Note that **pixel size**, referred to the object, is **inversely proportional to zoom** magnification.

It should be noted that, contrary to much legend, satisfying the sampling criterion often requires a **higher zoom** setting for **low magnification** objectives than for those with high magnification. The reason for this is that although a 100× NA 1.2 lens has three times the resolution of a 10× NA 0.4 lens, the latter produces an image in which all features are 10× smaller, so the smallest visible feature (an Airy disk) will be 3.3× smaller in any image plane when using the 10× lens.

The exact mirror movements that produce a given pixel size will vary somewhat with the field of view that characterizes the microscope being used, with the way the instrument has been adjusted, and (on older instruments) with the presence of accessories, such as epi-fluorescence or differential interference contrast (DIC) attachments, that increase the effective magnification of the objective lens. As a result, the scanning system must be re-calibrated if it is moved from one type of microscope to another.

Over-Sampling and Under-Sampling

Now that we have determined the correct zoom setting needed to record the full resolution in your image from the sampling point of view, we should point out that there may be circumstances where other settings may be more appropriate.

Under-sampling

Using a lower zoom setting has the advantage of reducing the bleach rate and increasing the field of view: bleaching rate = $k(\text{zoom factor})^2$. On living specimens, therefore, one may wish to work at a lower zoom setting to preserve the specimen. Under these circumstances, you will lose little more in terms of image resolution if you also open the detector aperture somewhat. This will make the *z*-resolution somewhat worse, but as you will probably also have decided to image fewer optical sections, the lower *z*-resolution will actually assist in coming closer to the Nyquist criterion for sampling in *z*. It will also permit more signal to be recorded at each pixel. As a result of this under-sampling, you can get sufficient image quality using less laser power (i.e., use a darker neutral density (ND) filter).

Over-sampling

Using a zoom setting higher than that needed to satisfy the Nyquist criterion leads to over-sampling. Although over-sampling implies a smaller field of view and causes more bleaching in fluorescent specimens, it provides a larger image that may be easier to view and it will provide data that are marginally more suitable for image deconvolution, a digital image processing technique that can be used to increase image contrast and reduce noise (see next section). Over-sampling is also needed when imaging a periodic specimen having features near the resolution limit.

Nyquist Reconstruction and Deconvolution

It is often forgotten that Nyquist digital sampling has two parts: first digitizing and recording the digital data and then reconstructing the analog output from this data. The latter process imposes important limits on digital microscopy. Because the Abbe equation places an absolute limit on the resolution of microscopical image data, it is relatively easy to calculate the proper size of a pixel if you know λ and NA. However, Nyquist reconstruction requires that these same resolution bandwidth limits also be applied when the digital data is turned back into an image. Of course, it is easy to assume that, because no information regarding features smaller than the resolution limit of the optical system can emerge from the microscope, such information cannot possibly be present in the data and therefore, that it need not be removed. Sadly, this is not so. Because pixel-to-pixel intensity variations associated with measurement and Poisson noise are added to the signal before it is digitized and because these are often big enough to produce contrast comparable to that produced by variations in dye concentration in the specimen, Nyquist-sampled confocal data sets always include single-pixel noise that appears to represent features 4× to 5× smaller than the optical system could possibly have imaged.

This spurious data can only be eliminated by deconvolving the raw digital data using any reasonable point spread function (PSF). Although deconvolution is usually thought of primarily as a means of removing out-of-focus blur from 3D widefield data sets, the process has many other useful functions. In the present context, the chief among these is that deconvolution effectively suppresses spatial frequencies above the bandwidth of the microscope optics.

This idea is important enough to be repeated: Poisson noise can introduce features into the data that appear to be ~1 pixel in size when the smallest Nyquist-sampled feature should be >5 pixels across. These noise features can and should be eliminated by deconvolving the data, preferably in 3D.

As an aside, because the confocal PSF can be approximated as a 3D Gaussian blob, 3D Gaussian filtering has almost the same

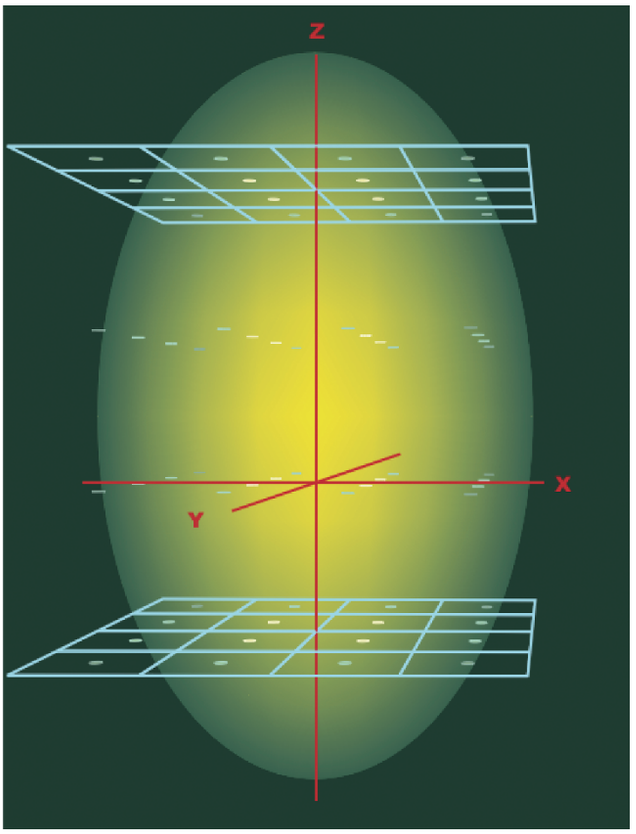

FIGURE 35.6. The relationship between resolution and the number of voxels needed to Nyquyist-sample the point-spread function. According to standard light microscopy theory, the Abbe resolution, δ, is defined as the radius of the first dark ring of the Airy disk. In confocal microscopy, this defines a blob with a diameter of 2δ. If we assume that a pixel is δ/2, then the 2D image of a point object will put signal into at least 12 to 16 bright pixels in the focus plane. The 3D image of a point requires collecting signal from at least four planes, separated by a distance equal to one half of the *z*-resolution. As a consequence of these factors, the 3D image of a point object requires measuring signal in 50 to 100 voxels. Averaging signal over these voxels (e.g., by deconvolution) does not reduce spatial resolution but does greatly improve the S/N of the resulting data. (See Chapter 25, this volume.)

effect as a full-scale 3D deconvolution, as least in terms of suppressing noise features.

Filtering the data in this way has another benefit: it effectively averages the voxel intensity data over the number of voxels containing significant counts in the Gaussian blob that represents the PSF. If we estimate that the blob is ~5 pixels wide in *x*, *y*, and *z* (see Fig. 35.6), then it will have significant counts in $5 \times 5 \times 5 = 125$ voxels. Therefore, 3D deconvolution will effectively average out Poisson noise over ~125 voxels: a significant factor (Chapters 19 and 25, *this volume*)!

Pixel Size Summary

To preserve all of the information in your data, you must use a pixel (or, in three dimensions, a voxel) size at least 2.3× smaller than the Abbe resolution limit of your optical system in (*x*, *y*, and *z*!!). This means that, for each objective, there is an optimal setting for both the zoom control and the interplane spacing of 3D data sets.

- The amount of light passing through the specimen is not affected by the zoom setting.
- At high zoom settings, the confocal microscope can easily illuminate the specimen with an intensity (and a bleaching rate!) that is 100× greater than in a normal fluorescence microscope using a Hg arc source.
- Fortunately, because the PMT detector is more sensitive than film, less illumination is actually required to record a usable confocal image.
- Set the zoom to produce a pixel size satisfying the sampling criterion for the lens in use.
- Don't forget to deconvolve (or 3D Gaussian filter) your data before viewing it.

USING A TEST SPECIMEN

Why Use a Test Specimen?

The tendency to use tried-and-true specimens when testing or learning how to operate the confocal microscope is so marked that it would be fruitless to recommend against it. However, it does have some disadvantages, and the chief of these is that one probably really does not know what a good confocal image of such a specimen would look like. This is because any stained biological specimen is probably too complex to understand so well that one can predict what a 3D image of it should look like and then adjust the instrument until this result is attained. Although we may have prepared hundreds of similar specimens and may recognize some as successes and others as failures, we seldom really know in a quantitative way either the exact size of any of the specific features that they contain, how much they have been stained, or how much this stain may have faded or bleached (Chapters 16 and 39, *this volume*). We may believe that such a specimen possesses a particular 3D structure when, if fact, this structure has been lost when it was inadvertently flattened during specimen preparation (Chapter 18, *this volume*). In short, such a specimen is not a test specimen, and one cannot really use it to measure either your own skill or the performance of the microscope.

Description of the Test Specimen

Microscope manufacturers have long produced a variety of test specimens for measuring the performance of their instruments. The most common of these is the stage micrometer, which usually consists of a graticle etched into a metal film on the lower side of a coverslip. Such patterns can be very useful for checking magnification but, because they are made using light optics, the finest spacings that can be produced on them are ~1 to 2 µm, and this is not fine enough to really test the ultimate **resolution** of a good optical microscope. To fill this void, we followed the lead of Oldenbourg and Inoué (Chapter 1, *this volume*) and designed a test specimen that we then fabricated at the National Nanofabrication Facility at Cornell University using electron-beam lithography (Pawley *et al.*, 1993).[6] The patterns were etched into a 50 nm Al film on the lower side of a 1 × 1 cm piece of #1.5 coverslip and

[6] Similar test specimens may soon be available again from Louie Kerr, at the Marine Biological Laboratory, Woods Hole, MA. Other test specimens are discussed in Chapter 36.

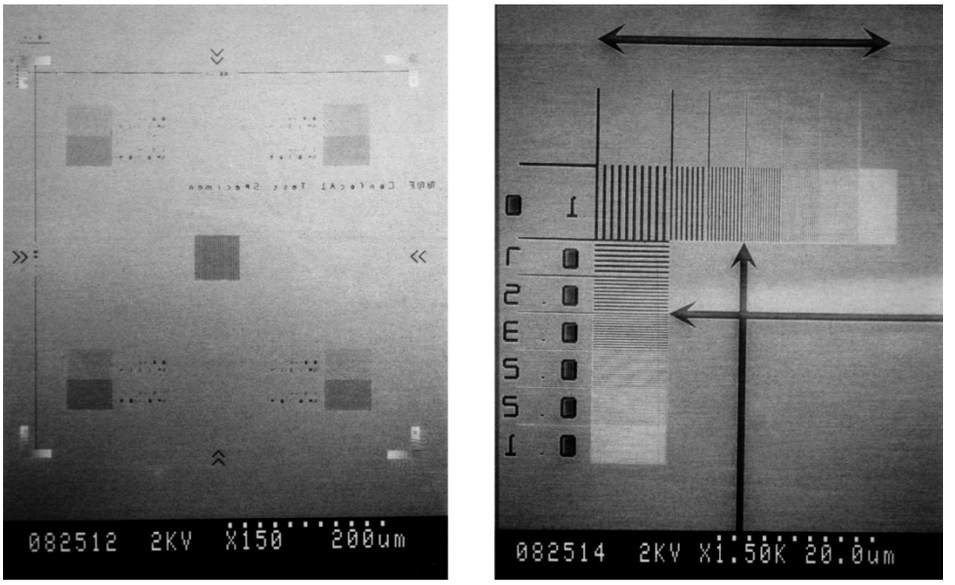

FIGURE 35.7. Scanning electron micrographs of a confocal test specimen fabricated at the National Nanofabrication Facility at Cornell University using electron-beam lithography. The pattern is etched into a 50 nm Al film on the lower side of a 1 × 1 cm piece of #1.5 coverslip and laid out as a square about 500 µm on a side (left). There is an L-shaped, resolution test pattern, 40 µm along each arm, at each corner with one 10 × 10 µm square of 1 µm period at the corner and six 5 × 10 µm rectangles of 0.7, 0.5, 0.35, 0.25, 0.20 and 0.17 µm period along each arm (right).

were laid out as a square about 500 µm on a side [Fig. 35.7(A)]. There is an L-shaped resolution test pattern, 40 µm along each arm, at each corner with one 10 × 10 µm square of 1 µm period at the corner and six 5 × 10 µm rectangles of 0.7, 0.5, 0.35, 0.25, 0.20, and 0.17 µm period along each arm [Fig. 35.7(B)]. The periods of the spacings are listed next to the pattern in the top right corner when the coverslip is mounted, Al-side down, so that the writing is legible. The procedure for measuring the optical transfer function of your microscope from images of such patterns is explained at the end of Chapter 1.

Such a test specimen can be used to record images using transmitted light, reflected light, or fluorescence. For the latter, the coverslip is mounted over a well into which dye-laced immersion oil has been introduced. As the test pattern is predominantly opaque, light can only reach the dye through the clear areas of the pattern [Fig. 35.8(A,B)]. Although the dye itself is essentially infinite in thickness, as long as the NA is reasonably high, most of the excitation is confined to a thin triangular region just under a clear space in the pattern and next to the coverslip [Fig. 35.8(C)]. Although some excitation light does reach the dye through adjacent clear lines in the pattern, this is only a minor effect as can be seen in the *xy*- and *xz*-images of such a specimen, shown in the upper half of Figure 35.8(A,B). Because the dye is a liquid, any bleaching that may occur is masked by the diffusion of new dye into the imaged area.

Using the Test Specimen

To image the test specimen in **reflected** light, treat it as you would any other. However, you may find it difficult to adjust the plane of focus to exactly coincide with the metal film. This is because, as is mentioned in Chapters 14 and 15, the **position** of maximum signal can be determined to a small fraction of the ***z*-resolution**, and in the case of reflected light, the stage-motion control on your instrument may not have sufficient precision to allow you to focus exactly on the metal surface.

Because you are attempting to view a periodic object, the zoom setting should be set about 2× higher than that normally required by the Nyquist criterion (i.e., 4 to 6 samples/resolution element). This is because the Nyquist analysis is based on information theory and, according to information theory, a periodic object contains only two items of information: the frequency and the phase. Although these can be determined from data sampled at the Nyquist rate, the image can look very poor. Figure 35.9 shows how,

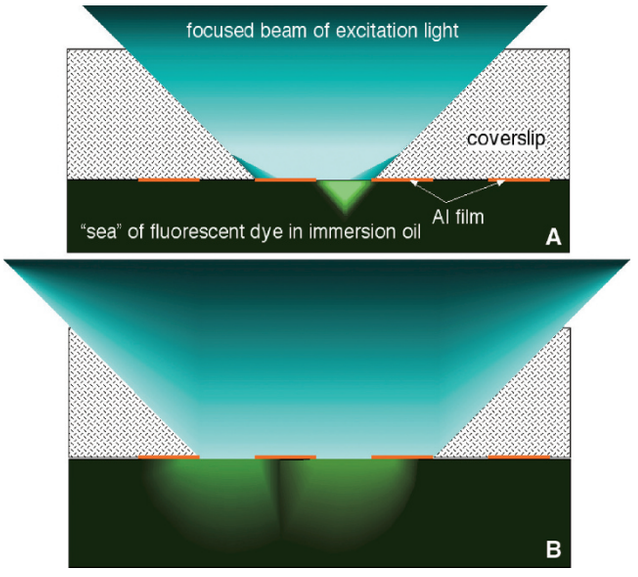

FIGURE 35.8. (A) Diagram of the fluorescent test object. Because it is difficult to fabricate a planar fluorescent test object that does not bleach, this device works by placing a pool of fluorescent immersion oil just below the etched metal pattern on the bottom of the coverslip. As long as the BFP of the objective is filled uniformly, much of the illumination will approach the specimen at relatively large angles, selectively exciting a triangular prism of dye beneath each etched line. (B) As the focus plane moves into the dye, some of the dye deeper in the specimen is excited by rays that pass through adjacent etched lines. However, this is only a minor effect that makes the *z*-response somewhat asymmetrical.

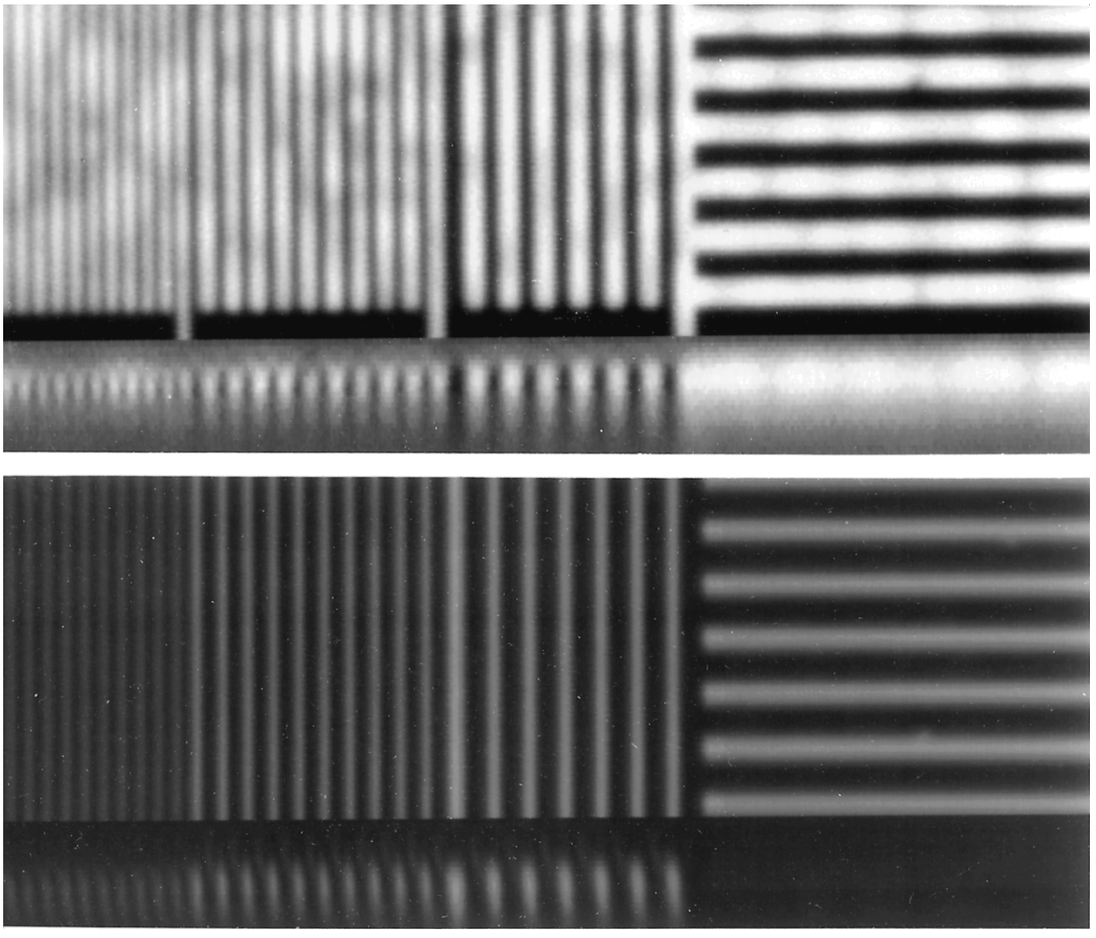

FIGURE 35.9. Images of the test specimen made using fluorescence (upper pair) or backscattered/reflected light (lower pair). The images were collected simultaneously and show mirror symmetry because the fluorescent light emerges from those areas not covered with the metal film that provides the reflected signal. The upper member of each pair shows an *xy*-image and the lower member shows an *xz*-image that makes more evident the asymmetry of the fluorescent signal. Nikon 60×/1.4 on a Bio-Rad 600/Optiphot. The vertical spacings have the following spacings left to right: 0.35, 0.5, and 0.7 µm (vertical) and 1.0 µm (horizontal).

when sampling at twice the frequency of the analog data but doing so just as the signal crosses zero, it is possible to miss the variations in a periodic signal entirely. As a result, one should choose 0.05 µm pixels or about 35 µm field width for a 768 pixel line for use with an NA 1.3 to 1.4 objective.

If you have done things correctly, the picture that you see should look like that in Figure 35.10(A). In these images, it can be seen that the edges of the vertical lines have a high-frequency wiggle (period about 4–5 raster lines = 16.6–20 ms), indicating the presence of mains-frequency electronic, magnetic, or mechanical interference. In Figure 35.10(B), the sets of horizontal lines above and below the axis of the computer-calculated Fourier transform (FT) below each image clearly demonstrate the presence of some other periodic instability present in these single-scan images. As the vertical spacings of the two finer periods are near the cut-off frequency of the contrast-transfer function, only a single spot is shown along the horizontal axis on either side of the vertical axis, but the spacing of all the first-order spots can be seen to be inversely related to the actual spacing of the features in each segment of the image. The horizontal bars in the FTs to the left of Figure 35.10(C,D) show the effect of Kalman averaging. Not only do the spots corresponding to the fine serrations of the edge get averaged out, but the S/N of the FT is markedly higher. The FT in the lower right of Figure 35.10(E) was made from a larger patch of the specimen having a 0.8 µm spacing. Three harmonics can be seen easily, but the fourth, representing 0.8/4 = 0.2 µm data, is barely visible.

If you have the equipment for simultaneous fluorescent/BSL imaging, the specimen can be imaged in **fluorescence** light simply by switching to the other channel. Otherwise the microscope must first be reconfigured for fluorescent light imaging. The image that you see should look like that in Figure 35.11.

The Diatom: A Natural 3D Test Specimen

The test specimens discussed have the disadvantage of being planar. Making a test specimen having precise structural features in the third dimension is more difficult.

The best solution so far is to immerse diatom frustules (North Carolina Biological) in fluorescent oil and view them as negative objects, as suggested by Roger Tsien (University of California, San Diego). Figure 35.12 shows a stereo view of such a preparation, imaged with BSL using the system mentioned above. Although careful attention to the exact species of diatom can result in highly reproducible spacings, this specimen is not without its problems.

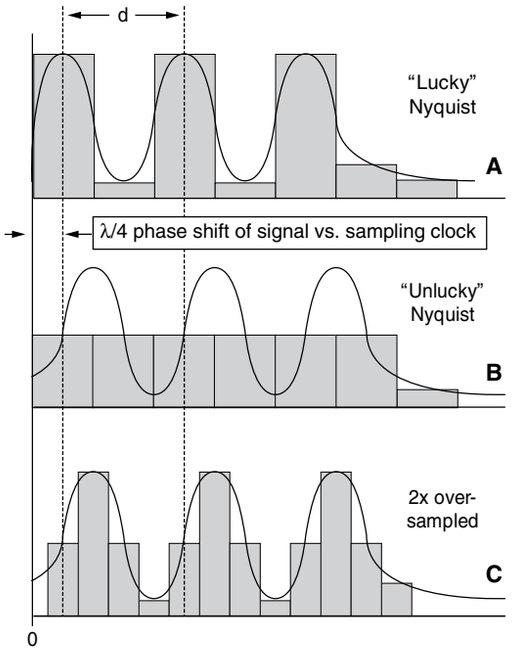

FIGURE 35.10. Data sampling of periodic objects. Although the Nyquist criterion states that only ~2 samples are required for each period of the highest spatial frequency in the data, this does not always work for periodic objects. Although a fortuitous phase relationship between the sampling instant and the positive and negative peaks of the highest frequency can lead to an accurate digitization (A), a phase relationship that sampled the signal only at the instant that the signal crossed the axis would not record this contrast at all (B). For this reason, it is safer to digitize images of periodic objects with at least four samples/period (C).

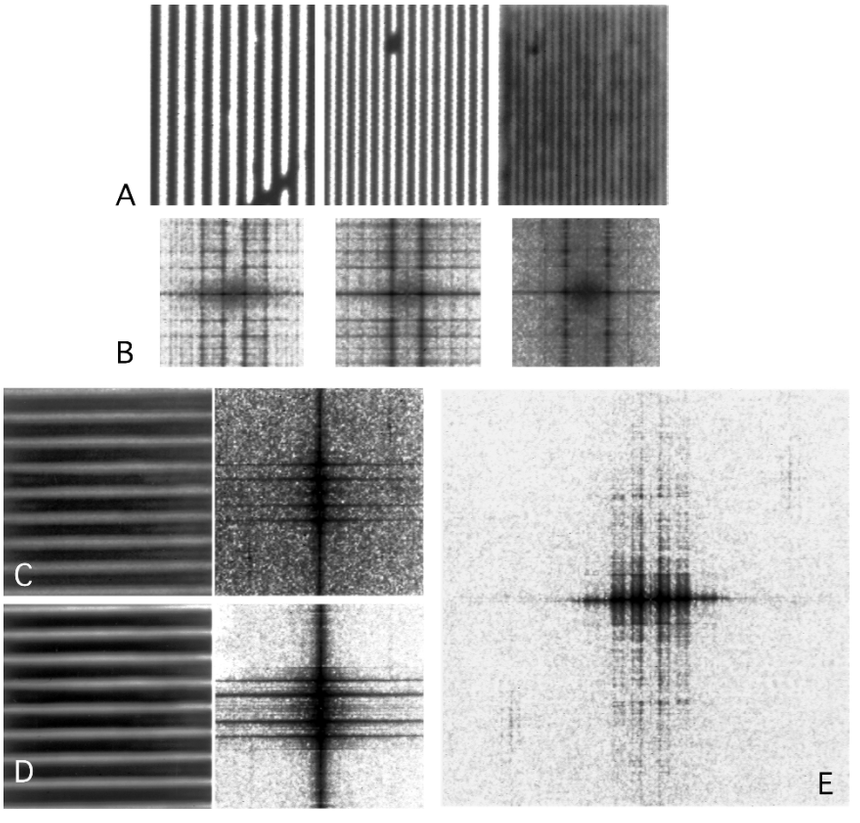

FIGURE 35.11. (A) High resolution reflected light image of test specimen recorded using a Nikon 60×/1.4 lens on a Bio-Rad 600/Optiphot. Spacing, left to right: 0.5, 0.35, and 0.25 µm. In (B), the sets of horizontal lines above and below the axis of the computer-calculated FT below each image clearly demonstrate the presence of a periodic instability in these single-scan images. As the two finer spacings are near the cut-off frequency of the contrast–transfer function, only a single spot is shown along the horizontal axis on either side of the vertical axis, but the spacing of all the first-order spots can be seen to be inversely related to the actual spacing of the features in each segment of the image. Panels (C, D) are reflected light images of 0.7 µm horizontal spacings made either live (C) or after averaging 20 scans (D). The horizontal bars in the FT plots to the left of each image show the effect of averaging. Not only do the spots corresponding to the fine serrations of the edge get averaged out, but the S/N of the lower FT is markedly higher. The FT in the lower right (E) was made from an image of a larger patch of the specimen having an 0.8 µm spacing. Three harmonics can be seen easily, but the fourth, representing 0.8/4 = 0.2 µm data, is barely visible.

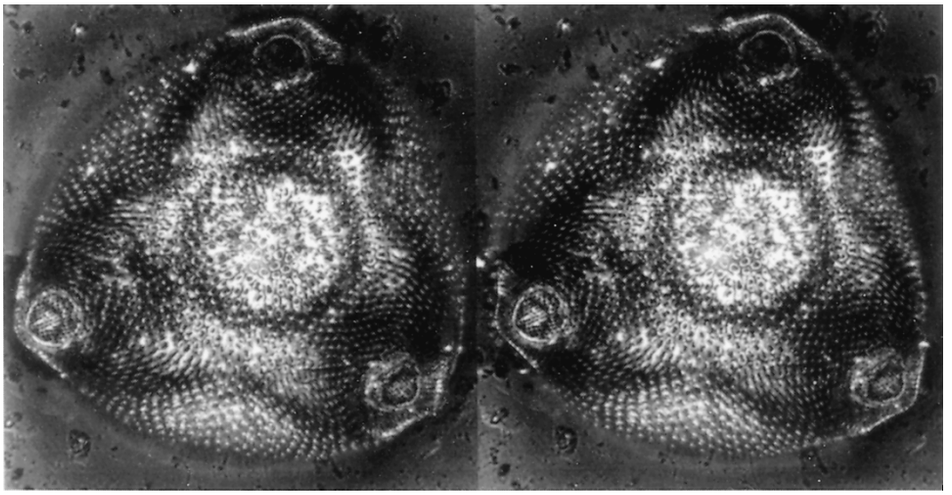

FIGURE 35.12. Stereo image of part of a diatom immersed in immersion oil made with BSL using a Nikon 60×/1.4 lens on a Bio-Rad 600/Optiphot equipped with the optimized BSL system described in the text and diagrammed in Figure 2.3. The diatom is resting on a microscope slide and is viewed from the top through the coverslip and two layers of immersion oil. The signal reflected from the slide surface can be seen around the edge of the image but the presence of the diatom distorts the focus plane away from the surface in the center of the field of view. (Specimen kindly provided by Nelson Navaro, C.U.P.R., Puerto Rico.)

Because the amorphous silica out of which the frustule is made has an index of refraction different from that of either water or immersion oil, it refracts and also scatters considerable light. While this is convenient for BSL imaging, it produces aberrations whenever the focus plane penetrates too far. This effect can be seen in Figure 35.12 where, around the edge of the image, one can see the signal reflected back from the flat surface of the glass slide on which the diatom is resting. However, in the center of the field the beam must pass the through the frustule to reach the surface and its presence distorts the focus plane away from the surface of the glass making it appear dark.

REASONS FOR POOR PERFORMANCE

Sampling Problems

As mentioned above, when imaging periodic objects, it is necessary to sample the image data at higher than the Nyquist rate. Failure to do so will result in the aliasing artifacts shown in Figure 35.13.

Optical Problems

Aberrations

As is mentioned above and covered in Chapters 1, 7, 8, and 20, one can only obtain diffraction-limited resolution if all optical aberrations are absent. The major aberrations are spherical aberration and chromatic aberration. In a high NA objective, spherical aberration is usually corrected for a number of wavelengths but **only** for one immersion medium. To get the recommended performance, you must use the immersion liquid for which the lens was designed or use a lens having a correction collar to adjust for different media.

As the focal length of any objective changes slightly with wavelength, its magnification also changes with wavelength, only in the opposite sense. As a result, when operating in fluorescence, an off-axis ray at the excitation wavelength will cross the intermediate image plane at a different point from the longer wavelength emission ray coming from the same point on the specimen. As a result, the emission ray may partially or totally miss the pinhole, resulting in loss of signal (Fig. 35.14)! Two other points are worth mentioning in regard to this second point:

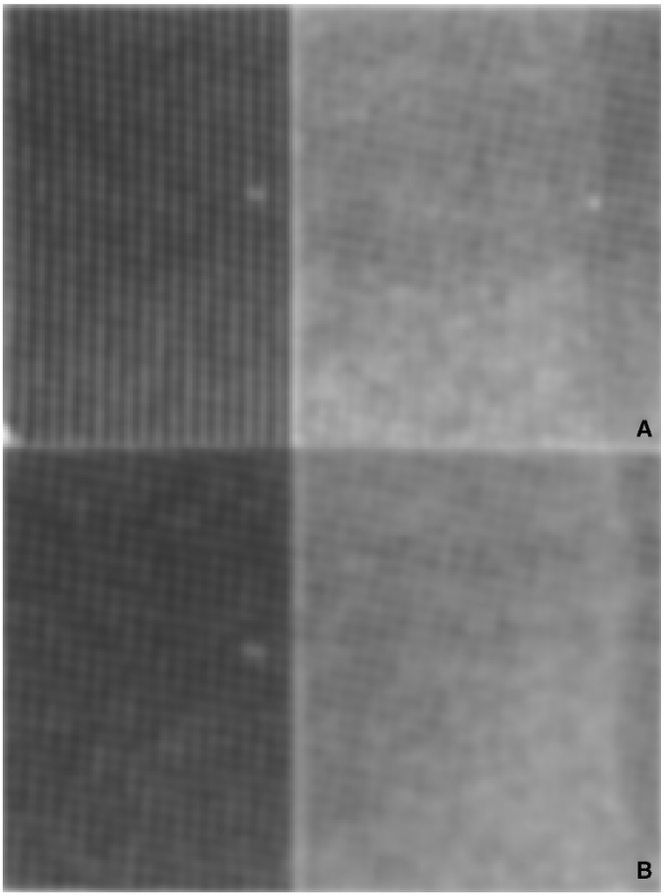

FIGURE 35.13. The effect of under-sampling on a periodic image. The upper image was recorded using reflected light with a Nikon 60×/1.4 lens on a Bio-Rad 600/Optiphot with 0.04 µm/pixel (twice the Nyquist rate for 0.2 µm structures) and then printed on a dye sublimation printer having pixels the equivalent of 0.018 µm in size. In it, both the 0.25 µm (left) and 0.2 µm (right) spacings are clearly seen. The lower image was made with identical optical conditions but the pixel size was 0.08 µm (exactly the Nyquist limit) and the image was recorded photographically from a video monitor having a screen pitch equivalent to 0.05 µm/elements. To some extent, the reduced contrast in the 0.25 µm spacing may be due to the bandwidth limitations of the monitor or the vagaries of the photographic process, but the virtual absence of the 0.2 µm spacings is probably due to sampling problems, as is evidenced by the presence of a faint aliasing pattern at about twice the period of the actual features in the image.

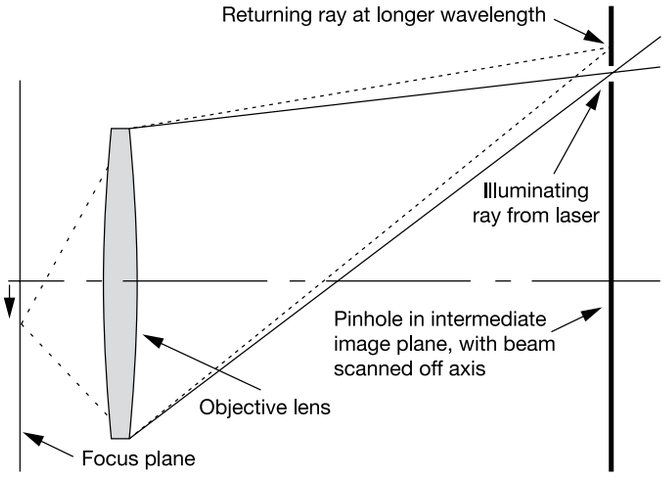

FIGURE 35.14. Lateral chromatic aberration. The effect of chromatic magnification error on signal level in the confocal microscope. The focal length and, hence, the magnification of any optical system varies with wavelength. Consequently, an off-axis ray of short wavelength light will not follow the same path as that of a ray of longer wavelength light originating from the same point. In the laser-scanning confocal microscope, the scanning mirrors are supposed to deflect both the source and the detector pinhole off-axis by the same amount. However, this will not happen if the optical system has high chromatic aberration and the system is used for fluorescence, because the exciting and emitting wavelengths are magnified by different amounts. As a result, the signal light will miss the pinhole and the image will become darker away from the axis. The problem can be reduced only by using optical systems that are highly corrected for chromatic aberration and by placing the field-of-view as near to the optical axis as possible.

- In many older microscopes, it was common to correct chromatic aberration in the eyepiece. Objectives from such microscopes will demonstrate totally unacceptable levels of chromatic aberration if they are not used in conjunction with the appropriate correcting eyepieces. Generally one should use only modern, highly-corrected objectives for fluorescence confocal microscopy and use them only with other components that are properly matched.
- The magnitude of the displacement caused by the chromatic magnification error is generally proportional to the distance that the point in the image is away from the optical axis. Therefore, the effect will be less severe if imaging is restricted to an area near the optical axis.

Curvature of Field

Unless concrete steps are taken to prevent it, simple lenses will focus a plane surface onto a segment of a sphere. Objectives designated "plan" are supposed to have flatter fields of view than most, but this feature is usually incorporated at the cost of additional elements and often lower light transmission. Figure 35.15 shows two images of a plane mirror, one made with a non-plan lens (upper) and one with a planapo-correction (lower). The images have been posterized to emphasize the variation in signal caused by field curvature. The variation in signal strength across the field is shown with greater precision in the *x*-profile plots at the bottom. We should emphasize that these plots show variation in the measured intensity of reflected light passing the confocal pinhole, not the actual geometrical curvature of the field! Although the upper image displays a variation in focus that could degrade the image of a flat test object such as that mentioned above, one

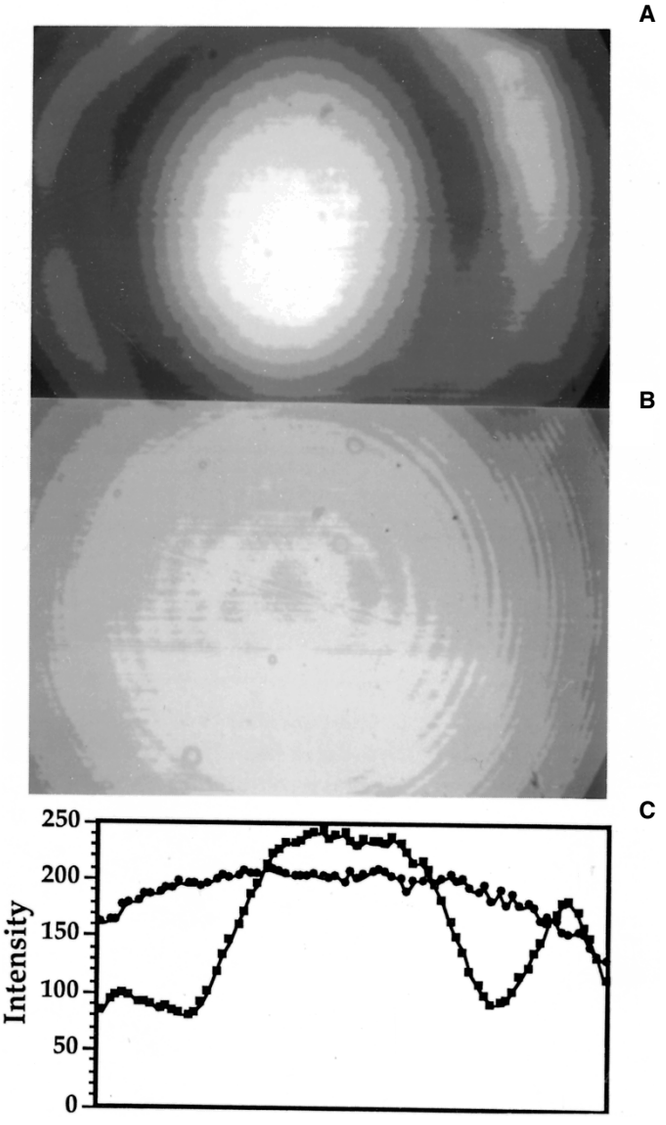

FIGURE 35.15. Curvature of field. In general, lenses focus a plane surface onto a spherical one. In objectives not specifically corrected for plan operation, the curvature of field has the effect that an image of a plane surface loses intensity off-axis. This figure shows two images of a plane mirror, one made with an uncorrected lens (A) and one with a planapo correction (B). The variation in signal strength across the field is shown with greater precision in the two *x*-profile plots (C). (We should emphasize that these plots show variation in the measured **intensity** of reflected light passing the confocal pinhole, and are not a measure of the actual geometrical curvature of the field!) Although field curvature can be a serious problem when viewing planar specimens, it is often less serious when making 3D fluorescent images of biological specimens because in the latter case there is no signal loss but simply a slight dish-shaped distortion in the final 3D data. However, if this distortion is to be kept symmetrical, it is important not to pan the scanned area away from the center of the field of view when using such a lens.

should remember that, with a *z*-resolution of ~0.6 µm, even an intensity drop of 90% between the center and the edge of the field would only indicate a field curvature of <1 µm. However, this image does show the penalty of panning the zoomed area away from the optical axis (e.g., to avoid reflection artifacts in BSL imaging) when using a non-plan lens.

Not Filling the Objective Pupil

We have mentioned above that one can only expect an optical system to reach its diffraction-limited performance if it is operated at its full NA. Check that the size of the laser beam at the BFP (upper part of Fig. 35.2) is equal to the diameter of the hole in the rear of the objective that you are using. If you are in doubt, compare its *x-y* resolution performance with an objective of the same NA but a higher magnification, as this will have a smaller BFP. If the performance is better with the higher magnification objective, the lower magnification one was probably being under-filled.

Dirty Objective

Common blemishes found on optical elements, such as dust, smudges, or old immersion oil, significantly reduce their optical performance and contribute to stray light. In fact, any smudge or smear that shifts the phase of the light passing through it by as little as $\frac{1}{4}\ \lambda$ will prevent those rays from contributing to a diffraction-limited image (see Chapter 11, *this volume*).

Especially disturbing are blemishes at or near the conjugate image planes or on the front surface of the objective. Specks of dust or fibers can be carefully removed by lightly sweeping the surface with a soft camel-hair brush or by blowing them off with a puffer made of a large rubber bulb fitted with a **plastic** micropipette tip. Canned-air blowers should be used with caution as these sometimes deposit oil or condensed impurities from the propellant onto optical surfaces.

Material that is more adherent may require wiping with a cleaning solution or solvent. The type of cleaning solution required depends on the specific material involved. Water, glycerol, and salt crystals can easily be removed using double-distilled water, while immersion oil and some mounting media respond well to the household glass cleaner Sparkle (A.J. Funk and Company, Elgin, IL), which, despite its humble origin, is recommended by several optical manufacturers. This cleaner is effective at removing oil, evaporates fairly quickly, and does not contain harsh solvents that might attack the cement holding the lens elements together. Because it is non-flammable and non-toxic, it can be stored right beside the microscope.

One should not resort to harsher solvents such as ether, benzene, xylene, or acetone until the more benign ones have proved ineffective. Although these solvents can damage some lens cements, the bigger danger is that they may infiltrate the lens to wet the interior glass/air surface, where they will then deposit impurities when they dry. When using such solvents, clean the surfaces quickly so that the solvent evaporates before it has a chance to leak inside the lens. **Never soak an objective in anything!** If organic solvents must be used, take proper safety precautions, such as working in a fume hood. Whenever wiping is necessary, one must use clean lens tissue (i.e., one that has not been exposed to dust deposit from the air), free from finger oils, and never use the same area of the lens tissue twice.

With hard surfaces such as oculars and camera lenses, it may be sufficient to fog the lens with your breath and gently wipe the surface with clean lens tissue. Either use the folded edge of the tissue or, if needed, apply slight finger pressure. Be sure to use several thicknesses of tissue so that finger oils do not penetrate to the glass.

To clean more delicate lens surfaces, a non-contact method is preferred. Apply a drop of the cleaning solution of choice at the near-edge of a piece of lens tissue held horizontally (be sure this area was not previously touched by your fingers). Lower the drop of cleaning solution to the surface of the lens standing upright on the tabletop and gently draw the lens tissue over its surface (Fig. 35.16). There is no need to apply pressure. Repeat this procedure three or four times. As the tissue is drawn across the surface, most of the cleaning solution and the oil and dirt are drawn off by capillary action, and the remaining solution evaporates.

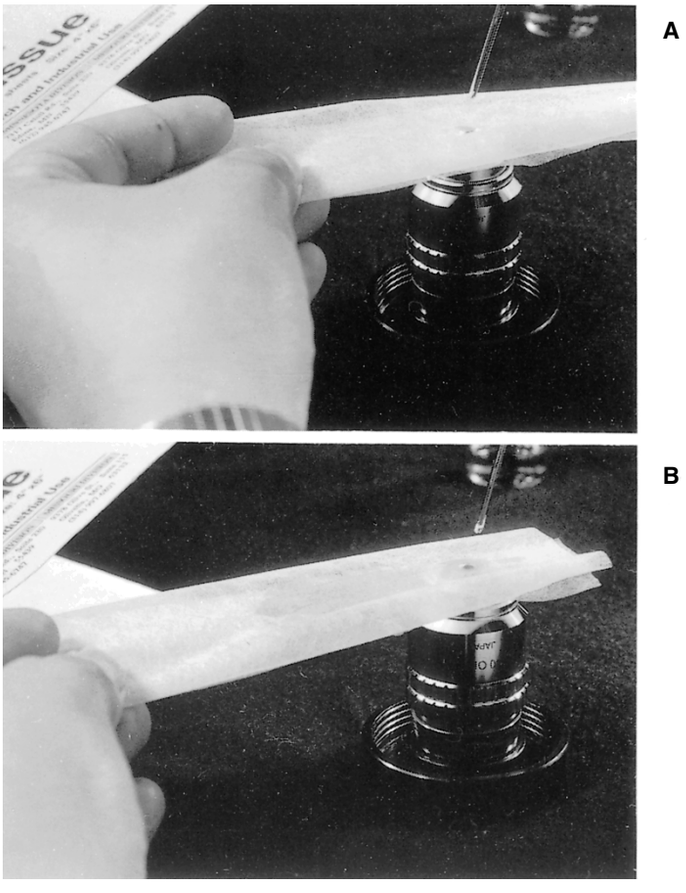

FIGURE 35.16. How to clean objectives. (A) Apply a drop of the cleaning solution of choice at the near-edge of a piece of lens tissue held horizontally (be sure this area was not previously touched by your fingers). (B) Lower the drop of cleaning solution to the surface of the lens and gently draw the lens tissue over the surface as shown. There is no need to apply pressure. Repeat this procedure as necessary.

Use an inverted ocular as a magnifier to check that the oil or dirt has been removed and that there are no streaks on the outer surface of the objective. Surface films are most easily seen if you orient the objective so that you are viewing an overhead light reflecting directly off the surface of interest. When a film of solvent dries onto a surface, any impurities in it will be deposited onto the surface. Use very clean solvents!

This cleaning method works well for all types of objective lenses, even those with recessed surfaces. To clean oculars, CCD camera sensors, or the back surface of the objective, you must make a swab of lens tissue. Wrap the end of a wooden applicator stick with lens paper or carefully washed cotton batting. Do not use a commercial cotton swab, even if wrapped with lens tissue, because it may contaminate the lens with the glue used to secure the cotton fibers. Wet the end of the applicator with cleaning solution and, starting from the center of the lens, gently sweep from

the center to the edge of the surface in a continuous spiral while twirling the applicator. Using a new lens tissue swab each time, repeat this procedure until the lens surface has been cleaned.

If one is using an inverted microscope with an oil- or water-immersion lens, there is the potential for oil or water to seep down the barrel and into the body of the lens. This can be an annoying problem and objectives can be expensive to clean (US$1,000–US$2,000). Some simple devices can prevent this problem. Placing a rubber O-ring around the upper part of the lens will act as an oil dam. As long as there are no cracks in the O-ring and excess oil is blotted away periodically, this remedy works exceptionally well. Some people replace the O-ring with a "scrunchie," an elasticated device normally used to confine hanks of human hair! Other remedies to the problem can compromise the spring shock absorber that is a feature of most short-working-distance lenses. One can also wrap the inner barrel of the lens with Teflon tape to seal the point where the oil enters the inside of the lens or place a rubber sleeve over the entire lens. Such a sleeve can be made by cutting one of the digits from a rubber surgical glove and making a small hole at the tip. The sleeve is stretched over the entire objective so that only the front element pokes through the small hole.

Air Bubbles

An air bubble in the immersion oil will act as a small, yet extremely powerful lens, which can have a pronounced and deleterious effect on the optical performance of the microscope. Fortunately, such bubbles can be detected by imaging the oil layer directly. It is possible to focus on a plane within the oil by adjusting either an internal Bertrand lens (also called a phase lens as it is used to observe the process of centering the phase-contrast rings) or an external phase-telescope fitted in place of one of the oculars.

The best plan is to try to avoid the formation of the air bubbles in the first place by making sure that any bubbles have a chance to float up, away from the tip of the dispenser before you express a drop or, perhaps even better, dip a drop of oil out of the oil container using a glass rod.

Imaging Depth

An important aspect of any microscope used for making 3D images is how far below the surface of the specimen it can obtain useful data. The most obvious limitation is the mechanical one imposed by the finite working distance of the objective (Chapter 7, Table 7.3, *this volume*). Once the objective touches the specimen surface, it clearly cannot be focused to yet deeper planes.

Unfortunately, the opacity and optical inhomogeneity of the specimen usually impose more stringent limits (for more details, see Chapters 17 and 20, *this volume*). To the extent that the illuminating beam is either scattered or absorbed as it passes through the upper layers of the specimen, it will fail to reach any plane of focus located farther into the specimen. Likewise, signal light that is scattered or absorbed between the focus plane and the detector cannot be measured (except when non-descanned detection is used with two-photon excitation). Finally, any optical inhomogeneity in the specimen between the focus plane and the objective will tend to defocus both the beam of incident illumination and the returning signal. This will increase the effective size of the focused spot that is returned to the pinhole, so that much of the light that should pass through it does not do so (Pawley, 2000; Chapter 21, *this volume*).

All three of these effects reduce the fraction of the signal that is detected as the focus plane is pushed progressively farther into the specimen. In addition, the last mechanism also reduces the spatial resolution. Together, these three factors usually place a practical limit on the effective penetration depth of the confocal light microscope, and all of them depend on the optical properties of the specimen.

When using an oil-immersion objective on a biological specimen that is only lightly stained and has been cleared by replacing the water with an imbibing medium having an index of refraction close to both that of the solid components of the cell and that of immersion oil, then the useful penetration depth can be several hundred micrometers, but on less ideal specimens it is usually much less. On the other hand, even deeper penetration is sometimes possible if the microscope is used at lower magnification with an immersion objective having an NA of <0.8, because the defocusing effects of the specimen are usually less serious under these circumstances (Chapter 20, *this volume*).

Singlet-State Saturation

The intensity of the light at the focus of a high-NA objective lens is so high that, if the beam power is more than ~1 mW, a large fraction of the fluorescent molecules within the focal region are in the excited state at any given time. As excited molecules are unlikely to absorb a second photon, they represent a reduction in the effective stain concentration.

Saturation can be avoided by using less input power. However, as the rate at which data is produced from the specimen is proportional to the excitation intensity, dye saturation places an absolute limit on the **rate** at which fluorescence information can be obtained. This limit is most severe when the instrument is operating at high spatial resolution because this implies a smaller focused spot and, therefore, a higher light flux density. Fortunately, many fluorescent specimens produce adequate images using as little as a few microwatts (Pawley and Centonze, 1997) or even nanowatts (Fig. 19.2, *this volume*) of laser power. At these levels it is often possible to make thousands of images before the specimen bleaches.

Because of fluorescence saturation, one cannot expect to scan a single beam rapidly [e.g., at television (TV) scan rate] and also produce a statistically well-defined, high-resolution, fluorescent, confocal image unless one also reduces the number of pixels and, therefore, the raster size of each image.

- Because the degree of saturation is directly proportional to the fluorescent lifetime, operating at or near saturation means that signal intensity may reflect environmental conditions that affect this parameter as well as reflecting the active concentration of the dye.
- As saturation is a function of the dye molecule, rather than, for example, the detector, it affects all parts of the image equally. As a result, areas with more dye will still appear brighter than areas with less, and the image does not look saturated in the same way as an over-exposed photograph does.
- As most photodamage processes do not saturate, operating near saturation produces relatively more photodamage for the amount of data that is obtained.
- As the degree of saturation depends on the intensity of the incident light flux, saturation losses are highest near the focus plane where the light beam is narrowest. As a result, an image recorded from a dye that is saturated tends to record relatively more signal representing light from planes that are out of focus. This reduces the z-resolution.
- The saturation problem decreases with the square of the resolution of the optical system.

WHICH 3D METHOD IS BEST?

Elsewhere in this book, you will find chapters describing all the major methods of 3D light microscopy: widefield/deconvolution, confocal, and two-photon (the latter two, in both single-beam and multi-beam versions). You will also find chapters pointing out the advantages of combining deconvolution with the other two methods and comparing their performance from a theoretical point of view. However, because each of these chapters is written by skilled practitioners of the particular method described, there is little discussion of some of the fundamental differences between them. This section sets out to fill this gap.

As making comparisons of any type is always invidious, we must start by explaining what we will not do. We will not recommend one method over another nor will we provide more than a rudimentary description of the operating principles. What we will do is point to the features of each one that makes it particularly suitable for certain studies, particularly studies of living specimens. Our discussions will cover differences in the shape of the bleach pattern, the power density of the excitation, the differences in the quantum efficiency and noise level of the detector, and how these affect resolution and S/N of the final image.

Because widefield techniques collect data in parallel and use CCDs with high effective quantum efficiency (QE), while single-beam scanning techniques are serial devices and use photomultiplier tubes with 5× to 10× lower QE, one can both elicit and detect many more photons/second with widefield techniques. Figure 35.17 shows the excitation aspect of this difference. Assuming a 1024 × 1024 pixels CCD, widefield will be exciting and recording data from 10^6 pixels, while the single beam of the confocal will be highly exciting about 12 Nyquist pixels and collecting data from most of these.[7]

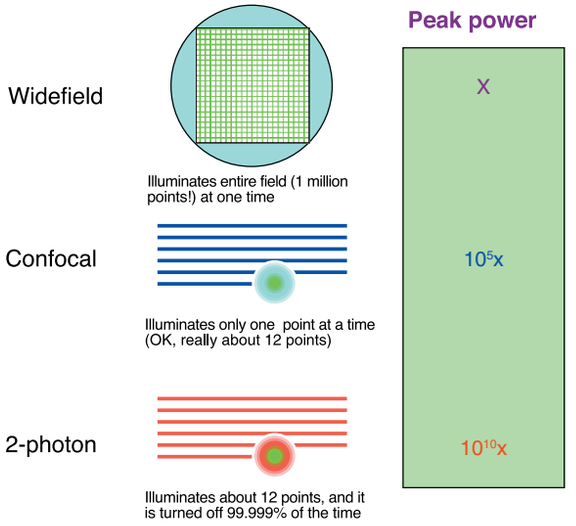

FIGURE 35.17. Variation in peak brightness of the excitation light in the three common forms of 3D fluorescence microscopy. Assuming that one needs to excite a fixed number of photons from the focus plane in the specimen in order to record a 1000 × 1000 image of a given quality, the level of excitation at the focus plane, in photons/square micrometer, must be much higher in a scanning microscope, where only about 12 pixels are excited at any one time, than with a widefield microscope in which 10^6 pixels are illuminated simultaneously. The low duty-cycle of femtosecond two-photon excitation produces a peak power that is about 10^5 to 10^6 times higher again. It is now clear that many dyes bleach more rapidly at these extremely high excitation levels (see Chapters 38 and 39, *this volume*).

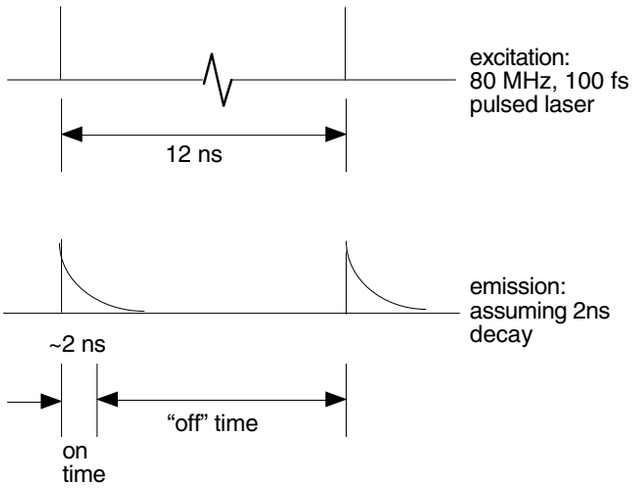

FIGURE 35.18. Excitation duty cycle for 80 MHz, pulsed illumination. Because the time between the excitation pulses of an 80 MHz pulsed laser is long (12 ns) compared to the decay time of most fluorescent dyes, such dyes are driven into singlet-state saturation at a signal level about 10× lower than they can produce with continuous illumination.

To collect an image in the same time period, the total flux of detected photons/s must be the same in both cases and this can only occur if the laser is 10^5× more intense (in photons/μm^2 at the focus plane) than the Hg illumination used in widefield microscopy.[8] When one moves to two-photon microscopy, there is another major increase in the peak flux required and this is provided by the fact that the duty cycle of a 100 fs pulsed laser operating at 100 MHz is a 10^{-5} and this means that the peak intensity is $10^{5\times}$ higher than confocal, for the same average beam power. In fact, two-photon systems are commonly operated with an average power about 10× to 50× higher than that used for confocal, but it is also true that, with a wavelength that is 2× longer, the area of the two-photon spot is 4× larger in area and the intensity is consequently 4× lower. Because excitation is proportional to the square of the intensity, the **area** from which the fluorescent light emerges is about 1.4× larger in diameter and 2× larger in area than would be the case with single-photon excitation.

A second factor relates to the fact that using a 100 MHz laser excites organic dyes inefficiently. Figure 35.18 shows that even if each pulse excites essentially all the dye in the focal volume, because the decay time of most common dyes is short compared to the time between pulses, the system is essentially turned off for about 90% of the time.

[7] Assuming that the pinhole is set to 1 to 2 Airy units, some of the photons emitted from all these 12 pixels on the specimen will be stored as the value for a single pixel in the computer (see Fig. 24.A6 in the appendix to Chapter 24, *this volume*, for more on this).

[8] Strictly speaking, to account for the lower detector QE, it might require 10^6× more brightness. However, because better image data is needed if one must later remove the out-of-focus light by deconvolution, things get complex and we will leave this argument to other chapters.

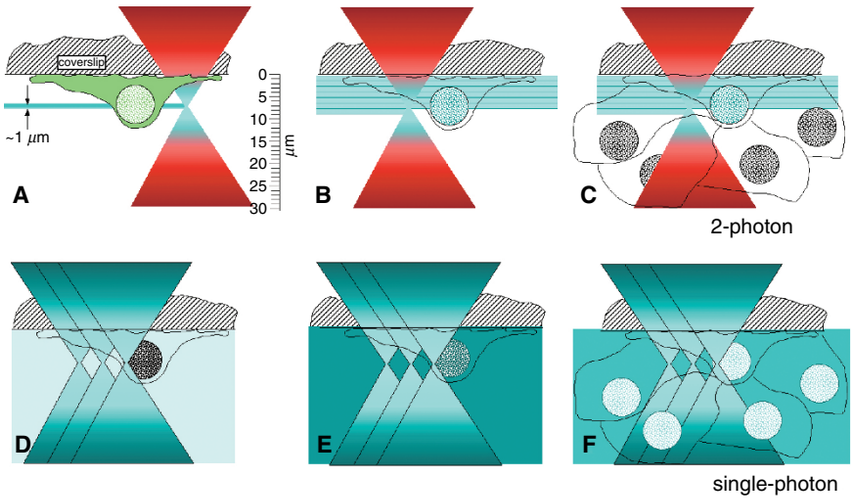

FIGURE 35.19. Dependence of total photodamage on damage/emitted-photon and specimen thickness in single- and two-photon excitation fluorescence imaging. One of the signature advantages of two-photon excitation is that excitation/damage is normally confined to the focus plane (A, D). However, if as seems likely, in many cases, the damage/excitation produced by two-photon excitation is greater than with single-photon excitation by a factor γ, then two-photon excitation will only be advantageous when the specimen is so thick that the number of single-photon excitations produced as the beam passes through the entire specimen exceeds the two-photon excitations produced in the focus plane by a factor of >γ (C, F).

It would be surprising indeed if excitation of the dye was entirely unaffected by these very marked differences in photon flux. At the very least, one can see that one must operate ~10^5 × closer to singlet-state saturation with single-beam confocal than with widefield and at least another factor of 10× closer with two-photon microscopy. The two simplest responses to this problem are: (1) to take more time to collect data from a single plane in confocal and two-photon microscopy, and (2) to settle for detecting 100× to 1000× fewer photons/pixel than is common in widefield/deconvolution.[9]

Numerous studies now connect phototoxicity to excitation of the dye. By themselves either the dye or the light are relatively innocuous. Together, energy is deposited and some of this energy produces damaging compounds. Although it is often said that both widefield and confocal systems do the same amount of bleaching above and below the plane of focus, this is not, strictly speaking, true. Figure 35.1(B) shows the situation for confocal in which the worst bleaching is confined to two pyramids, above and below the central focus plane. On the other hand, if widefield microscopy is performed using a light source that does fill the objective BFP and with the field diaphragm set to confine the illumination to a circle just large enough to contain the area imaged by the CCD,[10] then the excitation away from the focus plane depends only on defocus. Damage is not peaked towards the center of the out-of-focus planes. On the other hand, probably the best known feature of two-photon imaging is that excitation is confined close to the focus plane. What complicates the choice is that, for many dyes, the damage/excitation produced by two-photon excitation is higher than when the same dye is excited by a single photon (see Chapters 38 and 39, *this volume*). This differing dye-damage sensitivity is almost undoubtedly related to the marked differences in peak excitation intensity noted above.

Although the magnitude of the nonlinear damage effect is poorly understood, let us consider the implications if the damage/excitation is 10× higher for two-photon than it is for single-photon microscopy. Figure 35.19(A,B) diagrams the two-photon excitation of a tissue-culture cell on a coverslip. The damage at the plane of focus is worse locally but no damage occurs away from it. Figure 35.19(D,E) shows the situation for single-photon excitation: damage is less at any point but extends throughout the cell. Clearly, if the cell is less than 10× thicker than the two-photon excitation plane, the total amount of photodamage from a single scan will be more with two-photon excitation. Assuming a damage plane thickness of about 1 μm, single-photon excitation would have the advantage for most common tissue culture cell preparations.

However, given our assumptions, if the stained volume of the preparation is more than 10× thicker than the two-photon damage plane, there will be less damage with two-photon excitation [Figs. 35.19(C,F)].

If the damage/excitation discrepancy is less than 10×, the advantage will switch to two-photon excitation at a specimen thickness that is somewhat less than 10 μm.

Finally, there is the matter of how much power is needed for two-photon microscopy. In Chapter 38, Karsten Koenig points out that about 5 mW of femtosecond pulsed laser light is sufficient to prevent cells from dividing **if it is focused into a small spot by a high-NA objective** [Fig. 35.20(A)]. On the other hand, the popular titanium : sapphire (Ti : Sa) lasers only work properly if they are producing about a watt of optical output. Although some users have taken advantage of this surplus power to make many-beam two-photon excitation systems, it has also been utilized to overcome the losses imposed by imaging thick, scattering specimens where hundreds of milliwatts must be used to get sufficient intensity to produce two-photon effects in an aberrated spot located some hundreds of micrometers from the surface of the specimen. This topic is worth talking about here because a number of new femtosecond mode-locked lasers are now in development that couple great operating convenience with potentially much lower cost but that produce comparatively modest amounts of power (10–20 mW). It seems likely that these systems will find applications in which operation on specimens 50 to 100 μm thick is routine.

[9] These estimates are just that. Different users viewing specimens with widely different staining levels may find that they can produce acceptable results with recorded signals that vary over a range at least this large. The numbers used are meant to be illustrative, not definitive. It is also important to remember that, because of the difference in detector QE, this may only amount to a 10× to 100× difference in the number signal photons detected from near the plane of focus.

[10] This setup is recommended both to reduce damage to those parts of the preparation not being imaged and also to reduce stray and scattered light to a minimum.

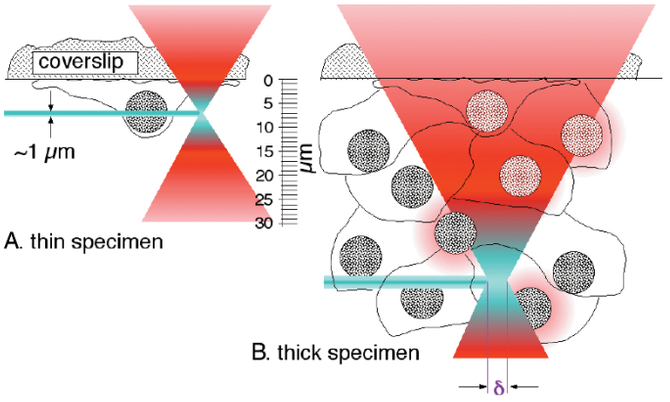

FIGURE 35.20. Total beam power required varies with specimen thickness in two-photon excitation. (A) When focused near the coverslip, scattering and aberrations are low and most of the laser light passing through the objective is focused into a small, high-intensity spot. (B) As the focus plane moves farther into the specimen, more light is scattered out of the beam and aberrations increase beam diameter (δ), further reducing peak intensity. Maintaining signal level under these circumstances can require 10× to 100× more beam power.

Optimal 3D Light Microscopy Summary

After all these words, important questions remain:

- How important are "dose-rate effects"?
  - — Are dye molecules that have been excited to the singlet state more likely to absorb a second, damaging photon in laser confocal microscopy? In two-photon microscopy? Why does this problem seem worse with some dyes than others?
  - — How fast does the risk of bleaching/emitted photon increase as the excitation gets more intense?
  - — Does crossing to the triplet state significantly reduce the effective dye concentration in laser confocal microscopy? In two-photon microscopy?
  - — If so, how does this depend on the type of dye and the local environment?
- When does staining geometry and density become a serious problem for widefield/deconvolution?
- Are there aspects of dye performance under two-photon excitation that may limit the use of certain dyes?
  - — Absorption spectra
  - — Absorption cross-sections
  - — Quantum efficiency
  - — Bleaching/phototoxicity
  - — New dye molecules developed for two-photon excitation
- Do confocal and two-photon excitation bleach dyes by different mechanisms? (Probably!)

Things to Remember About Deconvolution

- Deconvolution effectively averages signal over data stored in >64 voxels, reducing Poisson noise.
- Deconvolution separates in-focus from out-of-focus light by imposing PSF, non-negativity, and other constraints.
- Deconvolution has more problems if there is too much out-of-focus light.
- Deconvolution should be applied to both confocal and widefield data.
- Blind deconvolution techniques can be subject to bootstrap errors. (If you start with a "bad" first estimate, or iterate too long, you may "create" structure.)
- Deconvolution requires very high positioning accuracy and a relatively constant, known PSF. Remember, the widefield PSF varies with wavelength, NA, field and aperture diaphragm diameters, alignment of the arc (or use of ground glass), and the immersion medium.

Decision Time

Premise: **All** 3D microscopical data should be deconvolved before viewing and that the object is to get the best 2D (optical section) or 3D data possible from a fixed number of excitations in the specimen.

MULTI-PHOTON VERSUS SINGLE-PHOTON EXCITATION

The advantages of multi-photon excitation include:

- Only the focus plane subjected to short wavelength damage:
  - — Less damage as long as specimen is thick.
  - — Less absorption of the excitation beam by heavily-stained specimens.
- Optical sectioning without use of a pinhole:
  - — On thick, scattering specimens, non-descanned detection of scattered signal can be up to 300% more efficient than normal semi-confocal detection.
- Longer wavelengths are scattered less by the specimen: better penetration of excitation.
- Can excite ultraviolet (UV) dyes without needing UV lasers or UV optics.
- System acts as if the pinhole is infinitely small, and perfectly aligned.
- Huge Stokes shift means that dichroic beam-splitters and filters can be more efficient.
- Laser often tunable to optimal excitation wavelength.

The disadvantages of MP include:

- Longer wavelength, somewhat lower resolution: not 2× worse, more like 20% to 40%.
- Excitation duty cycle only ~10% forces one to work closer to singlet-state saturation.
- Hard to arrange >1 distinct excitation wavelengths.
- Requires expensive, pulsed laser: compare the productivity of one two-photon versus two confocals.
- Higher average beam power destroys cells that absorb infrared (IR) light.
- Many dyes emit >10× fewer photons/molecule before being destroyed.
- Hard to use with fiber-optics and acousto-optical deflectors.
- Laser-tweezer effects may displace some small particles.

- Nonlinear dependence of both signal and damage on laser power makes it crucially important to set this parameter accurately.

Widefield Versus Beam Scanning

Advantages of widefield scanning include:

- Well-optimized systems available.
- High detector QE and optical simplicity means more fluorescent photons are detected.

Disadvantages of widefield scanning include:

- Cannot view optical section directly (must collect several planes, then deconvolve).
- Image noise from out-of-focus fluorescence eventually swamps the advantage of higher detector QE.
- Rapid 2D imaging difficult.
- CCD read noise rises with square-root of the readout speed.

The question between widefield/deconvolution and confocal is: Assuming that you have set up both instruments in an optimal manner, to what extent does the statistical noise produced by out-of-focus light from your WF specimen offset the increased QE of the CCD detector used in widefield scanning?

- Thin, sparsely-stained specimens are good for widefield/deconvolution.
- Confocal is good if you only need to see one plane.

The questions between confocal and two-photon excitation are:

- Do you need to look more than 20 µm into your specimen?
- Are you content to excite at only one wavelength at a time?
- Will you get more successful live-cell experiments done on one two-photon setup than on two confocals?
- Are your specimens free from important structures that absorb in the near IR or that might be damaged by three-photon excitation at the wavelengths you plan to use?
- Do you have enough room for a two-photon system?
- Can you get along without video-rate imaging or rapid wavelength switching?
- Do you have the technical support needed to keep the pulsed laser happy or can you live with one of the automated femtosecond lasers with a reduced wavelength range? (See Chapters 21 and 40, *this volume*, for a discussion of the advantages of using wavelengths >1000 nm for multi-photon excitation.)

If YES to all of these, then the greater signal collection efficiency and reduced bleaching volume should make two-photon your best bet. These characteristics are summarized in Table 35.1

SUMMARY

As with any type of sophisticated instrument, the best results can only be obtained if the operator uses a confocal microscope correctly. Many aspects of proper practice are either similar or identical to those that govern the operation of the conventional light microscope. However, there are two controls, one contrast effect and one optical effect, that are unique to the confocal microscope. The limitations imposed on confocal imaging by these four factors — pixel size, pinhole size, fluorescence saturation, and chromatic magnification error — must be understood if one is to make best use of this highly productive instrument. In addition, an appreciation of the effects of photon statistics will enable users to get the most possible information from their specimens.

Remember that when using a confocal microscope, the resolution visible in the final image can be limited by three considerations in addition to the optics defining the volume of the specimen that is sampled by both the laser beam and the confocal pinhole. These three considerations are:

1. The choice of pixel size (referred to the specimen): Pixel size is a function of the zoom setting and the NA and magnification of the objective lens.
2. Deconvolving, or at least filtering, the 3D data before displaying or measuring suppresses artifactual single-pixel noise features and improves S/N by averaging the photon signal over many voxels.
3. The S/N of the intensity signal recorded can be optimized by maximizing dye contrast and counting as many of the photons produced by the specimen as possible.

TABLE 35.1. Three-Dimensional Microscopy Methods: Summary

	Widefield/Deconvolution	Confocal Single-Beam	Confocal Disk Scanner	Two-Photon
Effective detector QE	60%–80% (CCD)	3%–12% (PMT)	30%–40% (EM-CCD)	3%–12% (PMT)
Detector noise (RMS electrons pixel)	±4	<0.01	0.01	<0.01
Peak signal[a]	>20,000	20–100	1–3000	20–100 photons/pixel
Acquistition time (s/frame)	Depends on CCD readout	0.33–3	0.002–10	0.33–10
Excitation intensity power/µm^2	10 nW	0.1 mW	1–10 µW	10 W
Excitation wavelengths	350–650 nm (Hg arc)	Laser lines (fiber-coupled)	2–3 laser lines (dichroic hard to change)	700–900 nm Ti : Sa
Major limitation	Poisson noise from out-of-focus light	Singlet saturation limits data rate Scattering samples defocus signal Galvo limits scan speed	Less sectioning than single-beam Scattering samples defocus signal	Singlet saturation Hard to excite only one member of FRET pair Damage/excitation greater for many dyes

[a]These figures assume data collected from same volume of the specimen in same time period. Ideally, the raw data from all methods should be deconvolved (see Chapter 25, *this volume*).

Determine and use the optimal pixel and pinhole sizes for each objective and, if the confocal bleaching rate seems higher than that you have come to expect from normal, photographic epi-fluorescent microscopy, **you are probably doing something wrong!**

While the points that we have tried to emphasize in this chapter were chosen because they seemed to us to be those most often misunderstood by novice users of the confocal microscope, they certainly do not cover all of the possible problem situations that can occur in this field. A better understanding of more complex problems of a general and theoretical nature can be obtained by reading the other 50 chapters in this book while specific problems that apply to a certain type of instrument or dye are best forwarded either to the appropriate supplier or to discussion on the Confocal (LISTSERVER) E-mail Network. This network supports active, informal, and informative discussions of current topics in confocal microscopy by over two thousand scientists having a wide variety of experience. Anyone on the Internet can subscribe to this service by sending the message: "subscribe confocal <your name>" to the address "LISTSERV@UBVM.CC.BUFFALO.EDU". You will then receive a message describing the rules and purpose of the group as well as future postings to it.

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APPENDIX

Normal Köhler Illumination

Proper alignment of a light microscope begins with the microscopist setting up the optical components for Köhler illumination (see also Figs. 6.3 and 6.7, *this volume*). This mode of illumination establishes two important sets of conjugate image planes. Conjugate planes are sets planes perpendicular to the optical path that focus onto each other, that is, the same features will be visible in each plane of a set of conjugate planes, although the size may vary. There are an infinite number of such sets of conjugate planes in any optical system but only two of these sets are of particular interest in microscopy: aperture planes and image planes. In Köhler illumination, the aperture planes are at the light source, the iris diaphragm of the condenser, the BFP of the objective, and the eyepoint (i.e., where you are supposed to place your cornea). The image planes are the field diaphragm, the focus plane in the specimen, the field stop in the eyepiece (located 1 cm inside the tube holding the occular), and the retina of the eye or the camera film plane. These two sets of planes are reciprocally related to one another in the following way: light passing through any **location** (point) in any **image plane** will be found to have passed at a specific **angle** through any **aperture plane** and vice versa.

To produce maximum resolution, light must leave (or approach) the specimen from as many angles as permitted by the size (or NA) of the objective. This means that, for full performance, all aperture planes must be filled to the full NA of the system. In addition, the optical components for phase and DIC contrast enhancement need to be placed in aperture planes.

Setting up for Köhler illumination ensures that these conditions are met. If all specimens had exactly the same thickness (±1 µm) and refractive index, it would be possible to preset the microscope for Köhler illumination. As they do not, one must set it up manually every time a new specimen is mounted.

To achieve Köhler illumination for transmitted light microscopy, one must first center and focus the illuminator (this step is usually not required in modern microscopes in which the illuminator is built into the microscope base). Remove all filters from the lamp housing, close down the field diaphragm, and focus the collector lens to obtain a sharp image of the filament on a target placed at a distance of ~25 cm away. The lamp centering screws should then be adjusted to insure that the collector lens and the source are aligned and the image of the filament is centered. [Note: If using a Hg-arc source it is better to align the source while viewing an aperture plane in the microscope (and using a lot of neutral density filters!) in order to avoid exposure to UV light.]

Next, the condenser must be aligned. Using a low-power objective, focus on a specimen on the microscope stage, close down the field diaphragm (the field diaphragm is usually in the base of the microscope while the condenser aperture, described below, is actually incorporated into the condenser that is mounted under the stage), and adjust the height of the condenser until you obtain a sharp image of the edge of the field diaphragm superimposed on the focus plane in the specimen. Move the condenser adjustment screws to center the opening of the field diaphragm in the field of view. If the condenser is badly misaligned, you may not see any light if you initially close the field diaphragm down all the way. In this case, do it a little bit at a time, focusing and centering at each step.

The field diaphragm should then be opened to the edges of the field of view or at least until it surrounds the area of interest. Opening it more than necessary will let more light into the system than you need, and the extra light will cause reflections that produce flare and hot spots that reduce image contrast.

The working NA of an objective used in transmission is set by the aperture diaphragm. Closing the condenser iris reduces the angle of the illumination striking the specimen, lowering the effective NA of the system. Opening the condenser increases the working NA, increasing the resolution but usually reducing the depth of field, the contrast and sometimes introducing flare. Consequently, final adjustments to the condenser iris are a compromise between resolution and image contrast.

Because the condenser is often fixed to the stage, the objective and condenser usually do not maintain their spacing as the focus is adjusted or as additional optical components such as filters are introduced. Therefore, one must readjust for Köhler and reset the field diaphragm and the condenser iris to obtain the best image possible each time the optical system is changed (e.g., by changing objectives). When the microscope is properly adjusted for Köhler illumination, the result is maximum lateral and axial resolution, optimum contrast, a uniform level of illumination across the field, and a minimum amount of flare from internal reflections.

Strictly speaking, conventional, transmitted light, Köhler illumination is not necessary for epi-fluorescent confocal microscopy because both excited and emitted light travel through the same objective. However, one must set up the epi-illumination system for Köhler in order to ensure even illumination and to use the epi-field diaphragm to restrict the excitation to just the area being imaged, a habit that greatly reduces non-specific flare.

Transmitted Köhler is also necessary if one needs an optimal phase contrast or bright-field image to locate the most interesting part of one's specimen before beginning confocal work. In addition, the non-confocal transmitted-light detectors provided with many instruments will only work correctly if the condenser has been properly adjusted for Köhler illumination.