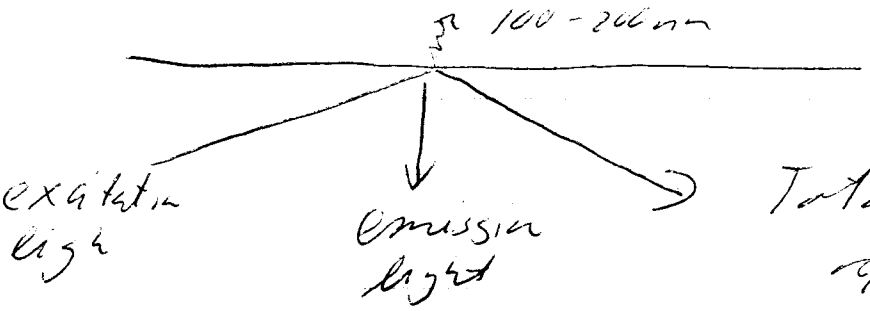
TIRF – Microscopy

Problem:

Visualization of a layer close to cover glass

Solution: TIRF, images only 100 nm – 200 nm above cover glass



$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

$$\theta_2 = 90°$$

$n_1 = 1{,}5$ Dodyglas

$n_2 = 1{,}33$ Wasser $\Rightarrow \theta_c = 61°$

$$\Rightarrow J(z) = J(0)\, e^{-z/d}$$

$$d = \frac{\lambda_0}{4\pi \sqrt{(\sin\theta_1 / \sin\theta_2)^2 - 1}}$$

$$d = 100\ \text{nm} - 200\ \text{nm}$$

96 Confocal Microscopy and Deconvolution Techniques

Multiplaton and TIRT

INTRODUCTION

Epifluorescence microscopy is an invaluable technique for both the research biologist and the clinician (see Section 9). It enables not just visualization, but also identification of structures within cells and tissues. The emitted signal is viewed against a black background providing high contrast. This increases the likelihood that even a weak signal can be detected. This is in contrast with bright-field microscopy where weak signals are viewed as small modulations of a bright background. In addition, fluorescence imaging can provide superb selectivity. Specific probes of known spectral properties may be imaged with appropriately specified chromatic filters and excitation wavelength while other signals with different spectral characteristics are rejected.

Fluorescence microscopy initially gained popularity as a biological research tool as a consequence of the development of immunofluorescence techniques. With these techniques, the enormous diversity and exquisite specificity of antibody molecules are used to generate specific fluorescent probes for practically any desired biological molecule. In addition, various naturally occurring ligands, such as the actin-binding, fungal metabolite phalloidin, may be conjugated with a fluorophore and used as a specific probe.

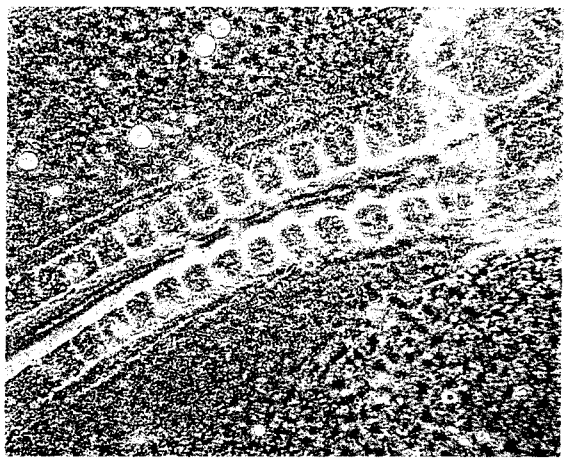
Immunofluorescence is generally applied to fixed specimens, but recently there have been a number of fluorescent techniques that can be applied to living specimens. Purified proteins can be derivatized with a fluorophore yet still retain their function. These derivatized proteins may be injected back into a cell thereby enabling the dynamic behavior of the protein to be observed by fluorescence microscopy (see Section 8). Cells or organisms may be transfected with the gene for the naturally fluorescent green fluorescent (GFP) protein (Chalfie et al. 1994). This technique may be used to observe patterns of gene expression in an organism by attaching the promoter of the gene being studied to the coding region of GFP. Alternatively, the GFP gene may be spliced to the coding region of the gene for a protein under investigation and the distribution of the chimeric protein within a cell observed by GFP fluorescence (see Chapter 78).

Fluorescent indicator molecules have been developed that change their optical properties in response to particular components of their environment. Probes for calcium, pH, ATP, and membrane potential are available. These probes allow the physiological state of a live cell to be monitored optically. Although useful for many applications, fluorescence or conventional microscopy is subject to significant limitations for some purposes: Thick specimens are not well-resolved and produce murky images, and extraneous excitation of the fluorophore may produce phototoxicity of the sample.

The following two chapters present two approaches for addressing these difficulties through modifications of the microscope or of the image. This chapter describes confocal microscopy and deconvolution. Typically, a confocal imaging system consists of a laser scanning microscope with a pinhole aperture in the image plane of the

Which Method Should Be Used?

Both confocal microscopy and deconvolution techniques are solutions to problems encountered in imaging thick specimens. For a thin specimen (<5 μm) it will probably be much quicker and less frustrating to avoid both techniques and use conventional fluorescence microscopy. If for some reason the best possible resolution is absolutely necessary, then either method is capable of delivering a small improvement over a conventional microscope (Wilson 1990; Hiraoka et al. 1991). However, for most thin samples, the small improvement will not be worth the large extra effort.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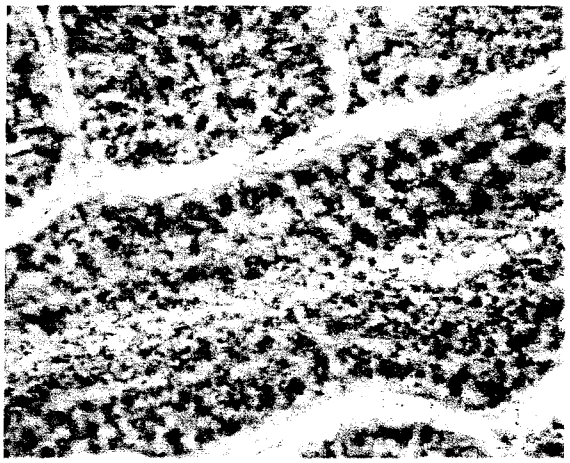
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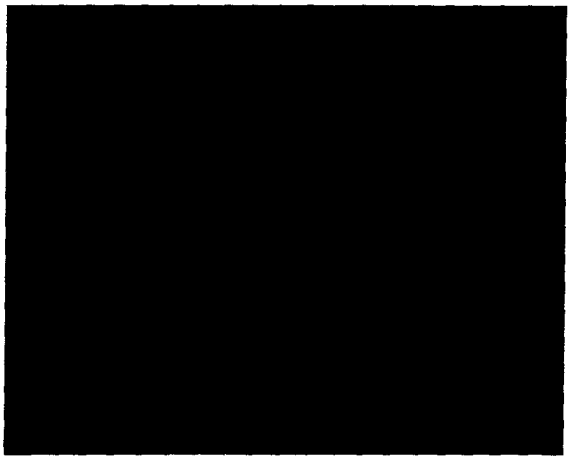
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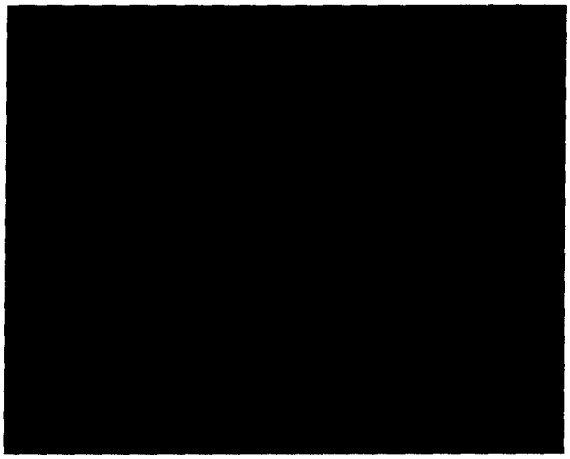
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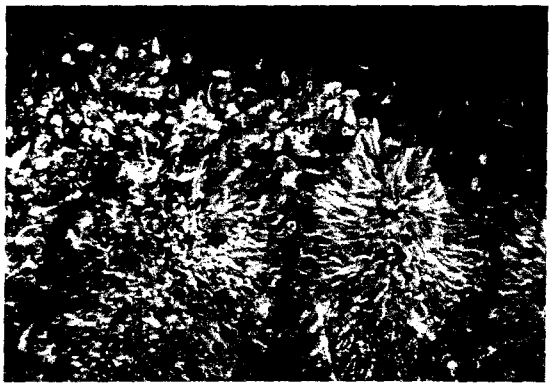
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For thicker objects that produce a "moderate" amount of out-of-focus light, either technique should give a dramatically better result than a conventional microscope. However, with very thick specimens that produce an overwhelming amount of out-of-focus light, probably only confocal microscopy will give a satisfactory result. How much is a "moderate" amount of out-of-focus light? Typically in such a specimen the image seen through a conventional microscope will be too blurred to be useful, but one will be able to locate the region of interest and at least roughly set the focus level. Thus it is possible to find the area to be imaged by visual observation, although the image will be too blurred to discern details. On the other hand, if the view through a conventional microscope is virtually featureless, giving no landmarks for choosing the appropriate area or for setting the focus, then the only choice is confocal microscopy. The confocal microscope can produce extremely useful images from astonishingly bad specimens, but in these typically very thick specimens it is not realistic to expect a final image quality comparable to the best that a conventional microscope produces with a thin specimen.

CONFOCAL MICROSCOPY

Confocal microscopes differ from conventional (*wide-field*) microscopes because they do not "see" out-of-focus objects. In a confocal microscope, most of the out-of-focus light is excluded from the final image, greatly increasing the contrast and hence the visibility of fine details in the specimen. Fig. 96.2 gives a schematic illustration of the operating principle. On the left of the drawing is a wide-field microscope. A light source, in conjunction with a condenser, distributes light uniformly across the area of the specimen under observation. Consider light arising from three locations in the specimen, passing up through the objective lens and eventually (ignoring some intermediate lenses that need not concern us here) reaching a detector of some sort: film, video camera, or retina. The first location is in the center of the field of view and in the focal plane of the objective lens. The heavy dashed lines represent the limits of the bundle of light rays that contribute to the image from this point. Similarly, the lighter dashed lines mark the path of rays from a second point in the same plane but displaced horizontally to one side of the first point. Finally, we have light, represented by the dotted lines, coming from a third point located below the first point (i.e., in an out-of-focus plane). The light from this third point contributes to the blurred background, which we wish to eliminate from the image.

The right-hand side of Figure 96.2 shows how this is done, simply by adding a pinhole aperture to the wide-field microscope. Notice that behind the objective lens, all of the light rays are brought together at a crossover point, the location of the intermediate image plane of the microscope. Normally, the microscope oculars are focused on this plane to form the final fully magnified image we observe. The location of this crossover plane along the vertical axis of the microscope is different for different light rays, depending on the distance of the corresponding point in the specimen from the front of the objective lens. The crossover point for light rays from the illustrated out-of-focus plane (dotted lines) is below that for rays from the in-focus plane (dashed lines). As illustrated, a pinhole aperture at the correct height will pass the concentrated light from the in-focus point, but block nearly all the dispersed rays from any points higher or lower than the focal plane. Out-of-focus

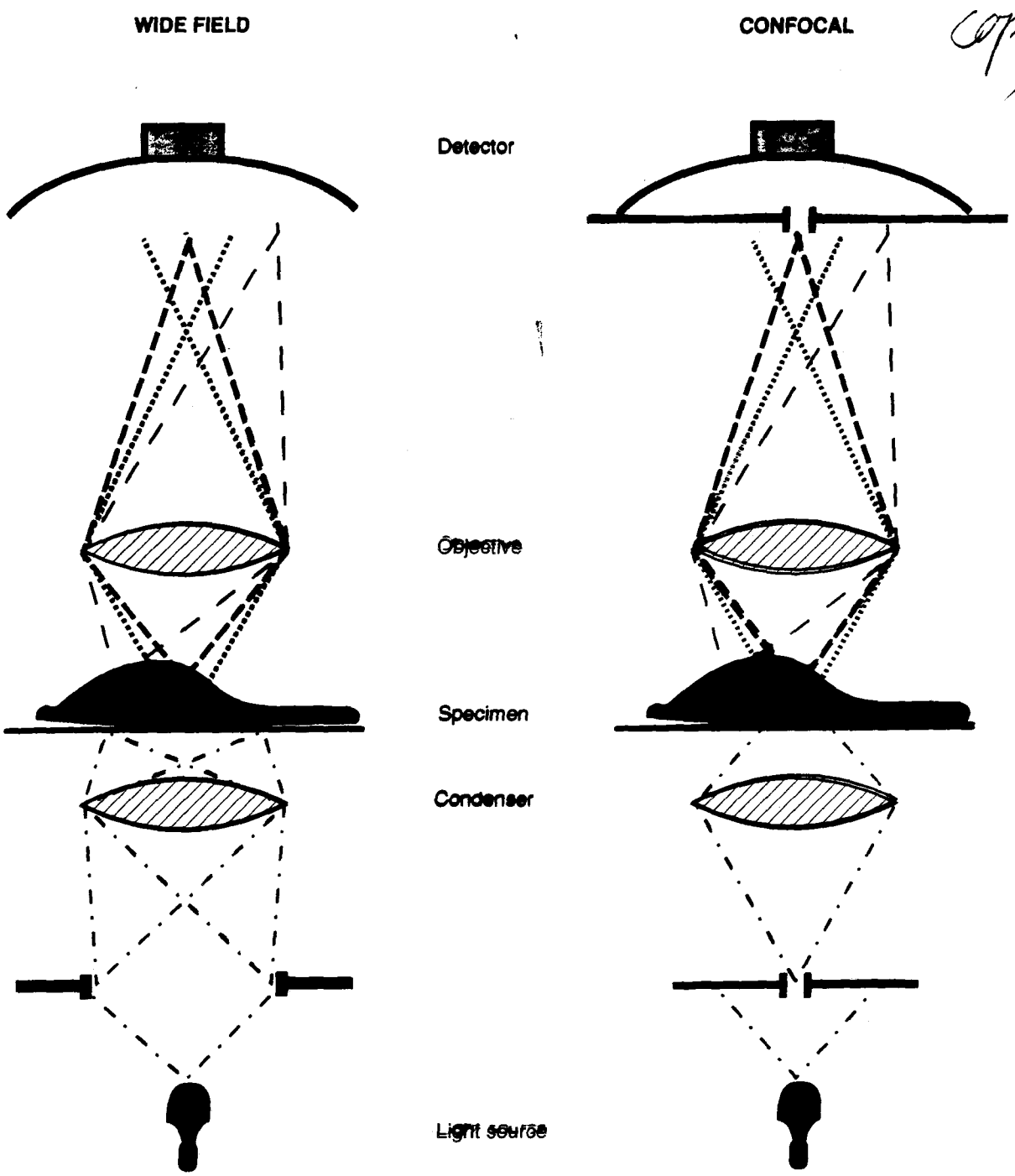


FIGURE 96.2
Schematic illustration of the operating principle of the confocal microscope. Left side—a conventional, or *wide-field* microscope. The specimen is illuminated over an extended region by a light source and condenser. Light rays arising from three points in the specimen are shown. The dashed lines emanate from two points in the focal plane, one centrally located (darker dashed line), the other off-axis (lighter dashed lines). The third point is on-axis but located below the plane of focus (dotted lines)—it gives a blurred image at the detector. The detector forms an image from the sum of all the simultaneously arriving light rays. Right side—a confocal microscope. Two pinhole apertures have been introduced. The upper aperture passes to the detector only the focused light rays from the on-axis in-focus point of the specimen. The lower aperture restricts the illumination so that it is focused on the point seen by the upper pinhole aperture.

points therefore contribute insignificantly to the final image; they are essentially invisible. An unfortunate side effect of the pinhole aperture is that most of the in-focus points also become invisible; only the rays from the central spot are passed by the aperture. We will see how to get around this problem shortly, but there is one more essential feature of the confocal microscope that we have to introduce first.

Since all regions of the specimen outside the spot being imaged through the pinhole aperture will be invisible, there is no need to illuminate them. Illumination is needed over only a small area at any one time (i.e., the area seen through the pinhole aperture), and there are three good reasons for restricting the incoming light to this minimum necessary area. First, light going to other parts of the specimens will be scattered, and inevitably some of it will leak through the pinhole aperture, degrading the contrast in our image. Second, all of the illuminated area will be subject to photobleaching. Third, restricting the illumination to a single focused point gives a dramatic improvement in the discrimination against points above and below focus; in other words it enhances the *vertical* resolution. The reason for this enhancement is as follows. If the incoming illumination is focused sharply to a point in the focal plane, then regions above or below this focal point will receive dispersed, much less intense, illumination. Thus, when we use this type of focused spot illumination, not only will the pinhole aperture be selecting against light from out-of-focus planes, but the intensity of the light originating from these planes will be less. By exactly the same reasoning, the *lateral* resolution of the microscope will also be enhanced if a focused spot of illumination is used. These two modifications, limiting the area "seen" by the detector and limiting the area illuminated by the light source, are the key ingredients of a confocal microscope. A confocal microscope is simply a light microscope in which both the field of view of the objective lens and the region of illumination have been restricted to a single spot in the same focal (*con*focal) plane.

To gain the optical sectioning capability of the confocal microscope, other aspects of the microscope's performance have been sacrificed. *Field of view* has been traded for increased *axial resolution* (Wilson 1990). The pinhole aperture effectively excludes light from out-of-focus planes, but it also restricts the field of view laterally to a spot the size of the demagnified pinhole. Thus, in order to gain the advantages conferred by the confocal pinhole, we have to give up the convenience of acquiring an image from an extended area in parallel. The confocal image has to be built up sequentially by scanning one or more spots over the specimen until the region of interest has been covered.

Instruments

In principle, one could build up a complete image by scanning the specimen to and fro under a fixed spot of illumination, or by scanning the objective lens, the incoming illumination, or the pinhole itself. In practice, because the scanning needs to be very fast to generate a useful image in an acceptable time, some types of scanning are much easier than others. There are three major types of confocal microscopes currently available, differing in the method they use to move the confocal spot relative to the specimen. In the simplest, a single diffraction limited spot is held stationary

on the optic axis of the lens system, while the specimen is moved (*specimen scanning*) (Brakenhoff et al. 1989). In the *tandem scanning* type, many carefully spaced beams derived from a single arc lamp source are scanned and detected in parallel using a rotating perforated disk (Egger and Petran 1967; Xiao and Kino 1987). In the more common *spot scanning* type, a single laser beam is deflected in a raster fashion by moving mirrors or with acousto-optical deflectors (Carlsson et al. 1985).

There are important optical advantages associated with the stationary beam of the specimen scanning type of instruments, but their primary disadvantage is that the specimen must be moved. If the scan is to be completed within a reasonable time, the mechanical accelerations required are large, and only certain specimens are suitable. Sweeping the illumination over a stationary specimen can be done much faster than moving the specimen under a stationary light beam. On the other hand, current versions of the scanned beam instruments are truly confocal in epi-illumination fluorescence or reflection modes only. In these modes a single lens and aperture are used for both the illumination and imaging light paths. When the illumination and imaging light travel separate paths, as in all forms of transmitted light imaging (e.g., bright-field, phase contrast, DIC), then only the stationary beam, specimen scanning, instruments are confocal.

In the tandem scanning and laser scanning confocal microscopes, the illumination is scanned, while the specimen is held stationary. Tandem scanning instruments use conventional broad spectrum light sources such as high pressure arc lamps. This has the advantage of allowing a wider selection of fluorescent probes, but the disadvantage of lower intensity illumination, compared to the laser scanning types. Another important advantage of tandem scanning is that for some bright specimens, direct visual observation in real time is possible.

The need for fast scanning places stringent demands on the source of illumination. If we want to collect a 512 by 512 pixel image in 1 second, then the scanning spot of light can dwell on each point for only 4 μsec. In this time one needs to collect as many photons as possible so that the statistical noise in the image is minimized. For this reason a very intense source of light is needed, which in most instances means we have to use a small laser. A schematic diagram of a typical laser scanning instrument is shown in Figure 96.3. As illustrated, the laser is used as an "epi-illuminator" in this as in most other types of confocal microscopes. The objective lens therefore also plays the role of condenser.

A third form of beam scanning microscope has been developed that is, strictly speaking, only partially confocal (Lichtman et al. 1989). These "slit-scanning" instruments replace the round pinhole aperture behind the objective lens with a long very narrow slit. The illumination is shaped into a single narrow line of light, focused to the same line on the specimen that is seen by the slit aperture. Scanning is necessary in only one direction, since the slit is long enough to admit light from points all the way across the field of view. The resolution and contrast in the image are no longer completely isotropic, but in practice the image quality is almost equal to that achieved with spot scanning instruments for some types of sample, and the images are collected in a fraction of the time.

Although the commercial beam scanning instruments do not currently give a truly confocal transmitted image, they do have the useful capability to form non-confocal transmitted light (e.g., phase contrast or DIC) images in parallel with the

reflectance or fluorescence image. Even though they are not truly confocal, the quality of these scanned transmitted images is usually higher than could be obtained using a conventional wide-field microscope, and they will be in perfect register with a simultaneously acquired fluorescence or reflectance confocal image.

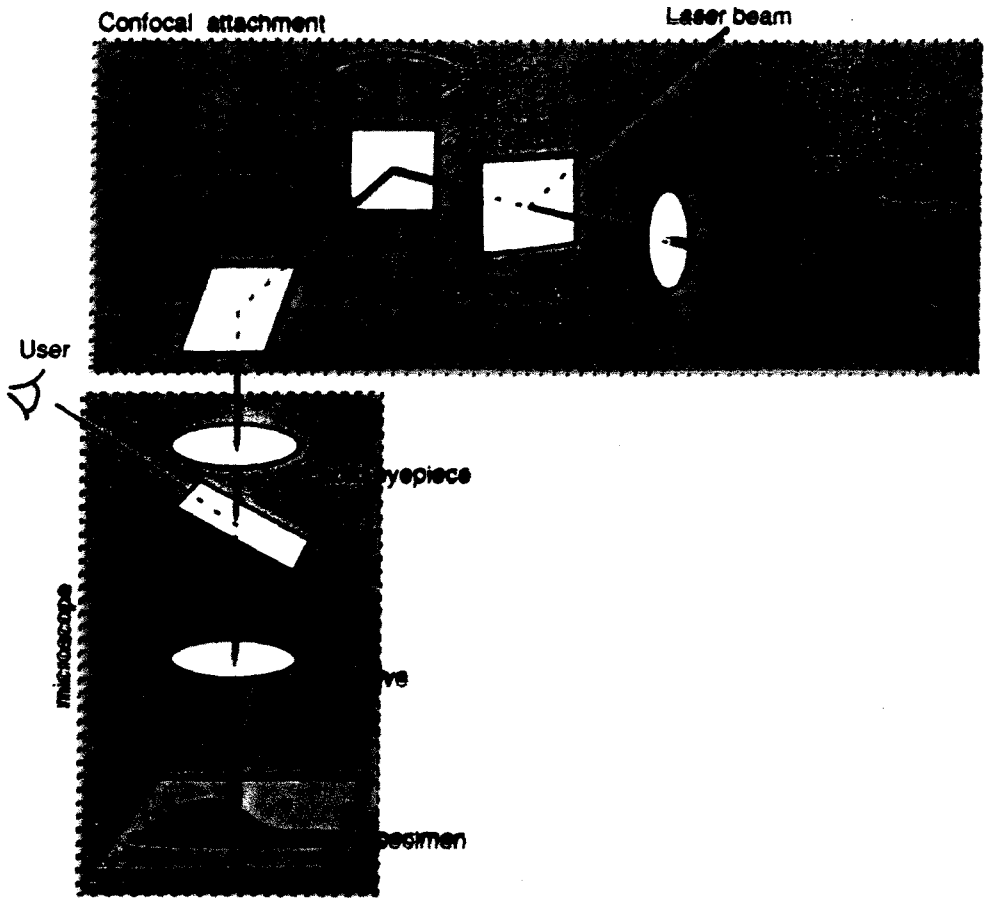


FIGURE 96.3
Diagram illustrating the configuration of a typical laser scanning confocal microscope. The instrument consists of a conventional fluorescence microscope (enclosed in the lower shaded rectangle) to which has been attached a confocal scanning unit (upper shaded rectangle), comprising a pair of scanning mirrors, a laser, some wavelength-selective filters, a pinhole aperture, and a photomultiplier detector. The laser illumination is directed down the phototube of the microscope, having been deflected by the rapidly oscillating scanning mirrors so that it sweeps across the specimen in a raster pattern. Fluorescent light emitted by the sample passes back up through the phototube, is "descanned" by the scanning mirrors, passes through the dichroic beam splitter (which removes any reflected laser light) to the pinhole aperture. Light originating from the focal plane passes through the pinhole to the detector, but all other light is blocked. For reflectance imaging, the dichroic beam splitter is replaced by a half-silvered mirror. By moving a sliding prism, the user can switch to visual (nonconfocal) observation through the usual binocular eyepieces, using the normal microscope lamps for illumination.

Simultaneous Imaging of Multiple Labels

The wavelengths available from the light source become especially important when two or more fluorophores must be imaged in the same specimen. In general, one can expect problems with cross-talk between two channels ("bleed-through") when the emission ranges of two fluorophores overlap significantly, and one of them is much more strongly excited than the other. The range of wavelengths over which emission intensity is at least 5% of the maximum for each fluorophore can be used together with the range of excitation wavelengths to predict when problems are likely to arise. For example, rhodamine and **fluorescein** are a popular pair of fluorescent labels for double-labeling experiments in conventional epifluorescence microscopy, using mercury arc illumination at 495 and 546 nm. However, these two fluorophores often give unsatisfactory results in confocal microscopes, because the commonly used lasers (argon and argon/krypton) do not emit appropriate wavelengths for efficient excitation of rhodamine. Some instruments attempt to use the 488-nm line of the argon laser to excite both fluorescein and rhodamine. Since fluorescein is efficiently excited at 488 nm but rhodamine is not, the weak long-wavelength emission from fluorescein is comparable in intensity to the rhodamine emission, causing severe problems with bleed-through. Fortunately, fluorescein can be used in combination with Texas Red to give excellent results with both conventional microscopes and confocal microscopes using the krypton or argon/krypton lasers.

Caution: **Fluorescein** (See Appendix 5 for Caution)

Specimen Preparation

Confocal microscopy is compatible with any of the conventional specimen preparation methods (see Sections 9 and 11), including imaging of unprepared living tissue or of fixed samples (Figs. 96.4 and 96.5). Modest resolution images of material down to a depth of approximately 0.5 mm below the surface can be obtained from

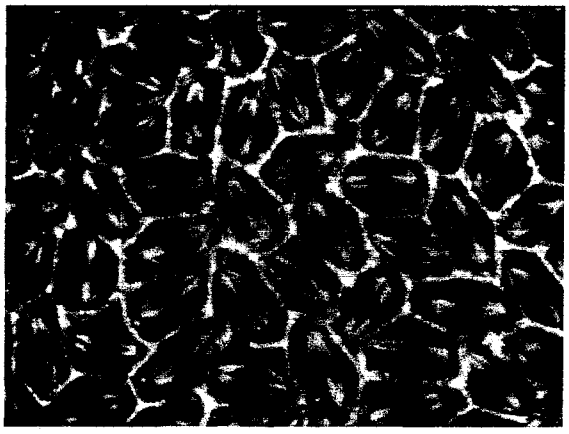


FIGURE 96.4
Optical section of a *Drosophila* embryo during 12th mitotic division showing the distribution of spindle microtubules *(red)* and actin *(green)*. (Photo provided by B. Theurkauf, SUNY, Stony Brook, New York.)

Oxyrase Inc., Ashland, Ohio) of the type normally used for anaerobic metabolic studies.

DECONVOLUTION METHODS

The goal of these techniques is to improve the images of thick objects by computationally removing the out-of-focus blur. The strategy is to use any and all prior information about the specimen and the optical system (in particular, a quantitative understanding of the effects of defocus) to predict the in-focus appearance of a specimen that would produce the observed partially focused image. The method commonly employed is to refine iteratively an initial guess of the true specimen appearance until this estimate, when appropriately blurred by the effects of defocus, yields the actual observed image.

The first requirement for applying this technique is that we understand what happens to the image when we defocus the lens through which we observe the specimen. For this purpose, it is helpful to introduce the concept of the *contrast transfer function* (CTF). This function describes the extent to which contrast variations in the specimen are faithfully replicated in the image. Perfect contrast transfer means that image contrast equals specimen contrast. The CTF is usually expressed as a ratio, so that perfect contrast transfer means that the CTF has a value of 1.0. In the real world, things are less than perfect, and the CTF is always less than 1.0.

It is reasonable to expect that some features of the specimen might be transferred into the image more faithfully than others. For instance, the image may be a nearly perfect representation of the large-scale features of the object, but contain much less information about the very smallest details. This will always be true for images obtained from an optical microscope, since we cannot see clearly those details of the specimen that are small compared to the wavelength of light. Thus the CTF is usually given as a function of the size of the feature being observed (Fig. 96.6). Normally the CTF is shown in graphical form, by plotting the ratio of image contrast to specimen contrast (vertical axis) against the *reciprocal* of size (i.e., spatial frequency).

The example of a typical good microscope CTF shown in Figure 96.6 represents the case where the specimen is thin and lies exactly in the focal plane of the objective lens. When the specimen is moved out of the focal plane, a very surprising thing happens: The CTF develops ripples, and in some regions becomes negative. This means that for some features of the specimen, the image will have reversed contrast. For features in the size range corresponding to these negative oscillations of the CTF, dark parts of the specimen will appear bright in the image and vice versa (Fig. 96.7). As the degree of defocus increases, the CTF becomes increasingly oscillatory, with the contrast reversals affecting ever larger features in the image.

It surprises most people that microscopes can produce such wildly "incorrect" images. An example is shown in Fig. 96.8, but it can be easily observed on almost any specimen. Find a small high-contrast feature in a specimen (a small dust particle or a scratch works well) using a good dry or oil-immersion lens. Carefully focus up and down by a small distance on either side of correct focus and you will see the particle oscillate from bright to dark and back again. If you can control the focus carefully enough, you may be able to find the position, halfway between a bright and a dark oscillation, where the particle becomes practically invisible. Evidently some care is required in interpreting microscope images! How can you tell which is the "correct" appearance?

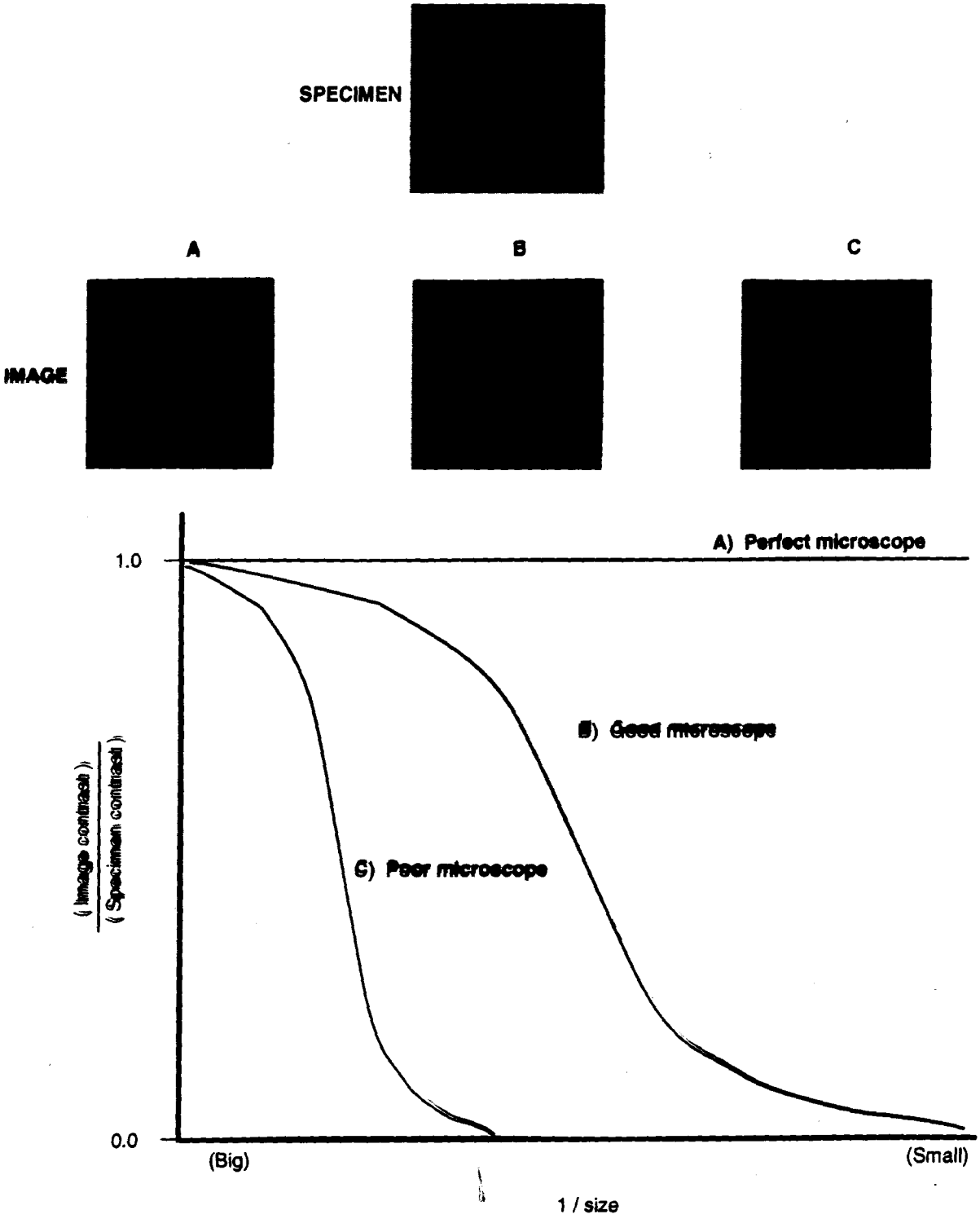


FIGURE 96.6
Schematic representation of some contrast transfer functions, and the corresponding object-image pairs. (*A*) A perfect (impossible!) microscope; (*B*) a typical good microscope; (*C*) a poor, or improperly used, microscope.

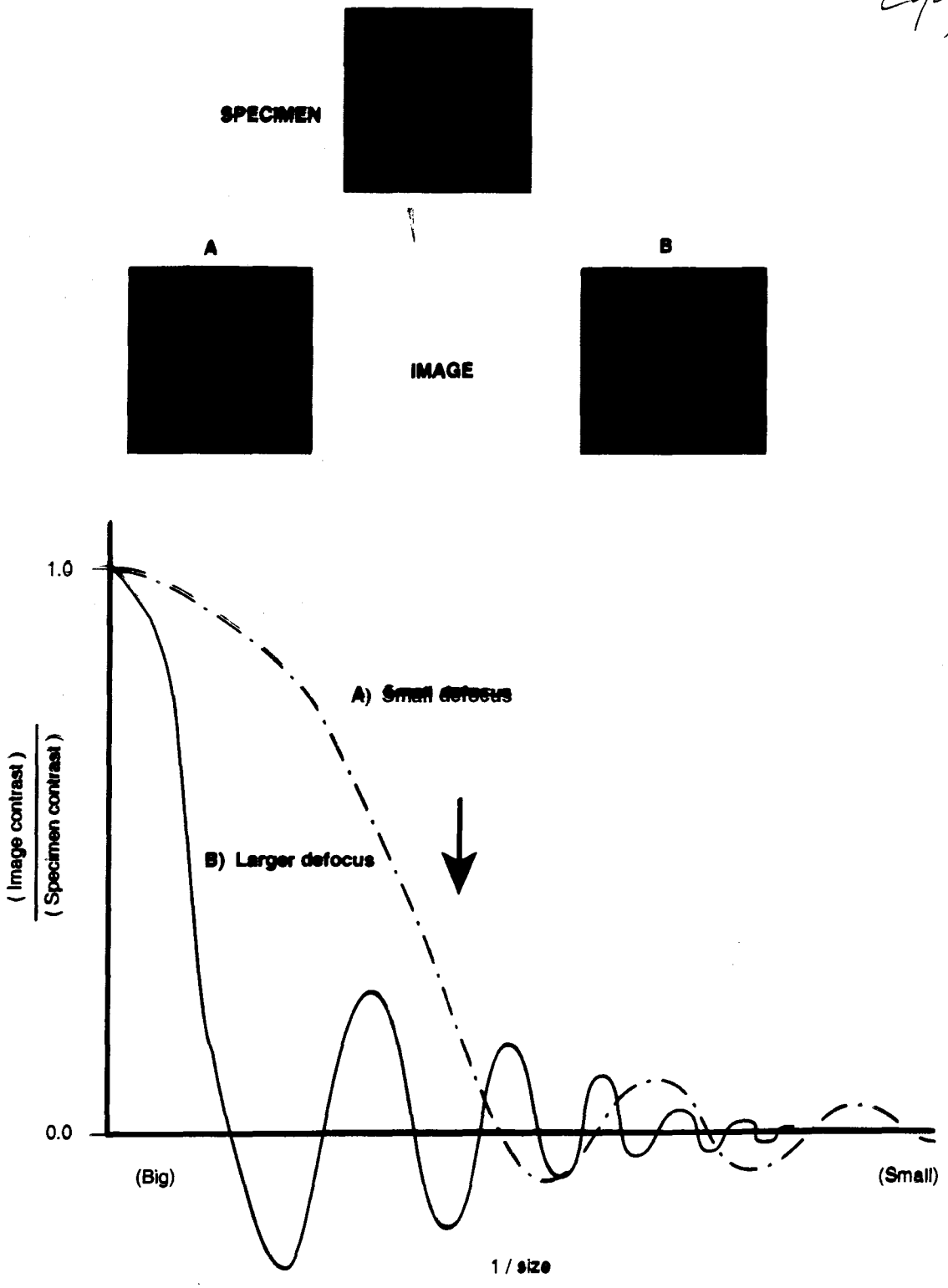


FIGURE 96.7
Images and CTF from a microscope at two different values of defocus. Objects in the size range indicated by the arrow would be seen to change from black to white or vice versa as the focus level changed between the two values indicated. An example of this contrast reversal is shown in Figure 96.8.

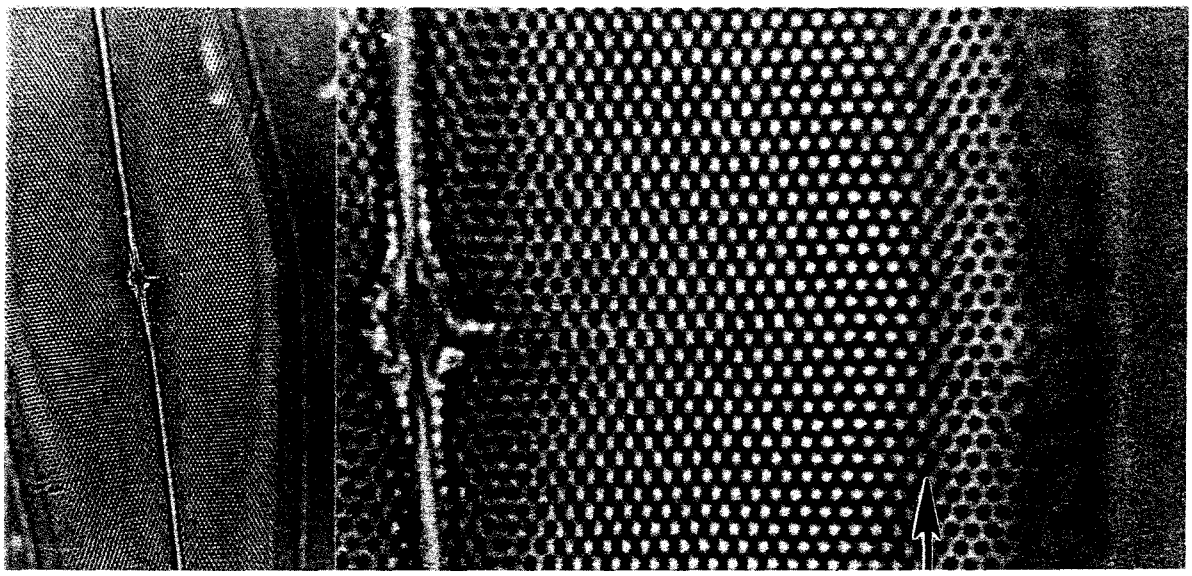


FIGURE 96.8
Contrast reversal due to defocus. (*left*) A bright-field image of a diatom taken through a conventional microscope using a 60× NA 1.4 lens. (*right*) An enlargement of a small portion of the image on the left, showing contrast reversals due to changing amounts of defocus. The diatom shell is curved, being thinner at the edges. As a result, the distance from the lens to the surface of the diatom varies; in other words, the view includes a range of defocus values. Over this range, the CTF changes sign twice: from left to right, the holes can be seen to change from black to white and then to black again. The arrow indicates a narrow band midway between a black and a white hole region where the contrast of the holes is low: i.e., the CTF is nearly zero for structures of this size at this value of defocus. (Photo provided by J. Murray, University of Pennsylvania.)

The CTF contains all the information we would need to predict the appearance of a known specimen when viewed through the corresponding optical system. However, our problem is the converse of this. We know the appearance, but we would like to know the real structure of the specimen. Although it is in principle possible to go backwards in one step from the observed appearance to the actual structure using the mathematical procedure of *deconvolution*, in practice it works better to carry out this calculation in an iterative fashion. For this procedure, we consider the specimen to be made up of a stack of discrete planes. Normally our data will also be a stack of images of the specimen, collected by changing the fine focus of the microscope by a small increment between successive images. To illustrate the iterative calculation, we will describe the steps for calculating one plane, say number 5, of a specimen that is 9 planes thick (Figs. 96.9 and 96.10)

Consider plane number 5 of the observed stack of images (Fig. 96.9). When this image was recorded, what the detector "saw" was the sum of an in-focus view of specimen plane number 5 and a view of specimen plane number 6 blurred by one increment of defocus, plus specimen plane number 4 blurred by one increment of defocus in the opposite direction, plus plane numbers 7 and 3 blurred by plus and minus two increments of defocus, respectively, and so forth. Assume that we have made an initial guess at the real structure of the specimen on planes 1–4 and 6–9.

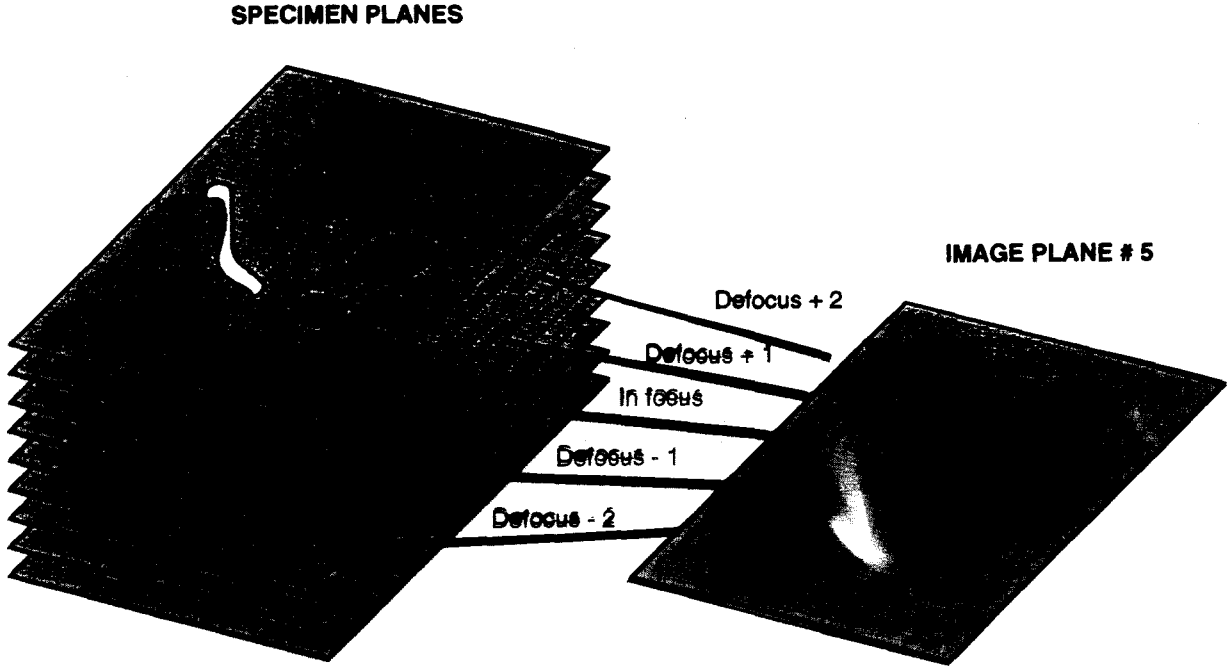
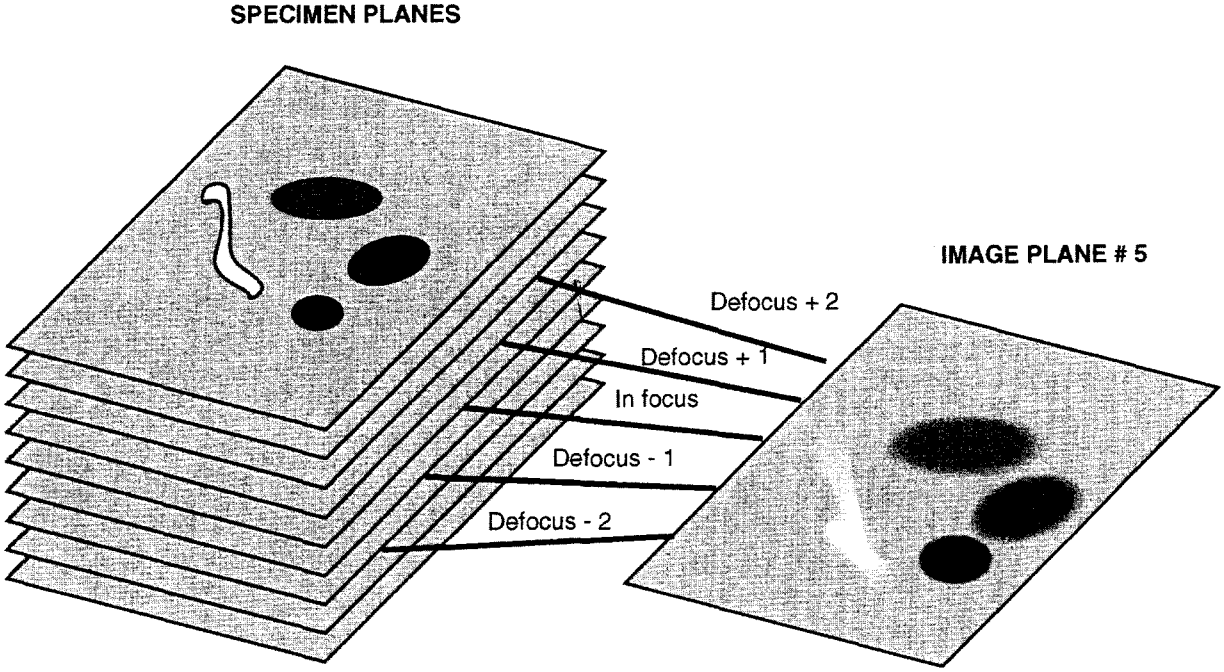


FIGURE 96.9
Formation of an image of a thick specimen by adding the in-focus image of one plane to the images of neighboring planes viewed at different amounts of defocus.

Using the known CTF appropriate for each plane's defocus, we can blur these initial estimates to predict their contribution to the observed plane number 5. Subtracting the sum of blurred specimen planes 1–4 and 6–9 from the observed plane number 5 then gives us an estimate for the in-focus appearance of specimen plane number 5. Repeating these steps for all 9 planes generates an improved estimate of the specimen. The entire sequence of operations on all planes is repeated in a loop until the specimen estimate no longer changes significantly (Fig. 96.10).

Fortunately, when using the high-numerical-aperture lenses necessary for fluorescence microscopy of biological specimens, the contributions to an image plane from distant specimen planes becomes insignificant, so we need not consider all specimens planes at every step of the cycle. The number of neighboring planes we need to include in the calculation depends on the size of the focus increment, the numerical aperture of the lenses, and the desired final resolution. Typically, for an effective numerical aperture of 1.33, defocus values greater than ± 4 μm can be safely ignored. For certain specimens, a substantial improvement can be gained by considering only the "nearest-neighbor" planes, which is a simple computation that runs quickly on small PC class computers.

The success of this iterative deconvolution procedure depends critically on the accuracy of the image data and of the CTF. Typical signal-intensified target (SIT) camera images, although digitized to eight bits, are rarely reliable to more than 5 or 6 bits. CCD camera data are far superior, and cooled CCDs have been used for most applications of deconvolution techniques.



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Fortunately, when using the high-numerical-aperture lenses necessary for fluorescence microscopy of biological specimens, the contributions to an image plane from distant specimen planes becomes insignificant, so we need not consider all specimens planes at every step of the cycle. The number of neighboring planes we need to include in the calculation depends on the size of the focus increment, the numerical aperture of the lenses, and the desired final resolution. Typically, for an effective numerical aperture of 1.33, defocus values greater than ± 4 μm can be safely ignored. For certain specimens, a substantial improvement can be gained by considering only the "nearest-neighbor" planes, which is a simple computation that runs quickly on small PC class computers.

The success of this iterative deconvolution procedure depends critically on the accuracy of the image data and of the CTF. Typical signal-intensified target (SIT) camera images, although digitized to eight bits, are rarely reliable to more than 5 or 6 bits. CCD camera data are far superior, and cooled CCDs have been used for most applications of deconvolution techniques.

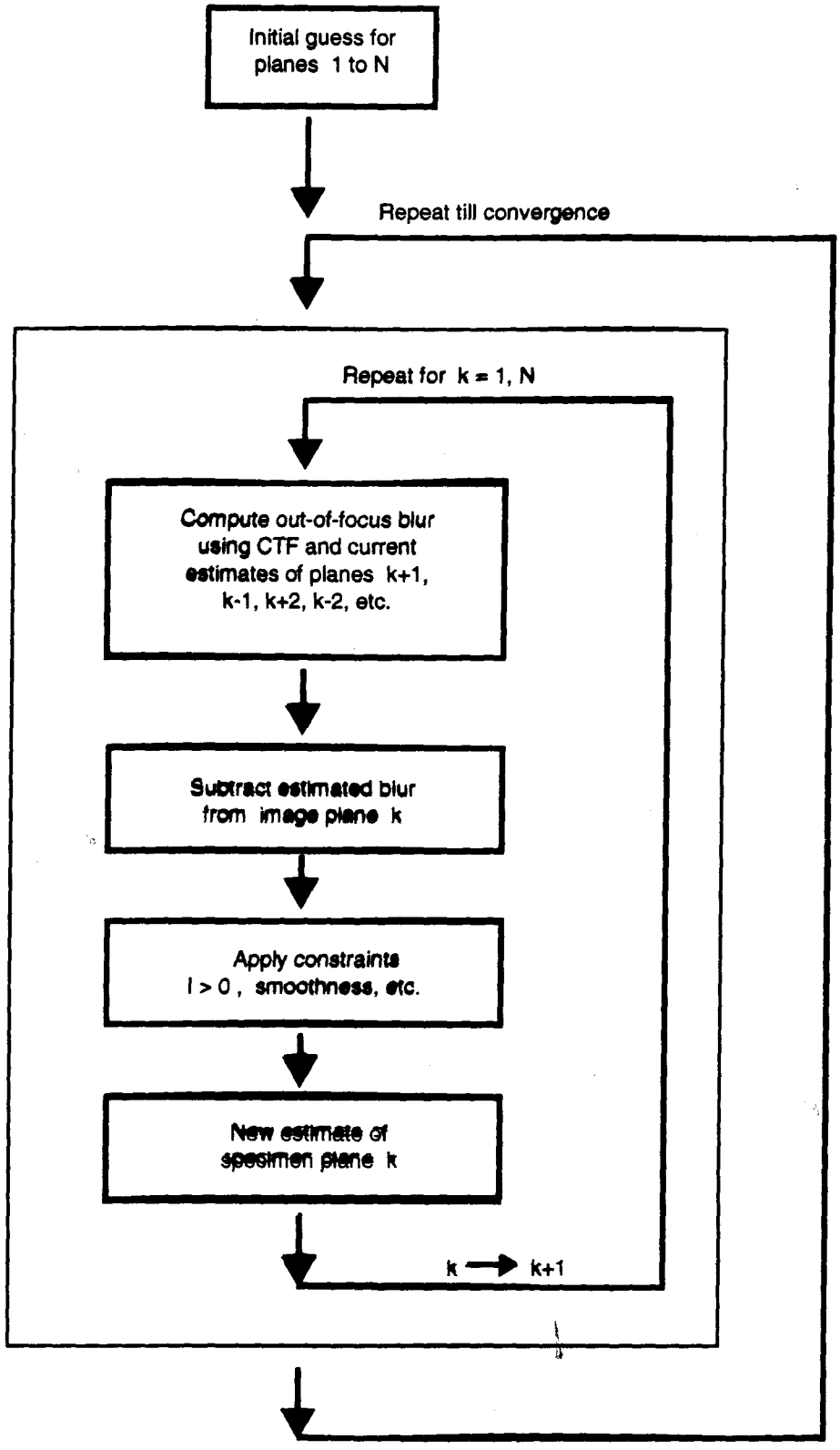

FIGURE 96.10
Flow chart for the constrained iterative deconvolution algorithm.

INTERPRETING THE RESULTS

Although the images produced by confocal microscopy and deconvolution methods are referred to as optical "sections," they differ from true sections in that their top and bottom edges are not sharply defined. In a true section as cut by a microtome, there is no ambiguity about which section contains each point of the original specimen, at least not at the resolution of the light microscope. A specified location in the cell either gets included in one particular microtome section or it does not, depending on its position, but there is no intermediate state. An optical section, however, includes some locations fully (i.e., present at their true intensity), and other locations above and below at less than their actual intensity. There is no sharp cutoff that demarcates what is included in the optical section and what is excluded. Instead, there is a continuous decrease in intensity for locations further and further away from the midpoint.

A measurement that is commonly used as the analog of section "thickness" for optical sections is the width at half amplitude (full width at half maximum, FWHM) of the curve that describes the relative intensity of points at different distances from the midpoint of the section. This curve can be easily measured by collecting a series of closely spaced optical sections [0.1 μm for the highest numeric aperature (NA) lenses] of a small bright object. A small fluorescent bead is a good specimen for this measurement, but any bright object that is small compared to the expected FWHM can be used. A plot of the total intensity of the image of the object in each optical section should give a curve similar to those in Fig. 96.11, referred to as the vertical point-spread function (PSF) for the optical system. For the highest NA objective lenses, confocal microscopes should give a vertical PSF having a FWHM equal to approximately 0.6 μm.

The width of the vertical PSF decreases with the square of the NA of the objective lens. For confocal systems, the width also decreases with decreasing pinhole size. For all microscopes, the vertical PSF is very sensitive to the presence of spherical aberration (see Fig. 96.11). Unfortunately, one is often forced to accept a certain amount of spherical aberration when examining thick specimens (see Section 9). In order to maximize the amount of light collected from weakly fluorescent specimens, it is necessary to use the highest NA oil immersion objectives. These lenses are designed for work with a specimen that is located immediately beneath a coverslip connected to the lens by immersion oil, which obviously cannot be true for all regions of a thick specimen. Consequently the image is increasingly degraded by spherical aberration as the focal plane is set deeper and deeper into the specimen. The problem is greatly ameliorated by using the recently developed, long working distance, water immersion objectives. The currently available water immersion lenses are much more expensive than the best oil immersion objectives, but they allow one to obtain excellent images to a depth of approximately 200 μm beneath the coverslip.

THREE-DIMENSIONAL RECONSTRUCTION

The output from confocal microscopy or deconvolution is typically a stack of optical sections, collected by changing the focus by a constant amount between each image acquisition. If the goal is to collect enough information to permit a three-dimensional (3D) reconstruction of a specimen at the highest resolution possible,

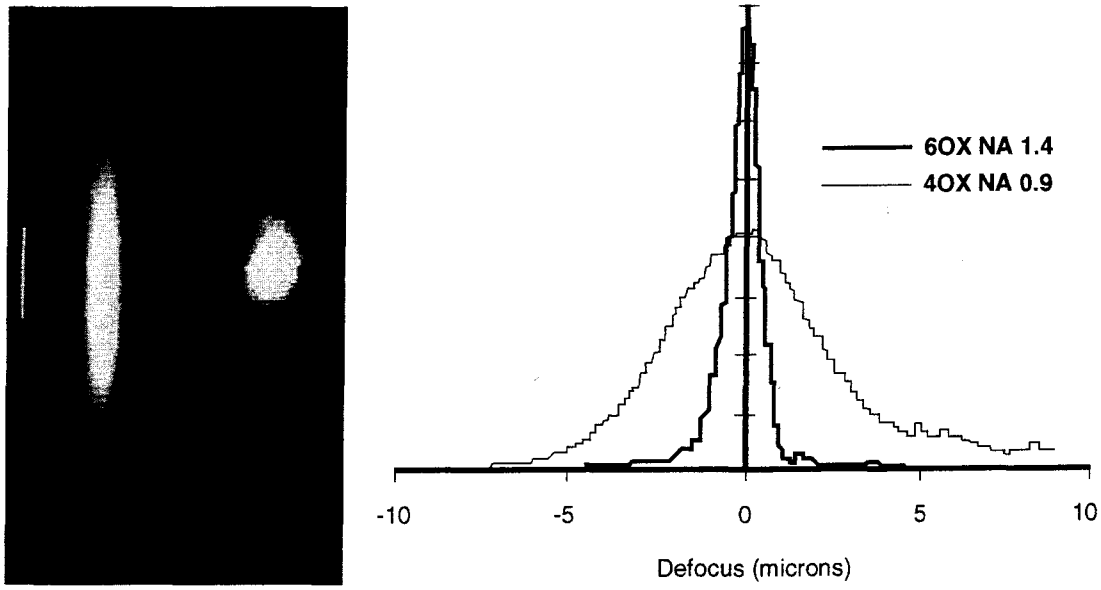

FIGURE 96.11
***X-Z* scans of 0.9 μm-diameter fluorescent beads and measured vertical point-spread functions.** The left half of the image shows a scan using a 40 × 0.9 NA objective lens. The right half is a scan of a different sample using a 60 × NA 1.4 lens. The round bead appears much more elongated with the lower NA objective because decreasing the NA affects vertical resolution much more severely than lateral resolution. The left scan is markedly unsymmetrical above and below focus, indicative of spherical aberration. The right scan (of a different sample) also shows a small amount of spherical aberration. Scale bar, 2 μm. The graphs show the measured vertical point-spread functions for these two situations. In these curves, spherical aberration is manifest as an unsymmetrical profile to the right and left of the peak.

then adjacent optical sections will have to be spaced at increments of roughly half the FWHM of the vertical PSF. The resolution in the vertical direction will always be worse than that in the horizontal direction, by approximately a factor of 3. Thus it is common in 3D reconstructions, particularly from confocal microscopes, for objects to appear elongated in the vertical direction (see Fig. 96.11). The software supplied for deconvolution often compensates for this effect, and a similar compensation (essentially a one-dimensional edge sharpening filter) is helpful on confocal images.

TROUBLESHOOTING GUIDE

When poor images are obtained, the first question to be answered is whether the problem is with the specimen or with the equipment. An enormous amount of wasted time and frustration can be avoided if standard samples are available that can be used to compare the present system performance with its performance in the past on a day when good images were obtained. Two simple specimens are useful for this purpose: small fluorescent beads, 0.2–0.5-μm diameter, and a solution of fluorescent dye. The beads should be spread into a film on a glass slide, allowed to dry, and mounted in a very thin layer of antifade solution. A generous layer of the fluorescent dye solution should be sealed under a coverslip. For multichannel instruments,