

The Specimen Illumination Path and Its Effect on Image Quality

Carol J. Cogswell and Kieran G. Larkin

INTRODUCTION

The unique imaging properties of all confocal microscope systems are based on the fundamental condition that the illumination and collection (detection) optical paths contribute equally to the formation of the final image. However, many of the optical and imaging properties of confocal microscopes can be analyzed effectively by considering the illumination and detection paths to be two distinct entities. In this chapter, we explore one of these optical paths—the illumination path—and describe the ideal function and practical limitations of the total path and the optical components therein. Several other chapters in this volume present additional specific information concerning some of these optical components (e.g., light sources, intermediate optics, objective lenses). Our purpose is to treat the illumination path as an integral system and describe its contribution to the overall performance of the confocal microscope.

Optical Components and Layout of the Illumination Path

Over the past few years, several different confocal microscope designs have become commercially available, including designs that employ some combination of rotating disks, scanning mirrors, or scanning stages that use either white light or laser illumination and that incorporate pinhole or slit detectors. To avoid exhaustive explanations in this chapter, we shall confine our discussion to confocal systems containing the following components or design features: (1) laser illumination (not white light), (2) beam-scanning and specimen-scanning *pinhole* designs (not rotating disks and not slits), and (3) visible wavelength lasers (not UV). It should be noted that the other confocal microscope designs, such as those using rotating Nipkow disks or scanning slits, require that similar constraints be met in their illumination paths in order to optimize image quality.

Figure 1 is a schematic layout of the illumination path of a typical laser scanning confocal microscope; however, some of the components in this diagram are not present in all of the different confocal instrument designs. The specific function of each of these components is described in detail later in this chapter.

Purpose of the Illumination Path

The ultimate purpose of the illumination path is to produce a diffraction-limited illuminating spot at some plane within the specimen and maintain the integrity of that spot during scanning. A laser provides a highly accurate source of parallel wavefronts for the confocal illumination path. To obtain optimal performance, the quality of this laser light must be maintained all the way to its focus within the object. Ideally the size, shape and intensity of the illuminating spot should be the same for any λ laser used or for any thickness of specimen. In practice, fixed aberrations in the microscope optical system or variable aberrations resulting from a complex specimen as it is scanned in depth will degrade the final image. This chapter will be devoted to a discussion of what constitutes the ideal illumination path and focused spot in a confocal microscope, which phenomena (in practice) commonly degrade this ideal, and what can be done to correct these resulting defects.

PROPERTIES OF THE ILLUMINATING SPOT

The illumination point-spread function (PSF) defines the distribution of irradiance* in the focal region (i.e., the size and shape of the illuminating spot) and is characterized by interrelated transverse and axial dimensions. Some simplified theory will be presented to clarify aspects of the diffraction PSF that often remain obscured in the more detailed theory. Many errors in diffraction calculations are due to inappropriate approximations for high numerical aperture (NA) systems. The equations presented in this section will give errors of less than 10% for all commonly encountered NAs (in fact for all semi-aperture angles, $\alpha < 72^\circ$).

*The term light intensity, or, more correctly, irradiance refers to the power (in Watts) per unit area (in m^2) incident upon a region. Strictly, the power is actually the power in the electric (not magnetic) field and is thus proportional to the square of the electric field vector.

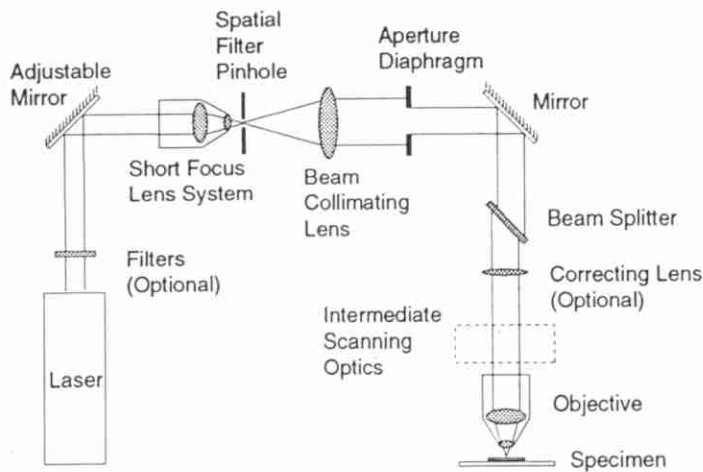


FIGURE 1. Schematic diagram of components that may be found in the illumination path of a confocal laser microscope.

Illuminating Spot Size and Shape

Small NA

The distribution of irradiance in the focal region for objectives with $NA < 0.5$ has the well-known Airy pattern in the focal plane and a simple sinc-squared distribution^{*} along the optical axis (commonly called the z -axis). Plots of the corresponding transverse and axial distributions are shown in the previous chapter in this volume. However, for $NA > 0.5$, the approximations involved in the above analysis are no longer valid and can lead to significant errors in the predicted irradiance. To avoid errors in prediction, it is prudent to use the appropriate theory outlined in the next paragraph which gives correct results for both large and small NAs.

Large NA

High-resolution microscopy typically involves the use of objectives with NA in the region 0.9–1.4. In this case, the detailed diffraction theory to predict the irradiance is more complicated for two reasons: (1) the extremely steep convergence angles modify the polarization of the marginal (outer) beam; and (2) the amplitude of the marginal beam is reduced by a fundamental geometric effect (i.e., it is **apodized**). In the case of classically designed objectives, which satisfy an optical design constraint known as the **sine condition**, the beam irradiance drops off as the cosine of the beam angle. Also, the focal plane irradiance distribution for a linearly polarized input beam[†] no longer has the radial symmetry associated with the Airy disk. Instead, the widths in the two transverse directions differ to some extent. The axial irradiance distribution also departs from the simple sinc-squared form associated with small NA.

Perhaps the simplest way to characterize the irradiance distribution near the focus is by utilizing the **full-width half-maximum**[‡] (FWHM) as measured in each of the three orthogonal

* $\text{sinc}^2(az) = (\sin(az)/az)^2$.

[†] Typically, the lasers used in confocal microscopes are linearly polarized.

[‡] FWHM is the full width of the irradiance distribution measured at one half the maximum irradiance value.

directions. Conceptually the advantage is to reduce a multidimensional, multi-variable distribution down to just three representative numbers which essentially define the width of the PSF along three axes. Many people may be familiar with other criteria for specifying the spot size (or equivalently the **resolution**) such as the Rayleigh criterion. Unfortunately such a criterion does not necessarily give meaningful results at very high NA.

In a **conventional** optical microscope, the three FWHMs are closely linked to the spatial frequency response of the **detection** path alone. The spatial frequency response of a **confocal** microscope is equally dependent upon both the illumination and detection paths. The FWHM can be calculated using the expressions in Table 1, where η is the index of refraction in the medium between the focal point and the objective and λ is the wavelength of light. (The beam polarization is assumed to be parallel to the y -axis.)

If there is a misalignment or other defect in the illumination path, the corresponding FWHM will increase to a value greater than that given in Table 1, with the result that resolution performance will be diminished.

Maximum Irradiance of the Illuminating Spot

An aspect of great importance to confocal microscopy is the peak irradiance, I_p , obtainable from an objective of known NA and input power. This is particularly important under conditions in which the amount of laser illumination is limited or when the signal emitted by the specimen is weak. Typically such conditions are found in fluorescence and multiwavelength studies or when examining very thick biological specimens. Perhaps worth remembering is the fact that any losses occurring in the objective occur both on the way in and on the way out, which means that an objective with low transmission will significantly reduce the signal detected from a specimen. Such an objective would be unsuitable for most fluorescence studies.

The precise expression for the peak irradiance is as follows (Sheppard and Larkin, 1994):

$$I_p = \frac{\pi}{2} PT \left(\frac{\eta}{\lambda} \right)^2 (1 - \cos \alpha) (3 + \cos \alpha)$$

where P is the input power in Watts; T is the transmission factor of the objective; $\alpha = \sin^{-1}(NA/\eta)$ is the semi-aperture angle of the objective; and η is the refractive index of the immersion medium. The above expression is valid for any value of NA and includes the

TABLE 1. FWHM of Illumination PSF Irradiance

FWHM	Scalar Approximation (Unpolarized)	Polarized
	W_x	$\frac{0.51\lambda}{\eta \sin \alpha}$
W_y	$\frac{0.51\lambda}{\eta \sin \alpha}$	For high NA: $\frac{0.55\lambda}{\eta \sin \alpha}$
W_z	$\frac{0.44\lambda}{\eta \sin^2(\alpha/2)}$	Slightly more for high NA

vector components of an incoming polarized beam. Paraxial theory predicts that I_p increases as the square of the NA and thus actually predicts a value up to 40% in error for large NA.

As an example of a peak irradiance calculation we consider the following microscope objective:

$NA = 1.4$, $\eta = 1.52$, $\alpha = \sin^{-1}(1.4/1.52) = 67^\circ$ and transmission factor $T = 0.6$ (see next section). Consequently, if $P = 1$ mW and $\lambda = 633$ nm, then:

$$I_p = \frac{\pi}{2} [0.001] 0.6 \left(\frac{1.52}{6.33 \times 10^{-9}} \right)^2 (1 - \cos 67^\circ) (3 + \cos 67^\circ) [\text{W/m}^2]$$

$$I_p = 11,227 [\text{W/mm}^2] = 11.2 [\text{mW}/\mu\text{m}^2]$$

The final choice of units for I_p , although not necessarily recommended as an SI standard, should be more meaningful to microscopists.

The transmission factor of an objective must be found by measurement because manufacturers do not quote the value. Generally, the power transmission factor can be expected to be well above 60% even for multiple-element lenses if good anti-reflection coatings are used on all air/glass interfaces. A simple way to measure T is shown in Fig. 2. Two identical lenses should be used so that the angular sensitivity of the power meter is eliminated from the results. If the power input into the lens pair is P_1 and the transmitted power is P_2 , the transmission factor is given by:

$$T = \sqrt{\frac{P_2}{P_1}}$$

DEFECTS THAT MAY OCCUR IN THE ILLUMINATION PATH

We have seen in the previous section that a high-NA objective with good power transmission is generally desirable for applications such as high-resolution confocal fluorescence microscopy. It is possible to calculate the idealized 3D irradiance distribution of the focused spot for such an objective (Sheppard and Cogswell, 1990). For example, Fig. 3 is a 2D section (xz) through a calculated 3D irradiance distribution for a high-NA objective. In practice, however, it is extremely difficult to actu-

ally observe or measure this 3D focused spot. The reason for this should be obvious: most attempts to image the tiny focused spot require using yet another optical system, which introduces its own diffraction effects and aberrations.* In light of this difficulty, the following section describes some **indirect** methods for observing the quality (i.e., size, shape, and intensity) of the illuminating spot in a confocal microscope. These methods give only relative (rather than absolute) measures of the lateral and axial size and shape of the focused spot, as well as the overall alignment and optical properties of the components in the illumination path. Nonetheless, as described in detail below, there are many sources of potential aberrations and misalignment defects in the illumination path, all of which degrade the focused spot, and hence the final image. It is therefore extremely important to have some methods (however imprecise) for locating defects and evaluating whether they can be reduced.

How to Measure or Observe Defects in the Illumination Path

A few easy-to-perform tests of the confocal system are described in this section. These may be useful to the biologist for gaining some insight into how well certain objectives and optical components are performing in conjunction with the particular specimen being observed. The effectiveness and effort involved in carrying out these tests will vary depending on the design of the particular confocal system being used.

Axial Response

A standard method for measuring the axial resolution of a confocal reflection system is to first illuminate a planar object (e.g., a front surface mirror) with a stationary diffraction-limited spot, and then step or scan the object through the focus of the microscope

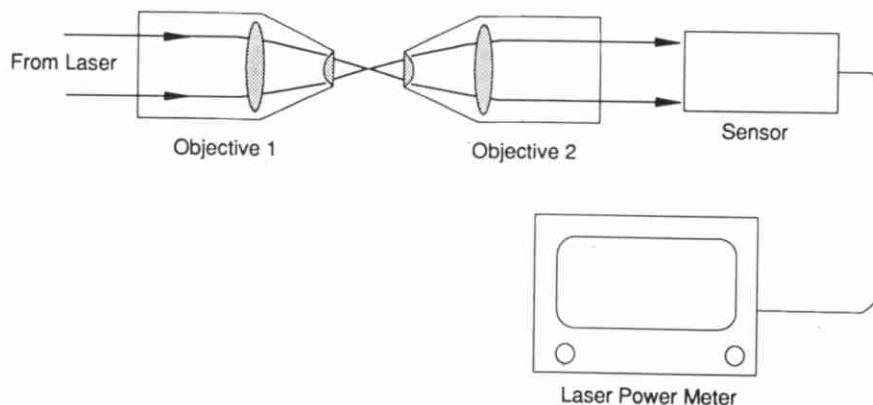


FIGURE 2. Method for measuring the transmission factor of objectives using a laser power meter and two identical axially aligned objectives.

*A notable exception is the technique recently developed by A. Roberts, K. Nugent and A. Barty in the School of Physics, University of Melbourne. They use an optical fiber probe with a precision-tapered tip that has been sheathed in aluminium to produce an aperture (diameter approximately 100 nm) at the very end of the probe. This fiber tip is mounted in the specimen plane of the confocal microscope while its opposite end is coupled to a photodetector. Because the tip diameter is small, it is possible to measure directly the irradiance of the focal distribution simply by scanning the fiber tip laterally and axially through the specimen field (Roberts, Nugent and Barty, personal communication).

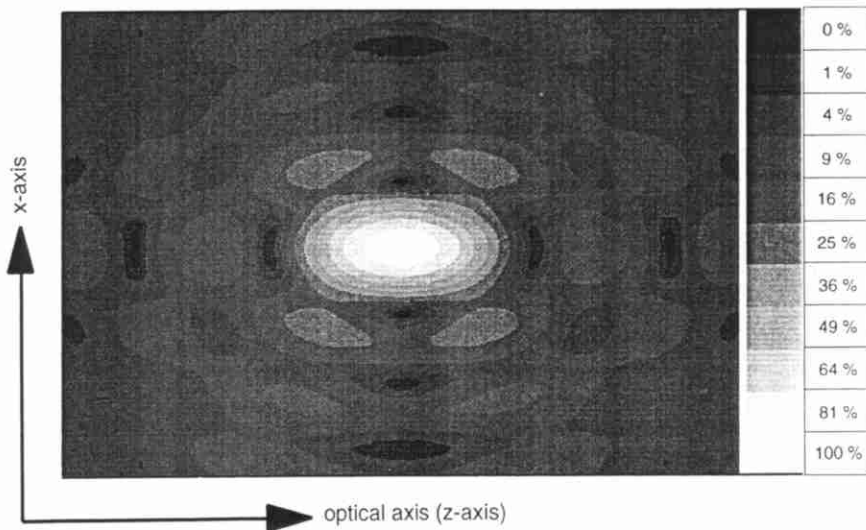


FIGURE 3. Contour plot of the intensity distribution of the illumination point-spread function (PSF) at the plane of focus in a typical confocal laser microscope.

(i.e., parallel to the z-axis). The intensity of the resulting signal, obtained from the pinhole detector, can then be plotted as a function of the position of the reflecting planar object along the z-axis, as shown in Fig. 4. The FWHM of this graph is commonly used as a measure of the axial resolution of the confocal system (Cogswell *et al.*, 1990).

For a confocal fluorescence configuration, a very thin layer of fluorochrome dye (approximately one-half λ in thickness) could be used instead of a front surface mirror and scanned through focus in the same way as above. However, this would be more difficult and is not really necessary for our purpose here, which is to identify defects in the illumination path of the microscope.

xz-Imaging and Axial Response

A variation on the above method may be necessary for certain commercial confocal beam-scanning microscope designs that do not allow the user to fully stop the scanning mechanism

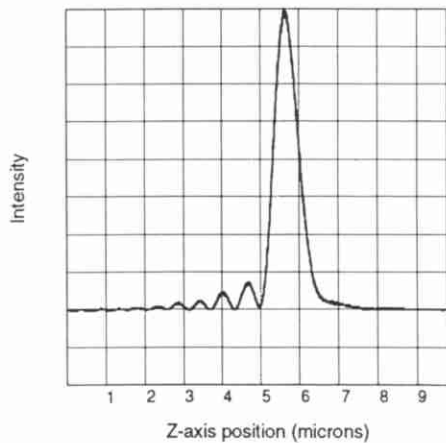


FIGURE 4. Typical axial response curve obtained by scanning a planar mirror through the focal region of the illuminating spot (parallel to the z-axis) and recording the signal intensity at the detector. FWHM (measured from this plot) = 0.7 μm .

in both the x- and y- directions. In these systems, it is usually possible to produce an image in the xz-plane (i.e., to repeatedly scan a single line of points parallel to the x-axis, while changing the focus in z). If a front surface mirror is used as the test object, the resulting image will be a 2D representation of the axial response. Using computer image analysis software, a graph of the image intensity vs. depth (z-axis position) can usually be obtained by plotting values from a line of pixels drawn parallel to the z-axis (i.e., through focus).

Figure 5 illustrates an xz-image of a front-surface mirror obtained using a beam-scanning commercial confocal microscope. In this example, the analysis software provided by the manufacturer allows the user to draw a line through any region of the image (in this case parallel to the z-axis) and obtain a graph of the pixel intensities along this line.

Indirect Imaging of the Focused Spot Using a CCD Detector

A very useful indirect method for gaining information about the illumination path of the microscope and the characteristics of the focused spot is to place a charge-coupled device (CCD) camera at the detector pinhole plane of the confocal system being studied. This can be done in many confocal microscopes by installing the camera (without lens) in place of one of the photomultiplier detectors. The CCD array detector must be placed at the same plane previously occupied by the detector pinhole (i.e., the rear conjugate focus of the illuminating spot). In some types of confocal microscopes, it may be easier to temporarily insert a mirror or beamsplitter into the detection path in order to redirect the light to a CCD. Figure 6 shows the image of a stationary illuminating spot (Airy disk), obtained by placing a CCD camera at the detector plane of a confocal microscope and turning off the scanning mechanism. In this example, the specimen was optically very uniform (front surface mirror, immersed in oil, NA 1.4 oil objective), and hence, most of the energy in the image is distributed in the central peak with fairly even radial symmetry.

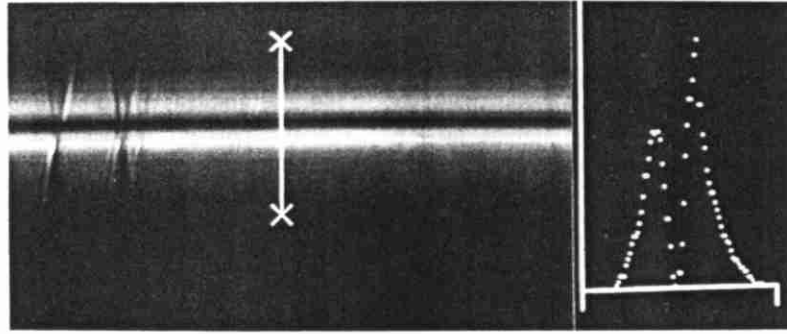


FIGURE 5. xz -image of a front-surface mirror obtained using a commercial confocal microscope is shown at left. On the right is an axial response graph of pixel intensities from along the vertical line in the xz -image showing the double peak typical of astigmatism.

Locating and Correcting Defects in the Illumination Path

In this section, we describe many of the commonly occurring problems encountered in the specimen illumination path, which can ultimately degrade the confocal image. For ease of understanding, we will begin at the laser source and trace through the optical path, describing the function of each component, its potential for producing aberrations or alignment defects, and some possible solutions to these problems.

Laser

The laser is a convenient source of near-monochromatic coherent radiation commonly used in confocal microscopy because its brightness is far greater than any other available source. Laser sources are considered in detail in Chapter 5 (*this volume*).

Possible problems and solutions:

- Some lasers may be susceptible to power fluctuations or may have pointing instabilities that will make them unsuitable for use as illuminating sources in confocal microscopy. These particular aspects of laser design and performance need to be carefully investigated before purchasing a laser for a confocal application. Pointing instabilities can be minimized by a properly designed beam



FIGURE 6. The (indirect) image of an illuminating spot obtained by placing a CCD camera at the detector plane of a confocal microscope with the scanning mechanism turned off. A front-surface mirror was used as a specimen.

expander/spatial filter (see section below). However, a poor design can have the result that pointing instabilities produce intensity variations.

- Another problem commonly encountered is vibration in the microscope (visible as jitters in confocal images of linear features). Vibration can be caused by a cooling fan being directly connected to the laser source which in turn is rigidly connected to the microscope. Even though the cooling fan may be separate from the laser, vibrations are often transferred through a wire-reinforced plastic hose used to connect the two or produced by turbulent flow of the air. In some cases, this problem can be remedied by decoupling the laser from the cooling fan using a vibration absorbing baffle of some flexible material at one end of the connecting hose (such as the wrist section of a rubber glove with the fingers cut off). In more extreme cases, the best solution may be to mount the laser at a distance from the microscope and couple the illuminating beam by way of an optical fiber. Optical fiber coupling is discussed in more detail later in this chapter and in Chapter 33 (*this volume*).
- For some optical modes of confocal microscopy [i.e., polarization and Nomarski differential interference contrast (DIC)] a polarized laser must be used or, alternatively, a polarizing filter will be required in the illumination path. The use of a polarizing filter will reduce the intensity of the illuminating source, which may be undesirable when examining weakly reflecting specimens. Fortunately, most lasers used in commercial confocal instruments are polarized.
- A second related problem can arise if the plane of polarization of the laser is not aligned to be either parallel or perpendicular to the planes of incidence of the mirrors and beamsplitters in the illumination path (see discussion below).

Spectral Filters

Spectral filters are not usually necessary in the **illumination** path of a scanning laser confocal microscope because the laser source already produces a highly monochromatic beam. Such filters are generally of great importance in the **detection** path for

selecting specific imaging λ s if multiple illumination frequencies are employed.

Possible problems and solutions:

- Occasionally spectral filters may be utilized in the illumination path to isolate specific λ s from a multimode laser (e.g., for dual- λ excitation fluorescence studies). In these cases, the optical quality of the filter is critical. Tests should be conducted to ensure that the filter does not have wedge-errors (i.e., does not deflect the beam because the two sides are not parallel) and that it is polished flat enough to prevent the introduction of optical aberrations into the illumination path.

Neutral Density (ND) Filters

Neutral density filters are frequently used in laser microscopy to vary the brightness of the illuminating beam.

Possible problems and solutions:

- The optical quality of ND filters is also variable. The built-in ND filters in most contemporary research quality microscopes and confocal systems can probably be relied on to be of sufficient quality not to cause beam misalignment or aberrations. However, this assumption should not be made for any additional ND filters acquired by the user for occasional insertion into the optical path of the confocal microscope.
- For best results, spectral and neutral density filters should be inserted between the laser source and the spatial filter/beam expander. In this position, aberrations and wedge-errors introduced to the beam by the filters can be reduced by the spatial filter (pinhole) which becomes the new, fixed source for the optical system.

Laser Beam Expander

This element of the illumination system generally consists of three parts: a low-power microscope objective (or an aberration-corrected short-focal-length lens), a circular pinhole, and a long-focal-length achromatic lens, which collectively form a reverse telescope system. The purpose of these components is to expand the laser beam (typically a gaussian profile of approximately 0.5-mm diameter) into a second collimated beam (5- to 10-mm diameter), which is large enough to fill the back focal plane (BFP) of the microscope objective. More precisely, as shown in Fig. 1, the first lens of the beam expander focuses the laser beam down to a diffraction-limited gaussian waist and onto a circular pinhole. As long as this pinhole has a diameter appreciably smaller (30–50%) than the waist, it acts as a spatial filter to remove the high spatial-frequency modes in the laser beam resulting from dust and reflections in the optics as well as from higher modes in the laser. Misalignments of the input beam, such as those caused by filter wedge errors or the pointing instability of the laser, produce a lateral displacement of the waist across the pinhole aperture. This may reduce the fraction of light that passes through the aperture. The output from the spatial filter pinhole is a smooth irradiance profile over the diverging cone of light, having the pinhole as its origin. An achromatic lens is generally used as the third component

of the beam expander because its spherical aberration on-axis is small, resulting in a highly collimated output beam.

Possible problems and solutions:

- The first lens in the beam expander must focus the laser beam to a precise diameter gaussian waist in order for the remaining optics in the illumination path to produce a diffraction-limited illumination spot at the specimen plane. If both the focused laser beam waist and the spatial filter aperture are too large, diffraction at the aperture will be insufficient to fill the BFP of the objective, the illuminating spot in the specimen will increase in diameter, and resolution will be reduced (i.e., diffraction-limited imaging will not be possible).
- Selection of an appropriate diameter of circular pinhole (spatial filter) in the beam expander is also critical for optimum illumination of the specimen and hence maximum resolution in the confocal image. For example, if the pinhole diameter is too large, no spatial filtering will occur and irregularities will still be present in the beam, resulting in a non-uniform irradiance profile. Conversely, if the pinhole diameter is too small, diffraction effects will result in significant loss of light and a reduction in intensity of the illuminating spot. A reasonable compromise is a spatial filter diameter that clips the gaussian beam at about the half-power point. In such a case, more than 80% of the desired spatial frequency mode of the laser is transmitted through the pinhole, and the angular distribution of the transmitted light is little affected by the truncation. In a system prone to beam pointing errors, a smaller pinhole transmitting ~50% laser power will reduce intensity variations.
- Selection of the third component of the beam expander—the longer focal length achromatic (collimating) lens—is based on the focal length of the first lens, the required diameter of the output beam and the tube length of the imaging objective. The output beam diameter, w_2 , is related to the input beam diameter, w_1 , by the following relation:

$$w_2 = w_1 f_2 / f_1$$

where f_1 and f_2 are the respective focal lengths of the input and output lenses of the beam expander.

Adjustable Diaphragm

Although the rear stop of the imaging objective determines the usable diameter of the collimated beam, it is still important for control of this beam diameter to be near the collimating lens of the beam expander. Scattered light in the system is reduced by limiting the collimated beam to slightly larger than the rear stop diameter of the objective.

Possible problems and solutions:

- Ideally, an adjustable diaphragm should be imaged by the intermediate optical system onto the rear stop of the imaging objective. However, this is not essential if the separation between diaphragm and stop is not too large. If the diaphragm is not imaged then the irradiance distribution

falling upon the rear stop is no longer a truncated gaussian but will show Fresnel diffraction effects especially at the outer edges of the beam. Consequently the focused beam will have a slightly modified irradiance distribution.

Mirrors

Fixed mirrors are used to fold the optical path into a compact or more convenient arrangement. (Mirror systems used in the intermediate optical systems of beam-scanning confocal microscopes are covered in more detail in Chapter 9, *this volume*.)

Possible problems and solutions:

- The potential depolarizing effects of mirrors are worth considering. Depolarization only occurs upon reflection when the electric field vector of the light (i.e., the **polarization vector**) is neither in the plane nor perpendicular to the plane containing the incident and reflected beam. In such a case the electric field components **in** the plane of incidence and **perpendicular** to the plane of incidence generally have different reflection (Fresnel) coefficients. The plane of polarization is thus seen to change in such a reflection and this effect can greatly degrade the performance of all types of interference and polarizing modes in the confocal microscope.*

Beamsplitters

Three main types of beamsplitters (BS) are used in confocal microscopy: the broad spectrum, the polarizing, and the dichroic BS. A typical broad spectrum BS actually has closely matched S and P polarization reflection coefficients and should cause little depolarization when used out of the plane of incidence as was the case mentioned previously for mirrors. These reflection coefficients also remain nearly constant over the full visible spectrum. The polarization BS is useful for selecting alternate paths for S and P polarized beams and is, again, effective over the full visible spectrum. The dichroic BS selects alternate paths for light of different λ s. There are two common varieties: one reflecting red and transmitting blue and green; and one reflecting blue and transmitting green and red. Such filters generally work at 45° incidence with minimum power loss.

Possible problems and solutions:

- Beamsplitters of all types are often designed with a slight optical wedge (to reduce internal interference effects). Care must be taken when using them in a confocal microscope where critical alignment is crucial for achieving good image resolution. This is particularly important when using beamsplitters that may be taken in and out of the microscope for different applications, such as those associated with fluorescence. In commercial confocal microscopes, realignment of the illuminating beam (as well as

the detector pinhole) should be carried out whenever a BS/filter block is interchanged.

- Dichroic beamsplitters are susceptible to depolarization if used out of the plane of incidence, so care must be taken if polarizing modes are used in the microscope. Also, when dichroic beamsplitters are used to separate excitation and emission λ s in fluorescence microscopy, additional barrier filters are necessary to achieve sufficient rejection ratios.
- Problems with wedge errors in beamsplitters have encouraged some manufacturers to use only a single beamsplitter for all purposes. This may be a single piece of uncoated glass that reflects between 2% and 10% of light incident at 45° (depending on polarization). In this case, the low reflectivity is seen as a small price to pay for the convenience of more stable alignment, especially as available lasers usually produce more power (~10 mW) than the specimen can withstand.

Polarizing Filters

These may be necessary in the illumination path if depolarization has occurred in parts of the system or if an unpolarized laser was used as a source. Even so, a polarizer will only be necessary for polarization-sensitive modes such as Nomarski DIC. A variety of polarizer designs are available including sheet materials and high-quality prism types.

Possible problems and solutions:

- As in the case of spectral and neutral density filters, wedging and aberrations can also be present in polarizing filters. As the filters themselves are made out of three separate layers (glass-Polaroid-glass), they have at least four surfaces that can be the source of interference effects.

Wollaston Prisms

Wollaston prisms are needed for the confocal Nomarski DIC mode, which is now possible using some types of confocal microscope designs. Prisms are matched to particular objectives and must be oriented at 45° to the incident polarization, a condition that can be difficult to satisfy even if the polarization direction of the laser is adjusted with a half-wave plate.

Additional Aberration Correcting Lenses

Additional correction lenses may be necessary in the illumination path of the confocal microscope, especially for the following cases:

1. Old style microscope objectives with 160-mm tube length in an infinity conjugate optical system (or vice versa)
2. An n mismatch between the immersion fluid and specimen mounting medium that produces spherical aberration
3. Any other intermediate optical component (e.g., an incorrect coverslip thickness) introduces a fixed aberration

Possible problems and solutions:

- Correction lenses used in the illumination path to correct for fixed aberrations should be of good optical quality and should have fairly long focal lengths (e.g., 1–4 m). A set of positive and negative opticians' lenses in quarter dioptre

*For experimental confocal microscope studies using Nomarski or polarizing modes, it is important to consider the alignment of the plane of polarization of the laser and the orientation of mirror and beamsplitter surfaces. Because many scanning-mirror confocal microscope designs cannot preserve the polarization of the beam, experimental studies conducted in our group use a specimen-scanning design in which all mirrors and optical components remain stationary on-axis, preserving the beam polarization (Cogswell and Sheppard, 1992).

increments can be quite useful for this purpose. The position of the correction lenses in relation to the imaging objective is also critical since they themselves will change the effective tube length of the illumination path. In addition, although simple (meniscus) correction lenses can be used for on-axis (specimen-scanning) confocal systems, they are also likely to introduce off-axis aberrations and thus are not well-suited for use in beam-scanning confocal microscope designs.

- The best way to determine the effects of correction lenses is to measure the axial response (outlined in a previous section) and compare the results when various correction lenses are present.
- Alternatively, a qualitative representation can be obtained using a CCD image of the focused spot at the detector pinhole plane. Figure 7a shows such an image acquired in a confocal microscope with an incorrect objective lens tube length. Figure 7b shows a corresponding image of the same specimen with a tube length correction lens inserted. The reduction in spherical aberration is clearly demonstrated.

Objective

The main properties of commercially available microscope objectives are described in the preceding chapter. Such objectives

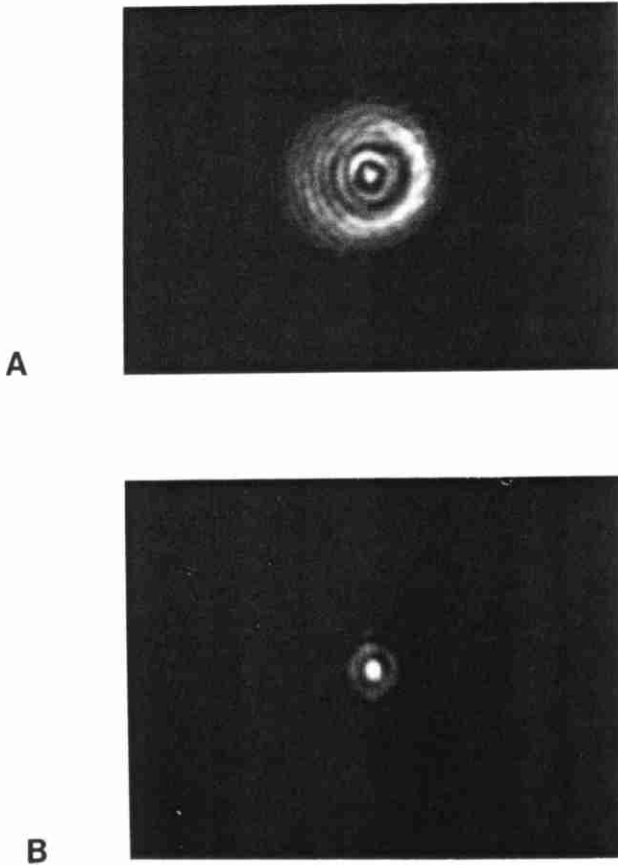


FIGURE 7. Effect of objective lens tube length on resolution of the illuminating spot. (a) CCD image shows an intensity increase in the outer rings (typical of spherical aberration), which results when an objective of improper tube length is inserted in the optical path of the microscope. (b) CCD image acquired in the same system as (a) after insertion of a tube-length correction lens.

are designed primarily for conventional microscopy but perform reasonably well in beam-scanning confocal designs, since the lens imaging requirements (i.e., near diffraction-limited imaging over a wide object field) are consistent with conventional microscopy. Confocal systems employing a specimen-scanning configuration, on the contrary, have quite different requirements. In these microscopes, the beam paths remain on-axis. In principle, this means that even high-NA objectives can have a much simpler design with far fewer elements and still achieve near-ideal diffraction-limited performance. However, specialized specimen-scanning objectives are not yet commercially available because of the present predominance of beam-scanning systems in the marketplace.

It is also worth mentioning that the point-spread function of a specimen-scanning system is spatially invariant, unlike that of a beam-scanning system. Invariance is a condition essential for the straightforward application of object reconstruction and deconvolution techniques.

Possible problems and solutions:

- Beam-scanning confocal microscope designs have the added complication that a variety of different objectives must be utilized in order to obtain a range of magnifications. For highest resolution at any one magnification, it is necessary to evaluate the performance of the particular objective being used and in some particular cases to minimize the effects of aberrations with image processing techniques such as boosting intensity of the periphery of an image to compensate for signal loss caused by off-axis aberrations.
- Specimen-scanning confocal designs, on the other hand, have the advantage of being able to use a single high-NA objective for a broad range of desired magnifications. This is accomplished by simply altering the distance over which the specimen is scanned. However, for low-power observations, the resulting image is not precisely comparable to the equivalently magnified image from a beam-scanning microscope. This is because a high-NA objective produces a higher resolution (and hence smaller diameter) illuminating spot. Thus, unless a very dense raster pattern is used, a specimen-scanning confocal microscope is likely to have gaps between each sampled point in the final, low-magnification image, because the actual area of illumination for each point on the specimen is smaller than the comparable area covered by each single pixel in the low-power image (see the discussion of aliasing, Chapter 4, *this volume*). A beam-scanning confocal system utilizing a lower NA objective will produce an illuminating spot with a larger area which will, in practice, be more comparable to the size of an image pixel. In all cases, when considering the size of the illuminating spot, one must also consider the **dwell time** per pixel and the **sampling frequency** which, depending on the particular confocal system used, may be available for control by the user. For example, decreasing the frequency bandwidth of the signal amplifier has the effect of smearing the illuminating spot in the horizontal scan direction. This may help alleviate the sampling gaps (blind spots, at least in one direction) when using a high-NA objective at a low-zoom magnification but it also reduces horizontal resolution. As a result, it must be used

with caution and consideration for the structural properties of the specimen (e.g., punctate, filamentous or continuous gradient) and the confocal optical mode used (e.g., fluorescence, backscattered light, DIC).

Objective Immersion Medium

It is extremely important in confocal microscopy to use the correct immersion medium for which the objective is designed. Usually this will be air, water, or immersion oil, although glycerin-immersion objectives are also available. In addition, there are some multi-immersion objectives on the market that incorporate a correction collar so that they can be used with either water, glycerin, or oil, as well as with or without a coverslip (Chapter 7, in *this volume*). For confocal microscopy, it is particularly important that the correction collar be set to the proper position for the immersion medium (and coverslip) being used in order to minimize spherical aberration. Slight variations in setting from the manufacturer's designation may be necessary. The optimum correction setting can be determined precisely using some of the system tests described earlier (i.e., in the section on how to measure or observe defects).

Possible problems and solutions:

- When using oil-immersion objectives, it is probably safest to obtain the immersion oil from the same company that produced the objective lens. Third-party immersion oils may, in practice, have small but crucial differences in η from the standard for which the objective was designed. This difference can introduce large amounts of aberration into any confocal system, making it impossible to obtain high-resolution images.

Coverslip

The coverslip can be considered the final optical component in the illumination path before the specimen. Objectives for biological applications are usually designed (or **corrected**) for a coverslip of known composition and thickness (usually $\eta = 1.518$ @ $\lambda = 546$ nm and 0.17-mm thickness). Some new water-immersion objectives can be used without a coverslip, while some very high-NA dry or multi-immersion objectives have a correction collar that can be rotated to set the internal lens elements for the particular coverslip thickness and immersing medium being used (Chapters 7, 20, *this volume*).

Possible problems and solutions:

- Like all other optical elements in the microscope, coverslips can be made from a variety of materials including glass, quartz, and even plastic. Obviously, these materials have different refractive indices as well as different dispersion and birefringence properties. Because high-resolution confocal microscopy is so dependent on the quality of the illuminating spot, it is extremely important to utilize coverslips that do not degrade the focused spot before it reaches the specimen. For example, even though a typical glass coverslip may have the same η as the immersion oil required for a high-NA objective, the dispersive properties of the glass will be different from that of the oil, and hence the characteristics of the illuminating spot may vary for different λ s and different coverslip

thicknesses. Plastic coverslips (e.g., those made of CY-TOP) might be deemed useful for water-immersion objectives because they have $\eta = 1.34$ which is close to that of water ($\eta = 1.33$). However, they can also create great problems because they are usually birefringent and can greatly alter the plane of polarization of the illuminating beam, making such modes as Nomarski DIC and polarizing microscopy virtually impossible to perform. Since the coverslip is one optical element in the microscope over which the user has some control, it is certainly worth spending some time testing a few brands and thicknesses (using the tests described in this chapter) to find an optimum type for the microscope objectives and specimens that are actually being used.

Specimen

To obtain a diffraction-limited image in a confocal microscope, properties of the specimen such as η and thickness must be considered. As the plane of focus of the objective is progressively moved deeper into a typical biological specimen, spherical and defocus aberrations become more prevalent (Sheppard and Cogswell, 1991). In practice, this causes an increase in the size of the 3D illuminating spot, which results in a rapid loss of resolution, particularly in the axial direction. Figure 8a shows the axial response for a specimen consisting of a 50 μm layer of water sandwiched between a mirror surface and a coverslip. The FWHM is slightly increased compared to Fig. 4, and the subsidiary maxima on one side of the peak are enhanced in a manner typical of spherical aberration. Another example of specimen induced aberration is shown in Fig. 8b, which uses the indirect method of imaging the focused spot on a CCD. The irregular ring pattern is a consequence of imaging through a thick complex biological specimen.

Possible problems and solutions:

- If a microscope user repeatedly observes specimens prepared using some standard methodology and thickness, it is advisable to start by performing an axial response test with the same preparation mounted on a mirror-coated slide and coverslip, similar to that shown in Fig. 9. The test simply entails measuring the axial response from the upper mirror coating and comparing it to that of the lower mirror coating (through the coverslip and specimen). This will help to determine the extent of the aberrations (such as spherical and defocus) introduced by the specimen and will indicate whether it may be desirable to use thinner preparations, a longer working-distance objective, correction lenses, and so forth.
- Repeating the test with the coverslip inverted (such that the mirror coating faces the specimen) can indicate if the coverslip itself is introducing aberrations. A mirror coating can easily be added to ordinary slides and coverslips by most scanning electron microscope laboratories.
- Another effect of specimen-induced spherical aberration is that the power density of the illuminating spot will progressively decrease as the depth within the specimen increases. This means that, for applications such as quantitative fluorescence investigations, the irradiance at any

point in the focused spot (and hence the amount of fluorescence excited) will diminish with depth. Similarly, these same aberrations will ensure that a smaller-than-expected fraction of the fluorescence signal will be focused back to the detector pinhole. This effect is in addition to the loss in irradiance due to scattering and absorption by the specimen, factors that also must be considered if quantitative fluorescence measurements are desired.

Overall Illumination Path

Each optical surface in the illumination system will deviate slightly from a perfect planar or perfect spherical shape. This so-called **form error** is most often of a spherical or cylindrical character, although more irregular forms are also common. The overall effect of such errors is to introduce a wavefront aberration in the light beam propagating through the system and consequently to enlarge the illumination PSF, thereby degrading resolution. Form errors on plane surfaces oriented at 45° (e.g., adjustable mirrors, beamsplitters and scanning mirrors) tend to produce astigmatism, characterized by a separation of the planes of focus for the

x - and y -directions. An example of the double-peaked axial response typical of astigmatism is shown in Fig. 5.

Possible problems and solutions:

- When all other causes of poor resolution in a confocal microscope have been explored, there remains the possibility that all of the system errors together are producing a fixed wavefront aberration. This can be largely compensated for by inserting a spherical or cylindrical correction lens (as described earlier). However, in a beam-scanning design the aberrations caused by overall system errors will probably vary over the image field, and correction lenses may be of limited use in this case.
- Many beam-scanning systems require the operator to adjust the beam centering, following the insertion of different filters or filter blocks. Alignment is simply accomplished with the aid of a prism that fits into the objective turret and allows the user to see the position of the beam relative to the rear stop in the BFP of the objective. The alignment mirrors should be adjusted such that the beam is stable and centered, and entirely fills the stop (Chapter 36, *this volume*).

Special Cases

Single Wavelength vs. Multiple-line Laser Sources

Many contemporary confocal microscopes use multiple wavelength laser sources. Because axial chromatic aberration is present to some extent in even the best quality apochromatic objectives (Cogswell *et al.*, 1992), it is essential to measure and compare the axial response of the objective at all of the λ s used. Figure 10 illustrates the axial response curve obtained using a front surface mirror, a NA 1.4 apochromatic objective and two laser sources (red and green). The double peak indicates that these two λ s focus at different planes in z . This effect will result in axial misregistration of images in applications such as multiple excitor fluorescence.

Optical Fibers

Optical fibers have become a popular way of delivering a coherent light beam from a laser source to the main body of the confocal microscope. Such connections are especially convenient for microscopes utilizing multiple laser sources. In addition, the

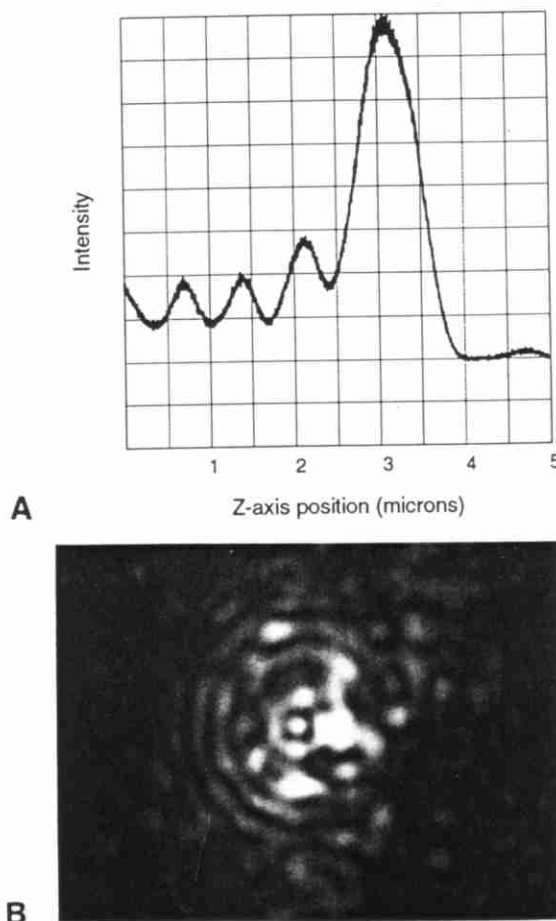


FIGURE 8. Examples of specimen-induced aberrations. (a) Axial response from a specimen consisting of a $50\ \mu\text{m}$ layer of water sandwiched between a plane mirror and a coverslip. (b) CCD image of a single illuminating spot reflecting from a region within a complex biological specimen. Spherical and other aberrations produce irregularities and an increase in signal strength in the outer rings.

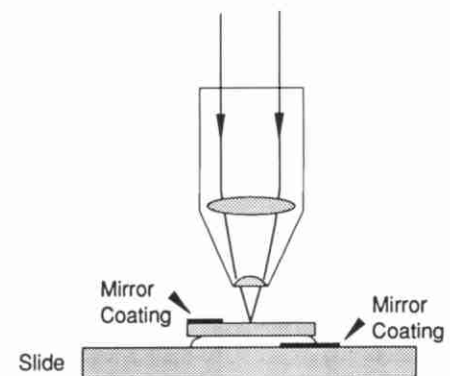


FIGURE 9. Schematic diagram of a sample prepared using a mirror-coated slide and coverslip which is useful for measuring axial response.

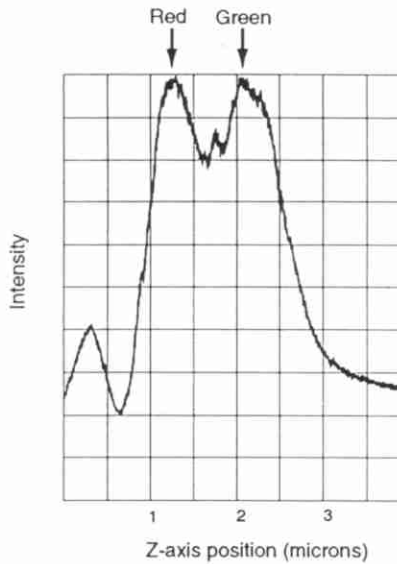


FIGURE 10. Axial response for a high-NA apochromatic objective using two illuminating laser λ s (red and green). The double peak indicates that axial chromatic aberration is present.

use of optical fibers allows bulky and noisy air-cooled lasers to be positioned some distance from the microscope, resulting in substantial vibration isolation.

The laser light is coupled to the input of a single mode optical fiber using a suitable short focal-length lens (generally an objective of about 25 \times). Efficient coupling depends upon matching the fiber NA with the input beam NA (Chapter 33, *this volume*). The output end of the fiber acts as the near-perfect coherent source of an (almost) gaussian divergent beam and can be coupled directly to the collimating lens of the beam expander shown in Fig. 1.

Possible problems and solutions:

- Systems using optical fiber coupling may exhibit illumination level variations. The cause of this is likely to be slight misalignment of the input coupling. Realignment of the input coupler is a complex task and generally should be left to qualified service personnel.
- Polarization is not necessarily preserved in a beam propagating through a fiber. Typical depolarization over 5 m of fiber may be on the order of 20%. If the microscope is to use polarization-sensitive modes, a polarization-preserving fiber should be used for best results. Alternatively a polarizing filter may be inserted in the illumination path.

- Finally, a fiber cannot be used to transmit the very short (100 fsec) pulses used in the two-photon technique (Chapter 28, *this volume*).

SUMMARY

- The optical design and alignment of the illumination path is of critical importance in any confocal system because it plays a major role in determining the final axial and lateral resolution of the microscope.
- Fixed aberrations introduced by optical components and varying aberrations introduced by complex thick specimens may be measured using techniques such as axial response, xz -imaging and indirect imaging of the focused illuminating spot with a CCD camera at a detector plane.
- A partially mirror-coated slide and coverslip arrangement can facilitate measurements of axial response and the detection of aberrations, both those in the microscope illumination path and those introduced by the specimen.

ACKNOWLEDGMENTS

The authors express their thanks to Keith Brain, a student member of the Physical Optics group, for providing the axial response data. The first author would also like to acknowledge the generous support for her position by the Nell and Hermon Slade Trust and the G. Hermon Slade Foundation. Other funding for this project is provided by the Australian Research Council.

REFERENCES

- Cogswell, C.J., and Sheppard, C.J.R., 1992, Confocal differential interference contrast (DIC) microscopy: Including a theoretical analysis of conventional and confocal DIC imaging, *J. Microsc.* 165:81–101.
- Cogswell, C.J., Hamilton, D.K., and Sheppard, C.J.R., 1992, Color confocal reflection microscopy using red, green and blue lasers, *J. Microsc.* 165:103–117.
- Cogswell, C.J., Sheppard C.J.R., Moss, M.C., and Howard, C.V., 1990, A method for evaluating microscope objectives to optimize performance of confocal systems, *J. Microsc.* 158:177–186.
- Sheppard, C.J.R., and Cogswell, C.J., 1991, Effects of aberrating layers and tube length on confocal imaging properties, *Optik* 87:34–38.
- Sheppard, C.J.R., and Cogswell, C.J., 1990, Three-dimensional imaging in confocal microscopy. In: *Confocal Microscopy* (T. Wilson, ed.), Academic Press, San Diego, pp. 143–169.
- Sheppard, C.J.R., and Larkin, K.G., 1994, Optimal concentration of electromagnetic radiation, *J. Mod. Opt.* 41:1495–1505.