9

The Intermediate Optical System of Laser-Scanning Confocal Microscopes

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INTRODUCTION

This text explains some of the basics of intermediate optical systems in confocal microscopes. Important microscope components are discussed in more detail in other chapters: lasers (Chapter 5) and other light sources (Chapter 6), objective lenses (Chapter 7), special optical elements (Chapter 3), fibers (Chapter 26), and detectors (Chapter 12). Although this chapter concentrates on laser-based single-spot scanning instruments while other types of instruments are covered elsewhere (Chapters 10 and 29), remarks on different approaches will guide the interested reader.

DESIGN PRINCIPLES

Overview

The basic optical layout of a confocal fluorescence microscope is found in Figure 9.1. A laser beam is focused into a pinhole, which acts as a point-like light source. The spatially filtered light is deflected by a dichroic mirror and focused by an objective lens into the fluorescent specimen. The fluorescence light is emitted in all directions; a fraction of the fluorescence emission is collected by the objective lens and focused into a pinhole in front of a detector.

The lateral discrimination capability of the confocal fluorescence microscope occurs because light that is emitted in the focal plane at a distance Δx from the focus will miss the pinhole in front of the detector by a distance $M\Delta x$. The main advantage of a confocal microscope is, however, its axial discrimination capability. If the sample is flat, this property is not required, but in a thick sample, fluorophores located in front and behind the focal plane ($\Delta z \neq 0$) will also be excited by the incoming laser beam. However, their fluorescence emission will be focused either in front of or behind the image plane that includes the detection pinhole ($\Delta z' \neq 0$). Hence their images are expanded at the pinhole plane and only a fraction of the fluorescence light passes the pinhole to reach the detector. If one removes the pinhole, the axial discrimination is lost and such a laser-scanning system has the properties of a conventional fluorescence microscope.

A slightly different way to understand Figure 9.1 is to realize that the objective lens and the two tube lenses form images of both the illumination pinhole and the detection pinhole in the object. Ideally, both pinholes are located in conjugate image planes and on the optical axis. Therefore, their images will overlap perfectly in the object and define the volume element that is observable. This description emphasizes symmetry considerations.

Because a confocal microscope observes only one spot in the object at a time and the main interest is to form a two- or three-dimensional image, the sampling light spot must be either scanned through the object (beam scanner) or the object must be moved through the light spot (object scanner).

In most confocal microscopes the scan unit is attached to a conventional microscope, and the stage that positions the sample and the objective lens are part of the conventional instrument. This chapter discusses the other opto-mechanical elements, that is, the illumination and detection units and the confocal scanner. Essentially it provides a detailed description of how to build a beam scanning confocal fluorescence microscope.

Telecentricity

Figures 9.1 and 9.2 show the basic geometrical optics of a confocal microscope. Figure 9.1 outlines the major elements of the confocal microscope in terms of the objective lens and its image and object planes (a complete description is found in Chapter 7, *this volume*). In practice, the user of the microscope needs to know only the magnification and the numerical aperture (NA) of the lens. Most of the other properties (e.g., the diameter of the entrance aperture) can be easily calculated (see Tables 9.1 and 9.2). The sketch in Figure 9.1 show an ideal setup as it is well known from many papers on confocal microscopy (Bacallao and Stelzer, 1989). The microscope objective is, however, a multi-lens system, and the locations of its principal planes are usually provided by the manufacturer. The image and object distances must remain fixed if the full correction of the objective is to be exploited. This fixes the positions of the image and the object planes relative to the position of the objective (Table 9.1).

Ordinary photographic cameras use a single lens to form an image. This has the property that the magnification varies with the distance of the object from the lens. As a result, in pictures of high buildings taken from ground level, the edges are tilted towards the image's top center. This phenomenon is also known as the converging lines effect. A single lens is, therefore, of limited use when one intends to perform measurements in objects that have depth. A system that uses two lenses spaced the sum of their focal lengths apart has a magnification that depends only on the ratio of their respective focal lengths. Such systems are called telecentric.

All microscope objective lenses are corrected telecentrically (Fig. 9.2). A telecentric system is arranged like a Keplerian telescope with a stop in the common focal plane of the two lenses. The entrance and exit pupils are both at infinity in object and image space, respectively. Telecentric systems are space invariant and linear. Space invariance means that the lateral and longitudinal

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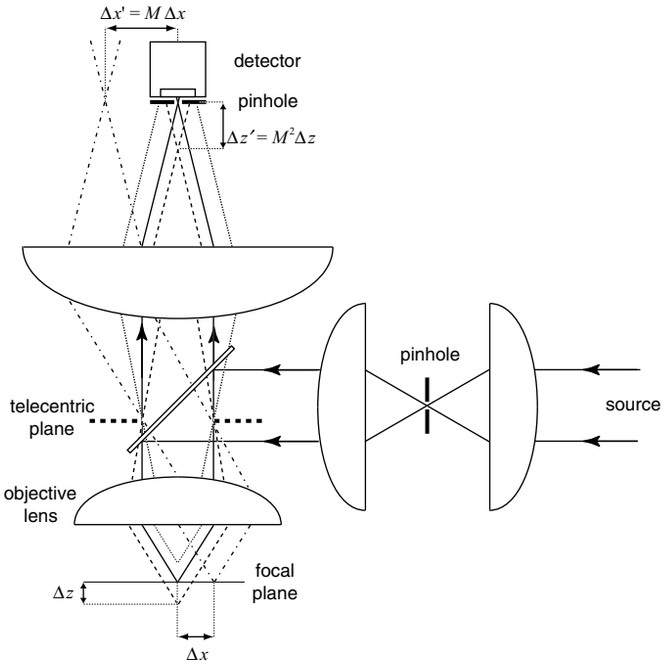

FIGURE 9.1. Principles of a confocal fluorescence microscope. The laser light of the excitation beam enters the instrument from the right-hand side. It is expanded by a telescope with the illumination pinhole in its center. A dichroic mirror deflects the laser beam towards the objective lens. The laser light excites the fluorophores in the focal plane. Some of the fluorescence emission is collected by the objective lens, passes the dichroic mirror, and is focused into the detection pinhole in front of a light detector.

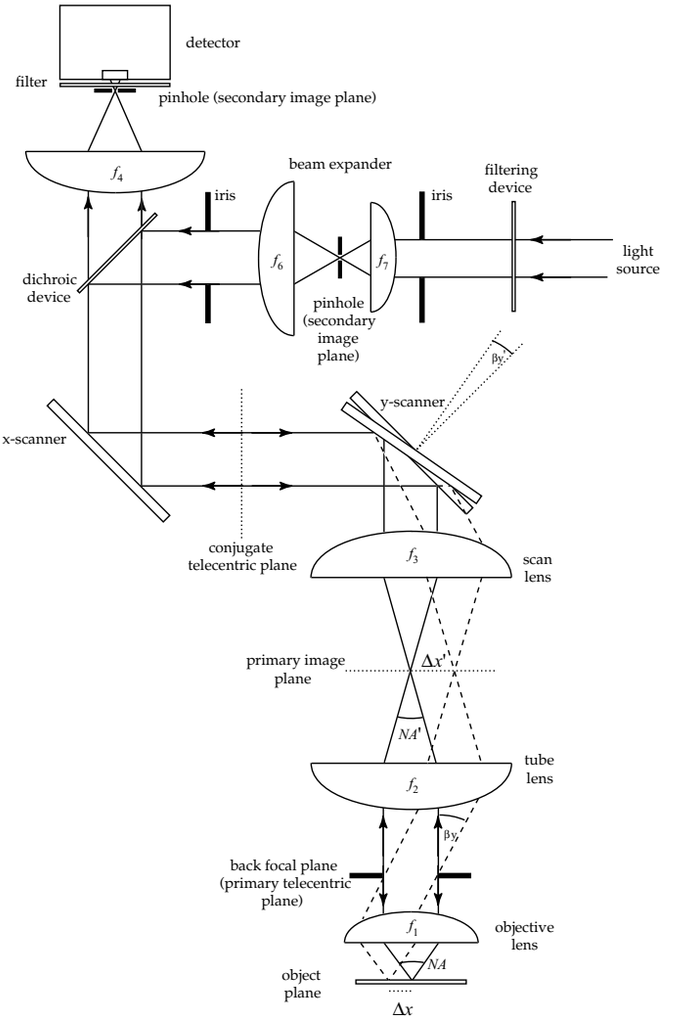

FIGURE 9.2. Simplified diagram of the components of a confocal fluorescence microscope. The light enters the instrument from the right-hand side. A filtering device selects at least one wavelength for the excitation of the fluorophores in the sample and also attenuates the light intensity to the required level. A beam expander adjusts the beam diameter to fit the diameter of the back-focal plane of the microscopes objective lens (Fig. 9.5). A dichroic mirror deflects the excitation light towards the scanner pair. Ideally both are located in conjugate telecentric planes of the microscope objective lens. By tilting the *y*-scanner by an angle β_y', a laser light spot is moved by $\Delta x'$ in the primary image plane of the microscope. The tube lens and the microscope objective lens form an image of the light spot in the object plane. The detected fluorescence light follows the same path backward and is descanned by the *y*-scanner. It passes the dichroic mirror and enters the pinhole. Filters in front of the detector pass at least one band from the sample response. The optical path is defined by irises that are also used to align the illumination and the detection paths. The two irises around the beam expander, which also remove any excess light that will not enter the sample anyway, and others (not shown) reduce stray light. Ideally they are located in telecentric planes, that is, always close by the focal plane of a lens. f_i are the focal lengths of the lenses (see Table 9.1).

magnifications of the optical system are constant throughout the whole space, and the shape of the point spread function (PSF) is independent of the absolute location of the point source. When observing two points near the focal plane of a microscope as the focal position is changed, the points become blurred, but their center–center distance does not change. Because of this particular property when defocusing, telecentricity is used in all optical measurement devices.

The most important feature of a telecentric system is that the magnification factor determines all the properties of the system. All beams pass the telecentric plane with an angle that is characteristic of the position of their focal point in the object plane. This property is very important, and the conjugate telecentric planes, like the conjugate image planes, are often shown in overviews of the optical paths of conventional microscopes. As we will notice later, it is important to know the positions of these planes if one wishes to extend optical paths.

The Scanning System

Because the position of the sampling light spot depends on the angle of the beam in the telecentric plane (Fig. 9.2, Table 9.1), the method of choice to scan the spot through the object plane is to change this angle. From the description above, it should be obvious that this can be achieved by placing a scanning mirror such that its pivot point (the center of the tilting movement) is in the center of a conjugate telecentric plane of the microscope objective. The stationary beam falling on this scanning mirror is reflected and, proportional to the tangent of the angle of the mirror, the spot moves to different positions in the object plane.

Any conjugate telecentric plane is an image of the telecentric plane of the microscope objective. An intermediate optical system forms an image of the scan mirror in the entrance aperture of the microscope objective. Not every arrangement is valid, however. As pointed out above, the microscope objective is designed to be used with fixed object and image distances. Otherwise it will not function correctly, in particular, spherical and chromatic aberration

TABLE 9.1. Mathematical Formulas

No.	Symbol/Equation	Description	Typical Value
	f_1	Focal length of microscope objective lens	1.9 mm
	f_2	Focal length of tube lens	160 mm
	f_3	Focal length of scan lens	50 mm
	f_4	Focal length of pinhole lens	50 mm
	$M = f_2/f_1$	Magnification of microscope objective lens	63
	NA	Numerical aperture of microscope objective lens	1.2
	$NA' = NA/M$	Numerical aperture of tube lens and scan lens	0.02
	n	Refractive index of immersion medium	1.33
	λ	Wavelength of light	
	λ_{ex}	Wavelength of excitation light (illumination path)	0.488 µm
	λ_{em}	Wavelength of emission light (detection path)	0.520 µm
	$\beta'_y = f_3/f_2 \cdot \beta$	Tilt angle of y-scanner	4°
	β_y	Tilt angle along y-axis in telecentric plane	11°
	$s_x \times s_y$	Size of first scan mirror	2 mm × 3 mm
	$\alpha = \sin^{-1} NA/n$	Angular aperture	64°
	$\Delta r = \frac{\lambda}{n\sqrt{3 - 2\cos\alpha - \cos 2\alpha}}$	Lateral resolution	0.22 µm
	$\Delta r_{img} = M \cdot \Delta r$	Lateral resolution in primary image plane	14 µm
	$\Delta z = \frac{\lambda}{n(1 - \cos\alpha)}$	Axial resolution	0.65 µm
	$\Delta r_{img} = M^2 \cdot \Delta z$	Axial resolution around primary image plane	2.6 mm
	$\Delta x = f_1 \cdot \tan\beta_y$	Field of view in object plane	0.4 mm
	$\Delta x' = \Delta x \cdot M$	Image size in primary image plane	24 mm
	$\phi_{BFP} = 320\ mm \cdot \frac{NA}{M}$	Diameter of back-focal plane of microscope objective lens	6.1 mm
	$\phi_{ill,ph} = \Delta r \cdot \frac{f_2}{f_1} \cdot \frac{f_4}{f_3} = \Delta r \cdot M \cdot \frac{f_4}{f_3}$	Diameter of illumination pinhole	23 µm
	$\phi_{det,ph} = \Delta r \cdot \frac{f_2}{f_1} \cdot \frac{f_6}{f_3} = \Delta r \cdot M \cdot \frac{f_6}{f_3}$	Diameter of pinhole in front of detector	30 µm

will no longer be properly corrected. If the (incident) light beam is collimated, the intermediate optical system must have its focal plane coincide with the image plane of the microscope objective (Fig. 9.2).

The imaging outlined so far can also be described as follows. Every point in the object plane has a conjugate point in the image plane. As the light spot is moved in the image plane, the conjugate light spot is moved in the object plane. To scan the object plane, light spots must be produced in the image plane. If the beam falling into the intermediate optical system is collimated, a spherically corrected beam will be achieved only if the focal plane of the lens and the image plane of the microscope objective coincide. The position of the light spot in the image plane then depends on the scan angle.

A single scan mirror will be fixed on a rotation axis and will, therefore, only be able to provide a motion of the focal point in one direction. However, this concept can be easily expanded to include more than one mirror and hence to scan along two perpendicular axes. As pointed out, the mirrors should be placed in conjugate telecentric planes. Any number of these planes may,

TABLE 9.2. Resolution of Different Objective Lenses in Conventional and Confocal Fluorescence Microscopy[a]

	Resolution[b] in Object Plane				Parameters in Image Plane			BFP
Lens[c]	Lateral (µm)	Lateral Conf. (µm)	Axial (µm)	Axial Conf. (µm)	Lateral Resolution[d] (µm)	Line Pairs/mm	Line Pairs/Image[e]	Diameter (mm)
100 × Oil/1.4	0.19	0.14	0.54	0.38	43.6	46	920	4.5
63 × Oil/1.4	0.19	0.14	0.54	0.38	27.5	73	1460	7.1
63 × W*/1.2	0.23	0.16	0.66	0.47	32	62	1250	6.1
40 × W*/1.2	0.23	0.16	0.66	0.47	20.3	98	1970	9.6
100 × W/1.0	0.28	0.20	1.10	0.78	61	33	660	3.2
63 × W/0.9	0.31	0.22	1.43	1.01	42.7	47	940	4.6
40 × W/0.8	0.35	0.25	1.87	1.32	30.5	66	1310	6.4
20 × /0.75	0.37	0.26	1.48	1.04	16.3	123	2460	12
20 × /0.6	0.47	0.33	2.50	1.77	20.3	98	1970	9.6

[a] For a wavelength of 500 nm. Refer to Table 9.1 for precise definitions and formulas.
[b] The resolution is the full width at half the maximum (FWHM).
[c] Oil refers to oil-immersion lens; W to water-immersion dipping lens; W* to a water-immersion lens corrected for usage with a cover glass.
[d] The resolution is given by $2M\ 0.61\ \lambda/NA$ (see Table 9.1).
[e] Assuming an intermediate image diameter of approximately 20 mm.

however, be easily generated by adding further optical elements. The image of a second mirror may then be formed on the first mirror. If the two mirrors move the beam in orthogonal directions, the object is scanned along orthogonal x and y axes.

What happens with the light emitted from the spot illuminated by the laser beam? As explained above, the images of the source and the detector pinholes overlap in a confocal microscope. The position of the illuminating light spot in the image plane is, therefore, linearly related to the position of the image of the light spot formed in the sample. Hence, the pinhole in front of the detector must be in a plane conjugate to all the image planes of the microscope (Fig. 9.2). In a scanning system, this means that we need an optical arrangement that will form an image of the image plane. The scan system will move this image across the pinhole, and the light from only one point in the object will enter the detector. If everything is set up correctly, this point will also be the point in the object that is illuminated by the laser. In most cases, the pinhole will be placed on the optical axis and in the focal plane of a lens. This detection lens and the intermediate optical system discussed above form a conjugate image plane.

The Back-Focal Planes

To work in the diffraction limit, the back-focal plane (BFP, also known as the entrance aperture or Fourier plane and defined by the stop in the telecentric plane) must be uniformly filled by a planar wave. Failing to do so results in a smaller effective NA and hence in a broader spot and in poorer resolution. As pointed out above, the BFP diameter of a lens is closely related to its resolution and its magnification (Table 9.2). The higher the resolution and the lower the magnification, the larger is the BFP diameter. This means that the diameter of the illumination pinhole must be matched to a specific lens, and the diameters of all optical elements throughout the illumination and detection paths must be large enough to transmit a beam of light this large without truncation. A microscope system with interchangeable objectives must either be adjustable or at least be usable with the lens having the largest BFP diameter. All other lenses will then be over-illuminated, which produces some stray reflections and involves some loss of illumination intensity. The best idea is actually to optimize the diameter for a set of lenses that have fairly similar M/NA ratios. Any design requires a decision regarding the specific lenses likely to be used and instrument performance in terms of illumination and detection efficiency. When working with lasers having a Gaussian beam profile, it is common to over-illuminate the BFP by a factor of 2, that is, the $1/e^2$ diameter of the Gaussian beam should be twice as large as the BFP diameter. This results in the beam transmitted having only about 70% of the light in the untruncated beam. Finally, the location of the BFPs on the optical axis varies by a few millimeters. Although in principle this causes vignetting, the effects can probably be neglected for all practical purposes in biology.

PRACTICAL REQUIREMENTS

Diffraction Limit

Illumination

The goal in confocal microscopy illumination is to make the light spot as small as possible. Its size should be determined only by the wavelength of the beam of laser light emerging from the single-mode fiber and the NA of the objective lens; that is, the system should be diffraction limited. According to Goodman (1968, p. 103), "An imaging system is said to be diffraction-limited if a diverging spherical wave, emanating from any point-source object, is converted by the system into a new wave, again spherical, that converges toward an ideal point in the image plane." The system, in our case, consists basically of the microscope objective lens and the tube lens. Because we have real elements, this requirement is never fulfilled. Every optical element will cause some degree of aberration that increases the spot size. The actual spot size Δr (Table 9.1) can be measured in the image plane of the microscope objective.

While Δr is approximately the full-width at half-maximum, $2\Delta r$ is approximately the diameter of the Airy disk in the image plane. In general, it should not be larger than the spot in the object plane times the magnification of the objective lens (e.g., an $M = 63$; NA = 1.2 lens at $\lambda = 488$ nm would have a Δr in the intermediate image plane of about 14 µm). Another important requirement to achieve the diffraction limit is that the aperture of the objective lens is uniformly filled with a planar light wave (if the objective lens is corrected for a finite distance, a spherical wave must fill its aperture).

Every optical element degrades the wavefront somewhat (a good description of aberrations introduced by optical elements is given in the Melles Griot catalogue (Chapter 1, 1999); the performance of real lenses is found in Melles Griot, Chapters 6 and 11, *this volume*). One goal of the optical system is to generate a light spot in the image plane that is smaller than actually required. This is equivalent to overfilling the microscope entrance aperture. Efficiency in the illumination path is often not of great importance, as lost light can be easily made up because most of the appropriate lasers have more power than is needed for fluorescence confocal microscopy. However, as applications such as fluorescence recovery after photobleaching (FRAP; Axelrod *et al.*, 1976) and other related techniques usually try to expose the sample to as much energy as possible within a short period of time, efficient light delivery has become a much more important issue.[1] Multiphoton microscopy (Denk *et al.*, 1990; Stelzer *et al.*, 1994; see also Chapter 28, *this volume*) is another field that requires an efficient illumination light path but has the additional requirement that group velocity dispersions should be under excellent control.

Detection

It is as important to achieve the diffraction limit in the detection path as it is in the illumination path. A pinhole that is somewhat larger than the ideal case produces only a modest reduction in the out-of-focus discrimination of the confocal setup (Wilson and Carlini, 1987; see also Chapter 22, *this volume*). Increasing the pinhole diameter is complementary to over-illuminating the entrance aperture of the microscope objective and can be used to correct the same errors mentioned above. If, however, the optical arrangement of the detection path is close to the ideal case, nothing can be gained in terms of detecting light from the focus plane by increasing the pinhole size beyond 1 or 2 Airy units. A particularly good test is that the intensity of the signal from a single point-like object (e.g., a 100-nm fluorescent latex bead or a non-resolvable concentration of some dye) should not increase by more than a factor of 3 as the pinhole diameter is increased. When one observes the image of a single point object that is in focus, a well-corrected

[1] In response, Olympus recently introduced a microscope incorporating an entirely separate intermediate optical system for use in FRAP and uncaging experiments.

confocal arrangement will collect at most (i.e., at the highest NA) 30% of the available light produced by the object and focus it at the pinhole.

Detection efficiency is always crucial in fluorescence microscopy. The basic rule is to have as few optical elements as possible in the detection path. Sticking as closely as possible to this rule helps avoid losses due to reflection or absorption and prevents unwanted degradation of the wave front. It is a particularly bad idea to place annular apertures into the detection path that obscure large fractions of the fluorescence light as is sometimes suggested to improve *z*-resolution (Martínez-Corral *et al.*, 2002). The light loss caused by such devices reduces detectability limits by increasing the effect of Poisson noise.

Because only the light emitted from the focal plane in the sample is collimated and properly focused into the detector pinhole, most stray light is excluded by the pinhole and is usually not a serious problem. However, any laser light that passes the dichroic beam-splitter should be captured in a simple beam stop to prevent it being returned and reflected up the axis from the back side of the dichroic. Exposed surfaces that could reflect light (especially laser light) should be covered with black paint, self-sticking velvet, or black anodized aluminum. The intensities are usually too low to cause heating.

Geometric Distortion

As geometric distortion is introduced by any inaccuracies in the scanning process, it is important to keep these as small as possible. Distortions can arise from deficiencies in either the mirror positioning system or the intermediate optics. The movement of the scanning mirror is known only approximately, and it was very common to run the horizontal galvanometers in a sine function that is linear to ± 4% only over a small part of the sine waveform (= ± 30%, see Chapter 3, *this volume*). The scanning system and the way it is used obviously define the geometric distortion to a very large degree. These factors also influence the efficiency of the illumination and the average energy penetrating a unit area. Much effort has been made to drive the scanners accurately, for example, by calculating sums of harmonics, changing the acceleration in a systematic manner, etc., in order to achieve a reasonably high duty cycle. The advent of DSP-controlled, lightweight ultra-precise galvanometers has made this task very easy. An essentially undistorted speed of more than 1 kHz even at large scanning angles is now readily available (see Chapter 3, *this volume*). Finally, great care must be taken when the intermediate lens is selected. The optimal solution is an f-theta lens.

EVALUATION OF THE ILLUMINATION AND DETECTION SYSTEMS

Influence of Optical Elements

All optical elements in the confocal microscope influence the characteristics of the light beam:

- its energy spectrum,
- its polarization,
- its modal distribution,
- its waist diameter,
- its divergence,
- its direction,
- its position relative to the optical axis.

In a fully corrected optical imaging system, all these parameters are considered, but in confocal microscopy this is usually not necessary. After all, a confocal microscope does not form an image, and the wavelength used at any time is only a small fraction of the total optical bandwidth.

Several rules govern the properties of the incoming beam (i.e., the beam entering the entrance aperture pupil of the objective lens), and another set of rules determines the properties of the beam entering the detector. The optical elements in either the illumination or the detection path are used to shape the beam, that is, to ensure that the beam will have the desired properties. The actual problem is that the optical elements are not perfect and that they influence to a varying degree more than one of the properties of the beam.

Errors

Beam Shift

The beam is shifted every time it traverses a flat optical component that is tilted relative to the optical axis or a lens that is not correctly centered. If the optical layout is designed for collimated beams, shifts can usually be compensated for in the detection path, but any shift in the illumination path can cause vignetting. On the other hand, in laser arrangements with collimated beams, flat surfaces are often purposely tilted by 0.5 to 1° to avoid interference effects caused by specular reflections. However, if these tilts are too large, refraction and dispersion can cause a visible wavelength-dependent shift of the beam. If the tilt is in the range of 1°, the shift is about 6 µm/mm of glass while, in the tilt range of 45° (as for a beam-splitter), the shift is about 330 µm/mm.

Angular Deflection

In the confocal microscope, any angular deflection of the beam is very critical and practically not correctable. Deflections are caused by flat optical components that have surfaces that are not parallel (i.e., they have wedge errors) or by lenses that are tilted relative to the optical axis. As pointed out above, in telecentric systems the angular deflection determines the position of the light spot in the object plane. Every deflection of the beam except that produced by the scanning system causes a shift of the observed area. The typical planarity achieved on a flat component is <1 second of arc. If the thickness varies 1 µm over a length of 25 mm, and the collimated beam is focused with a 90 mm lens, the axial shift at the focal plane is 2 µm. The amount of shift that is critical depends on the diameter of the pinhole, and an estimate of the tolerable wedge error depends to a large extent on the magnification of the objective lens. The dichroic mirror is in this respect the most critical part in a confocal fluorescence microscope. Even slight changes in its position cause the image of the source and detector pinholes not to overlap anymore. This is particularly important as most fluorescence microscopes operate with several excitation lines, and this means that, unless a multiband dichroic or an acousto-optical beam-splitter (AOBS, see Chapter 3, *this volume*) is used, the component must be frequently changed, a process that must not affect the angle of the beam. In addition, all the different dichroic mirrors must have similar surface characteristics (Fig. 9.2).

Polarization

The state of polarization does not seem to be very critical. However, a number of optical elements, especially the filters and beam-splitters with a dielectric coating, have a reflection coefficient that is polarization dependent as are the imaging characteristics of microscope objectives. Changing a dichroic mirror can

therefore have surprising effects on the image and on the amount of light that penetrates the sample. In fluorescence microscopy, it seems best to work with light that is either circularly polarized or phase randomized (Table 9.2).

Intensity Loss

Every element in the detection path causes some intensity loss. If the elements are coated (broadband, anti-reflection coating in transmission; broadband, high-reflection coating for mirrors), the energy losses can be as low as 0.5% per surface for wavelengths within the bandwidth.

The various filters cause the largest intensity losses. Filters come in three general types (Chapter 3, *this volume*): those made from absorbing/colored glass, interference filters made from transparent optical flats coated with several dielectric layers, and hybrids made from absorbing optical flats coated with dielectric layers. Dichroic filters are always of the interference type. An interesting alternative is to take advantage of the polarization dependence of the reflectivity of flat surfaces and to use an uncoated optical flat instead of a dichroic. At an incidence angle of 45°, an *s*-polarized beam can be reflected by 40% while the randomly polarized fluorescence emission may be transmitted up to 80%. A single reflective glass plate with an anti-reflection coated back will provide an excellent performance across a relatively large bandwidth with no need for substitutions that might produce misalignment. The Achrogate beam-splitter/scan-mirror found in the Zeiss LSM5 line scanner is a modification of this idea in which use is made of the fact that the diffraction pattern of a line is another line. Therefore, while a small silvered line in the center of the beam-splitter is sufficient to reflect the excitation line of any wavelength towards the objective, most of the returning signal bypasses it towards the detectors.

The narrow-band interference filters used to select a laser line transmit between 50% and 90%, depending on the batch, the manufacturing process, and the design bandwidth. Dichroic filters reflect 80% to 90% of a single laser line and should transmit more than 80% of the light 12 to 20 nm above the maximum reflection. Long-pass color filters transmit more than 90% of the light 12 to 20 nm above the 50% cut-off wavelength. Special dichroics can be designed that have a particularly high reflectance for the excitation laser line. In these cases the 50% cut-off can be pushed further and a difference of less than 15 nm can be achieved (see also Chapter 3, *this volume*).

Specifications of the most suitable filter depend on the excitation and emission spectra of the fluorophore and therefore on the laser line used. Thus, compromises can hardly be avoided, and carefully adapting the filters for any experiment involving more than one dye is worth both effort and time. For single-channel operation, the goal is to use a long-pass filter or a broadband interference filter that comes close to the emission maxima in the detection channels. If necessary, a change of the laser wavelength (Kr/Ar-ion lasers offer several lines between 458 nm and 647 nm) or a different dye should be considered. Note that dyes often change their spectral properties when coupled to antibodies or incorporated into the target cell (Chapters 16 and 17, *this volume*) and filters can age from prolonged exposure to intense light, particularly that from an Hg-arc lamp without an ultraviolet (UV) filter.

Evaluation of Optical Arrangements

This section discusses and evaluates different optical arrangements for confocal fluorescence microscopes, shows a drawing of the compact confocal camera built at EMBL, and shows the spectroscopic center of such an instrument, that is, the assembly that is used to separate the excitation from the emission light (Fig. 9.3).

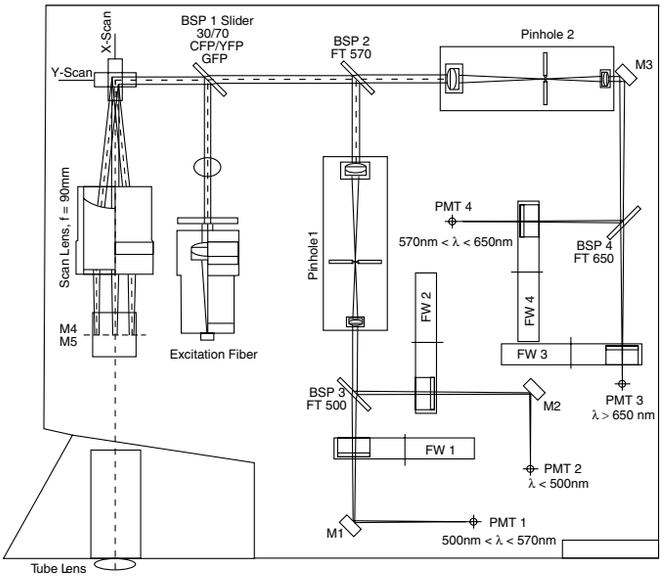

FIGURE 9.3. Schematic drawing of the compact confocal camera built at EMBL. Several lasers (not shown) provide light through an excitation fiber. The collimated beam is deflected by a dichroic mirror (BSP 1) towards the scan unit (upper left corner). The scan unit consists of two mirrors that deflect the beam in two orthogonal planes to scan it in the object plane. The fluorescence light emitted by the sample (not shown) is descanned by the scan unit, transmitted by the dichroic mirror BSP 1, and split into a short- and a long-wavelength component by another dichroic mirror (BSP 2). Each of the two beams is focused into a pinhole (pinholes 1 and 2). Behind the pinhole, each divergent beam is focused by a lens and split once more by a dichroic mirror (BSP 3 and 4). The resulting four convergent beams each pass a detection filter (filter wheels FW 1–4) and eventually hit a photomultiplier tube (PMT 1–4).

Class 1

In a class 1 system [Fig. 9.4(A)], the illumination pinhole is at the focal point of the first two lenses that spatially filter and expand the collimated laser beam. The expansion is determined by the ratio of the focal lengths of the two lenses and should be chosen to fill the entrance pupil of the objective lens. The spatially filtered beam is then deflected by the dichroic mirror in the direction of the scan unit. Fluorescence light emitted from the sample passes the dichroic mirror, and a lens focuses the beam into a pinhole in front of the detector (Fig. 9.4).

Refraction in the dichroic mirror causes the emission beam to shift as it passes through. This shift is intrinsic. But, because the light beams from the focal plane are collimated, any such shift can be corrected by displacing the detection lens and the detector by a similar amount. However, this arrangement cannot correct for any tilt produced by wedge error in the dichroic mirror, which must be carefully controlled. In order to obtain a backscattered light signal a polarizing beam-splitter and a quarter-wave plate can be placed between the beam expander and the dichroic mirror without decreasing the efficiency of the detection path. This setup is optimal, as it uses a small number of elements, guaranteeing high detection efficiency. Furthermore, it uses each optical element as it should be used; for example, a collimated beam passes the dichroic mirror and a planar wavefront enters an intermediate lens.

Class 2

In a class 2 system [Fig. 9.4(B)], the excitation light is focused into a pinhole that is at the focal point of two lenses arranged as

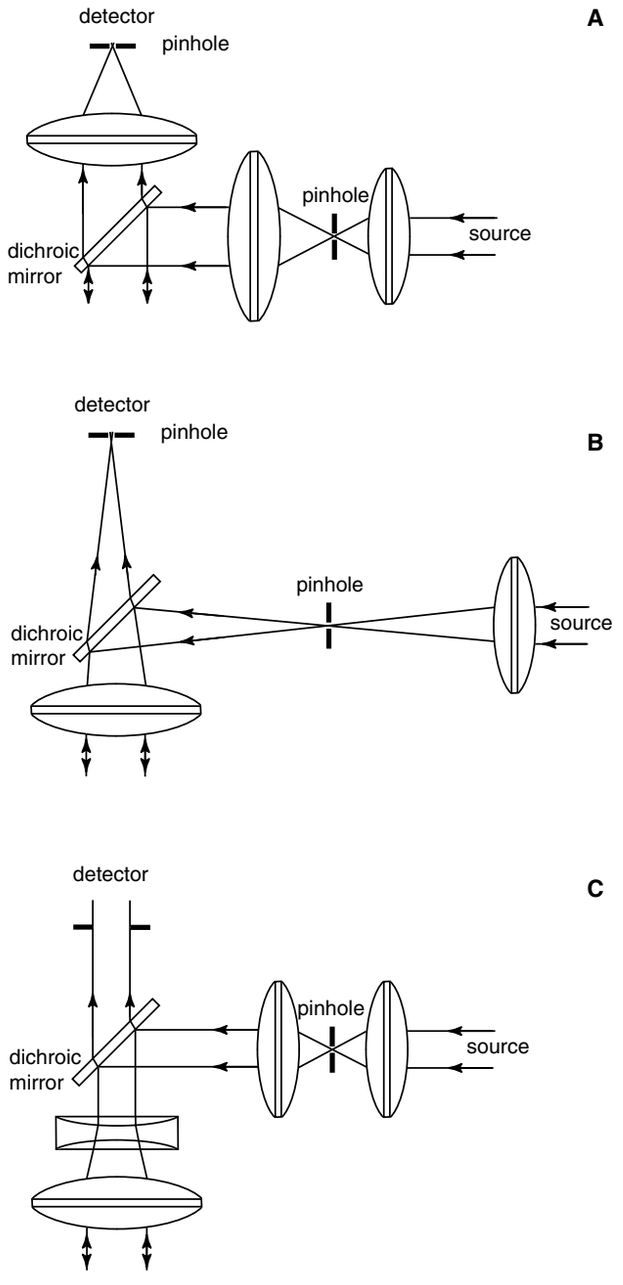

FIGURE 9.4. Three different optical arrangements. In all arrangements the laser beam comes in from the right-hand side, is spatially filtered by a pinhole, and deflected by a dichroic mirror in the direction of the tube lens (not shown). Note that refraction of the fluorescence light when passing the dichroic mirror causes a shift of the light path. The illumination and detection pinholes are placed in conjugate image planes. (A) In a Class 1 arrangement, the laser beam is expanded and collimated *before* it reaches the dichroic mirror. The collimated fluorescence light is focused into the detector pinhole by a separate lens *after* passing the dichroic mirror. This is the optically ideal arrangement. (B) In a Class 2 arrangement, the laser beam is expanded and collimated *after* it hits the dichroic mirror, so the beam is divergent at the dichroic mirror. The fluorescence light is focused into the detector pinhole by the same second lens of the beam expander *before* passing the dichroic mirror. (C) In a Class 3 arrangement, the laser beam is collimated before it reaches the dichroic mirror, and is expanded in the light path common to illumination and detection. Therefore, the beam expander (shown as a Galilean telescope) reduces the diameter of the fluorescence light beam that enters the detector placed at a long distance from the image plane.

a beam expander. However, the dichroic mirror is placed such that the divergent beam is deflected by its surface before being collimated. The second lens of the beam expander is also used to focus the fluorescence emission into the detector pinhole. A convergent detection beam therefore passes the dichroic mirror, which causes a shift and a reduced geometric focal length. This arrangement is, therefore, not able to correct for either a shift of the beam or a tilt of the beam at the dichroic mirror. It is also not possible to insert a beam-splitter arrangement to make use of the back-scattered or reflected light. The arrangement is sparse and, therefore, efficient but the dichroic mirror is placed in a convergent beam, which might cause trouble because the transmission/reflection properties of the dichroic mirror depend on the angle of incidence, and the spectral response will be different for axial and non-axial rays.

Class 3

In a class 3 system [Fig. 9.4(C)], the fluorescence light passes the dichroic mirror and enters a detector that is some distance away. The beam is shifted in the dichroic mirror. Any further shift of the beam can be corrected in this arrangement, whereas a tilt at the dichroic mirror cannot. The setup is obviously efficient, as it uses the minimal number of optical elements, and a collimated beam passes the dichroic mirror. Not having to use a detection lens reduces chromatic effects, but this must be balanced against the need for distances of more than 1 m before the beam can enter the photomultiplier tube (PMT). This "optical lever" is usually implemented by placing three, four, or more mirrors into the detection path, a process that makes the device, at least in principle, more sensitive to mechanical vibrations. However, to a first approximation, the distance between two conjugate image planes divided by the diameter of the detector is a constant, so the performance of this arrangement should be equivalent to that described as Class 1 [see Fig. 9.4(A)]. A detection lens will decrease the path length but, unless it is set up as a telescope, it also decreases the effective area of the detector and hence makes the introduction of a small pinhole necessary.

The advisability of using a beam expander [as shown in Figs. 9.4(A) and 9.4(B), but not in Fig. 9.4(C)] is debatable. The beam can, of course, be expanded elsewhere in the system (e.g., in a scan lens such as the eye-piece). It is, however, inefficient to spatially filter the beam in the common detection/illumination path. The main problem is that the laser may not run in TEM_{00} mode (Chapter 5, *this volume*). If it does not do so, it must be spatially filtered and then it may also be expanded in the same process. If the laser does run in TEM_{00} mode, the light need not be spatially filtered because it would all pass the filter anyway. A single-mode polarization preserving fiber can be regarded as a pinhole with a distinct entrance and a distinct exit. Thus it will always select the TEM_{00} mode of a laser.

Not shown in Figure 9.4 are filters and special equipment for double-fluorescence experiments. Although all could be placed in front of the second lens, it is better to put them behind the detector pinhole (see Fig. 9.3). It is, therefore, not necessary to include these parts in this discussion. A practical problem that may arise if a single pinhole is used for both channels is that one channel may receive less signal than the other. If two pinholes are used, one can be opened to increase the signal while the other remains set to the optimal size (Chapters 22 and 35, *this volume*).

Evaluation of Scanner Arrangements

The simplest of all confocal optical systems is exemplified by that used in the Optiscan confocal endoscope. In this system, light emerging from the end of a single-mode optical fiber is focused by an objective lens into a spot inside the specimen. Returning

light is picked up by the same fiber tip that also acts as the detector pinhole. Fluorescence light is separated from excitation light using interference filters mounted at the far laser end of the fiber. Scanning is accomplished by actually moving the fiber tip in an *xy*-raster using an electromagnetic drive. Although this system is extremely compact, the detector pinhole dimensions are fixed and there must always be some back-reflection from the scanning tip that will be added to any backscattered light signal from the specimen (see also Chapter 26, *this volume*).

However, conventional beam-scanning confocal microscopes use scan mirrors, and this section describes and evaluates four different arrangements of *xy*-scanners. Note that not all optical arrangements described above should be combined with all scanner arrangements.

The galvanometer mirrors used for confocal scanning microscopy must be custom-made for high efficiency in fluorescence applications. These mirrors have conflicting specifications: they should be small, lightweight, mechanically stable, and flat to a fraction of the wavelength, and the reflecting surface should be centered on the rotation axis of the scan unit. The compromise depends to a large degree on the application of the confocal fluorescence microscope and the arrangement of the scan unit. The emphasis should always be on efficiency and instruments that are tuned to perform best with high-NA high-magnification lenses. An excellent choice is currently the VM500 produced by GSI Lumonics. Its specifications meet essentially all requirements if one uses mirrors that are smaller, thicker, and flatter than the standard parts (see also Chapter 3, *this volume*).

The scan mirrors are effectively also aperture stops so their diameter must match the diameter of the conjugate BFP (see Table 9.2). Therefore, high NA and low magnification that requires a large beam diameter needs large scan mirrors to fill the BFP, a factor that limits horizontal (actually fast axis) scan speed (Fig. 9.5).

Two Scan Mirrors + Relay Optics

The two scanning mirrors rotate on perpendicular axes, so their combined movement produces an *xy*-movement of the light spot in the image plane [Fig. 9.5(A)]. As the relay optics form an image of the first mirror on the axis of the second mirror, both mirrors will be in conjugate telecentric planes of the microscope, as long as the axis of either mirror is in such a telecentric plane. This arrangement is optically perfect. The difficulty is that the requirements for the relay optics are very strict. They must produce perfect images over a broad range of wavelengths, and this is quite expensive and probably also not very efficient.

To have a well-corrected image across a widefield, one can also use two plane-scanning mirrors with two intermediate concave mirrors, as was done in some now obsolete Bio-Rad scan heads (e.g., Amos, 1991).

Two Closely Spaced Scan Mirrors

Two mirrors can also be used without any relay optics [Fig. 9.5(B)], as implemented, e.g., by Zeiss in the LSM 5 Series and more recently by Leica and almost all other companies). Again the mirrors rotate on perpendicular axes, but now they are placed as close together as possible with the telecentric plane located at the midpoint between them. The closer the two mirrors come together, the more this arrangement approximates perfect telecentricity. The actual error depends on the magnification of the intermediate optical system and is probably about 4 mm over 100 mm. In general, this will only have an effect at large scan angles (low zoom magnification) where optimal optical performance is less essential. The advantages of small size are obvious.

Center Pivot/Off-Axis Pivot

The arrangement described above can be improved by moving the axis of one mirror off the beam axis [Fig. 9.5(C), as implemented

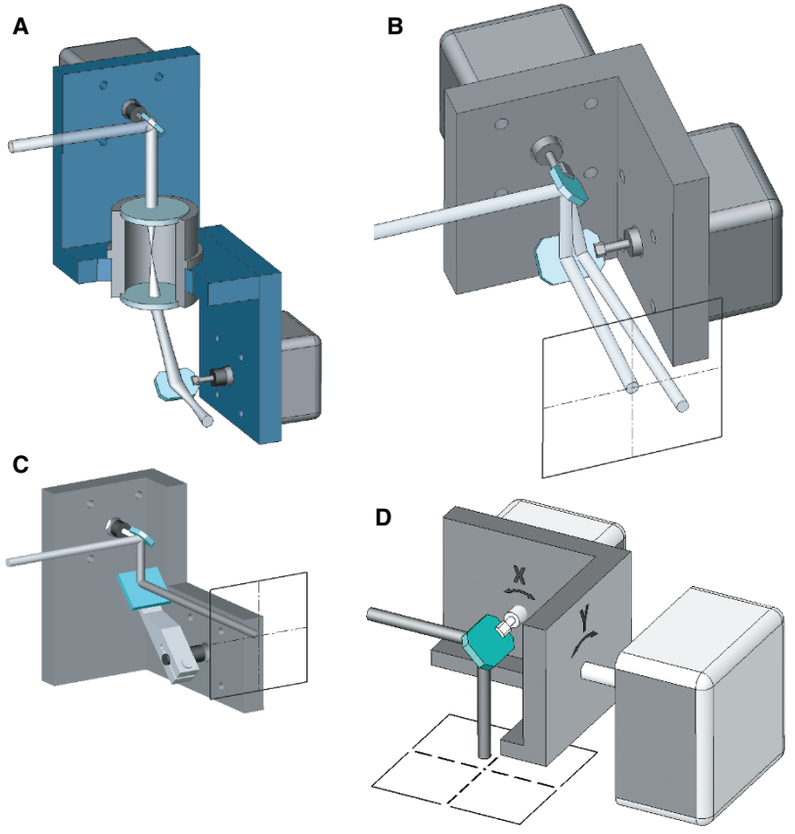

FIGURE 9.5. Four different scanner arrangements. (A) A system consisting of two scan units and intermediate imaging optics. Two mirrors are used to scan the beam along the *x* and *y* axes. The optical system is arranged such that an image of one scan mirror is formed on the other mirror. If either mirror is in a telecentric plane, both mirrors are in conjugate telecentric planes of the microscope. This is the basic and ideal optical arrangement. (B) The scan mirrors are placed as closely as possible and the geometric midpoint of the two mirrors is in a conjugate telecentric plane of the microscope. Both mirrors are, therefore, only approximately in a telecentric plane. (C) An arrangement with off-axis and on-axis scan units. While one mirror rotates around the center of its axis, the other has a pivot point that is off-axis. This latter movement compensates for the missing intermediate imaging optics. The two mirrors are, therefore, both in an approximate conjugate telecentric plane of the microscope. (D) A single scan mirror is used to scan the beam along both the *x* and *y* axes. This is accomplished by mounting the mirror on a fast galvanometer, which is itself mounted on a second, orthogonal scan unit whose center of rotation is in the center of the mirror.

initially by Molecular Dynamics]. The second mirror shifts as it rotates. The combined movement can reduce the apparent position error of the mirror by a factor of up to 25 (Carlsson and Liljeborg, 1989). It also changes the path length slightly as the *y*-scanner moves to the next line. This scanner arrangement may, therefore, not be used with the class 2 optical arrangement [see Fig. 9.4(B)].

Single Mirror/Double Tilt

The simplest solution is to use a single mirror that tilts around two orthogonal axes [Fig. 9.5(D), as implemented in the early Leica systems]. A small galvanometer is mounted on a larger, slow-scan unit. The galvanometer performs the fast movement and the scan unit provides the tilt along the slow axis. The center of the single mirror is easily placed in a telecentric plane of the microscope, and the efficiency is only limited by the reflection coefficient of this mirror (Stelzer *et al.*, 1988). However, this solution does not allow interchanging the *x* and *y* axes for scanning arbitrarily formed areas (Trepte, 1996).

Another possibility is to mount a single mirror on three linear translators. By appropriately driving the three devices one can tilt a mirror along any axis and can even place the point of rotation on the mirror surface. Such piezo-driven devices are available from a number of manufacturers. Although they currently lack either a sufficiently high speed or a large enough rotation angle, they are definitely an excellent choice if these issues are not critical and a compact device is anticipated.

Scanner arrangements can be evaluated on a number of criteria. The most important parameter for a confocal fluorescence microscope is photon efficiency. Other requirements might be the speed or precision with which the raster is scanned, the size and shape of the raster area, the size of the scan unit, *xy*-distortion, long-term stability/repeatability, and its flexibility in scanning nonstandard patterns.

Scanners

Z-Scanners

To get a three-dimensional image of a sample, it has to be scanned in three directions. Therefore, we need to discuss the options for scanning the sample axially, that is, up and down in a standard microscope. The two principal solutions are moving the beam while keeping the object stationary, and moving the object while keeping the beam stationary.

A movement of the focus by some special intermediate optics can be ruled out in microscopy. The reasoning is quite simple. In any telecentric system, the axial magnification M_z, is the square of the lateral magnification M. This relation tells us that, when using a 40× lens, an axial movement of the focus in the object by 10 µm produces a shift of the focus in the intermediate image plane of 16 mm![2] Therefore, it does not make sense to change focus position by moving, for example, the tube lens of the microscope or the scan lens along the optical axis. The required movement distances are by far too large. Therefore the only solution for beam scanning along the optical axis is the axial movement of the objective lens.

The most common method for producing *z*-motion is to drive the fine-focusing control of the microscope. Some manufacturers add an internal fixed reference for full control over the exact axial position. Another technique is to mount the lens (or the slide holder in case of object scanning) on a piezoelectric driver. This offers excellent, backlash-free operation over a range of ≤100 µm. Several manufacturers have mounted an extra galvanometer on a standard specimen stage. This moves the specimen by tilting a substage a few degrees.

The following points are to be considered: any movement along the optical axis affects the immersion-oil film. A rapid movement that increases the distance between the objective lens and the coverslip may cause the sample to "stick" to the lens or to break the film so that air bubbles penetrate the volume between the coverslip and the objective lens. All this could result in a shift of the absolute position of the object. If the absolute position of the object is important, one needs either a "hard" connection between the driving mechanism and the motor or an independent position feedback sensor. A potentiometer connected to the motor is insufficient.

The resolution of the *z*-axis motion must be determined according to the needs. A confocal fluorescence microscope for biological applications needs not be more precise than ±50 nm, whereas an instrument for confocal reflection microscopy should be able to determine axial position with a resolution better than ±4 nm.

Disk Scanners

As pointed out above, the pinholes in a beam-scanning device are located in the conjugate image planes of a conventional microscope. They can be placed directly into the first image plane. In the tandem scanner (Petráň *et al.*, 1968; see also Chapter 10, *this volume*) part of a rotating disk with holes that serve as both sources and detector pinholes is located in the intermediate image plane. The holes are arranged such that the object is uniformly covered by light passing through them as the disk rotates. The separation between adjacent holes guarantees little overlap between the detection and the illumination path when viewing specimens in which the dye is confined to a relatively thin layer. Atto Bioscience's CARV module, that converts a conventional fluorescence microscope into a real-time confocal system, uses a Nipkow disk with a set of spirally arranged pinholes to produce about 1000 beams in the field of view at one time. These can scan the entire field simultaneously at a rate of 400 to 1000 Hz. Because disk scanners form a real image, a charge-coupled device (CCD) camera is placed in an image plane of the microscope to detect the photons with a quantum efficiency that is 5 to 10 times better than that of a PMT. The main problems are the limited brightness of a conventional light source and the loss of 97% to 99% of the excitation light at the disk. Source brightness can easily be increased by using a higher-powered lamp or a laser as the light source, but eventually absorption by the disk causes it to overheat, limiting the effectiveness of this approach (see also Chapter 10, *this volume*).

There can also be a problem in matching the pinhole diameter to the specifications of the objective lens (e.g., 30 µm diameter for 63×/1.4 or 45 µm diameter for 100×/1.4). As a result, one disk is optimal for only a few lenses. In addition, if the disk resides in the intermediate image plane, the source and detector pinholes cannot be optimized independently (Chapter 11, *this volume*). Ideally, pinholes of this size should also be very thin, but this is in conflict with the mechanical stability of the disk.

However, the most challenging aspect of the intermediate optics of the tandem scanner is the difficulty of adjusting the various mirrors so that the returning pattern of points exactly coincides with the pattern of holes on the detector side of the disk. Assuming 40 µm holes and a 160 mm fixed conjugate objective lens, each hole in the field should be aligned with an accuracy of ±10 µm in *x* and *y* over a field of ~25 mm. Apart from the need to

[2] Some confocal endoscopes utilize special-purpose objectives that contain moveable elements capable of shifting the focus plane (see Chapter 26, *this volume*).

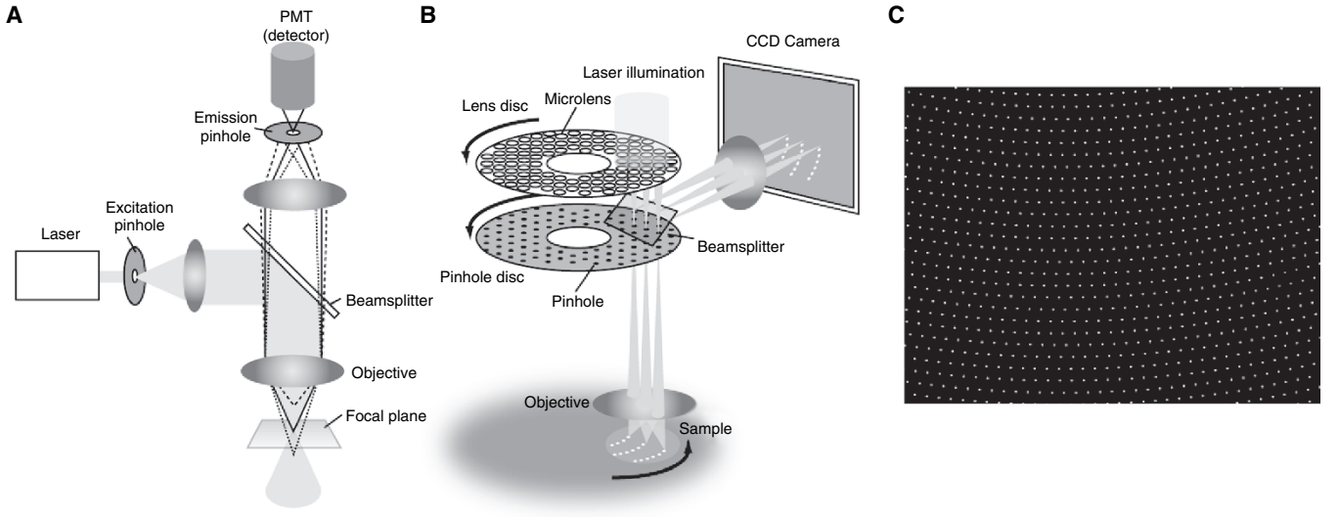

FIGURE 9.6. The optical system of the Yokogawa spinning disk microscope. An array of 20,000 microlenses is mounted on a disk. Approximately 1000 of them are illuminated at one time, and each of them focuses the light into a particular pinhole below it on a second disk. Both disks rotate synchronously at 30 Hz, and because 12 complete images are formed per rotation (a segment of 30° gives a complete image), an image acquisition rate of 360 Hz is achieved (Fukui *et al.*, 1999; Maddox *et al.*, 2003).

exactly orient the mirror angles so as not to distort the pattern of dots, the total length of the illumination and detection paths must match to about ±10 µm to prevent change in magnification from causing the outer spots to miss their holes. Not surprisingly, tandem scanners tend to use fairly large pinholes.

Some of the problems of the tandem scanner, such as disk thickness and alignment, are avoided by the single-sided disk scanner, which uses a tilted quartz disk coated with an opaque metal film except where the pinholes are located (Chapter 10, *this volume*). As the same area of the disk is used for both excitation and detection, there is almost no alignment problem, but there can be problems with the illumination light reflecting off the disk and obscuring the image data coming through the pinholes. Clever polarization techniques can reduce these reflections to a remarkable extent, and they are less of a problem when recording fluorescence images because then the light reflected from the disk can be excluded on the basis of its wavelength. However, it is still difficult to convey to the specimen sufficient light in a narrow waveband to permit high-resolution fluorescence images to be recorded in a reasonable time span.

The optical efficiency problem has been solved by Yokogawa in a design that is used by PerkinElmer, Solamere, Andor (Fig. 9.6), and others. A microlens array is mounted on a rotating disk. Each lens focuses the light from a laser into a particular pinhole located one focal length below it on a second disk, such that more than 40% of the light passes the pinholes (Tanaami *et al.*, 2002). The dichroic beam-splitter is placed between the disks, such that the detection light does not pass the microlenses (Fig. 9.6).

Object Scanners

The first operating confocal microscopes were stage scanners (Minsky, 1961; 1988; Brakenhoff *et al.*, 1979; Marsman *et al.*, 1983; Stelzer and Wijnaendts-van-Resandt, 1985), and those which scanned the beam by moving the objective lens (Wilke, 1985). One source and one detector pinhole are placed in conjugate image planes. The optical arrangement is stationary, and the object is scanned through a single beam that is located on the optical axis of the lens. Any geometrical distortion in the recorded image can come only from the imperfect control of the motion of the object. Object scanners, therefore, offer the highest quality image for data processing purposes and, because of their simplicity, they are very photon efficient. Disadvantages include a low scanning speed (10–150 lines/s) and the special specimen preparation techniques necessary to keep the moving mass low.

The two overriding considerations that have limited the use of this approach in biological confocal microscopy are that early instruments employing this technique did not permit the observer to switch to normal non-confocal operation easily, in order to scan the specimen and find the area of interest, and that biologists were concerned that mechanical resonance within the specimen would cause it to move relative to the stage, causing disruption of the cell and distortion of the image. Although the force on the specimen due to the scanner's acceleration is usually very low (about 1% of the gravity for an amplitude of 100 µm and a frequency of 10 Hz, but 1 G at 100 Hz), it can affect the behavior of living specimens suspended in viscous media (Fig. 9.7).

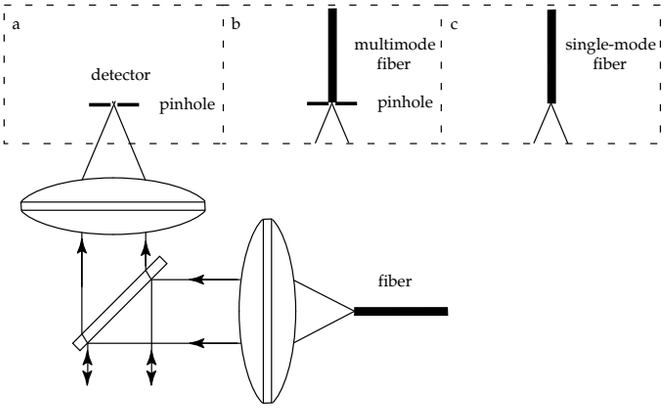

FIGURE 9.7. Optical arrangements using fibers. The arrangement of Class 1 uses illumination with an SMPP fiber, whose tip is in the focal point of a lens that collimates the beam. Complete plugs for laser-light delivery systems consist of the tip and a short-focus achromatic lens. The detection can be performed with a conventional system, employing a multi-mode fiber placed behind a detector pinhole, or using the same single-mode fiber used as the source.

Attachment to Microscopes

A conventional microscope offers several ports through which a confocal setup can be attached to it and through which the excitation beam can enter and the emission beam can leave the instrument. Commonly used are the video port, the ocular, or the port normally used for the photographic camera. Having mentioned efficiency as one of the main issues in confocal fluorescence microscopy, it should be obvious that it is preferable to use the port that causes the beam to pass the smallest number of optical elements. Many (but not all) inverted microscopes guide the detection light over long distances and many optical elements between the object and the ocular (or video port). If for some important reason such an inverted microscope is the system of choice, one should consider having the instrument modified to permit confocal operation using a simpler, separate path. Such modifications are now easier because both Nikon CF objectives and Zeiss ICS objective lenses (plus a tube lens) are fully chromatically corrected, and the attachment can be performed through the intermediate image plane without the need for any additional optics.

Among upright microscopes, the video port of the Zeiss Axioimager series provides direct access to the intermediate image plane. A confocal setup may be interfaced to this plane with no extra optical elements. Microscopes from other manufacturers also have this capability. The advantage of scanning through video ports and eyepieces is that no modification of the microscope is required.

Merit Functions

Functions that determine the performance of an instrument can be object dependent and object independent. An object-dependent merit function is the product of all transmission and all reflection coefficients weighted with the emission and absorption spectra of a particular fluorophore (Tables 9.3 and 9.4). The sum over all surface errors and the sums over all tilts, thickness variations, and deviations from planarity are object independent.

Object-dependent functions can be used to measure the performance of an instrument and to compare the data with those generated at later times or by other instruments.

This is not so easy with the other four merit functions that are more likely to be used in the design phase of an instrument to decide which development path should be followed. The sum over all surface errors should include all elements beyond the source pinhole. It determines how much the beam deviates from a perfect planar wave front, hence how well the beam can be focused and how much the objective lens must be over-illuminated. As a benchmark, it is important to remember that a wavefront error of only $\lambda/4$ reduces the contrast at the midpoint of the spatial frequency plot by almost 50%.

TABLE 9.3. Typical Intensity Losses in the Illumination Path

Element	Loss (%)	Remainder (%)
Laser		100
Mirror	1–3	98
1–3 dichroic mirrors for several lasers	3–10 per mirror	88
AOM	10–15	75
Fiber	25	56
Collimator/beam expander	4	54
Mirrors	1–3 per mirror	52
Polarizing beam-splitter and $\lambda/4$-plate	4	50
Dichroic mirror	10–20	42
Scanner	4	41
Relay lens	10	37
Inside microscope	10	33
BFP of objective lens	80	7
Total intensity entering the sample		7

TABLE 9.4. Typical Intensity Losses in the Detection Path

Element	Loss (%)	Remainder (%)
Sample emission		100
Collection by high-NA lens	75	25
Inside microscope	10	23
Relay lens	10	20
Scanner	4	19
Dichroic mirror	25–30	15
Collimator/beam expander	4	14
Dichroic mirror	20	11
Filter	40	7
Detector	85	1
Total intensity detected		1

MULTI-FLUORESCENCE

Multi-fluorescence experiments are those in which one observes specimens containing more than one fluorophore labeling different targets. Such experiments can be performed by observing each target separately or by observing two or more targets at the same time. In the first case, specific filter combinations would be selected for each fluorophore, matching the excitation line and the emission band. In the second case, one excitation line could excite emission from two or more fluorophores and this signal would have to be separated later in the detection path.

The main disadvantage of the first approach is the difficulty of inserting several different filter sets without changing the illumination or detection paths. Any change prevents the individual images from being properly aligned with each other. The amount of shift that can be tolerated depends on the resolution of the objective lens used. In addition, as was pointed out above, the images of the source and the detector pinholes must overlap in the object plane. If all the dichroic mirrors used do not have the same orientation and position, the signal will not fall into the pinhole and will be lost. On the other hand, observing each fluorophore separately does provide the necessary degrees of freedom needed for the optimal choice of filter sets to observe each dye.

Separating two emission bands for measurement after simultaneous excitation with a single line is possible only with a relatively low efficiency because the narrow bandpass of the two filter sets will cut off a lot of otherwise usable spectrum. In addition, if only a single excitation wavelength is used, one of the dyes will almost always be excited less efficiently than the other. However, the advantage of simultaneous excitation is that the corresponding pixels of the two images are definitely in register because a single spot of incident light is the source of both of them. If the color separation filters are placed behind the pinholes, this setup is also unaffected by changes of filter sets. A problem that cannot be dealt with so easily is the cross-talk between channels, caused by the limited spectral performance of the filters.

Another solution that also adds to the stability of the instrument is the use of double and triple dichroics, that is, dichroic mirrors that are designed to deflect several laser lines and let

several emission bands pass (or vice versa). They need not be moved in order to use different laser lines, whether these are used simultaneously or not, and they can even be used in combination with an acousto-optical element to switch laser lines on a pixel-by-pixel basis. The most critical element for the proper overlap of the illumination and the detection paths is the dichroic mirror, and therefore using such a double or triple dichroic is ideal for this purpose. The problem is that multiple dichroics (tri-chroics, tetra-chroics, . . .) have slightly reduced transmission between the reflection bands because of the design compromises needed to produce their complex spectral properties, and that they only match a small number of fluorophore combinations. As with every design, the user has to decide between efficiency and alignment.

The acousto-optical beam-splitter (AOBS, see Chapter 3) is a system recently invented by Leica (Birk *et al.*, 2002), which can replace the dichroic mirrors that usually separate the illumination and detections paths in confocal microscopes. The advantage of the AOBS is that it does not have fixed transmission and reflection bands, but that it can be programmed to adapt from single to multi-chromatic characteristics of any type. This adds flexibility for working with different combinations of laser lines and fluorophores. In practice, the AOBS is programmed such that it leaves most of the fluorescent light undeflected, while the laser lines are deflected by a fixed angle. In addition, the AOBS is more efficient in terms of transmittance and reflection. This allows one to work with less laser power and to detect with an improved signal-to-noise ratio. These benefits are particularly valuable when working with multiple detection channels.

An interesting option is the use of two-photon excitation (Denk *et al.*, 1990; Stelzer *et al.*, 1994; see also Chapters 28 and 37, *this volume*) to excite not only UV dyes but also practically any currently used visible dye (Fischer *et al.*, 1994). The required filter sets are of a very simple design and extremely efficient because excitation and emission lines are spectrally very far apart. It should be no real problem to use this technique in any of the existing confocal instruments in many implementations — however, because no fluorescence light is generated away from the plane of focus, the detector pinhole is unnecessary. Instruments used for a combination of single-photon and two-photon excitation should probably have provisions for both confocal and widefield (non-descanned) detection.

SPECIAL SETUPS

Setups for Fluorescence Recovery After Photobleaching Experiments

Bleaching experiments use lasers to bleach a spot, an area, or an arbitrary pattern into a fluorescent sample. Bleaching refers to the removal of the fluorescent response of the target, ideally without damaging the target itself. The experiment consists of the observation of time-dependent changes in the bleached pattern, that is, recording changes in the structure of the bleach patterns or recording the fluorescence intensity recovery in certain locations. The confocal beam scanner is in principle an ideal device for generating bleach patterns or for applying a laser light pulse of a well-defined intensity to a particular location. It is also quite well suited to observe those locations before and after the application of the bleaching light pulse. For the bleaching process, the main requirement is the capability to drive the scanners in a more or less arbitrary path across the sample and to control the laser power. Although bleaching occurs unavoidably during any observation process (excitation consumes a fluorophore with a certain probability) it is usually rather slow. For typical FRAP experiments one wishes to bleach fast. The whole pattern should be generated within much less than a second. This typically requires lasers that provide a higher power than those required for simply imaging (i.e., milliwatts, not microwatts). For FRAP experiments it also makes sense to provide for an efficient illumination path. So, apart from needing a higher-powered laser and appropriate software, all confocal beam-scanning fluorescence microscopes should be suited for FRAP experiments.

It should not be forgotten that single-photon bleaching occurs along the entire illumination path. Hence it is not restricted to the focal volume, but is more intense there if only a small area is exposed. Multi-photon excitation can be used to bleach in a well-defined three-dimensional location (Brown *et al.*, 1999; Chapters 28 and 35, *this volume*).

Setups for Fluorescence Resonance Energy Transfer Experiments

In a fluorescence resonance energy transfer experiment (Clegg, 1996), one tries to determine whether two fluorophores and hence two proteins are located within a few nanometers of each other. Under certain circumstances, an excited fluorophore can be quenched by a neighboring fluorophore if its own emission band overlaps the excitation band of the neighbor. In the spectral domain, this is, in principle, a multi-fluorescence experiment where only one fluorophore is excited directly by the laser light, and any confocal fluorescence microscope should be equipped to perform such experiments. In the temporal domain, one requires special equipment (not only software but also hardware) that controls the illumination and detection timing.

Setups for the Integration of Optical Tweezers

A particularly interesting application of lasers in biology is the use of focused beams to capture and move small objects such as cells or bacteria (Rohrbach and Stelzer, 2001; Rohrbach *et al.*, 2004). One usually employs lasers that provide powers in the milliwatt range. To reduce damage due to absorption and heating, these lasers operate in the near-infrared range (NIR, 800–1100 nm). Once again in principle, the beam-scanning confocals are equipped for such purposes. However, optical elements are optimized for the visible range of wavelengths (400–750 nm) and may fail to work well at the particularly important wavelength of Nd:YAG lasers, 1064 nm. It is, therefore, more appropriate to design a separate optical path with a separate scanner pair, optimized mirrors, well-adapted beam expanders, and an appropriate scan lens and to feed the NIR light into the system through a separate port.

Used in continuous mode, multi-photon lasers provide the right laser powers for optical tweezers. When operated in a slow scanning mode (<100 lines/s) they can move a lot of biological material towards the borders of the scan area.

Setups for the Integration of Laser Cutters

Pulsed UV laser beams and, to a certain extent, the pulsed NIR laser beams used for multi-photon microscopy, can be used to cut biological material (see Chapter 38, *this volume*). Once again being able to position the laser beam and to control its power are the

crucial aspects. However, the wavelength of the UV lasers (~355 nm) is outside the specifications of the visible path and a separate optimized path should be employed. A serious issue is that the axial foci of the UV as well as of the NIR beams can be several tens of micrometers away from the focal plane of the visible light. Using slightly divergent or convergent beams, respectively, is a mediocre solution. Much better is to refocus the beam during the cutting procedure, that usually lasts much less than a second, by placing the lens on a piezo-driven positioner.

Optical tweezers as well as laser cutters work very well with regular widefield microscopes. It makes perfect sense to use a beam steering device for either or both of these applications and to observe the effect in the considerably faster widefield video mode (Grill *et al.*, 2001; Grill *et al.*, 2003; Colombelli *et al.*, 2004).

Setups for the Observation of Living Specimens

The main issue in the observation of living specimens is to reduce the exposure of the sample to light as far as possible. The sample should only be illuminated when a signal that is relevant for the experiment is recorded or when the system is set up prior to the experiment. Hence, speed and precision of operation and ease of control are crucial elements of a high-quality instrument. An efficient light path, a precise control of the laser power, and the ability to turn off the laser beam during retrace or between frames are just a few of many effective procedures.

MINIATURIZATION AND COMPUTER CONTROL

Usually, confocal microscopes are built around a conventional microscope by attaching a new intermediate optical system to its body. However, because image acquisition in a confocal microscope is computer controlled, it is questionable whether this solution is appropriate. Utilizing the body and the basic mechanical and optical elements of a conventional microscope puts mechanical and physical constraints on the design of the optical system that are mostly dictated by anatomical considerations. Indeed, the size of the instrument (and thus the length of the optical paths) is adapted to the posture of the user. Optical corrections are performed to ensure that the image of the sample is well perceived through the observer's eyes. Because the most important issue in confocal fluorescence microscopy is efficiency, it seems reasonable to put these constraints aside and to start a new, computer-centered design, in which principles such as miniaturization and computer-delegation are put first. This also provides a number of other advantages such as higher precision and long-term stability, features that are required for high-resolution studies of living biological specimens. In addition, the state of the microscope at any point in time is known to the computer. Each setting is documented and therefore reproducible. Manual intervention other than placing the sample in the microscope is not necessary and not desirable.

Important points:

- Use of digital detectors/cameras
- Corrections made by software
- Instrument is not bigger than a pile of books
- Feasibility of quantitative microscopy
- Good temperature control due to small size
- Suitable for implementation of structured illumination (Gustafsson, 1999)

THERMAL STABILITY

The increasing use of confocal microscopes for the acquisition of large three-dimensional data sets and for time-lapse experiments generate a concern about the long-term thermal stability of the instruments. Recent systematic investigations (Adler and Pagakis, 2003, of the effect of temperature fluctuations) have shown that images are distorted due to movements of the microscope stage, in particular along the optical axis. For room temperature variations of several degrees, focal shifts of about 2 µm have been observed. This can result in a stretched or compressed image of the sample, depending on the direction of the shift. Such temperature variations can easily occur in laboratories with many heat sources (computers, lasers, lamps, etc.), or where the temperature is cooled down cyclically by air conditioning. For long time-lapse experiments that run during the night, one should remember that the heat load will inevitably be lower when the laboratory is less busy.

In addition to the large heat sources already mentioned, heaters close to the sample are obviously a concern, in particular sample chamber heaters, stage heaters, and objective lens heaters. Other temperature-sensitive parts that can be sources for thermal distortions of the image are the immersion oil, mounting liquids, the light sources, the acousto-optic tuning filter (AOTF), and fiber-optics (Swedlow *et al.*, 2002).

But, generally speaking, the thermal stability of modern commercial microscopes is quite well taken care of.

VIBRATION ISOLATION

Although, vibration-isolation tables just mechanically isolate the instrument from the environment, usually some more sophisticated versions can actively damp noise sources created by the instrument itself. The only real vibration sources in a confocal microscope are the scan mirrors and the cooling system of the laser head. The latter source can be eliminated if the laser head is not mounted on the same table as the microscope and the light is carried to the microscope by an optical fiber. Figure 9.7 shows how an arrangement of Class 1 uses illumination and detection with fibers (see also Chapter 26).

One should also remember that mechanical damping only works when all the optical elements, particularly heavy ones, are firmly fixed to the table. Otherwise the movement of the different elements will be independent and not in phase. Under any circumstances, damping mechanical vibration is a complicated task, and it is often fairly easy to do something that seems straightforward but that actually makes things worse.

CONCLUSIONS AND FUTURE PROSPECTS

The most important issue in any kind of light microscopy is photon efficiency, as this determines the gap between photo-damage and useful contrast in the recorded image. As long as the chosen optical arrangement maintains the necessary diffraction limit, the general rule is that the more sparse an optical path, the more efficient it will be. The scan system should be chosen in accordance with photon efficiency. Because a number of different scanning microscopes have been realized, each of them employing different optical and scanning arrangements, time should show which are the most efficient and the most versatile to use. My personal preferences are the optical arrangement shown in Figure 9.4(A) and the scanner arrangements shown in Figures 9.5(B) and 9.5(D).

Sparsity, relative simplicity, and good illumination efficiency are advantages of single-beam scanners over disk scanners. The disadvantage of single-beam scanners is that they have to use relatively inefficient PMT detectors to achieve high recording speeds. It is also a mistake to assume a technological standstill. The large number of companies and the short product lifetimes indicate that much can still be done. In the short term, major improvements are more likely to be found in the fluorescent dyes, in the biological application, or in other biochemical developments. Particularly useful alternatives are the disk scanning-devices. They offer an excellent compromise between speed and efficiency, and the new electron-multiplying charge-coupled device (EM-CCD) cameras give the disk scanners a detector that is very comparable to the PMT (see Chapters 10 and 12, *this volume*).

Ceterum censeo that conventional fluorescence microscopes are excellent, reliable, and easy-to-use optical instruments. A high-quality, conventional optical microscope should always be the first instrument of choice. Those scientists who do not achieve the expected image quality should consider attaching a high-quality, possibly cooled CCD camera (Hiraoka *et al.*, 1987) to their conventional widefield microscope. Using an efficient CCD camera, a powerful computer, and appropriate software to discriminate against out-of-focus light will in some cases be a simpler, and perhaps an even more efficient, solution to many relevant biological problems (Agard and Sedat, 1983; Fay et al., 1989; see also Chapters 23 and 24, *this volume*).

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REFERENCES

Adler, J., and Pagakis, S.N., 2003, Reducing image distortions due to temperature-related microscope stage drift, *J. Microsc.* 210:131–137.

Agard, D., and Sedat, J., 1983, Three-dimensional architecture of a polytene nucleus, *Nature* 302:676–681.

Amos, W.B., 1991, Achromatic scanning system, US Patent 4,997,242.

Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E., and Webb, W.W., 1976, Mobility measurement by analysis of fluorescence photobleaching recovery kinetics, *Biophys. J.* 16:1055–1069.

Bacallao, R., and Stelzer, E.H.K., 1989, Preservation of biological specimens for observation in a confocal fluorescence microscope and operational principles of confocal fluorescence microscopy, *Methods Cell Biol.* 31:437–452.

Birk, H., Engelhardt, J., Storz, R., Hartmann, N., Bradl, J., and Ulrich, H., 2002, Programmable beam-splitter for confocal laser scanning microscopy, *SPIE Proc.* 4621:16–27.

Brakenhoff, G.J., Blom, P., and Barends, P., 1979, Confocal scanning light microscopy with high aperture immersion lenses, *J. Microsc*. 117:219–232.

Brown, E.B., Wu, E.S., Zipfel, W., and Webb, W.W., 1999, Measurement of molecular diffusion in solution by multiphoton fluorescence photobleaching recovery. *Biophys. J.* 77:2837–2849.

Carlsson, K., and Liljeborg, A., 1989, A confocal laser microscope scanner for digital recording of optical serial sections. *J. Microsc.* 153:171–180.

Clegg, R.M., 1996, Fluorescence resonance energy transfer, In: *Fluorescence Imaging Spectroscopy and Microscopy* (X.F. Wang and B. Herman, eds.), John Wiley and Sons, New York, p. 179–252.

Colombelli, J., Grill, S.W., and Stelzer, E.H.K., 2004, UV diffraction limited nanosurgery of live biological tissues, *Rev. Sci. Instrum*. 75:472–478.

Denk, W., Strickler, J.E., and Webb, W.W., 1990, Two-photon laser scanning fluorescence microscopy, *Science* 248:73–76.

Fay, F.S., Carrington, W., and Fogarty, K.E., 1989, Three-dimensional molecular distribution in single cells analysed using the digital imaging microscope, *J. Microsc.* 153:133–149.

Fischer, A., Cremer, C., and Stelzer, E.H.K., 1994, Fluorescence of coumarines and xanthenes after two-photon absorption with a pulsed titanium-sapphire laser, *Appl. Opt.* 345:1989–2003.

Fukui, Y., de Hostos, E., Yumura, S., Kitanishi-Yumura, T., and Inoue, S., 1999, Architectural dynamics of F-actin in eupodia suggests their role in invasive locomotion in *Dictyostelium*, *Exp. Cell Res.* 249:33–45.

Goodman, J.W., 1968, *Introduction to Fourier Optics*, McGraw-Hill: San Francisco.

Grill, S.W., Gönczy, P., Stelzer, E.H.K., and Hyman, A.A., 2001, Polarity controls forces governing asymmetric spindle positioning in the Caenorhabditis elegans embryo, *Nature* 409:630–633.

Grill, S.W., Howard, J., Schäffer, E., Stelzer, E.H.K., and Hyman, A.A., 2003, The distribution of active force generators controls mitotic spindle position, *Science* 301:518–521.

Gustafsson, M.G.L., 1999, Extended resolution fluorescence microscopy, *Curr. Opin. Struc. Biol.* 9:627–634.

Hiraoka, Y., Sedat, J.W., and Agard, D.A., 1987, The use of a charge-coupled device for quantitative optical microscopy of biological structures, *Science* 238:36–41.

Maddox, P.S., Moree, B., Canman, J.C., and Salmon, E.D., 2003, Spinning disk confocal microscope system for rapid high-resolution, multimode, fluorescence speckle microscopy and green fluorescent protein imaging in living cells, *Methods Enyzmol.* 360:597–617.

Marsman, H.J.B., Stricker, R., Wijnaendts-van-Resandt, R.W., Brakenhoff, G.J., and Blom, P., 1983, Mechanical scan system for microscopic applications, *Rev. Sci. Instrum.* 54:1047–1052.

Martínez-Corral, M., Caballero, M.T., Stelzer, E.H.K., and Swoger, J., 2002, Tailoring the axial shape of the point spread function using the Toraldo concept, *Opt. Express* 10:98–103.

Melles Griot, 1999, *The Practical Application of Light*, Irvine, CA, Melles Griot. (The Melles Griot catalogue is very informative and contains numerous excellent comments on optical elements and performance characteristics.)

Minsky, M., 1961, Microscopy Apparatus, US Patent 3,013,467.

Minsky, M., 1988, Memoir on inventing the confocal scanning microscope, *Scanning* 10:128–138.

Petráň, M., Hadravský, M., Egger, M.D., and Galambos, R., 1968, Tandem-scanning reflected-light microscope, *J. Opt. Soc. Am.* 58:661–664.

Rohrbach, A., and Stelzer, E.H.K., 2001, Optical trapping of dielectric particles in arbitrary fields. *J. Opt. Soc. Am. A* 18:839–853.

Rohrbach, A., Tischer, C., Neumayer, D., Florin, E.L., and Stelzer, E.H.K., 2004, Trapping and tracking a local probe with a photonic force microscope, *Rev. Sci. Instrum.* 75:2197–2210.

Stelzer, E.H.K., and Wijnaendts-van-Resandt, R.W., 1985, Applications of fluorescence microscopy in three dimensions — microtomoscopy, *SPIE Proc.* 602:63–70.

Stelzer, E.H.K., Hell, S., Lindek, S., Stricker, R., Pick, R., Storz, C., Ritter, G., and Salmon, N., 1994, Nonlinear absorption extends confocal fluorescence microscopy into the ultra-violet regime and confines the illumination volume, *Opt. Commun.* 104:223–228.

Stelzer, E.H.K., Stricker, R., Pick, R., Storz, C., and Hänninen, P., 1988, Confocal fluorescence microscopes for biological research, *SPIE Proc.* 1028:146–151.

Swedlow, J.R., Hu, J., Andrews, P.D., Roos, D.S., and Murray, J.M., 2002, Measuring tubulin content in *Toxoplasma gondii*: A comparison of laser-scanning confocal and widefield fluorescence microscopy, *Proc. Natl. Acad. Sci. USA* 99:2014–2019.

Tanaami, T., Otsuki, S., Tomosada, N., Kosugi, Y., Shimizu, M., and Ishida, H., 2002, High-speed 1-frame/ms scanning confocal microscope with a microlens and Nipkow disks, *Appl. Opt.* 411:4704–4708.

Trepte, O., 1996, Enhanced scanning control in a confocal scanning laser microscope, *Scanning* 18:356–361.

Wilke, V., 1985, Optical scanning microscopy — the laser scan microscope, *Scanning* 7:88–96.

Wilson, T., and Carlini, A.R., 1987, Size of the detector in confocal imaging systems, *Optik* 72:109–114.