# 5 The Confocal Microscope

## THE SCANNING OPTICAL MICROSCOPE

Instead of forming an entire image at one instant, a scanning optical microscope (SOM) scans a beam of light across specimen in a regular pattern, or *raster,* and forms its image point by point. A point of light is focused to a diffraction-limited spot on the sample by the objective lens. An image is built up point by point by a detector, either below the slide (if the microscope is exclusively an SOM) or below the condenser (which is more practical if the microscope is also used for conventional imaging). SOMs have been used for about 50 years, and the image they produce is formally and in practice equivalent to the image given by a conventional microscope. Why, then, use a scanning microscope? The most common reason is that image processing and analysis are facilitated by having the picture broken up into a series of points (pixels); before the advent of digital cameras, the SOM was the simplest way to achieve this. Scanning also facilitates contrast management when dealing with samples that have either very high or very low contrast. However, what made the SOM an essential part of cell biology was the simple modification that introduced the *confocal* imaging technique. The transformation from SOM to CSM (confocal scanning microscope) is summarized in Figure 5.1.

## THE CONFOCAL PRINCIPLE

In a confocal microscope, as in the SOM, a point light source is imaged on the specimen by the objective. This image is no longer a point, but an Airy disk, its size depending on the numerical aperture (NA) of the lens: the larger the NA, the smaller the spot will be, and hence the better the resolution (Chapter 1). This spot is then scanned over the specimen; historically this was done by moving the slide, but commercial microscopes normally move the beam of light, as discussed later in this chapter. For *confocal* imaging (meaning that there are two coincident focal points), we then collect the light with the objective lens and once again bring it to a focus in front of the detector. At this point, we place a pinhole: in principle, the closest approximation we can make to a geometrical point. Figure 5.1 shows this as an epi-fluorescence microscope, but by using a half-silvered mirror instead of a dichroic mirror for the beamsplitter, we can use reflected light instead, with identical geometry. In either case, the transmitted light can be collected, too, but that will *not* give a confocal image.

What is the point of doing this? This simple layout has the surprising (but easily understood) property of rejecting information from outside the plane of focus, as

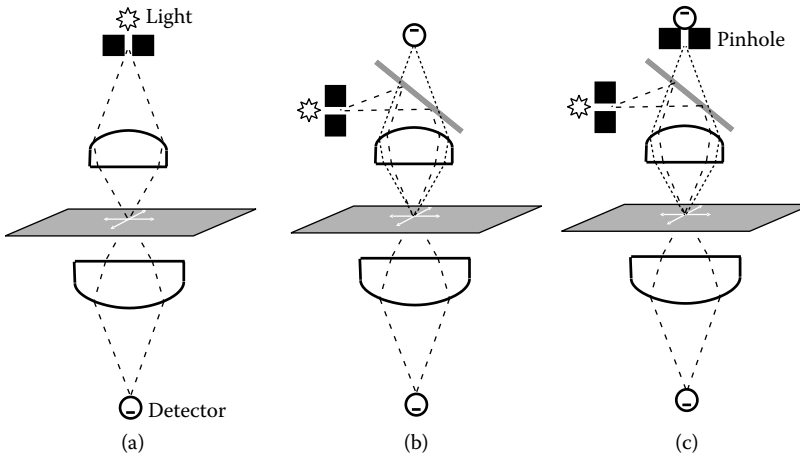



**FIGURE 5.1** (a) A simple scanning optical microscope (SOM). A point of light is focused to a diffraction-limited spot on the sample by the objective lens. A transmission image is built up, point by point, with a detector below the condenser, while the slide or the beam is scanned in two directions. (b) The system modified for fluorescence imaging, with one detector recording the fluorescence image and another capturing the transmission image. (c) The addition of a pinhole now turns the microscope in (b) into a CSM, whereas the bottom detector still collects a nonconfocal image.

Figure 5.2 shows. The light that is in focus (solid lines) is brought to a small spot at the pinhole, and therefore all goes through to the detector. Light falling on an out-of-focus plane (dotted lines) is bought to a spot in front of (or behind) the plane of the pinhole. At the pinhole, the out-of-focus light spreads over a large area, so that very little of this light passes through the pinhole. The improvement this makes is dramatic to the fluorescence image of a thick sample (Figure 5.3).

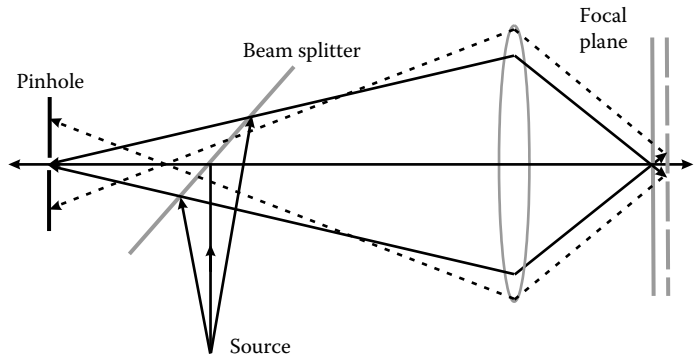
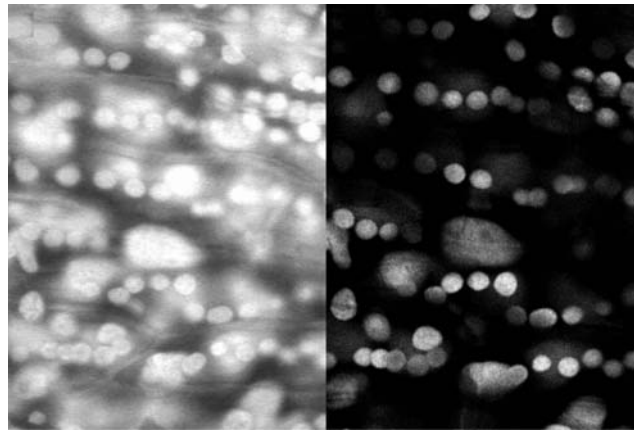



**FIGURE 5.2** A basic confocal optical system, showing paths of in-focus and out-of-focus light.



(a) (b)

**FIGURE 5.3** Fluorescence images of part of a *Selaginella* leaf (chlorophyll autofluorescence). (a) Conventional wide-field image. (b) Confocal image. Both images are focused on the same plane.

When we use a conventional microscope, we are restricted to one plane, the plane of best focus, but this restriction does not apply to a confocal microscope. With a confocal microscope, we can carry out *optical sectioning*: imaging individual planes of a thick object (Figure 5.4). The confocal technique transforms optical microscopy into a fully three-dimensional imaging medium. With a suitable motor-

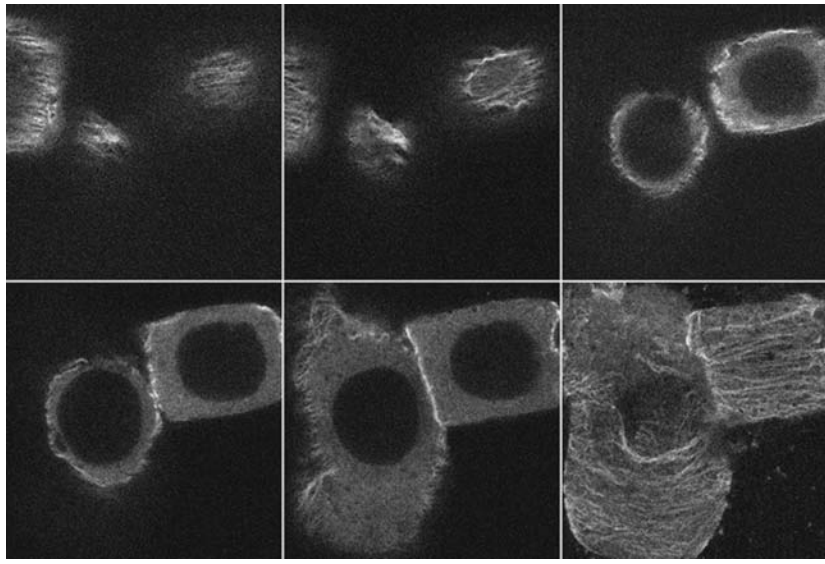


**FIGURE 5.4** Six planes from a set of 74 optical sections through an onion root-tip squash, showing the cortical microtubule array stained with FITC — anti α-tubulin.

ized stage, we can collect automatically a complete, three-dimensional data set of our sample. With suitable software, we can then extract information either by resectioning in arbitrary planes or by constructing projections from different views.

## RESOLUTION AND POINT SPREAD FUNCTION

With the optical microscope, we are accustomed to thinking of resolution in the horizontal plane only. As we saw in Chapter 1, that resolution depends directly on the NA of the lens and is given (in fluorescence) by the Rayleigh formula:

$$r = 0.61\ \lambda / NA$$

where r is the minimum resolved distance and $\lambda$ is the wavelength of the light.

But confocal microscopes also resolve structures in the axial (Z, or vertical) direction, and the factors controlling the resolution in this direction are not quite the same. What controls the resolution in the Z direction is the amount of light rejected by the pinhole. *Out-of-focus light can never be totally rejected.* However large the out-of-focus spot, some proportion of the light will go through the pinhole, as Figure 5.5 shows. So we can never completely exclude out-of-focus objects, and if these objects are very bright, their influence on adjacent planes may be quite noticeable.

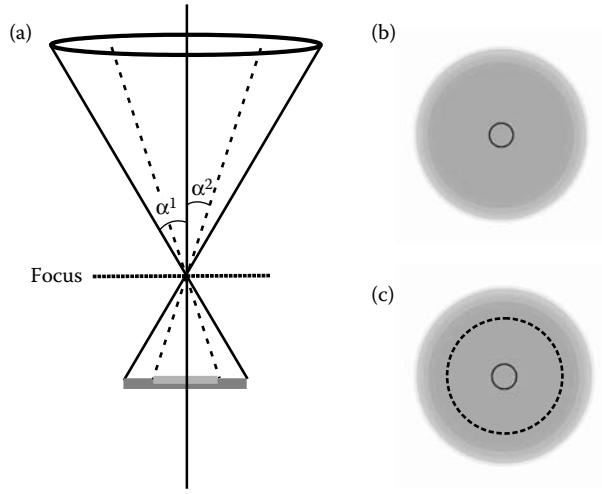



**FIGURE 5.5** The size of an out-of-focus spot in relation to numerical aperture. (a) The diameter of the spot at an arbitrary plane depends linearly on the half-angle of the lens $\alpha^1$ or $\alpha^2$, that is, on the NA. (b) The spot in relation to the pinhole. Even though the vast majority of the light is rejected, some always passes through. It is the ratio of the *area* of the spot to the *area* of the pinhole that determines how much is rejected. (c) The spot of the lower NA $\alpha^2$ superimposed (dotted circle). Because the diameter of each circle depends on the NA, the area — and thus the rejection of out-of-focus light — depends on the square of the NA.

What should also be clear from Figure 5.5 is that the amount of the light that is rejected depends on the *area* of the spot of out-of-focus light compared to the *area* of the pinhole. So what determines the size of the out-of-focus spot? Looking at Figure 5.2 and Figure 5.5a, we can see that the amount that the light spreads out beyond or before the plane of focus depends directly on the angle $\alpha$ at which the rays converge on the focal point — in other words, on the numerical aperture of the lens. Thus, the *diameter* of the out-of-focus light depends *directly* on the NA of the lens, and the *area* of that spot therefore depends on the *square* of the NA: Although the lateral resolution improves linearly with increasing NA, the axial resolution improves as $NA^2$. Using a high NA lens is critically important if we want good optical sectioning. For this reason, many manufacturers now make lenses with relatively low magnification and high NA, so that we can get good 3D confocal stacks of reasonably large areas.

Because the axial resolution depends on the square of the NA and lateral resolution is determined by the NA directly, the two are never likely to be the same. In fact, the resolution is always worse in the axial direction. How, then, can we summarize the overall resolution of a confocal microscope? A point, imaged on a plane, appears as an Airy disk. The way that same point is imaged in three-dimensional space is called the *point spread function* (PSF), which is an ellipsoid (egg shape) (Figure 5.6). Its cross section through the center is the familiar Airy disk, and a vertical section shows it as an elongated ellipse. The ellipse is at its shortest with the highest NA lenses, where it is a little more than twice its width. With an NA 1.4 lens, we can expect an axial resolution of about 500 nm and a lateral resolution about 200 nm. With NA 0.65, as in Figure 5.6, the axial resolution will be 4 times worse, at ~2 $\mu$m.

Although we can apply the Rayleigh criterion in depth and give our resolution that way, in practice it is hard to find a suitable specimen with points directly above each other in a known spacing. So it is common to use a different criterion, the full-width half maximum (FWHM). Fluorescent beads are common and simple test specimens, and if we take an XZ section of one, we see something like the second image of Figure 5.6. We can then, with the microscope software, take a line trace

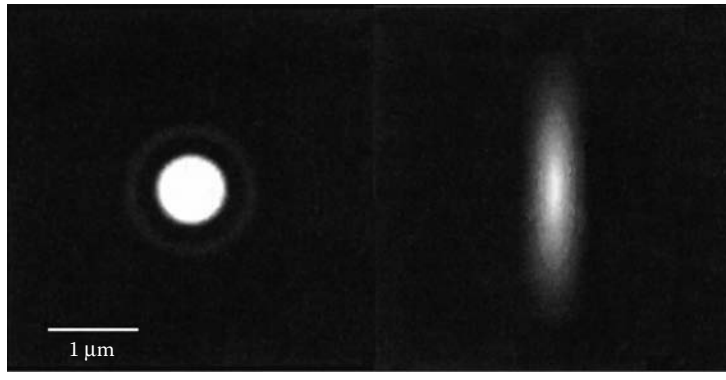



**FIGURE 5.6** Horizontal and vertical sections through the PSF of an NA 0.65 lens.

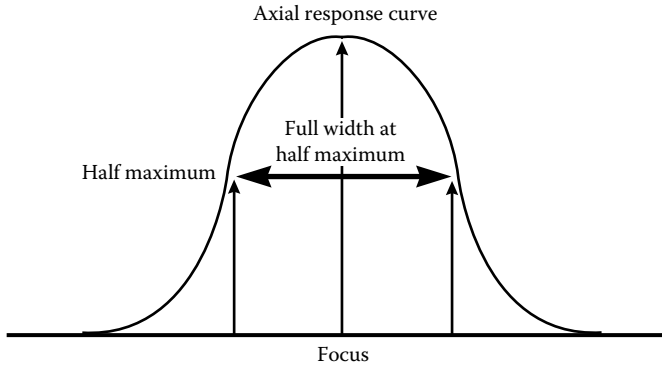



**FIGURE 5.7** The definition of full-width half maximum (FWHM).

of the intensity vertically through the image and get something like the trace seen in Figure 5.7. The width of this curve, measured halfway between the peak and the background level, is the FWHM. As we saw in Chapter 1, it is a less stringent condition than Rayleigh's (Figure 1.17).

## Lateral Resolution in the Confocal Microscope

The confocal microscope can also offer a small but useful increase in lateral resolution, especially in reflection imaging. This is a bit tricky to understand. As the beam scans across a point in the specimen (lower line of Figure 5.8), its Airy disk gradually intersects that point. The illumination of that point is therefore determined by the distribution of intensities in the Airy disk. The edge of the Airy disk illuminates the point weakly, and the image Airy disk (upper line) is therefore dim. As the

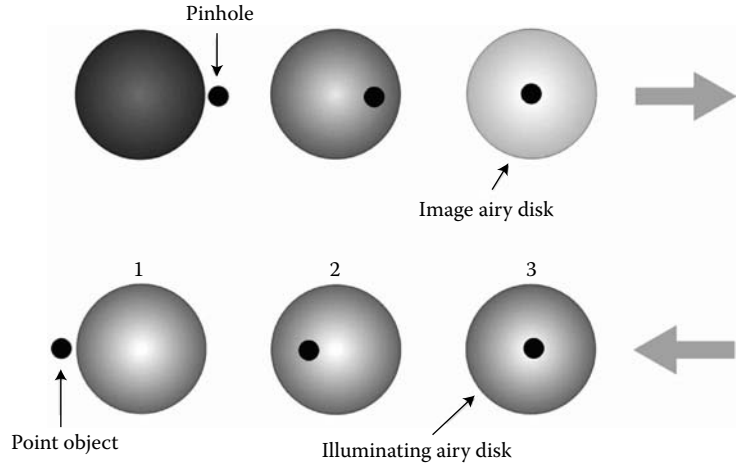



**FIGURE 5.8** As the illuminating Airy disk scans across a point, the image Airy disk becomes progressively brighter.

illuminating spot moves further onto the point, it illuminates the point more and more strongly, and the image Airy disk therefore becomes brighter as it moves progressively over the pinhole.

Thus the intensity seen by the detector is a product of the gradually increasing overall intensity of the *image* Airy disk (resulting from the distribution of intensity in the *illuminating* Airy disk) and the intensity distribution in the *image* Airy disk itself. This means that the intensity seen by the detector at any one time is the illuminating intensity multiplied by the imaging intensity. The two curves, multiplied together, give a final curve of intensity vs. distance, with much steeper sides (Figure 5.9).

What does this mean? Strictly speaking, we could say that the size of the Airy disk has not changed: The *minimum* remains in the same position. But the curve of intensity is now much steeper. If we adopt Rayleigh's criterion in its original form (the center of one Airy disk on the first minimum of the next), our resolution has not changed. However, as Figure 1.17 shows, using Rayleigh's criterion on normal Airy disks, the intensity at the point of overlap is 0.735 times the peak intensity. What if we accept the same value with our "squared" Airy disks? In this case, the centers of the disks are closer together, and we get a value for the resolution that is better than that of a conventional microscope by about $\sqrt{2}$ (1.414). In other words, our minimum resolved distance is now the Rayleigh value divided by $\sqrt{2}$.

We have to be a little cautious about this since the resolution cannot have an actual point detector. If our detector is larger than a point, improvement is reduced. Fortunately, optical sectioning is not much affected by a finite-sized pinhole, at least until the pinhole size exceeds the Airy disk. In practical terms, therefore, the optical sectioning property of CSM is often more important than its resolution improvement in fluorescence mode, where we usually set the pinhole to be more or less equal to the Airy disk diameter. In reflection mode, with more light to play with, we can close the pinhole down and get better lateral resolution.

As far as fluorescence is concerned, optical sectioning is the key factor in any case. Strong fluorescence above and below the plane of focus is the major degrading factor in conventional fluorescence microscopy, and practically useful reso-

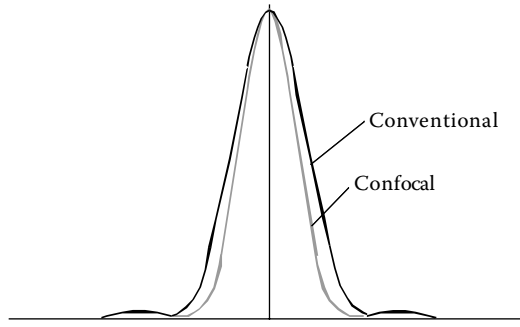



**FIGURE 5.9** The intensity distribution in the confocal Airy disk is the square of the intensity distribution in the wide-field Airy disk.

lution in biological confocal microscopy will therefore be improved, even if the lateral resolution is unchanged in theory.

As we saw in Figure 5.1c, we can have an additional detector below the specimen, which gives a nonconfocal, scanned image acquired simultaneously with the confocal image. Optical theory tells us that this image will be identical to a wide-field image, and we can therefore use contrasting techniques such as phase-contrast and differential interference contrast. This can be extremely convenient in practice, because it reveals other cell structures that are not labeled with our fluorescent probes, enabling us to relate labeled structures to the rest of the cell.

## PRACTICAL CONFOCAL MICROSCOPES

Figure 5.10 shows a schematic of a conventional laser-scanning confocal microscope (LSCM or CLSM), in which one spot scans the specimen in a regular pattern, or raster. Other implementations of the basic confocal principle have been devised — often sacrificing some degree of confocality in the interest of speed — but these are covered in Chapter 9. Commercial confocal microscopes from Leica, Zeiss, Bio-Rad, Nikon, and Olympus all conform (more or less) to this basic plan. (Bio-Rad microscopes are no longer made, but many are still in use.)

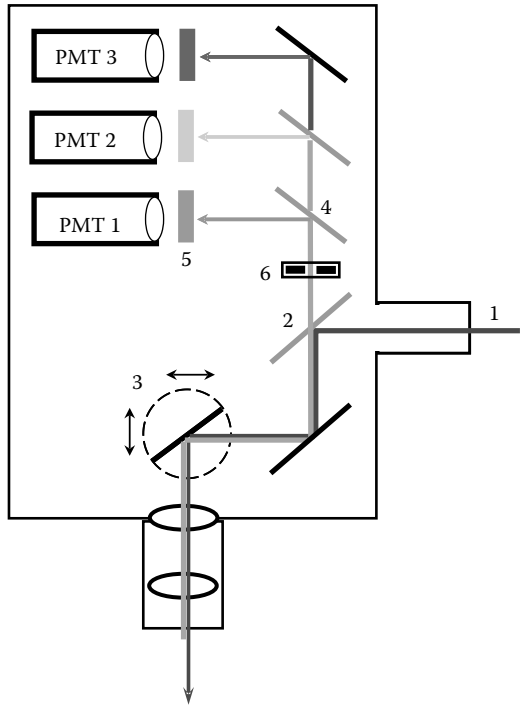



**FIGURE 5.10** **(See color insert following page 174.)** Diagram of a simple confocal microscope. 1: laser beam entry; 2: primary beamsplitter; 3: scanning mirrors; 4: channel-selecting dichroics; 5: barrier filters; 6: pinhole.

The light source is always a laser because nothing else can deliver enough light to one spot. The light from the laser enters the confocal head [1] (either directly or through an optical fiber) and is reflected by the beamsplitter [2] and another mirror down to the scanning mirrors [3], which scan the beam across the specimen, and thence into the microscope itself. The returning fluorescence is descanned (returned to a stationary beam) by the scanning mirrors and passes through the primary dichroic or beamsplitter [2]. It is focused to a point at the pinhole [6] and is then split by more dichroic mirrors [4] into two, three, or more channels, each containing a particular wavelength band. Barrier filters [5] further select the detected wavelength range and block any stray laser light before the signal is detected by the photomultiplier tubes (PMTs).

There are various, specific components to consider in such a design, and the choice in each department can make substantial differences to the performance — and price — of a confocal system:

- The laser or lasers
- The delivery system for the laser light
- The scanning mirrors
- The detection pinhole
- Splitting the signal to different detectors
- Detecting that signal

## The Light Source: Lasers

The word *laser* is an acronym for *light amplification by stimulated emission of radiation*. Lasers amplify light and produce coherent light beams, which can be made extremely intense, highly directional, and very pure in frequency. Electrons in atoms or molecules of a laser medium are first pumped, or energized, to an excited state by an energy source, either light or electric energy. When one electron releases energy as a photon of light, that photon stimulates another to do the same, which in turn stimulates more in a chain reaction, a process known as *stimulated emission*. The photons emitted have a frequency characteristic of the atoms and travel in step with the stimulating photons. The photons move back and forth between two parallel mirrors, triggering further stimulated emissions and amplifying light. The mirrors used must be an integral number of half-wavelengths apart, so that the light continues in phase as it travels back and forth (the total distance between the mirrors can be anything from meters to a few wavelengths, but must nevertheless be a precise number of half-wavelengths). Provided that the pump mechanism continually reexcites atoms or molecules that have lost energy, the light builds up into a very powerful beam. The intense, directional, and monochromatic laser light finally leaves through one of the mirrors, which is only partially silvered.

Figure 5.11 sums this up in a generalized diagram. There are many widely different possibilities for the medium that generates the lasing action: solid, liquid, or gas. Most lasers can be assigned to one or other or to the categories of solid state, gas, semiconductor, or dye (liquid). Semiconductor lasers are solid-state devices, of course, but a distinction is made because their mode of action is different from solid-

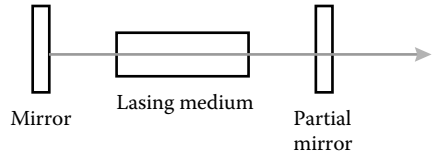



**FIGURE 5.11** Schematic of the components of an idealized laser.

state crysal lasers. All except dye lasers are routine components of confocal microscopes.

## Gas Lasers

The laser medium of a gas laser can be a pure gas, a mixture of gases, or even metal vapor. A cylindrical glass or quartz tube usually contains the medium. The ends of the tube are set at the angle (the Brewster angle) at which light of one direction of polarization is transmitted without loss. If the tube ends were not set in this way, as *Brewster windows,* reflections from them would interfere with the laser action. With Brewster windows, one direction of polarization is lost and takes no part in the lasing action, but the window is invisible to light polarized in the other direction. The output beam is therefore plane polarized. Two mirrors are located outside the ends of the tube to form the laser cavity (Figure 5.12). The gas lasers used in confocal microscopes are pumped by a high-voltage electric discharge in the low-pressure gas.

Argon lasers are very common in confocal microscopy because their 488 nm line is ideal for exciting fluorescein; their second line, 514 nm (blue–green) was once neglected but is now very useful for yellow fluorescent protein (YFP). More powerful argon lasers offer extra lines — 457 nm is very useful for green fluorescent protein (CFP) and also aldehyde-induced autofluorescence. Very powerful argon lasers offer a family of close-spaced lines in the near UV; these were used in the past in some confocal microscopes, but they introduced substantial technical difficulties and have been superseded by diode lasers.

Krypton lasers have a yellow–green line at 568 nm and a 647 nm red line. These are very useful complements to the argon lines, and some makers add a krypton laser to the standard argon laser. Another alternative is a mixed-gas argon–krypton laser, which offers "white" light (actually a mixture of three discrete wavelengths: 488 nm, 568 nm, and 647 nm). Helium–neon gas lasers are another common alternative, providing 543 nm and 633 nm lines, although the green 543 nm HeNe lasers are rather low in power.

## Solid-State Lasers

Until recently, gas lasers were almost universal on confocal microscopes, but semiconductor and solid-state lasers are now taking over and becoming the new standard.

Solid-state lasers are essentially rods of fluorescent crystal pumped by light at the appropriate wavelength to excite the fluorescence. A ruby laser powered by a flash tube was the first laser ever made, producing pulses of light at 697 nm, but the

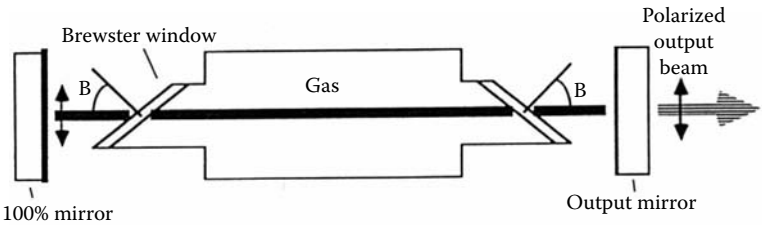



**FIGURE 5.12** The components and layout of a gas laser.

most common lasers in confocal microscopes are doped sapphire crystals and neodymium-doped garnets. The ends of the rod may be two parallel surfaces coated with a highly reflecting nonmetallic film to form the laser mirrors, or may be cut at the Brewster angle for use with external mirrors. Solid-state lasers may be operated either in a pulsed manner, to generate rapid bursts of light, or in continuous-wave (CW) mode. Pumping is always from other lasers — usually semiconductor diodes, a combination known by the acronym DPSS (diode pumped solid state). *Frequency doublers*, special crystals that double the frequency of light (Chapter 8), are often used to turn the 1064 nm wavelength of neodymium lasers into the more useful 532 nm (green); lasers are compact, powerful, and low in cost and are now common on confocal microscopes. Recently, similar doubled DPSS lasers operating at 561 nm have become available. This is in many ways a more convenient wavelength, because it permits a wider window for detection of green fluorescence, so doubled DPSS lasers may become even more popular.

### Semiconductor Lasers

The most compact of lasers, the semiconductor laser, usually consists of a junction between layers of semiconductors with different electrical conducting properties. The laser cavity is confined to the junction region by means of two reflective boundaries. Gallium arsenide and gallium nitride are typical semiconductors used. Semiconductor lasers are pumped by the direct application of electrical current across the junction, and they can be operated in CW mode with better than 50 percent efficiency. Pulsed semiconductor lasers are also available (Chapter 15 deals with their place in confocal microscopy). Common uses for semiconductor lasers include CD and DVD players and laser printers. Red semiconductor lasers operating at around 640 nm and violet ones at 408 nm or 405 nm are routine in confocal microscopes, and blue diodes working at around 488 nm are beginning to challenge argon lasers. Also available are 440 nm diode lasers (ideal for CFP) and near-UV 370 nm diode lasers (useful for many dyes, but a challenge optically).

It seems certain that DPSS and diode lasers will take over from gas lasers in the confocal market. The advantages DPSS and diode lasers offer are compact size and much lower heat output into the microscope room. In principle, these lasers should offer longer life, but only time will tell if this is borne out in practice. Before the company's demise, Bio-Rad Microscience offered a low-cost microscope, the Cell Map, with diode and DPSS lasers as the only option. Other manufacturers have

not been so prescriptive, but most offer systems with no gas lasers as an option. Diode lasers are typically not as strictly monochromatic as other laser types, and individual lasers can diverge by several nanometers from the nominal wavelength. Although this divergence is rarely a problem in the cell biology, it does mean that more latitude may be needed in filter specification.

Using a laser rather than a lamp means that only certain defined wavelengths are available, and ideally our fluorochromes should be tailored to the laser lines. In practice, the use of lasers means that, in many cases, we are not exciting the fluorochrome optimally. Table 5.1 shows the excitation and emission peaks of various common fluorescent stains and how they match with the lines available from argon and argon–krypton lasers.

Careful selection can make a big difference to the effectiveness of our labeling. Thus, among the rhodamine-derivative dyes, TRITC is by far the best choice for a green HeNe laser, whereas Lissamine is excellent with krypton excitation.

## Laser Delivery

In simpler, low-cost, and older LSCMs, the laser line selection is carried out by bandpass filters, shutters, or both, and neutral density filters control the proportion of laser light allowed through. This design is simple, cheap, and effective, but it is slow if we want to collect parallel images with different excitations. Modern high-end systems use an acousto-optical tunable filter (AOTF) to control both line selection and intensity.

An AOTF is a special crystal (tellurite, tellurium oxide), which is birefringent (Chapter 2) and has the unusual property that, if it is deformed by a high-frequency ultrasound signal, its birefringence is reduced *for one particular wavelength*. Because laser light, as we have seen, is polarized, if the polarization direction is matched to the crystal axis, the ultrasonic vibration changes the direction in which the crystal refracts that one wavelength (Figure 5.13). The frequency of the ultrasound determines the *wavelength* affected, and the sound's amplitude (volume) determines the *proportion* of the wave affected. An AOTF can therefore very rapidly

**TABLE 5.1**
**Common Fluorochromes and Their Compatibility with Popular Laser Lines**

| | | | Percent Max Excitation at: | | | |
|---|---|---|---|---|---|---|
| Fluorophore | Excitation Peak | Emission Peak | 488 nm | 514 nm | 568 nm | 647 nm |
| FITC* | 496 | 518 | 87 | 30 | 0 | 0 |
| TRITC** | 554 | 576 | 10 | 32 | 61 | 1 |
| Lissamine rhodamine | 572 | 590 | 5 | 16 | 92 | 0 |
| Texas red | 592 | 610 | 3 | 7 | 45 | 1 |
| Allophycocyanin | 650 | 661 | <1 | 4 | 5 | 95 |
| CY518 | 649 | 666 | 1 | <1 | 11 | 98 |

* Fluorescein iso-thiocyanate.

** Tetra-methyl rhodamine iso-thiocyanate.

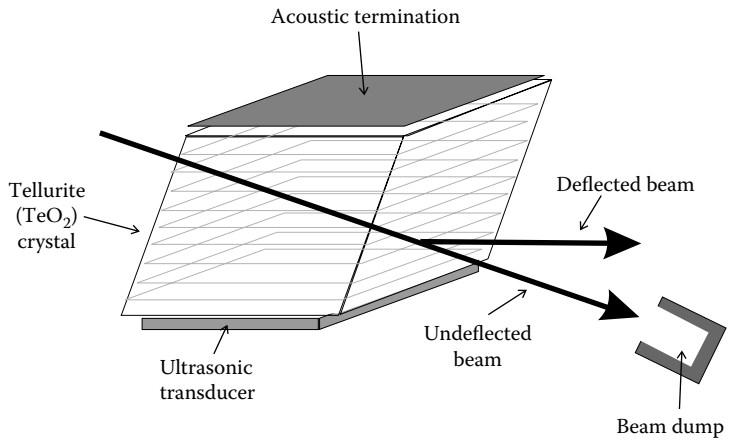



**FIGURE 5.13** Above, diagram of an acousto-optical tunable filter (AOTF). The frequency of the signal generated by the transducer determines which wavelength is deflected and therefore escapes the beam dump and enters the microscope. By varying the strength of the signal, a greater or lesser proportion of the selected wavelength will be deflected.

change both the laser line and the intensity of the light. Multiple frequencies of ultrasound can be used simultaneously, allowing us to have several different laser wavelengths entering the microscope, each at a different strength.

This high-speed control allows us to carry out many useful tricks. By turning the beam on and off during the scan, we can irradiate a defined shape, as irregular as we like, for bleaching or photoconversion (Chapter 14). We can switch excitation wavelength on a line-by-line basis to obtain live, two-channel imaging while minimizing bleed-through. Also, the light can be blanked as the beam flies back at the end of the scan line. With most microscopes, no data is collected during this time, so by blocking excitation we lose no signal but substantially reduce bleaching.

## The Primary Beamsplitter

Single dichroic mirrors are highly efficient. However, changing dichroics for different laser wavelengths requires that very precise alignment be maintained between them. Also, changing between single dichroics is limiting if we want to collect multiple channels at reasonable speed. One popular solution to this problem is to use a *triple dichroic*, which reflects at three distinct wavelengths and transmits the rest of the spectrum (Figure 5.14; see also Figure 3.13). Because, as Figure 5.14 shows, a triple-dichroic beamsplitter inevitably stops a lot of the returning fluorescent light; several manufacturers have looked for alternatives.

One clever approach has been to use a polarizing beamsplitter, taking advantage of the fact that the laser light is polarized, but (generally speaking) the returning fluorescence is not. This technique is wasteful to some extent: Some incoming light is lost, but there is usually plenty of laser light to spare, and the loss to the returning fluorescence, with a claimed 80 percent transmittance, is substantially less than with a triple dichroic. However, the beamsplitter does little to reject returning laser light,

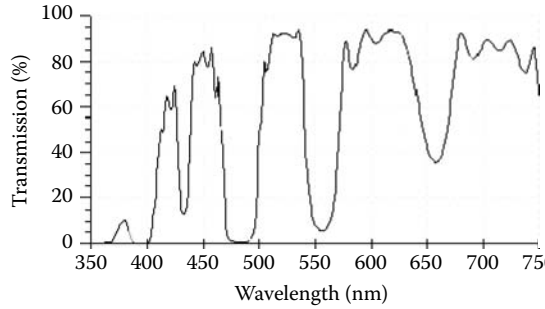



**FIGURE 5.14** Transmission curve of a triple dichroic for confocal use (argon-krypton laser) from Chroma. (Courtesy of Chroma Technology Corp.)

so the detection filters must be more efficient. Both Bio-Rad and Nikon have used this technique in some of their microscopes.

Leica introduced a system that uses an AOTF to provide a beamsplitter. The company calls this the *acousto-optical beamsplitter* (AOBS). Up to eight wavelengths can be selected with only a few nanometers lost to each. The price for this convenience is considerable complexity; the birefringence of the tellurite crystal splits returning fluorescence into two components, of opposite polarization and propagating in different directions. Further, the dispersion of the crystal separates the fluorescence into its component wavelengths. Both effects require compensation with matching (unmodulated) tellurite crystals, so that all wavelengths and polarizations finally emerge as one beam.

## Beam Scanning

The beam is scanned by deflecting it with mirrors, normally at a rate between 0.25 and 10 seconds per frame, though some manufacturers have made faster systems. A fast (line) scan in the X direction and a slower (frame) scan in the Y direction are required. The mirror configuration is an important design consideration. Ideally, the mirrors should be light to permit rapid scanning, especially in the X direction. However, there is only one correct spot for the mirror: the plane conjugate with the back focal plane of the objective; only here is the angular movement given to the beam correctly transferred to a spatial movement at the specimen (Chapter 1). Failure to meet this condition creates a risk of *vignetting*: the image is darker at the edges and in the corners, because the whole beam does not enter the pupil of the objective at large scan displacements. Use of a single mirror is thus theoretically ideal; it maximizes efficiency, because there is only one reflecting surface and the mirror can be positioned in the optimal plane to avoid vignetting. However, the mechanical complexity of making a mirror move in two planes increases both cost and the potential for inaccuracy, so that dual-mirror scanning has been the preferred option in most designs.

Some manufacturers simply place two mirrors as close together as possible and hope for the best. A better approach is to introduce optics, forming an image of one of the scanning mirrors onto the other, so that both appear at the same plane to the

objective. This can be done with a lens, but Bio-Rad (Figure 5.15) always used two convex mirrors, a relatively bulky system but with very low losses and no risk of chromatic aberration. Although in principle the Y scan mirror, which moves slowly, does not need such a high-performance galvanometer as the X (fast) scan mirror, there are advantages to making the mirrors identical. In this case, the horizontal and vertical scan directions can be interchanged, so that we can have the fast scan either horizontal or vertical. And by cunningly adjusting the scan signal to the mirrors so that a proportion of the voltage going to the X mirror is sent to the Y, and vice versa, the actual scanned frame, or raster, can be rotated without changing the position of either mirror.

Yet another approach is to duplicate the slow scan mirror. By using two linked mirrors for the Y scan, one above and one below the fast scan mirror, a motion is generated that *appears* to come from the plane of the single X-scan mirror. Effectively, the second mirror corrects the errors of the first. This technique provides a theoretically correct scan motion with a minimum of optical elements, but to rotate the direction of scan, you must rotate the whole mirror assembly.

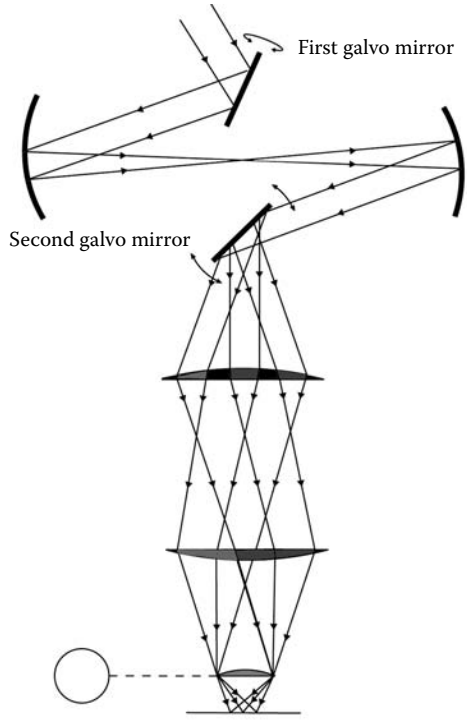



**FIGURE 5.15** A pair of convex mirrors image one scan mirror on to the other, so that both are congruent with the back focal plane of the objective. Using mirrors eliminates chromatic aberration. This system was used on all Bio-Rad confocal microscopes. (Courtesy of Bio-Rad Microscience.)

## Pinhole and Signal Channel Configurations

The next design consideration is where and how to arrange the pinhole. Typically, we want to separate and detect two or three channels of fluorescence, so we could have one pinhole and split the light passing through it into different channels, or we could split the returning beam of light and then send it to two or three different pinholes, each directly in front of a detector. The first approach places some limits on size; if the splitting takes up too much space, the beam will spread out too far and part will not enter the detector. The second places a premium on alignment; all three pinholes must be precisely aligned, and we may have to realign them if we change the beam splitting for different fluorochromes. However, it does mean that we can set each pinhole precisely relative to the Airy disk size for the relevant wavelength — a refinement that is probably more significant in theory than in practice. There is no consensus on pinhole configuration, and in the end the choice is probably dictated by other aspects of the optical design: Are we aiming for a compact scanhead, or do we want multiple devices in the detection pathway?

In a simple LSCM design, the size of the Airy disk at the plane of the pinhole is a function of the magnifications of the objective and projector (eyepiece) lenses. A 100× oil-immersion lens of NA 1.3 has an Airy disk with an equivalent diameter of 500 nm at the plane of the specimen. The disk is therefore 50 $\mu$m in diameter at the first image plane. A dry lens of 0.7 NA and 40× magnification gives an Airy disk of ~40 $\mu$m at the same plane. Some confocal microscopes, such as the Nikon C1, use small, fixed pinholes of these dimensions. Using a 10× projection "eyepiece," the Airy disk becomes ~0.4 nm to 0.5 mm in diameter, simplifying manufacture and alignment at the expense of having more optic elements in the beam path. Making a small pinhole adjustable is another problem, and often pinholes are not round but square, made up of two L-shaped pieces of metal, moved by a galvanometer.

Another approach, used in all Bio-Rad microscopes and some Olympus models, is to make the Airy disk very much larger — several millimeters — by projecting it to a considerable distance or by using a telephoto lens to enlarge it. Both systems potentially reduce efficiency by introducing additional optical elements, but offer the great advantage that a conventional iris diaphragm can be used as the pinhole. This gives the user extremely flexible control over the size of the confocal aperture.

In recent years, most manufacturers have introduced high-end systems that have abandoned the simple dichroic mirror and barrier filter approach to separating the channels shown in Figure 5.10 (though this remains standard on lower-priced systems). The alternative approach is spectral detection: introducing a dispersive device, either a prism or diffraction grating, and directing different parts of the spectrum to different detectors, with no dichroic mirrors or filters involved. This approach allows the user to specify exactly which parts of the spectrum should be recorded in each channel of the image, without being constrained by the dichroics and barrier filters installed in the scanhead. It is therefore highly versatile, and in principle at least very efficient, because a prism and mirror pass more light than a dichroic and barrier filter. It can also give an emission spectrum — for example, of an unknown fluorochrome. Indeed, it can give a true "spectral image" with a complete spectrum acquired at each pixel.

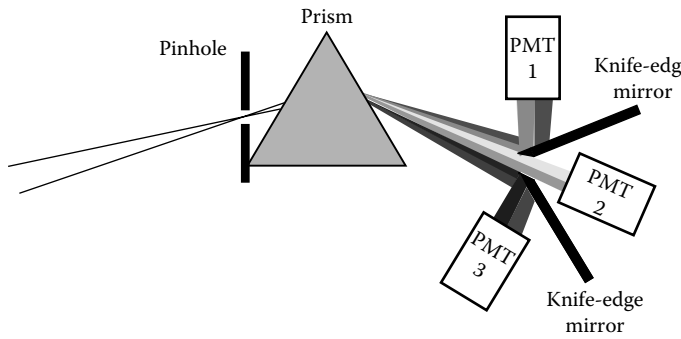



**FIGURE 5.16** **(See color insert following page 174.)** Spectral detection using movable knife-edge mirrors and single photomultiplier tubes (PMTs).

There are essentially two approaches to spectral detection used in confocal microscopes, which can be regarded as serial and parallel. The *serial* approach, typified by the Leica SP series of instruments, is shown in outline in Figure 5.16. A prism after the pinhole splits the light into a spectrum, and movable knife-edge mirrors divert the different parts of the spectrum to different detectors. No part of the spectrum need be lost; every wavelength can be collected by one or other detector. In a real system, additional mirrors are used so that we can have the equivalent of "bandpass" collection at each detector, and there may also be more than three detectors. To acquire a spectrum, the mirrors would be moved together to give a fine slit, and then moved in tandem to scan the required spectral range, acquiring an image at each position. This generates a lot of data, especially since the Nyquist criterion (Chapter 6) applies to spectra just as much as to images, so if the slit is made 5 nm wide (spectral width, not physical), the step size between images should be 2 nm. Serial collection will, however, give a very high-resolution spectrum, which parallel systems cannot match. The serial approach also offers very high sensitivity, because there are few losses and conventional photomultiplier tubes can be used (below). Its disadvantage is that acquiring a full spectral image is slow.

The *parallel* system, shown in Figure 5.17, uses a linear array of detectors, so that the entire spectrum can be collected at once. The Zeiss Meta is an example of this type. Figure 5.17 shows a diffraction grating rather than a prism (of course, either could be used with either system). Diffraction gratings lose more light than a prism but provide a spectrum that is closer to linear. This may be preferable in parallel collection, since the spacing of the detectors in the array is uniform and fixed. The detector is typically a 32-channel photomultiplier: essentially 32 tubes, miniature versions of that shown in Figure 5.18, arranged in a line. Any number of elements can be pooled to give the equivalent of a normal two- or three-channel image, or each can be stored separately to give a full spectral image. This gives a huge speed improvement over serial collection, but with only 32 channels, the spectral resolution is much worse. Some designs seek to overcome this problem by having a variable geometry, so that either the whole spectrum or only part of it lies across the detector array, making it possible to acquire a high-resolution spectrum

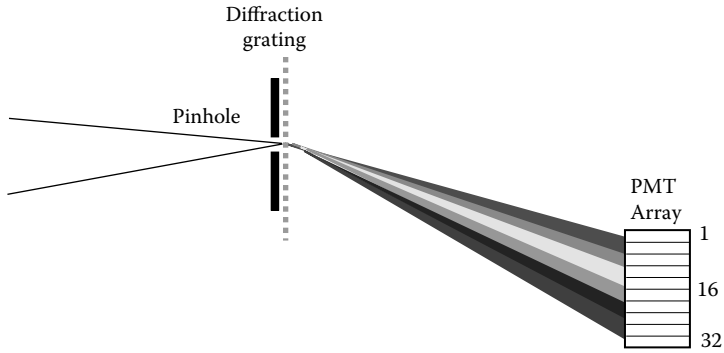



**FIGURE 5.17** **(See color insert following page 174.)** Spectral detection using a linear PMT array.

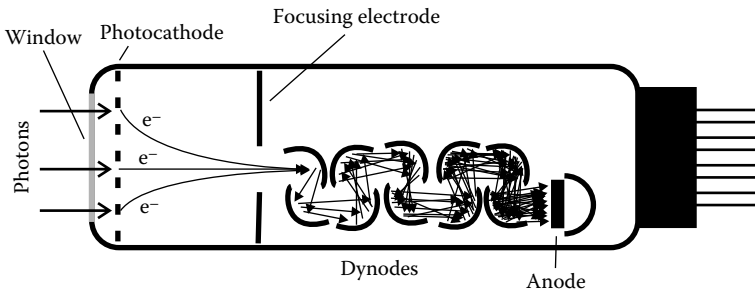



**FIGURE 5.18** An end-window PMT.

in several passes. The major disadvantage of parallel detection is that the array detector's sensitivity is significantly worse than that of a conventional photomultiplier tube (PMT). One loses a lot of signal for the sake of speed. This often means that conventional dichroic-based splitting and PMTs must be provided as an alternative within the scanhead, adding significantly to the cost and complexity — and inevitably adding further losses along the way.

Spectral systems of both types also offer utilities for linear unmixing of the spectral data, that is, correction for bleed-through according to known or measured spectra of the fluorochromes. Once the correction has been calculated, from known data or a spectral image, it can be applied to conventional multichannel images, not only spectral ones. This technique gives very good channel separation, even when fluorochromes have quite substantial overlap.

## Detectors

Detectors for a confocal microscope are typically single elements because they do not have to form an image. The only type to have gained any commercial acceptance is the PMT. Conventional photodiodes are not sensitive enough for fluorescence

detection, athough they have been used for transmitted images. *Avalanche photodiodes* have been used in some experimental applications, but not commercially.

Charge-coupled devices (CCDs), as used in digital cameras, offer better quantum efficiency (they capture nearly all the photons that hit them) but do not offer any gain, so turning this efficiency into sensitivity is not easy. The amplification necessary for such tiny signals hugely magnifies any noise that is present, so the CCD array in low-light cameras is always cooled to minimize random electronic noise. In fluorescence microscopy, it is normal to acquire an image for several seconds. CCDs have not yet been made in a suitable form for confocal microscope use, because the signal level is too low at any reasonable scan rate. Dwelling a second or more on each pixel would hardly be practicable.

Photomultipliers detect photons arriving at a *photocathode,* which is maintained at a high negative potential (Figure 5.18). This is made of a photoelectric substance, so that it emits electrons when light falls on it. These electrons are accelerated away towards the first *dynode*, which is at a positive potential relative to the cathode. To ensure that the electrons hit the first dynode, they are focused by a *focusing electrode*. This is negative with respect to the cathode, so that electrons approaching it are repelled and follow a curved trajectory. Because these electrons have been accelerated across a potential difference, they have considerable energy when they hit the dynode, and each knocks out a shower of low-energy *secondary electrons*. These secondary electrons are accelerated in their turn toward the next dynode, which is at a more positive potential, where further showers of secondary electrons are ejected, and so on through the chain of dynodes. As a result, for each electron that hits the first dynode, thousands or millions travel from the final dynode to the anode (which, for convenience is at earth potential, so that everything else is negative relative to it). The PMT therefore has an extremely high gain, giving a strong signal from a small amount of light. The number of electrons — that is, the current passing between cathode and anode — is proportional to the number of photons arriving over several orders of magnitude, an important feature for quantitative imaging.

Figure 5.18 shows an *end window* tube: The light enters at the end of the tube, and the anode is at the opposite end. Another configuration is the *side window*, in which the light enters at the side of the tube and the dynodes are arranged in a ring around the tube. Both forms are used in confocal microscopes; any difference is more an issue of design convenience than performance.