

Special Optical Elements

Jens Rietdorf and Ernst H.K. Stelzer

INTRODUCTION

The amazing array of microscopic instrumentation for generating three-dimensional images of biological specimens that are described elsewhere in this volume rely on a relatively small number of optical and mechanical components. Although most biologists have a general idea of their function and how to operate them, some mysteries remain.

It is the purpose of this chapter to provide the reader with a description of how components are specified, which components are used in commercially available equipment, and how they operate, along with some insight in terms of their specific applications to confocal or multi-photon microscopy. We will try to emphasize those specifications and limitations that impinge directly on the performance of the entire instrument so that the reader can understand the various trade-offs and raise sensible questions during the purchasing process. Those wishing to make their own confocal from scratch will need to consult more detailed sources.

The comments provide insights developed over many years during which we designed, built, and used a large number of different confocal microscopes. Our comments are meant to improve the understanding of the fine points of microscope operation among users, and should not be construed as recommendations either in favor of or against any particular brand. If some of our remarks seem vague or inconclusive this may reflect the fact that some elements make sense in this but not in that design. Parts that might have been important in the past often become less important due to technical improvements in other areas.

The best approach to optical design is: "Efficiency through simplicity." Use as few optical elements as are absolutely necessary to achieve a certain goal and place them in the optimal location along the optical path. In general, flat surfaces are more easily manufactured and easier and less expensive to coat than curved surfaces. Lenses with long focal lengths usually have fewer aberrations and are less expensive than those with short focal lengths. Additionally, the actual performance of an optical instrument always seems to reflect engineering and financial compromises.

This chapter describes the operation of some of the important components used in confocal microscopes: filters, scanners, acousto-optical devices, electro-optical modulators, and polarizers. It complements in particular Chapter 9, which discusses the intermediate optics of confocal microscopes.

REGULATING THE INTENSITY

In modern laser confocal microscopes, the intensity of the light is typically regulated using acousto-optical tunable filters (AOTFs; see below), combinations of several AOTFs, or combinations of AOTFs and neutral-density (ND) filters. A polarizer can also serve as a continuously variable attenuator as most lasers emit polarized light (Callamaras and Parker, 1999). However, if the polarization axis needs to be preserved [e.g., to maintain proper differential interference contrast (DIC) operation] a second polarizer must be included and adjusted to pass the original beam. Neutral-density filters are either absorptive gray glass filters (for low-power applications) or reflective metallic filters (for high-power applications) that attenuate the intensity of the transmitted light independent of the wavelength (λ). Circular, neutral-density filters, which have a band of optical density around their perimeter that increase linearly or logarithmically with the angle, also allow one to control the intensity continuously, but the intensity profile of a large beam passing such a filter becomes slightly inhomogeneous.

Because most lasers used in confocal microscopy produce only milliwatts of power, there is little danger of overheating the ND filter. However, this does not apply to filters used with arc sources or lasers for multiphoton excitation, where the beam power may reach several watts. ND filters come in two general types, those made of darkened glass that absorbs light and those coated with a metal film that reflects light. The former are more likely to overheat and the latter more likely to produce stray light or to deflect potentially damaging power elsewhere.

WAVELENGTH SELECTIVE FILTERING DEVICES

The following sections discuss the different filter types available for use as excitation or emission filters or as dichroic mirrors. In fluorescence microscopy, filtering devices (e.g., Melles Griot Catalog, Chapter 13) are used to separate light beams on the basis of their wavelengths. Four different types of filters are used to selectively transmit or block a desired range of wavelengths (Fig. 3.1): (a) short-pass filters (blue line) that cut-off wavelengths longer than a certain wavelength (λ_0) — heat filters are used to exclude infrared light to reduce specimen heating by the illumination; (b) long-pass filters (red line) that only transmit light longer than a certain wavelength — fluorescence emission filters are often long-pass filters used to prevent the excitation light from reaching the detector; (c) bandpass filters (light green line) that only trans-

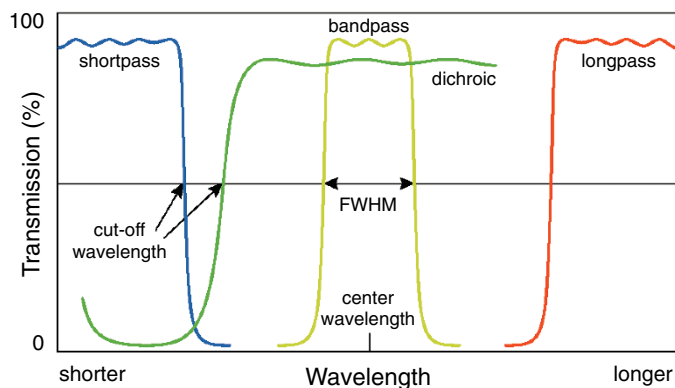


FIGURE 3.1. Types of filters. Filters are flat optical components designed only to transmit light of certain wavelengths. There are four general types: short-pass (dark blue), long-pass dichroic (dark green), bandpass (yellow-green), and long-pass (red).

mit light between a cut-on and a cut-off wavelength — these may be used to select either excitation or emission wavelengths especially when one is trying to image signals from more than one fluorophore simultaneously; and (d) dichroic mirrors (dark green line) that separate the emitted light from the excitation light. In fluorescence microscopy, dichroic mirrors are long-pass filters designed to operate when oriented at 45° to the optical axis.

Note that active optical devices such as acousto-optical (AO) elements can also fulfill these functions (see description of AODs and AOBs below) and some confocal systems have now replaced all filter components by AO devices and dispersive elements (e.g., Leica AOBs SP2 and RS).

Selecting the Wavelength of the Illumination and the Detected Light

While filament-based lamps emit a continuous spectrum of light that is very similar to black-body radiation, high-pressure, plasma-based light sources emit a large number of distinct lines on top of a black-body radiation background (see Chapter 6, *this volume*). Lasers come in various types and may emit one or a number of lines, each with an extremely narrow bandwidth (see Chapter 5, *this volume*). Finally, femtosecond pulsed lasers emit bands that can be up to several tens of nanometers wide.

The spectral problems posed by epi-fluorescence microscopy can be understood by looking at Figure 3.2, which shows the important variables schematically. The excitation spectrum of a fluorophore (blue) is skewed so that the peak is towards its long- λ side while the emission spectrum (green) is peaked near its short- λ side. The difference in nanometers between the peaks of these spectra is referred to as the Stokes shift. A large Stokes shift has the advantage that it is relatively easy to separate the excitation light from the much fainter signal light, but it also “uses up” more of the spectrum, making it more difficult to excite and record signal from two or more dyes at the same time.

The object is to excite the dye as efficiently as possible while collecting as much of the emitted light as possible without transmitting any excitation light into the detection channel, a task complicated by the fact that the excitation light is usually about 10^5 to 10^6 times brighter than the fluorescence emission.

Ideally, one should illuminate with a narrow wavelength band that matches the peak excitation wavelength of a fluorophore selected. This requires both that the excitation filter pass only this

band and that the dichroic beam-splitter reflect it. Although this can work well if one has a laser line that coincides with the peak of the excitation spectrum, more commonly, the excitation is from a Hg or Xe arc. As such sources are far less bright than lasers, in terms of being able to concentrate a lot of photons onto a single point in the specimen (see also Chapter 6, *this volume*), one normally chooses to excite the dye over a band of wavelengths that matches the “half-power” points of the excitation spectrum. Although this allows more photons to strike the dye, it also means that the window of wavelengths available for detecting the fluorescent light must now start at a slightly longer wavelength if it is to exclude all the excitation light.

On the other hand, no matter what the bandwidth of the excitation, fluorophores emit light over a spectral band and the wider the detection bandwidth, the more signal will be recorded.¹ An emission filter (sometimes called a barrier or rejection filter), is necessary to block any excitation light from reaching the detector. This filter is at least a long-pass filter, but may be a bandpass filter if this is needed to block undesired autofluorescence or when the emission from two or more fluorophores must be discriminated.

Generally speaking, each fluorophore requires different illumination and detection filters, and yet other filter packages when two or more fluorophores are present in the same specimen. Although total signal is maximized when large filter bandwidths are used, spectral discrimination is enhanced if the filters are chosen to provide a good separation of the bands. In addition, narrowing the illumination spectrum to match the excitation peak will increase the excitation efficiency while reducing the bleaching and excitation of non-specific fluorescence, by avoiding wavelengths that excite the fluorophore less efficiently (Kramer, 1999).

Separating the Light Paths

In fluorescence microscopes, part of the light path is used by both the excitation and the fluorescent light and these two components must be separated, based on their spectral properties and their

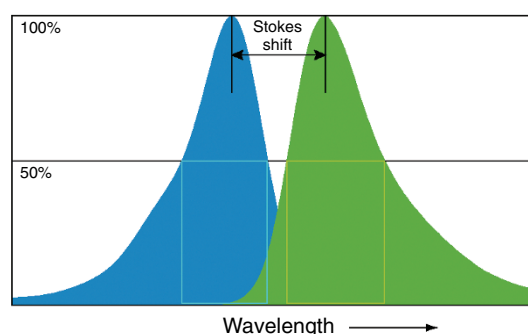


FIGURE 3.2. Fluorescence parameters. To a first approximation, the excitation and emission spectra are mirror images of each other. The wavelength difference between their peaks is called the Stokes shift and varies with the dye and its environment. The bandwidth (light blue and light green rectangles) is a measure of the width of each spectrum, defined as the wavelength difference between the half-power points of each spectrum.

¹ The fact that all common photodetectors cease to respond once the energy of each photon drops below a fixed level, places a practical limit on the long- λ end of the detection bandwidth.

direction of propagation. This separation is produced either by dichroic mirrors, which transmit one part of the spectrum and reflect the other part when placed at 45° in the light path, or by acousto-optical beam-splitters (see AOBS below). For single fluorophores, a long-pass dichroic that reflects the short- λ illumination light and transmits the fluorescent light is generally chosen. Note that, in a two-photon microscope, the fluorescent light has a shorter λ than the excitation light. Therefore, if a single-photon setup is also used for two-photon excitation, the long-pass dichroic must be replaced by a short-pass dichroic.

Common fluorophores usually have a Stokes shift of 20 to 100 nm, that is, the peak of the emission spectrum occurs at a λ that is 20 to 100 nm longer than that of the absorption peak (e.g., *Molecular Probes*, 2002, pp. 903–905; Chapter 16, *this volume*). Therefore, in order to transmit most of the short- λ part of the fluorescence to the detector, dichroic mirrors must have a very sharp edge between their reflection and transmission bands. While a loss of illumination intensity due to reduced reflection by the dichroic mirror can often be compensated for by increasing the intensity of the light source, fluorescence mistakenly reflected towards the source rather than proceeding to the detector represents a loss that cannot be remedied without subjecting the specimen to more light.

Modern confocal microscopes may have several detectors to allow the simultaneous detection of signals in different wavebands.

Conventional Filters

Conventional filters generally consist of colored glass, metallic films, or polymers that absorb unwanted wavelengths. Colored glass filters are relatively inexpensive and easy to handle, but have some disadvantages (Reichman, 2000, p. 11): the cut-off between the transmission and absorptions bands is rather gradual and the peak transmittance is usually low. Additionally, because they absorb light, they may overheat and crack if subjected to high light intensities (i.e., in the illumination path). In addition, many of the glasses involved are autofluorescent. In recent years, advances in the manufacture of interference filters have made them the λ -selective components of choice for all filter-based microscopes.

Interference Filters

It is not an overstatement to say that the development of epifluorescence microscopy owed more to the perfection of interference filters, and more particularly of dichroic beam-splitters, than to that of any other optical component.

When light passes from a smooth medium having a refractive index (RI) of n_1 to a second medium with an RI = n_2 , an amount of the light proportional to $(n_1 - n_2)^2$, is reflected [Fig. 3.3(A)]. When light strikes a thin layer of high-RI material covering a substrate of low-RI, optical-grade glass or fused silica/quartz, some light reflects back from each interface. If the thickness of the layer is such that the light reflected by the top surface is out-of-phase with the light reflected from the next surface down (i.e., the layer is $\lambda/4$ (or $3\lambda/4$, $5\lambda/4$. . .) thick,² these two beams will be out of

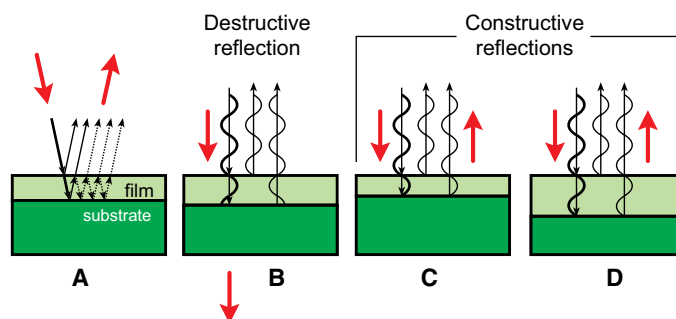


FIGURE 3.3. Interference filtering. (A) When light strikes a transparent surface covered by a thin transparent film having a different refractive index, some light is reflected by each interface. (B) If the thickness of the film is such that these two wave-trains are out-of-phase as they leave the surface, they will tend to cancel out, reducing the overall reflectivity (and increasing transmission). (C, D) On the other hand, for light with a wavelength such that the waves leaving the surface are in phase, the total reflectivity will be increased. (These diagrams modified from originals kindly provided by Turan Erdogan, Semrock, Rochester, NY.)

phase and will tend to cancel each other out, with the result that less total light will be reflected [Fig. 3.3(B)]. Alternatively, if either λ or the thickness of the layer is changed so that the two reflected waves are now in phase (i.e., the layer is $\lambda/2$ thick), then the total reflectivity at this wavelength is increased, although still quite low [about 10% from only two surfaces, Fig. 3.3(C,D)]. The total reflectivity can be increased almost arbitrarily by adding more layers of alternating high- and low-RI materials (Fig. 3.4).

Traditionally, the materials used for the high-index layers were zinc sulfide (RI = 2.35), zinc selenide (RI = 2.67), and sodium aluminum fluoride, or cryolite, was used for the low-index material. Because these materials are both soft and hygroscopic, they must be protected, usually by sticking the coated sides of two glass substrates together with epoxy [Fig. 3.5(A)]. This has the disadvantage of making it very difficult to make all the flat surfaces parallel with the result that most such filters suffer from “wedge-error” and light passing through them is deflected at a slight angle.

More recently, these coating materials have been replaced by semi-transparent layers of metal oxides. Although more complex and time-consuming to deposit,³ these so-called **hard coatings**, are unaffected by water, temperature changes, or normal, careful handling, and because they deposit as denser, more uniform layers, the interfaces are more uniform and hence scatter less light. As a result, more light is transmitted. Best of all, because they are deposited on a single piece of glass [Fig. 3.5(B)], both the wedge error and wavefront error (“flatness”) can be reduced substantially (respectively to <0.1 minute of arc and $<\lambda/\text{inch}$ of surface), and this reduces pixel shift when the filters (or dichroics) must be changed for sequentially recording several dyes in a single specimen.

² One must remember that, when measuring film thickness in terms of wavelength, we are referring to optical thickness (i.e., geometric thickness $\times$ RI) but that, as RI changes with wavelength, some subtlety is needed in determining the correct actual thickness. In practice this is accomplished by actually monitoring the transmission of the filter as each layer is deposited.

³ The process involves ion-assisted deposition, a recent modification of evaporation methods that utilizes either direct thermal or electron beam to vaporize source materials and an energetic ion source that ionizes the evaporated materials and then bombards the substrate with them. This process increases the packing density in the film layers, which increases the RI and improves the mechanical characteristics of the coat. Fewer voids reduces water absorption, a common cause of mechanical failure and unstable optical properties. Depending on the films being deposited, various ion species may be employed, including oxygen or inert gases such as argon.

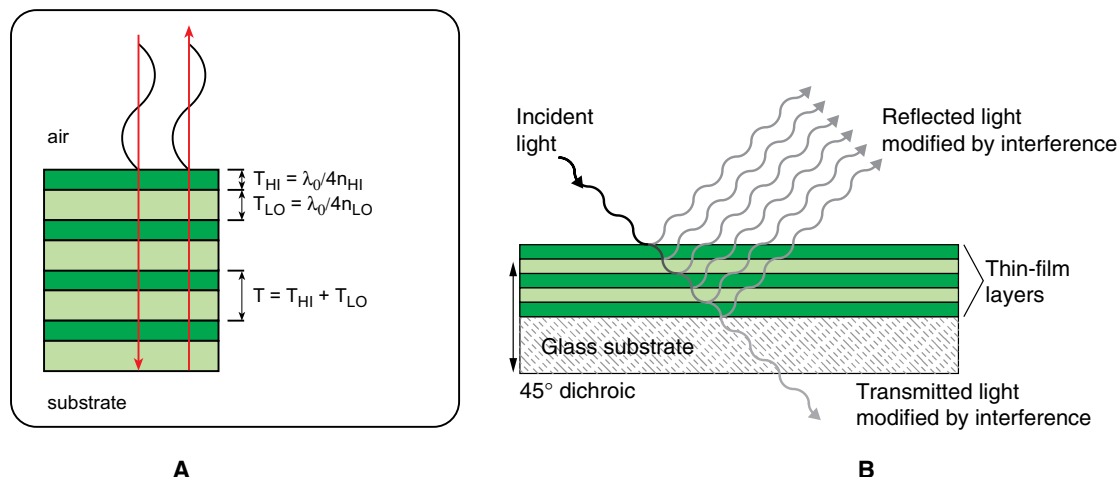


FIGURE 3.4. Interference mirrors. (A) Light impinging on a stack of transparent layers of alternating high and low refractive index, will be highly reflected if each layer is $\lambda/4$ of a wavelength in thickness. (B) This also applies to surfaces struck at an angle of 45° , although in this case, shorter wavelengths will be reflected for a given layer thickness.

Traditional dielectric coatings are often stacked into units called **cavities**, which are constructed of groups of alternating layers separated by a wider layer of one of the materials called a **spacer** (see Fig. 3.6). The spacers are produced with a thickness that corresponds to even multiples of a half wavelength in order to reflect or transmit light in registration with the dielectric layers. Increasing the number of cavities utilized to build an interference filter produces a proportional increase in the slope of the cut-on and cut-off wavelength transmission boundaries. Conventional interference filters featuring up to 15 stacked cavities can have a total of over 75 individual dielectric layers and provide a bandwidth only a few nanometers wide. However, because the surfaces of soft, evaporated films are not perfectly smooth, they produce incoherent scattering that reduces peak transmission substantially when many layers are used. Because the surfaces formed by the hard coating materials are smoother [Fig. 3.6(C)] and can be deposited more precisely, it is possible to deposit more than 200 of them without reducing peak transmission substantially and while restricting cumulative thickness errors so that the order of the array is maintained.

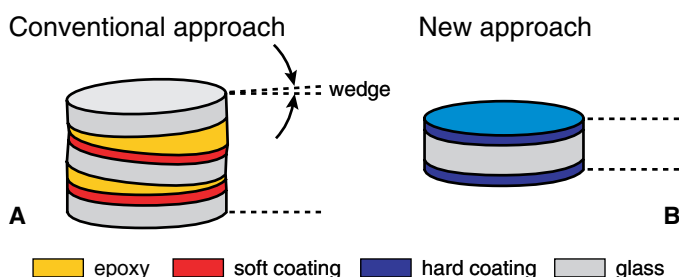


FIGURE 3.5. Fabrication methods. (A) As the materials conventionally used for the thin films that make up interference filters are both soft and hygroscopic, they must be protected from the environment by placing them on the inside of a filter assembly composed of at least two pieces of glass held together with epoxy. (B) As the coatings used on more modern filters are hard enough to be applied to the outside of a single glass blank, the assembly can both be thinner and have surfaces that are more parallel. (These diagrams modified from originals kindly provided by Turan Erdogan, Semrock, Rochester, NY.)

Types of Interference Filters

Virtually any type of filter can be designed and constructed with thin-film interference coating technology, including bandpass, short-pass, long-pass, dichroic beam-splitters, neutral density, and a variety of mirrors, including those used to confine laser cavities (Fig. 3.1). We will start by considering a **long-pass cut-off beam-splitter** (Fig. 3.7)⁴ that reflects all light $<\lambda_0$. Clearly, this could be made by depositing onto the substrate a stack of cavities, each with $\lambda/2$ -thick layers tuned to different wavebands longer than λ_0 . Those cavities reflecting light near to λ_0 must have steeper cut-offs (i.e., more layers) than those centered farther away but the result is that **only wavelengths shorter than λ_0 will be reflected**. It is important to remember that, as any light that is not transmitted by an interference filter is reflected rather than absorbed or scattered, provision must be made to absorb it lest it become a source of stray light.

A **short-pass filter** is made using the reverse logic (**reflect λ longer than λ_0**), while a bandpass filter could be made by essentially coupling a short-pass and a long-pass filter. Alternatively, one can deposit $\lambda/4$ layers for the wavelengths that one does want to transmit with the assumption that other wavelengths will be reflected. As the latter approach involves thinner layers that take less time to deposit, it is therefore more commonly used. Figure 3.8 shows the performance of both “hard” and “soft” bandpass filters as might be used as the excitation and emission filters for a fluorescein cube. When the “hard” curves are combined with the filter shown in Figure 3.7 (now used as a beam-splitter), the performance of the resulting set can be seen in Figure 3.9.

Although in fact most filters are made using film-thickness patterns considerably more complex than those just described, state-of-the-art methods now allow one to fabricate a component as complex as the triple-dichroic beam-splitter designed to reflect 364, 488, and 633 nm light so accurately that the measured performance closely approximates the theory (Fig. 3.10). One can see

⁴ Because they have been a leader in introducing this new technology to microscopy, this chapter uses many illustrations provided by Semrock, Inc. (Rochester, NY). However, it seems likely that, by the time you read this text, other filter companies will also be offering similar products fabricated using the new hard-coating techniques.

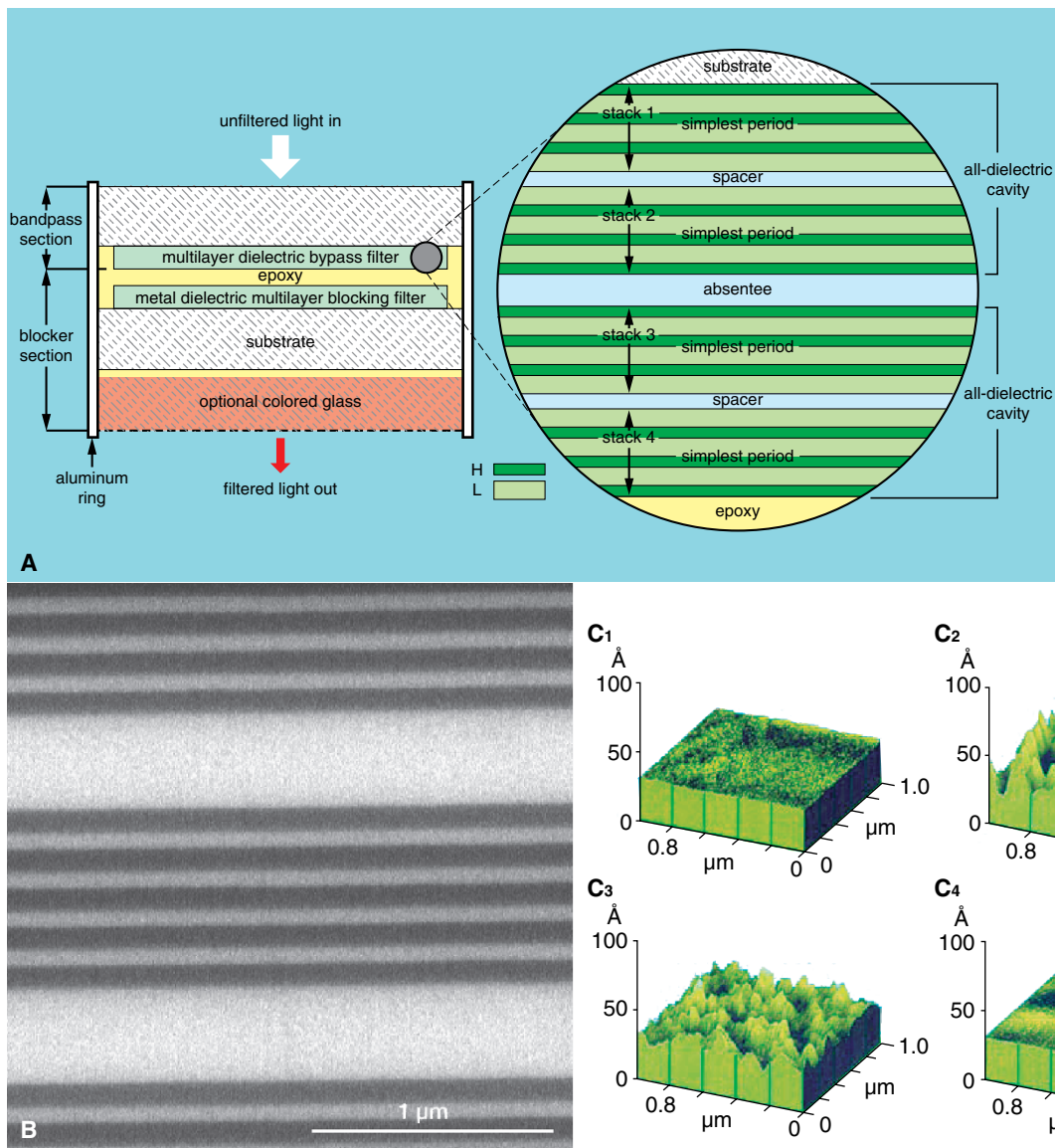


FIGURE 3.6. Layers, cavities, filters and surfaces. (A) Most interference filters use tens or even hundreds of layers. These layers are often grouped in sets of 5, with a thicker spacer layer between them. Larger sets are referred to as cavities. (B) Scanning electron microscope image of the edge-view of a part of a stack of layers. The lighter layers are thinner and have a higher refractive index. (C) Atomic force micrographs of the surface of a variety of optical surfaces: (C1) “super-polished” substrate with a surface quality of $0.5 \text{ \AA}$ rms; (C2) same substrate with a 25-layer, mirror coating deposited by electron-beam (EB) evaporation, $10 \text{ \AA}$ surface quality; (C3) same substrate with a 25-layer, mirror coating deposited by continuous, plasma-enhanced or ion-assisted EB coating, $4 \text{ \AA}$ surface quality; (C4) same substrate with a 50-layer, ion-beam-sputtered coating, $0.5 \text{ \AA}$ surface quality. The improvement in surface roughness is clear and this results in lower inter-layer and overall scattering. (The diagram modified from an original kindly provided by Melles Griot, Rochester, NY. The micrograph is from Turan Erdogan, Semrock, Rochester, NY.)

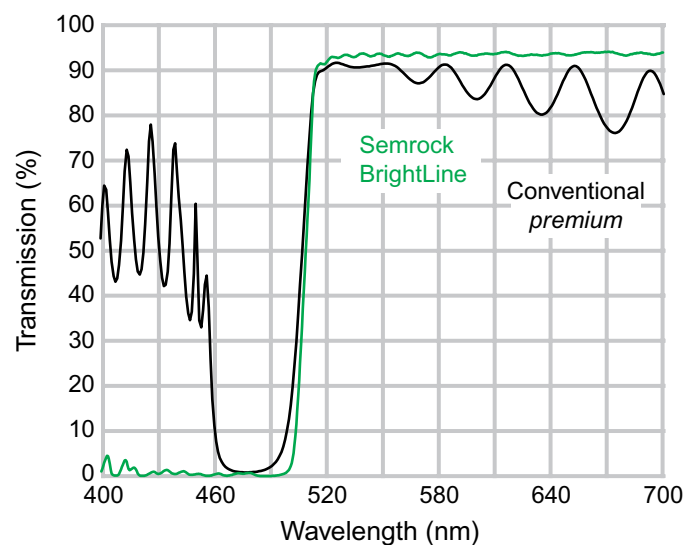


FIGURE 3.7. Performance of a 45° beam-splitter. The black line represents the transmission characteristics of a traditional, high-quality dichroic beam-splitter. The green line represents the performance of a newer, hard-coated filter. The superior optical properties of the hard coatings allow one to use more layers to produce steeper cut-offs while still maintaining very high peak transmission. (This diagram modified from an original kindly provided by Turan Erdogan, Semrock, Rochester, NY.)

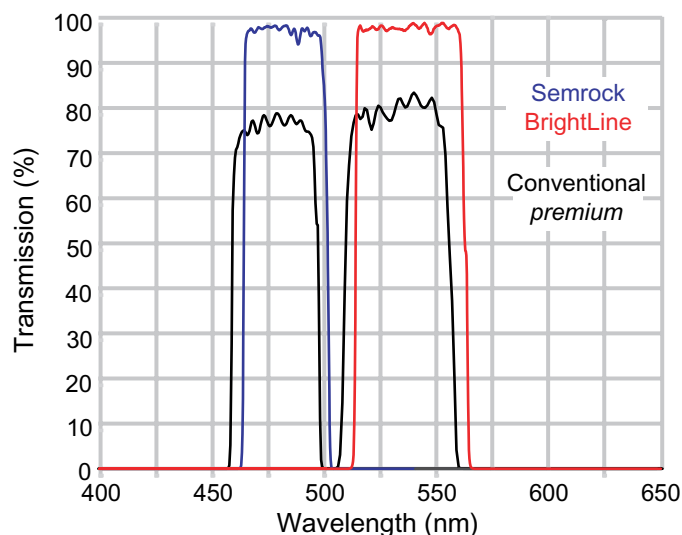


FIGURE 3.8. Excitation and emission bandpass filters. The difference in performance between the older “soft” coatings and the newer “hard” ones is evident. (This diagram modified from an original kindly provided by Turan Erdogan, Semrock, Rochester, NY.)

the close agreement between designed and measured performance that is possible when using the new hard coatings.

Although the average biological microscopist is unlikely to try to manufacture an interference filter, one factor worth remembering is that “reflections add.” In other words, cavities shape the spectral properties of the incident light by subtraction: by reflecting unwanted wavelengths. Once these wavelengths have been removed from the beam, they cannot be “restored” merely by depositing a subsequent layer that would have transmitted them. In practice, this means that it is easier to make a filter that transmits only one specific wavelength.

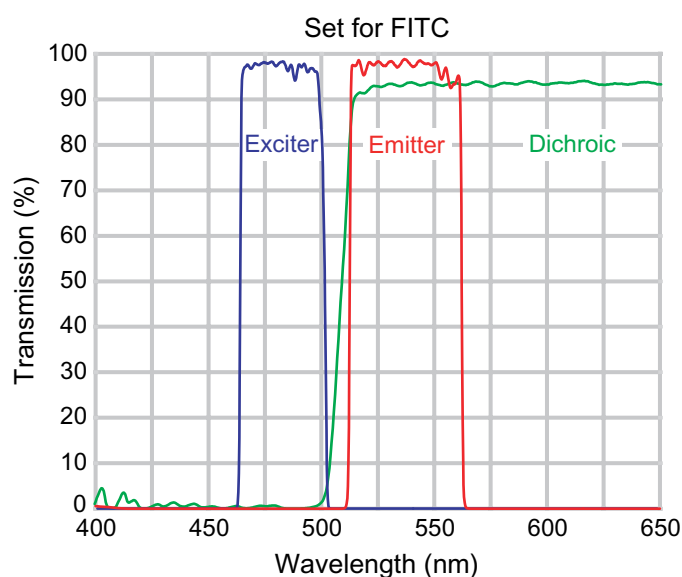


FIGURE 3.9. Filter cube for FITC. Combined performance of the filters diagramed in Figures 3.7 and 3.8. (This diagram modified from an original kindly provided by Turan Erdogan, Semrock, Rochester, NY.)

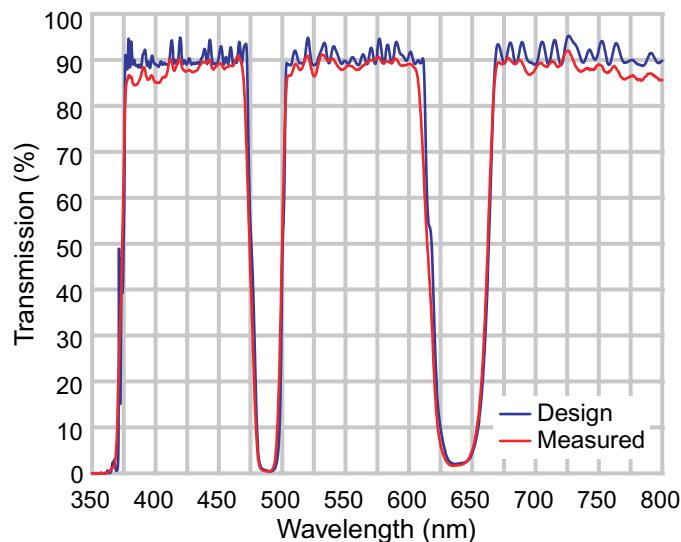


FIGURE 3.10. Theory and practice in filter design. The hard optical coating performance is so predictable that theoretical and actual performance can be very similar, even on a component as complex as a triple-bandpass dichroic. (This diagram modified from an original kindly provided by Turan Erdogan, Semrock, Rochester, NY.)

The number of layers and cavities deposited is the variable used to control the nominal wavelength, bandwidth, and blocking level of the filter with very high precision. Figure 3.11 shows the performance of a pair of “hard” filters made for use in Raman spectroscopy. In this technique, photons scattered by individual molecules in a transparent specimen (or even in the optics!), are found to have lost very small amounts of energy, and the amount of this loss is related to the electronic structure of the scattering material. As a result, the scattered light contains spectral data that allows one to identify the scattering molecules. Because Raman scattering is produced by every element in the optical system and

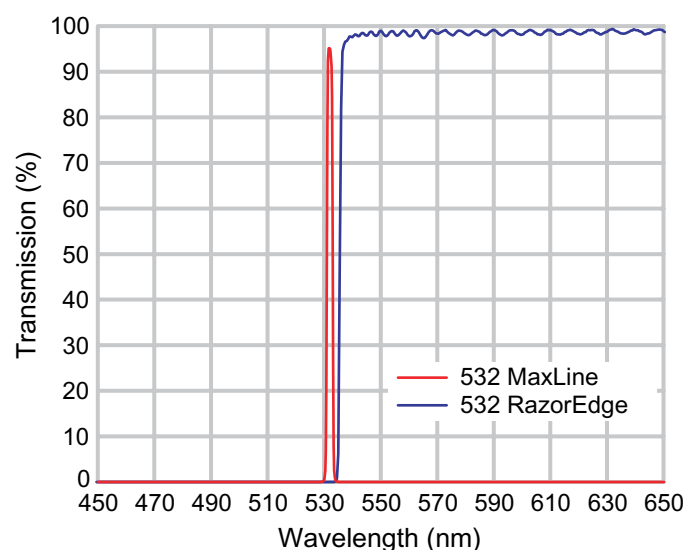


FIGURE 3.11. Laser-line mirrors. Modern hard-coating mirrors can be made with enough layers to produce very narrow reflection bands (red line), and long-pass filters with extremely steep cut-offs (blue line). (This diagram modified from an original kindly provided by Semrock, Rochester, NY.)

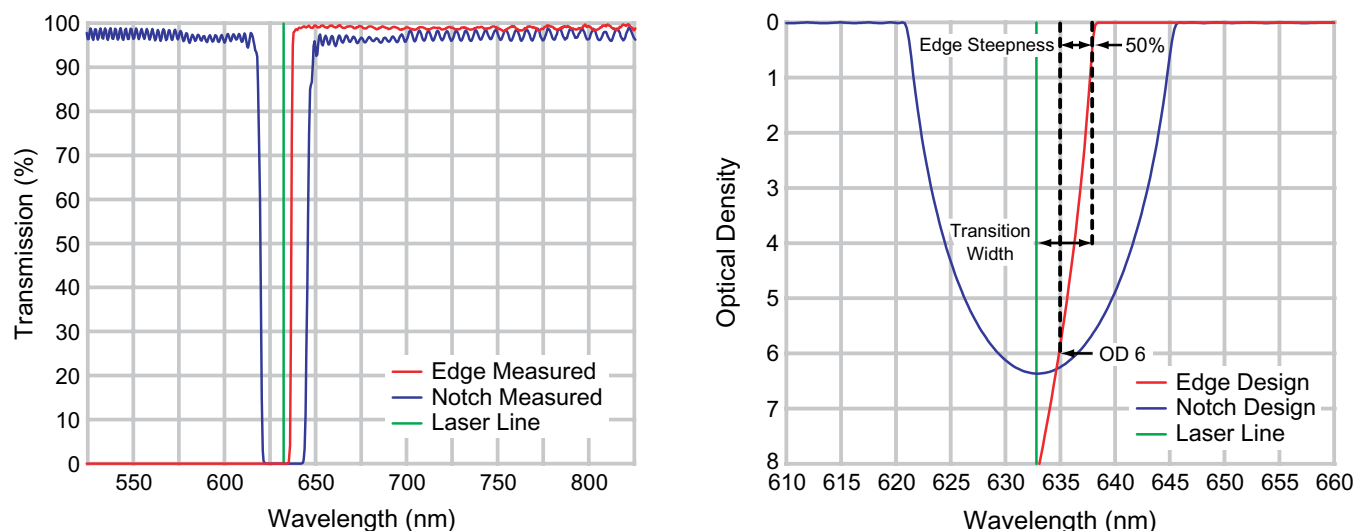


FIGURE 3.12. Linear versus logarithmic plots of transmission. Although transmission graphs (left) are easy to understand, they don't give the whole story because one can only read them to about 1% transmission. As the emitted light is about one million times dimmer than the excitation, great effort is required to produce dichroic and barrier filters with sufficient "blocking" to prevent any excitation light from reaching the detector. As a result, one can only usefully estimate filter suitability if one views a transmission plot displayed in logarithmic, "optical density units" (OD, right). Here one can see that a laser-line "notch" filter with a transmission bandwidth of ± 12 nm has a transmission ratio of $>10^6$, and that the cut-off of the long-pass barrier is similarly steep. (These diagrams modified from originals kindly provided by Turan Erdogan, Semrock, Rochester, NY.)

because the specimen itself produces very little Raman-scattered light, filters such as those shown in Figure 3.11 are needed to ensure that the light striking the specimen is uncontaminated with Raman wavelengths picked up "in-transit" (red line), using a "laser-line" filter composed of a very large number of $\lambda/4$ layers that effectively transmits only the laser light. A steep-cut-off, long-pass filter is used so that only light with $\lambda > \lambda_{\text{laser}}$ can reach the spectrometer (blue line).

If the optical configuration requires that the filter pass the laser line rather than block it, a notch filter is used. Figure 3.12 shows the performance of this combination in two different ways: (a) as a linear plot (as is the case in all the other figures so far) and (b) with the vertical scale in optical density (OD) units. As OD units are logarithmic, the figure shows that the blocking level of this filter at its center wavelength is $>10^6\times$. Such a filter (or at least one centered at a longer wavelength!) could be used to block near-IR from a pulsed laser from reaching a transmitted light detector set up to measure the second- or third-harmonic signal (see Chapter 40, *this volume*). More generally, because the fluorescence signal is commonly $10^6\times$ less intense than the excitation, it is often even more important that emission filters have good blocking than that they have a sharp cut-off, because without this feature, a lot of stray, reflected excitation light may reach the detector, effectively reducing image contrast.

Because light striking a thin film at an angle will need to have a slightly shorter wavelength in order for the next interface to be encountered in exactly $\lambda/2$ or $\lambda/4$, the spectral properties of interference filters and beam-splitters vary with incidence angle. Figure 3.13(A) shows both how $\lambda_{\text{cut-off}}$ of a short-pass filter changes by about 10% as the incidence angle increases to 45° and also how this effect depends on the polarization direction of the incident light. At angles greater than normal incidence, light waves undulating parallel to the plane containing the incident and reflected rays (*p*-polarization) exhibit a different transmission profile from waves undulating perpendicular to the plane of incidence and

reflection (*s*-polarization). Except when performing anisotropy measurements, it is usually desirable to reduce the polarization-dependent behavior of filters in the detection path because light emanating from the sample is typically less polarized. However, in the excitation light path this pol-dependence can be exploited to achieve a higher reflectance for polarized excitation laser light.

Figure 3.13(A) makes clear how the intensity of the beam striking the specimen in a single-beam, confocal microscope can vary substantially if the light emerging from the (supposedly) polarization-preserving fiber connecting the scan head to the laser skips between modes. Changing the pol-direction of the fiber output changes the amount of this light that is reflected by the 45° dichroic⁵ and proceeds to the objective (see Chapter 26, *this volume*).

Figure 3.13(B) shows how this angle dependence can be utilized intentionally to tune the bandpass of a narrow, notch filter, or other high cut-off component, by tilting it up to 14° from normal incidence.

It is widely understood that the prime advantage of using infinity-conjugate objectives is that, in the space between the objective and the tube lens, light rays originating at the front focus plane will be parallel to the optical axis and therefore, that such rays will strike any filter or dichroic introduced into this region at a fixed angle. However, as light rays originating from above or below the plane-of-focus will not be parallel to the optical axis in this space, the effective cut-off wavelength of these components may shift slightly when applied to such light. Although such light is excluded by the pinhole in a confocal microscope, it can reach the detector in the widefield microscope.

⁵ A dichroic beam-splitter is very similar to an interference filter except that the reflective, interference layers are deposited only on a single outside surface and the substrate is often thinner. The "back" side of the substrate is often coated with a broadband, anti-reflection coating.

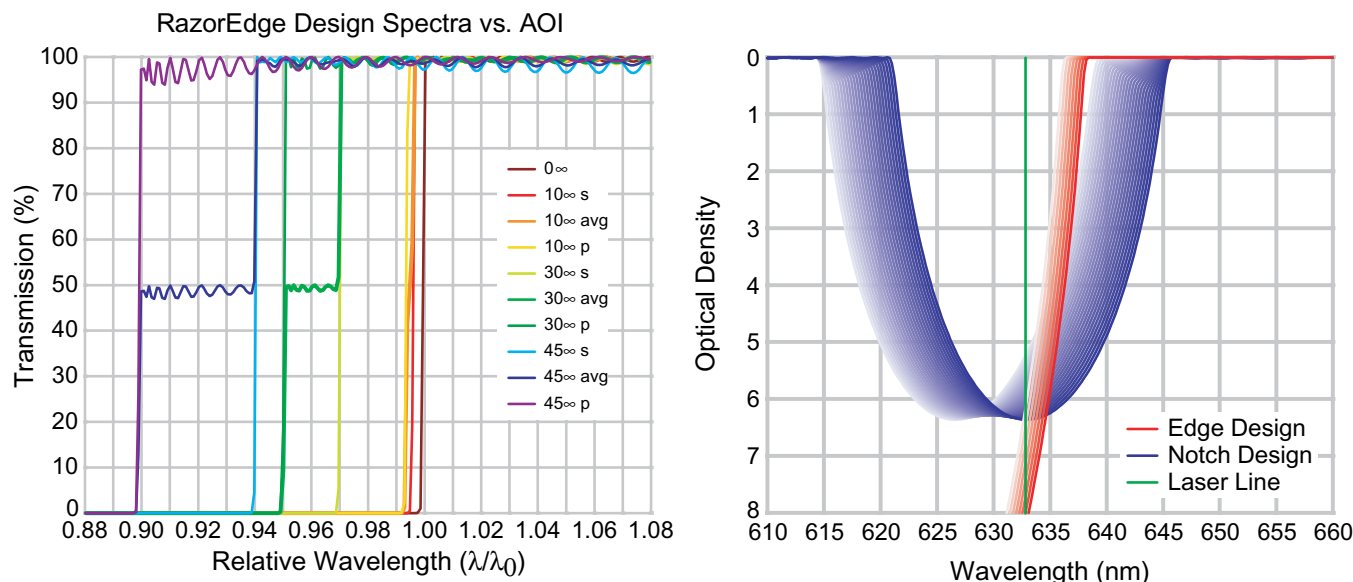


FIGURE 3.13. Reflectivity and incidence angle. As the angle at which the incident light strikes the filter becomes less than 90° , the cut-off wavelength becomes shorter (left). The change in wavelength is about 10% at 45° although the exact amount depends strongly on the polarization of the incident light (6% vs. 10%). This angular dependence can be used to ‘tune’ the cut-off wavelengths of notch and edge filters (right). (These diagrams modified from originals kindly provided by Turan Erdogan, Semrock, Rochester, NY.)

Dichroic and Polarizing Beam-Splitters

Dichroic mirrors or beam-splitters are special interference filters that reflect light in defined bands while transmitting light in other bands when placed at an angle in a light path (in distinction to dichroic filters, dichroic mirrors are placed at an angle to the incident light and their reflective properties are just as important as their transmission). To insure that the image made with one dichroic is aligned with that made with others, the glass blanks (substrates) used to produce them must be polished to very fine tolerances for thickness and parallelism (see above). This is necessary because, in epi-fluorescence microscopy, the imaging rays pass through a beam-splitter oriented at 45° , and the position of the transmitted light bundle is displaced by an amount proportional to the thickness and RI of the substrate on which the interference layers are deposited. If the thickness is reduced much below 3 mm, the flatness of the substrate may be compromised and, of course, the refractive index (RI) of glass is a function of the wavelength: for BK7 glass RI = 1.507 @ 1060 nm but RI = 1.536 @ 365 nm. This difference is so large that the variation in offset will be very noticeable in any design that covers a range from the ultraviolet to the near-infrared. Instead of ignoring these effects, it is probably wise to work with them and for example, to use a thicker piece of glass instead of a thin glass plate and create shifts that are so large that secondary or ghost images are easily removable with a regular aperture.⁶

To circumvent the spectral problems of interference-based beam-splitters, it is possible to use a polarizing beam-splitter that reflects half of the light polarized in one direction and transmits all the light polarized in the other direction (Cox, 2002). About

80% of the fluorescent light is transmitted for all visible wavelengths. There is no spectral distortion because the transmittance is independent of the wavelength, and the beam-splitter does not need to be changed, simplifying alignment.

The beam-splitter in the LSM 5-Live line-scanning confocal microscope (Carl Zeiss, Jena, DE) uses a linear mirror in the center of a glass blank located in an optical plane corresponding to the back-focal plane and placed to only reflect a narrow “line” of laser illumination (Fig. 3.14). As only a small fraction of the blank is coated as a mirror, more than 95% of the entire bundle of fluores-

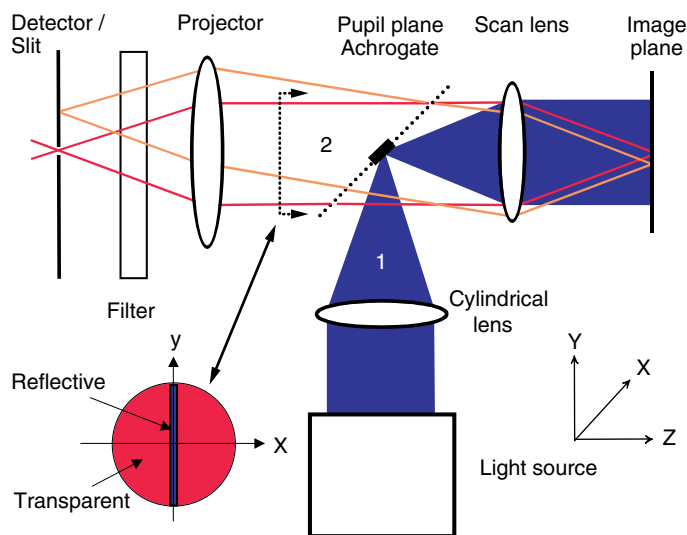


FIGURE 3.14. Operation of the “Acrogate.” The beam-splitter in the Zeiss 5-Live takes advantage of the fact that, in a line-scanner, only a line of illumination exists in the back-focal plane. Consequently, a piece of glass with a high-reflectivity linear mirror across the center will reflect all of a line of coherent laser light down to the specimen but obstruct only about 5% of the returning fluorescent light, and this performance is not affected by wavelength.

⁶ Indeed, a very useful achromatic beam-splitter can be made by using such a piece of glass with no coating on the rear surface. Such a surface will reflect about 5% of all light impinging at 45° . Although, this “wastes” laser power, there is usually more than one needs of this in any case. Care must be taken that any excitation light not reflected is absorbed before it can become stray light.

cent light returning from the objective can be transmitted (see also Chapter 10, *this volume*).

Filters and Dispersive Elements for Multi-Channel Detection

As light emitted by fluorophores in the sample is mainly unpolarized, polarization-sensitive beam-splitters can only separate half of it from the polarized excitation light. Instead, the emitted light is either sequentially split by a combination of short-pass and long-pass dichroic mirrors before being passed through a bandpass or long-pass emission filter to the detector (see Fig. 3.9), or it strikes a dispersive element (e.g., a grating or a prism). Light split by a dispersive element can either be projected directly onto a fixed array of mini-photomultiplier tubes (PMT) from which a relatively continuous spectrum is recorded (Fig. 3.15, as in the Zeiss META), or the light passes through a system of slits and/or mirrors that can be adjusted to pre-select the segments of the spectrum directed to each detector (Fig. 3.16, as in the Leica TCS SP II). The former system has the advantage of more channels (32 vs. 4 detectors, 8 vs. 4 channels digitized simultaneously) but the disadvantages that the width of each spectral channel is quantized by the spacing of the PMTs, that some signal is lost in the dead zone between photocathodes and because mini-PMTs have lower quantum efficiency and more multiplicative noise than the more conventional PMT. In both systems, optimal spectral resolution requires that the light passed by the pinhole originate from a very small area of the specimen (i.e., it must be collimated light: opening the pinhole reduces spectral resolution), a factor that is not important for systems that use interference filters to separate the light. More details about

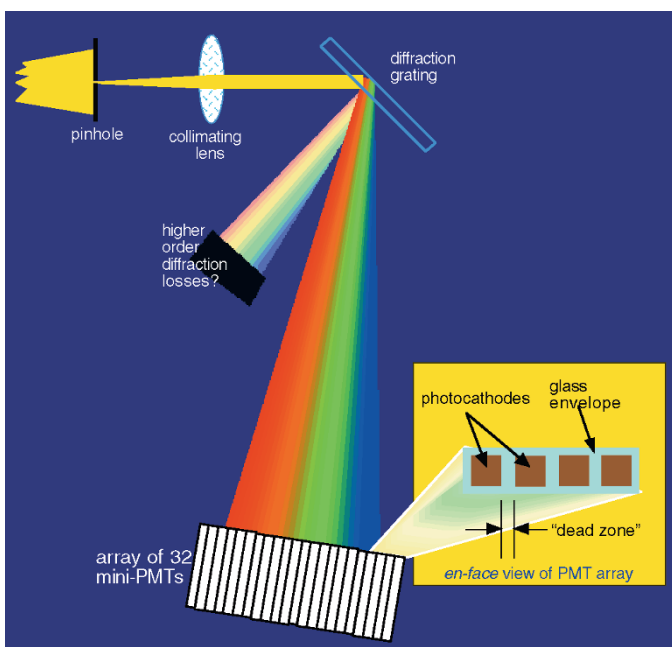


FIGURE 3.15. Schematic diagram of spectral detector in a Zeiss META confocal head. Light passing through the pinhole is made parallel by a collimating lens and diffracted by a diffraction grating. Light of different colors strikes different segments of a 32-channel mini-PMT array. Light that strikes the active area of one of the photocathodes produces photoelectrons that are amplified by the adjoining electron-multiplier section. Up to eight signals can be digitized from eight sets of adjacent PMT channels. Metal pins are placed in front of the PMT array to prevent light at laser wavelength from reaching the detector.

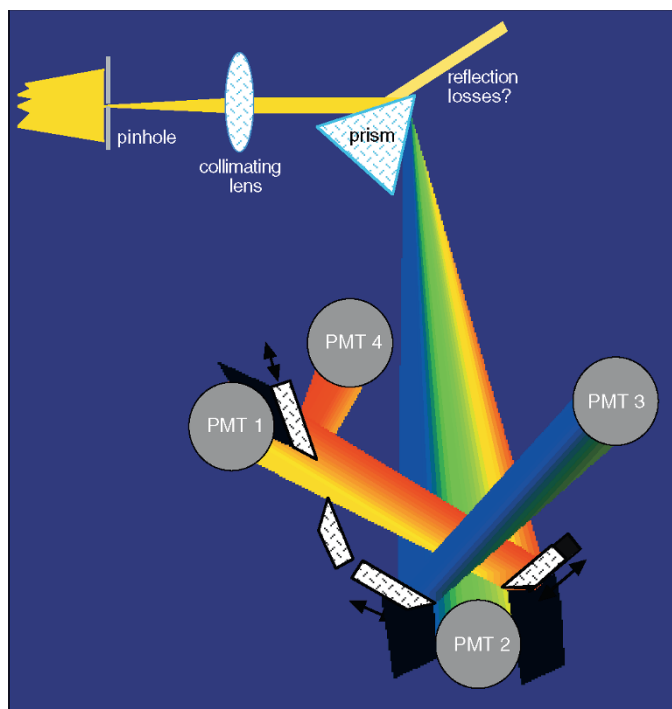


FIGURE 3.16. Schematic diagram of the spectral detector in the Leica TCS SP II. Light passing through the pinhole is made parallel by a collimating lens and refracted by a prism. Light of different colors is reflected to one of four photomultiplier tubes (PMTs) by a series of movable mirrors that can also be arranged to form shutters or slits. Up to four signals can be digitized from the four PMTs.

these detectors and tests that can be applied to validate their performance can be found in Chapter 36 (*this volume*; Lerner and Zucker, 2004).

MECHANICAL SCANNERS

In most of the commercial confocal microscopes used in biology, the beam scans, not the specimen. The laser light is scanned over the image plane by changing the angle at which the laser beam passes through the back-focal plane (BFP) of the objective. If the rotational axis of a scan mirror coincides with both its own surface and the optical axis at a telecentric plane (i.e., any plane that is conjugate to the back-focal plane of the microscope objective lens), rotation of the mirror moves the focused spot across the focus plane in a straight line (Fig. 3.17). The devices used to tilt the mirror in a rapid and precise manner are called galvanometers.

While the first generation of scanning microscopes could only scan the beam over rectangular areas of the specimen using lines running in a fixed orientation, modern instruments are equipped with scanners that allow one to rotate the scanned rectangle or even scan arbitrarily shaped areas. The scan angle can be rotated by apportioning some of the x -scan signal to the y -galvo and vice versa. Scanning an arbitrary area is particularly useful for experiments involving FRAP or the release of caged compounds, where the high-intensity illumination area can be precisely matched to a region of interest defined using biological criteria applied to the recorded image.

Flexible scanning, integrated to a system to shift excitation and emission wavelengths rapidly, allows a much greater flexibility

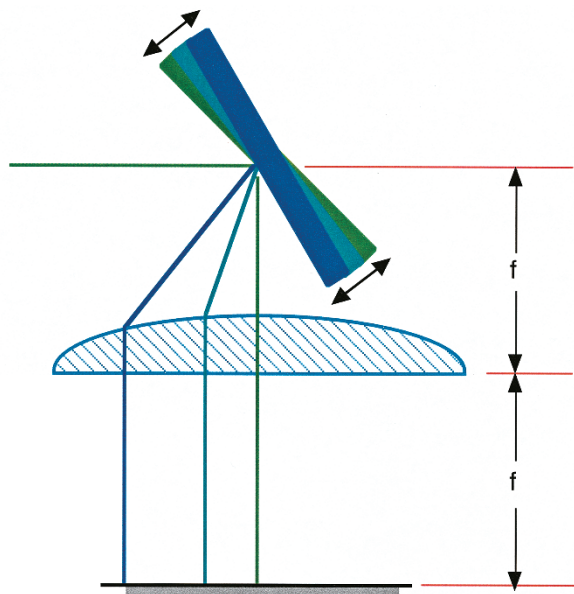


FIGURE 3.17. How rotation of a mirror in an aperture plane causes the linear motion of the focused spot. Rotation of the mirror changes the angle at which light leaves the focal point. The green line at 45° represents the neutral mirror position. Light blue and dark blue lines show the effect of rotating the mirror clockwise by one half of the total deflection angle. Because it is not possible to mount the galvanometer at the actual back-focal plane of the objective, a scan lens is used to image this plane to a more convenient location. Because this lens has some magnification (M), the angles actually scanned by the galvanometer mirror are less than those implied by this diagram by a factor of $1/M$. f , focal length.

regarding the sequential acquisition of signals from different labels (line-by-line or frame-by-frame). This section describes the different scanner types. More detailed information can be found on the Web pages of Cambridge Technology (Cambridge, MA) and GSI-Lumonics (formerly, General Scanning, Bellerica, ME).

Galvanometer Scanners

Beam scanning is generally implemented using mirrors mounted on galvanometer “motors” (see Chapter 9, *this volume*). The mirror surface is deposited on a substrate of silicon, fused quartz, or, for highest performance and cost, beryllium. Such a rotating mirror device can control the scan angle with exquisite accuracy and has the additional advantage that the scanning angle is insensitive to wavelength, something not true of the faster acousto-optical scanners.

Like a motor, the actuator in a galvanometer has a rotor and a stator and currents circulating in coils in one or the other interact with the magnetic field of a permanent magnet in the other to produce the forces that move the rotor (Fig. 3.18). The responsiveness of the actuator depends on the torque/inertia ratio. In general, moving-coil actuators have a lower moment of inertia (I), lower torque, and better positioning accuracy than moving-magnet actuators, while the latter can move faster because of their superior stiffness. Consequently, they are the type most often used in confocal microscopes.

Although some cheap scanners achieve high scan speed by rotating continuously (rotating multi-mirror scanners), the angular scanning range of such scanners is fixed and consequently, the scanned area cannot be “zoomed.” In addition, the scan motion is not linear and it is difficult to fabricate and mount a multi-segment

mirror in which all the facets are an equal distance from the axis. To avoid these limitations, the scanners used in confocal microscopes oscillate around a fixed neutral position. Such galvanometers come in two flavors: resonant scanners that vibrate at a fixed frequency and in which much of the force needed to oscillate the mirror is provided by energy stored in a torsion spring, and linear galvanometers that operate at lower but variable frequencies and in which the spring is much weaker or absent.⁷ In both cases, the required scan forces increase with the square of both the scan frequency and the scan angle and the limit on both is usually set by the amount of power that can be dissipated by the actuators. The important difference is that, as a resonant actuator requires far less power than a linear one at a given scan frequency and scan angle, it can attain more of either (or both) of these before overheating.

Resonant galvanometers usually operate at 4 kHz and above, often near to a fractional multiple of the horizontal scan frequencies of the NTSC video standard. The mirror angle varies in a sinusoidal manner at a frequency determined by the mechanical resonance of the device. This in turn is determined by the spring constant k and the moment of inertia I of the rotor/mirror assembly: larger mirror or higher I , lower frequency; stiffer spring, higher frequency but smaller scan angle.

The Olympus SIM scanner runs sinusoidally in “tornado” mode. The Leica RS uses a resonant scanner but drives it with a sawtoothlike waveform. However, the angular velocity of a resonant galvanometer varies, reaching a maximum at a phase angle of 0° and a minimum at $\pm 90^\circ$, where the scan direction changes. As the signal intensity per pixel is inversely proportional to scanner speed, in order to keep geometrical scan nonlinearity to $<4\%$, recording is restricted to the two $\pm 30^\circ$ intervals of the total 360° waveform. As $\sin 30^\circ = 0.5$, the actual amplitude of the mirror motion has to be twice as large as is actually required to scan the field being digitized and, because of the time lost in turnaround, signal can only be collected 15% to 30% of the time. To avoid

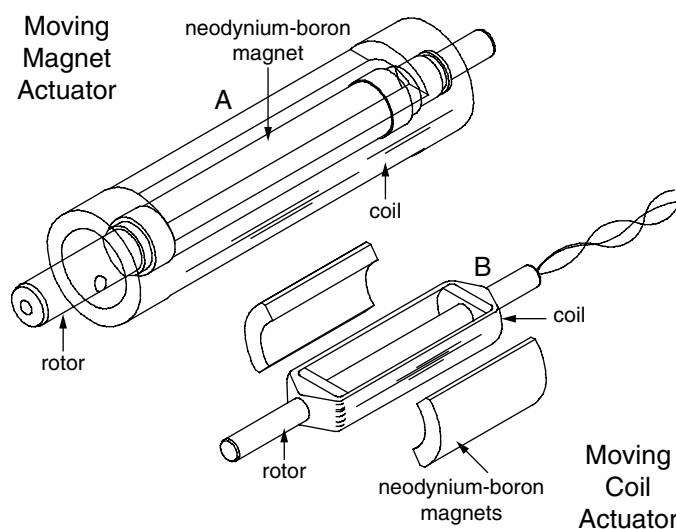


FIGURE 3.18. Two types of galvanometer actuator. Although moving-magnet actuators (A) are smaller and more rigid, moving-coil actuators (B) are more precise. (These diagrams modified from originals kindly provided by R. Aylward, Cambridge Technology, Cambridge, MA.)

⁷ Mechanical stops of some kind must be used to restrict rotor movement to <1 rotation.

bleaching outside the recorded area during the remainder of the waveform, stops are inserted at an image plane to exclude light that would otherwise land outside the digitized area.

The effective scanning speed can be increased by collecting data during both the forward and the backward movements of the mirror. However, because the backward movement also reverses the direction in which pixels are recorded, additional electronics or appropriate software is needed to repackage the data into a standard image format. This alignment process is seldom totally successful, especially when used to perform confocal imaging at video rate, and the result is that every other line is displaced slightly sideways with respect to its neighbor.

Here at the EMBL, two high-resolution methods have been implemented to solve this problem: use of a position signal from the galvanometer to detect the zero-crossing voltage line. This event starts a counter. By running the galvanometer symmetrically and using two independently adjusted counters, the forward and backward movements can be accurately synchronized, although special software is still needed to rearrange the pixel order. However, even this trick does not overcome the fact that sinusoidal bidirectional scanning permits one to record quasi-linear data during only ~33% of the scan time.⁸

For example, the galvo inside the Leica TCS SP2 RS runs at 4000 lines/s (8000 in bidirectional mode) and this translates into 150 fps @ 32×512 pixel @ zoom 1. Using a galvanometer from Cambridge Technology, Olympus claims 4000 lines or 16 fps @ 256×256 for both their confocal microscopes in the Fluoview 1000.

Resonant scanners accelerate fast enough to actually cause the mirror to bend or flex. This has two negative effects: (a) the wavefront can become distorted, reducing the resolution of the instrument and (b) if a high-reflectivity coating is used, it may age more rapidly than on a mirror at rest. The latter problem can be avoided by using the less-efficient “enhanced full-metal” coatings.

Note that flexing is more of a problem for large mirrors so choosing a mirror is always a compromise between rigidity/scan speed and the mirror size required to reflect a beam large enough to fill the BFP of all the objective lenses used. Clearly these requirements can interact in that a mirror big enough to reflect the entire ray bundle may also flex enough to negate the resolution improvement obtained by so doing. In this case it is important to remember that, at a given NA, the ray bundle required will be smaller when using an objective with a higher magnification.

In a linear galvanometer, almost all of the torque needed to move the mirror is provided by the magnetic forces produced by currents flowing in coils. The maximum speed is limited by the amount of RMS power the galvo can dissipate without the permanent magnet being heated beyond its Curie point (100–110°C) where it becomes demagnetized. Although linear galvanometers try to follow a distorted sawtooth waveform, in this electro-mechanical system, much more time is lost to overscan than is common in the electron-magnetic systems used to scan the electron beam in a cathode-ray tube (CRT).⁹ Time lost in retrace is one of the reasons that, assuming that the microscope is designed properly, one should get better results recording signal from a single,

3-s scan than by Kalman-averaging three, 1-s scans.¹⁰ If the mirror is driven with a triangular wave and the signal collected in both directions, it is possible to increase both the “usable/unusable” duty cycle and the line-scan rate, as long as provision is made for inverting the pixel order and aligning the two sets of scans (see above).

In contrast to resonant galvanometers, linear galvanometers follow the input signal at any drive frequency although with a phase delay that is not negligible under normal operating conditions. Ideally, a sawtooth motion produces a relatively slow, linear recording line followed by a fast retrace (mono-directional scans). Problems occur because of the high angular acceleration needed during the transition between the end of one line to the beginning of the next (i.e., the retrace). The disparity between the linear sawtooth or triangular-wave input and the actual motion of the mirror is best reduced by using closed-loop negative feedback: the universal engineering prescription for “distortion” (Aylward, 1999, 2003). This requires a means by which the position of the rotor can be measured and the information fed back to the control circuitry driving the coils.

Figure 3.19 diagrams four methods of detecting rotor position: two involving changes in capacitance between fixed and moving

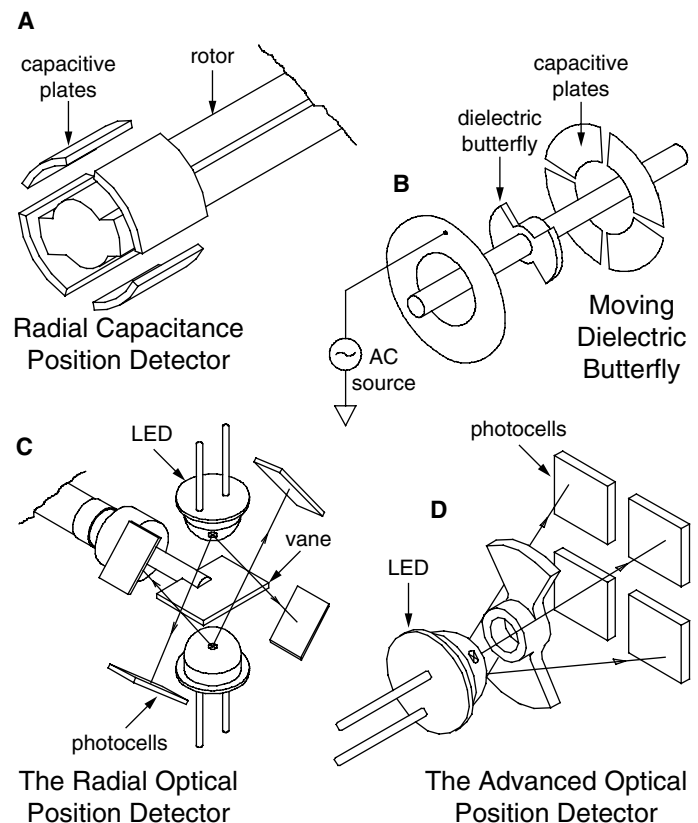


FIGURE 3.19. Four types of galvanometer position sensor. Rotor position can be determined by ascertaining changes in the capacitance between structures attached to it and other electrodes mounted on the stator. The dielectric butterfly design (B) is more precise than that shown in (A) because it is easier to align. [Note that in (B), the electrode spacings have been exploded for clarity.] (These diagrams modified from originals kindly provided by R. Aylward, Cambridge Technologies, Cambridge, MA.)

⁸ Two $\pm 30^\circ$ scans cover 120° , or 33% of the 360° sine-wave cycle.

⁹ In other words, when you use a higher line rate, a significant fraction (20%–50%) of your collection time can be wasted by retrace. This reduces the signal-to-noise ratio (S/N) of the data beyond that which comes about merely because the beam spends less time in each pixel and therefore excites fewer photons.

¹⁰ On the other hand, from the sampling point of view, if one is recording three-dimensional data, it is better to record three 1-s scans at slightly different defocus positions, if doing so is needed to meet Nyquist conditions in the z-direction.

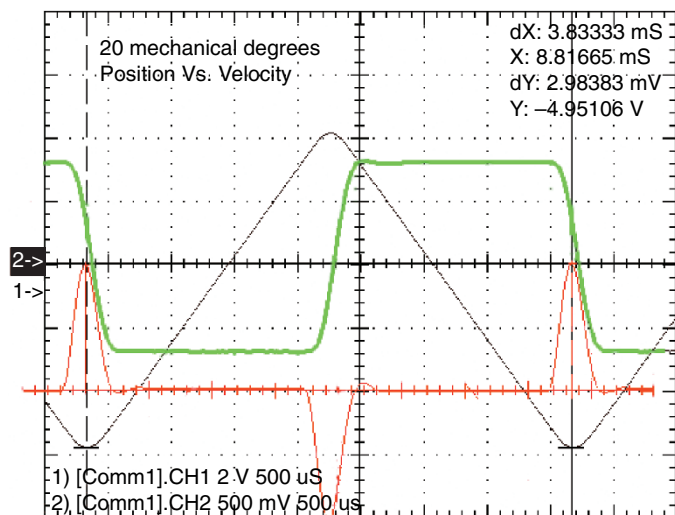


FIGURE 3.20. Galvanometer mirror performance. Measured plots of mirror position (black), angular velocity (green), and drive current (red). (These diagrams modified from originals kindly provided by Steven Sequeira, Cambridge Technologies, Cambridge, MA.)

electrodes and two involving the modulation of light from a light-emitting diode (LED) by the action of a shutter attached to the rotor. Of the capacitance methods, the newer “dielectric butterfly” method provides repeatability of 1 micro-radian (i.e., 1 mm at a range of 1 km). Although the LED methods are less precise, they are also much smaller and less expensive. In its efforts to make the actual motion of the mirror mimic the sawtooth waveform, the feedback circuit produces a current acceleration/deceleration waveform that hardly resembles a “sawtooth” at all (Fig. 3.20 red line).

As useful signals cannot be recorded during retrace, the beam should be blanked to avoid unnecessary bleaching and this can be implemented using mechanical stops such as those mentioned above or by using laser-blanking systems utilizing an AOD or a Pockels Cell (see below). Not only do these devices reduce the average light dose to the sample by 10% to 20%, they also eliminate the high-dose damage done in the region to either side of the raster where the beam slows down, stops, and re-accelerates. As this process may deposit 10 to 20 times more photons/ μm^2 in these localized areas, it is wise to ensure that living cells do not extend beyond the side borders of the recorded area if appropriate beam-blanking is not available.

An advantage of using linear actuators responding to sawtooth drive signals is that the actual recording time can be 2 to 2.5 times higher than the 33% mentioned above. The advantages of one type of galvanometer over the other in terms of stability and long-term performance are not known.

Olympus drives their optional SIM scanner in a special “tornado” mode in which a damped, sinusoidal signal is applied to both scanners, producing a spiral, Lissajous pattern. This pattern is used to generate a circular bleaching spot without wasting any time turning the scanner around, as happens when it is driven with sawtooth signals. However, because the beam paths overlap in the center of this circle, more energy is deposited there, creating a non-uniform bleach.

General Specifications

Scanners wobble above and below the scan line and jitter as they go along it. The wobble specification tells how well a scanner remains on the line and the jitter how accurately the scanner returns

to a specific pixel of that line at a given instant of the scan cycle. In both cases, performance depends on the quality of the bearings and the care with which the moving mass has been mass balanced.¹¹ In a scanning microscope, these specifications can affect the resolution limit of the microscope.

Let us assume an optical scan angle of $\pm 3^\circ$ in a beam scanner (i.e., a complete mechanical scan of 6° or 100 mrad). If there are 2500 pixels/line, the jitter (and the wobble) must be lower than $40 \mu\text{rad}$ ($100 \text{ mrad}/2500$), which is about four times more than the 5 to $10 \mu\text{rad}$ specification for the Cambridge Technology 6210H [with advanced optical feedback, Figure 3.16(D), used in many confocal microscopes. Cambridge Technology scanners have $15 \mu\text{rad}$ drift per $^\circ\text{C}$]. Fortunately, common experience has shown that the scanner specifications tend to be conservative and their actual performance is good enough that it does not limit microscope performance. The actual test is to use the galvanometers at the very small scan angles appropriate to high-NA, low-magnification lenses. The minimal scan angle of the instruments built at EMBL is $\sim 20 \mu\text{rad}/\text{line}$, but even here, the stability is so good that an indicator must be used to tell the user that the scanner is operating. More recent scanners have even better specifications and use even larger mirrors, a factor that allows designers to use simpler scanning lenses.

Current devices represent a highly optimized product that is the culmination of intensive research over many years. Commercial units perform at or near the limits imposed by physics (e.g., galvo mass vs. planarity, mirror diameter, drive power vs. overheating) and materials properties (density vs. stiffness). In spite of this long development history, last year Cambridge Technology introduced the 6215H galvanometer with scan rates 50% to 100% higher (1.5 kHz triangular wave @ $\pm 3^\circ$) than the previous model.

The situation is less settled in the field of resonant galvanometers where the number of applications for very fast scanners is increasing rapidly and not all of the requirements can be fulfilled using electro-optic or acousto-optic devices. With resonant scanners, one-directional line scanning can now be performed at up to 8000 Hz with present commercial scanners but research scanners have gone faster. However, resonant scanners have the disadvantage that their scan speed is fixed and their duty cycle short. To improve the signal-to-noise ratio of weak fluorescence signals, one can only line average, and this process accumulates readout noise. Finally, there is also no simple way to move the beam within a specific region or along a line that isn’t horizontal. For FRAP experiments, one must bypass the laser light around the resonant scanner to a second linear scanner.

ACOUSTO-OPTICAL COMPONENTS

The performance of acousto-optical components is based on the optical effects of acoustic fields on birefringent crystals. The acoustic field is generated and controlled by a piezoelectric

¹¹ Mass balancing is both crucially important to reduce vibration and difficult to achieve if one adheres to the optical requirement that the axis-of-rotation pass through the plane of the mirror. If the mirror surface is on the axis, it follows that the material making up the mirror assembly can only be distributed symmetrically on both sides of the axis if additional structures are added solely to counterbalance it. Such additions lead in turn to complex mirror geometries that increase both I and the chance that the mirror will flex. As a result, the scanners used in confocals are likely to have silicon mirrors about 1-mm thick mounted symmetrically (i.e., with the axis going up the middle of the silicon). Moral: One pays in many ways for trying to scan fast. Do not do it unless you need to.

crystal, excited at 50 to 150 MHz, attached to one side of the crystal and a vibration absorber attached to the facing side. At a specific frequency of the acoustic wave, the periodic pattern of compression and rarefaction associated with it affects the local density, and therefore the RI, of the crystal to form a three-dimensional periodic diffractor. Light passing through the crystal is diffracted at an angle (θ) that depends on the frequency (and hence the wavelength) of the acoustic wave and on the wavelength of the light¹² and is defined by the equation:

$$\theta = \lambda f_a / V_a$$

where λ is the optical wavelength in air, V_a is the acoustical velocity of the material, and f_a is the acoustic frequency. In a TeO_2 crystal excited at 100 MHz, the diffraction angle for 500 nm light is $\sim 4.3^\circ$.

Normally the crystal is shaped like a rhombus, with the undiffracted beam entering and leaving normal to the two sides and the piezo generating plane waves that are parallel to the other two sides (Fig. 3.21). The waves are tilted with respect to the laser beam by an angle that is roughly half of the expected diffraction angle.

Although fused and crystalline quartz can be used as the active material, the preferred AO material is TeO_2 . Most AO materials operate well only over a limited wavelength range: generally less than 400 nm (Chang, 1995). They also affect the polarization of the light. Light passing through a TeO_2 AOD, along the “slow” direction, will emerge with its polarization direction rotated by 90° .

By sweeping the drive frequency of the pressure waves, an acousto-optical deflector (AOD) will deflect a beam of monochromatic light over a range of angles, making it useful as a scanning device. Acousto-optical modulators (AOM) are used to blank a monochromatic light beam intermittently, to create a new beam with a lower (time-averaged) intensity. In an acousto-optical tunable filter (AOTF), the pressure wave pattern in the crystal is

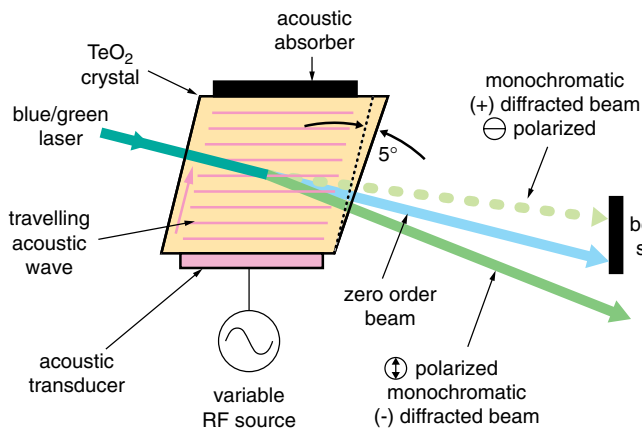


FIGURE 3.21. Schematic diagram of acousto-optical device. Light entering from the left strikes the surface of the TeO_2 crystal at normal incidence. Acoustic waves propagating upwards from the piezoelectric transducer diffract the light beam, either up or down, depending on the polarization of the light beam. Because diffracted light would suffer dispersion on leaving the crystal, the far face is cut at a steeper angle than would be needed if it were a rhombus. Therefore, the device shown would work optimally only with light polarized parallel to an acoustic beam that diffracts into the 1st order.

¹² In TeO_2 , the crystal most commonly used, the speed of sound is 660 m/s. As a result, a 100 MHz wave will have a period of about $6.6 \mu\text{m}$, about 10 to 15 time greater than the wavelength of the light being diffracted.

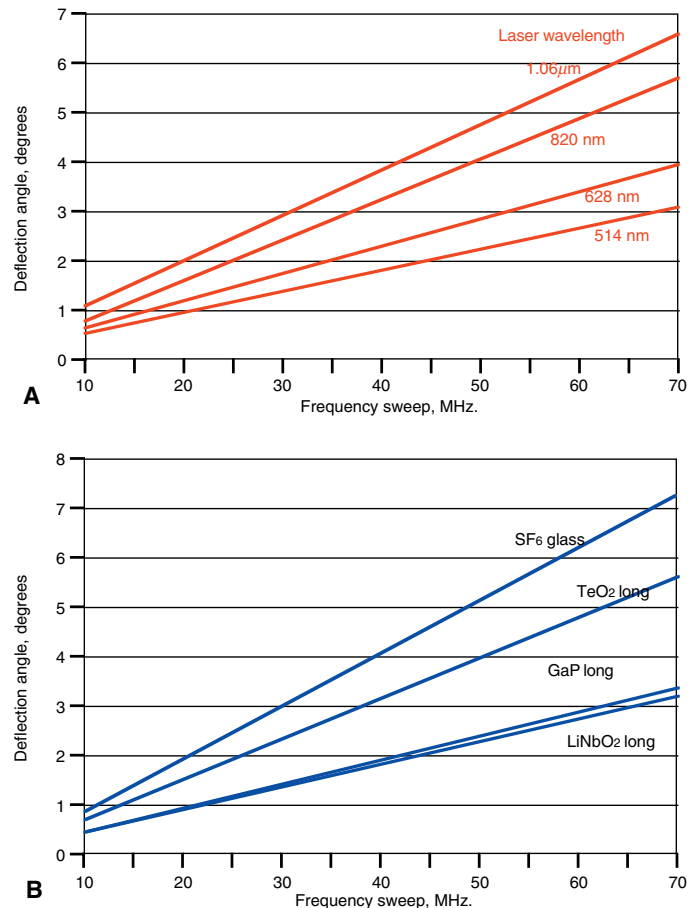


FIGURE 3.22. AOD performance. The amount of deflection produced by an AOD varies with the amount of change in the acoustic frequency, the wavelength of the incoming light (A), and the material in which the deflection takes place (B).

used to spectrally filter the light. An acousto-optical beam-splitter (AOBS) is really just an AOTF used to spectrally combine or separate the light at specific laser wavelengths from all other light.

As the frequency of the acoustic wave can be changed on the microsecond time scale, acousto-optical components can change their deflection properties much faster than when using mechanical parts. For a given frequency sweep, the amount of deflection depends on the type of material used in the AOD and the wavelength of the incident light (Fig. 3.22). The response time is limited by the time it takes for a new acoustic wave field to propagate across the width of the crystal and, therefore, depends linearly on the width of the light beam that has to be modulated. Typical rates are $\sim 150 \text{ ns/mm}$ of beam width. Only a narrow band of wavelengths are diffracted and out-of-band contributions are reduced, a feature that is especially suitable for filtering devices. Also, as acousto-optical components do not have any moving parts, they require little maintenance.

At root, all AO components are very similar. They use the same crystals, operating at the same acoustic frequencies and diffract over similar angles. As AOTFs and AOBSs operate on smaller beams, they can be faster and because they operate at fixed input and output angles, the crystals are smaller and less expensive. As AODs operate at a fixed input angle but a variable output angle, they must be larger, slower, and more expensive. Otherwise they are just AOTFs operating with a swept acoustic frequency. Increas-

ing the acoustic frequency increases the deflection angle: increasing the acoustic power increases the fraction of the light that is deflected from the incident beam. The preferred crystal, TeO_2 , gives the most deflection for a given input wavelength and acoustic power level. It is only replaced by one of the other materials when deflecting high-power laser beams¹³ or when it cannot be cooled sufficiently well.

Acousto-Optical Deflectors

Acousto-optical deflectors (AOD) can scan a laser beam at up to 100kHz, compared to about 500 to 1000Hz for linear galvanometer scanners and about 4 to 8kHz for resonant galvanometers. In addition, they allow zooming and varying the scan speed. As they do not have the momentum associated with moving parts, they require little retrace time and are capable of rapidly accessing random scan areas.

However, one cannot simply replace the faster of the two scanners in your confocal (as described in Chapter 9, *this volume*) with an AOD because, as the deflection is produced by diffraction, the scan angle depends on the wavelength of the light beam. As a result, the longer wavelength fluorescent light will be deflected by a different amount on its way back through the crystal and will therefore fail to pass through the pinhole. Although this dispersion effect can be theoretically compensated for by a chromatic correction system, such systems involve so many additional expensive, lossy elements, they are not practical for confocal use. AOD dispersion is an even more serious problem when working with pulsed radiation in a multi-photon microscope (Lechleiter *et al.*, 2002).

A workable AOD scan system can be developed for confocal fluorescence microscopy if the incident laser beam is first *x*-deflected by the AOD and then, after passing the dichroic mirror, *y*-deflected by a slower, galvanometer scanner. As the returning fluorescence emission is deflected by the slower scanner and reflected by the dichroic mirror in the direction of a linear detector, it does not reach the AOD. A lens focuses light from the dichroic onto a detector slit, which must be used because the movement along the fast axis is not being descanned. Although a linear CCD detector behind the slit might be used to “track” the moving signal spot, this solution is still confocal only along one axis. However, it gives better sectioning than slit-illumination/slit-detection systems, such as the Zeiss LSM-5Live and in practice it seems to work quite well (Draaijer and Houpt, 1988; Tsien and Bacskaï, 1995).

Compared to galvanometers, AODs provide small deflection angles ($<3.5^\circ$ vs. $\sim 6^\circ$ needed for most objectives), a limited angular resolution,¹⁴ a non-circular aperture, and a low “reflection” efficiency ($<70\%$, i.e., only 0%–70% of the incident beam is deflected vs. 95%–99% for a mirror). These parameters depend on each other. A higher angular resolution can be achieved, but only at the expense of a spatial variation in the diffraction efficiency (10% at 2000 lines). Finally, the drive signals fed to an AOD must be readjusted for each excitation wavelength. Although this can be

arranged for a small number of discrete laser lines, it cannot be used to deflect “white” light in a useful manner.

Acousto-Optical Modulators

Acousto-optical modulators (AOM) are used to modulate the intensity of a beam and can thus also function as laser-line selectors. In FRAP experiments, for example, they are used to switch the laser on and off rapidly (Flamion *et al.*, 1991). It is common to choose the deflected beam as the one that is used by the optical system because it can be switched on and off with high extinction ratio (typically $>40\text{ dB}$ or 100:1) and its intensity can be varied from zero to more than 85% of the incident beam (Fig. 3.17).

Acousto-Optical Tunable Filters

Acousto-optical deflection causes light of different wavelengths to exit the crystal at different angles. In an optical system designed so that input light strikes the crystal at only one angle and diffracted light re-enters the optical system only at one diffraction angle, then one can choose the wavelength of the light diffracted at this angle by varying the frequency of the acoustic wave. This makes it a “tunable filter.” Because it is not possible to diffract all the light (and therefore turn off the input beam), the acousto-optical tunable filters (AOTF) is usually configured so that one uses the light in the diffracted beam.

As temperature affects both the RI of the crystal and the speed of sound, it also affects the wavelengths of both the light and the acoustic waves. Therefore, it also affects the diffraction angle. If the angle changes enough so that the diffracted beam “misses” the entrance of the optical system (i.e., wanders off the input pupil of the optical fiber), this will result in a large reduction in the signal entering the fiber (and finally striking the specimen).

Because the diffraction properties of acousto-optical filters (Harris and Wallace, 1969) can be switched within microseconds, they are a tremendous improvement over using filter wheels to choose excitation wavelengths (Nitschke *et al.*, 1997). AOTFs can be used to select the illumination waveband when working with either a broadband light source (Lewis *et al.*, 1992) or a multi-line laser (Wachman *et al.*, 1997). They can also be used to select a light detection band (Wachman *et al.*, 1997).

When one has signals from two or more fluorophores, it is easier to separate them if they are excited individually and sequentially. To accomplish this, the AOTF can be used to change the laser lines after each line scan, that is, each line is scanned several times, sequentially — once for each of the excitation wavelengths needed. However, one can only take full advantage of this high-speed switching of the excitation if the other components in the light paths can also be adapted to operate with similar speed and flexibility.

AOTFs are also used for switching off the illumination light during retrace (see above).

Acousto-Optical Beam-Splitters

The acousto-optical beam-splitter (AOBS) is actually an AOTF used in an imaginative manner by Leica to replace the dichroic mirror usually used to separate the illumination and detections paths in a confocal fluorescence microscope (Engelhardt *et al.*, 1999; Birk *et al.*, 2002; it is implemented in Leica’s Spectral Confocal Microscopes, TCS SP2 AOBS). In practice, the AOBS is programmed so that it leaves most (90%–95%) of the fluorescent light undeflected, and only deflects light at specific laser lines.

¹³ Such as those used for laser light shows.

¹⁴ About 500–1500 lines. This is set by time-bandwidth product of the crystal: faster scans have less precision and hence fewer distinct lines in the raster. This occurs because if the acoustic frequency varies too rapidly, the acoustic wavelength will be different on the near and far sides of the light beam, diffracting these rays differently.

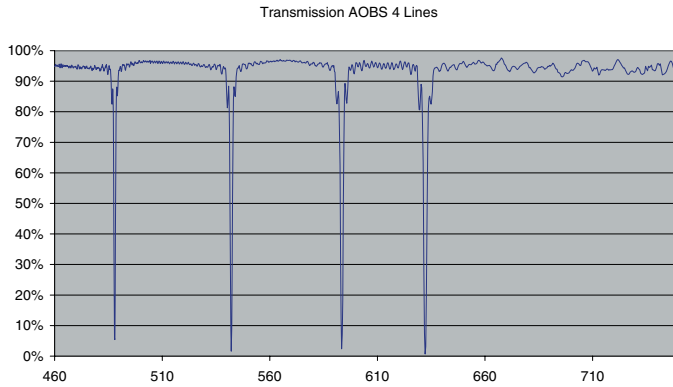


FIGURE 3.23. Transmission of Leica acousto-optical beam-splitter (AOBS). This plot shows the transmission of the beam-splitter to light originating from a point in the specimen. The notches in the transmission at 488, 542, 593, and 623 nm show that the AOBS has been adjusted to diffract these wavelengths of laser light into the beam and, consequently, remove them on the return trip. The position of these bands can be adjusted to suit any visible laser that might be used.

Because it is possible to excite the piezo with a signal that is the sum of several specific frequencies, and because the resulting pressure field then diffracts several wavelengths simultaneously, the AOBS allows efficient, simultaneous deflection of up to 8 laser lines towards the sample, while the average transmission of the crystal (away from the reflection bands) is about 95% (Fig. 3.23). The intensity of each line can be controlled independently over a range of more than 20:1.

The advantages of the AOBS over systems that use several, multi-band, dichroic mirrors as the main beam-splitter are its high efficiency, its lack of moving parts, and its flexibility in terms of being able to set up the system to use new laser lines simply by changing the software. On the other hand, because the proper operation of the AOBS depends on the light input being parallel as it passes through the crystal, light originating above or below the plane-of-focus may see slightly different transmission characteristics. Fortunately, in confocal operations, most out-of-focus light is removed at the pinhole.

ELECTRO-OPTICAL MODULATORS

Electro-optical modulators (EOM) are crystals utilizing the Pockels effect to modulate the polarization, the intensity, the phase, the frequency, or the direction of propagation of laser beams. They are faster (1 GHz) than acousto-optical devices but they not only are sensitive to electro-magnetic interference but they also generate quite a lot of it (Draaijer *et al.*, 1996). In addition, they have a lower extinction ratio and are more sensitive to temperature changes (Chang, 1995). EOMs can be used to modify the polarization (Hofkens *et al.*, 1998) and the intensity or the frequency of laser beams (Helm *et al.*, 2001). Although, in principle they can also be used as scanners, they are generally not used as such in microscopes. They are usually optimized for performance at a single wavelength (Maldonado, 1995).

Piezoelectric Scanners

Piezoelectric scanners are mostly used to move the objective lens axially for three-dimensional imaging (Callamaras and Parker,

1999) or to move the object, either up and down or in all three directions (in a stage-scanning microscope).^{*} Because massive objects are being scanned, settling times are in the millisecond range and horizontal scan speeds are limited to 10 to 100 Hz. Piezoelectric scanners utilize the piezoelectric effect: the generation of a voltage across some crystals when they are compressed or alternatively, the deformation of a crystal when a voltage is applied. In piezoelectric scanners, voltages are applied to electrodes on a tube piezoelectric ceramic and this elongates and moves an object fixed to the end of the tube. Typically, extensions of about 100 to 200 μm are possible with a resolution in the order of 1 nm (sub-nanometer resolution can be achieved if smaller travel ranges are acceptable). Like galvanometer scanners, piezoelectric scanners can be driven in a resonant mode with frequencies in the kilohertz range.

Piezoelectric scanners suffer from two problems: hysteresis, in which a given voltage produces a different displacement when approached from a lower voltage than when approached from a higher voltage (i.e., the position does not track when reversing the scanning direction), and creep, in which the position changes with time, even though the applied voltage remains fixed. As a result, piezoelectric elements are best used in conjunction with an element that measures the position, such as a capacitance-based translation/rotation sensor. In addition, hysteresis effects are easily avoided by always approaching the final position from only one direction, rather than moving the system back and forth. In this case the illumination is best switched off when driving the objective back towards the start position. Position drifts with time are seldom a serious limitation for most scanning light microscopy applications.

POLARIZING ELEMENTS

The combination of a polarizing beam-splitter and a quarter-wave plate is very useful in confocal reflection microscopy because it provides access to a backscattered (reflected) light signal without any loss of photon efficiency (Pawley *et al.*, 1993). A horizontally polarized beam is reflected by a polarizing beam-splitter and then circularly polarized with the quarter-wave plate before being focused into the sample. The light scattered back from inhomogeneities is rotated by another 45° when passing the quarter-wave plate on the return journey and, now vertically polarized, deflected by the polarizing beam-splitter. This beam can be used to generate backscattered light contrast.

It should not be forgotten that the signal derived from small features in a biological specimen is usually incoherently backscattered and not coherently reflected. From a physical point of view, backscattered and reflected light have different properties. The detection system described above will only produce a useful signal if the process of contrast generation preserves the polarization of the incident light. The arrangement does not reduce the efficiency of the fluorescence detection path, and there are good reasons to believe that a circularly polarized beam is better focused by standard objective lenses than is a linearly polarized beam (van der Voort and Brakenhoff, 1989).

^{*} Recently piezo activators have been used to provide small deflections of the galvo mirror along an axis perpendicular to the galvo axis. This is used to provide rapid XY scanning in multi-beam scanners using a single galvo.

REMOVING EXCESS LIGHT

It is always a good idea to introduce adjustable apertures (e.g., irises) into the optical path. The best locations for these are, of course, at conjugates of the aperture and field planes. On one hand these apertures can be used to adjust the beam because they essentially define the optical paths in the instrument (i.e., NA and field of view). On the other hand, once the instrument is aligned for Köhler illumination (see the Appendix of Chapter 36, *this volume*), these apertures should be closed as far as possible so that stray light is reduced, preventing any light from entering the optical system beyond that needed (1) to fill the back-focal plane and (2) to fill the field-of-view.

Some optical elements require more effective care of unwanted light. Good examples are AOMs and AOTFs. These components control not only the laser light intensity, they also “switch” laser light sources on and off by deflecting the beam. However, light deflected so that it does **not** enter the optical path is likely to reflect to places where it is unwanted unless steps are taken to absorb it.

Flip mirrors can also be used to keep a laser from entering an optical device. Particularly, when lasers in excess of a few tens of milliwatts are used, any deflected beam should be guided towards a strong absorber. A blackened surface on the inner endplate of a tube works very well for lower laser powers, while a 20-mm high stack of razor blades whose cutting edges are oriented towards the beam will do an excellent job for higher laser powers. Powers above 1 W are rarely used in microscopy but if they are, acousto- and electro-optical devices may start having problems and beam dumps will require extra cooling (improved air circulation and even water cooling).

SOME USEFUL LINKS

<http://www.cambridgetechnology.com/news/Choosing%20A%20Galvanometer.html>
<http://www.brimrose.com>
<http://www.gsilumonics.com/>
<http://micro.magnet.fsu.edu/primer/techniques/fluorescence/interferencefilterintro.html>
http://www.neotech.com/new_content.asp?content=AO_Introduction
<http://www.semrock.com>

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REFERENCES

- Aylward, R.P., 1999, The advances and technologies of galvanometer-based optical scanners, *SPIE Int. Soc. Opt. Eng.* 3787:158–164.
- Aylward, R.P., 2003, Advanced galvanometer-based optical scanner design, *Sensor Rev.* 23:216–222.
- 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.
- Callamaras, N., and Parker, I., 1999, Construction of a confocal microscope for real-time x-y and x-z imaging. *Cell Calcium*, 26:271–279.
- Chang, I.C., 1995, Acousto-optic devices and applications. In: *Handbook of Optics*, Vol. II (M. Bass, ed.), McGraw-Hill, New York, pp. 12.1–12.54.
- Cox, G., 2002, Biological confocal microscopy, *Mater. Today*, March:34–41.
- Draaijer, A., and Houpt, P. M., 1988, A standard video-rate confocal laser-scanning reflection and fluorescence microscope, *Scanning* 10:139–145.
- Draaijer, A., Gerritsen, H.C., and Sanders, R., 1996, Time-domain confocal fluorescence lifetime imaging. Instrumentation, *Scanning* 18:56–57.
- Engelhardt, J., Ulrich, H., and Bradl, J., 1999, Optische Anordnung. German patent application DE 199 06 757 A1.
- Flamion, B., Bungay, P.M., Gibson, C.C., and Spring, K.R., 1991, Flow-rate measurements in isolated perfused kidney-tubules by fluorescence photobleaching recovery, *Biophys. J.* 60:1229–1242.
- Harris, S.E., and Wallace, R.W., 1969, Acousto-optic tunable filter, *J. Opt. Soc. Am.* 59:744–747.
- Helm, J.P., Haug, F.M.S., Storm, J.F., and Ottersen, O.P., 2001, Design and installation of a multimode microscopy system, *SPIE Proc.* 4262:396–406.
- Hofkens, J., Verheijen, W., Shukla, R., Dehaen, W., and De Schryver, F.C., 1998, Detection of a single dendrimer macromolecule with a fluorescent dihydropyrrroloporroledione (DPP) core embedded in a thin polystyrene polymer film, *Macromolecules* 31:4493–4497.
- Kramer, J., 1999, The right filter set gets the most out of a microscope, *Biophotonics Intl.* 6:54–58.
- Lechleiter, J.D., Lin, D.T., and Sieneart, I., 2002, Multi-photon laser scanning microscopy using an acoustic optical deflector, *Biophys. J.* 83:2292–2299.
- Lerner J.L., and Zucker, R.M., 2004, Calibration and validation of confocal spectroscopic imaging systems, *Cytometry* 62A:8–34.
- Lewis, E.N., Treado, P.J., and Levin, I.W., 1992, Visible/near-infrared imaging spectrometry — applications of an acousto-optic tunable filter (AOTF) to biological microscopy, *FASEB J.* 6: A34.
- Maldonado, T.A., 1995, Electro-optic modulators. In: *Handbook of Optics*, Vol. II (M. Bass, ed.), McGraw-Hill, New York, pp. 13.1–13.35.
- Melles Griot, 1999, *The Practical Application of Light*, Melles Griot, Irvine, CA. The Melles Griot catalogue is very informative and contains numerous excellent comments on optical elements and performance characteristics.
- Molecular Probes, 2002, *Handbook of Fluorescent Probes and Research Products*, Molecular Probes, Eugene, Oregon.
- Nitschke, R., Wilhelm, S., Borlinghaus, R., Leipziger, J., Bindels, R., and Greger, R., 1997, A modified confocal laser scanning microscope allows fast ultraviolet ratio imaging of intracellular Ca^{2+} activity using Fura-2, *Pflug. Arch. Eur. J. Phys.* 433:653–663.
- Pawley, J.B., Amos, W.B., Dixon, A., and Brelje, T.C., 1993, Simultaneous, non-interfering, collection of optimal fluorescent and backscattered light signals on the MRC-500/600, *Proc. Microsc. Soc. Am.* 51:156–157.
- Reichman, J., 2000, *Handbook of Optical Filters for Fluorescence Microscopy*, Chroma Technology Corp., Brattleboro, Vermont.
- Tsien, R.Y., and Baskai, B.J., 1995, Video-rate confocal microscopy. In: *Handbook of Biological Confocal Microscopy*, 2nd ed., (J.B. Pawley, ed.), Plenum Press, New York.
- van der Voort, H.T.M., and Brakenhoff, G.J., 1989, Modeling of 3D confocal imaging at high numerical aperture in fluorescence, *SPIE Proc.* 1028:39–44.
- Wachman, E.S., Niu, W., and Farkas, D.L., 1997, AOTF microscope for imaging with increased speed and spectral versatility, *Biophys. J.* 73:1215–1222.