Optical Filters

Impact Fluorescence Fidelity

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SUMMARY

Optical filters are crucial in fluorescence experiments and instrumentation. Recent advances in filter technology offer a way to improve fluorescence techniques.

In optical fluorescence, fluorophores are specifically attached to biological molecules, to cell regions and to other targets of interest to enable quantification, identification and even real-time tracking of activity on a microscopic scale. Fluorescence forms the basis of a wide variety of applications in modern biotechnology, from research through clinical diagnosis, including genomics and proteomics. Fluorescence instruments range from optical microscopes and more sophisticated imaging systems to DNA microarray scanners, flow cytometers and high-throughput screening systems.

The sensitivity afforded by fluorescence enables smaller sample volumes, miniaturization of instrumentation and increased specificity; in some cases, single molecules can be reliably detected. These features are important for applications such as the sequencing of DNA, RNA or proteins. The speed with which fluorescence assays can be performed is critical for such applications as high-throughput screening, in which each day of delay in introducing a new blockbuster drug can mean up to $1 million in lost revenue for a pharmaceutical company. And because fluorescence techniques are nonradioactive and often simpler than other methods, they also are safer and less expensive.

To maximize the performance of the instrumentation for all of these applications, it is critical to understand some of the basic principles of signal (sometimes called brightness), noise and optical signal-to-noise ratio (sometimes called contrast). These instruments are generally based on optical filters, and there is usually more flexibility in choosing filters than other aspects of the optical system (such as the light source or detectors), so an understanding of how filters affect these three properties is key. The proper use and specification of optical filters can be crucial to the success of a particular experimental test or assay as well as to the optimal design of robust instrumentation.

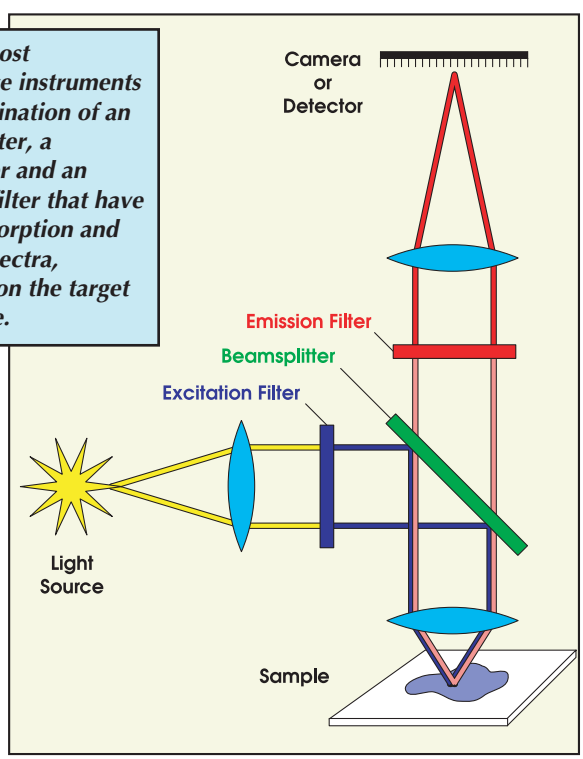

Figure 1. Most fluorescence instruments use a combination of an emission filter, a beamsplitter and an excitation filter that have various absorption and emission spectra, depending on the target fluorophore.

A system with a broadband light source, such as a fluorescence microscope, has three basic filters: an excitation filter, a dichroic beamsplitter and an emission filter (Figure 1). The exciter is typically a bandpass filter that passes only the wavelengths absorbed by the fluorophore, thus minimizing excitation of other sources of fluorescence and blocking light in the fluorescence emission band.

The dichroic is an edge filter used at an oblique angle of incidence to efficiently reflect light in the excitation band and to transmit light in the emission band. The emitter is also typically a bandpass filter that passes only the wavelengths emitted by the fluorophore and blocks all undesired light outside this band — especially the excitation light.

Systems with laser illumination might or might not use an exciter or a dichroic, but most include some variation of these filters. The

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fluorescence filters function as a set to provide the optimum signal with minimal noise.

In most fluorescence instruments, the best performance is obtained with thin-film filters, as opposed to other types of fixed or tunable filters, such as those based on diffraction gratings. Thin-film filters comprise multiple thin layers of materials with high and low indices of refraction on a glass substrate. The complex layer structure determines the spectrum of light transmission by a filter — the more layers and the more precisely they are deposited, the more complex and accurately reproduced a desired spectrum can be made.

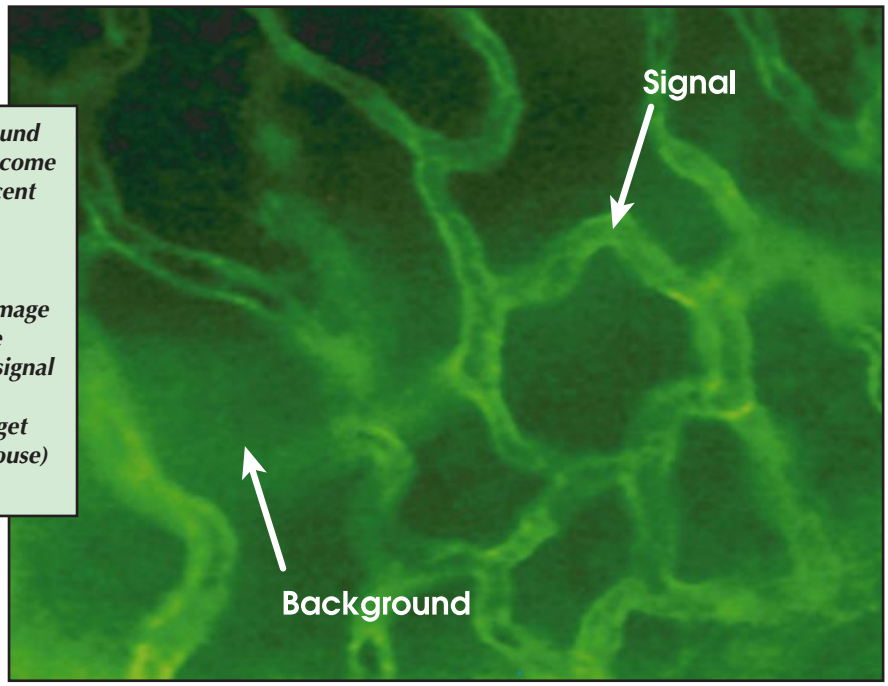


Figure 2. Background fluorescence can come from autofluorescent molecules in the sample, such as NAD(P)H in this epifluorescence image of mouse prostate vasculature. The signal is from FITC conjugated to target CD41 marker (mouse) antibodies.

Thin-film filters are simpler, are less expensive and provide excellent optical performance: high transmission over an arbitrarily determined bandwidth, steep edges and high blocking of undesired light over the widest possible wavelength range. Recent advances in thin-film filter technology permit even higher performance while resolving the longevity and handling issues that can affect filters made with older technology.

Signal, noise, signal-to-noise ratio

The signal in a fluorescence instrument is a measurable change in the detector output caused by emission light derived only from a desired fluorescent probe bound to a desired target molecule or species. The detector might be an observer's eye, a photodiode, a photomultiplier tube or a CCD camera. Noise, on the other hand, is any other change in the detector output. It can be caused by background fluorescence, unblocked excitation light and detector noise.

The signal-to-noise ratio, as the name implies, is the ratio of the signal level to the noise level, as measured by the detector. An increased signal-to-noise ratio raises sensitivity because a lower signal level can be discriminated from noise. Specificity also is improved because thresholds can be set with a higher degree of certainty, minimizing the error rate for false level determinations. For example, to properly count and sort cells in a quantification application such as flow cytometry, a positive or negative determination based on fluorescence level is made for each cell.

Although the following statement might seem obvious, it is fundamental. There are only two ways to improve the signal-to-noise ratio of an instrument: Increase the signal, or decrease the noise. To increase the signal, one may increase the source intensity, but this can expose the sample to too much light, especially

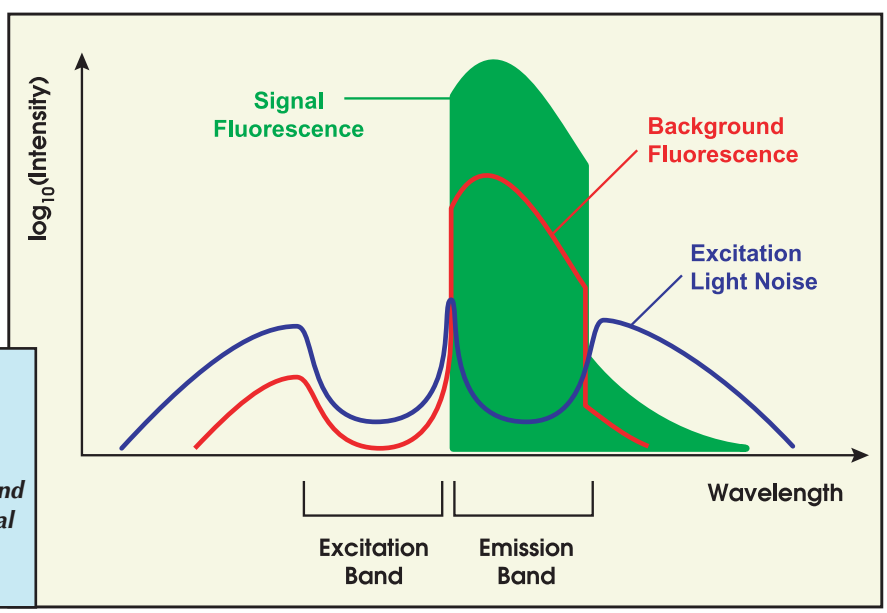


Figure 3. Filter edge steepness, placement and extended blocking are crucial for suppressing out-of-band noise and for controlling the relative level of desired signal fluorescence to undesired background fluorescence, as seen in this typical spectral distribution of noise in a fluorescence system.

if it is susceptible to photobleaching. It is also possible to improve the fluorescence efficiency by choosing a fluorophore with the highest quantum yield and least quenching for the desired target molecule or species, and increasing the fluorophore concentration as much as permitted by the particular test or experiment. A third means of substantially affecting the signal level is through the proper specification of the optical filters.

Looking for noise

Scientists and engineers often overlook the possibility of reducing noise, largely because noise in fluorescence systems is not widely understood.

The first major source of noise is background fluorescence — all fluorescence that doesn't originate from the fluorophore of interest bound to the desired target. Background can arise from nonspecific binding and/or excess desired fluorophore molecules in the sample, which can be minimized only by careful specimen preparation. The second source is autofluorescence from other constituents of the sample (Figure 2). Examples include aromatic amino acids (such as tryptophan, tyrosine and phenylalanine) and proteins, enzyme cofactors [e.g., NAD(P)H], flavins (e.g., FAD) and pyridoxal phosphate derivatives.

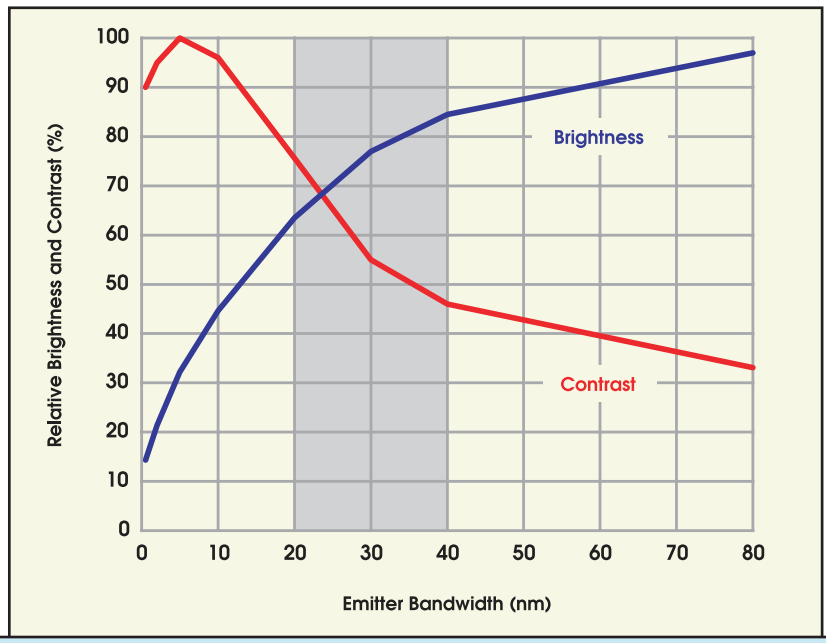
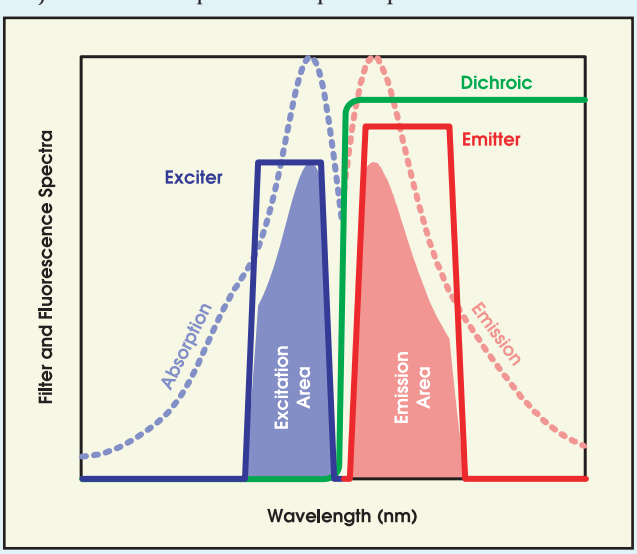


Figure 4. One filter parameter (emitter bandwidth) in a fluorescence instrument can greatly affect the brightness (signal) and contrast (signal-to-noise ratio) of an instrument, demonstrating the frequent fundamental trade-off between these two aspects of system performance. In this example, displaying a reasonably high brightness while maintaining high contrast would require an optimal filter bandwidth between 20 and 40 nm.

Thirdly, autofluorescence from optical components and glassware — such as lenses, optical filter substrate glass and glass sample slides — also can cause background noise. Microscope objectives are a particularly large source of autofluorescence in epifluorescence microscopes because the excitation light that

Calculating Signal and Noise

The combined excitation and emission processes determine the intensity of the detected signal fluorescence. The signal intensity is proportional to the product of the "excitation area" and the "emission area." The excitation area is the integrated area under the product of the source spectrum, the excitation filter spectrum (exciter transmission multiplied by dichroic reflection) and the fluorophore absorption spectrum. The emission area is the integrated area under the product of the detector response, emission filter (emitter transmission multiplied by dichroic transmission) and fluorophore emission spectra (see figure). The net product, called the fluorescence spectral throughput, is the key to determining the brightness exhibited by a given filter set.



The intensity of the noise can be calculated by independently determining background fluorescence and excitation light noise — the two main causes of intensity noise — and then adding them. To estimate the background noise, a quantity analogous to the fluorescence spectral throughput can be computed, except that the target fluorophore absorption and emission spectra are replaced by very broadband (almost uniform) background fluorescence absorption and emission spectra, the relative efficiency of which can be determined empirically from measurements.

To determine the excitation light noise, the absorption and emission spectra can be replaced with a wavelength-independent factor that represents the efficiency with which the excitation light remains in the optical path — typically this number is up to several percent. □

passes through a large volume of glass excites fluorescence in the emission passband that is not blocked by the emitter filter.

Excitation light noise also presents problems. Any light from the excitation source that makes it to the detector is noise. It could be stray light scattered from rough edges or inhomogeneous optics, or excitation light reflected from the sample and not sufficiently blocked by the emitter. Optical filters are the only line of defense.

The detector also can contribute noise. This is mainly electronic noise in the detector itself (e.g., thermal and shot noise). However, it is typically a concern only for the lowest detection thresholds. Using the right detector or a cooled detector can reduce this effect.

In terms of the spectrum, the majority of noise tends to come from background in the emission band and from excitation light noise just outside or between the excitation and emission bands (Figure 3).

Impact of filters

Better optical filters can increase the signal, or brightness; reduce background outside the emission band; and reduce excitation light noise. But they cannot reduce background noise inside the emission band. In other words, any adjustment to the filters that increases the signal brightness also increases the background within the emission band. Whether or not such an adjustment affects the signal-to-noise ratio favorably depends on the details of the system, including the background.

The ideal set of fluorescence filters consists of perfectly rectangular bandpass filters with 100 percent transmission and vertical slopes, and an ideal edge filter for the dichroic with 100 percent reflection in the excitation band and 100 percent transmission in the emission band. To prevent excitation light noise between the bands, the exciter and emitter filters must not overlap.

Although filters do not possess these ideal properties, manufacturers must try to come as close as possible. Key specifications that distinguish filters are average passband transmission, bandwidth, edge steepness and edge wavelength accuracy. The last of these is not apparent from looking at a single plot of the filter spectra, but rather is based on a statistical sample of a large number of filters. It is crucial for guaranteeing consistency in high-volume instrumentation applications as well as in end-user systems such as microscopes.

The performance of a given set of filters in an instrument can be modeled (see sidebar), and this can greatly aid in the design of filter specifications for any fluorescence instrument. Calculating the relative brightness and contrast achieved as the bandwidth of an emitter filter varies shows the trade-off often faced between brightness and contrast (Figure 4). The narrower the filter, the more selectively it transmits the desired fluorescence signal relative to the background and excitation light noise, and therefore the higher the contrast. But if the filter is too narrow, the signal itself becomes too small.

Optical thin-film filters, including those designed for fluorescence applications, have been around for decades. Yet even today many instruments utilize outdated thin-film filter technology. Just recently, this technology has taken a giant leap forward as a result of several advancements.

Ion-assisted, ion-beam sputtering — a relatively new vacuum deposition technology — enables high-performance filters that are tens of microns thick with hundreds of thin-film layers. Further advances in filter design and deposition control systems allow such a large number of layers with arbitrary thicknesses to be made in a reproducible manner. This added flexibility has increased filter spectral accuracy and sophistication.

These advances resulted from the rise of the telecommunications industry, which demanded unprecedented filter performance and rigorously proven reliability and which brought with it the money to drive the development efforts to attain these lofty goals. Now the biological field, which might not have driven such advances on its own, can benefit from the substantial investment made in this telecom filter technology.

Benefits of advances

For the end user, what are the benefits of all of these advances? First, filters can more closely approximate the best possible brightness and contrast. As an example, one challenging type of filter set — a multiband fluorescence filter set — can now achieve high transmission, steep edges and nearly complete blocking between the passbands, thanks to thin-film technology (Figure 5). The filters enable an increase in signal but also a decrease in noise, an effect particularly pronounced when viewed directly in the microscope (Figure 6).

Because these filters are made by ion-beam sputtering, they are based on hard

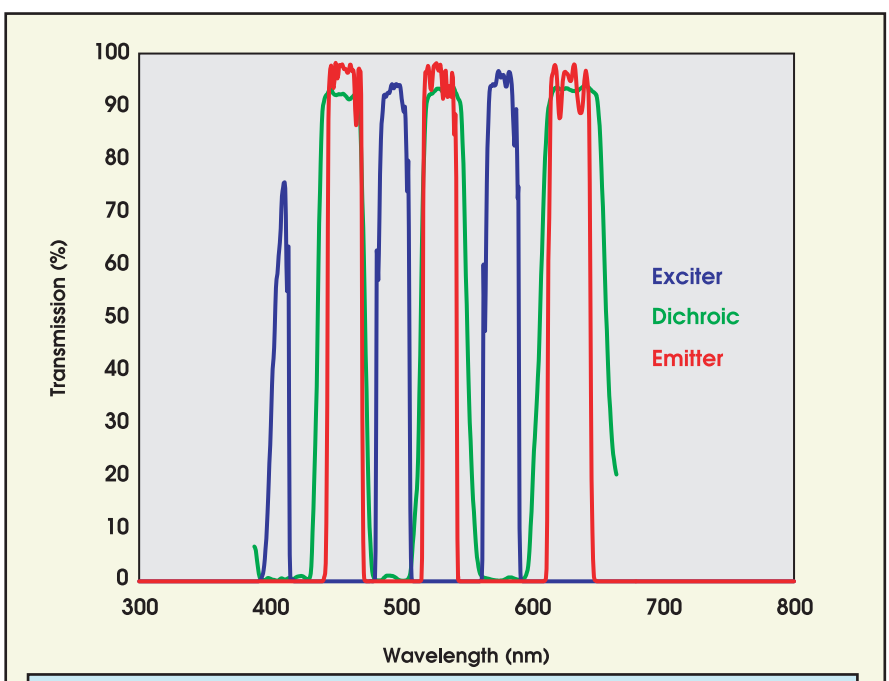


Figure 5. These transmission spectra for a DAPI-FITC-Texas Red multiband filter set illustrate the effect of optical thin-film filter technology. Note that the exciter short-wavelength transmission has been deliberately reduced to minimize photobleaching and to balance the colors.

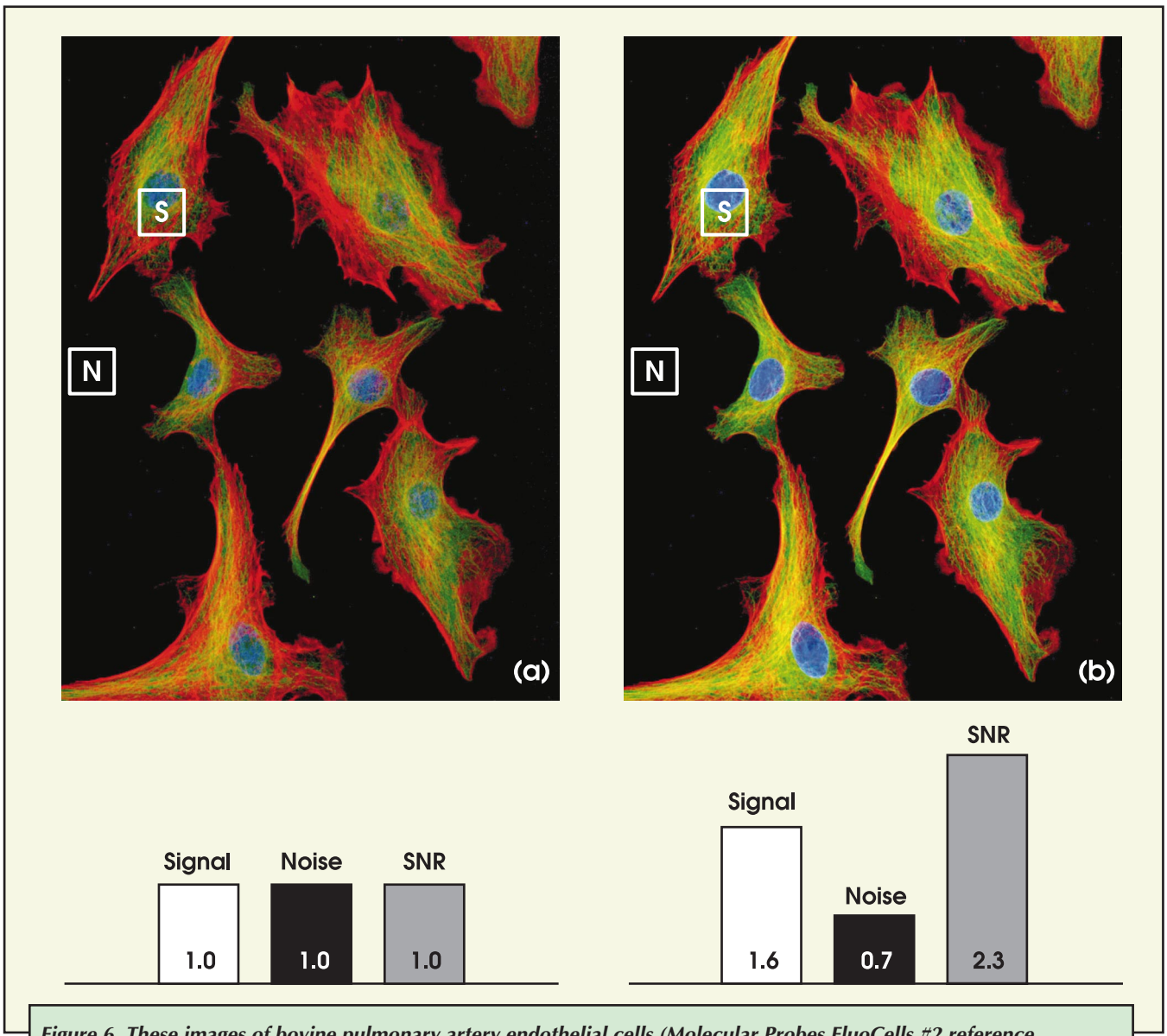


Figure 6. These images of bovine pulmonary artery endothelial cells (Molecular Probes FluoCells #2 reference standard) were taken with (a) older, conventional optical filters and (b) filters based on the new technology. Also shown below the images are the relative signal (S), noise (N) and signal-to-noise ratio (SNR) as determined from the square boxes with the same label on the images. These measurements were normalized to the values measured on the left image. The images were captured by a Spot Insight Color camera from Diagnostic Instruments, and the data were measured on identical monochrome images captured by a QImaging Retiga cooled monochrome 12-bit camera.

oxide materials that can withstand rigorous environmental durability requirements. The coatings are "nonshifting" when exposed to damp heat and can operate over a wide range of temperature and humidity conditions without failure. This is important for many fluorescence filter applications, particularly instruments used in high-throughput diagnostic or research laboratory settings.

The oxide coatings are as hard as the glass substrate itself. The filters also can be cleaned using standard solvent techniques without fear of damage. In addition, because of the single-substrate design, no epoxies are in the optical path, so one need not worry about damage or degradation from excessive optical power or the effects of prolonged high temperature and humidity.

The filters also can improve imaging quality. In devices such as microscopes that have traditional filters, it is difficult to eliminate beam deviation caused by the wedge angle of the overall filter structure. When multiple images produced by different filter sets are overlaid, any beam deviation causes poor image registration. However, by putting hard coatings on a single piece of glass, these problems are virtually eliminated because single glass substrates are manufactured to high tolerances of parallelism. □

Meet the authors

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