FLUORESCENCE IMAGING

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New Optical Filters Improve High-Speed Multicolor Fluorescence Imaging

by Dr. Turan Erdogan, Semrock Inc.

Based on ion-beam sputtering of hard oxide glass materials, the novel filters provide higher system brightness and sensitivity.

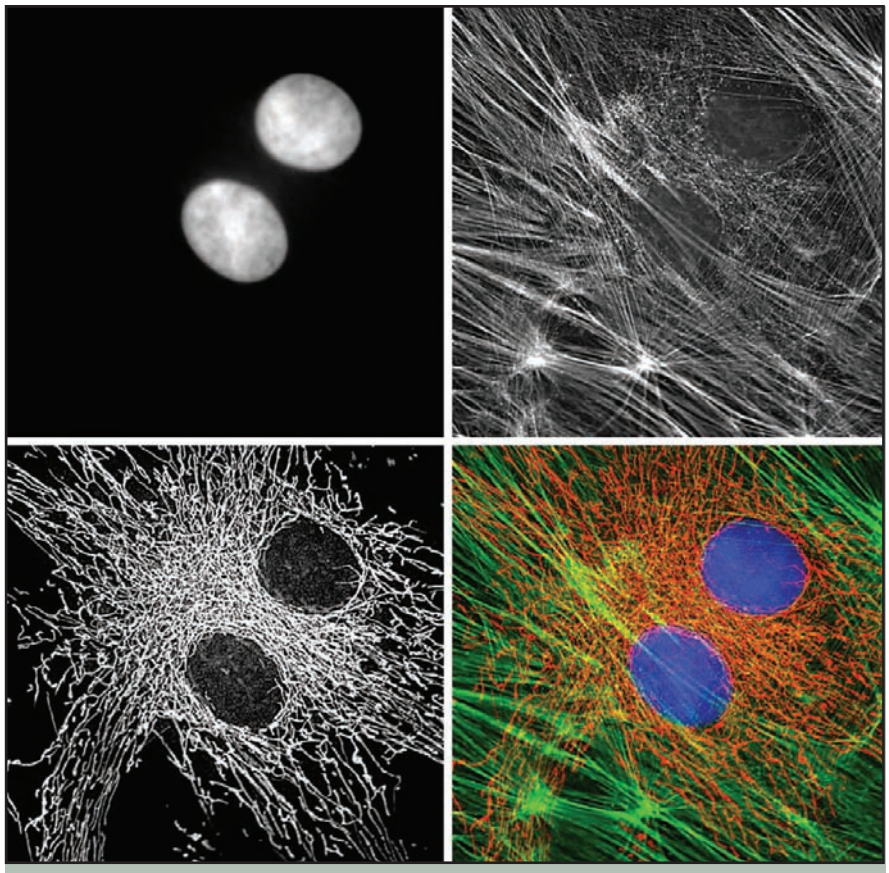

Figure 1. Images of a triple-labeled sample were captured using three separate single-band filter sets in sequence (BrightLine DAPI-5060B, FITC-3540B and TXRED-4040B from Semrock Inc.). An Olympus BX41 microscope with a 40× objective and a QImaging Retiga monochrome CCD camera were used. The sample is bovine pulmonary artery endothelial cells, where DAPI is labeling the nucleus, F-actin is labeled with BODIPY FL phallacidin, and the mitochondria are stained with MitoTracker Red CMXRos.

Fluorescence microscopy is ideal for biological imaging because it reveals only objects of interest in an otherwise dark background, while permitting selective tagging. This technique continues to develop rapidly, enabling the capture of faster, brighter, more highly selective and more complex images of an increasingly large number of objects in a single sample. Modern optical filter technology is developing just as rapidly and is playing a vital role in making these new imaging methods a reality.

The ability to label multiple, distinct targets of interest in a single biological sample greatly enhances the power of fluorescence imaging. For a long time, the only way to achieve high-quality images of multilabeled samples was to take multiple photographs while switching single-band optical filter sets between photographs. The photographs are combined electronically to yield a single, composite image. This approach can come with pixel shift among the multiple, single-color images, and it can take a significant amount of time to capture a complete multicolor image. Pixel shift has been addressed (see "Achieving the Best Alignment for Fluorescent Images," *Biophotonics*

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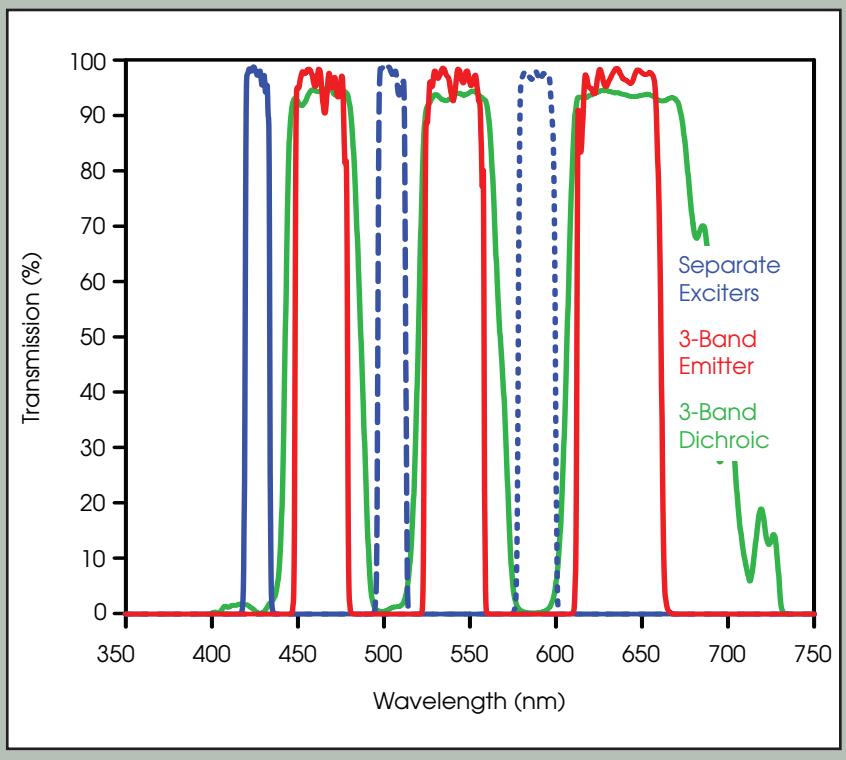

Figure 2. In a Pinkel filter set, single-band exciters are used in conjunction with multiband dichroic beamsplitter and emission filters, as in this example of a triple-band filter set designed for high-speed sequential imaging of cyan, yellow and red fluorescent proteins. Filter set shown is a CFP/YFP/HcRed-3X-A set from Semrock Inc.

International, August 2005), and this single-band filter set approach is still often the best method for achieving images with the highest contrast and lowest bleedthrough possible.

However, many researchers want the ability to achieve high-speed, multicolor imaging while maintaining good images. Fluorescent proteins allow precise and real-time imaging of protein localization in living cells and of the structure and function of living tissue. As a result, there is an ever-increasing demand for an alternative to the single-band filter set approach without sacrificing image fidelity (the most accurate reproduction of the actual sample). In this article we will look at how recent advances in multiband optical filter technology have brought high-speed multicolor imaging to a new level.

Single-band vs. multiband

Most fluorescence instruments, including microscopes, use optical filters to control the spectra of the excitation and emission light. Filters make it possible for light only within the absorption band of a fluorophore to reach the sample, and light only within the emission band to reach the detector. Without filters, the detector could not distinguish the desired fluorescence from other sources of light, including scattered excitation light; autofluorescence from the sample, substrate and other optics in the system; and ambient light in the laboratory.

A fluorescence instrument with a broadband light source, such as most wide-field microscopes, has three basic filters: an excitation filter (or exciter), a dichroic beamsplitter and an emission filter (or emitter). The exciter is typically a bandpass filter that passes only the band of wavelengths most highly 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 a 45° angle of incidence to efficiently reflect light in the excitation band and to transmit light in the emission band.

The emitter also is typically a bandpass filter that passes only the band of wavelengths most efficiently 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. In the absence of any fluorescence, these filters ideally allow no light to reach the detector, and thus create a dark-field imaging system. In practice, they function together as a set to provide the optimum signal with minimal noise.

When the sample has two or more fluorescent labels that absorb and emit light in different wavelength regions, two or more sets of filters can be used independently to image the labels in sequence. Typically, each filter set is held as a unit in a microscope filter holder — often called a cube — and the cubes are exchanged via a turret that rotates either manually or with motorized control. Individual images are captured with different filter sets, and the composite image is created electronically (Figure 1).

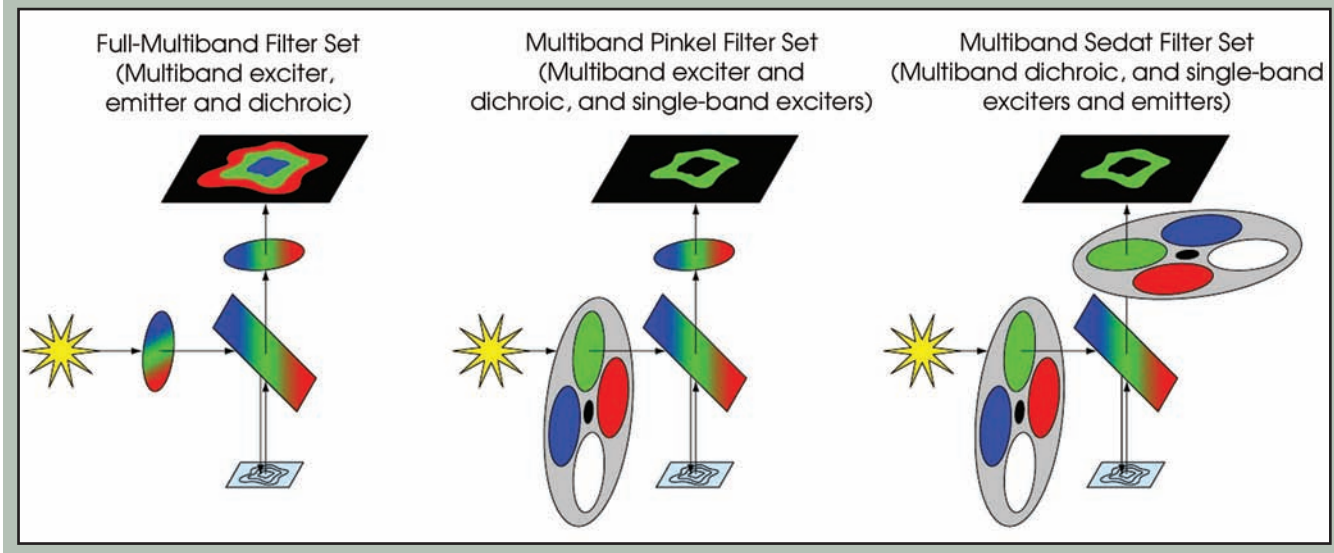

Figure 3. There are three types of multiband filter sets.

Alternatively, it is possible to make optical filters with multiple passbands so that more than one fluorophore can be excited, or the emission from more than one fluorophore observed, with a single filter. For these filters to enable the relatively weak fluorescence emission to be observed above all the other possible sources of light noise, the dark-field imaging property of the complete filter set must be preserved. In other words, the exciter must provide sufficient blocking in the wavelength regions where the emitter transmits light, and vice versa.

To achieve such a high level of blocking in multiple bands while maintaining very high transmission in the passbands requires very complex filters with a very large number of thin-film layers precisely deposited with extreme control over the layer thicknesses. Fortunately, recent advances in the technology used to fabricate fluorescence filters have made it possible to produce ones with such spectral performance features.

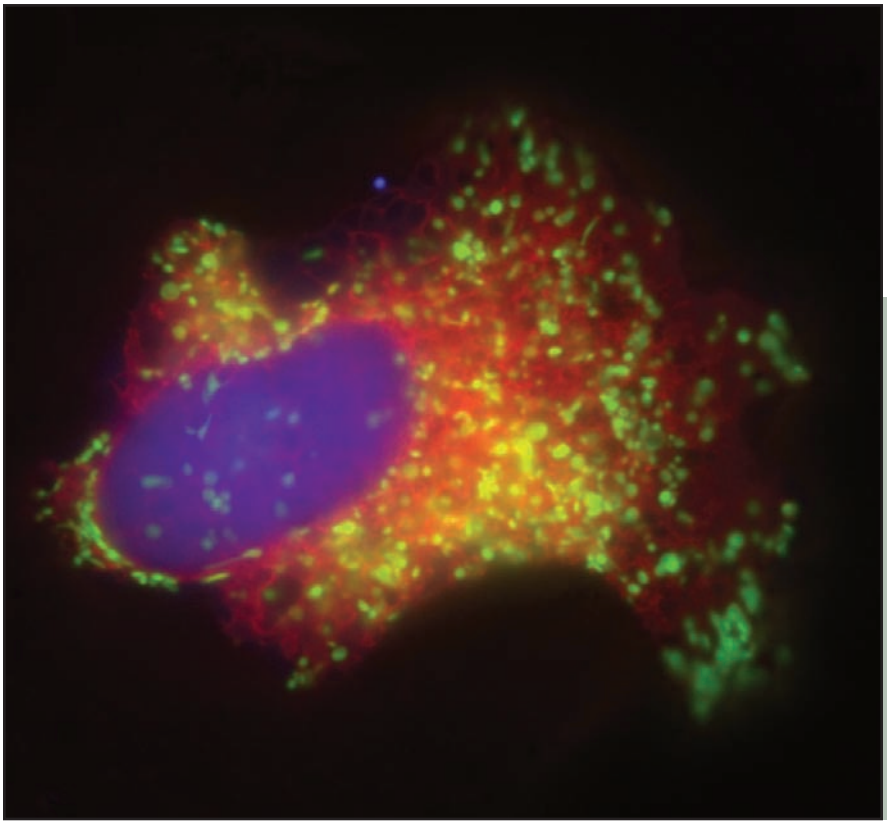

Figure 4. This composite image was created from separate monochrome images captured using a Pinkel filter set. The sample is a HeLa cell expressing SECFP, Venus and mRFP targeted to the endoplasmic reticulum, the mitochondria and the nucleus, respectively. The wide-field image was taken on a Nikon TE2000E inverted microscope with a 60×, PlanApo, 1.4-NA, oil-immersion objective, and a cooled monochrome CCD camera (Orca-ER, Hamamatsu Photonics) using a BrightLine CFP/YFP/HcRed-3X-A filter set. Courtesy of Takeharu Nagai and Kenta Saito, Laboratory for Nanosystems Physiology, REIS, Hokkaido University.

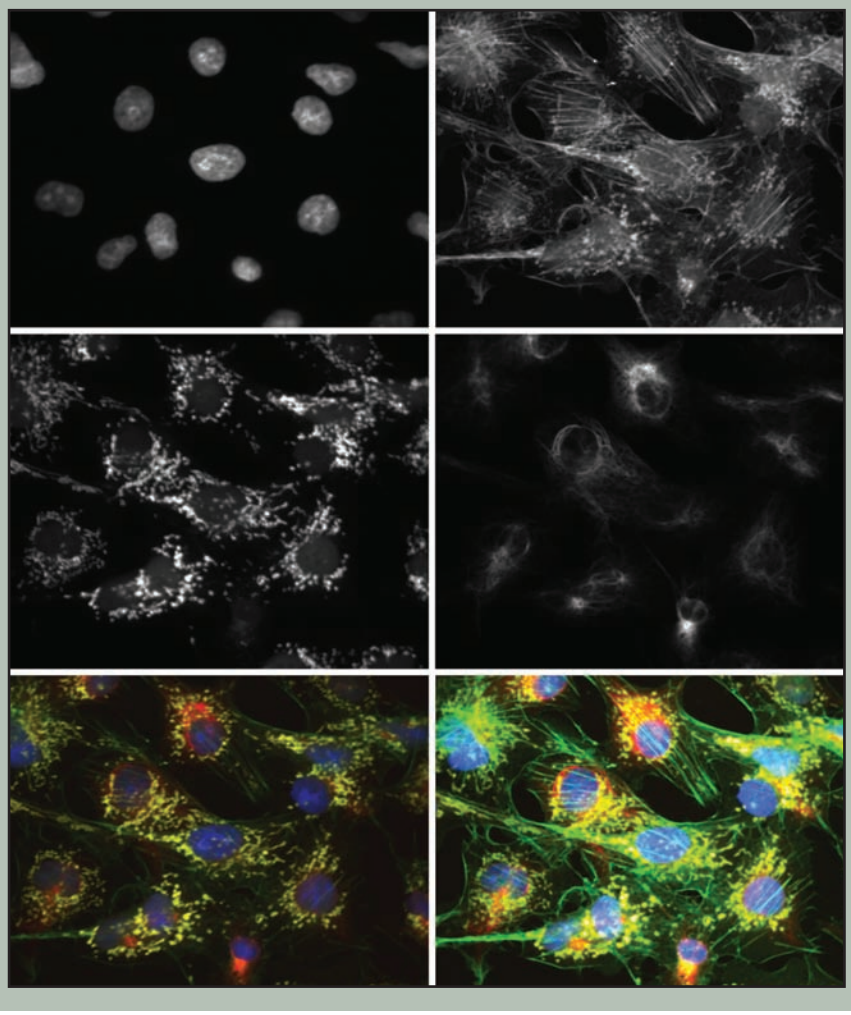

Figure 5. Quadruple-band images of a sample of rat kidney mesangial cells are labeled with various fluorophores. Images captured by a Hamamatsu Orca-ER monochrome CCD camera using a BrightLine quadruple-band DA/FI/TR/Cy5-4X-A filter set on an Olympus BX61WI microscope with a DSU spinning-disk confocal unit. Individual monochrome images of each color channel are shown, along with the resulting composite image (lower right). The image on the lower left was created with a multiband filter set based on older technology, clearly demonstrating the improvements afforded by the new filter technology. Sample courtesy of Michael Davidson, Molecular Expressions.

Ion-beam sputtering

The new filter technology is based on ion-beam sputtering of hard oxide glass materials (as hard as the glass substrates on which they are coated). Ion-beam sputtering coupled with state-of-the-art deposition control technology permits highly optimized filter designs that provide new levels of filter transmission and discrimination (Figure 2). These filters, in turn, drive higher system brightness and sensitivity. Full triple-band sets made with this technology have been measured to have 2.5 times the contrast of the previously best available triple-band sets. The latest quadruple-band sets have been independently tested and have shown up to four times the brightness and twice the contrast of equivalent quadruple-band sets that were, until recently, leading the industry.

Such dramatic advances are forcing a re-evaluation of multicolor imaging techniques. In addition, these new hard-coated filters are extremely durable. They can be cleaned like any glass optics, do not burn out even under prolonged exposure to intense arc-lamp (and laser) sources and are not affected by humidity.

There are three basic types of multiband filter sets for high-speed multicolor imaging. A full-multiband configuration uses all multiband filters — exciter, dichroic beamsplitter and emitter — and is ideal for direct visualization (Figure 3). (Here we refer to the beamsplitter as a dichroic by convention, despite the fact that it separates two colors — the meaning of dichroic — multiple times.)

Researchers often use the full-multiband filter set to visually scan a sample and locate the areas of interest on which they want to focus more carefully and perform more detailed analysis. These sets are quick and easy to implement and are compatible with all standard fluorescence microscopes.

However, they require a good-quality color camera to create reasonably high fidelity electronic images. Such cameras tend to be costly and do not yet have low-noise performance as good as monochrome CCD cameras. Furthermore, even with ideal multiband filter sets, it is not possible to totally eliminate fluorophore bleedthrough (see page 6) because the extended tails of the fluorophore absorption and emission spectra tend to cause light from more than one fluorophore to show up in a given emitter passband. Nevertheless, it is possible to create electronic images

of reasonable quality.

In contrast, the Pinkel configuration uses single-band exciters — typically exchanged with a motorized filter wheel —with a multiband dichroic beamsplitter and a multiband emitter. This approach offers a straightforward and economical way to achieve very high speed multicolor imaging (Figure 4). The configuration is named after professor Daniel Pinkel of the University of California, San Francisco, who first proposed using this configuration. Because only a single excitation passband is lit up at any given time, only a single fluorophore is predominantly excited at a time, making it possible to use a monochrome CCD camera to capture images in sequence.

Monochrome cameras are less expensive and tend to offer better noise performance than color cameras. Some bleedthrough is still possible, however, because all emission bands are imaged simultaneously through the multiband emitter and are indistinguishable by the monochrome camera. Nevertheless, improvements afforded by the new filter technology continue to make the Pinkel approach popular for high-speed imaging for two-, three- and now even four-color labeling because it offers excellent image fidelity (Figure 5).

Finally, the Sedat configuration uses single-band exciters and single-band emitters — usually exchanged by dual, electronically synchronized filter wheels —along with a multiband dichroic beamsplitter. It is named after professor John W. Sedat of the University of California, San Francisco, who first proposed using this configuration. The approach provides the best image fidelity for high-speed multicolor imaging. The only downside relative to the other multiband configurations is that it requires a larger investment in systems hardware and software.

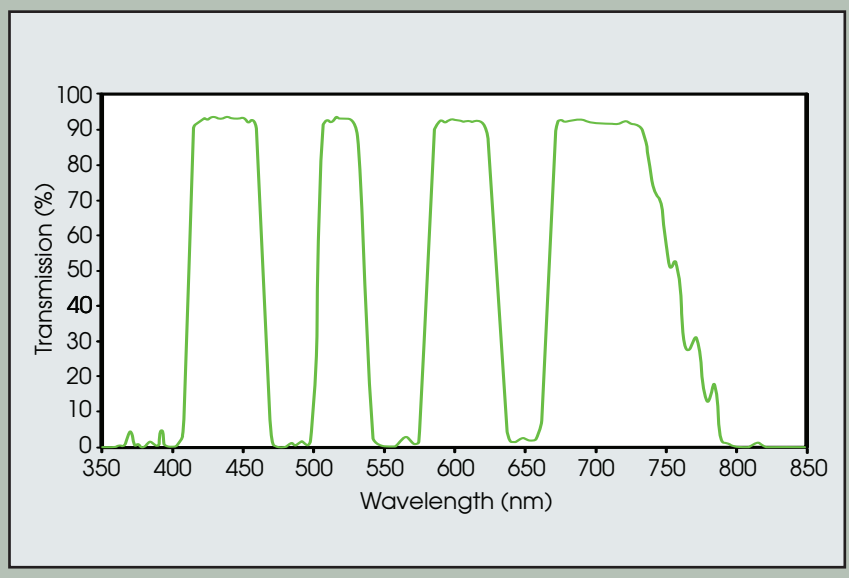

Figure 6. This transmission spectrum of a new, high-performance quadruple-band dichroic beamsplitter (BrightLine FF410/540/582/669-Di01) illustrates how improvements in filter technology have made it possible for Sedat set imaging to match the fidelity of single-band set imaging. Note the steep transitions and the high and flat transmission and reflection bands, exceeding 90% and 98%, respectively.

In comparison with the multiple single-band filter set approach — which is limited in speed — the ability of the Sedat approach to achieve comparable brightness, contrast and bleedthrough performance is limited only by the spectral quality of the multiband dichroic beamsplitter (Figure 6). Fortunately, the recent advances in filter technology described above have made it possible to achieve high and flat transmission and reflection bands, and very steep edges, even for triple- and quadruple-band beamsplitters. As a result, the images achieved with multiband Sedat filter sets rival those achievable with even the best single-band filter sets.

High-speed multicolor imaging applications — especially those involving real-time analysis of live-cell activity — can now achieve the same levels of image fidelity previously enjoyed only with static imaging of fixed-cell samples, in large part because of the improved quality of multiband fluorescence filters. □

Acknowledgments

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Meet the author

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What Is Bleedthrough?

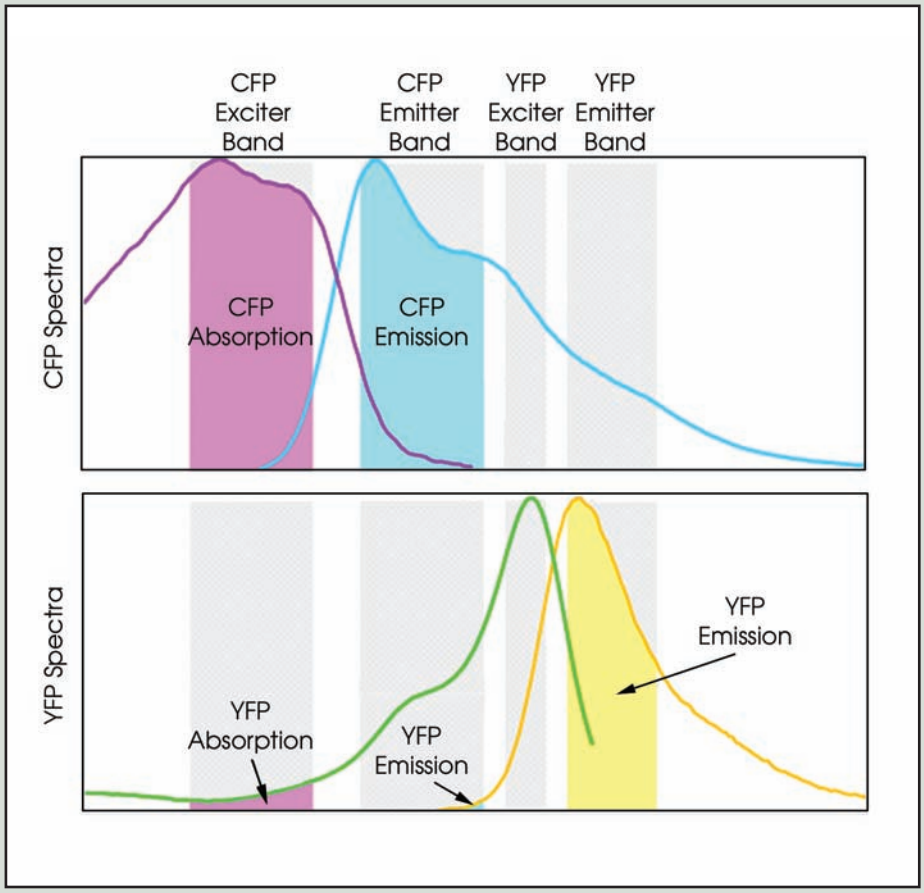
Bleedthrough (also known as crosstalk) occurs when the emission of one fluorophore is detected in the filter passband that is reserved for a different fluorophore. It arises because most fluorophores have fairly broad spectral profiles that tend to overlap. The contribution from the undesired fluorophore is more deleterious than other possible sources of noise, such as autofluorescence or detector electronic noise, because it contains spatial information linked to the sample of interest that can mask or confuse interpretation of the image created by the desired fluorophore. It is particularly problematic for subcellular imaging techniques, such as colocalization, or when quantitative measurements are necessary, such as for Förster resonance energy transfer (FRET) or fluorescence recovery after photobleaching (FRAP).

The figure illustrates what bleedthrough looks like in the spectral domain. In this example, two fluorophores are to be imaged on the same sample: cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The CFP absorption (magenta) and emission (cyan) spectra are shown in the top graph, while the YFP absorption (green) and emission (yellow) spectra are shown in the bottom graph. Possible excitation and emission filter bands associated with these two labels are illustrated by the solid gray bands.

When one is interested in imaging only the CFP-stained portions of the sample, only the CFP exciter and emitter filters are used (and, hence, only these bands are present) for both the single-band filter set and the multiband Sedat configurations. For the Pinkel configuration, only the CFP exciter band is present, but both the CFP and YFP emitter bands are present because of the multiband emitter. For the full-multiband configuration, both exciter bands and both emitter bands are present simultaneously. In all cases, the main signal of interest comes from the large overlap between the CFP absorption spectrum and the CFP exciter band and between the CFP emission spectrum and the CFP emitter band.

Also, in all cases, bleedthrough arises from the overlap of the YFP absorption spectrum with the CFP exciter band, which gives rise to YFP emission in the CFP emitter band. However, because the overlap in both cases is small, the total bleedthrough, which is proportional to the product of the overlap values, is very small. This result explains why the image fidelity is so good for single-band sets and for the Sedat configuration.

For the Pinkel and full-multiband configurations, the overlap of the YFP absorption spectrum with the CFP exciter band also gives rise to YFP emission in the YFP emitter band, which can be substantially larger than the bleedthrough described above. Thus, it is critical to minimize the overlap of the absorption of the undesired fluorophore with the exciter filter band associated with the desired fluorophore. High-performance filters with steep, precisely positioned edges are necessary to minimize overlap without sacrificing overall throughput. □



When only CFP is intended to be imaged, a small amount of YFP emission can bleed through into the CFP emitter band for all filter set approaches. The Pinkel and full-multiband approaches also may allow YFP emission to bleed through into the YFP emitter band. In all cases, the bleedthrough is minimized by high-performance, optimally positioned filters.