FLUORESCENCE FILTERS

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Achieving the Best Alignment for Fluorescent Images

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New filter technology can eliminate pixel shift in multicolor fluorescence imaging.

Image registration is important whenever multiple images are superimposed. For example, if the colored and black cartridges of an ink-jet printer are not well-aligned, a reader's eyes are drawn to the defects instead of to the more important story the page is trying to tell. Achieving excellent image registration can be even more critical in fluorescence microscopy because poor registration is not merely distracting, but also can inhibit determination of how molecules interact or how a cell functions.

Many fluorescence imaging applications involve samples labeled with multiple fluorophores. To distinguish the multiple colors, they are typically imaged sequentially onto a high-resolution monochrome camera by exchanging fluorescence filter sets that correspond to each fluorophore's emission color. The images of each fluorophore are given false colors and combined to produce a complete picture that allows testing for traits such as collocalization.

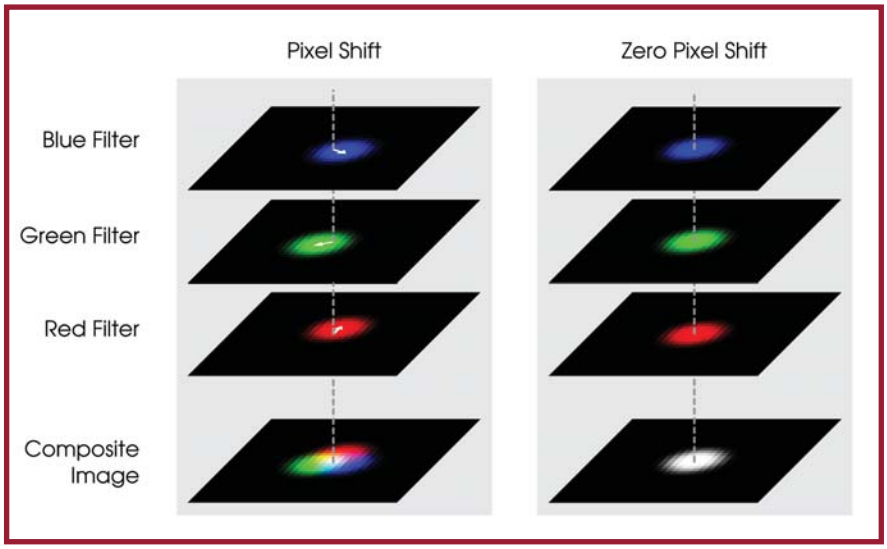

Figure 1. When imaging a multicolor spot (a multistained microsphere) with three separate filter sets, pixel shift (left) causes the various colored spots to be out of alignment in the merged image at the bottom; whereas with zero pixel shift, the spots are perfectly aligned (right).

Because the optical filters are the only part of the imaging system that changes from image to image, imperfections in the filters can cause the image associated with one fluorophore to shift on the CCD camera relative to that of another fluorophore. This phenomenon is called pixel shift (Figure 1).

What causes pixel shift?

The major imperfection in optical filters that causes pixel shift is beam deviation that is created by a nonzero wedge angle (nonparallelism) of either the emission filter or the dichroic beamsplitter, be-cause both filters are in the optical imaging path (Figure 2). When a beam of light travels through a glass plate that has surfaces that are not perfectly parallel, the direction of the emerging beam is deviated, or no longer parallel to that of the incident beam.

These imperfections occur because fluorescence filters have very demanding requirements: They must transmit as much light as possible over a specific band of wavelengths, and then very quickly transition outside that band to very high blocking over an incredibly large spectral range. The approach developed several decades ago to meet these requirements was to utilize "soft" coating materials (largely because of their very high refractive index contrast) and multiple coatings applied to multiple glass substrates that are laminated together with optical epoxy (Figure 3). The coatings must be encased within glass, and the resulting structure must be epoxied in an aluminum ring to achieve a quasi-hermetic seal that blocks moisture from reaching the hygroscopic coatings. Besides diminishing the trans-

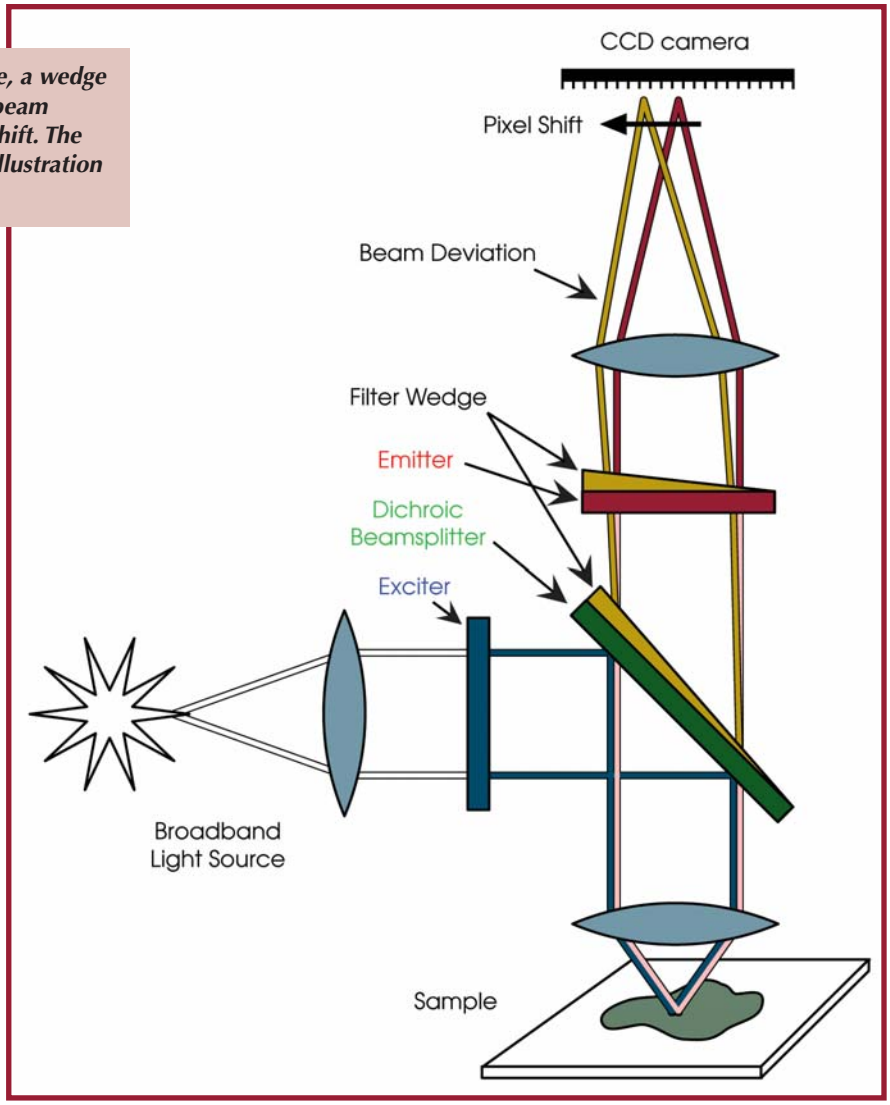

Figure 2. In an epifluorescence microscope, a wedge angle on the dichroic or emitter causes a beam deviation (gold path) that results in pixel shift. The wedge angles are greatly exaggerated for illustration purposes.

mission, the epoxied interfaces create undesirable scattering and autofluorescence, can absorb water vapor and can photodarken.

But for the purposes of the discussion here, the main drawback is the difficulty of achieving a low overall wedge with this composite structure. Although the finished filter can be postprocessed — before mounting in the aluminum ring — to improve the parallelism, this is laborious and therefore costly, and such esoteric filters may not be kept as stock items.

Beam deviation

The beam deviation — usually measured in arc seconds or degrees/3600 — caused by the dichroic beamsplitter is about 81 percent of the wedge angle, and for the emitter, about 52 percent. The dichroic beam deviation is larger because the filter is at a 45° angle. In an infinity-corrected microscope, the number of pixels of shift at the CCD camera is about equal to 5×10^{-3} times the product of the tube lens focal length (in millimeters) and the beam deviation (in arc seconds), divided by the pixel spacing of the camera (in microns).

For example, in a microscope with a typical tube lens focal length of 200 mm and a modern CCD camera with a pixel spacing of 6.7 µm — and absent any fortuitous cancellation effects between the wedge angles of the two filters — the wedge angles must be limited to the range of a few arc seconds to achieve zero-pixel-shift performance (Figure 4). As the pixel spacing decreases for a higher-resolution camera, the requirements on the filters become even more demanding.

Pixel shift also can be caused by imperfections in the microscope and, to a lesser extent, by imperfections in the microscope filter holders (filter cubes). This source is often referred to as mechanical noise. It is particularly a problem in upright microscopes because just rotating the filter turret to exchange filter sets requires a small amount of torque on the upper arm of the microscope, which is only remotely mechanically connected to the stationary sample stage. If the upper arm, which holds the camera, does not return to precisely the same position after the filters have been exchanged, the image of the stationary sample will be shifted on the camera. Thus, it is highly recommended that an inverted microscope and low-stress mounting of the filters in the cubes be used to achieve very low pixel-shift performance.

It is always desirable to obtain a multicolor image that is as precise a representation of the actual sample as possible, but certain applications are particularly sensitive to pixel shift.

For example, various techniques employed to study cellular genetics, such as karyotyping, comparative genomic hybridization and multicolor FISH, are particularly dependent on low pixel shift. Multicolor FISH is especially challenging. The method sometimes uses five or more fluorophores to tag various DNA probes.[1] These probes are hybridized to complementary target DNA sequences in a sample set of chromosomes. Five fluorophores can be used in various combinations to label a particular DNA probe and to provide many more effective colors. This can allow 24-color experiments, in which the 22 human autosomes and both sex chromosomes can be visualized simultaneously.

Without correction, DNA sequences can be misidentified with these cytogenetic techniques. Sophisticated software algorithms can correct for pixel shift in the images, but this technique is time-consuming, has limited accuracy and often cannot be fully automated (requiring an operator to manually click on reference points on the images to help the computer implement the correction algorithms). Automation is especially important for these applications because they are used not only in research, but also in clinical diagnostics.

Collocalization analysis using fluorescence imaging is another technique that is sensitive to pixel shift. This analysis de-

termines whether two or more molecules tagged by different colored fluorophores are attaching to one another or whether they are simply migrating to the same location. By studying a statistically significant number of molecules in a given sample, distance correlation analysis can be performed to determine if collocalization is occurring.

Researchers use collocalization to study, for example, the dynamics of and the interactions among the various components that a cell employs to accomplish endocytosis.[2] Unfortunately, the variance of the correlation coefficients can be dramatically magnified by pixel shift, sometimes to the point of appearing statistically insignificant when there truly is a correlation.

In another example, researchers Mohan Gupta and David Pellman at Dana-Farber Cancer Institute and Harvard Medical School in Boston are studying microtubule interactions with chromosomes and with the cell cortex to understand how cell signals regulate chromosome segregation and polarized morphogenesis, which relates to asymmetric cell shape and orientation. By combining the genes for certain proteins with different color variants of GFP, they can follow the localization and dynamics of several proteins at the tip of an individual dynamic microtubule in a living cell.

The researchers imaged two fixed cells that had microtubules labeled with CFP and karyogamy protein labeled with YFP. Karyogamy protein is required for correct positioning of the mitotic spindle and for orienting cytoplasmic microtubules, and it localizes at the tip of the microtubules in certain situations. To determine whether the YFP-labeled karyogamy protein is at the tip of the linear CFP-labeled microtubule, very high resolution and accurate pixel registration are required, and they achieved this (Figure 5). If pixel shift had been present, they would not have achieved accurate distance measurements (Figure 6).

Correcting/eliminating pixel shift

Software can be used to correct pixel shift after the images are acquired, but this is undesirable for many applications. It can be done perfectly only if one knows exactly what the two or more images should look like, which can be the case when imaging, for example, multicolor fluorescent microspheres designed for aiding image alignment. These spheres are smaller than the diffraction-limited resolution of the microscope. But in practical applications, one must rely on the assumption that a pixel-shift calibration done prior to the actual experiment is sufficiently accurate for use during the experiment. Microscope component or sample changes as well as mechanical drift over time can reduce the accuracy. Furthermore, software calibration is complex and time-consuming, thus limiting the speed with which experiments can be carried out.

Pixel shift can be eliminated altogether by using a multiband beamsplitter and a multiband emitter filter, switching only the exciter filters (positioned in a filter wheel) to achieve independent sequential imaging of the various color fluorophores on a sensitive monochrome camera. This multi-exciter approach was first

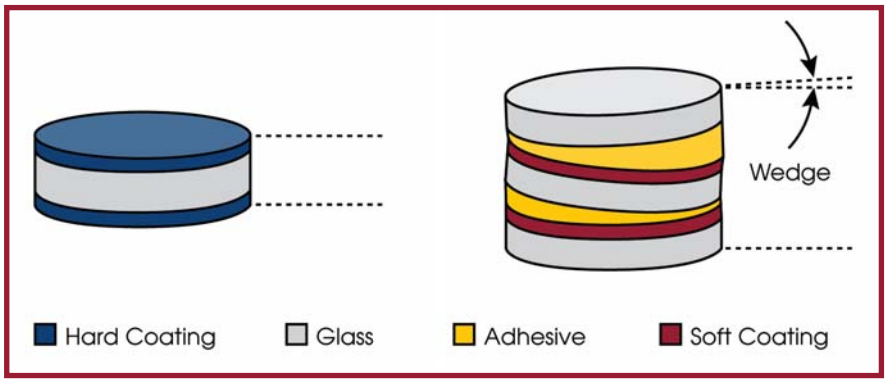

Figure 3. Fluorescence filters (left) manufactured with hard oxide coatings using ion-beam sputtering have a simple structure that makes it straightforward to achieve low wedge and zero-pixel-shift performance. Traditional fluorescence filters (right) are based on laminating multiple substrates with soft coatings using optical adhesives, making it difficult to achieve a low overall wedge. Diagrams are not to scale, and the angles are greatly exaggerated for illustration purposes.

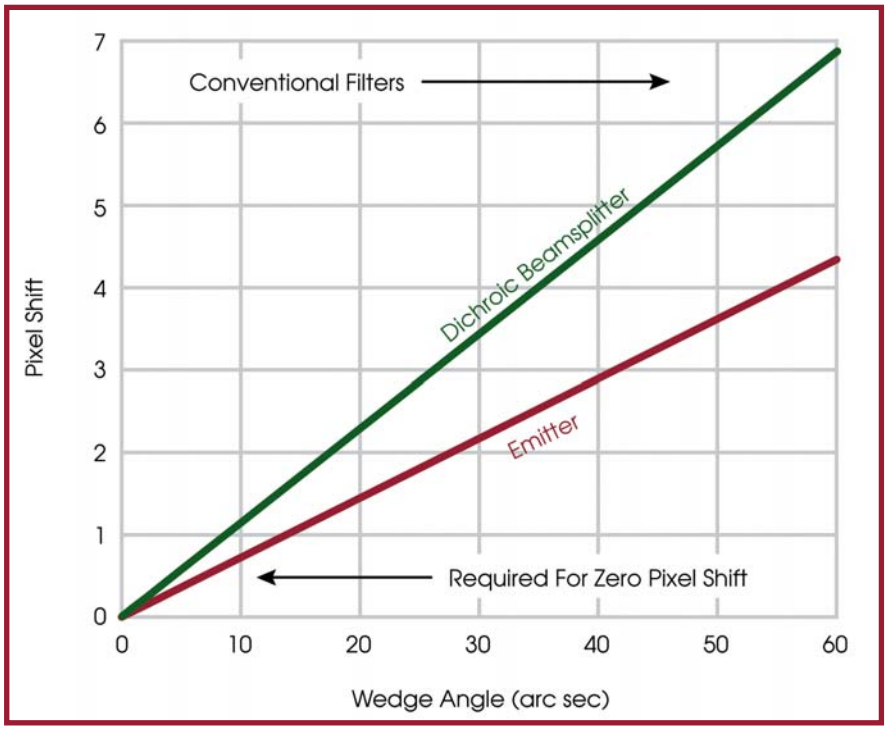

Figure 4. As the wedge angle in the dichroic beamsplitter or the emitter increases, it results in pixel shift at the CCD camera. An infinity-corrected microscope with a 200-mm-focal-length tube lens and 6.7-µm pixel spacing are assumed. To guarantee less than one pixel of shift for any filter mounting in a cube, the sum of these curves must be considered.

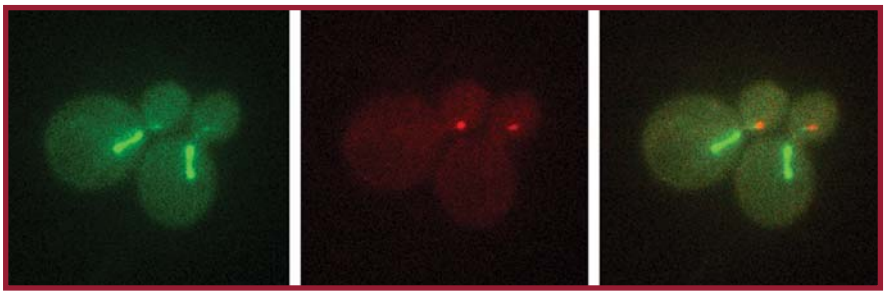

Figure 5. Images of two Saccharomyces cerevisiae *("baker's yeast") cells that contain tubulin labeled with CFP and karyogamy protein labeled with YFP were obtained using a Carl Zeiss microscope with a 100×, 1.45-NA objective. Image (a) was taken with a low-pixel-shift CFP filter set and falsely colored green, and image (b), with a YFP filter set and falsely colored red. There is almost no pixel shift between the CFP (green) and YFP (red) images, as evidenced by the fact that the karyogamy protein clearly appears at the very tip of the microtubule in the merged image (c). Each image is 250 × 250 pixels.*

proposed by Daniel Pinkel, a professor of laboratory medicine at the University of California, San Francisco.

Although it works for some applications — especially those that require very high speed filter changes — image fidelity suffers from the fluorophore crosstalk that occurs because all emission bands are present in every measurement. For applications that cannot tolerate the increased crosstalk, users can put single-band emitters into a filter wheel that is synchronized to the exciter wheel. This method is expensive, and the pixel shift caused by imperfections in the emitters must still be corrected or eliminated.

The most straightforward and effective way to eliminate pixel shift is to use filter sets that do not suffer from this problem. One technique is hand-selecting pairs of emitter and dichroic filters with similar beam deviations and carefully aligning (orienting) the filters with respect to one another in the filter cube so that the two beam deviations cancel each other. However, once carefully aligned, the filters cannot be removed or replaced in the cube except by the filter vendor, for whom this approach can be prohibitively expensive, as the yield for achieving just the right matches of emitters and dichroics is low.

Fortunately, recent advances in the technology used to fabricate fluorescence filters have made it possible to produce filters that deliver superior spectral performance, that do not suffer from reliability problems such as burnout, and that can be designed for zero-pixel-shift imaging performance via a straightforward, highly manufacturable process.

Durable filters

Filters manufactured with the new technology are much simpler in structure than previous ones. The new coating is based on ion-beam sputtering of "hard" oxide glass materials (as hard as the glass substrates on which they are coated). The simpler structure eliminates epoxies that often scatter light or autofluoresce. And hard oxide materials provide the highest possible brightness (Figure 7). The filters are extremely durable — they can be cleaned like any glass optics, will not burn out even under prolonged exposure to intense arc-lamp (and laser) light sources and are not affected by humidity.

A key difference between standard versions of these new filters and those specified to deliver zero-pixel-shift performance is that the manufacturing process for low-pixel-shift filters starts with very low wedge substrate glass. The increased cost of this glass accounts for the small price premium associated with the low-pixel-shift versions.

With these filters, almost any microscope user can achieve zero-pixel-shift imaging, even in an older microscope. Current specifications guarantee that the image will shift less than one pixel relative to a "correct image" when exchanging these filter sets, based on a 200-mm-focal-length tube lens and 6.7-µm CCD camera pixel spacing. Because the pixel shift is proportional to the tube lens focal length and inversely proportional to the pixel spacing, the specification in pixels varies slightly for different systems.

Many benefits

There is no special alignment or assembly required to install the filters in a cube, so a microscope user can populate his or her own cubes, or exchange filters as often as desired. Because the filters are manufactured using a straightforward process, the added zero-pixel-shift performance is affordable, and the filters are readily provided from stock. In fact, given their low cost and long lifetime, it may be prudent for microscope users to add the zero-pixel-shift option whether or not they need it today.

The choice of the appropriate filters affects all aspects of fluorescence imaging systems and can, by itself, eliminate

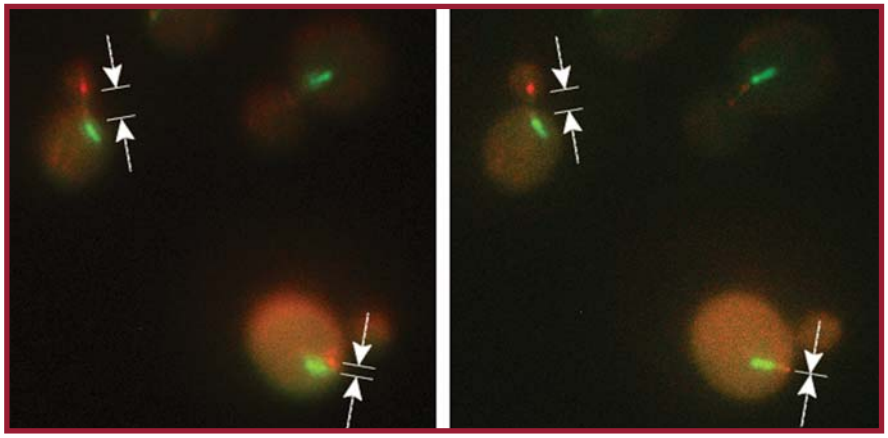

Figure 6. Separate images measured with standard CFP and YFP filter sets that exhibit appreciable pixel shift (left), and zero-pixel-shift CFP and YFP filter sets (Semrock's BrightLine Zero filter sets) (right) are merged. The clearly observable pixel shift (left) is predominantly vertical, as evidenced by the significant lateral offset of YFP-labeled karyogamy protein (red) from the CFP-labeled microtubule (green) in the cell on the lower right, and in the artificially long separation of the karyogamy protein from the microtubule labeled region in the cell on the upper left. Pixel shift is not observable in the image on the right.

pixel shift from the list of imaging artifacts with which microscope users must be concerned. □

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Meet the authors

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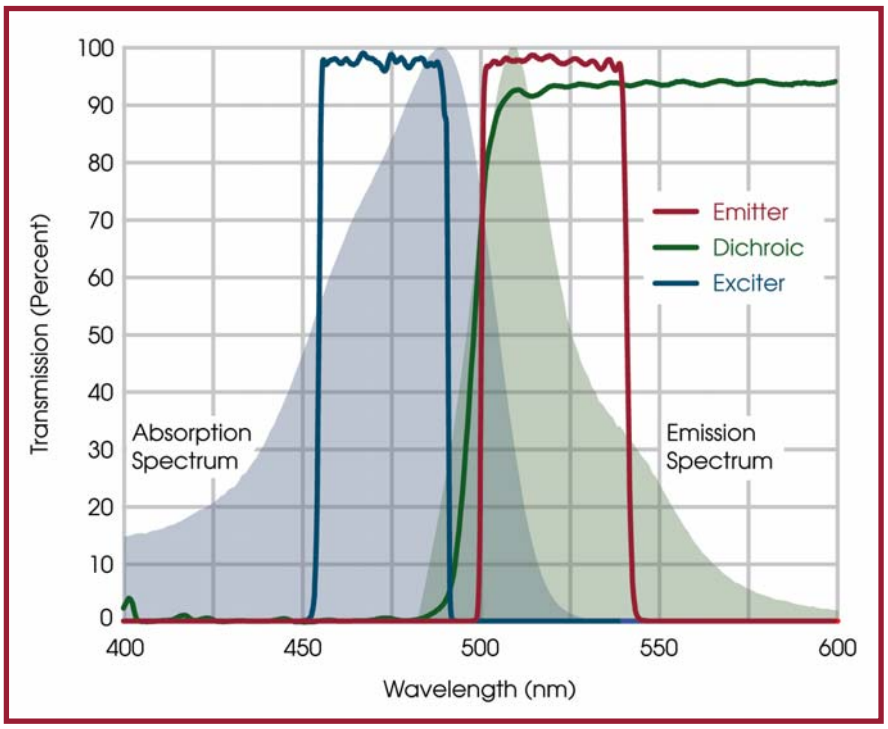

Figure 7. The spectral performance of the three filters from Semrock's BrightLine GFP-3035B set is shown overlaying the absorption and emission spectra of GFP.