Optimize Your System with the Right Filter Set

by Roy K. Kinoshita

SUMMARY

Fluorescence imaging requires the user to know how to make the right filter selection. Filter sets are designed around the system and the application. The light source, fluorophore(s) and detector drive the spectral requirements, and the microscope make and model dictate the physical requirements.

Choosing a filter set for a fluorescence application can be difficult, but knowledge of the microscope, light source, detector and fluorophore(s) can make the decision easier. The optical properties of filter sets correspond to the application-specific fluorophore excitation and emission spectra. The physical dimensions — size and thickness — are tailored to the specific instrumentation hardware.

Filter sets

Successful fluorescence imaging requires three filters mounted as a single unit in a filter cube or holder, secured in a fluorescence microscope with the proper light source and detector. The excitation filter, positioned normal to the incident light, has a bandpass design that transmits the wavelengths. The filtered excitation light reflects off a long-pass dichroic mirror placed at 45° and excites the fluorophore. The mirror has the unique ability to reflect more than 90 percent of the light within the reflection band while passing more than 90 percent of the light in the transmission region. This directs excitation light and fluorescence emission appropriately within the optical setup.

Following excitation, the fluorophore emits radiation at some longer wavelength, which passes through the dichroic mirror and emission filter into a detector. The emission filter blocks all excitation light and transmits the desired fluorescence to produce a quality image with high signal-to-noise ratio (Figure 1).

Filters are manufactured to rigorous physical and spectral specifications and tolerances. For example, emission filters have physical specifications that take into account transmitted wavefront distortion, parallelism and flatness. They also are ground and polished to an identical

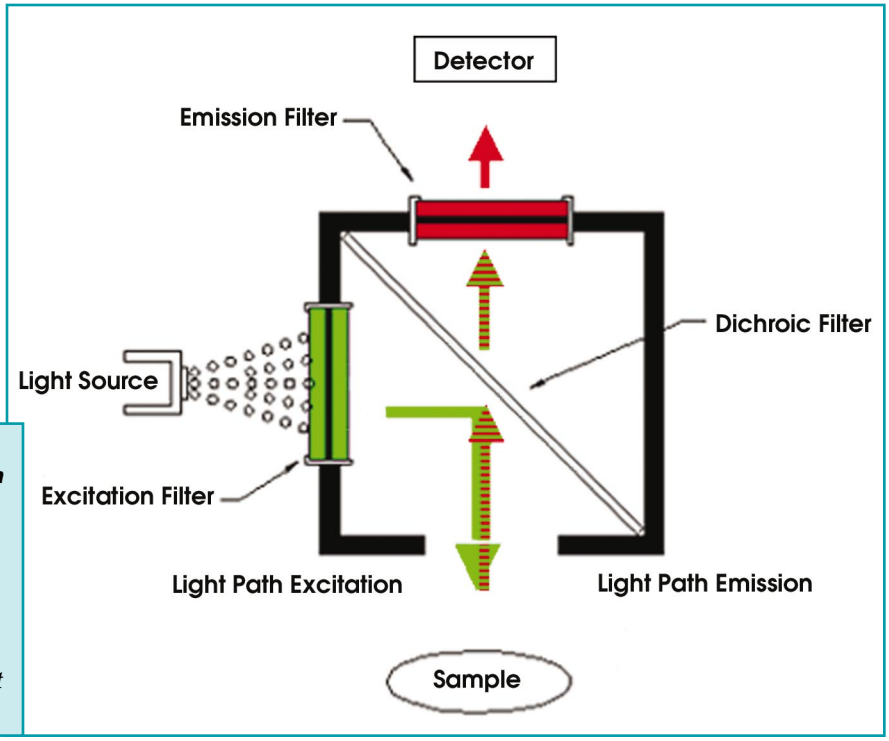

Figure 1. In a fluorescence filter cube, the incident light passes through the excitation filter. The filtered light reflects off a dichroic mirror, striking the fluorophore. The longer-wavelength fluorescence emission passes through the dichroic mirror and emission filter to the detector. The emission filter blocks stray excitation light, providing bright fluorescence against a dark background.

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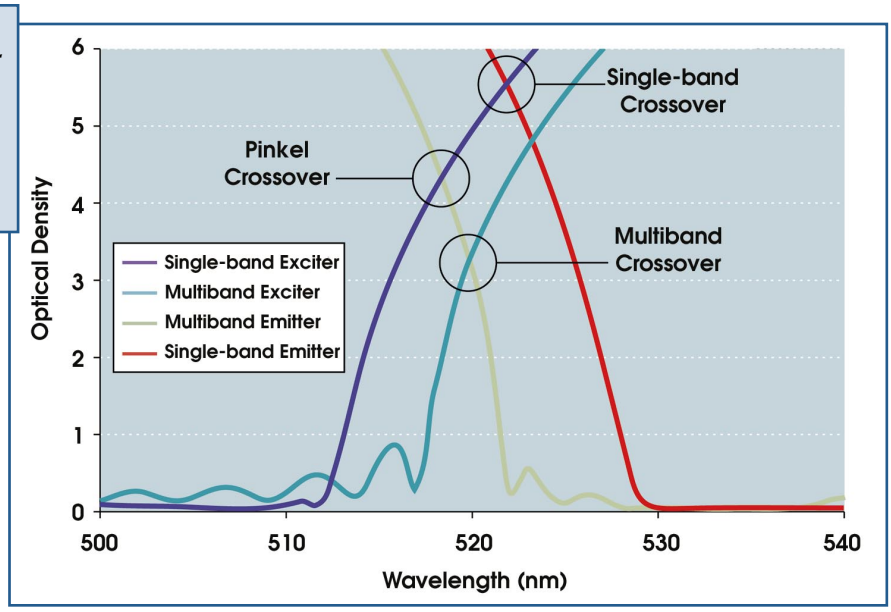

Figure 2. Higher optical density crossover darkens the background. Single-band filter sets have the highest crossover, resulting in the best signal-to-noise ratio. Pinkel and multiband sets produce moderate blocking with compromised signal-to-noise.

thickness to maintain clarity and focus when interchanged. A filter set is designed so that the tolerances of the three filters are compatible. It is important to note that filters cannot be randomly interchanged without the possibility of compromising performance.

Tight tolerances

To ensure an optimal signal-to-noise ratio, which produces bright fluorescence emission against a black background, excitation and emission filters must meet tight transmission and blocking tolerances. Dichroic mirrors also must meet tight tolerances Optical density, OD, the degree of blocking, is calculated as −log (transmission). For example, OD1 = 10 percent transmission, OD2 = 1 percent transmission and OD3 = 0.1 percent transmission. Background blackness is controlled by attenuating excitation light through the emission filter. The degree of attenuation is determined at the wavelength where light passes through both filters, known as the point of crossover blocking, and toleranced to ≥OD5 (Figure 2). Multiband sets cross over at ≥OD3 because multiple passbands yield moderate blocking, resulting in a lower signal-to-noise ratio.

Bandpass filters consist of either a single interference coating or a combination of a short-pass filter, which transmits a given wavelength and all shorter ones to about 300 to 400 nm, and a long-pass filter, which blocks a given wavelength range and transmits longer wavelengths (Figure 3). The steepness of the transition between the transmission and near-band blocking — important design and performance features — depends on the filter design and phase thickness (number of optical interference coating layers). More filters are placed in series to extend blocking to the UV and/or the IR.

Coatings with a high phase thickness produce the steepest transition region available, characterized by a 1 percent, five-decade slope factor. This means that a 500-nm long-pass filter (the wavelength at 50 percent transmission) will achieve OD5 blocking (0.001 percent transmission) at 495 nm, or 500 nm minus 1 percent. Less demanding and less expensive designs have five-decade slope factors of 3 to 5 percent. Designs and manufacturing technologies produce filters of radically different band shape and throughput, allowing application-specific properties.

To extend blocking, coated and absorption glasses can be added to the filter assembly. A typical filter consists of multiple glass stacks laminated together. Arrows printed on the filter rings indicate the orientation at which the incident light is selectively reflected prior to absorption (if applicable). This minimizes thermal load and optimizes filter life. In addition, autofluorescing substrates are placed downstream in the glass stacks to minimize their excitation. Because detectors are sensitive to energy in the near-IR, excitation filters must be fully blocked out of band from the UV to 1150 nm. Emission filters, on the other hand, must be blocked only on the low side. Adding a red-side blocker is usually unnecessary and will reduce transmission. For applications that exclude the excitation filter, or for multiphoton microscopy, blocking strategies differ and should be reviewed prior to purchase.

System-based needs

Epifluorescence systems are the most common in fluorescence microscopy. Standard filter sets for this have transmission and blocking optimized for the application's fluorophore(s) and the white light used for excitation, usually a mercury arc or xenon arc lamp. The mercury arc lamp is most commonly used because of its brightness. Its five energy peaks — 365, 405, 436, 546 and 577 nm — affect application performance and are considered in the filter set designs. The xenon lamp, though not as bright, irradiates fairly uniformly between 300 and 800 nm with energy peaks beginning at ~820 nm; it is recommended for ratio imaging.

The Nipkow disc scanning confocal

Decoding filter descriptions

Filter descriptions are identified by center wavelength, filter design code and full width half maximum. The values are nominal; actual values vary within accepted manufacturing tolerances. For example, a 490DF20 filter has a 490-nm center wavelength, a Fabry-Perot design and a 20-nm bandwidth, or a 480- to 500-nm passband (all values are approximate). A 490DF20 passband is 490 ±10 nm, not 490 ±20 nm. The blocking characteristic of the filter is inadequately explained in the description alone.

microscope contains optics similar to those in the epifluorescence system and thus requires similar filters. However, laser scanning confocal microscopes require filters designed for the specific laser used for excitation. The secondary lines and other unwanted background signals caused by lasers demand customized excitation filters. Emission filters must have much greater than OD5 blocking and antireflection coatings on both sides to minimize skew rays reflecting off secondary surfaces. As in epifluorescence systems, dichroic mirrors must efficiently reflect specific laser wavelengths and transmit the desired fluorescence.

Multiphoton microscopy, another laser-based fluorescence technique, requires a tunable pulsed Ti:sapphire infrared laser. This light source excites shorter-wavelength fluorophores, contrary to conventional fluorescence systems. At the focal point, a fluorophore absorbs two photons simultaneously. The combined energy elevates the fluorophore's electrons to a higher energy level, causing it to emit a photon of lower energy when the electrons return to the ground state. For example, a 900-nm laser pulse will excite at 450 nm and yield fluorescence emission at ~500 nm, depending on the fluorophore. This technique generally uses a combination of a short-pass dichroic mirror and an emission filter with deep blocking at the laser line. An all-purpose multiphoton short-pass dichroic mirror reflects radiation between 700 and 1000 nm — the range of Ti:sapphire lasers — and transmits visible light. The emission filter must transmit fluorescence and block the laser light to more than OD6.

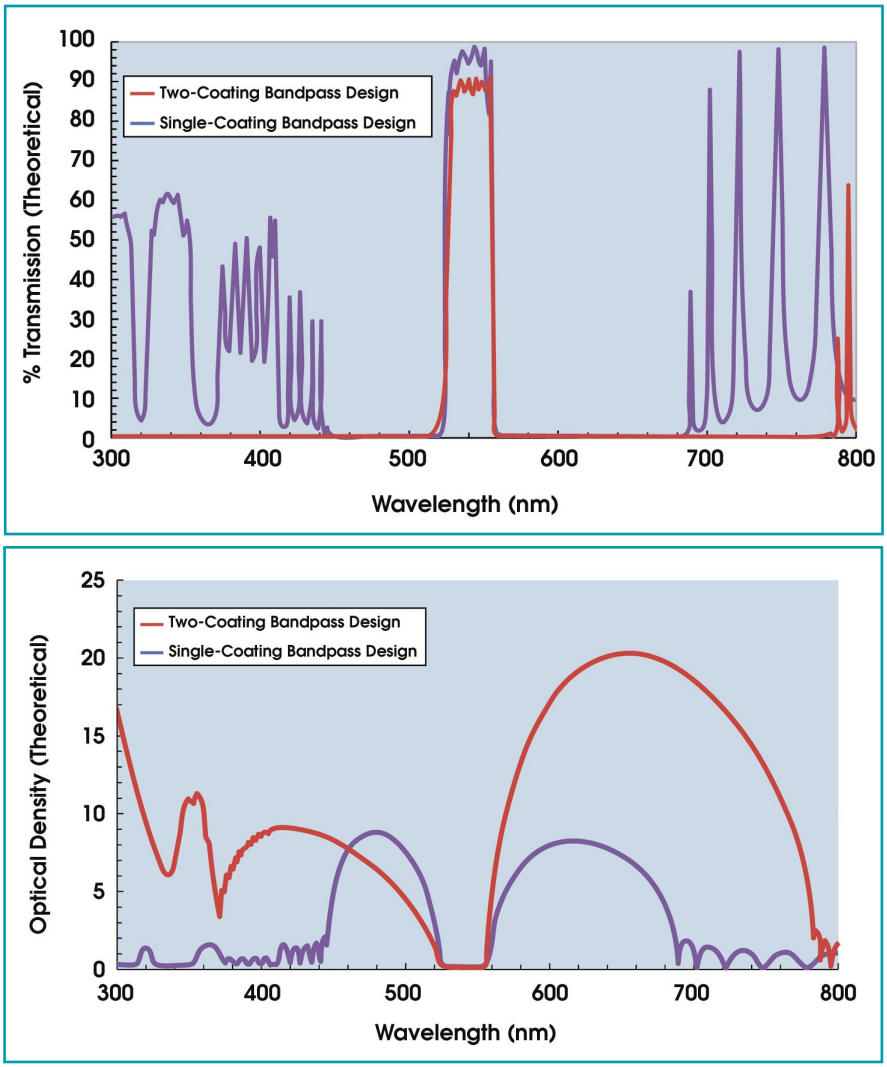

Figure 3. In the theoretical transmission of two bandpass designs (top), the differences in passband transmission are moderate, but the out-of-band transmission is more attenuated using the two-coating design. The corresponding theoretical blocking curves (bottom) show that the single-coating design has moderate near-band blocking, while the short-pass/long-pass design has deeper and more extensive blocking.

Application relevance

A number of applications have been developed around epifluorescence, and some are being extended to confocal and multiphoton. For instance, the ratio imaging technique can be used to quantify environmental parameters such as calcium-ion concentration, pH and molecular interactions, and it demands a unique set of filters. For example, Fura-2, a calcium-dependent fluorophore, has excitation peaks at 340 and 380 nm, necessitating excitation filters that coincide with the peaks and a dichroic mirror that reflects them. The xenon arc lamp is an ideal excitation source for epifluorescence because of its uniform intensity over the excitation range. A mercury arc source may require additional balancing filters to attenuate the effects of the energy peaks.

In fluorescence resonance energy transfer (FRET), energy is transferred via dipole-dipole interaction from a donor fluorophore to a nearby acceptor fluorophore. The donor emission and acceptor excitation must spectrally overlap for the transfer to happen. A standard FRET filter set consists of a donor excitation filter, a dichroic mirror and an acceptor emission filter. Separate filter sets for the donor and acceptor are recommended to verify dye presence, but most importantly, single-dye controls are needed because donor bleedthrough into the acceptor emission filter is unavoidable (Figure 5).

Multicolor imaging is extending to 800 nm, with far-red fluorophores readily available and CCD camera quantum efficiencies being pushed to 1200 nm. There are a multitude of filter combinations from which to choose, depending on the application. Each has its own advantages and disadvantages. A standard multiband filter set allows simultaneous color detection by eye and is designed

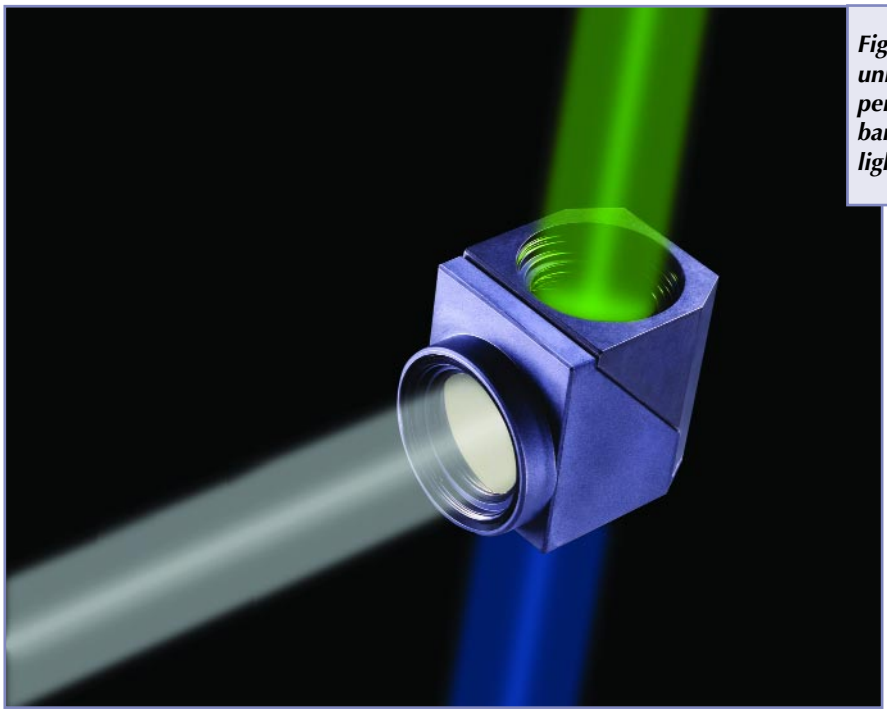

Figure 4. The dichroic mirror has the unique ability to reflect more than 90 percent of the light within the reflection band while passing more than 90 percent of light in the transmission region.

for conventional fluorophores such as DAPI (blue), fluorescein (green) and rhodamine/Texas red (orange/red). Two- and three-color sets are most common, while the fourth "color" in a four-color set includes a fluorophore in the 650- to 800-nm range. Multiple passbands limit the deep blocking achieved in single-band filter sets, resulting in a lower signal-to-noise ratio from multiband sets.

For an increased signal-to-noise ratio and better fluorophore-to-fluorophore discrimination, Pinkel filter sets for the camera consist of single- and multiband filters. For microscopes that are equipped with an excitation slider or filter wheel, changing single-band excitation filters allows single-color imaging of multilabeled samples. The Pinkel filter holder and sample slide remain fixed, minimizing registration errors.

A Pinkel set hybrid combines a similar suite of single-band excitation filters in a filter wheel; a set of single-band emission filters, along with an emission filter slider or wheel; and a multiband dichroic housed in a filter holder. Such a hybrid setup will increase the signal-to-noise ratio and discrimination even more than a traditional Pinkel set. The disadvantages of Pinkel sets to multiband sets include increased filter cost and the inability to image multiple colors simultaneously. Instead, commercially available imaging software can be used to merge separate images.

Fluorescence in situ hybridization (FISH) applications attempt to image as many colors as possible in a single sample. For example, multiple fluorescently labeled DNA probes can identify genes colorimetrically on a single chromosome. Optimized signal-to-noise ratio and color discrimination require narrowband single-dye filter sets. The filters must conform to tighter spectral tolerances than standard bandpass filter sets to minimize excitation/emission overlap of spectrally close fluorophores. Minor passband edge shifts may significantly compromise fluorophore discrimination. In addition, these narrowband filters must be optimized for transmission to provide adequate signal. Tight tolerances and strict blocking strategies tend to increase manufacturing costs for FISH filter sets.

Choosing optical filter sets for fluorescence microscopy can be confusing. Proper bandwidths, degree and extent of blocking, and the type of filter design for your application are also important considerations. A filter manufacturer can help with your decision-making. □

Meet the author

Roy K. Kinoshita, fluorescence microscopy product manager at Omega Optical Inc. in Brattleboro, Vt., received a PhD in biomedical sciences from the State University of New York at Albany.

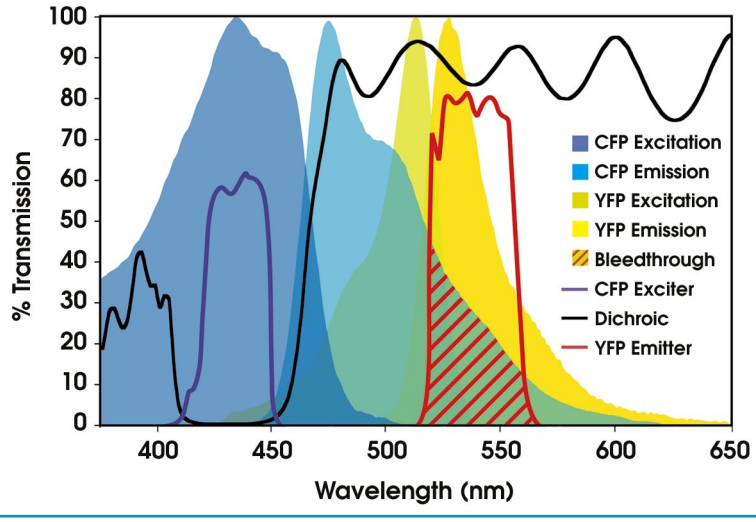

Figure 5. In FRET applications, it is important to quantitate cyan fluorescent protein bleedthrough and subtract the background from the FRET analysis. This is because the cyan fluorescent protein emission (donor) overlaps the yellow fluorescent protein excitation (acceptor) in the area where energy transfer occurs and cyan emission overlaps the yellow emission, causing bleedthrough of cyan into the yellow emission filter.