



The Right Filter Set Gets the Most out of a Microscope

By Jennifer Kramer

SUMMARY

Knowing the basics of how to select a filter set for different fluorescent labels or multiple labels will help researchers get the best data from fluorescence experiments.

Filter sets, a basic component of fluorescence microscopy, are extremely important to the success or failure of experiments. Often researchers spend months choosing the correct microscope, camera and image processing equipment, but give short shrift to choosing the correct filter set. That decision can compromise the optical performance of the system.

A filter set has three components: an exciter filter, a dichroic mirror and an emission filter. These parts are located in a filter cube, so they can be moved easily in and out of a microscope. To choose the best filter set for any given application, users need to understand a few basic

concepts. Although there are exceptions, understanding the guidelines will enable users to cope with the exceptions and will prevent filter selection from becoming confusing and intimidating. (For a list of common filter terminology, see the box on page 55.)

Know the light source

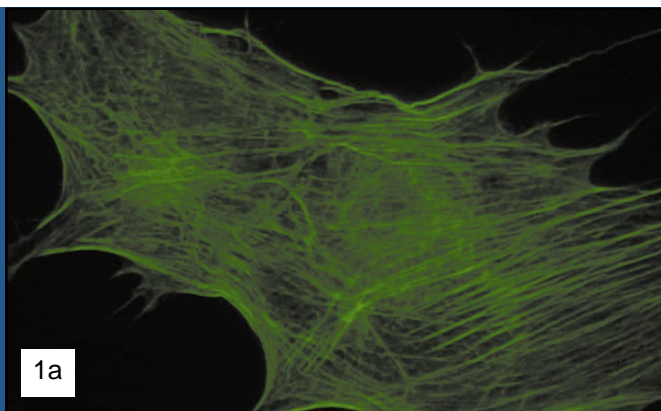
Typically, an epifluorescence microscope will have either a mercury or a xenon arc lamp. Usually, the lamp type and its wattage rating are marked on the microscope's lamp housing or its power supply box.

Microscopes for general fluorescence imaging typically come with a mercury arc lamp and are marked with "Hg" or "HBO" and/or wattage

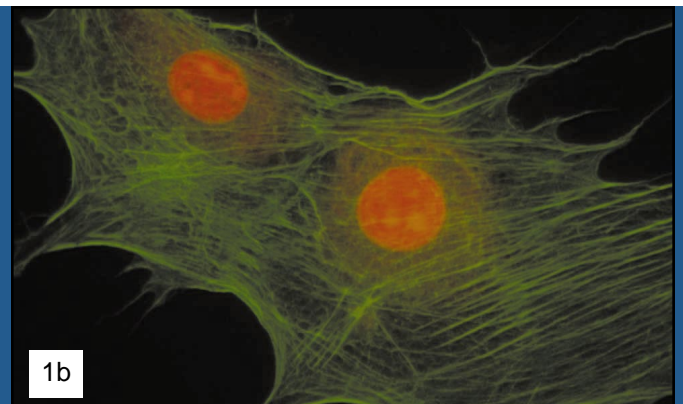
ratings of 50 or 100 W. The mercury arc lamp's power output spectrum contains energy peaks at various wavelengths that can be exploited in imaging. These peaks can allow the excitation filter to be of a narrower bandwidth, which will reduce photobleaching at less important wavelengths. For example, an excitation filter that passes only a narrow slice of the spectrum around the 546-nm spike in the mercury power spectrum allows researchers to get the maximum signal when using rhodamine or Cy₃ markers.

Microscopes used more for ratio imaging or imaging in the far red may have a xenon arc lamp. They usually are marked "xenon," "Xe" and "XBO" and have wattage ratings of 75 or 150 W. Xenon's power curve is very flat through the visible light regions, but it increases significantly at 750 nm and continues to be strong through 1000 nm. The relative evenness of power throughout the visible spectrum makes this light source attractive for ratio imaging.

Some microscopes have both types of lamps; in this case, the users must decide which light source they plan to use for their applications. Also, if the microscope has no indication of the light source type,



A bandpass filter (1a) excludes the red signal from mouse fibroblasts that have the actin filaments labeled with Bodipy FL phalloidin (green) and the nuclei with ethidium homodimer 1 (red). The same image seen through a long-pass emission filter (1b) shows bleedthrough of the red signal.



FILTERS FOR MICROSCOPY

users should get that information from the manufacturer.

For the most part, only confocal microscopes use lasers for illumination. The two most common are the argon-ion and krypton-argon lasers. Various types of helium-neon lasers are also used in imaging. Researchers who use a laser should know which line of the laser they intend to employ for excitation. In some cases, a laser line excitation filter helps isolate a particular laser line. In other cases, foregoing the excitation filter is useful, particularly for monochromatic sources, because the standard narrowband filters used to isolate laser lines often significantly reduce laser transmission.

Detection method

The detection method is important because different detectors are sensitive to different wavelength regions. Most charge-coupled device (CCD) cameras, for example, are sensitive to wavelengths of light much farther red than the human eye. As a result, a filter set designed for eye viewing alone may allow infrared signal to bleed through to the CCD camera. With the wrong filter set, an image that looks great through the eyepieces may look saturated to a camera. Usually, this is more of a problem with multiband filters than with single dye filters, and it can be solved by adding an infrared-blocking filter in front of the camera.

Single- or multicolor labeling

Excitation and emission filters are designated bandpass, long-pass or short-pass. Although bandpass and long-pass filters are common types for fluorescence microscopy, the short-pass filter is not.

Before selecting a filter set, researchers should know whether they will be labeling samples with only one fluorescently tagged target or will have multiple targets and markers.

A bandpass filter passes wavelengths within a certain range and absorbs or reflects wavelengths outside its range. This range of wavelengths, called the passband, is specified using the full-width half-maximum designation. Full width half maximum refers to the points on the cut-on and cutoff edges where the transmission is one-half of the maximum transmission. The bandpass filter also is defined by the

Definitions of filter terminology

Absorption glass: It behaves as a normal long-pass, bandpass or short-pass filter, except that it absorbs light at wavelengths outside the transmission region. Absorption glass filters are very hardy for harsh environments because they have no coatings to burn or scratch. However, they can be autofluorescent, and this can be particularly noticeable with low-light-level imaging. Furthermore, these filters tend to have very long cutoff and cut-on profiles, meaning that they take a long time to move from transmission to absorption of light. This can be a disadvantage in imaging with dyes with narrow Stokes shifts.

Background: What the area without the sample looks like; for example, the area in between cells or outside of the tissue section.

Bandpass filter: These filters pass a given set of wavelengths of light only, while reflecting or absorbing light at other wavelengths.

Bleedthrough: This is the term used when, in multiple labeling, more than one dye is visible through a single-color filter set. Bleedthrough is also sometimes used to describe a bright background caused by light leaking from the excitation source into the emission filter.

Center wavelength: This is the center of the bandpass region of a filter, but not necessarily the region with maximum transmission.

Dichroic mirror: Placed at a 45° angle relative to the incoming excitation light, the dichroic mirror performs two functions: It reflects the excitation light to the sample and transmits the emission signal to the microscope eyepieces.

Emission (or barrier) filter: This filter goes between the objective lens and the eyepieces or camera port on the microscope. It screens out excitation light and any other light not related to the sample's fluorescence.

Exciter (or excitation) filter: This filter goes between the light source and the objective lens in the microscope's illumination. Typically it is located in a filter wheel, in an excitation slider or in the filter cube itself. The coatings on this filter are often tough enough to stand up to the harsh light from the light source.

Filter cube: The filter cube is a filter holder specifically designed for a given microscope. It holds the excitation and emission filters and the dichroic mirror in the correct positions for performing fluorescence microscopy.

Full width half maximum: The midpoint of transmission as a band of a filter begins to transmit and as it begins to end transmission. This is sometimes referred to as the 50 percent transmission point, or 50 percent of the maximum transmission. Therefore, if a filter transmits maximally at 80 percent, the 50 percent transmission point would occur at 40 percent of the absolute transmission.

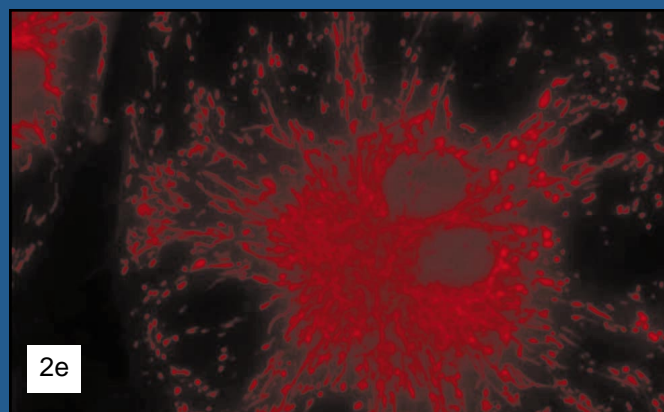
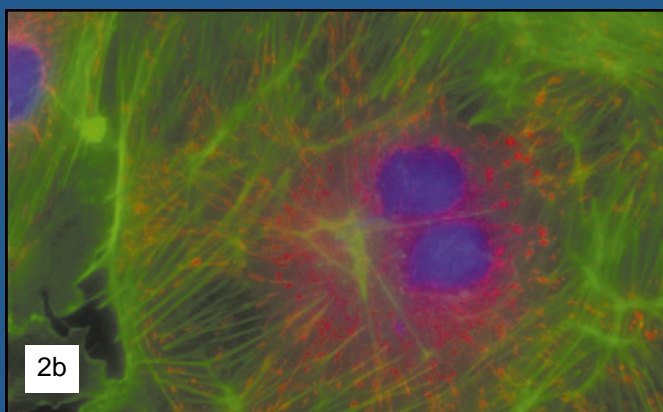
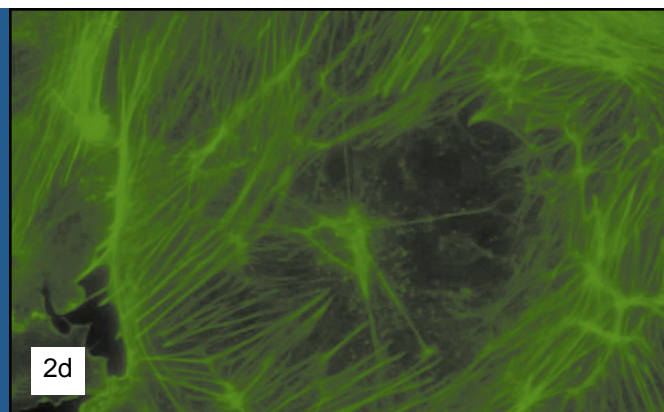
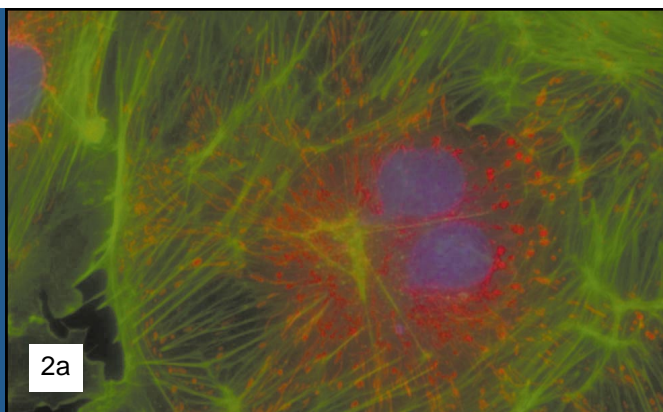
Interference filter: This type of filter contains coatings in layers that filter light by causing reflectance or destructive interference of specific wavelengths.

Long-pass filter: These filters pass all wavelengths of light from a given wavelength to the red of that wavelength (or to all longer wavelengths of the designated point).

Narrowband: A bandpass filter with a very narrow band, typically 1 to 3 nm wide. It can be used effectively only with a laser or other high-intensity light source, because the narrow bandwidth often reduces transmission significantly.

Short-pass filter: These filters pass all wavelengths of light from a given wavelength to the blue of that wavelength (or to all shorter wavelengths of the designated point).

Stokes shift: The distance between the peak absorption and emission of a dye, usually in nanometers.



A single exposure using a multiband filter (2a) of the fluorescent labels DAPI (blue), Bodipy (green) and MitoTracker (red) has poorer color balance than an overlay (2b) of three images (2c, d and e) taken with individual filter sets.

For instance, a filter that is marked 515EFLP or 515LP has half its maximum transmission capacity at 515 nm, and all wavelengths above 515 nm will transmit through the filter.

Excitation filters are almost always bandpass filters. Emission filters can be bandpass or long-pass filters. A long-pass filter set usually contains a bandpass excitation filter, a long-pass dichroic mirror and a long-pass emission filter. A bandpass filter set contains a bandpass excitation filter, a long-pass dichroic mirror and a bandpass emission filter.

Single-label filters

Labeling only one target or antigen provides the most flexibility in choosing a good filter set. Either a long-pass or bandpass set will work for these applications. Researchers who are not planning to

expand from single labeling into multiple labeling may want to purchase a long-pass set because these filters maximize the amount of signal passing to the detector. This reduces the exposure time needed by the detector, decreasing the time the sample is illuminated and, in turn, helping to reduce the amount of photobleaching in the sample.

Long-pass filters have some drawbacks, such as bleedthrough. For example, a researcher who combines rhodamine labeling with fluorescein labeling but uses a long-pass fluorescein filter set will likely see rhodamine signal through those filters. Long-pass filters also tend to have a brighter background than bandpass filters, which have blacker backgrounds. Although some filter sets have a completely black background, it is not uncommon for long-pass filters to have a gray or slightly colored background. In the case of a fluorescein long-pass set, for example, the background might be pale green, whereas a rhodamine filter set may have a pale orange background.

wavelength that occurs in the center of the passband. This wavelength, however, is not necessarily the one of maximum transmission.

In epifluorescence microscopy, bandpass filters are typically 20 to 35 nm wide, but passbands as wide as 70 nm or as narrow as 10 nm are also common. The markings on bandpass filters typically designate the center wavelength, followed by the width of the passband — for example, 530DF30 or 530/30 means that at full width half maximum, the filter transmits wavelengths at 530 ± 15 nm, or 515 to 545 nm.

A long-pass filter passes all wavelengths at or longer than a specific wavelength.

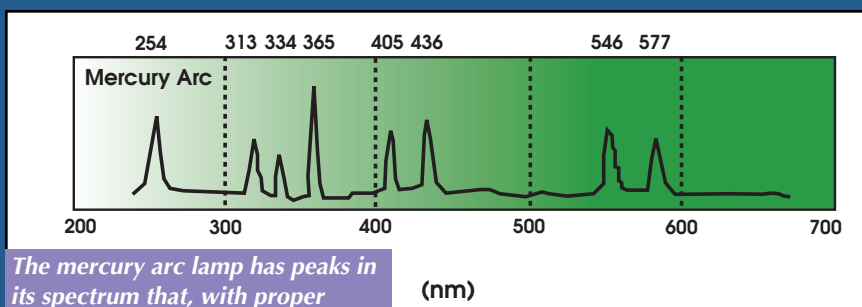
Researchers who plan to expand into multiple labeling, or who want more flexibility, should purchase a bandpass filter set. Imaging samples that are double-labeled with rhodamine and fluorescein and visualized with a bandpass fluorescein filter set should produce a minimum of rhodamine bleedthrough.

Unfortunately, there is a trade-off in the amount of signal that reaches the detector. The bandpass filter cuts off a piece of the dye's emission spectrum, reducing

necessitates computer work (in the case of a CCD or photomultiplier tube) or waiting for film to come back from the developer. Of course, if the eye is the only detector, the colors never will be visible simultaneously. In addition, sometimes a slight shift in the image is observed as filter sets are changed. A variety of small problems can cause this shift in registration, most relating to manufacturer's tolerances when making filter cubes. However, registration can be easily

or oil immersion lens, all of the labels might not be in focus at the same time because they might not all be in the same plane of focus for the lens. This is less of a problem with low-NA dry lenses (usually 40 \times or lower in magnification). Third, there is less that can be done to prevent one color from overwhelming the others. Finally, it is important to remember that with most multiband filter sets, the bands are very narrow, and dim fluorescence might be impossible to see.

In the search for the best of both worlds, some researchers have tried using a multiband dichroic mirror and emission filter with a series of individual excitation filters in a filter slider or a filter wheel. This way, a computer can change the excitation filter while imaging through the same dichroic and emission filter. The excitation filters can be of the single- or a multiband variety. Most image processing programs (shareware and



The mercury arc lamp has peaks in its spectrum that, with proper filtration, can be used to benefit fluorescence microscopy.

the amount of signal going to the detector. The result is that exposure times are typically longer and the risk of photo-bleaching increases.

Multiband filters

Labeling more than one antigen or target gives rise to more considerations in choosing the correct filter set. Imaging can be performed with a series of individual bandpass filters, for viewing each color sequentially without the signals from other labels. However, a multiband filter set enables imaging of all colors at the same time. Also, there is the possibility of a hybrid of the two: exciting with individual excitation filters and imaging through a multiband dichroic mirror and emission filter.

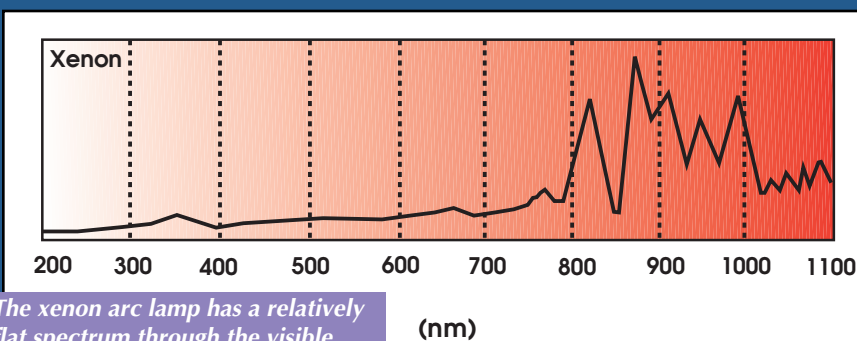
The simplest option is to select a series of individual bandpass filters to see each color by itself, one at a time. This method requires a series of multiple exposures of the sample to get all the color in one image. It has several advantages: Each color's exposure time can be adjusted so no one color overwhelms the others; dim labels are more easily seen; and it can be used with a color detector (film, color CCD) or with a black-and-white detector (CCD or photomultiplier tube).

The disadvantage to using the individual bandpass filter sets is that only one color is visible at a time. To see what all the colors look like at the same time

corrected in many image processing software packages.

Multiband filter sets, which allow a researcher to see two or more colors simultaneously through the eyepieces of the microscope, also have become popular in recent years. There are advantages to using these sorts of filters. If the eye is the only detector, this is the only way to see all of the labels at the same time. It can be convenient to photograph the samples with a multiband filter as well, because getting the full image requires only one photograph.

Multiband filters also have some drawbacks. First, to see all wavelength bands simultaneously, one must have a color detector. This usually means that the eye, film or a color CCD camera are the only detectors that can be used. Second, at high magnification using an objective lens with a high numerical aperture, such as a water



The xenon arc lamp has a relatively flat spectrum through the visible wavelengths that is good for ratio imaging.

commercially available) contain a simple program for taking a series of photographs with varying exposure times using a filter wheel. This combination of filters is often referred to as a Pinkel set, after its inventor, Dan Pinkel of the University of California at San Francisco.

This method is advantageous in many ways. First, the colors of the specimen are visible individually or all at once, depending on which excitation filter is in place. Second, it makes possible individual pictures of a sample, allowing for individual exposure times for each color to create a more balanced photograph. Third, the computer can be programmed to do the same series of exposure times at any point in the sample, streamlining the data collection process. Finally, the registration of the individual colors is usually better with a single filter cube.

The biggest disadvantage to this method is cost. Typically, a researcher will need to purchase a filter wheel and software to

control it, as well as several extra excitation filters. A less expensive method is to place the individual excitation filters in a neutral density slider in the microscope, but this cannot be computer-controlled.

Signal-to-noise ratio

A final consideration in filter selection is the signal-to-noise ratio. Some applications, such as a neuron microinjected with DiI, a very bright membrane stain, likely will have high signal-to-noise ratio. The sample background will be nearly black, and the neuron will glow so brightly that a researcher might need a neutral density filter to dim the image for eye comfort. In this case, a typical interference filter set will probably work fine.

Other applications have a low signal-to-noise ratio. For instance, a researcher imaging poorly expressed proteins with

green fluorescent protein will want to collect every last possible photon. For these applications, companies have designed a series of filters with very black backgrounds, because in many sets, the excitation light is blocked to a greater degree than with a typical interference bandpass set. The bandpasses are typically a bit wider than with other interference filters, to improve transmission. A wider bandpass allows more signal to get through the filter to the detector while maintaining the blacker background.

These filters also feature very sharp cut-on and cutoff points in transmission. In the case of dyes with very small Stokes shifts, this means that the spectra of excitation and emission filters can be positioned more closely together, allowing the filter to capture more of the dye peak. The excitation and emission filters can't

be positioned too closely together, however, or excitation light will bleed through to the emission filter, causing a high background.

The nature of fluorescence microscopy means that researchers need to understand the fundamentals of filter technology so that they can optimize their experiments. Proper filter selection can take advantage of dye and illumination selection to improve image quality by minimizing stray light while maximizing the transmission of the desired fluorescence signal. □

Meet the author

Jennifer Kramer is manager of microscopy products at Omega Optical Inc. in Brattleboro, Vt., a manufacturer of optical filters for microscopy and other applications. She received a BS in biology from the University of North Carolina at Chapel Hill.

Manufacturer	Microscope	Holder	Exciter	Dichroic	Emitter
Nikon	Labophot, Diaphot, FXA, Optiphot, Microphot	Original Holder	18 mm R	18 × 26 mm	18 mm R
		Modified Holder	20 mm R	18 × 26 mm	22 mm R
	Quadfluor Attachment Eclipse Series	Quadfluor Holder	25 mm R	25.7 × 36 mm	25 mm R
Olympus	IMT-2	IMT-2 Holder	22 mm R	21 × 29 mm	20 mm R
	BH-2, BHT, BHS AHBS 3, AHB 3, AMMT 3	BH-2 Holder	18 mm R	18 × 26 mm	18 mm R
	AX, BX, IX series	BX Holder	25 mm R	25.7 × 36 mm	25 mm R
Zeiss	Axio Series	Axio 3 (3-Position Slider)	25 mm R	25.7 × 36 mm	25 mm R
		Axio 2 Cube (On Spinning Turret)	25 mm R	25.7 × 36 mm	25 mm R
	Standard, IM-35, 3RS Photo III, Universal		18 mm R	22 mm R	18 mm R
Leica	BioMed, DMIL, Diaplan Dialux 20, 22, Diavert, Fluovert, Laborlux, Laborvert, Orthoplan 1, Ortholux 2	Ploemopak Holder	18 mm R	18 × 26 mm	18 mm R
	DMR	DMR Holder	22 mm R	21 × 29 mm	22 mm R
	Aristoplan, Orthoplan II		20 mm R	20 × 28 mm	20 mm R
	DMIRB	DMIRB Holder (XC122)	20 mm R	18 × 26 mm	20 mm R