Multichannel System Filter Selection

Introduction to Fluorescence Microscopy

Fluorescence is a quantum mechanical event whereby a molecule will absorb a photon of light and then emit a photon of light at a higher wavelength. In fluorescence microscopy, a sample containing a fluorescent probe is imaged by exciting the probe with light of a specific wavelength and then monitoring the higher-wavelength light emitted from the probe. The most common way to select the excitation and emission wavelengths for a given experiment is to use a specific fluorescent filter set that comprises three basic components: an excitation filter, an emission filter, and a dichroic beamsplitter (see Figure 1).

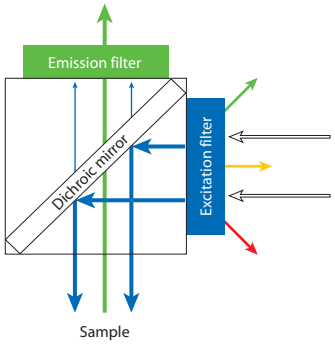

Figure 1. Schematic of a basic filter

The excitation filter blocks all wavelengths except those that excite the sample (blue in this example). The dichroic mirror reflects the excitation light 90 degrees to the sample. The sample then emits light of a higher wavelength (green in this example). This green light passes through the dichroic mirror, along with some of the blue light, towards the emission filter. Contrary to the schematic in Figure 1, the intensity of the excitation light is many times brighter than the light emitted from the sample. The primary role of the emission filter is to block this overwhelmingly bright excitation light from reaching the detector (either your eyes or your camera) while transmitting the specific wavelengths emitted from the sample to the detector.

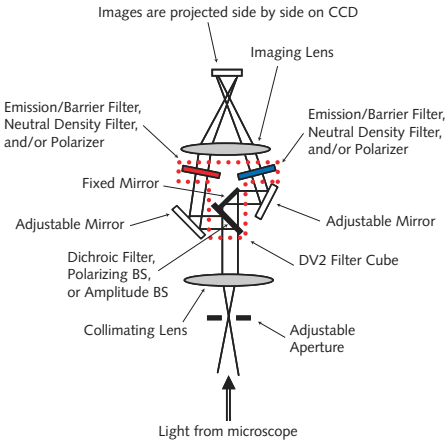

Figure 2. Schematic of the emission path of the DV2

The Photometrics® DV2™ / DC2™ / QV2™ emission filters are not typically found in the microscope, rather they are located in the emission-splitting unit, external to the microscope body (see Figure 2). As a result, the emission filters are located in the optical path *after* the light is split and sent to the eyepieces. Therefore, unfiltered light comes through the eyepieces (see Figure 3), thus rendering the eyepieces unusable unless separate emission filters are placed in the eyepieces themselves. (Note that the same result occurs if the emission filters are placed in external emission wheels.) This has serious implications when UV light and/or lasers are used for excitation, as both of these types of excitation light can potentially damage your eyes. Care should always be taken when using any optical system in which the emission filters are located after the eyepiece split.

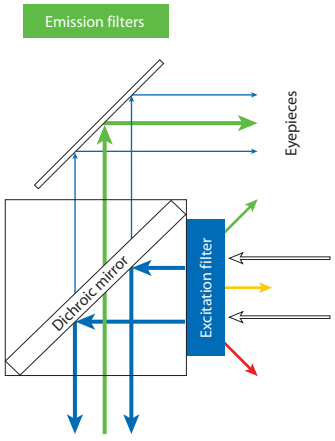

Figure 3. Schematic of a typical filter set with emission filters after eyepieces

How to Choose the Correct Filter Set for the DV2 System

The most simple, straightforward way to ensure that you select the correct filter set for the DV2 is to choose one of our standard filter sets. We have standard filter sets to work with many of the fluorochrome combinations commonly used in biological imaging.

To make sure that your filter set will result in the highest possible signal-to-noise ratio, several factors must be taken into consideration. First and foremost is that the excitation and emission filters must be chosen such that there is minimal cross-talk between these two filters.

As previously explained, the primary role of the emission filter is to block the excitation light, which will be many times brighter than the fluorescent emission. *Blocking*, when used to describe the ability of a filter to block unwanted light, is defined in terms of optical density (OD). OD is a logarithmic scale that describes how much light at a specific wavelength is transmitted through a filter (see Table 1). For example, a filter that blocks 90% of light at a specific wavelength (10% transmission) is said to be OD1 at that wavelength.

% Transmission	OD value
10%	1
1%	2
0.1%	3
0.01%	4
0.001%	5
0.0001%	6

Table 1. OD values

For an emission filter to effectively block the light passing through the excitation filter, it should block the exciter to at least OD4. Another way to visualize this is to plot the spectra of the excitation and emission filters in OD and look at the point at which the two filters overlap. In Figure 4, a typical excitation (black) and emission (blue) filter pair is shown. As you can see, the excitation and emission filters cross at ~OD5. It is also essential that the emission filter blocks the excitation filter throughout its entire bandpass region. In Figure 4, note that the emission filter has a blocking level greater than OD5 throughout the entire transmission band of the excitation filter.

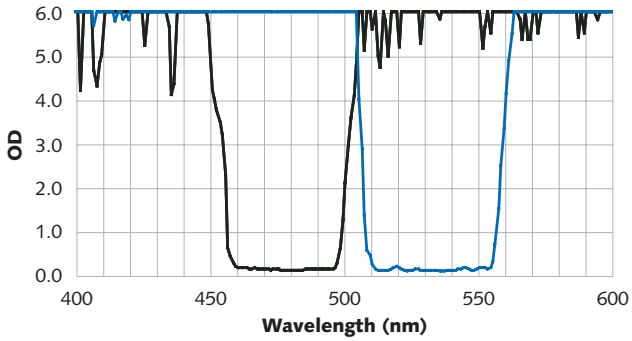

Figure 4. Spectra of an excitation and emission pair shown in OD

The second factor in designing a filter set is the correct choice of the primary dichroic mirror in the microscope. As shown in Figure 1, the role of the primary dichroic mirror is to reflect the excitation light to the sample and to transmit the emission light to the detector. The spectrum of a typical dichroic mirror is shown in Figure 5. A dichroic mirror has a defined reflection region and a defined transmission region. It is essential that the dichroic selected can optimally reflect the excitation wavelengths and transmit all of the emission wavelengths. For the DV2, this means that the primary dichroic *must* transmit both emission regions used in your experiment.

For some experiments, a wider reflection band is required. An example of this type of experiment is dual imaging with fura-2 and fluo-4. For this experiment, a dichroic mirror is needed that can reflect both blue light (for fluo-4 excitation) and UV light (for fura-2 excitation). This requirement can be met by using a dichroic mirror with extended reflection. (These mirrors are typically utilized as the emission-splitting dichroic in the DV2.) An example of this type of dichroic is shown in Figure 6. Note that, as in Figure 5, the cut-on of this dichroic is at 505 nm. The dichroic in Figure 6, however, maintains its reflection far below 450 nm. Therefore, whenever ordering a custom dichroic, it is essential to know the exact wavelengths you will need it to reflect and transmit in order to ensure that you receive the correct optic.

The same considerations are involved when choosing the correct emission-splitting dichroic for the DV2. The emission-splitting dichroic in the DV2 must reflect the lower-wavelength channel and transmit the higher-wavelength channel. Typically, utilizing an extended-reflection dichroic as the emission splitter is beneficial, as it allows a greater degree of flexibility in choosing emission filters. If the standard dichroic in Figure 5 is used, then the choice of the short-wavelength emission filter is limited to the wavelengths from 450 nm to 500 nm. If the extended-reflection dichroic in Figure 6 is used, then the short-wavelength filter can span from 350 nm to 500 nm.

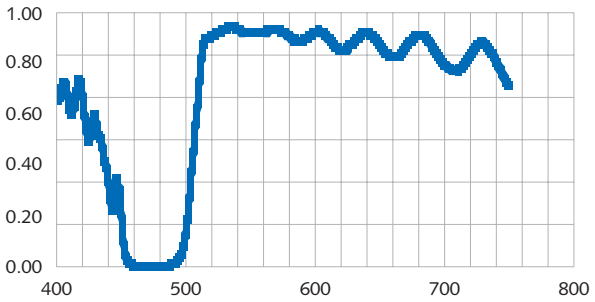

Figure 5. Spectrum of a typical dichroic mirror

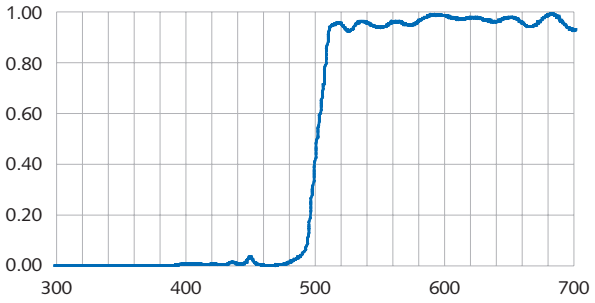

Figure 6. Spectrum of extended-reflection dichroic mirror

How to Choose the Correct DV2 Filter Set to Use with Laser Excitation

The use of lasers to excite fluorescent samples is becoming more and more common. The introduction of a laser into an optical system places additional requirements on the filters being used in both the microscope and the DV2. We have developed several filter sets designed specifically to work with many of the commonly used laser lines; however, when filter sets are needed to work with laser configurations other than those currently listed, then the following requirements must be met.

In the microscope (or somewhere in the excitation path), the typical excitation filter must be replaced with a laser cleanup filter. This substitution is necessary because most laser sources used in biological imaging do not produce light of a single discrete wavelength. Instead, they either produce multiple discrete emission lines (e.g., argon lasers) or their emission tends to be broad (e.g., diode lasers).

Laser cleanup filters ensure that only the desired laser line(s) reach the sample. Typically, a laser cleanup filter is a bandpass filter with two important characteristics. First, a laser cleanup filter needs to be ground and polished to a high degree of parallelism in order to minimize distortion of the laser beam as it passes through the filter. Second, a laser cleanup filter must have an antireflection (AR) coating on both surfaces. The AR coating minimizes back reflections of the laser off of the two outer surfaces of the filter. When the laser reflects off of the filter, it may pass back through the incident beam, thus resulting in both positive and negative interference of the incident laser. These interference patterns will result in uneven illumination, thereby degrading the final image. In addition, the back-reflected laser light may also re-enter the laser cavity, resulting in severe damage to the laser itself.

As with the laser cleanup filter, the primary dichroic mirror in a laser-based system must also be AR coated. Since the reflection of the primary dichroic is not 100% efficient, some of the laser light will pass through the coated surface and, if the back surface of the dichroic is not AR coated, be partially reflected. The consequences of this effect may vary. First, the secondary reflection may result in an additional laser spot illuminating the sample. Second, the secondary reflection may be reflected again by the front-reflected coating, producing a standing wave within the dichroic that results in additional interference patterns.

As with the excitation optics, the emission filters used with laser excitation also have specific requirements. Since laser light is typically much brighter than the light produced by a white-light source (e.g., a mercury lamp), the emission filters used in a laser system must have very high blocking at the laser wavelengths being used. To ensure that the emission filters will sufficiently block the laser line to a high OD, we only supply "HQ"-type emission filters for use with laser optics.

When ordering DV2 emission filters for use with laser excitation, it is critical that you know all of the possible laser lines that will be used with these filters. Again, it is important to reiterate that when using the DV2, the emission filters are located past the eyepiece split, resulting in potentially dangerous laser light reaching the eyepieces. Therefore, it is advisable to add an emission filter to the eyepieces to prevent unfiltered laser light from reaching your eyes.

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