Selecting Filters for Fluorescence measurements

Basic Principles: We provide several principles to follow in order to improve assay performance. The essence is to match the spectra of the light source and the spectral sensitivity of the detector with the excitation and emission spectra of the fluorophore, respectively.

Introduction: The Analyst systems use bandpass filters and dichroic mirrors to select and deliver light of chosen wavelengths. The direct approach to evaluating new fluorophores is to use the simple off-the-shelf band pass filters and dichroic mirrors available from any one of several optical manufacturers. This can be a good starting place, and often yields acceptable performance, but on occasion more tailored optical equipment is necessary to achieve the desired performance such as a superior lower limit of detection. Some consideration of optical principles can minimize the effort to achieve satisfactory performance.

Points to consider:

1. Know the excitation and emission spectra of the fluorophore. Often the supplier can provide a spectrum upon request. Be aware that environmental influences such as solvent, pH, chemistry of linkage can cause deviations in the pattern shown by the manufacturer. The separation (in nm) between the peak excitation and emission wavelengths is known as the Stokes’ Shift. Fluorophores with a narrow Stokes’ Shift (less than 20 nm) pose special challenges in developing sensitive assays, as the construction of suitable excitation and emission bandpass filters often results in individual filters with low light transmission (see section 5, below).

2. Assure that the light source provides light of the wavelength necessary to excite the fluorophore. The wavelength of a laser source is very precise, but allows little flexibility, as only fluorophores whose excitation spectrum overlap the laser light band can be excited. In contrast, broad-spectrum sources such as the xenon arc lamps used by Analyst and Acquest can provide light of all wavelengths from the UV to the near IR. Power varies with wavelength and some lamps use hot mirrors and other filters to reduce IR heating and ozone production by UV. The spectrum of the Analyst flash lamp is suitable for UV excitation; the hot mirror of the continuous lamp does remove UV light below 360 nm.

3. Assure that the optical system can transmit the light of interest. This is particularly relevant for the UV. Analyst components transmit UV. Also, because Analyst systems use epifluorescence, the role of the dichroic mirror should be considered: this longpass filter reflects short (excitatory) wavelengths, and transmits longer (emission) wavelengths. It is crucial to use a dichroic mirror designed for the paired excitation and emission bandpass filters. In addition to the longpass dichroic mirror, there are also 50-50 mirrors (beamsplitters). These are relatively wavelength insensitive, transmitting about half the light at any wavelength (within a given range). The half-silvered "polka dot" beamsplitter provides flexibility but causes diminished sensitivity.
4. Assure that the detector is suitably sensitive to the emitted wavelength. The Analyst PMT for fluorescence detects to 700 nm. Longer wavelengths may be detectable with diminished sensitivity.

5. Select filters that transmit the light of interest and reject light which does not excite or emit from the fluorophore.

Filters impart specificity by preferentially passing wavelength of interest, increasing the signal:background ratio. Transmitted irrelevant light increases the background signal, and increases the noise about that background. Analyst uses bandpass filters which are defined by the central wavelength (CWL), the bandpass (the distance in nm between 50% transmission cut-on and cut-off) and the rejection of light of undesired wavelength. Typically, filter quality differences can be shown in the accuracy of the CWL assignment and the rejection in adjacent wavelengths.

It is a good practice to keep the CWL of the two filters separated by the sum of the bandwidths (not ½ the sum of the bandwidths). In this way the separation is twice the distance to the half power points (wavelengths at which there is 50% transmission). Thus, for fluorescein absorbing (exciting) at 490 and emitting at 514 nm, LIL has selected a 485-20 and a 530-25, respectively. The difference between the CWL’s is 45 nm and the sum of the bandwidths is 45. Also the best dichroic would have a 50% cut-on that is nearly in the middle between the two filters. In addition to knowing the excitation and emission spectra of the fluorophore, it is important to know how much light will be available to detect. The light available is a function of quantum yield of the fluorophore and of the concentration of the fluorophore. In cases where little light is expected, it is preferable to use broader bandpass filters. This is at the cost of passing irrelevant light.

Background counts have several sources, including the biochemical components used in the assay. The background measured by using buffer only examines the contributions of the buffer components, the microassay plate and the optical system. Selection of filters determines the amount of fluorophore-specific light and irrelevant light measured. Increasing the magnitude of the background signal will also increase the magnitude of the noise around the background. Shot noise reflects the randomness in sampling a source of signal. The standard deviation of the signal contributed by counting imprecision is the square root of the signal. Increasing the signal increases the standard deviation while decreasing the coefficient of variation (CV, the normalization of the standard deviation obtained by dividing the standard deviation by the mean and multiplying by 100%).

Therefore use only filters designed for fluorescence having the deep level of blocking needed; a factor of 6 decades (logs) is usually sufficient. Thus an important strategy is to block the intense multicolored light so that only fluorescence passes through both filters. This is why there must be so much separation between the two bandpass filters (see above). The dichroic does not provide blocking but is useful because it steers the light where it is needed.
A procedure for selection:
Start with a filter set from MDS, Omega, Corion or Chroma. Avoid any mixing of filters from different sets of the same vendor. Note that the Omega excitation filters and Omega emission filters are not interchangeable, but are built as specific sets.

To improve results, attempt a careful approach to mixing of filters.

Evaluate by making a careful dilution series of the fluorophore in the assay buffer and using the plate planned for the assay. Measure the signal of empty wells, buffer alone and determine the lower limit of detection. LJL recommends using the concentration equivalent to the signal of the buffer plus 3 standard deviations of the buffer signal for the lower limit of detection.

Discussion: Our experience has been that (1) off the shelf optical components may deviate unacceptably from the nominal performance of such attributes as CWL, (2) that filter sets made for microscopy may not be optimized for analytical instrumentation, and (3) that mixing components from different selected "sets" may diminish performance (i.e., lower limit of detection). There is often a 2-3 week delay in receiving filters from component sources in the United States. Custom manufacture may take 6-8 weeks.

Useful numbers of vendors of optical products:

Newport 800-598-6783 www.newport.com
Omega 802-254-2690 www.omegafilters.com
Chroma 800-824-7662 www.chroma.com