

PB-FRET™: ILLUMINATION & DETECTION WINDOWS FOR FILTER-BASED INSTRUMENTS

In FRET assays (see TechNote TNPJ100 *Considerations for Development of FRET Assays*), the selection of specific wavelengths for excitation and emission detection can significantly impact the strength of the signal obtained and the level of background fluorescence. Here, we discuss the choice of filters for use in PB-FRET™ assays (see TechNote TNPJ100.10 *PB-FRET vs. TR-FRET*) on filter-based fluorescent plate readers.

Background fluorescence in FRET comes from three major sources:

- Off-peak excitation of the acceptor pigment

Fluorescent pigments have excitation spectra that tend to drop sharply above the excitation maximum, but may tail significantly below the maximum (see Figure 1). Thus, when illumination is applied to the donor pigment, photons may also be absorbed directly by the acceptor in this "tail" of its excitation spectrum, leading to acceptor fluorescence that is not due to FRET.

- Off-peak emission of the donor pigment

Emission spectra tend to tail off gradually at wavelengths above the emission maximum (Figure 1); thus, there may be residual donor emission even at the emission maximum for the acceptor.

- Background fluorescence of unknowns

Candidates added to assay reaction mixtures occasionally have fluorescence of their own which can lead to background fluorescence in the detection window, although such sample auto-fluorescence is relatively rare at the long wavelengths used for PB-FRET detection.

The first two of these background sources can be minimized through selection of illumination and detection windows that minimize the contribution of background to overall fluorescence. The latter source of background counts is not amenable to such treatment, but can in most cases be distinguished from actual signal through appropriate analysis of FRET data.

Selection of illumination windows must be based not only on obtaining strong fluorescence signals, but also on maximizing the ratio of FRET fluorescence to off-peak background fluorescence. Figure 1 shows the ratio of donor excitation to acceptor excitation for the FRET pair, R-Phycoerythrin (RPE) and Allophycocyanin (APC).

The complex excitation spectrum of RPE provides several options for excitation illumination, but the figure clearly shows that the maximum enhancement of donor illumination versus acceptor illumination is found in the vicinity of the 495 nm excitation peak. Thus, while the exact characteristics of

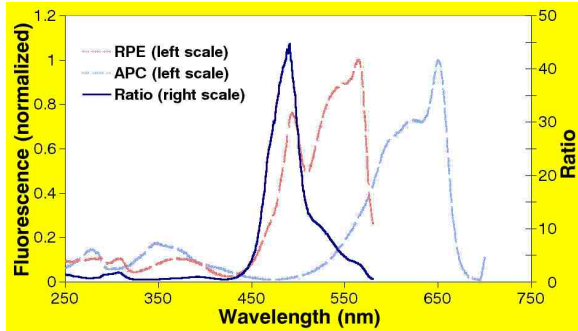


Figure 1 - Excitation spectra for RPE and APC normalized to a maximum value of 1, and the ratio of RPE excitation to APC excitation as a function of wavelength.

the excitation filter employed will be somewhat specific to the nature of the illumination source and the transmission characteristics of the filter, it is clear that optimal illumination will be in the range of 475 - 500 nm. In this case, examination of the excitation ratio significantly alters the selection of an illumination window over what would be selected if only the RPE excitation spectrum were examined.

On the other hand, Figure 2 shows that the optimum detection window for PB-FRET is shifted only slightly from the detection window for APC alone; best results are obtained in the range of 650 nm and above.

Where no other sources of interference or extraneous fluorescence are involved, a wide detection window starting at 650 nm can provide enhanced sensitivity compared to a typically narrow window.

Table 1 shows some commercially available filters that meet the emission filter criteria outlined above. In Figure 3, the emission spectrum of APC is shown, along with the spectra obtained when APC emission is passed through various filters. Percentage values noted under the curves indicate the proportion of the complete APC emission spectrum transmitted by the various filters.

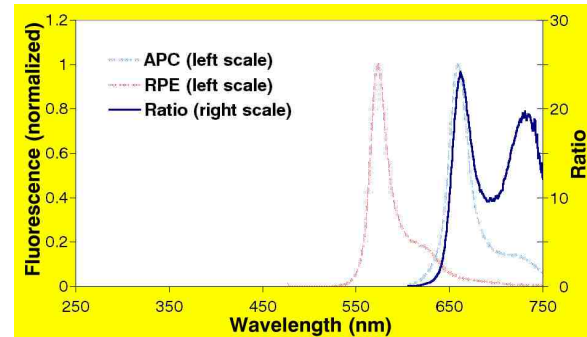


Figure 2 - Emission spectra for RPE and APC normalized to a maximum value of 1 and the ratio of APC emission to RPE emission as a function of wavelength.

Table 1 - Detection of APC emission by various filters

Parameter	Filter				
	650LP	670/40	694/45	665LP	668LP
% Transmission of APC Emission	69	49	31	48	41
% Transmission of APC Emission <700 nm	66	61	26	39	32
% Transmission of RPE Emission	2.2	2.4	1.8	2.4	2.8
% Transmission of RPE Emission <700 nm	1.6	1.8	17	20	13
APC:RPE Transmission	32	21	17	20	13
APC:RPE Transmission <700 nm	41	34	20	16	12

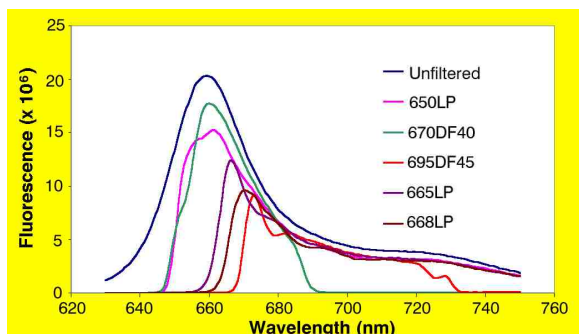


Figure 3 - Emission spectrum of APC unfiltered and viewed through filters listed in Table 1

Due to limitations of optics and photomultipliers, many fluorescence readers lack sensitivity above about 700 nm; percentage transmission of APC emission for the range less than 700 nm is also given in Table 1.

The best filters for PB-FRET detection were the 650LP (long-pass filter with cuton at approximately 650 nm) or the 670/40 (bandpass centered at 670 nm with a width at half-maximum of 40 nm). Particularly for wavelengths less than 700 nm, these two filters have quite similar performance, as would be expected.

These two filters were compared in a PB-FRET application that detected tyrosine kinase phosphorylated peptide. Table 2 shows the limit of detection calculated as the standard error of the estimate of peptide concentration from PB-FRET fluorescence (see Tech Note TNPJ100.11 *Detection of Tyrosine Kinase Phosphorylated Peptide With FRET*).

A third filter, a 665/10 nm filter normally used for the detection of APC fluorescence in TR-FRET and supplied with the Wallac Victor² 1420 Multilabel Counter (Victor), was also employed as an example of a narrow-band filter. The former two filters were, as expected, quite similar in their performance, with no statistically significant

Table 2 - Limit of detection of the phosphorylated peptide (nM) under test conditions

Filter	Plate 1	Plate 2	Mean
670/40	0.54	0.60	0.57
650LP	0.67	0.58	0.62
665/10	1.45	----	1.45

difference between the results, and were both significantly superior to the narrow bandpass filter.

The results summarized in Table 2 were obtained with a 485/15 nm excitation filter factory-installed on the Victor, which provides illumination consistent with the requirements defined above. A 490/10 filter gave similar results.

Filters were supplied by Omega Optical, Inc., Brattleboro, VT, a manufacturer of filters for analytical instrumentation and fluorescence microscopy. The filters referred to here include: 650 AELP (longpass), custom part; 670 DF40 (bandpass), XF3030; 665 EFLP (longpass), custom part. Contact Omega Optical at www.omegafilters.com.

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