Molecular Devices

MAXline™
Microplate Readers

Application Note
31

Optimizing Excitation and Emission Wavelengths for Narrow Stokes' Shift Fluorophores Using the SPECTRAmax® GEMINI and SOFTmax PRO

INTRODUCTION

The basic strategy for optimization of excitation and emission wavelengths using the SPECTRAmax GEMINI microplate spectrofluorometer is outlined in MAXline Application Note No. 30. The easiest case is when the fluorophore has a relatively large Stokes' shift (> 80 nm); the optimal wavelengths are those giving maximal signal, assuming no background interference. If the Stokes' shift is narrow, the selection/optimization process is less straightforward because scattered excitation light interferes with the fluorescent signal. The following Application Note gives details of an optimization procedure for fluorophores with Stokes' shifts less than 80 nm and includes custom SOFTmax PRO formulas to assist in the selection process. The fluorophore used in this example is fluorescein.

OVERVIEW

An excitation scan is performed as previously described (Application Note No. 30) and the excitation lambda max is noted. If the Stokes' shift is < 80 nm, the recommended excitation wavelength is not lambda max. Instead, a wavelength lower than lambda max is chosen such that excitation light carryover is decreased, but there is still ample fluorescent signal. For the first iteration, Molecular Devices recommends using the lowest possible wavelength that will yield 90% maximal RFU. Emission scan(s) and background scan(s) are then performed with one or more cutoff filters. Plots of signal/background versus wavelength are created to help in the selection of the combination of emission wavelength plus cutoff filter giving the highest possible signal/background ratio.

EXAMPLE OF OPTIMIZATION PROCEDURE USING FLUORESCEIN IN PBS, PH 8.5

Excitation Scan

A fluorescein solution (10 nM in PBS, pH 8.5) was dispensed in quadruplicate into microplate wells (200 µL/well) and PBS alone was dispensed into other wells to serve as the blank (referred to as "background" in the discussion below). Based on a literature value, the emission wavelength was set initially at 540 nm. An excitation scan with no cutoff filter revealed a peak at 488 nm (Figure 1).

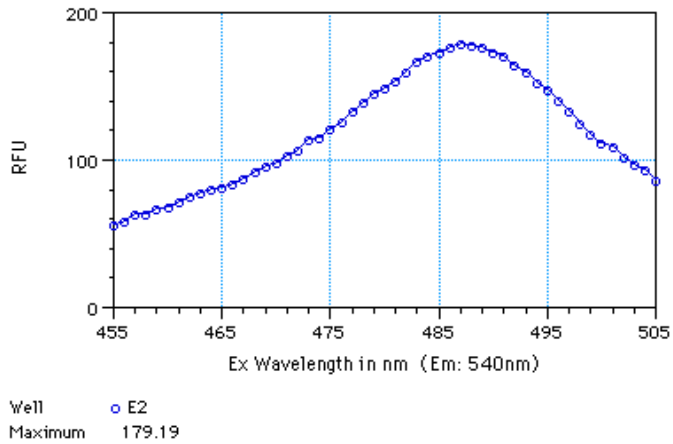

Figure 1: Excitation scan of 10 nM fluorescein in PBS pH 8.5 with emission set to 540 nm.

Selection of Excitation Wavelength.

The excitation/emission separation was clearly less than 80 nm, so a wavelength lower than the lambda max was selected (482 nm). SOFTmax PRO automatically determined that lambda max was not the best choice and calculated the lowest excitation wavelength giving 90% maximal RFU. The detailed procedure and custom formulas are given below.

A group called "Excitation Optimization" was created in the Template Editor and the fluorescein samples were assigned to it. The original Group table was modified and expanded to include new columns and summaries, as shown in Figure 2. The *Ex. lambda Max* column gives the excitation wavelengths with maximum fluorescence signal in the wells. The *RFU max* and *90%MAX RFU* columns are self-explanatory. The *Max.Index* column contains the wavelength increment above the starting wavelength (455 nm) at which maximum RFU occurred, and is used by SOFTmax PRO to link wavelength and maximum RFU value. The final column (*Lambda at 90% Max*) contains the wavelength corresponding to 90% Max RFU.

Excitation Optimization

Sa	W	Ex. Lambda Max	RFU Max	90%MaxRFU	Max.Index	Lamba at 90%Max
Flu	E1	487	180	162	33	482
	E2	487	179	161	33	482
	F1	488	186	167	34	482
	F2	488	184	165	34	482

Emission wavelength used = 540
Excitation Lambda Max Observed = 488
Average Excitation Wavelength at 90%Max = 482
Excitation/Emission difference: < 80 nm
Excitation Wavelength Chosen = 482

Figure 2: Excitation Optimization Group table used for automatic selection of optimal excitation wavelength.

The actual selection of excitation wavelength was accomplished in the summary lines below the Group table. The custom formulas used in the columns and summaries of Figure 2 are listed in Table 1 below (for further details, see the *SOFTmax PRO Formula Reference Guide*.) The term 'val' in S#1 converts the emission wavelength from text to a numerical value. The final summary (S#5) contains the conditional formula for selecting the wavelength. If the excitation/emission difference is less than 80 nm, the chosen wavelength is S#3, otherwise it is S#2. In this example using fluorescein, the excitation/emission wavelength difference was less than 80 nm, so the chosen excitation wavelength was 482 nm.

Column Name	Column Formula
Ex. Lambda Max	!WellValues
RFU Max	Max(!WellL1)
90%MaxRFU	'RFU Max'*0.9
Max.Index	IndexOfMax(!WellL1)
Lambda at 90%Max	NthItem(!WellWavelengthRun,(IndexOfNearest (NullOutside(!WellL1,1,Average(Max.Index)),'90%MaxRFU')))

Summary Lines	Summary Description	Formula
S#1	Emission Wavelength used	Val(!EmWavelengths@ExScan)
S#2	Excitation lambda Max Observed=	Average(!WellValues)
S#3	Average Excitation Wavelength at 90% Max)	Average('Lambda at 90% Max')
S#4	Excitation/Emission difference:	If ((S#1-S#2)<80, "< 80 nm", "> 80 nm")
S#5	Excitation Wavelength Chosen	If ((S#1-S#2)<80,S#3,S#2)

Table 1: Formulas for Excitation Optimization table

Note: For fluorophores with extremely narrow Stokes' shifts, it may be necessary to decrease the excitation wavelength even further than the 90% maximum RFU.)

Emission Scans

Having selected 482 nm as the excitation wavelength, emission scans (500 - 580 nm) were performed with a) no cutoff filter (Emscan1), b) 515 nm cutoff (Emscan2), and c) 530 nm cutoff (Emscan3). It was imperative that all scans began at exactly the same wavelength so that different scans could be subsequently overlaid as shown in Figure 3 (if they did not start at the same wavelength, plots

would be erroneous because the RFU values would not correspond to wavelength.) The following section gives the detailed procedure for overlaying spectral plots in SOFTmax PRO.

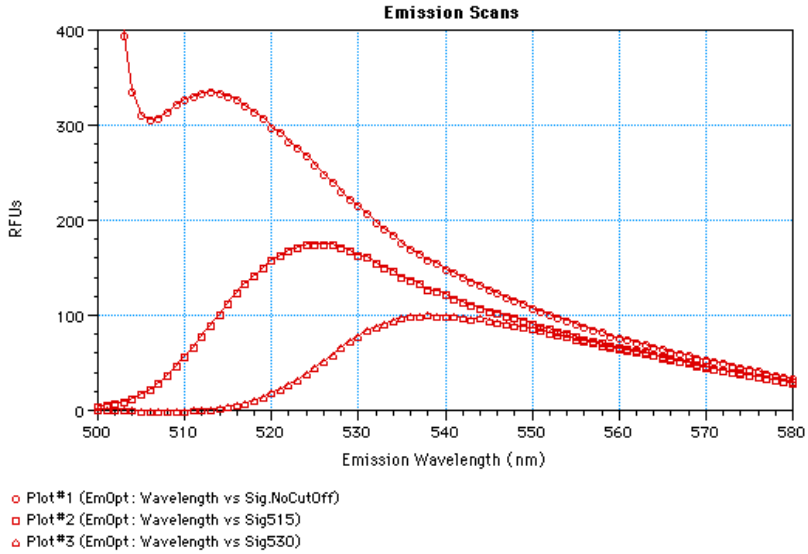

Figure 3: Emission scans of 10 nM fluorescein in PBS pH 8.5 with excitation set to 482 nm. Plots from left to right are: No cutoff filter, 515 cutoff, 530 cutoff.

Creating a Group Table Containing Raw Spectral Data using SOFTmax PRO Custom Formulas.

In order to overlay plots from different scans as shown in Figure 3, a Group table was created to contain raw spectral data from individual wells. The Group table was artificial in the sense that it was not used for the usual SOFTmax PRO purpose– to link sample information and well location. Instead, raw spectral data from individual wells in different plates and wells were put into the columns using special SOFTmax PRO plate accessors (See "Kinetic and Spectrum Data Accessors" in Chapter 4 of the *SOFTmax PRO Formula Guide*.) In this example, a group named *EmOpt* was created and a single well in one of the emission scan plates was assigned to it. (The choices of plate and well location were not important; the assignment was simply necessary to create a Group table.)

From the original Group table, all but the sample column were deleted and new columns with custom formulas were added. A representative portion of the final Group table is shown in Figure 4.

EmOpt

Wavelength	Sig.NoCutOff	Sig515	Bkg515	Sig/Bkg515	Sig530	Bkg530
!WavelengthRun	!F2@EmScan1	!F2@EmScan2	!B1@EmScan2	Sig515/Bkg515	!F2@EmScan3	(!B1@EmScan3)+
490	#Sat	9	9.09	1.03	5	5.6
491	#Sat	9	8.82	1.00	5	5.1
492	#Sat	8	8.18	1.03	5	5.0
493	#Sat	8	7.73	1.01	5	4.7
494	#Sat	7	7.09	1.05	4	4.4
495	#Sat	7	6.43	1.06	4	4.0
496	#Sat	6	6.04	1.02	4	3.7
497	#Sat	6	5.35	1.08	3	3.3
498	#Sat	5	4.71	1.16	3	2.8
499	1696	5	3.99	1.37	3	2.5
500	1082	6	3.34	1.70	2	2.1
501	750	6	2.95	2.07	2	1.8
502	502	8	2.51	3.03	1	1.5
503	395	10	2.05	4.90	1	1.2

Figure 4: Portion of Emission Optimization Group table (with formulas revealed).

The column called *Wavelength* lists the individual wavelengths read from the emission scan. It was obtained from the *Emscan2* plate (but could have come any of the emission scans). The column *Sig515* lists the corresponding RFU values for a fluorescein-containing well (F2) in the *Emscan2* plate (which used the 515 nm cutoff filter). Similarly, the *Bkg515* column lists the RFU values from a PBS-containing well (B1) in the same scan. The *Sig/Bkg515* column gives the ratio of the two previous columns at each wavelength. The formulas for the other scans were similar, except the plate was *Emscan1* for no cutoff filter and *Emscan3* for the 530 nm cutoff filter. Examples of the formulas used to create the columns are given in Table 2.

Column	Formula
Wavelength	!WavelengthRun@Emscan2
Sig515	!F2@Emscan2
Bkg515	!B1@Emscan2
Sig/Bkg515	'Sig515'/Bkg515
Sig530	!F2@Emscan3
etc.	

Table 2: Formulas for emission optimization (partial list)

Overlay of Emission Plots

The emission plots were overlaid in a SOFTmax PRO Graph section by designating the wavelength column as the X variable and the *Sig515*, *Sig530* and *Sig.NoCutoff* columns as Y variables. Predictably, the cutoff filters shifted the peak to longer wavelengths (525 and 539 nm, compared to 513 nm with no cutoff filter) and lowered its intensity (Figure 3.) The background scans were similarly plotted in a second Graph section (Figure 5) by selecting the *Bkg515* and *Bkg530* columns as Y variable. Below approximately 550 nm, the 515 nm cutoff plot was distinctly higher than the 530 nm cutoff plot. Above that region the two plots were essentially superimposable.

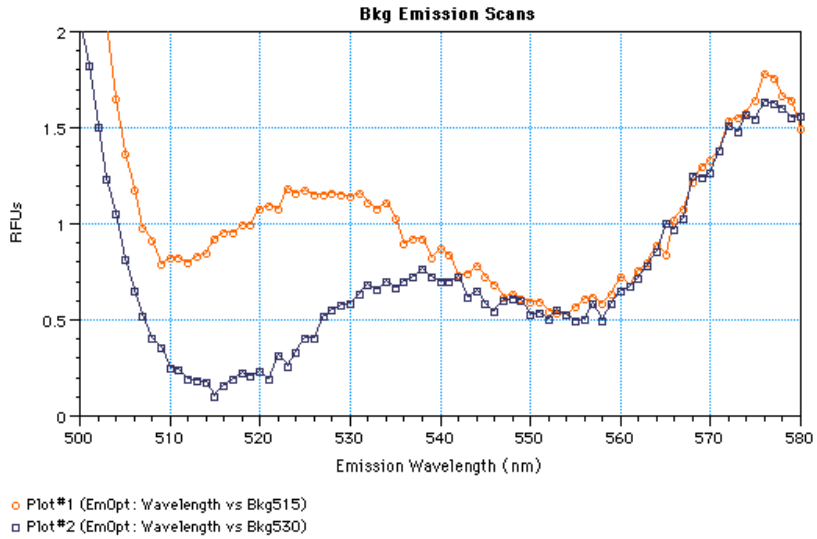

Figure 5: Emission scans of blank solution (PBS pH 8.5) with 515 nm and 530 nm cutoff filters

Final Choice of Emission Wavelength and Cutoff Filter

Signal/background plots were prepared to help identify the combinations of emission wavelengths and cutoff filters giving the highest ratios. The plots revealed more than one possible wavelength/filter combination (Figure 6). With the 515 nm cutoff filter, there were two roughly comparable choices: 520-525 nm and approximately 550 nm. With the 530 nm cutoff filter, the optimal region was approximately 545 nm. Thus, several choices were available to accommodate samples with potential interference at one of the wavelengths. Such flexibility could extremely beneficial in the optimization of multi-fluorophore assays.

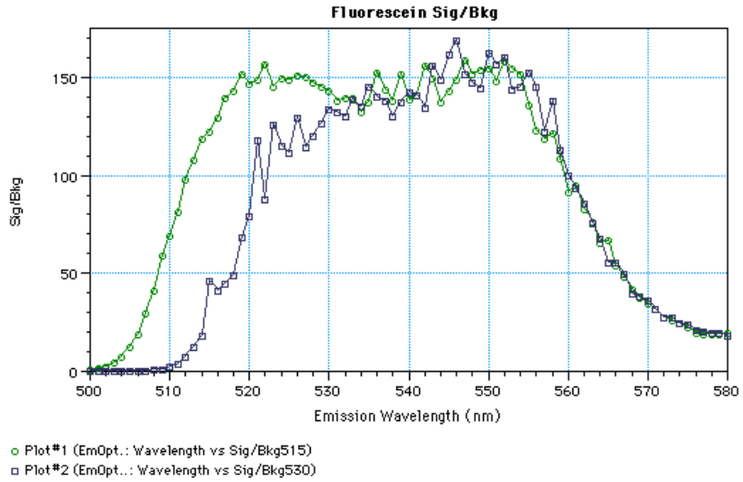

Figure 6: Plots of signal/background for emission scans with 515 nm and 530 nm cutoff filters.

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