4.6.2. USING SPECTRAL SCANNING TO OPTIMIZE EXCITATION AND EMISSION WAVELENGTHS FOR FLUORESCENCE ASSAYS

1 Put 200 µL of sample that includes the fluorophore and 200 µL of a buffer control into separate wells of a microplate.

2 Perform an excitation scan:

a Using SoftMax Pro, set up a Plate section for a fluorescence read, spectrum mode, Em Fixed/Ex Scan, with no cutoff filter (default), and medium PMT.

b Set the emission wavelength based on the tentative value from the literature (or from a customary filter set used to measure your fluorophore). If the emission wavelength is not known, select a tentative emission wavelength about 50 nm greater than the absorbance maximum of the fluorophore. If necessary, the absorbance maximum can be determined by performing an optical density spectral scan first.

c Set the excitation scan to start/stop approximately 50 nm below/above the tentative excitation value obtained from the literature (or customary excitation filter).

d Set the step increment to 2 or 3 nm. (You may choose to do a preliminary scan with a 10-nm increment to determine the approximate peak location, and then repeat the scan over a narrower wavelength range with a 2-nm or 3-nm increment.)

e Perform the scan and view the results as a plot of emission fluorescence vs. excitation wavelength. Note the excitation wavelength at the emission peak and the maximum RFU value.

If an error message reporting missing data points occurs, it may be due to possible saturation reported by SoftMax Pro at the end of the spectral scan. Reset the PMT to "low" and re-scan the sample (scan the buffer blank with the PMT set to "medium" or "high"). If the error occurs after scanning with the PMT set to "low," it may be necessary to dilute the sample.

If the excitation scan shows no apparent peak, change the PMT setting to "high" and re-scan the sample. If the spectral scan still shows no apparent peak, adjust the Y-scale of the zoom plot so that the plot fills the graph.

f Select the optimal excitation wavelength. If the excitation peak wavelength and emission wavelength are separated by more than 80 nm, use the excitation peak wavelength value. If the excitation and emission wavelengths are less than 80 nm apart, use the shortest excitation wavelength that gives 90% maximal emission. (Follow the plot to the left of the peak until the RFU value falls to approximately 90% of the maximum, and then drop a line from the 90% point on the plot to the x-axis—see Figure 4.1.)

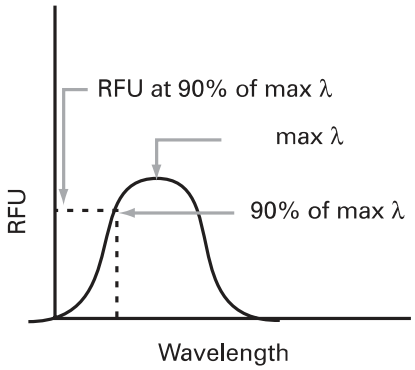

Figure 4.1: Plot of RFU vs. wavelength.

3 Perform emission scan #1:

a In SoftMax Pro, set up a second plate section for a fluorescence read, spectrum mode, Ex Fixed/Em Scan, with no cutoff filter (default), and medium PMT.

b Set the excitation wavelength to the value determined in step 2f above.

c Set the emission scan to start/stop approximately 50 nm below or above the tentative emission value obtained from the literature (or existing filter pair). Note: If the Stokes shift is less than 50 nm, then start the emission scan above the excitation wavelength.

d Set the step increment to 2–3 nm (or do a preliminary scan with a 10-nm increment to determine the approximate peak location and then repeat the scan over a narrower wavelength range using a 2–3 nm increment.)

e Perform the scan and view the results as a plot of fluorescence vs. emission wavelength.

4 Choose the emission filter:

a Select an emission cutoff filter that blocks as much of the residual excitation light as possible without unduly reducing the fluorescence signal. The cutoff wavelength choices are 325, 420, 435, 475, 495, 515, 530, 550, 570, 590, 610, 630, 665, or 695 nm. The cutoff value should be near the maximum emission wavelength (preferably between the excitation wavelength and the maximal emission wavelength) but at least 10 nm less than the emission wavelength. If you have questions about this procedure please contact MDC Technical Support and ask to speak to an applications scientist.

5 Perform emission scan #2:

a In SoftMax Pro, set up a third plate section for an emission scan as specified in step 3 above, except selecting Manual Cutoff Filter and setting the wavelength to that determined in step 4.

b Perform the scan and view the results as a plot of fluorescence vs. emission wavelength. Note the wavelength giving the maximum emission (the optimal emission wavelength).

c Compare the spectra of the sample containing the fluorophore to the spectra of the buffer blank to get an estimate of the signal-to-noise ratio. If there is significant background interference, repeat steps 5a and 5b with another choice of cutoff filter.

6 The optimal excitation and emission wavelengths are those determined in steps 2f and 5b, above.

7 Comments:

In endpoint or kinetic fluorescence modes, the "Autofilter" feature generally selects the same cutoff filter wavelength as the above optimization method. If desired, however, you may specify the cutoff filters manually.

For emission wavelengths less than 325 nm, experimental iteration is usually the best method of determining the optimal emission and excitation wavelengths. Begin optimization by performing steps 2–5 above. Try emission and excitation wavelength combinations with the 325 nm cutoff or with no cutoff filter. Similarly, for excitation wavelengths greater than 660 nanometers, try emission and excitation wavelength combinations with the 695 nm cutoff or with no cutoff.

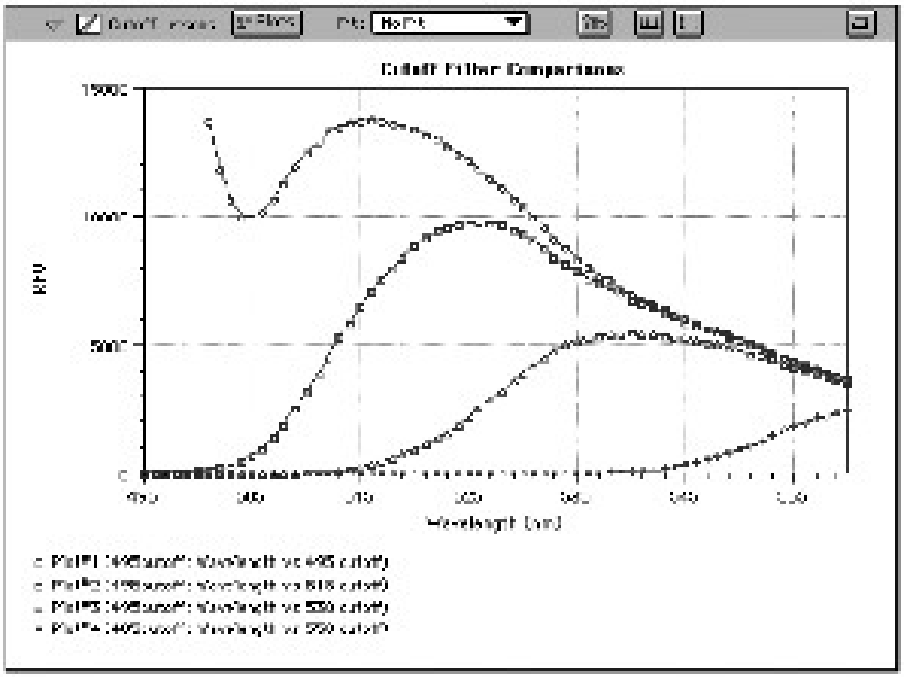

Figure 4.2: Effects of cutoff filters on fluorescein. Emission was scanned from 490 to 560 nm; excitation was fixed at 485 nm.

Figure 4.2 shows the effects of different cutoff filters on a scan of fluorescein where excitation was fixed at 485 nm and emission was scanned from 490 nm to 560 nm (buffer blanks are not shown in this plot). Table 4.1 (following) lists default settings for the emission cutoff filters. For spectrum mode, the default is "manual" (no automatic cutoff).

Table 4.1: Emission cutoff filter default settings.

#	Automatic Cutoff Selection	Endpoint and Kinetic Modes
	Wavelength (nm)	Emission Wavelength (nm)
1	None	< 415
2	420	415–434
3	435	435–454
4	455	455–474
5	475	475–494
6	495	495–514
7	515	515–529
8	530	530–549
9	550	550–569
10	570	570–589
11	590	590–609
12	610	610–629
13	630	630–664
14	665	665–694
15	695	695–850