A Guided Inquiry Approach to Understanding Fluorescence Spectroscopy

This experiment is designed with four parts where each section builds on the previous part. The experiment starts off with exploring the instrument and the data collection parameters. You will explore how data collection parameters play off of each other to generate a spectrum.

The second part actually starts fluorescence data collection. You will determine the optimum wavelength for excitation by looking at the absorbance spectrum, manipulating the data collection parameters, and manipulating the LED excitation with a known sample. The goal of this is purely to get a strong, clear spectrum while manipulating the parameters you just learned about, plus a few more.

In part three, you prepare serial dilutions from a stock solution to investigate the most vital, and perhaps trickiest part of fluorescence spectroscopy, the effect of concentration on fluorescence emission. Finally, in part four, you start to learn about the properties of molecules through fluorescence spectroscopy.

Materials

Part I Operating a Spectrometer: Exploring the Instrument

LabQuest or computer

LabQuest App or Logger *Pro*

Go Direct SpectroVis Plus Spectrophotometer

Part II Obtaining an Fluorescence Spectrum

materials from Part I

cuvette

fluorescein solution (1.2x10-5 M in 0.1 M NaOH)

NaOH (0.1 M)

Part III Conducting a Fluorescence Experiment: Concentration

materials from Part II

fluorescein solution (6.0x10-4 M in 0.1 M NaOH)

Part IV Learning about Molecules with Fluorescence Spectroscopy

your instructor will provide additional materials as applicable

INVESTIGATION

Part I Operating a Spectrometer: Exploring the Instrument

1. Connect the spectrophotometer to the USB port of LabQuest or a computer. Start the data-collection program, and then choose New from the File menu.
2. Switch to Uncalibrated Data mode and observe the data collection parameters. Uncalibrated Data mode simply reports the raw signal from the lamp onto the detector.
3. To switch to Uncalibrated Data mode in Logger *Pro*, from the Experiment menu choose Change Units ► Spectrometer ► Select Spectrometer Mode. This opens the Spectrometer dialog box. Select Uncalibrated Data from the list of options. Note: This dialog box can remain open during data collection.   
   In LabQuest App, from the Sensors menu choose Change Units ► Spectrometer ► Uncalibrated. Tap on the Mode button to access the Spectrometer dialog box.
4. Note the Sample Time, Wavelength Smoothing, and Samples to Average parameters.
5. Start data collection. Once a spectrum appears, end data collection.
6. Return to the Spectrometer dialog box, if necessary. Modify the Sample Time, Wavelength Smoothing, and Samples to Average parameters systematically to determine what, if any, impact the values have on the lamp output.
7. Stop and Start data collection as appropriate. Note: You will not need these spectra for the remainder of the experiment. When you are finished, save data as instructed and select New from the File menu.

Part II Obtaining an Fluorescence Spectrum

1. Obtain a sample of 1.2x10-5 M fluorescein in 0.1 M NaOH from your instructor. **WARNING**: *Sodium hydroxide solution,* NaOH: *Causes skin and eye irritation*.
2. Set the spectrophotometer to Absorbance mode and calibrate it.
3. The spectrophotometer should be in Absorbance mode. If it is not, choose the appropriate instruction below:   
   To switch to Absorbance mode in Logger *Pro*, from the Experiment menu choose Change Units ► Spectrometer ► Select Spectrometer Mode. This opens the Spectrometer dialog box. Select Absorbance from the list of options.   
   In LabQuest App, from the Sensors menu choose Change Units ► Spectrometer ► Absorbance.
4. To calibrate the spectrophotometer, choose Calibrate ► Spectrometer from the Experiment menu of Logger *Pro* or the Sensors menu of LabQuest.
5. Prepare a blank by filling the empty cuvette 3/4 full with the solution used to prepare the sample.
6. Place the cuvette in the spectrophotometer.
7. Select Finish Calibration. Select OK.
8. Collect absorbance *vs.* wavelength data.
9. Remove the blank cuvette from the spectrophotometer. Empty the cuvette.
10. Fill a cuvette with ~3 mL of the known sample.
11. Place the sample in the spectrophotometer.
12. Start data collection. Once the absorbance spectrum is displayed, stop data collection.
13. Examine the plot of absorbance *vs.* wavelength. Make sure the absorbance values are between 0.1 and 1.0 absorbance units. Any values outside this range may introduce an error. If your sample is outside this range, dilute or concentrate it and repeat Step 6.
14. Use the Statistics feature in Logger *Pro* or LabQuest App, determine the wavelength of maximum absorbance.
15. Store the run. In Logger *Pro*, do this by choosing Store Latest Run from the Experiment menu. In LabQuest App, you can store a run by tapping the file cabinet icon.
16. Based on your absorbance spectrum, determine the best LED to excite your samples for the fluorescence spectrum. The Go Direct SpectroVis Plus has built-in excitation LEDs centered at 405 nm and 500 nm.
17. Switch to Fluorescence mode
18. To switch to Fluorescence mode in Logger *Pro*, from the Experiment menu choose Change Units ► Spectrometer ► Select Spectrometer Mode. This opens the Spectrometer dialog box. Select Fluorescence from the list of options. Note: This dialog box can remain open during data collection.   
    In LabQuest App, from the Sensors menu choose Change Units ► Spectrometer ► Fluorescence. Tap on the Mode button to access the Spectrometer dialog box.
19. Set the Sample Time to 20 ms. The samples to average should be set to 6 and the wavelength smoothing to 1.
20. Collect fluorescence *vs.* wavelength data.
21. Fill a cuvette with ~3 mL of the known sample.
22. Place the sample in the spectrophotometer.
23. Start data collection.
24. Examine the plot of fluorescence *vs.* wavelength. Make sure the fluorescence values are above 0.1 relative units. Any values below this may introduce an error. If your sample is outside this range, adjust the sample time, samples to average, or LED intensity based on your investigations in Part I. **Note**: If you adjust these values too much, your baseline will no longer be around zero. If it floats too far from the zero line, you should stop data collection and recalibrate with the new settings.
25. Once the fluorescence spectrum is displayed, stop data collection. Record the sample time, samples to average, and wavelength smoothing values that resulted in the cleanest fluorescence spectrum.
26. Hint Logger *Pro* users: To display the absorbance and fluorescence spectra on the same graph, utilize the double-y-axis feature in Logger *Pro*. Select the absorbance *vs.* wavelength graph. From the Options menu, select Graph Options. Click on the Axes Options tab and check the Right Y-Axis box. Select the Fluorescence runs you would like displayed from the options listed under Right Y-Axis Columns.
27. Measure the fluorescence emission of your sample with the other excitation wavelength.
28. Remove the solution from the spectrophotometer and discard as directed.

Part III Conducting a Fluorescence Experiment: Concentration

1. Set the spectrometer mode to the correct LED excitation.
2. Obtain the 6.0x10-4 M fluorescein in 0.1 M NaOH. Accurately prepare serial dilutions of this standard to the following concentrations: 6.0x10-5 M, 1.2x10-5 M, 9.6x10-6 M,   
   7.2x10-6 M, and 6.0x10-6 M. Dilute with 0.1 M NaOH.
3. Collect fluorescence vs. concentration data.  
   To set this mode in Logger *Pro, c*lick Configure Spectrometer Data Collection, . Select Fluorescence *vs.* Concentration as the Collection Mode. Select the wavelength of maximum fluorescence (λ max) by clicking on the peak fluorescence on the graph or by choosing a wavelength from the list. Select OK.   
   To set this mode in LabQuest App, Note the fluorescence peak maximum. On the Meter screen, tap Mode and change the data-collection mode to Events with Entry. Enter the Name (e.g., Concentration) and Units (e.g., mg/L). Select OK. To enter the wavelength you wish to measure, tap on the meter itself, and select Change Wavelength. Enter the wavelength of your choice and select OK. If the wavelength you type in is not measured by the spectrophotometer, the LabQuest will automatically choose the wavelength closest to your choice.
4. Fill the cuvette with the most concentrated sample first and place in the spectrophotometer. Adjust your spectrometer settings to the values that gave you the spectrum in Part II. If necessary, recalibrate with your blank solution.
5. Start data collection. After the fluorescence reading stabilizes, select Keep. Enter the concentration of the solution and select OK. Collect data for all the solutions. Note: Do not adjust spectrometer settings during data collection; if you wish to adjust them, stop data collection and start again.
6. Examine the plot of fluorescence *vs.* concentration. Note deviations from expected behavior, if any. Perform a linear regression analysis on all or part of the data.

Part IV Learning about Molecules with Fluorescence Spectroscopy

* Using the skills you have learned from Parts I through III, measure the pH dependence of fluorescein fluorescence. Adjust the pH of five samples between pH 2 and pH 6. The concentration of fluorescein should be the same in each solution.
* Using the skills you have learned from Parts I through III, measure the halide quenching of fluorescein fluorescence. Prepare five solutions of 1.2x10-5 M fluorescein in 0.1 M NaOH with the following concentrations of KBr: 0 mM, 2.5 mM, 5.0 mM, 7.5 mM, and 10.0 mM. Measure the fluorescence of the five solutions. The concentration of fluorescein should be the same in each solution.

ANALYZING RESULTS

Part I Operating a Spectrometer: Exploring the Instrument

1. Discuss the changes in your spectral output that resulted from adjusting the Sample Time.
2. Discuss the changes in your spectral output that resulted from adjusting the Samples to Average.
3. Discuss the changes in your spectral output that resulted from adjusting the Wavelength Smoothing.

Part II Obtaining an Fluorescence Spectrum

1. How did you determine the excitation wavelength for your fluorescence spectrum?
2. Discuss why a graph showing absorbance and fluorescence on a double-y-axis is useful.
3. Did other excitation wavelengths produce an accurate fluorescence spectrum? Why or why not?

Part III Conducting a Fluorescence Experiment: Concentration

1. Discuss deviations in linearity in your fluorescence intensity *vs.* concentration data.
2. If you were to perform a fluorescence experiment using fluorescein, what concentration would you use and why?

Part IV Learning about Molecules with Fluorescence Spectroscopy

1. Discuss the changes in fluorescence emission of your sample as a function of pH.
2. Discuss the changes in fluorescence emission of your sample as a function of halide concentration.