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# **Experimental Procedure and Lab Report Instructions for Introduction to Fluorescence Spectroscopies I.**

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(Partly Adapted from Clarke RJ, Oprysa, A. Fluorescence and Light Scattering J. Chem. Ed. 81(5) 705-707 2004

## **1. Introduction**

This document is the experiment part going with “Introduction to Fluorescence Spectroscopies I. Theory”

Objectives: Gain a working familiarity with the Horiba Fluoromax4C Fluorometer, including making observations of

1. fluorescence emission and excitation spectra
2. types of light scattering often seen in these spectra
3. learn about artifacts due to incorrect use of the instrument
4. measure quantitatively the effect of quencher concentration on the fluorescence intensity (quantum yield)

## **2. Experimental Procedure**

### **2.1 Examine the Spex Fluorolog Instrument Monochromator**

The instructor will help you “get under the hood” to see the simple inner workings of the optical part of the older fluorimeter to examine the gratings, and see how the different wavelengths are selected.

### **2.2 Watch the Transmittance, i.e., $10^{-A}$ change on the Horiba FluoroMax-4P**

With the room darkened, watch the light beam as it passes through a cell filled with a “high” concentration  $\sim 1 \times 10^{-4}$  M of fluorescein as the wavelength is scanned from 250 to 600 nm. Roughly note the wavelength of maximum absorption (minimum transmittance). Estimate the absorbance at this wavelength.

## 2.3 Fluorescence spectrum and light scattering from a very low concentration of fluorescein.

When concentrations are low, one usually sees scattering in addition to fluorescence. The aim here is to measure all peaks in the emission spectrum of a fluorescent solution that is fluorescing somewhat weakly, and to identify their origin as: fluorescence, Rayleigh scattering, or Raman scattering).

**2.3.1** For a  $\sim 10^{-7}$  M solution of fluorescein, obtain fluorescence emission spectra using excitation at 400, 440 and 480 nm.

Settings: start = 380 nm

end = 600 nm

Excitation. Band pass = 2 nm

Emission band pass = 2 nm

*These slit widths should be decreased if the signal is more than  $10^6$  counts per second (cps) on the S detector photomultiplier tube at the peak.* (The reason is that intensity in this instrument is done by *photon counting*, which is more accurate than photocurrent—but like a human—the electronics is overwhelmed by having to count large numbers in a short time.)

The instructor will ensure that concentration and settings are such that fluorescence, Rayleigh, and Raman scattering are visible in the spectra.

## 2.4 Fluorescence excitation spectrum

### 2.41 Background

In an excitation spectrum, one positions the emission monochromator to wavelengths near the peak of the fluorescence spectrum and scans the excitation wavelength with the emission detector signal (S) divided by the reference signal (R), i.e., the S/R mode.

The reason for obtaining excitation spectra is usually to ensure that the fluorescence comes from the molecule of interest (whose absorption spectrum is known) and not some impurity. The S/R excitation spectrum can be the shape of the absorbance (A) spectrum of a pure sample—providing the intensity *that the emission monochromator sees* is proportional to A. This can only be the case at low A (high transmittance, T), because the total amount of light given off is proportional to the amount of light *absorbed*, which is  $1-T$ , i.e., what is not transmitted.

Under what conditions is this proportional to A, given that  $T = 10^{-A}$ ? The mathematics to answer this is quite simple, but may seem unfamiliar. It is an exercise seen very often in scientific literature called “linearizing” the exponential. This happens only when the exponent is small, as seen below.

Recall that  $10 = e^{2.303}$ . So  $T = 10^{-A} = e^{-2.303A}$ . An important fact for *all* scientists is that

$$e^x = \sum_n \frac{x^n}{n!} = 1 + x + \frac{x^2}{2} + \frac{x^3}{6} + \dots$$

Therefore, since  $x = -2.303A$ ,  $1-T = 1 - (1 - 2.303A) = 2.303A$ , **but only if  $(2.303A/2)^2$  is much smaller than  $2.303A$** . Only then will the excitation spectrum be proportional to absorbance spectrum. By inserting different values of A, discover at what value of A and T will make  $2.303A > 10$  times  $(2.303A/2)^2$ .

#### 2.4.2 Procedure.

Run 3 excitation spectra with concentrations corresponding to A approximately =10, 0.1 and 0.01, and compare to the expected shape. **Which is the mirror image of the fluorescence spectrum?**

#### 2.5 Quenching of fluorescein by iodide:

Quenching of fluorescence is the basis for many investigations using fluorescence. The introduction provided in the following is also instructive of basic first and second order kinetics, which you will be studying in Lecture.

We will use the equations and data from the Theory handout.

##### 2.5.1 Solutions

1. Prepare ~ 20 ml of fluorescein solution buffered to ~pH 9, dilute enough such that with the excitation wavelength near the peak absorbance, the beam is not visibly attenuated as it passes through the sample.
2. Use about half of this fluorescein solution to make a ~0.5 M solution of by weighing KI solid such that the molar concentration is known to 3 significant figures.

##### 2.5.2 Procedure

1. With the excitation wavelength near the peak absorbance, and emission monochromator near the peak emission, adjust the slits so that the S signal is between  $10^5$  -  $10^6$  cps for the solution of fluorescein buffered at pH ~9 without quencher. Note: we don't need to know the fluorescein concentration because that does not occur in the quenching rate equation. We are only concerned with the ratio of unquenched emission to the quenched emission.
2. By mixing precise ratios of the pure solution with the iodide quencher solution, obtain fluorescence spectra of 3 or 4 solutions ranging in quencher concentration from the maximum to a concentration that causes about a 10 % reduction in the spectrum.

### 3. DATA ANALYSIS AND DISCUSSION TOPICS

**3.1** Copy the data files of all spectra taken that are needed below to your flash drive before leaving the lab. Show plots of these as needed for your lab report.

### 3.2 Fluorescence Spectrum in solvent

For spectra from 2.3, assign the peaks as fluorescence, Rayleigh scattering or Raman scattering, justifying your assignments.

#### 3.2.1 Kasha's Rule

Do the spectra from **Section 2.3** obey Kasha's Rule? Compare the typical emission spectrum with an absorption spectrum. How well is the mirror image rule obeyed for your spectra?

#### 3.2.2 Raman frequency

Using the wavelength maxima of the Raman bands and the exciting light, calculate the vibrational frequency causing this scattering. Compare with the observed OH stretching frequency of water in liquid water.

#### 4.2.2 Excitation Spectra

- Compare the excitation spectra to the absorption spectrum in solvent for the 3 excitation spectra for the different absorbance values. Explain why some of these are grossly distorted.
- Sketch the expected excitation spectrum shape if the emission monochromator was able to capture 100 % of the light emitted for the cases of  $A = 0.001$  and  $A = 5$ .

#### 4.2.3 Fluorescence Quenching by Iodide

- Calculate the quantum yield for each concentration of the iodide,  $[Q]$ , using numbers from the Theory document and:

$$\Phi_f = \frac{k_{rad}}{k_{rad} + k_{ic} + k_{isc} + k_q[Q]}$$

Compare the measured ratio of the peak height of the quenched cases to the unquenched peak with the calculated values.

- If this equation is turned upside down and multiplied by the same expression for no quencher, the resulting equation is the widely used Stern-Volmer equation:

$$\frac{I_0}{I} = 1 + k_q \tau_0 [Q]$$

where  $\tau_0$  is the lifetime in the absence of the quencher,  $(k_{rad} + k_{ic} + k_{isc})^{-1}$ ,  $I$  and  $I_0$  are the intensities in the presence and absence of quencher, respectively, and  $k_q$  is the diffusion-controlled quenching rate. The product  $k_q \tau_0$  is known as the Stern-Volmer constant,  $K_{sv}$ .

- Plot  $I/I_0$  vs.  $[Q]$ , which should give a straight line. **[If the line is curved, this would indicate that some sort of permanent association is occurring between the quencher and fluorophore, causing what is called "static" quenching.]** What is the Stern-Volmer constant determined from your best straight line plot?