HUMBOLDT-UNIVERSITÄT ZU BERLIN

Institut für Physik, Physik von Makromolekülen

Practical coarse Polymer Science, 2nd quarter

Fluorescence Spectroscopy of Dyes in Solution

Tutor: See announcement in guidelines.

Keywords

Fluorescence, excitation-, emission spectroscopy, dye molecules, Franck-Condon principle,

Literature

[1] P.W. Atkins, *Physical Chemistry*, Oxford University Press. (basics of Fluorescence)
[2] C.R. Cantor and P.R. Schimmel, *Biophysical Chemistry, PartII: Techniques for the study of biological structure and function*, Freeman, New York. (Technique and principles of fluorescence spectroscopy)
[3] J.R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Plenum Press, 1983 [UH 5870]

Introduction

An introductory chapter about the principles of fluorescence is attached to the manual, taken from [3]. Please read it carefully.

Instrumentation

In Fig 1 the principle setup of a fluorescence spectrophotometer is presented. It consists of a light source (L) which is passed through a monochromator (M_1) and focussed on the sample (S). Since the light is used to excite the fluorescence, M_1 is usually referred to as the excitation monochromator. The fluorescence emission is detected under an angle of 90^o through the emission monochromator (M_2) using a sensitive photomultiplier tube. For each monochromators there are two slits (at the entrance and at the exit) to cut out part of the spectrum. A spectrum is recorded by scanning one of the monochromators over a certain range of wavelengths and monitoring the emission intensity.

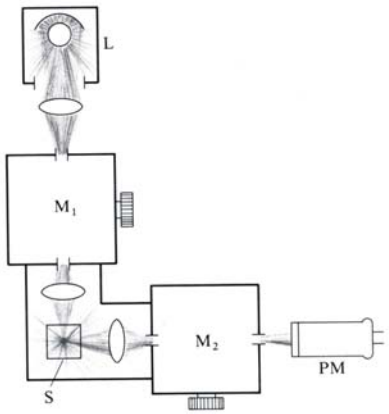


Fig. 1

There are two different modes to record a spectrum:

1. **Emission spectrum:**
   In this case the excitation wavelength is kept at a fixed value and the emission monochromator is scanned over a preset wavelength range. An emission spectrum provides the information about the transition energies from the excited to the ground state (spontaneous emission).
2. **Excitation spectrum:**
   In this case the emission wavelength is kept at fixed value and the excitation monochromator is scanned. The excitation spectrum yields the information about the transition energies from the ground to the excited state (absorption).

The intensity of the detected light depends on several factors:

1. The **concentration** of the samples, because emission is proportional to the absorbance of the excitation light. If the concentrationis too high, part of the light emitted will be reabsorbed by the sample which deforms the spectrum. There is almost no lower limit, it is only given by detection efficiency. In principle, single molecules can be observed.
2. The **slit width**. The slits determine how much of the spectral range of the light is passed through the monochromators and to the detector. For every slit, the intensity is roughly proportional to the slit width.
3. The **detection efficiency**. The light is detected using a photomultiplier. The sensitivity of the photomultiplier can be adjusted. In the extreme case, single photons can be counted (not with our instrument!).

One has to be aware, that the emission spectrum of the lamp is not homogenous, but there are several intense peaks. Therefore, excitation spectra may show artificial peaks that are due to the lamp spectrum. For a better analysis, those spectra should be corrected. The same holds for the detection path: the sensitivity of the photomultiplier also depends on the wavelength.

Experimental details

The Instrument: Hardware

The optical system of the instrument used here (JASCO) is shown in Fig 2.

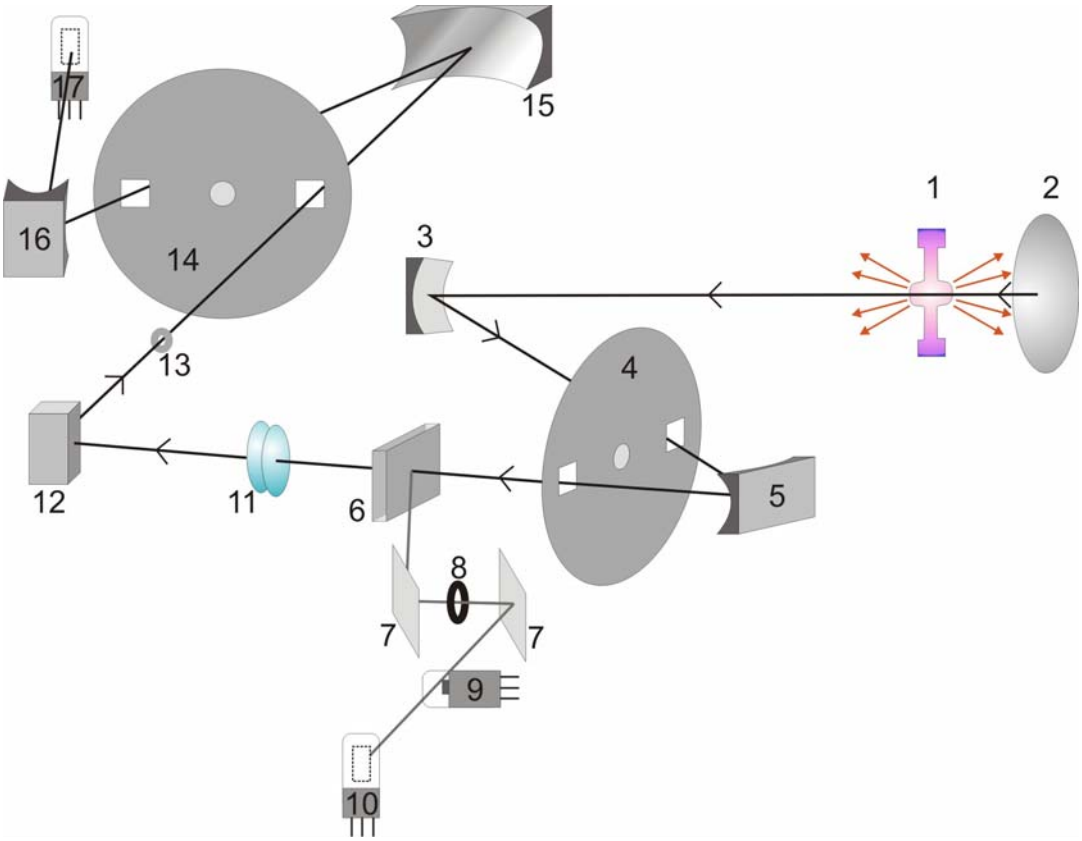


Fig. 2 Optical System of the JASCO

(1) Xenon Lamp, 15OW (2) Condenser Ellipsoidal Mirror. (Si02 Coated) (3) Concave Mirror (4) Excitation Slit Assembly (5) Concave Diffraction Grating (for Excitation) (6) Beam Splitter Quartz Plate (7) Teflon Reflector (8) Light Beam Balance Aperture (9) Optical Attenuator (10) Monitor Photomultiplier (11) A Couple of Light-collecting Lenses	(12) Cell (Sample) (13) Light-collecting Lens (14) Emission Slit Assembly (15) Concave Diffraction Grating (for Emission) (16) Concave Mirror (17) Photometric Photomultiplier

Note that the slits are mounted on a wheel. When you change the slit width, the entrance and exit slit is changing in parallel for one monochromator!

The Instrument: Polarisation dependent measurements

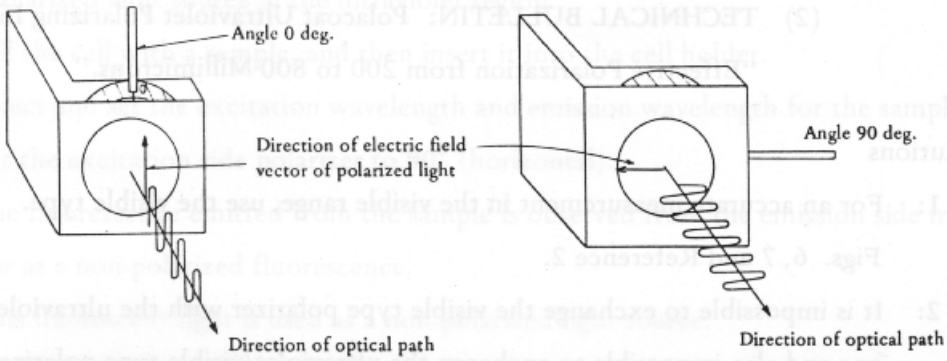
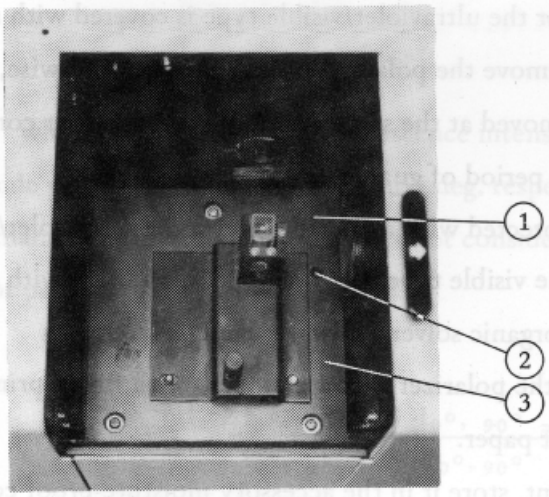


Fig. 3 Relation Between Polarizer Angle and Electricfield Vector of the Transmitted Light



1 Tapped hole
2 Positioning pin hole
3 Sample compartment base

Fig. 4 RF-540 Sample Compartment Base

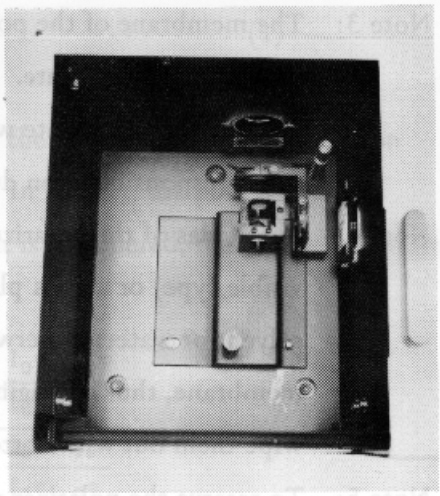


Fig. 5 Polarization Attachment Assembly Installed in RF-540 Sample Compartment

The Fig. 3 to 5 show how to insert the polarizers and how the polarisation directions are oriented.

The Instrument: Software operation

The spectrometer used for this experiment is a JASCO brand. It is operated by a personal computer and the software is running under MS Windows XP. For the details of operation please read the manual. If the program hangs up you must restart the computer by common Windows restart. F
Follow the short instructions below.

For short, you have to proceed as follows:

1. Start the JASCO by pressing the power button. The lamp needs approximately 30min to reach the operating temperature.

2. After computer is powered on, MS Windows XP is started. Please start the the Spectroscopy Program by double-clicking on the Spectroscopy-link on the Windows desktop.
   A menu will appear where you choose “Spectrum Measurement” – usually the second option.
   The measurement program will start.

3. Before starting a measurement, you have to set the instrument parameters. You can enter the **Parameter Dialogue** by a single click on the parameters option under measurement menu. Please look at the manual for a detailed description of the parameters.
   The necessary options are:
   - Type of measurement
   - Excitation (set value or set range)
   - Emission (set value or set range)
   - Slit width (both monochromators)
   - Scanspeed
   - Scan-stepsize
   - Detection sensitivity

   There are more options to choose, but actually not necessary for your tasks.

   Don’t forget to enable the auto-save function, so that all your obtained spectra are stored automatically.
   You will have to convert all spectra later on into a common ASCII-code file. Such files can be opened by any data-analysis program.

Lab tasks

From the tutor you will receive two stock solutions of different fluorescent probes. One is **Rhodamine** (red solution) and the other one is **Fluorescein** (yellow solution). A data sheet about the dyes will be provided. It is the aim of this lab course to become familiar with the method of fluorescence detection and the underlying physics.

If not stated otherwise, solutions are 10 times diluted from the stock solution. The solvent is pure water. You can dilute directly in the cuvette using 2 ml of water and 0.2 ml of dye solution.

1. Get familiar with the instrument:
    a) Measure the complete emission and excitation spectrum of each dye.

    b) Measure for one dye probe the emission spectrum at different slit width (excitation and emission, use different sensitivity or excitation wavelength, if necessary. Don't forget to calibrate against each other!)

    c) Make a series of decreasing concentrations and measure the emission spectra. Try to find the lowest concentration where the emission still can be detected. Select proper slit width, sensitivity, time constant etc.

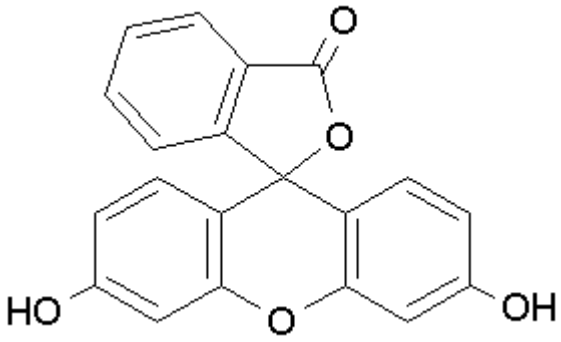
2. Fluorescence depolarisation:
    a) Measure the emision spectrum of one dye probe for all four major polarisation directions.

Data Analysis:

1. Plot the excitation and emission spectrum in one graph. Assign the vibronic levels.
2. Plot the peak intensity against the product of both slits on a log-log scale. What do you expect? Note, that each monochromator has two slits that are changed in parallel!
3. Plot the emission intensity against the concentration on a log.log.scale. If you change slit width or sensitivity, you have to recalibrate the intensity accordingly.
4. Evaluate *r* and *P* for solution (don't forget G-factor!) following the instructions of added paper. Is the result in accordance with your expectations?

Substances:

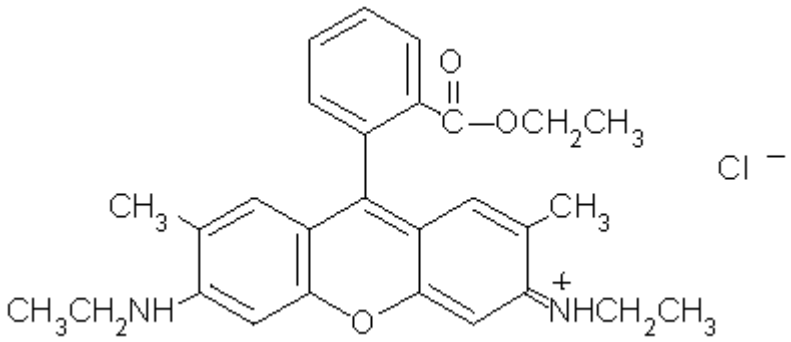
Fluorescein (free acid):



FW = 332.31

Concentration of stock solution: …… mol/l

Rhodamine 6G:



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Concentration of stock solution: …… mol/l