Spectro-Fluorimetric Analysis of Biomolecules

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**1. INTRODUCTION**

Fluorescence spectroscopy is one of the most widely used spectroscopic techniques in the fields of biochemistry and molecular biophysics today. Although fluorescence measurements do not provide detailed structural information, the technique has become quite popular because of its acute sensitivity to changes in the structural and dynamic properties of biomolecules and biomolecular complexes. Like most biophysical techniques, fluorescence spectroscopic studies can be carried out at many levels ranging from simple measurement of steady-state emission intensity to quite sophisticated time-resolved studies. The information content increases dramatically as various fluorescence observables are time resolved and combined in global analyses of the phenomena of interest. Nonetheless, quite a good deal of information is available from steady-state measurements for which the requirements in instrumentation are quite modest. Consequently, steady-state fluorimeters are routinely used to measure complexation and conformational phenomena of biological molecules.

**The Fluorescence Process**

Fluorescence is the result of a three-stage process that occurs in certain molecules called fluorophores or fluorescent dyes. A fluorescent probe is a fluorophore designed to localize within a specific region of a biological specimen or to respond to a specific stimulus. The process responsible for the fluorescence of fluorescent probes and other fluorophores is illustrated by the simple electronic-state diagram (Jablonski diagram) shown in Figure 1.

![Jablonski Diagram](image)

**Figure 1.**

**Stage 1: Excitation**

A photon of energy $h\nu_{EX}$ is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state.
(S1'). This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction.

**Stage 2: Excited-State Lifetime**

The excited state exists for a finite time (typically 1–10 nanoseconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, the energy of S1' is partially dissipated, yielding a relaxed singlet excited state (S1) from which fluorescence emission originates. Second, not all the molecules initially excited by absorption (Stage 1) return to the ground state (S0) by fluorescence emission. Other processes such as collisional quenching, Fluorescence Resonance Energy Transfer (FRET) and intersystem crossing may also depopulate S1. The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted (Stage 3) to the number of photons absorbed (Stage 1), is a measure of the relative extent to which these processes occur.

**Stage 3: Fluorescence Emission**

A photon of energy hνEM is emitted, returning the fluorophore to its ground state S0. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than the excitation photon hνEX. The difference in energy or wavelength represented by (hνEX – hνEM) is called the Stokes shift. The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons. In contrast, absorption spectrophotometry requires measurement of transmitted light relative to high incident light levels at the same wavelength.

**Fluorescence Spectra**

The entire fluorescence process is cyclical. Unless the fluorophore is irreversibly destroyed in the excited state (an important phenomenon known as photobleaching), the same fluorophore can be repeatedly excited and detected. The fact that a single fluorophore can generate many thousands of detectable photons is fundamental to the high sensitivity of fluorescence detection techniques. For polyatomic molecules in solution, the discrete electronic transitions represented by hνEX and hνEM in Figure 1 are replaced by rather broad energy spectra called the fluorescence excitation spectrum and fluorescence emission spectrum, respectively. The bandwidths of these spectra are parameters of particular importance for applications in which two or more different fluorophores are simultaneously detected. With few exceptions, the fluorescence excitation spectrum of a single fluorophore species in dilute solution is identical to its absorption spectrum. Under the same conditions, the fluorescence emission spectrum is independent of the excitation wavelength, due to the partial dissipation of excitation energy during the excited-state lifetime, as illustrated in Figure 1. The emission intensity is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength (Figure 2).
Fluorescence Detection

Fluorescence Instrumentation

Four essential elements of fluorescence detection systems can be identified from the preceding discussion: (1) an excitation source, (2) a fluorophore, (3) wavelength filters to isolate emission photons from excitation photons and (4) a detector that registers emission photons and produces a recordable output, usually as an electrical signal or a photographic image. Regardless of the application, compatibility of these four elements is essential for optimizing fluorescence detection.

Fluorescence instruments are primarily of four types, each providing distinctly different information:

- **Spectrofluorimeters and microplate readers** measure the average properties of bulk (µL to mL) samples.
- **Fluorescence microscopes** resolve fluorescence as a function of spatial coordinates in two or three dimensions for microscopic objects (less than ~0.1 mm diameter).
- **Fluorescence scanners**, including microarray readers, resolve fluorescence as a function of spatial coordinates in two dimensions for macroscopic objects such as electrophoresis gels, blots and chromatograms.
- **Flow cytometers** measure fluorescence per cell in a flowing stream, allowing subpopulations within a large sample to be identified and quantitated.

Other types of instrumentation that use fluorescence detection include capillary electrophoresis apparatus, DNA sequencers and microfluidic devices. Each type of instrument produces different measurement artifacts and makes different demands on the fluorescent probe. For example, although photobleaching is often a significant problem in fluorescence microscopy, it is not a major impediment in flow cytometry or DNA sequencers because the dwell time of individual cells or DNA molecules in the excitation beam is short.
Fluorescence Signals

Fluorescence intensity is quantitatively dependent on the same parameters as absorbance — defined by the Beer–Lambert law as the product of the molar extinction coefficient, optical path length and solute concentration — as well as on the fluorescence quantum yield of the dye and the excitation source intensity and fluorescence collection efficiency of the instrument. In dilute solutions or suspensions, fluorescence intensity is linearly proportional to these parameters. When sample absorbance exceeds about 0.05 in a 1 cm pathlength, the relationship becomes nonlinear and measurements may be distorted by artifacts such as self-absorption and the inner-filter effect. Because fluorescence quantitation is dependent on the instrument, fluorescent reference standards are essential for calibrating measurements made at different times or using different instrument configurations. To meet these requirements, Molecular Probes offers high-precision fluorescent microsphere reference standards for fluorescence microscopy and flow cytometry and a set of ready-made fluorescent standard solutions for spectrofluorimetry. A spectrofluorimeter is extremely flexible, providing continuous ranges of excitation and emission wavelengths.

2. EXPERIMENTAL

Aim

(1) To understand Principles of Fluorescence.
(2) Determining $\lambda_{EM}$, $\lambda_{EX}$, calibration curves and unknown concentrations of proteins

Equipment

The instrument used in this experiment is Cary Eclipse fluorescence spectrophotometer which is controlled by Cary Eclipse software on a Pentium PC. The instrument consists of two Czerny-Turner monochromators (excitation and emission), a Xenon light source, a range of fixed width selectable slits, selectable filters, attenuators and two photomultiplier tubes as detectors.

Figure 4 illustrates the optical system of the Cary Eclipse fluorescence spectrophotometer. A high intensity Xenon flash lamp operating at 60-75 kW power is used as the light source. The Schwartzchild collector mirrors collect the light energy from the Xenon Flash Lamp module. This light is then focused through a lens onto the excitation entry slit. The Xenon flash lamp flashes at a rate of up to 80 flashes per second and has a pulse width of approximately 2 to 3 microseconds.
The Excitation monochromator module has a motorized grating and it selects a suitable wavelength from the white light emitted by the xenon lamp. The filter flywheel (F.F) helps the removal of higher wavelength light. The available wavelength range for both excitation and emission spectra is 190-1100 nm.

For light source compensation, a portion of the excitation light is reflected by a beam splitter (B.S) and directed to a reference detector. This improves the signal to noise ratio. The other portion of the excitation light illuminates the sample cell.

The light containing the fluorescent signal from the sample is viewed at 90° with respect to the incident beam. The emitted light from the cell is directed into the emission monochromator, consisting of a motorized grating. The detector (photomultiplier tube) measures the spectral light intensity.

**Cell Handling**

Absorption cells (sometimes called cuvettes) should receive the same care given for a lens or other optical component. The optical surfaces of cells that are placed in the light beam must be absolutely clean, or serious errors in spectrophotometric measurements will result.

In the handling of cells, the following well-known rules should be followed without exception.

1. *Never* touch the optical surfaces of the cell. Contact with the skin will invariably leave a film that, though invisible to the eye, will change the light transmission and reflection characteristics of the cell windows, especially in the ultraviolet
region.

2. Handle cells only at the top portions of the side plates that do not face the optical axis.

3. When filling cells with sample solutions, a dropper, or preferably a pipette, should be used rather than direct pouring from a beaker or test tube.

4. Rinse the cell with several portions of the solution before filling. Avoid overfilling the cell.

5. Do not spill liquid on the outside of a cell. Before inserting a cell into the holder, carefully wipe the cell windows with a clean lens tissue or suitable absorbent lint-free disposable wiper.

6. Always orient cells in the same direction in the cell holder. When using a matched pair of cells, always use the same cell for the reference.

7. For the disposable plastic cells, solvents like methanol and ethanol can be contained for a maximum time of 5 min. Never use the plastic cells for toluene.

![Figure 5: Cell Handling Techniques](image)

**Preparation of Solutions:**

**Protein:** Lysozyme Egg White (6x crystallized), Seikagaku corporation, Tokyo.

**Solvent:** 1x PBS buffer (pH = 7.4)

**Stock solution:** 3 mg/ml: i.e., dissolve 30 mg of protein in 10 ml of buffer.

Using the stock solution prepare the following samples (3 ml each) by dilution in plastic cuvettes:
<table>
<thead>
<tr>
<th>Volume of stock (µl)</th>
<th>volume of buffer (µl)</th>
<th>Total volume (ml)</th>
<th>Protein Conc. (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>2900</td>
<td>3.0</td>
<td>0.1</td>
</tr>
<tr>
<td>200</td>
<td>2800</td>
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</tr>
<tr>
<td>1500</td>
<td>1500</td>
<td>3.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Emission Spectrum**

In proteins, the fluorescence is dominated by Tryptophan, which has an absorption peak at 280 nm. By UV absorption spectra, the absorption peak of lysozyme is found to be 285 nm. So use this as the excitation wavelength to study the emission spectrum.

Click on Cary Eclipse icon on desktop. Select **Scan**.

Select Setup button to display the setup dialog.

Set the data mode to Fluorescence;

- Scan setup to Emission;
- X mode to Wavelength (nm).

Enter the value of excitation wavelength, i.e., 285 nm.

- excitation slit: 20 nm.
- Emission slit: 2.5 nm

Start: 308 nm (sum of excitation wavelength and the slit widths)

Stop: 500 nm (greater than start value by 150-200 nm)
Scan control: medium
Select options to display the options page.

Set Excitation filter to Auto
Set Emission filter to Open
Set PMT voltage to Medium
Make sure that no Accessories are selected
Select the Reports. Enter your name and comments.
Set up your report style by selecting the appropriate check boxes.
Set up the Peaks reporting options
Select Auto Store options for data saving
Finish the setup by pressing OK.

Place the blank solution (3 ml of buffer) in the sample compartment. Click Zero button to zero the system.
Select the Start button to commence data collection.
Place the sample (0.1 mg/ml protein) solution in the sample compartment.
In the sample name dialog enter the name for your sample and click OK.
The Scan will commence and the trace will appear on the graphics area.
Make sure that the peaks are labeled. Print the emission spectrum and also save the file in the folder specified to you.

Inner Filter Effect
In this experiment, you will monitor the fluorescence intensity of protein solution as a function of concentration.
Click on Cary Eclipse icon on desktop. Select Concentration.
Select Setup button to display the setup dialog.
Set the data mode to Fluorescence
Enter the value of excitation wavelength, i.e., 285 nm.

Enter the value of emission wavelength obtained in the **SCAN** experiment described above.

Excitation slit: 20 nm.

Emission slit: 2.5 nm

Ave Time: 0.1 sec

Scan control: medium

Select options to display the options page.

Enter the minimum and maximum Y values to be displayed on the graph.

Set Excitation filter to Auto

Set Emission filter to Open

Set PMT voltage to Medium

Make sure that no Accessories are selected

Set up the calibration

Select standards page and enter the units for the concentration, number of standards, and the concentration values. Curve fitting is not required as explained below

Since this exercise is only for calibration, set the No. of Samples to zero.

Select the Reports. Enter your name and comments.

Set up your report style by selecting the appropriate check boxes.

Select Auto Store options for data saving

Select the Status display to view the information on screen.

Finish the setup by pressing OK.

Place the blank solution (3 ml of buffer) in the sample compartment. Click Zero button to zero the system.
Select the Start button to commence data collection.

The Standard/Sample selection dialog will be displayed. Select suitably.

Present Standard dialog will be displayed to prompt you to place the appropriate standard in the sample compartment. Press OK to measure the standard.

Repeat the above step till you finish measurement of all the solutions.

Ignore any curve fitting given by the software.

Once the run is finished, select Save As command from File menu and save the file in the folder specified to you.

**Note:** The concentration values are selected such that the fluorescence intensity increases linearly for low concentrations, deviates from linearity, reaches maximum and then decreases, as concentration is continuously increased. This result demonstrates the Inner Filter Effect.

### 3. REFERENCES