Teaching Biophysics

Approaches to Teaching Fluorescence Spectroscopy

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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 fluorometers are routinely used to measure complexation and conformational phenomena of biological molecules.

Despite the widespread use of fluorescence in research, courses in biophysical chemistry rarely cover fluorescence in any detail, if at all. Often students learn fluorescence techniques on the fly, and a number of mistakes and oversights can result in subsequent investigations using the technique. The following addresses primarily approaches to teaching fluorescence in the context of an introductory course in biophysical chemistry and biophysical techniques. Clearly, when expert faculty are available it can enrich a graduate program immensely to provide students with a comprehensive course in fluorescence spectroscopy and its more sophisticated applications to the understanding of biomolecules. Because the field has undergone a number of advances in instrumentation, analysis, and data interpretation in recent years, a discussion is also presented of some of the possible subjects that could be covered in an advanced course.

One of the first ideas that must be brought home to students when presenting a new technique is its usefulness. Their enthusiasm for learning the theoretical underpinnings of fluorescence spectroscopy will ultimately be linked to their perception of what it can tell them about the biological systems in which they are interested. One should not rely on any intrinsic interest in photophysics, however fascinating the subject may be. For this reason, before any discussion of the theory of emission of light by molecules it is advisable to begin with a brief, general discussion of the kinds of information that fluorescence measurements can provide.

BASIC FLUORESCENCE THEORY

The goal of teaching fluorescence theory in an introductory survey course in biophysical techniques is that students come away with a basic understanding of the phenomenon of spontaneous emission and the molecular and environmental properties that govern the intensity, color, and polarization of the emission. Because many of the students who take these courses are not majors in biophysics but in related fields such as biochemistry or molecular biology, their background in physical chemistry can be somewhat limited. One of the great difficulties inherent in organizing such a course is adjusting the level of detail of the discussion of the theory of fluorescence to the students' background in particular because this may vary widely within the same class. Both in the context of a survey course as well as in a more detailed treatment of fluorescence techniques the four basic rules of fluorescence should be covered. These are as follows:

1. The Franck-Condon principle: the nuclei are stationary during electronic transitions, and so excitation occurs to vibrationally excited levels of the excited electronic state.
2. Emission occurs from the lowest vibrational level of the lowest excited singlet state because relaxation from the excited vibrational levels is much faster than emission.
3. The Stokes shift: emission is always of lower energy than absorption due to nuclear relaxation in the excited state.
4. The mirror image rule: emission spectra are mirror images of the lowest energy absorption band.

A reasonably thorough and straightforward treatment of basic fluorescence theory can be found in a book on fluorescence spectroscopy by Lakowicz (1983). As a means of illustrating these principles, the Jablonski diagram and the Lennard-Jones potential diagram in chapter 1 of Lakowicz (1983) are quite useful. In the Jablonski diagram, the ground and two excited electronic states of a hypothetical molecule are diagrammed on the energy axis. The approximate time scales for the various events are also given in this diagram. The internal conversion of energy resulting in fast, nonradiative transitions from the second (S_2) to the first excited singlet state (S_1) is represented by the dashed lines.

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The Stokes shift can be explained using the Jablonski diagram by pointing out the longer length of the arrow from the zeroth level of the ground state to the second vibrational level of the excited state as compared with the arrow length of transitions from the zeroth level of the first excited state to the second vibrational level of the ground state.

The L-J diagram helps to bring home the point about the Stokes shift by linking the length of the emission arrow to the degree of nuclear relaxation that occurs in the excited state before emission. If the internuclear coordinates of the excited state are much different from those of the ground state, then the Stokes shift is large. This diagram also helps illustrate the concept that the probability of the transition between the zeroth level of either the ground or excited state and the various vibrational levels of the excited or ground state, respectively, depends on the overlap of the square of the wavefunctions of the energy levels. Once the concept of the overlap of wavefunctions has been introduced it is not difficult to explain the mirror image rule, because the form of the wavefunction in any vibrational level of the excited state is quite similar to its form in the ground state, and thus probabilities are comparable in excitation and emission.

FLUORESCENCE OBSERVABLES

Once these basic theoretical concepts regarding electronic transitions have been addressed, the various fluorescence observables can be covered. These include steady-state intensity and the steady-state spectrum, steady-state anisotropy, time-resolved intensity decay and decay-associated spectra, time-resolved anisotropy decay, and excited-state reactions.

In a one to two-lecture session as part of an introductory course, the detail with which these phenomena can be covered is relatively limited, whereas in a five to eight-lecture section one has the opportunity to expand the discussion and use numerous examples. When introducing these observables it is important to cover what they are, what environmental or molecular factors contribute to their ultimate values and properties, and how they are measured. This last area requires presentation of instrumentation diagrams. Good examples of such can be found in the book by Lakowicz (1983). This book also covers the measurement and analysis of anisotropy, solvent relaxation, and time-resolved fluorescence in reasonable detail. For presentation of even greater detail, the next series of books edited by Lakowicz (1991a–c) is very complete. There are three volumes covering, respectively, techniques, principles, and biochemical applications. In the first volume, chapters 1 and 5, which explain the two methods (time and frequency domain) for time-resolving fluorescence signals, are particularly applicable to a five to eight-lecture series on fluorescence. Another useful reference for an in-depth treatment of frequency domain fluorometry is given by Jameson et al. (1984).

EXAMPLES OF FLUORESCENCE APPLIED TO BIOPHYSICAL CHEMISTRY

After a general introduction to the observables listed above, explanation of their analysis is perhaps most easily accomplished through the presentation of some examples from the literature of the use of fluorescence in biophysics. The number of examples covered and the detail in which they are presented depends on the time available. In a one to two-lecture segment, presentation of only one example is practical. In a longer segment, three or four examples could be covered and the data analysis could be treated in greater detail. Many other examples in addition to those listed here can be found discussed in a very complete review on fluorescence techniques for studying protein structure by Eftink (1990), as well as an earlier review by Beechem and Brand (1985) specifically treating tryptophan fluorescence in proteins. Likewise in Vols. 2 and 3 of the Lakowicz series (1991b, c) are found numerous examples of biophysical applications.

Intrinsic tryptophan fluorescence in proteins

If one has the time for presenting only one example of the use of fluorescence in biophysics from the literature, the study of the fluorescence properties of the single tryptophan residue in phosphofructokinase by Kim et al. (1993) is a good choice. It deals with the origin of the multi-exponential decay characteristics of tryptophan in proteins, which has been a topic of much debate in the field of fluorescence. It is now widely, although not universally, accepted that the complexity of fluorescence decay of single-tryptophan proteins is indicative of multiple conformational states that interconvert on a time scale that is slow compared with emission. The study of Kim and co-workers (1993) from Mary Barkley's group provides an extremely thorough and well-presented argument for the conformational heterogeneity hypothesis. This article is well suited for use in teaching fluorescence because the investigation includes many different fluorescence approaches. Thus the students are exposed to all of the fluorescence observables listed above in the context of an important biophysical problem. The authors first characterized the emission wavelength dependence of the fluorescence decay and the calculation of decay-associated spectra. The work also includes examination of the temperature dependence of the emission and the calculation of the frequency factors and the activation energy for the quenching process using two analysis approaches (linear analysis of the steady-state data and global analysis of the time-resolved data). Solvent isotope effects and quenching by iodide and acrylamide solutes were performed, and the conclusion of all of these experiments is that solvent quenching and relaxation do not contribute to the decay. Interestingly, the two lifetime components exhibit different Stern-Volmer quenching rates. (See Eftink (1990) for an explanation of the Stern-Volmer quenching constants.) Kim et al. (1993) interpret the two quenching rate constants as strong evidence for two conformational states of the protein. In addition, the authors investigated the quaternary structure by anisotropy decay and light scattering and demonstrate that it is homogeneous. They consider the allosteric transition as well and convincingly demonstrate that this phenomenon cannot be responsible for the complex decay. The discussion of the conclusion that conformational heterogeneity is the basis for the decay properties is well

reasoned and thorough. Moreover, many of the important studies of tryptophan emission properties that have appeared over the years are cited and discussed, so this article provides a solid resource for explaining fluorescence decay in proteins.

Fluorescence quenching and protein structure and dynamics

The dynamic quenching of fluorescence emission of tryptophan in proteins arises from excited-state encounters of the tryptophan with the functional groups of the amino acids in the surrounding protein matrix or from encounters with the solvent. An article by Harris and Hudson (1990) provides a good discussion of the quenching properties of particular amino acids. This excited-state encounter implies that the tryptophan and/or its surroundings move during the lifetime of the excited state and thus in principle, tryptophan lifetime data carry information about the dynamic properties of the protein. Lakowicz and Weber (1973) published one of the early demonstrations of protein motions on the nanosecond time scale by oxygen quenching of intrinsic tryptophan residues in a number of proteins. Ross et al. (1981) used solute quenching to demonstrate the accessibility of the two tryptophan residues in lactate dehydrogenase, and thus provide some structural information about the protein.

Intrinsic tryptophan emission and protein folding

The literature abounds with fluorescence studies of protein denaturation and renaturation both kinetic and equilibrium. In addition, two reviews of the subject have recently appeared (Eftink, 1994; Royer, 1994). The fluorescence properties of intrinsic tryptophan residues in proteins invariably changes on denaturation of the protein. This said, it is not usually possible to predict how the intensity of fluorescence will change. Some proteins exhibit large increases in their intensities and lifetimes and others, decreases, on unfolding of the chain. Proteins with heme prosthetic groups typically exhibit an increase in intensity on unfolding due to loss of the heme group, which quenches the tryptophan emission in the native state by Forster energy transfer. Comparisons of quantum yields for a number of proteins in their native and denatured states (Kronman and Holmes, 1971; Grinvald and Steinberg, 1976) reveals that these vary widely. The average energy of the emission of the tryptophan residues usually shifts to the red on unfolding because the solvent exposure and thus solvent relaxation is augmented in the unfolded state. The magnitude of the shift in energy depends on the extent to which the protein is buried in the native state. In the unfolded state most tryptophan residues in proteins have spectra similar to that of *N*-acetyl tryptophanamide in water, with a maximum of ~355 nm. Because the motions of the tryptophan residues are significantly less constrained in the denatured states of proteins, one also observes a large decrease in the anisotropy of the fluorescence emission on denaturation. The extent to which one can interpret the changes observed in the fluorescence parameters of a particular protein on unfolding depends on the understanding one has about the structural factors that contribute to the fluorescence characteristics in the native state. In addition, it usually proves difficult to draw detailed structural conclusions from fluorescence unfolding experiments of proteins containing multiple tryptophan residues.

Anisotropy of extrinsic probes and biomolecular complexation

Fluorescence anisotropy is another fluorescence observable that can be very useful in molecular biophysics. The anisotropy is defined as the ratio of the difference between the emission intensity parallel to the polarization of the electric vector of the exciting light ($I_\parallel$) and that perpendicular to that vector ($I_\perp$) divided by the total intensity (I_T).

$$A = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$$

Because the anisotropy of emission (A) is related to the correlation time of the fluorophore (τ_c) through the Perrin equation

$$A_o/A - 1 = \tau/\tau_c$$

where A_o is the limiting anisotropy of the probe, which depends on the angle between the absorption and emission transition dipoles, and τ is the fluorescence lifetime, these measurements can be used to obtain hydrodynamic information concerning macromolecules and macromolecular complexes. Because the tryptophan residues intrinsic to proteins and covalently attached dye molecules nearly always exhibit local rotational motion in addition to depolarization through global Brownian tumbling of the macromolecule, it is wise to measure the time-resolved anisotropy as well as that observed in the steady-state mode. Motions of probes on macromolecules are quite complex and have been the subject of numerous reports. The reader is referred to Lakowicz (1985, 1993a–c) for a more complete discussion of fluorophore motion and the analysis and interpretation of fluorescence depolarization in macromolecules.

Despite the potential complicating factors, it is nearly always observed that complexation between two macromolecules or between a small fluorescent ligand and a macromolecule leads to an increase in the steady-state fluorescence anisotropy. If no changes in quantum yield occur on complexation one can directly fit the anisotropy data in terms of the equilibrium constant for the interaction. The laboratory of Gregorio Weber has long employed steady-state fluorescence anisotropy to study the dissociation of protein subunits by dilution or by the application of high hydrostatic pressure. The bulk of this work is referenced in a recent review by Silva and Weber (1993), as are many papers by other investigators who have used the technique. In cases where the fluorophore lifetime changes, then the steady-state anisotropy and the fractional intensity weighted average lifetimes can be used in the Perrin equation to calculate the average correlation time of the fluorophore. Despite the fact that this value represents an average of the depolarization due to local and global motions, it is nonetheless useful for monitoring complexation events, if not for calculating the molecular weights of the species involved. For this latter, one must carry out time-resolved anisotropy measurements. A good example of

this type of experiment in the time-resolved mode is that of Neyroz et al. (1987). These authors studied the temperature dependence of the monomer-dimer equilibrium of enzyme I of the sugar transport system and demonstrated the general rule that correlation times that are >10-fold the fluorophore lifetime cannot be recovered from anisotropy data with any reasonable accuracy. Other complicating factors include considerations of the irregular shape of macromolecules and macromolecular complexes, although reasonably good approximations can usually be made. Heyduk and Lee (1990) first used fluorescently labeled DNA for protein binding titrations observing the increase in anisotropy of the fluorophore as the protein binds to DNA. The sensitivity of this approach has recently been significantly increased such that it is now quite applicable to the study of very high-affinity protein-nucleic acid interactions (LeTilly and Royer, 1993).

Fluorescence resonance energy transfer and protein unfolding or complexation

Resonance energy transfer is a very powerful method for obtaining both structural and dynamic information about macromolecules and macromolecular complexes. It is based on the through-space dipolar transfer of excited-state energy to acceptor molecules up to 75 Å removed from the donor fluorophore. The efficiency of transfer is related to the inverse of the sixth power of the distance between the donor and acceptor molecules. Other factors such as the overlap of the emission of the donor with the absorption of the acceptor and the relative orientations of the two chromophores also affect the energy transfer. Thus distances, distance distributions, and even dynamic distance distributions can be obtained using this approach. Lakowicz (1983) and Eftink (1990) provide good introductions to the phenomenon of energy transfer. A more detailed treatment can be found in the chapter by Cheung in Vol. 2 of the Lakowicz series (Cheung, 1991). One can find in the literature many examples of the use of energy transfer in biophysics. Important structural information on the hammerhead rybozyme was recently presented in an energy transfer study by Tusch and co-workers (1994). The work in the laboratories of Amir and Haas (1988) and Brand and co-workers (James et al., 1992) provide excellent examples of the elegant use of fluorescence energy transfer to study protein folding phenomena.

Ideally, the instructor can tailor the biophysical examples to fit in with particular areas of concentration of the biophysics program in his or her institution or department. For example, none of the studies discussed above deals with membranes, membrane proteins, or receptor-ligand interactions. The number of examples is so large there is no attempt here to give a complete overview. The general reviews and books listed in the reference section should give the reader a much broader range from which to choose.

LABORATORY EXPERIMENTS AND IN-CLASS DEMONSTRATIONS

One particularly advantageous property of fluorescence is that, because it involves the emission of photons, one can actually see it. This may appear to be stating the obvious, but from a pedagogical point of view, visualization of a physical phenomenon can be reassuring to students. It is not difficult to devise laboratory experiments for such an introductory class, and steady-state fluorometers are not incredibly expensive. However, justification of spending $30,000 on teaching equipment usually depends on the number of students taking the course. Wherever possible, inclusion of laboratory sections in addition to in class demonstrations is highly advisable. Some examples of experiments include the unfolding of a protein with GuHCl monitoring the tryptophan emission spectrum, which changes in intensity and average emission energy as the tryptophan residue(s) become exposed to solvent; titration of bovine serum albumin with the hydrophobic probe, anilino-naphthalene sulfonate (ANS), increasing the quantum yield of ANS enormously on binding; and titration of a fluorescently labeled DNA double strand with a protein that recognizes a specific sequence on that DNA causing the anisotropy of fluorescence of the label on the DNA to increase as the protein binds.

If laboratory sessions are not practical, in-class demonstrations can be quite useful and even inspiring to the students. Solvent relaxation phenomena and their effect on the energy (color) of the emission spectrum provide an excellent example for in-class demonstrations. 6-Propionyl-2-(dimethylamino)naphthalene (PRODAN) exhibits a very large excited-state dipole that renders the emission spectrum quite sensitive to the relaxation of the solvent (Weber and Farris, 1979). Although the maximum in cyclohexane is ~400 nm it shifts to ~540 nm in water. The probe can be dissolved in these solvents in glass test tubes. Then a small ultraviolet hand lamp can be used for excitation. One observes the purple fluorescence from the red edge of the spectrum in cyclohexane. As one moves to dimethylformamide, glycerol, ethanol, and then water, the color of the emission changes from purple to yellow because of the greater effect of relaxation for solvents of high polarity. To bring home the point that these shifts are due to molecular motion, i.e., reorientation of the solvent molecules around the excited-state dipole, a tube containing PRODAN dissolved in glycerol, which fluoresces green at room temperature, is plunged into a bath of dry ice/ethanol to reduce the temperature to near −60°C. After a time, the emission has shifted from green to purple because of the slower reorientation of the solvent dipoles at such high viscosity. On heating the tube by holding it in one's hand, the emission eventually shifts back to green and the temperature gradient in the tube can be observed at intermediate times.

THOUGHTS ON CONTENT FOR AN ADVANCED GRADUATE COURSE ON FLUORESCENCE

Of course, if one has the luxury of devoting an entire semester (20 to 30 lectures) to fluorescence spectroscopy and its biophysical applications, then each of the topics mentioned above can be explored in much greater depth. One may want to begin with lectures on the physical characteristics of light. This can be followed by in-depth discussion of electronic absorption transitions in conjugated and

aromatic hydrocarbons, the effects of substituents and solvents, and the polarization of the transitions. These are well explained in the works of Platt (1949), Bayliss (1952), and Suzuki (1967), in which the authors describe the free electron approximation for benzene, naphthalene, and anthracene, and the assignments and polarizations of the electronic transitions. The content of the course dealing with emission wavelength properties could include much greater detail than presented here, such as the Lippert equations and time-resolved spectra for solvent relaxation phenomena (Weber and Farris, 1979; Bismuto et al., 1987), derivation of the expressions for anisotropy decay, and excited-state reactions from the differential equations of the change in the polarized intensity components with time (see the chapter by R. Steiner in Vol. 2 of the Lakowicz series (Steiner, 1991). One can cover the use of rotation matrices and the Legendre polynomials to derive the expression for anisotropy and anisotropy decay. A good discussion of this topic can be found in application notes 5 and 16 of Zare (1988). One can also include the derivation of the relationships of frequency domain and time-domain fluorometry, and a discussion of the information content in fluorescence decay (see Lakowicz, 1991a). Analysis approaches to heterogeneous decay, anisotropic rotations, time-resolved solvent relaxation phenomena, and excited-state reactions can also be discussed. The compartmental analysis approach for complex systems using a numerical eigenvector-eigenvalue solver is presented by Beechem and co-workers in the second volume of the Lakowicz series (Beechem et al., 1991). Also of use to the students is a presentation of lasers and synchrotron radiation as excitation sources, and ultrafast and parallel detectors. These are discussed in chapters 1 and 2 of the first volume of the series (Lakowicz, 1991a). Finally, in such a class, many lectures or student presentations could be devoted to uses of fluorescence in the recent or current literature.

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