Fluorescence Spectrophotometry

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Fluorescence spectrophotometry is a class of techniques that assay the state of a biological system by studying its interactions with fluorescent probe molecules. This interaction is monitored by measuring the changes in the fluorescent probe optical properties.

The Electronic Excited State

Fluorescence and phosphorescence are photon emission processes that occur during molecular relaxation from electronic excited states. These photonic processes involve transitions between electronic and vibrational states of polyatomic fluorescent molecules (fluorophores). The Jablonski diagram (**Figure 1**) offers a convenient representation of the excited state structure and the relevant transitions. Electronic states are typically separated by energies on the order of $10\,000\,cm^{-1}$. Each electronic state is split into multiple sublevels representing the vibrational modes of the molecule. The energies of the vibrational levels are separated by about $100\,cm^{-1}$. Photons with energies in the ultraviolet to the blue-green region of the spectrum are needed to trigger an electronic transition. Further, since the energy gap between the excited and ground electronic states is significantly larger than the thermal energy, thermodynamics predicts that molecule predominately reside in the electronic ground state.

The electronic excited states of a polyatomic molecule can be further classified based on their multiplicity. The indistinguishability of electrons and the Pauli exclusion principle require the electronic wave functions to have either symmetric or asymmetric spin states. The symmetric wave functions, also called the triple state, have three forms, multiplicity of three. The antisymmetric wave function, also called the singlet state, has one form, multiplicity of one.

To the first order, optical transition couples states with the same multiplicity. Optical transition excites the molecules from the lowest vibrational level of the electronic ground state to an accessible vibrational level in an electronic excited state. Since the ground electronic state is a singlet state, the destination electronic state is also a singlet.

After excitation, the molecule is quickly relaxed to the lowest vibrational level of the excited electronic state. This rapid vibrational relaxation process occurs on the time scale of femtoseconds to picoseconds. This relaxation process is responsible for the Stoke shift. The Stoke shift describes the observation that fluorescence photons are longer in wavelength than the excitation radiation.

The fluorophore remains in the lowest vibrational level of the excited electronic state for a period on the order of nanoseconds, the fluorescence lifetime. Fluorescence emission occurs as the fluorophore decay from the singlet electronic excited states to an allowable vibrational level in the electronic ground state.

The fluorescence absorption and emission spectra reflect the vibrational level structures in the ground and the excited electronic states, respectively. The Frank–Condon principle states the fact that the vibrational levels are not significantly altered during electronic transitions. The similarity of the vibrational level structures in the ground and excited electronic states often results in the absorption and emission spectra having mirrored features.

The electronic excited state also has specific polarization properties. Fluorophores are preferentially excited when the polarization of light is aligned along a specific molecular axis (the excitation dipole). Further, the fluorescence photons subsequently emitted by the molecule will have polarization orientated along another molecular axis (the emission dipole). In general, the excitation and emission dipoles do not coincide.
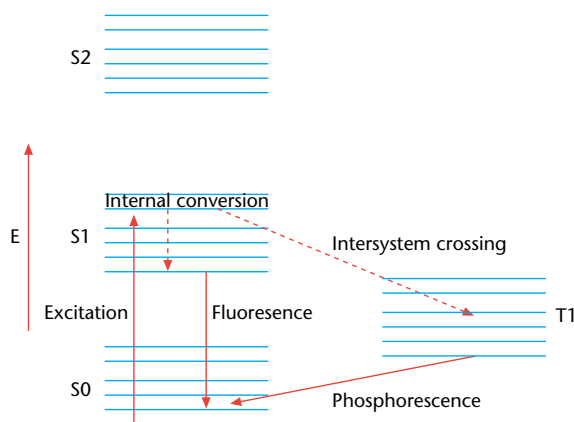

Figure 1 The Jablonski diagram of fluorophore excitation, radiative decay and nonradiative decay pathways. E denotes the energy scale; S0 is the ground singlet electronic state; S1 and S2 are the successively higher energy excited singlet electronic states. T1 is the lowest energy triplet state.

Radiative and Nonradiative Decay Pathways

Radiative decay describes molecular deexcitation processes accompanied by photon emission. Molecules in the excited electronic states can also relax by nonradiative processes where excitation energy is not converted into photons but are dissipated by thermal processes such as vibrational relaxation and collisional quenching. Let Γ and k be the radiative and nonradiative decay rates respectively and N be the fraction of fluorophore in the excited state. The temporal evolution of the excited state can be described by:

$$\frac{dN}{dt} = -(\Gamma + k)N \qquad [1]$$

$$N = N_0 e^{-(\Gamma + k)t} = N_0 e^{-t/\Gamma} \qquad [2]$$

The fluorescence lifetime, τ, of the fluorophore measures the combined rate of the radiative and nonradiative pathways:

$$\tau = \frac{1}{\Gamma + k} \qquad [3]$$

In the absence of nonradiative decay processes, one can define the intrinsic lifetime of the fluorophore:

$$\tau_0 = \frac{1}{\Gamma} \qquad [4]$$

The 'efficiency' of the fluorophore can then be quantified by the fluorescence quantum yield, Q:

$$Q = \frac{\Gamma}{\Gamma + k} = \frac{\tau}{\tau_0} \qquad [5]$$

Factors Affecting Fluorescence Intensity

A number of factors contributes to the nonradiative decay pathways of the fluorophores and reduces fluorescence intensity. In general, the nonradiative decay processes can be classified as:

$$k = k_{ic} + k_{ec} + k_{is} \qquad [6]$$

where k_{ic} is the rate of internal conversion, k_{ec} is the rate of external conversion, and k_{is} is the rate of intersystem crossing.

Internal conversion is a process where the electronic energy is converted to the vibrational energy of the fluorophore itself. Since vibrational processes are driven by thermal processes, the internal conversion rate typically increases with temperature, which accounts for the commonly observed decrease in fluorescence intensity with rising temperature.

External conversion describes the process where the fluorophore loses electronic energy to its environment through collision with other solutes. Collisional quenching processes are particularly interesting as they allow the biochemical environment of the fluorophores to be measured. A number of important solute molecules, such as oxygen, are efficient fluorescence quenchers. Upon collision, the fluorophore is deexcited nonradiatively. The collisional quenching rate can be expressed as:

$$k_{ec} = k_0[Q] \qquad [7]$$

where k_0 is related to the diffusivity and the hydrodynamics radii of the reactants and $[Q]$ is the concentration of the quencher.

When collisional quenching is the dominant nonradiative process, eqn [1] predicts that fluorescence lifetime decreases with quencher concentration:

$$\frac{\tau_0}{\tau} = (1 + k_0 \tau_0 [Q]) \qquad [8]$$

The steady state fluorescence intensity, F, also diminishes relative to the fluorescence intensity in the absence of quencher, F_0. This effect is described by the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + k_0 \tau_0 [Q] \qquad [9]$$

Fluorescence signal reduction can also result from ground state processes – steady state quenching. A fluorophore can be chemically bound to a quencher to form a 'dark complex' – a product that does not fluoresce. Fluorescence intensity decreases with steady state quenching as:

$$\frac{F_0}{F} = 1 + K_s[Q] \qquad [10]$$

where K_s is the association constant of the quencher and the fluorophore. Fluorescence lifetime is not affected by steady state quenching as the excited states are not involved.

Phosphorescence

Intersystem crossing is another process where fluorescence signal is reduced and phosphorescence is generated. Spin-orbit coupling is a quantum mechanical process that is responsible for intersystem crossing. Intersystem crossing describes the relaxation of the molecule from a singlet excited state to a lower energy, triplet excitation state. Since spin-orbit coupling is a weak effect, the intersystem crossing rate is low. The relaxation from the triplet state to the singlet ground state requires another change of multiplicity. Hence, the decay from the triplet states also has a very low rate. However, radiative relaxation, phosphorescence, does occur due to spin-orbit coupling. The typical phosphorescence lifetime is on the order of

microseconds to seconds. Phosphorescence has larger Stoke shift than fluorescence owing to the triple state having lower energy.

Since phosphorescence rate is often much lower than thermally activated nonradiative decay processes such as collisional quenching, phosphorescence is rarely observed in aqueous systems at physiological temperature. However, a number of protein conformation studies at cryogenic temperatures have utilized phosphorescence spectroscopy.

Instrumentation for Fluorescence Spectrophotometry

The measurement of fluorescence signals provides a sensitive method of monitoring the biochemical environment of a fluorophore. Instruments have been designed to measure fluorescence intensity, spectrum, lifetime and polarization.

Fluorescence intensity measurement allows the determination of the presence of fluorophores and their concentrations. Fluorescence intensity measurement is used in numerous biochemical assays. The instrument designs for these assays are rather straightforward but are as varied as the applications.

Fluorometers are general-purpose instruments designed to measure fluorescence spectrum, polarization and/or lifetime. A typical fluorometer includes a light source, a specimen chamber with integrated optical components, and high sensitivity detectors (**Figure 2**). The most common light source for fluorometers are lamp sources, such as xenon arc lamps. These lamps provide a relatively uniform intensity over a broad spectral range from the ultraviolet to the near infrared. The optical paths of the excitation and the detection light paths are along the orthogonal axis. The orthogonal arrangement ensures minimal leakage of excitation light into the detection side. High sensitivity photodetectors such as photomultipliers or charge-coupled device cameras are commonly used.

For spectral measurement, monochromers or band-pass filters are placed in the excitation and emission light paths to select a specific spectral band. The excitation spectrum is defined as the fluorescent intensity measured as a function of excitation wavelength at a constant emission wavelength; the emission spectrum is the fluorescent intensity measured as a function of emission wavelength at a constant excitation wavelength.

Fluorometers have also been designed to measure fluorescence lifetime. Given the typical lifetime of fluorophores, accurate lifetime measurement requires photodetectors and signal processing electronics with subnanosecond resolution. High-precision fluorometers have been designed in both the time and frequency domains. In the time domain, femtosecond or picosecond
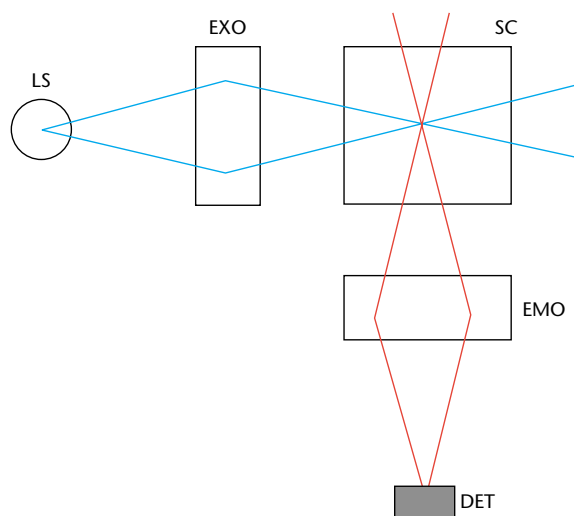

Figure 2 A typical fluorometer design. LS is the light source, EXO is the excitation optical train, SC is the specimen chamber, EMO is the emission optical train, and DET is the optical detector. Both the excitation and emission optical trains contain beam-shaping and collimation optics. For wavelength-resolved measurements, spectral selection optical components such as monochromers and filters are included in the EXO and EMO. For polarization measurement, polarizers are added to EXO and EMO. For lifetime measurement, a laser light source is often used and high-speed electronics are integrated into the detector subsystem.

pulsed lasers are often used as excitation light sources. A time-correlated single photon counting method is frequently employed, in which the time delay between the excitation light pulse and the resultant fluorescence photon is measured by high-speed electronics. In the frequency domain, the specimen is excited by a light source with high-frequency content. The resultant fluorescence signal is also modulated at the same frequency but is phase-delayed and amplitude-demodulated. The phase delay and demodulation contain the lifetime information of the fluorophore and can be measured using heterodyning or homodyning detection techniques.

For polarization measurement, polarizers are inserted into the excitation and emission light paths. With the excitation polarizer fixed, the emission polarizater can be rotated to measure the perpendicular ($I_\perp$) and parallel ($I_\parallel$) components of the fluorescence emission. The steady state polarization is defined as:

$$P = \frac{I_\perp - I_\parallel}{I_\perp + I_\parallel} \qquad [11]$$

and an equivalent measure is the steady state anisotropy:

$$r = \frac{I_\perp - I_\parallel}{I_\perp + 2I_\parallel} \qquad [12]$$

Applications of Fluorescence in the Study of Biological Structure and Function

The use of fluorescence in biology and medicine is ubiquitous. In particular, the measurements of fluorescence spectrum, lifetime and polarization are powerful methods of studying biological structure and function.

The fluorescence spectrum is highly sensitive to the biochemical environment of the fluorophore. Fluorophores have been designed such that their spectra change as a function of the concentration of metabolites, such as pH and calcium. Fluorescence spectral changes resulting from solvent relaxation of fluorescent amino acids, such as tryptophan and tyrosin, are important reporters of protein structure and folding. Protein domain structure and motion on the subnanometer scale can be spectrally monitored using fluorescence resonance energy transfer (FRET). FRET is a nonradiative process where the energy is transferred between two fluorophores. FRET requires that the emission spectrum of one fluorophore (the donor) overlaps the absorption spectrum of a second fluorophore (the acceptor). The efficiency of this process is a strong function ($1/r^6$) of the molecules' relative distance, r. Protein conformation can be monitored by labelling the relevant structures with a FRET pair. The distance between the two fluorophores can be quantified by spectrally resolving the relative fluorescence intensities of the donor and the acceptor.

Fluorescence lifetime provides complementary information to spectral measurement. Many fluorophores may respond to environmental changes with lifetime variations. An important example is oxygen concentration measurement based on the dynamic quenching of long-lifetime fluorophores. Lifetime measurements are also used to distinguish dynamic and static quenching mechanisms. Lifetime-resolved FRET measurement allows the determination of distance distribution of a population of FRET pairs.

Fluorescence polarization measures the rotational diffusion rate of macromolecules. Rotational diffusion contains information related to the shape of the proteins. Diffusional restrictions of molecules in biological macrostructures, such as cellular membrane or the cytoskeleton, can also be quantified based on polarization measurement. The most common application of fluorescence polarization spectroscopy is the monitoring of protein–ligand binding and oligomerization. The combination of lifetime and polarization measurements allows the quantification of rotational rate and has been used to study protein domain motion.

The limited scope of this article precludes a complete discussion on the use of different spectroscopic methods to deduce biological structures and functions. However, as a demonstration of the general principles, we will examine the use of fluorescence polarization to monitor protein–ligand interactions. In a typical molecular binding assay, the smaller ligand molecules are labelled by a fluorophore. The binding of the small ligand to a larger protein results in a significant increase in the hydrodynamic radius of the composite particle and a slower rotational diffusion rate. The change in rotational diffusion rate can be measured using fluorescence polarization assay. The fraction of bound molecules can be estimated by quantifying the optical signal contributions from the fast and slow diffusers. The association constant of this protein–ligand interaction can also be measured by quantifying the fractions of bound and free proteins at different protein–ligand mixing ratios.

Fluorescence spectophotometry is widely used in many areas of biology and medicine. A basic understanding of fluorescence principles, fluorophores properties, instruments and techniques is a prerequisite to the study of a wide range of biological systems.

Further Reading

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