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Fluorescence Spectroscopy

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Fluorescence spectroscopy is the study of radiative emission of a fluorescent molecule (fluorophore). This is a sensitive technique and its high sensitivity is due to the fact that the emitted radiation is measured directly and can be increased by increasing the incident power. Since the fluorescence intensity is dependent on the choice of excitation as well as emission wavelengths, it is more selective. It has a number of measurable fluorescence characteristics making it inherently information rich. It is a powerful tool employed in various diverse fields like physics, chemistry, biology and medicine. The local environments (polarity or hydrophobicity, viscosity, pH, etc.) affect the spectral, temporal and polarization characteristics of the fluorescent molecules. Fluorescence techniques are more sensitive(up to 10^{-9} M) compared to absorbance based techniques which are sensitive only up to 10^{-6} M. Moreover, emission based techniques are measured directly and they are zero background techniques which make them more attractive. Certain steady-state and time-dependent parameters characterize a fluorescence spectrum. Emission maxima (λ), fluorescence intensity, and average anisotropy (r) are steady-state parameters. The intensity decay lifetime (τ) and the time dependent anisotropy (r_t) are the temporal parameters.

1. CHARACTERISTICS OF FLUORESCENT EMISSION

The phenomenon of fluorescence is normally understood by the following schematic diagram called 'Zablonskii Diagram'.

In the diagram the notation 'S' implies a singlet state, 'IC' is 'internal conversion' and 'ISC' is 'intersystem crossing'. When light is incident on matter, the molecule absorbs light of proper wavelength and is excited to a higher energy level. On absorption (hνa) a molecule can be excited to an upper electronic state such as S_1 or S_2. The decay of this molecule to the ground state (S_0) can occur in a number of ways. The excited molecule can rapidly relax from the S_2 state to the lowest vibrational level of S_1 without emitting a photon. This process is called internal conversion and generally occurs in 10^{-12} s or less The possible decay pathways from S_1 state are: a) Fluorescence ($h\nu_f$), a transition to the S_0 state with the emission of a photon, b) Internal Conversion (IC), a nonradiative transition to the S_0 state and c) Intersystem Crossing (ISC), a transition to an excited triplet (T_1) state in which the electron spins are no longer paired. T_1 state may return to the ground state by a radiative transition

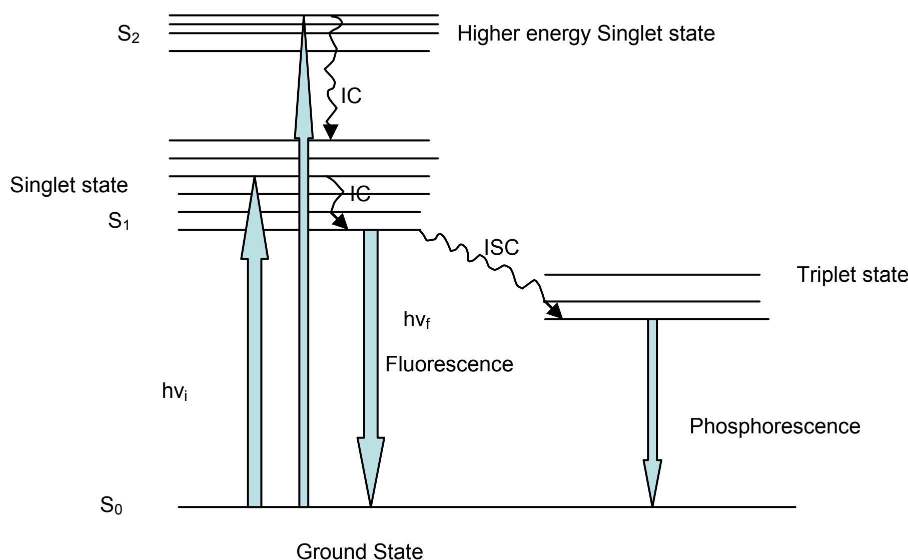


Fig. 1

(phosphorescence) or by a non- radiative transition (intersystem crossing). The excited photon in the S_1 state may transfer its excitation energy to other chromophores or participate in a photochemical reaction.

Since fluorescence lifetimes are typically near 10^{-8} s, internal conversion is generally complete prior to emission and hence fluorescence emission generally results from a thermally equilibrated excited state, that is, the lowest- energy vibrational state of S_1.

Return to the ground state typically occurs to a higher vibrational ground- state level, which then quickly reaches thermal equilibrium. Since electronic excitation does not greatly alter the nuclear geometry, this results in an emission spectrum, which is typically a mirror image of the absorption spectrum of the $S_0 \rightarrow S_1$ transition.

The following are some general characteristics of fluorescence:

1. *Kasha's rule*— In condensed media generally the same fluorescence is observed irrespective of excitation wavelength.
2. *Stokes shift*— Except for atoms in the vapor phase, there is invariably a shift to lower wavelength.
3. *Mirror symmetry*— If the ground and excited geometries of the fluorophore are similar, a mirror symmetry exists between the absorption and emission spectra. The excitation and fluorescence spectra of perylene below show such mirror symmetry.

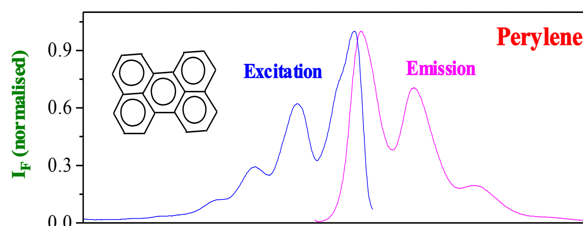
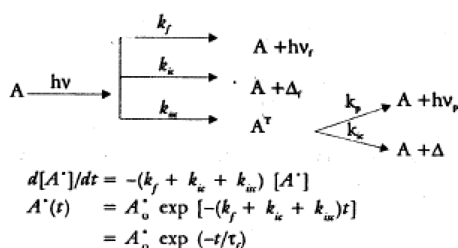


Fig. 2

4. *Quantum yield and lifetime*— Quantum yield of fluorescence(φ_f) is defined as the ratio of the number of molecules fluorescing to the number of molecules absorbing light. Lifetime of fluorescence(τ_f) is defined as the time for the excited molecules to 1/e th of the total number of such molecules formed immediately after excitation. For a molecule:



$\tau_f = 1 / (k_f + k_{ic} + k_{isc})$ and $\phi_f = k_f / (k_f + k_{ic} + k_{isc}) = k_f\tau_f$

($k_{ic} + k_{isc}$) is also sometimes designated as $\sum k_{nr}$.

The fluorescence decay plot then looks like

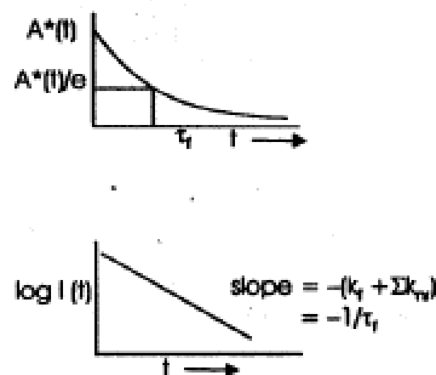


Fig. 3

5. *Fluorescence Anisotropy*— Fluorophores preferentially absorb photons whose electric vectors are aligned parallel to the transition moment of the fluorophore and thus on excitation with polarized light only selected molecules get excited leading to partially polarized fluorescence emission. The parametrization of the comnept is done the following way:

$$P = \frac{I_{\|} - I_{\perp}}{I_{\|} + I_{\perp}} \text{ ; and } r = \frac{I_{\|} - I_{\perp}}{I_{\|} + 2I_{\perp}}$$

Where 'P' represents 'fluorescence polarization' parameter and 'r' represents 'fluorescence anisotropy' parameter. $I_{||}$ is the fluorescence intensity observed when the polarizer is oriented parallel to the direction of the polarized excitation, and $I_{\perp}$ is the intensity observed the polarizer is perpendicular to the excitation. 'r' is the more commonly used parameter and gives information on the rigidity of the nano-environment around the fluorophore.

2. MEASUREMENT TECHNIQUES

2.1 Steady State Measurements

A schematic block diagram for a steady-state fluorimeter is given below.

Conventionally a 100 W xenon lamp is used as a light source. Observation is made at right angle to excitation so as to avoid the transmitted light entering the emission monochromator. Emission spectrum is obtained by exciting the sample at a suitable excitation wavelength and excitation spectrum is obtained by fixing the emission wavelength and scanning through the absorption spectral range. The spectra thus obtained are not the true spectra as instrument parameters like intensity of excitation light,

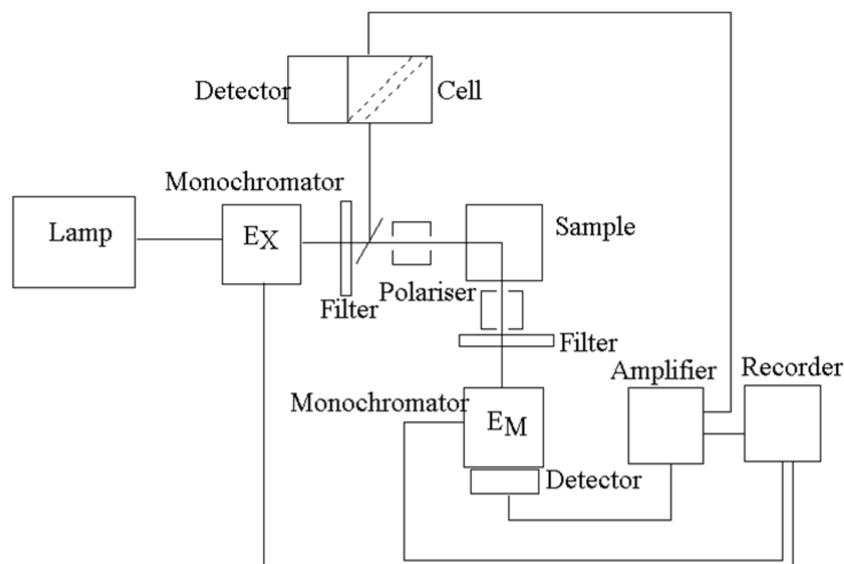


Fig. 4

response of the monochromators and the sensitivity of the photomultiplier tube are all wavelength dependent. Thus it is necessary to obtain suitable correction factors for an instrument and modify the observed spectrum to obtain the corrected spectrum. Determination of absolute quantum yield of fluorescence is difficult, however, relative quantum yield is easily obtained by using fluorophore standards of known quantum yield. Fluorescence polarization measurements are easily done by mounting a polarizer and an analyzer in the optical path.

2. Time Resolved Measurements

Three Different Methods are Usually Employed to Obtain Fluorescence Lifetimes and Time Resolved Spectrum. They are (A) Pulse Sampling Method, (B) Single Photon Counting and (C) the Phase Shift Method. A Physical or Chemical Event Occurring in the Nanosecond and Picosecond Time Range Competes with the Fluorescence Emission Time and Hence Often Time-Resolved Fluorimetry is Extremely Useful to Study a Fairly Large Number of Processes Occurring in the Excited state.

3. Recent Developments

With the progress in electronics and optics and the advent of personal computers, the instrumentation of fluorescence spectroscopy has improved tremendously. Some of the recent applications include time-resolved fluorescence microscopy, synchronous and excitation-emission matrix spectroscopy, fluorescence based sensors etc.

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