Chapter 1: General Introduction

1.1. Nature of Electromagnetic Radiation

The electric and magnetic fields ($\mathbf{E}$ and $\mathbf{B}$) generated by an electric dipole (i.e. by a pair of positive and negative charges) are simply the sum of the fields from the individual charges. If the orientation of the dipole oscillates with time, the fields in the vicinity will also oscillate at the same frequency. The oscillating components of $\mathbf{E}$ and $\mathbf{B}$ at a given position are perpendicular to each other, and at large distances from the dipole, they also are perpendicular to the position vector ($\mathbf{r}$) relative to the center of the dipole. They fall off in magnitude with $1/r$, and with the sine of the angle ($\theta$) between $\mathbf{r}$ and the dipole axis. Such a coupled set of oscillating electric and magnetic fields together constitute an electromagnetic (EM) radiation field.

A spreading radiation field like that mentioned above can be collimated by a lens or mirror to generate a plane wave that propagates in a single direction with constant irradiance (measure of the strength of EM radiation and expressed in terms of the amount of energy flowing across a specified plane per unit area per unit time). The electric and magnetic fields in such a wave oscillate sinusoidally along the propagation axis as illustrated in figure 1.1, but are independent of position normal to this axis. Polarizing devices can be used to restrict the orientation of the electric and magnetic fields to a particular axis in the plane. The plane wave illustrated in figure 1.1 is said to be linearly polarized because the electric field vector is always parallel to a fixed axis. Because $\mathbf{E}$ is confined to the plane normal to the axis of propagation, the wave also can be described as plane-polarized. An unpolarized light beam propagating in the $y$-direction consists of electric and magnetic fields oscillating in the $xz$ plane at all angles with respect to the $z$-axis.

The properties of EM fields are described empirically by four coupled equations that were set forth by J.C. Maxwell in 1865 [1]. These very general equations apply to both static and oscillating fields, and they encapsulate the salient features of EM radiation. In words, they state that: (i) Both $\mathbf{E}$ and $\mathbf{B}$ are always perpendicular to the direction of propagation of the radiation (i.e., the waves are transverse); (ii) $\mathbf{E}$ and $\mathbf{B}$ are perpendicular to each other; (iii) $\mathbf{E}$ and $\mathbf{B}$ oscillate in phase; and, (iv) If we look in the direction of propagation, a rotation from the direction of $\mathbf{E}$ to the direction of $\mathbf{B}$ is clockwise. Instead of putting too much stress on the Maxwell’s equations themselves; we can focus on a solution to these equations for a particular situation such as the
plane wave of monochromatic, polarized light illustrated in figure 1.1. In molecular spectroscopy, one is interested mainly in the time-dependent oscillations of the electrical and magnetic fields in small, fixed regions of space. Because molecular dimensions typically are much smaller than the wavelength of visible light, the amplitude of the electrical field at a particular time will be nearly the same everywhere in the molecule. One can also take the average of the field over many cycles of the oscillation initially and assume that it does not depend on coherent superposition of light beams with fixed phase relationships. With these restrictions, the dependence of $E$ on position and the phase shift can be neglected, and written as (taking $v$ as the frequency of oscillation)

$$E(t) = 2E_0 \cos(2\pi vt) = E_0(e^{2\pi ivt} + e^{-2\pi ivt})$$  \hspace{2cm} (1.1)

![Electric and Magnetic Fields](image)

**Figure 1.1** Electric (bold curve) and magnetic (light curve) field components as a function of position at a given time in a linearly polarized plane wave propagating in the direction of $y$-axis.

1.2. Interaction of Electrons with Oscillating Electric Field

In this section, we will see how an oscillating electric field of linearly polarized light (with frequency $v$), interacts with the molecule to cause electronic absorption. The oscillating electric field adds a time-dependent term to the stationary state Hamiltonian operator ($\hat{H}$) for the
electron. To an approximation that often proves acceptable, we can write the perturbation ($\hat{h}$) as the dot product of $E$ with the dipole operator, $\mu$:

$$\hat{h}(t) = -E(t) \cdot \mu$$  (1.2)

The dipole moment operator, $\mu$, for an electron can simply be written as:

$$\mu = e \mathbf{r}$$  (1.3)

where, $e (-1.602 \times 10^{-19} \text{ C})$ is the electronic charge, $\mathbf{r}$ is the position vector and $r$ is the position of the electron. Putting $E(t)$ in equation (1.2) from equation (1.1), one can write

$$\hat{h}(t) = -eE(t) \cdot \mathbf{r} = -e|E_0|(e^{2\pi i t} + e^{-2\pi i t})|r| \cos \theta$$  (1.4)

where, $r$ is the position of the electron and $\theta$ is the angle between $E_0$ and $\mathbf{r}$.

In the ground state, the wavefunction of the molecule (electron) can be described by $\psi_a$. However, in presence of an oscillating radiation field, this and the other solutions to the Schrödinger equation for the unperturbed system become unsatisfactory; they no longer represent the stationary states. Normally, the wavefunction of the electron in the presence of the field can be expressed by a linear combination of the original wavefunctions; $C_a \Psi_a + C_b \Psi_b + ...$, where the coefficients $C_a$ are functions of time. As long as the system is still in $\Psi_a$, $C_a = 1$ and all the other coefficients are zero; but if the perturbation is sufficiently strong $C_a$ will decrease with time, while $C_b$ or other coefficients increases. The expected rate of growth of $C_b$ can be given by:

$$\frac{\partial C_b}{\partial t} = \left(\frac{2\pi i}{\hbar}\right) e^{\frac{2\pi i(E_b-E_a)t}{\hbar}} (e^{2\pi i t} + e^{-2\pi i t})E_0 \langle \psi_b | \mu | \psi_a \rangle$$  (1.5)

$$= \left(\frac{2\pi i}{\hbar}\right) e^{\frac{2\pi i(E_b-E_a+\hbar t)}{\hbar}} + \left(e^{\frac{2\pi i(E_b-E_a-\hbar t)}{\hbar}} \right)E_0 \langle \psi_b | \mu | \psi_a \rangle$$  (1.6)

where, $E_a$ and $E_b$ are the energies of states $a$ and $b$ (represented by $\psi_a$ and $\psi_b$), respectively.

The probability that the electron has made a transition from $\Psi_a$ to $\Psi_b$ by time $\tau$ is obtained by integrating the above equation from time $t = 0$ to $\tau$ and then evaluating $|C_b(\tau)|^2$. The result gives:
\[ C_b(\tau) = \left[ \frac{1}{E_b - E_a + \hbar \nu} \times \left\{ e^{\frac{2\pi i (E_b - E_a + \hbar \nu) \tau}{\hbar}} - 1 \right\} + \frac{1}{E_b - E_a - \hbar \nu} \times \left\{ e^{\frac{2\pi i (E_b - E_a - \hbar \nu) \tau}{\hbar}} - 1 \right\} \right] \times E_0 \cdot |\psi_b \rangle \langle \mu | \psi_a \rangle \] 

(1.7)

It is to be noted that the two fractions in the first term differ only in the sign of the term \( \hbar \nu \). Let us assume the situation where \( E_b > E_a \), which means that \( \Psi_b \) lies above \( \Psi_a \) in energy. The denominator in the second fraction of the first term then becomes zero when \( E_b - E_a = \hbar \nu \). The numerator of this term is a complex number, but its magnitude also goes to zero under this condition, and the ratio of the numerator to the denominator becomes \( 2\pi i \tau / \hbar \). On the other hand, if \( E_b < E_a \) (i.e., if \( \Psi_b \) lies below \( \Psi_a \)), then the ratio of the numerator to the denominator in the first term in the large parentheses becomes \( 2\pi i \tau / \hbar \) when \( E_a - E_b = \hbar \nu \). If \( |E_b - E_a| \) is very different from \( \hbar \nu \), both terms will be very small. So something special evidently happens if \( \hbar \nu \) is close to the energy difference between the two states. Basically, the second term in the large bracket in equation (1.7) accounts for absorption of light when \( E_b - E_a = \hbar \nu \), and that the first term accounts for induced or stimulated emission of light when \( E_a - E_b = \hbar \nu \). Stimulated emission, a downward electronic transition in which light is given off, is just the reverse of absorption.

1.3. Regions of Electromagnetic Spectrum

The regions of the electromagnetic spectrum that will be the most pertinent for the discussion of molecular spectroscopy involve wavelengths between \( 10^{-9} \) and \( 10^{-2} \) cm. Visible light fills only the small part of this range between \( 3 \times 10^{-5} \) and \( 8 \times 10^{-5} \) cm (figure 1.2). Transitions of bonding electrons occur mainly in this region and the neighboring UV region; vibrational transitions occur in the infra-red (IR). Rotational transitions are measurable in the far-IR (microwave) region in small molecules, but in macromolecules these transitions are too congested to resolve. Radiation in the X-ray region can cause transitions in which \( 1s \) or other core electrons are excited to atomic \( 3d \) or \( 4f \) shells or are dislodged completely from a molecule. These transitions can report on the oxidation and coordination states of metal atoms in metalloproteins.

The inherent sensitivity of absorption measurements in different regions of the electromagnetic spectrum decreases with increasing wavelength because, in the idealized case of a molecule that absorbs and emits radiation at a single frequency, it depends on the difference between the populations of molecules in the ground and excited states. If the two
populations are the same, radiation at the resonance frequency will cause upward and downward transitions at the same rate, giving a net absorbance of zero [2].

![Figure 1.2](image)

**Figure 1.2** Regions of electromagnetic spectrum shown in logarithmic wavelength, wavenumber, frequency and energy scales. The upper panel is the enlarged ultraviolet (UV), visible and infra-red (IR) region presented in a linear scale.

1.4. Electronic Absorption

1.4.1. Quantification of light absorption: The Beer-Lambert law

A beam of light passing through a solution of absorbing molecules transfers energy to the molecules as it proceeds, and thus decreases progressively in intensity. The decrease in the intensity, or irradiance (I), over the course of a small volume element is proportional to the
irradiance of the light entering the element, the concentration of absorbers (c), and the length of the path through the element (dx):

\[
\frac{dl}{dx} = -\varepsilon' lc
\]  

(1.8)

The proportionality constant (\(\varepsilon'\)) depends on the wavelength of the light and on the absorber’s structure, orientation and environment. Integrating equation (1.8) shows that if light with irradiance \(I_0\) is incident on a cell of thickness \(l\), the irradiance of the transmitted light will be:

\[
I = I_0 \times e^{-\varepsilon'cl} = I_0 \times 10^{-\varepsilon cl} = I_0 \times 10^{-A}
\]  

(1.9)

where, \(A\) is the absorbance or optical density of the sample \((A = \varepsilon c l)\) and \(\varepsilon\) is called the molar extinction coefficient or molar absorption coefficient \((\varepsilon = \varepsilon' / \ln 10 = \varepsilon'/2.303)\). The absorbance is a dimensionless quantity, so if \(c\) is given in units of molarity \((1\text{M} = 1\text{ mol dm}^{-3})\) and \(l\) in cm, \(\varepsilon\) must have dimensions of \(\text{M}^{-1} \text{ cm}^{-1}\). Equations (1.8) and (1.9) are statements of Beer’s law, or more accurately, the Beer–Lambert law [3].

### 1.4.2. Techniques of measuring absorbance

A spectrophotometer for measuring absorption spectra typically includes a continuous light source, a monochromator for dispersing the white light and selecting a narrow band of wavelengths, and a chopper for separating the light into two beams (figure 1.3). One beam passes through the specimen of interest; the other through a reference (blank) cuvette. The intensities of the two beams are measured with a photomultiplier or other detector, and are used to calculate the absorbance of the sample as a function of wavelength \([\Delta A = \log_{10}(I_0/I_s) - \log_{10}(I_0/I_r) = \log_{10}(I_s/I_r)\], where \(I_0, I_s,\) and \(I_r\) are the incident light intensity and the intensities of the light transmitted through the specimen and reference, respectively\]. The measurement of the reference signal allows the instrument to discount the absorbance due to the solvent and the walls of the cuvette. Judicious choice of the reference also can minimize errors resulting from the loss of light by scattering from turbid samples.
The conventional spectrophotometer sketched in figure 1.3 has several limitations. First, the light reaching the detector at any given time covers only a narrow band of wavelengths. Because a grating, prism, or mirror in the monochromator must be rotated to move this window across the spectral region of interest, acquisition of an absorption spectrum typically takes several minutes, during which time the sample may change. In addition, narrowing the entrance and exit slits of the monochromator to improve the spectral resolution decreases the amount of light reaching the photomultiplier, which makes the signal noisier. Although the signal-to-noise ratio can be improved by averaging the signal over longer periods of time, this further slows acquisition of the spectrum. These limitations are surmounted to some extent in instruments that use photodiode arrays to detect light at many different wavelengths simultaneously.

1.4.3. Effect of surrounding on electronic transition energy

Interactions with the surroundings can shift the energy of an absorption band to either higher or lower energies, depending on the nature of the chromophore and the solvent. For example, considering an n→π* transition, in which an electron is excited from a nonbonding orbital of an oxygen atom to an antibonding molecular orbital distributed between oxygen and carbon atoms. In the ground state, electrons in the nonbonding orbital can be stabilized by hydrogen-bonding or dielectric effects of the solvent. In the excited state, these favorable interactions are disrupted. Although solvent molecules will tend to reorient themselves in response to the new distribution of electrons in the chromophore, this reorientation is too slow to occur during the excitation itself. An n→π* transition therefore shifts to higher energy in more polar or hydrogen-bonding solvents relative to less polar solvents. A shift of an absorption
band in this direction is called a “blue-shift.” On the other hand, the energies of \( \pi \rightarrow \pi' \) transitions are less sensitive to the polarity of the solvent but still depend on the solvent’s high-frequency polarizability, which increases quadratically with the refractive index. Increasing the refractive index usually decreases the transition energy of a \( \pi \rightarrow \pi' \) transition, causing a “red-shift” of the absorption band.

1.4.4. Relaxation of electronically excited molecules

The mechanisms by which electronically excited molecules come to ground state are given by the Jablonski diagram as shown in figure 1.4. The absorption of a photon takes the molecule from ground singlet state \( (S_0) \) to either first or second excited singlet states (represented by \( S_1 \) or \( S_2 \), respectively). From this Franck-Condon (FC) excited state, the molecule relaxes to the lowest vibronic level of the \( S_1 \) state through internal conversion (IC). Further relaxation from this state to \( S_0 \) can occur via three mechanisms. First, by radiative emission of a photon and the process is called as fluorescence. The other two non-radiative channels involve either direct relaxation to \( S_0 \) state (internal conversion) or passage to the triplet state \( (T_1) \) by intersystem crossing (ISC). The radiative transition from \( T_1 \) to \( S_0 \) state is quantum mechanically forbidden and hence is a very slow process relative to fluorescence and is called phosphorescence emission. The fluorescence photons have the information about energy, time, polarization and intensity at a given wavelength. Each of the above parameters of the fluorescence photon gives information about the local environment surrounding the fluorophore under investigation. So, fluorescence intensity, spectrum, polarization and their dependence are important parameters that one can use for the characterization [4].

The maximum of phosphorescence spectrum is generally shifted to longer wavelength relative to the fluorescence. Also, the \( S_1 \) state can be deactivated by a quenching mechanism, in which a quencher \( Q \) quenches the excited state of the fluorophore \( (S_1) \) through an excited state reaction. Reverse intersystem crossing from \( T_1 \) to \( S_1 \) can occur when the energy between \( S_1 \) and \( T_1 \) is small and when the lifetime of \( T_1 \) is long enough. This results in emission with the same spectral distribution as normal fluorescence but with a much longer decay time constant because the molecules stay in the triplet state before emitting from \( S_1 \). This fluorescence emission is thermally activated; consequently, its efficiency increases with temperature and is called delayed fluorescence of E-type. Again, in concentrated solutions, a collision between two molecules in the \( T_1 \) state can provide enough energy to allow one of them to return to the \( S_1 \)
state. Such triplet-triplet annihilation thus leads to a delayed fluorescence emission, and termed as *delayed fluorescence of P-type*.

![Figure 1.4 Schematic representation of Jablonski diagram.](image)

In table 1.1, all possible photochemical pathways those can occur during a photoexcitation and de-excitation process, are shown. If the excited state decayed solely by fluorescence, its population would decrease exponentially with time and the time constant of the decay would be $\tau_r$ (reciprocal of the radiative rate constant, $\kappa_r$). However, other decay mechanisms compete with fluorescence, decreasing the radiative lifetime of the excited state. The alternatives include formation of triplet states (intersystem crossing) with rate constant $\kappa_{isc}$, nonradiative decay (internal conversion) to the ground state ($\kappa_{ic}$). Radiative decay from the triplet state (phosphorescence with corresponding rate constant, $\kappa_p$) can also be another alternative relaxation pathway. Further, several excited state processes like transfer of energy to other molecules (resonance energy transfer, $\kappa_{et}$), electron and proton transfer ($\kappa_{et}$ and $\kappa_{pt}$, respectively) can also contribute hugely in the de-excitation mechanism.
Table 1.1 Different photophysical processes, associated transition and other relevant parameters

<table>
<thead>
<tr>
<th>Process</th>
<th>Transition</th>
<th>Rate constant</th>
<th>Timescale (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Absorption (Excitation)</td>
<td>( S_0 \to S_1 ) or ( S_n )</td>
<td>Instantaneous</td>
<td>( \sim 10^{-15} )</td>
</tr>
<tr>
<td>(b) Internal Conversion</td>
<td>( S_n \to S_1 )</td>
<td>( \kappa_{ic} )</td>
<td>( 10^{14} \sim 10^{10} )</td>
</tr>
<tr>
<td>(c) Vibrational Relaxation</td>
<td>( S_1 \to S_1 )</td>
<td>( \kappa_{vr} )</td>
<td>( 10^{12} \sim 10^{10} )</td>
</tr>
<tr>
<td>(d) Fluorescence</td>
<td>( S_1 \to S_0 )</td>
<td>( \kappa_f )</td>
<td>( 10^{-7} \sim 10^{-7} )</td>
</tr>
<tr>
<td>(e) Intersystem Crossing</td>
<td>( S_1 \to T_1 )</td>
<td>( \kappa_{isc} )</td>
<td>( 10^{10} \sim 10^{-8} )</td>
</tr>
<tr>
<td>(f) Non-radiative Relaxation, Quenching</td>
<td>( S_1 \to S_0 )</td>
<td>( \kappa_{nr}, \kappa_q )</td>
<td>( 10^{-7} \sim 10^{-5} )</td>
</tr>
<tr>
<td>(g) Phosphorescence</td>
<td>( T_1 \to S_0 )</td>
<td>( \kappa_p )</td>
<td>( 10^{-3} \sim 10^{-2} )</td>
</tr>
<tr>
<td>(h) Non-radiative Relaxation, Quenching</td>
<td>( T_1 \to S_0 )</td>
<td>( \kappa_{nr}, \kappa_q )</td>
<td>( 10^{-3} \sim 10^{-2} )</td>
</tr>
</tbody>
</table>

1.5. Molecular Fluorescence

1.5.1. Fluorescence intensity and spectra

The amount of fluorescence photons emitted per unit time and per unit volume is called the steady state fluorescence intensity \( I \). The steady state fluorescence intensity per absorbed photon as a function of the wavelength of the emitted photons represents the fluorescence spectrum or emission spectrum which reflects the distribution of the probability of the various transitions from the lowest vibrational level of \( S_1 \) to the various vibrational levels of \( S_0 \). The intensity versus wavelength plot (fluorescence emission spectrum) is characteristic of a fluorophore and sensitive to its local surrounding environment and consequently used to probe the structure of the local environment. The fluorescence emission spectrum is generally independent of excitation wavelength. This is because of the rapid relaxation to the lowest vibrational level of \( S_1 \) prior to emission; irrespective of excitation to any higher electronic and vibrational levels.

1.5.2. The stokes shift

The fluorescence emission spectrum of a molecule in solution usually peaks at a longer wavelength than the absorption spectrum because nuclear relaxations of the excited molecule and the solvent transfer some of the excitation energy to the surroundings before the molecule fluoresces. The red-shift of the fluorescence is called the Stokes shift after George Stokes, a
British mathematician and physicist and generally represented in the unit of wavenumber (cm$^{-1}$) with the symbol $\Delta \nu_{ss}$. This important parameter provides information on the excited state. As an example, when the dipole moment of a fluorescent molecule is higher in the $S_1$ state than in $S_0$ state, the magnitude of $\Delta \nu_{ss}$ increases with increase in solvent polarity. The Stokes shift reflects both intramolecular vibrational relaxations of the excited molecule and relaxations of the surrounding solvent.

![Figure 1.5](image)

**Figure 1.5** *Potential energy curves for a harmonic oscillator in the ground and excited electronic states. The vibrational reorganization energy ($\Lambda$) and the bond length displacement ($\Delta$) in the ground and excited states are also shown.*
The contributions from intramolecular vibrations can be related to displacements of the vibrational potential energy curves between the ground and excited states. Figure 1.5 illustrates this relationship. Suppose that a particular bond has length $b_{gr}$ at the potential minimum in the ground state, and length $b_{ex}$ in the excited state. The vibrational reorganization energy ($\Lambda$) is the energy required to stretch or compress the bond by $(b_{ex} - b_{gr})$. Classically, this energy is $(K/2)(b_{ex} - b_{gr})^2$, where $K$ is the vibrational force constant. The quantum-mechanical coupling strength ($S$) for a vibrational mode is defined as $\Delta^2/2$, where $\Delta$ is the dimensionless displacement of the potential surface in the excited state, $2\pi(m\nu/h)^{1/2}(b_{ex} - b_{gr})$, and $\nu$ is the vibrational frequency. It is well known that in cases where $|\Delta| > 1$, the Franck-Condon factors for absorption peak occurs at an energy approximately $Sh\nu$ above the 0-0 transition energy. The quantum-mechanical reorganization energy for a strongly coupled vibrational mode thus is approximately $Sh\nu$, or $\Delta^2h\nu/2$. If this is the only vibrational mode with a significant coupling strength, fluorescence emission will peak approximately $Sh\nu$ below the 0-0 transition energy, so the Stokes shift ($h\nu_{abs} - h\nu_{fl}$ in figure 1.5) will be roughly $2Sh\nu$, or $\Delta^2h\nu$. If multiple vibrational modes are coupled to the transition, the vibrational Stokes shift is the sum of the individual contributions: $h\nu_{abs} - h\nu_{fl} \approx \sum |\Delta_i|^2h\nu_i$ where $\Delta_i$ and $\nu_i$ are the displacement and frequency of mode $i$.

A plot analogous to those in figure 1.5 also can be used to describe the dependence of the energies of the ground and excited states on a generalized solvent coordinate. Neglecting rotational energies, the overall reorganization energy is the sum of the vibrational and solvent reorganization energies, and the total Stokes shift is the sum of the vibrational and solvent Stokes shifts.

1.5.3. The mirror-image rule

The fluorescence emission spectrum of a molecule is approximately a mirror image of the absorption spectrum, as illustrated in figure 1.6. Several factors contribute to this symmetry. First, if the Born-Oppenheimer approximation holds, and if the vibrational modes are harmonic and have the same frequencies in the ground and excited electronic states (all significant approximations), then the energies of the allowed vibronic transitions in the absorption and emission spectra will be symmetrically located on opposite sides of the 0-0 transition energy, $h\nu_{00}$. The solid vertical arrows in figure 1.5 illustrate such a pair of upward and downward
transitions whose energies are, respectively, $h\nu_{00} + 3h\nu$ and $h\nu_{00} - 3h\nu$, where $\nu$ is the vibrational frequency. In general, for fluorescence at frequency $\nu = \nu_{00} - \delta$, the corresponding absorption frequency is $\nu' = \nu_{00} + \delta = 2\nu_{00} - \nu$. Conversely, absorption at frequency $\nu'$ gives rise to fluorescence at $2\nu_{00} - \nu'$.

![Figure 1.6 Mirror symmetry in the absorption and fluorescence emission spectrum.](image)

Mirror-image symmetry also requires that the Franck-Condon factors be similar for corresponding transitions in the two directions, and this often is the case. In addition to requiring matching of the energies and Franck-Condon factors for corresponding upward and downward transitions, mirror-image symmetry requires the populations of the various vibrational sublevels from which downward transitions embark in the excited state to be similar to the populations of the sublevels where corresponding upward transitions originate in the ground state. This matching will flow from the similarity of the vibrational energies in the ground and excited states, provided that the vibrational sublevels of the excited state reach thermal equilibrium rapidly relative to the lifetime of the state. If the excited molecule decays before it equilibrates, the emission spectrum will depend on the excitation energy and typically will be shifted to higher energies than the mirror-image law predicts. The mirror-image relationship can break down for a variety of reasons, including heterogeneity in the absorbing or emitting molecules, differences between the vibrational frequencies in the ground and excited states, and failure of the vibrational levels of the excited molecule to reach thermal equilibrium.
1.5.4. Fluorescence yield and lifetime

Because the rate constant of different parallel processes (mentioned in section 1.4.4) add, the actual lifetime of the excited state (the fluorescence lifetime, $\tau_f$) is less than the radiative lifetime ($\tau_r$):

$$\tau_f = \frac{1}{\kappa_{total}} < \tau_r = \frac{1}{\kappa_r}$$  \hspace{1cm} (1.10)

where,

$$\kappa_{total} = \kappa_r + \kappa_p + \kappa_{isc} + \kappa_{isc} + \sum \kappa_{reactions}$$  \hspace{1cm} (1.11)

The fluorescence yield ($\phi_f$) is the fraction of the excited molecules that decay by fluorescence. For a homogeneous sample that emits exclusively from the first excited singlet state, this is simply the ratio of $\kappa_r$ to $\kappa_{total}$:

$$\phi_f = \frac{\text{photon emitted by fluorescence}}{\text{photons absorbed}} = \frac{\kappa_r}{\kappa_{total}} = \frac{\tau_f}{\tau_r}$$  \hspace{1cm} (1.12)

The fluorescence yield from a homogeneous sample is, therefore, proportional to the fluorescence lifetime and can provide the same information. However, the situation is little more complicated in real situations even for the simplest fluorophore and needs proper care before making a conclusion.

1.5.5. Fluorescence anisotropy

The fluorescence emission, emitted from the samples excited with polarized light is also polarized. This polarization is due the photo-selection of the fluorophores according to their orientation relative to the direction of the polarized excitation. This photo-selection is proportional to the square of the cosine of the angle between the absorption dipole of the fluorophore and the axis of polarization of the excitation light. The orientational anisotropic distribution of the excited fluorophore population relaxes by rotational diffusion of the fluorophores and excitation energy transfer to the surrounding acceptor molecules. The polarized fluorescence emission becomes depolarized by such processes. The fluorescence anisotropy measurements reveal the average angular displacement of the fluorophore which
occurs between absorption and subsequent emission of a photon. The steady state fluorescence anisotropy \( r \) is defined by the following equation:

\[
\begin{align*}
    r &= \frac{F_\parallel - F_\perp}{F_\parallel + 2F_\perp} \\
    &\quad \text{(1.13)}
\end{align*}
\]

where \( F_\parallel \) and \( F_\perp \) represent the fluorescence intensities when the orientation of the emission polarizer is parallel and perpendicular to the orientation of the excitation polarizer, respectively.

The fluorescence anisotropy \( r \) is a measure of the average depolarization during the lifetime of the excited fluorophore under steady state conditions. If a sample contains a mixture of components with different anisotropies, the observed anisotropy is simply the sum:

\[
\begin{align*}
    r &= \sum_i \xi_i r_i \\
    &\quad \text{(1.14)}
\end{align*}
\]

where \( r_i \) is the anisotropy of component \( i \) and \( \xi_i \) is the fraction of the total fluorescence emitted by this component.

The denominator in the equation (1.13) is proportional to the total fluorescence, \( F_T \), which includes components polarized along all three cartesian axes: \( F_T = F_x + F_y + F_z \). If the excitation is done along \( z \)-polarization and the fluorescence is measured with polarizers parallel to the \( z \)- and \( x \)-axes, then \( F_\parallel = F_z \) and \( F_\perp = F_x \). Because the emission must be symmetrical in the \( xy \) plane, \( F_y = F_x \). Thus, \( F_T = F_z + 2F_x = F_\parallel + 2F_\perp \). The total fluorescence also can be obtained by measuring the fluorescence through a polarizer set at the “magic angle” 54.7° from the \( z \)-axis, as shown in figure 1.7. This is equivalent to combining \( z \)- and \( x \)-polarized measurements with weighting factors of \( \cos^2(54.7°) \) and \( \sin^2(54.7°) \), which have the appropriate ratio of 1:2. Fluorescence measured through a polarizer at the magic angle with respect to the excitation polarization is not affected by rotation of the emitting chromophore.

But the time resolved measurements of fluorescence anisotropy using ultrafast polarized excitation source (laser) give insight into the time dependent depolarization. The time dependent fluorescence anisotropy or fluorescence anisotropy decay, \( r(t) \), is defined as follows:

\[
\begin{align*}
    r(t) &= \frac{F_\parallel(t) - F_\perp(t)}{F_\parallel(t) + 2F_\perp(t)} \\
    &\quad \text{(1.15)}
\end{align*}
\]
Figure 1.7 If a sample is excited with light polarized parallel to the z-axis, the total fluorescence is proportional to the fluorescence measured at right angles to the excitation through a polarizer at the “magic angle” of 54.7° with respect to z. This measurement weights z and x polarizations in the ratio of 1:2.

where $F_{||}(t)$ and $F_{\perp}(t)$ are the time-dependent fluorescence intensity decays collected with the polarization of the emission polarizer kept parallel and perpendicular to the polarization of the excitation source respectively. For a fluorophore in a simple solvent, the fluorescence depolarization is simply due to rotational motion of the excited fluorophore and the decay parameters depend on the size and shape of the fluorophore. For spherical fluorophores, the anisotropy decay is a single exponential with a single rotational correlation time and is shown in the following equation.

$$r(t) = r_0 \exp(-t/\Theta)$$  \hspace{1cm} (1.16)

Where, $r_0$ is initial anisotropy (anisotropy at time $t = 0$ or anisotropy observed in the absence of any depolarizing processes) and $\Theta$ is the rotational correlation time. The initial anisotropy $r_0$ is
related to the angle ($\delta$) between the absorption and emission dipoles of the fluorophore under study and the relation is given as:

$$r_o = \frac{2}{5} \left( \frac{3}{2} \cos^2 \delta - 1 \right)$$

(1.17)

here, the value $r_0$ vary between 0.4 and -0.2 as the angle ($\delta$) varies between 0° and 90° respectively. The rotational correlation time $\Theta$ of the fluorophore is governed by the viscosity ($\eta$) and temperature ($T$) of the solution and the molecular volume ($V$) of the fluorophore. This is given by the Stokes-Einstein relation [5] as shown below:

$$\Theta = \frac{\eta V}{K_B T}$$

(1.18)

Where, $K_B$ is the Boltzmann constant.

The relation between the steady state anisotropy ($r$), initial anisotropy ($r_0$), rotational correlation time ($\Theta$) and fluorescence lifetime ($\tau_f$) is given by the Perrin equation as follows.

$$\frac{r_0}{r} = 1 + \frac{\tau_f}{\Theta}$$

(1.19)

The Perrin equation is very useful in obtaining the correlation time without the measurement of polarization dependent fluorescence decays. The theory developed for more complicated shapes of the fluorophore shows that a maximum of five exponentials are enough to explain the fluorescence anisotropy decay.

1.5.6. Detection of fluorescence

The principal fluorescence measurement arrangement is depicted in figure 1.8, where the most important properties (parameters) are listed for both exciting radiation and fluorescence emission. Not all of these parameters are necessarily known or well specified for every spectrofluorometric instrument; any attempt at sophisticated analysis and interpretation of the fluorescence data should be accompanied by a rigorous measurement of all the listed
parameters that are relevant to the interpretation. The following examples of fluorescence spectroscopy applications also indicate this aspect of practical fluorescence measurements.

![Diagram](image)

**Figure 1.8** Summary of main variables and read-out parameters of fluorescence experiments.

The fluorescence of an object of interest can be detected in various ways. Besides the classical solution phase fluorescence measurement in different types of cuvette, there are several advanced ways of detecting the fluorescence signal [6]. The use of fiber optics allows measurement of fluorescence even in biological organs *in vivo*. When looking at cells, one can use cell culture plates or flow cytometry in combination with optical microscopy. Selected spots within a cell can be monitored using classical, confocal, or multiphoton microscopy. Advanced techniques of single molecule spectroscopy, total internal reflection fluorescence microscopy, fluorescence correlation spectroscopy and others are also described recently. New technology combining, for example, NFOM or STM/AFM with high-resolution photon timing, when each detected photon is tagged with all other information related to it, allows multi-dimensional fluorescence lifetime and fluorescence correlation spectroscopy to be performed during one measurement. Single molecule fluorescence characterization can thus now be done with unprecedented accuracy and depth with the combination of ultrafast excitation sources, high sensitive detectors/electronics and analyzing the output with complex mathematical models.
1.6. Quenching of Fluorescence

A fluorescence quencher is a compound, the presence of which in the vicinity of a fluorophore leads to a decrease of the fluorescence quantum yield and/or lifetime of the latter. For example, those molecules or ions can function as a quencher that are added to the solution and introduce new or promote already existing non-radiative deactivation pathways (solute quenching) by molecular contact with the chromophore. Further possibilities are self-quenching by simply another fluorophore molecule of the same type, and quenching by solvent molecules. In the following sub-sections we give a brief description on each of these types.

1.6.1. Solute quenching

Solute quenching reactions are a very valuable tool for studies of proteins, membranes and other supra- or macromolecular assemblies, providing information about the location of fluorescent supra- or macromolecular groups in the examined molecular structure [7-16]. A fluorophore that is located on the surface of such a structure will be relatively accessible to a solute quencher. A quenching agent will quench the chromophore that is buried in the core of the molecular assembly to a lesser degree. Thus, the quenching experiment can be used to probe topographical features of the examined structure and to detect structural changes that may be caused by addition of external compounds or changed physical conditions. In usual quenching experiments, the quencher is added successively to the fluorophore containing solution. The analysis of the dependence of fluorescence intensity (F), quantum yield (ϕ), or lifetime (τ) on the quencher concentration gives quantitative information about the accessibility of the chromophore within the macro- or supra-molecular structure.

Depending on the chemical nature of both the quenching agent and the chromophore, one has to distinguish between two forms of quenching: static and dynamic quenching. Static quenching results from the formation of a non-fluorescent fluorophore-quencher complex, formed in the fluorophore’s ground state. Characteristic for this type of quenching is that increasing quencher concentration decreases the fluorescence intensity or quantum yield but does not affect the fluorescence lifetime. An important feature of static quenching is its decrease with increasing temperature, as the stability of the fluorophore-quencher ground state complexes is generally lower at higher temperatures. On the other hand, if the quenchers act (e.g. through collisions) by competing with the radiative deactivation process, the ratio of the
quantum yield in the absence, $\phi_0$ and the presence, $\phi$ (or the fluorescence intensity $F_0$ and $F$, respectively), of the quencher will be equal to the ratio of the corresponding lifetimes, $\tau_0/\tau$ [equation (1.20)]. The concentration dependence of this so-called dynamic or collisional quenching is described by the Stern-Volmer equation, where the Stern-Volmer constant $K_{SV}$ is equal to $k_q\tau_0$.

$$\frac{\phi_0}{\phi} = \frac{F_0}{F} = \frac{\tau_0}{\tau} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \tag{1.20}$$

The other mechanism of the dynamic fluorescence quenching is connected with the chemical nature of the chromophore and the solute quencher: quenchers containing halogen or heavy atoms increase the intersystem crossing (isc) rate (generally induced by a spin-orbit coupling mechanism). Acrylamide quenching of tryptophans in proteins is probably due to the excited state electron transfer from the indole to acrylamide. Paramagnetic species are believed to quench aromatic fluorophores by an electron spin exchange process.

In many instances a fluorophore can be quenched by both dynamic and static quenching simultaneously. The characteristic feature for mixed quenching is that the plot of the concentration dependence of the quantum yield or intensity ratios shows an upward curvature. In this case the Stern-Volmer equation has to be modified, resulting in an equation which is second order in [Q]. More details on the theory and applications of solute quenching can be found in an excellent review by M. Eftink [17].

1.6.2. Solvent quenching

The influence of solvent molecules on the fluorescence characteristic of a dye solute is certainly one of the most complex issues in fluorescence spectroscopy. Eventually every chromophore shows some dependence of its quantum yield on the chemical structure of the surrounding solvent. This observation is to some extent due to fluorescence quenching by the solvent. One possibility is that the interaction of the chromophore with its solvent shell can promote non-radiative pathways by changing the energy of the $S_0$, $S_1$ and $T_1$ states. Transition probabilities for the internal conversion and intersystem crossing processes are governed by the energy-gap law [18]. This law states that the rate constants $\kappa_{ic}$ and $\kappa_{isc}$ increase exponentially as the energy gap between the corresponding $S_1$, $S_0$ and/or $T_1$ states decreases. Consequently, any
change in those energy levels will strongly influence the fluorescence lifetime and quantum yield.

1.6.3. Self quenching

Self-quenching is the quenching of one fluorophore by another one of the same kind. It is a widespread phenomenon in fluorescence, but it requires high concentrations or labeling densities. The general physical description of the self-quenching processes involves a combination of trap-site formation and energy transfer among fluorophores, with a possibility of trap-site migration which results in quenching [19]. Trap sites may be formal fluorophore complexes or aggregates, or they may result from sufficiently high concentrations of fluorophores leading to close proximity of the dye molecules.

1.6.4. Trivial quenching

Trivial quenching arises from attenuation of the exciting beam and/or inability of the fluorescence photon to reach out of the sample, which occurs mainly when other compounds are added that strongly absorb in the UV region. Though the added concentration may be small, they might block the excitation light completely. Another reason for trivial quenching can be the turbidity of the sample. True and trivial quenching, however, are easily differentiated, since in trivial quenching the lifetime and quantum yield remain constant.

1.7. Fluorescence Solvatochromism

A variety of environmental factors affect fluorescence emission, including interactions between the fluorophore and surrounding solvent molecules (dictated by solvent parameters), other dissolved inorganic and organic compounds, temperature, pH, and the localized concentration of the fluorescent species. The effects of these parameters vary widely from one fluorophore to another, but the absorption and emission spectra, as well as quantum yields, can be heavily influenced by environmental variables. In fact, the high degree of sensitivity in fluorescence is primarily due to interactions that occur in the local environment during the excited state lifetime [4]. In this section we will briefly discuss the origin of different types of solvent effect and how it affects the fluorescence behavior. Also, we will put forward different
empirical approaches which are commonly used to model the effect solvent on fluorescence properties of excited chromophores [20].

1.7.1. Non-specific interaction with the solvent

In addition to the solvent polarity effect, the change in fluorescence properties due to non-specific interaction of the excited fluorophore with solvent can arise due to several other factors like viscosity, solvent relaxation etc. However, it is usually the solvent polarity parameter that comes first into the picture. In this sub-section, we will mainly describe the fluorescence solvatochromism originated due to the solvent polarity effect.

In solution, solvent molecules surrounding the ground state fluorophore have dipole moments that can interact with the dipole moment of the fluorophore to yield an ordered distribution of solvent molecules around the fluorophore. Energy level differences between the ground and excited states in the fluorophore produce a change in the molecular dipole moment, which ultimately induces a rearrangement of surrounding solvent molecules. However, the Franck-Condon principle dictates that, upon excitation of a fluorophore, the molecule is excited to a higher electronic energy level in a far shorter timeframe than it takes for the fluorophore and solvent molecules to re-orient themselves within the solvent-solute interactive environment. As a result, there is a time delay between the excitation event and the re-ordering of solvent molecules around the solvated fluorophore (as illustrated in figure 1.9), which generally has a much larger dipole moment in the excited state than in the ground state.

After the fluorophore has been excited to higher vibrational levels of the first excited singlet state \( S_1 \), excess vibrational energy is rapidly lost to surrounding solvent molecules as the fluorophore slowly relaxes to the lowest vibrational energy level (occurring in the picosecond time scale). Solvent molecules assist in stabilizing and further lowering the energy level of the excited state by re-orienting (termed solvent relaxation) around the excited fluorophore in a slower process that requires between 10\(~\)100 ps. This has the effect of reducing the energy separation between the ground and excited states, which results in a red shift (to longer wavelengths) of the fluorescence emission. Increasing the solvent polarity produces a correspondingly larger reduction in the energy level of the excited state, while decreasing the solvent polarity reduces the solvent effect on the excited state energy level. The polarity of the fluorophore also determines the sensitivity of the excited state to solvent effects. Polar and charged fluorophores exhibit a far stronger effect than non-polar fluorophore.
1.7.2. Specific solvent interaction

The general solvent effect discussed above is often depends on the polarizibility of the solvent (described by the refractive index, $n$) and the molecular polarizibility resulted from the re-orientation of the solvent dipole. The later property is a function of the static dielectric constant, $\varepsilon$. The mathematical expression is the so called Lippert-Mataga (LM) equation (see chapter 3 for details). In contrast, specific interactions are produced by one or a few neighboring molecules, and are determined by the specific chemical properties of both the fluorophore and solvent. Specific effects can be due to hydrogen bonding, preferential solvation, acid–base chemistry, or charge-transfer interactions, to name a few. The spectral shifts due to such specific interactions can be substantial, and if not recognized, limit the detailed interpretation of fluorescence emission spectra. Specific solvent–fluorophore interactions can often be identified by examining emission spectra in a variety of solvents. For example, addition of low concentrations of ethanol, which are too small to alter the bulk properties of the solvent, result in substantial fluorescence spectral shift in 2-aminonaphthalene [4(a)]. Less than 3% ethanol causes a shift in the emission maximum from 372 to 400 nm. Increasing the ethanol
concentration from 3 to 100% caused an additional shift to only 430 nm. A small percentage of ethanol (3%) caused almost 50% of the total spectral shift.

Specific solvent–fluorophore interactions can occur in either the ground state or the excited state. If the interaction only occurred in the excited state, then the polar additive would not affect the absorption spectra. If the interaction occurs in the ground state, then some change in the absorption spectrum is expected. The indication for the presence of specific solvent effect is often seen by a substantial shift in fluorescence peak in presence of trace amount of protic solvents and/or deviation of LM plot from linearity in protic solvents. Quantitative interpretation of the specific solvent effect is often difficult and requires a careful analysis of the fluorescence spectra in a variety of solvents.

1.7.3. Modeling solvent interaction

(i) Single - parameter approach

Kosower [20(a)] was the first to use solvatochromism as a probe of solvent polarity that was based on the solvatochromic shift of 4-methoxycarbonyl-1-ethylpyridinium iodide. Later, Dimroth and Reichardt [20(b-c)] suggested using betain dyes whose negative solvatochromism is exceptionally large, which are the basis of famous Eₜ(30) scale. These compounds are considered as zwitterions having dipole moment of about 15 D in the ground state whereas it is nearly zero in the excited state. The Eₜ(30) value for a solvent is simply defined as the transition energy for the longest wavelength absorption band of the dissolved pyridinium-N-phenoxide betaine dye and normally expressed in kcal mol⁻¹. It has been observed that correlations of solvent-dependent properties; especially, positions and intensities of absorption and emission bands with Eₜ(30) scale often follow two distinct lines, one for non-protic solvents and one for protic solvents. The sensitivity of the betaine dyes to solvent polarity is exceptionally high, but unfortunately they are not fluorescent. So, the search for polarity-sensitive fluorescent dyes continues as they offer distinct advantages, particularly in biological studies.

(ii) Multi-parameter approach

In most of the cases, the spectral shift cannot be correlated only in terms of solvent polarity. Specific interaction of the probe with solvent, for example hydrogen bonding, can also contribute to the solvent dependent spectral shift. In these case a multi-parameter approach is preferable and the π* scale of Kamlet and Taft [20(d)] deserves special recognition because it has been successfully applied to the positions or intensities of maximal absorption in IR, NMR,
ESR and UV-Visible absorption and fluorescence spectra and many other physical or chemical parameters. The advantage of the Kamlet-Taft treatment is to sort out the quantitative role of properties such as hydrogen bonding. It is remarkable that the $\pi^*$ scale has been established from the averaged spectral behavior of numerous solutes. It offers the distinct advantage of taking into account both non-specific (general) and specific interactions.

1.8. Time-resolved Fluorescence Measurement

Time-resolved measurements are widely used in fluorescence spectroscopy, which contain more information than is available from the steady-state measurement. They were particularly used for studies of biological macromolecules and increasingly for cellular imaging [4]. For instance, a protein containing two tryptophan residues cannot be distinguished by the steady state measurement; whereas, these can be distinguished easily by time-resolved data giving two decay times. One can also distinguish the presence of static and dynamic quenching behavior using lifetime measurements [21]. Recently, an important application has been found in cellular imaging using fluorescence microscopy which can create lifetime images. Time resolved fluorescence measurements were widely used in two different methods namely - Time domain (TD) and Frequency domain (FD) methods.

1.8.1. Time-domain measurement

In time-domain or pulse fluorimetry, the sample is excited with a pulse of light. The width of the pulse is made as short as possible, and is preferably much shorter than the fluorescence decay time ($\tau_f$) of the sample. The time dependent intensity is measured following the excitation pulse, and the decay time $\tau_f$ is calculated from the slope of a plot of $\log I(t)$ versus $t$, or from the time at which the intensity decreases to $1/e$ of the intensity at $t = 0$. The intensity decays are often measured through a polarizer oriented at $54.7^\circ$ from the vertical $z$-axis. This condition is used to avoid the effects of rotational diffusion and/or anisotropy on the intensity decay.
1.8.2. Frequency-domain measurement

In frequency domain or phase modulation method, the sample is excited with intensity-modulated light, typically sine-wave modulation. The intensity of the incident light is varied at a high frequency typically near 100 MHz, so its reciprocal frequency is comparable to the reciprocal of decay time. When a fluorescent sample is excited in this manner, the emission is forced to respond at the same modulation frequency. The lifetime of the fluorophore causes the emission to be delayed in time relative to the excitation and this delay is measured as a phase shift (φ), which can be used to calculate the decay time. Magic-angle polarizer conditions are also used in frequency-domain measurements. The lifetime of the fluorophore also causes a decrease in the peak-to-peak height of the emission relative to that of the modulated excitation. The modulation decreases because some of the fluorophores excited at the peak of the excitation continue to emit when the excitation is at a minimum. The extent to which this occurs depends on the decay time and light modulation frequency. This effect is called de-modulation, and can also be used to calculate the decay time. FD measurements typically use both the phase and modulation information. At present, both time-domain and frequency-domain measurements are in widespread use.