1.1 INTRODUCTION

The study of the way that matter interacts with light is called spectroscopy. The origins of spectroscopy as a practical scientific discipline can be traced back to Newton’s famous experiments on the spectrum of sunlight using prisms in 1665. The inability of physicists in the nineteenth century to quantitatively explain and predict their spectroscopic observations led to the foundation of the theory of quantum mechanics. Quantum theory and spectroscopy have since evolve hand in hand and underpin the way we currently understand the structure of molecules. For example, electronic structure, as well as molecular size and shape, has been learned from quantum mechanical interpretation of experimental spectroscopy. Optical microscopy has likewise evolved over a period of many centuries, allowing us to elucidate physical structures down to the micrometer scale. More recently, advanced approaches have allowed resolution on the nanometer scale.

The fusion of the disciplines of microscopy and spectroscopy over the past three decades, spawning microscopy applications such as fluorescence lifetime imaging microscopy (FLIM), fluorescence resonance energy transfer (FRET), and fluorescence recovery after photobleaching (FRAP), has made both disciplines more powerful, more sensitive, and more selective, though more complicated. Researchers now must have a sound understanding of the physics of optics, spectroscopy, and nonlinear processes and the chemistry of dyes, antifade agents, and intermolecular interactions and yet somehow remain experts in their native discipline!

The objective of this chapter is to provide a simple understanding of molecular spectroscopy, with a focus on concepts and techniques relevant to microscopy. The
inherently quantum mechanical concepts of phenomena, including energy levels, radiative and nonradiative processes, and quantum yields, will be developed from a non-mathematical framework that is often used by experts in spectroscopy when discussing general principles. This simplified framework is developed into a practical and useful understanding of optical spectroscopy aimed at nonspecialists. This groundwork supports the more detailed discussion of complex techniques in the following chapters.

To begin the chapter we will outline basic features and properties of light and energy states of molecules. We then connect the two by exploring the interaction of light with molecules, initially for an isolated molecule. We will consider the key properties of absorption and emission and also those properties that are the bane of microscopy—the so called nonradiative mechanisms. After considering the isolated molecule, we will discuss interactions of molecules with their environment, which develops the concepts underpinning photobleaching and quenching, and also the more recent techniques of FRET and FLIM. En route we will examine the excitation and emission spectra of typical dyes, which can be found in any dye catalogue (see also Jameson et al. [1] for further reading).

1.1.1 Properties of Light

**Light as an Electromagnetic Wave** Light is a form of electromagnetic (EM) radiation, consisting of an oscillating electric field with an oscillating magnetic field perpendicular to it (Figure 1.1). These oscillating fields behave like waves travelling in the direction of propagation at the speed of light ($c$), which in a vacuum is $c = 3.00 \times 10^8$ m/s. In optical spectroscopy and microscopy, the wavelength of the light ($\lambda$), measured typically in nanometers ($1 \text{ nm} = 10^{-9} \text{ m}$), is its most commonly used characteristic. Light can be distinguished by many other characteristics, including the frequency ($\nu$), which is defined as the number of wavefronts passing a fixed point every second (Figure 1.1). The frequency is therefore inversely proportional to the wavelength; at a fixed speed, more wavefronts of shorter wavelengths will pass a fixed point in a given time than will wavefronts of longer wavelengths.

![Figure 1.1 Electromagnetic ray showing wavelength and frequency.](image-url)
The mathematical relationship that defines these three properties is

$$\nu = \frac{c}{\lambda}$$

(1.1)

The wavelength of light can vary over many orders of magnitude, without limit, as illustrated in part in Figure 1.2. The optical, or visible, wavelengths form a narrow range, extending from approximately 400 to 750 nm. The wavelength range immediately following the longer wavelengths is called infrared (IR) while the adjacent region with shorter wavelengths is the ultraviolet (UV). Historically, although UV illumination has been used to excite fluorescence emission in the visible region, optical microscopy has dealt exclusively with the visible wavelength region because our eyes were the detectors. This was supplemented with emulsion film photography; furthermore, more recently, with the development of IR- and UV-sensitive detectors and the advent of nonlinear optical techniques, these neighboring wavelength regions have also become important.

**Dual Nature of Light** About one hundred years ago, Planck postulated that the energy of an EM wave could not have any value but was quantized. This smallest unit of energy is proportional to its frequency $\nu$ and has a magnitude

$$E = h\nu$$

(1.2)

where $h$ is now called the Planck constant. This smallest amount, or quantum, of energy is associated with one photon of light. It is important to note that molecules usually absorb or emit one photon at a time. The exception to this rule is under conditions of very intense radiation, where two or more photons can be absorbed simultaneously, leading to multiphoton absorption (see Section 1.2.3).
The dichotomy of the two descriptions of light (the wave and particle descriptions) was the source of enormous debate for the first part of the twentieth century and was eventually united in quantum mechanics. Both definitions are helpful in different situations. For example, reflection, refraction, diffraction, and polarization are best described using wave properties. Absorption and emission of light by molecules are more intuitively described using the particle (photon) description. In the discussion and chapters that follow, we will use the wave and particle description of light interchangeably, depending on the process.

**Reflection, Refraction, Diffraction, and Polarization** Reflection, refraction, and diffraction of light are of obvious importance, especially when the instrumentation is considered. Although seemingly simple, these phenomena are actually quite complex, both in the intellectual sense and the mathematical sense of being described by complex numbers. Here, we will define and describe these three phenomena only in the simplest terms.

All of these processes involve a beam of light encountering a (nonabsorptive) substance with a different refractive index, for example, a mirror or a simple piece of glass. **Reflection** of light is the most straightforward of these three processes. As illustrated in Figure 1.3a, a beam of light striking a surface such as glass or water will reflect from that surface at the same angle as it is incident on the surface. Clearly, this is the process that underpins the behavior of all mirrors.

A spoon in a glass of water that is apparently bent is a good example of the second process, **refraction**. When light passes from one medium to another of a different refractive index, its speed changes. This causes it to bend if it enters the second medium at an angle, as shown in Figure 1.3b. The light always bends toward the medium of higher refractive index.

When light passes through a small opening, it diffracts from the edges of the hole, as shown in Figure 1.3c. When the hole approaches the same size as the wavelength of light, the **diffraction** becomes significant. This is the main phenomenon limiting the resolving power of a conventional light microscope. A conventional light or fluorescence microscope can only resolve features as small as the wavelength of light used to view the object. This is called the **diffraction limit**.

One of the complicating but powerful features of reflection, refraction, and diffraction is that the phenomena depend on many other factors, including the wavelength of light, the polarization of the light (see below), the refractive index of the materials, and the angles involved. These features are all used to advantage in the construction of a fluorescence microscope but are otherwise beyond the scope of this introductory chapter. The reader is referred to [2] for further information on these areas.

**Figure 1.3** Simple depictions of (a) reflection of a beam of light from a surface; (b) refraction of light through a medium of higher refractive index; and (c) diffraction of light through a small hole.
The polarization of light also has important implications for some types of fluorescence spectroscopy and microscopy, for example, the determination of rotational mobility of components in biological systems, which is commonly assessed by measurement of fluorescence emission polarization. Briefly, polarization of light refers to the following characteristics. As noted above, EM radiation consists of oscillating electric and magnetic fields, each perpendicular to the other and also perpendicular to the direction of propagation (Figure 1.1). If we consider the electric field only (for simplicity), the electric field vector can assume any direction of oscillation perpendicular or normal to the light propagation direction. If one was to view the light “end on” (i.e., along the axis of propagation), then the electric vector could be found at any angle within $360^\circ$ (as shown for the light at the left side of Figure 1.4). If all such angles are equally likely, then this is unpolarized light.

It is possible to use polarizing optics, including filters or prisms, to only pass light of one polarization (i.e., electric vector oscillating in only one plane), as shown in Figure 1.4. The light that passes through such a polarizing filter is now linearly polarized. Polarized sunglasses work in the same way and utilize the fact that unscattered light from the sun is randomly polarized. Reflections of a horizontal smooth surface such as water or the road are highly polarized, mostly horizontally (parallel to the reflective surface). The polarized sunglasses block this horizontally polarized light while passing vertically polarized light and therefore preferentially block the light scattered from horizontal surfaces. It is also possible, by turning the head on the side, to observe the reverse—we will then preferentially see the scattered light.

### 1.1.2 States of Molecules

When a molecule is placed in an electric field, any charged part of the molecule will experience a force. If the field is oscillating, as it is in light, then parts of the molecule will be pushed and pulled at that frequency. Just as in musical instruments, molecules

![Figure 1.4](image.png)  
**Figure 1.4** Representation of unpolarized and polarized light. In natural light, shown by a light bulb on the left, the electric field oscillation as seen along the direction of propagation can be aligned in any direction, as represented by the arrows on the plane next to the bulb. Using a polarizing device, light can be selected with electric field oscillation in a single plane, as shown propagating to the right of the polarizer and represented on the plane at the far right.
also have specific resonant frequencies at which they like to oscillate. Each of the resonant frequencies $\nu$ (or energies, as $E = h\nu$) is related to states of the molecule, each with an associated energy level. For example, vibrational states correspond to particular resonant frequencies of the atoms in a molecule vibrating together. Electrons in a molecule oscillating with a resonant frequency do so by making periodic transitions back and forth between two electronic states and the resonant frequency is given by the energy separation between these states. Because there are multiple atoms in a molecule, most with multiple electrons, there are a large number of energy levels; in fact, the number of each electronic and vibrational energy level is infinite. Fortunately, only a few are practically important.

There are many other states in molecules that give rise to different forms of spectroscopy, including nuclear spin [nuclear magnetic resonance (NMR) spectroscopy], rotation (microwave spectroscopy), and electron spin [ESR or electron paramagnetic (EPR) spectroscopy]. However, it is the interaction of light with the electrons and nuclei that is important in optical spectroscopy and microscopy, so we shall restrict our discussion to these interactions.

**Vibrational States**  If a group of molecules is cooled to near absolute zero, then they will all have the minimum energy possible; that is, they will all be in their ground state. The concept of the ground state comes from the idea that an elevator at the ground level is at the lowest possible level. As the temperature is raised, some molecules will start to vibrate after collisions with the walls or each other or by absorbing background thermal radiation. Vibration of the atoms in a molecule is one means by which a molecule can store energy.

In a simple but accurate picture, the vibrating atoms in a molecule can be treated as balls joined by springs (the bonds). To take the simplest such example, consider two balls joined by one spring to represent a diatomic molecule. These two balls can vibrate with a characteristic frequency that depends on the mass of the balls and the “tightness” of the spring holding them together. Mathematically, the equation that describes the characteristic oscillation frequency $\nu$ and how it depends on mass $\mu$ and spring (or force) constant $k$ is given by

$$\nu = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \quad (1.3)$$

Note here that $\mu$ is called the “reduced mass” and allows for the balls to be of different masses. The reduced mass is defined as

$$\frac{1}{\mu} = \frac{1}{m_1} + \frac{1}{m_2} \quad (1.4)$$

According to Eq. 1.3, stiffer springs (or stronger bonds) result in a higher frequency. Heavier atoms result in a lower frequency. This is consistent with what we know about vibrations of strings in musical instruments: For example, the heavy E string on a guitar is a much lower note (82.4 Hz) than the lighter E string, which is four times higher in frequency (329.6 Hz), while tightening a string increases the pitch (frequency).

This simple system of either two balls and a spring or two atoms and a bond has a single characteristic frequency. The balls, or atoms, can only vibrate with this
characteristic frequency or an integer multiple of this frequency. These integer-multiple frequencies are called overtones, both in music and spectroscopy.

A more complex molecule can still be modeled quite accurately as balls of different masses joined by springs of different tensions (representing different atoms with different bonds). Although it might not be as intuitively obvious, there are still a limited number of characteristic frequencies for a polyatomic molecule. For example, benzene has 30 characteristic vibrations, while MitoTracker red, a common fluorescent dye, has 243 (the number of characteristic frequencies is given by $3N - 6$, where $N$ is the number of atoms in the molecule). While the number of characteristic vibrations increases only slowly with the size of the molecule, the number of overtones rises more rapidly. Additionally, now that there are different types of vibrations, these vibrations may occur together, giving rise to combination vibrations. The number of these combinations now increases almost exponentially with increasing energy. The result is that for spectroscopically “large” molecules, including all microscopy dyes, the number of vibrational states is too high to count or even to portray accurately in a diagram. This can be a significant complication for a spectroscopist, but for the purposes of this introductory chapter, we do not have to be concerned with the specifics of vibrational states and only need to recognize that there are an incredibly large number of vibrational states and that these states are a temporary store of energy in a molecule (see below).

**Electronic States**

Electrons, being over 1000 times lighter than nuclei, oscillate at much higher frequencies. Oscillations of the valence electrons (those involved with chemical bonding) correspond to transitions between electronic states induced by EM waves with wavelengths in the ultraviolet and visible (UV/Vis) region of the spectrum. Oscillations (transitions) of the core electrons, which are bound very tightly to the nucleus, occur at a much higher frequency, corresponding to the X-ray region of the spectrum (remember a tight spring vibrates with a higher frequency than a loose spring). Optical spectroscopy and microscopy, involving radiation in the UV/Vis region of the spectrum, therefore involve the excitation of valence electrons in a molecule.

A property of electrons called spin is also associated with specific energetic states (spin represents a form of energy inherent in the spinning of a particle about an axis within itself, also called its intrinsic angular momentum). Most stable molecules have an even number of electrons. Because each electronic energy level (i.e., orbital) can only have two electrons with opposite spin (the Pauli principle), the lowest (ground) electronic state of most molecules has a zero total electron spin, as shown schematically in Figure 1.5. When an electron is excited to a higher electronic state, its spin is usually

![Figure 1.5](attachment:figure15.png)

**Figure 1.5** Electron spin for ground state, first singlet state, and first triplet states of the hydrogen molecule as a simple example.
unaffected. Therefore, although two of the electrons are now unpaired (the one excited to a higher orbital and the one left behind), the total electron spin on the molecule is still zero. All states with zero total electron spin are called singlet states and are given the label $S$. The labels are appended with a numerical subscript that simply counts from the ground state, $S_0$, through the first excited singlet state, $S_1$, and so on for higher states.

If the electron spin is flipped after being excited to a higher state, then the total electron spin on the molecule will be 1. In the same way that an orbital angular momentum of $l = 1$ gives rise to the three $p$ orbitals, $S = 1$ in a molecule gives rise to three close-lying electronic states. This collection of states is called a triplet state and is given the label $T$. If more than one electron is excited, then more “unpairings” can occur, giving rise to quintet states, and so on. A molecule with an odd number of electrons will have a half integral total spin, which gives rise to doublet and quartet states, and so on. However, for most molecules used in practical UV/Vis spectroscopy and microscopy, only singlet and triplet states are important.

**Depicting States and Transitions (Perrin–Jablonski Diagrams)** When a molecule absorbs a photon of light, it gets excited to a higher energy state. Unless the absolute energies of the molecular states are particularly important, then the energy levels of a molecule are depicted in a diagrammatic way, often called a *Jablonski diagram* but more correctly termed a *Perrin–Jablonski diagram* in recognition that Perrin was the first to formalize this diagrammatic analysis [1], a simple example of which is shown in Figure 1.6. Energy in this diagram is increasing along the vertical axis. This axis is usually not depicted because the energies are not drawn to scale. The horizontal axis in this diagram is meaningless and never drawn. Electronic states are represented by thick lines. In Figure 1.6, only three such states are drawn: the ground state $S_0$ and two excited electronic states labeled $S_1$ and $T_1$. As discussed above, these latter two states are the lowest excited singlet and triplet states. Notice that $T_1$ is of lower energy than $S_1$. This is always the case and crucial to the fluorescence characteristics of various dyes (see below).

The vibrational states in a Perrin–Jablonski diagram are represented by lightweight lines. Although there are a multitude of vibrational states, the vibrational energy-level structure is only drawn in a cartoon fashion. Often this is just a set of equally spaced lines, or sometimes a set of lines that get closer together in spacing to represent that the density of vibrational states is increasing with energy.

![Figure 1.6](image_url)

**Figure 1.6** Simple Perrin–Jablonski diagram showing three electronic states (thick lines) and several vibrational states (thin lines).
One of the key aspects of a Perrin–Jablonski diagram is that it is only drawn as complicated as it needs to be to get across the concept under discussion. For example, if triplet states were unimportant to a topic, then they would be omitted.

1.2 ABSORPTION AND EMISSION OF LIGHT

1.2.1 Interaction of Light with a Molecule

When a molecule is placed in an oscillating EM field (i.e., light is shone on the molecule), the oscillating fields can push and pull the molecule around. In particular, charged parts of the molecule are affected by the oscillating electric field of light; the effect of the oscillating magnetic field is much weaker. If the charges on the molecule can oscillate in synchrony with the oscillating field (this is called resonance), then the molecule can absorb energy from the field. As a physical analogy, we can consider a child on a swing. If the child is randomly pushed, then the swing never really gets going. But if it is pushed in resonance with the oscillation of the swing, then the swing can absorb a lot of energy.

This concept of resonance is frequently encountered in our daily life, from musical instruments to radio reception. One thing we should notice is that there is often one specific resonant frequency for simple systems. We can only push the child on the swing at one frequency. If we play middle C on a piano, then the piano string vibrates 262 times per second. Molecules likewise have specific resonant frequencies. A typical C–H bond in a molecule vibrates about $10^{14}$ times per second ($10^{14}$ Hz), while a C–C bond vibrates at about $3 \times 10^{13}$ Hz. Although this is incredibly fast compared to middle C above, Eq. 1.3 provides the reason: H and C atoms are around $10^{24}$ times lighter than the piano string. This mass difference on its own causes a $\sqrt{10^{24}} = 10^{12}$ difference in vibrational frequency. An oscillating EM field at $10^{14}$ Hz corresponds to light in the mid-IR region of the spectrum (see Figure 1.2). The corresponding range of wavelengths is about 3–30 μm (see Eq. 1.1). A molecule exposed to mid-IR radiation of the right frequency can therefore absorb one photon from the field to become vibrationally excited.

Electrons are over 1000 times lighter than nuclei and oscillate at even higher frequencies. Again, simple application of Eq. 1.3 suggests that this frequency is a factor of about 30 times higher. Indeed, a typical frequency of light absorbed by valence electrons is $10^{15}$ Hz, which is 30 times faster than the C–C bond vibration. Light with this frequency corresponds to the near-UV region of the spectrum. More generally, the range of wavelengths absorbed by valence electrons varies from about 1000 nm (near IR) through the visible and UV down to about 100 nm (far UV).

Absorption of EM radiation and excitation of electrons take place more or less instantaneously ($\sim 10^{-18}$ s) relative to nuclear motion ($\sim 10^{-15}$ s), according to the Franck–Condon principle. Despite this difference in time scales, excitation of electrons in a molecule can also cause a change in molecular structure. The absorbed energy leads to a redistribution of the electron cloud of the molecule, resulting in altered vibrational levels, an altered dipole moment, and a change in the shape of the molecule. Quite simply, the bonds are not the same after an electron has been removed and put into a different orbital. A consequence of this is that electronic absorption is often accompanied by vibrational motion as the atoms move to their new positions.
Excited states of molecules are unstable. They relax (lose their energy) by a number of mechanisms, including collisions with other molecules, for example, the solvent, or reaction with other species, for example, dissolved oxygen. The excited molecule may also relax by emitting a photon of light to return to a lower state, though not always the same lower state from which it came. The emitted photon will have an energy corresponding to the difference in energy between the initial and final states of the molecule. This emission of a photon is known as fluorescence or in some cases phosphorescence, as we shall see below.

1.2.2 Absorption and Emission of Light Depicted on a Perrin–Jablonski Diagram

An enduring picture that should be remembered for absorption and emission of radiation is that they occur in coincidence with a molecule undergoing a transition between two specific energy levels. On a Perrin–Jablonski diagram, these transitions are represented by arrows, as shown in Figure 1.7. At room temperature, molecules are almost always in their ground electronic state, \( S_0 \). Even for vibrational states, the ground state is still the most likely state of a molecule, although low-frequency vibrations may have significant population. Therefore, on the Perrin–Jablonski diagram, the absorption transition is shown as starting in the ground state. There is no such restriction on the excited state; therefore absorption may occur to a variety of vibrational states in the excited electronic states. The typical amount of vibrational excitation, as discussed above, depends on the change in geometry of the molecule between the two different electronic states. Small changes in geometry are accompanied by small vibrational excitation (arrow 1) whereas large changes in geometry are accompanied by large vibrational excitation (arrow 2). Essentially, to be absorbed, the energy of the incident radiation must exactly match one of the available energy-level transitions. Given the many possible combinations of electronic and vibrational energy levels for a polyatomic molecule, a range of light energies may be absorbed. As noted above (Eq. 1.2), the energy of the light is proportional to its frequency (thus inversely related to its wavelength), so a given molecule will absorb a specific set of wavelengths of light, giving rise to its absorption spectrum.

![Figure 1.7](image_url)
The Perrin–Jablonski diagram in Figure 1.7 shows absorption to both $S_1$ and $T_1$. In reality, the transition from $S_0$ to $T_1$ is many orders of magnitude weaker than from $S_0$ to $S_1$. Absorption into triplets states, as shown in the figure, is therefore practically unimportant in most cases and is only observed in specialized situations in spectroscopy. However, this is not to say the population of triplet states is unimportant; quite the contrary. Population of triplet states is one of the main mechanisms for photobleaching, which will be discussed later. It is simply, and fortuitously, that triplets are not populated to any great extent by absorption of light by molecules commonly used in fluorescence spectroscopy and microscopy.

Emission of light is shown on a Perrin–Jablonski diagram as an arrow from the excited state back to the ground state. Emission that does not change electron spin, just like absorption, is much preferred over emission in which the electron spin is flipped. This spin-conserving emission is called fluorescence. Because the process is favorable, it happens readily and quickly with the result that excited electronic states survive for only a very short period of time, typically a few nanoseconds, before emitting. Emission between triplet and singlet states is called phosphorescence. Because this is a weak and therefore slow process, the molecule can remain excited much longer, typically milliseconds to seconds. Although this might seem still like a very rapid process, it is slow compared to molecular events and can give rise to unusual chemistry in the excited state leading to reduction in fluorescence efficiency, photobleaching, and other processes (see later).

1.2.3 Multiphoton Excitation

The discussion above on absorption of light and excitation of fluorescence could be termed “one-photon” excitation; that is, one photon is absorbed, leading to one photon being emitted. The reader may already have heard about laser-scanning microscopic techniques designated as “two photon” or more generally as “multiphoton.” Such technology has several advantages and is becoming increasingly common. Multiphoton laser-scanning microscopy involves using pulsed (femtosecond, $1 \text{ fs} = 10^{-15} \text{ s}$) laser beams and optics to focus an intense incident beam of coherent photons to a precise spot. Using such intense radiation allows the possibility of more than one photon to (essentially) simultaneously excite a fluorophore.

A simple understanding of two-photon (or multiphoton) excitation can be achieved using either the particle or wave description of light. Consider first a photon (particle) with exactly half the energy required for a specific electronic transition. This photon, at the speed of light, will impinge upon the fluorophore, for approximately $10^{-18} \text{ s}$. If a second photon impinges on the molecule within this interaction time, then the molecule can absorb both photons together to impart twice the amount of energy into the molecule, as would be expected. The likelihood of two photons impinging on the molecule at the same time is proportional to the number of photons squared (akin to second-order kinetics for those familiar with kinetic theory). Therefore, the (very low) number of two-photon absorption events scales with the square of the laser power. Hence, two-photon microscopy is only possible with very intense lasers.

Above, we described the conventional absorption of light as arising from the interaction of the oscillating electric field of a wave of light with charged regions of the molecule. If the electric field becomes very intense, then the molecule will experience overtones of the field. To use a music analogy again, if you blow a recorder modestly,
you should blow a fairly pure note. If you blow too hard, then you will also generate
the next octave higher, which is exactly one overtone (twice the frequency). This is
a convenient analogy for what a molecule experiences in a very intense laser field; it
also experiences the overtone at twice the frequency of the field, which can then be
absorbed. Again, it can be shown that the amount of overtone scales as the intensity
squared, so the probability of two-photon absorption also scales as the laser intensity
squared using the wave description of the process.

No matter which picture of two-photon absorption we are more comfortable with, the
total energy of the two (or more) photons must still correspond to a difference of real
energy levels of the molecule, as shown in the Perrin–Jablonski diagram in Figure 1.8.
This diagram also shows that the resultant fluorescence emission, following two-photon
absorption, is still the conventional one-photon emission. Two-photon spontaneous
fluorescence has never been observed because the intensity of the fluorescence is much
too weak.

The generalized term “multiphoton” refers to the fact that excitation can be effected
by absorption of two or more photons; two-photon systems are now becoming common,
and commercial three-photon systems are under development. Multiphoton excitation
provides some interesting and useful variations from the one-photon case. If we con-
sider as an example two-photon excitation, coincident absorption of two photons, each
with half the energy required for single-photon excitation, results in the excited state.
This produces the same fluorescence output, but at approximately double the wave-
length of the incident light. Consider Eqs. 1.1 and 1.2; combining them gives

\[ E = \frac{hc}{\lambda} \quad \text{or} \quad \lambda = \frac{hc}{E} \]  

(1.5)

A practical consequence of this doubling of the wavelength of the incident light is
that the energy of the illumination is reduced from the high-energy UV region to
the lower energy red to near-IR region of the EM spectrum (700–1050 nm). This
provides a number of advantages over conventional one-photon fluorescence excitation.
Photobleaching is dramatically reduced by the use of lower energy red to near-IR

![Figure 1.8 Perrin–Jablonski diagram showing two-photon absorption followed by conventional fluorescence.](image-url)
irradiation, as excitation occurs only at the focal point of illumination of the specimen (only here is the laser intensity high enough for two-photon absorption). This also enables studies of UV-excitable dyes with red laser systems without the additional expense and maintenance of UV laser systems. In addition, due to reduced susceptibility to scatter, the penetration of longer wavelength radiation into turbid media such as biofilms of microorganisms and animal or plant tissues is much greater, allowing analysis of events at much greater depth.

1.3 NONRADIATIVE DECAY MECHANISMS

Excited molecules are inherently unstable. As noted above, fluorescence and phosphorescence are two radiative mechanisms for returning the molecule to a stable state. There are also a variety of nonradiative processes that can deactivate a molecule. As the name suggests, these processes do not result in the emission of light and therefore are not useful for fluorescence spectroscopy and microscopy. However, an understanding of them is essential to explain characteristics of fluorophores, including their optical properties, fluorescence efficiency, and bleaching.

1.3.1 Vibrational Relaxation

Molecules are continuously interacting with their surroundings by virtue of their translational, vibrational, and rotational motions. As the atoms collide with their neighbors, they can transfer vibrational energy to and from the vibrations of the surrounding molecules. This is an important process which leads to excitation of cold molecules until they react or deexcitation of hot molecules until they reach thermal equilibrium. The vibrational energy transfer process from hot molecules into their surroundings, called vibrational relaxation (VR) or thermalization, is very efficient and takes place on a picosecond (10$^{-12}$ s) timescale.

Vibrational relaxation occurs in both the ground and excited electronic states. Recall that fluorescence occurs on a nanosecond scale and phosphorescence on an even longer scale, and thus VR will deexcite any excess vibrational energy caused by the absorption

![Diagram](image-url)

Figure 1.9 Perrin–Jablonski diagram showing four steps in absorption/reemission of light.
basics of fluorescence

1. Absorption of light from $S_0$ to a high vibrational level in $S_1$.
2. Thermodynamics dictates that energy will flow from the vibrationally hotter molecule to the cooler surroundings. Therefore VR removes the excess vibrational energy, leaving the molecule in the lowest vibrational level of the $S_1$ state.
3. Fluorescence emission, decreasing the electron’s energy to a high vibrational level in $S_0$. This is not a random choice of vibrational state; if a large change in geometry between $S_0$ and $S_1$ causes significant vibrational excitation upon absorption, then the reverse geometry change will occur upon emission, again causing significant vibrational excitation.
4. VR removes this excess energy, leaving the molecule back where it started.

1.3.2 Internal Conversion

An interesting question concerns why the electronic energy is not also removed by the solvent in the same way that vibrational energy is. In fact it is! The difference is that the relaxation of an excited electronic state requires a much larger energy exchange with the surroundings. It is well known in the energy transfer literature that larger energy exchanges between molecules are much more difficult than small exchanges of energy. A small exchange of energy can occur between an excited fluorophore and the solvent if the fluorophore swaps its electronic energy for the same amount of vibrational energy. This process is shown schematically in the left panel of Figure 1.10, where an excited molecule has undergone several VR steps until it is left in the lowest vibrational state of $S_1$—the same as Figure 1.9. Now, however, the excited state is converted to a high vibrational state of $S_0$—a state of similar total energy. This is indicated on the Perrin–Jablonski diagram by a horizontal (meaning energy is constant), wavy arrow. This process is called internal conversion (IC). Although Figure 1.10 shows IC between $S_1$ and $S_0$, the process can occur between any states of the same multiplicity, that is, between singlet states or between triplet states. In fact, IC between higher states is even more likely and often occurs with a much faster rate than IC between $S_1$ and $S_0$. This is because higher electronic states lie closer in energy to each other than do the $S_1$ and $S_0$ state; the gap between $S_0$ and $S_1$ is usually the largest gap between any two electronic states. As a result, the energy exchange between fluorophore and solvent is less and the IC process is more efficient between higher states. The right-hand side of Figure 1.10 shows this other common fluorescence cycle: absorption to a higher electronic state, $S_2$, followed by IC to $S_1$, then fluorescence. This illustrates a process known variously as Vavilov’s law or Kasha’s law, which states that fluorescence only occurs from the lowest excited electronic state, irrespective of which state is initially excited. A consequence of this law combined with VR is that a fluorophore usually fluoresces at exactly the same wavelength, no matter which electronic or vibrational state is excited. This is shown in Figures 1.9 and 1.10. In both cases the fluorescence is exactly the same, even though $S_1$ is excited in one case and $S_2$ in the other. Specific examples of Vavilov’s or Kasha’s law are shown for fluorophores later in this chapter.
1.3.3 Intersystem Crossing

We have already seen that singlet and triplet manifolds do not interconvert readily by absorption or emission of light. Collisions with surrounding molecules seem to be more efficient at flipping a molecule from singlet to triplet states (and vice versa). The nonradiative transfer between singlet and triplet states is called **intersystem crossing (ISC)**. Figure 1.11 shows how to represent ISC in a Perrin–Jablonski diagram. Once in the triplet manifold, all the normal processes occur with similar efficiency, including VR and IC. After the initial ISC event the molecule will usually relax downward in energy until it reaches the lowest vibration level of the lowest triplet state. This state is **metastable**, which means that there is no efficient process to remove energy, even though the molecule is still in an excited state. This is a dangerous place for a molecule to be! The lowest triplet state, remember, has two unpaired electrons, which makes for a very reactive molecule. One of the main decomposition (or photobleaching) mechanisms for fluorophores is to react from a triplet state.

1.4 PROPERTIES OF EXCITED MOLECULES

1.4.1 Quantum Yield

With so many processes that can occur after the absorption of one photon, it becomes crucial to know which are the most important. One measure of the importance of each process is the **quantum yield**. Mathematically, the quantum yield $\phi_i$ for a process $i$ is

$$\phi_i = \frac{k_i}{\sum k}$$

where $k_i$ is the rate of process $i$ divided by the sum of all rates. Conceptually, the quantum yield is simply the fraction of times that a molecule undergoes a particular process. For example, a quantum yield for fluorescence, $\phi_f = 0.90$, means that after
absorption of a photon, 90% of the molecules will reemit the energy as fluorescence. Clearly, the higher the quantum yield for fluorescence, the brighter a fluorophore will appear (more photons out for the same photons in). In terms of degradation of the fluorophore, the quantum yield for intersystem crossing, $\phi_{ISC}$, is the important value, and clearly this should be as small as possible.

### 1.4.2 Excited-State Lifetime

The lifetime of fluorescence or phosphorescence is the time of persistence of electronic state excitation. The lifetime, denoted by the Greek letter tau ($\tau$), is a practically important parameter that may be affected by changes in the environment of a fluorophore, thereby affecting other measured parameters such as polarization. The lifetime is specifically defined as the inverse of the sum of the rate constants that deplete the excited state, and thus for the singlet state the relative contributions of internal conversion, intersystem crossing, and the radiative process will determine the lifetime value. The magnitude of the rate constants that deplete $S_1$ is typically around $10^8$ s$^{-1}$, indicating a fluorescence lifetime on the order of 10 ns ($10 \times 10^{-9}$ s), although known fluorophores have lifetimes varying from picoseconds to hundreds of nanoseconds. In the case of phosphorescence emission, the rate processes depleting the triplet state are generally slower (between $10^3$ and 1 s$^{-1}$), leading to lifetimes around milliseconds to seconds.

### 1.5 SPECTROSCOPY AND FLUOROPHORES

#### 1.5.1 Absorption, Excitation, and Emission Spectra

In this section we bring together all the earlier concepts into a concrete discussion of the spectra of dyes. Three different kinds of spectra are often reported, and the ability to interpret these spectra is crucial to the correct selection of instrumental parameters for fluorescence spectroscopy and microscopy and/or of an appropriate dye for the application at hand. These three spectra are the absorption, emission, and excitation spectra and we will deal with them separately.
1. Absorption Spectra

The absorption spectrum of a dye is the most fundamental of the three spectra to the extent that all molecules will have an absorption spectrum, though they might not fluoresce. The innate strength with which a molecule absorbs light is characterized by the absorption coefficient (or extinction coefficient, \( \varepsilon \)). The amount of light absorbed (absorbance \( A \)) is described by the Beer–Lambert law, which relates this absorbance to the concentration of the compound \( c \) and the length of the path through which the light travels \( (l) \):

\[
\text{Absorbance} = \log_{10} \frac{I_0}{I} = \varepsilon cl
\]  

(1.7)

As Eq. 1.7 shows, the absorbance is defined in terms of the intensity of incident light, \( I_0 \), and the intensity of transmitted light, \( I \). The unit of the pathlength is usually the centimeter (indeed, the standard absorption cell is 1 cm in length). If the units of \( c \) are moles per liter or grams per liter, then \( \varepsilon \) has units of liters per mole per centimeter or liters per gram per centimeter. Figure 1.12 shows the absorption spectrum, in black, of the fluorophore pyrenesulfonic acid (PSA). The spectrum at first may appear complicated but can readily be understood using the discussion in the previous sections. The main peaks, labeled \( S_1, S_2, \) and \( S_3 \), are the transitions corresponding to the lowest three excited singlet states of PSA. A subsidiary peak, displaced about 20 nm to the shorter wavelength of each labeled peak (blue-shifted), is absorption to a specific vibrational level within each electronic state. In this case the assignment is quite straightforward based largely on the emission spectrum.

2. Emission Spectra

The emission spectrum of PSA, excited by 347-nm light (i.e., the peak of the \( S_1 \) transition), is shown in blue in Figure 1.12. We should notice that the emission spectrum is shifted to a longer wavelength than the absorption spectrum. This is always true and Figure 1.9 shows why. Vibrational relaxation always removes some of the energy of the photon into the environment as heat. The energy of the emitting photon is therefore always lower than the photon that was absorbed (or at best the same energy). Lower energy corresponds to longer wavelength (Eq. 1.4). This difference between the absorption peak and the fluorescence peak is known as the Stokes shift (named after Sir George Stokes, who in 1852 described this phenomenon and also coined the term fluorescence). Different dyes have a different Stokes shift; this is related simply to the change in molecule geometry upon electronic excitation. As described above, a large change in geometry leads to a large vibrational excitation, which in turn leads to a large amount of VR both in \( S_1 \) and \( S_0 \) and ultimately to a large Stokes shift. Large Stokes shifts are very useful in microscopy and spectroscopy because it is easy to filter out the exciting light in favor of the fluorescence. However, large Stokes shifts are also associated with more energy dissipated as heat and therefore heating of the sample and faster degradation of the fluorophore.

The second thing to notice is that the emission spectrum has some structure to it. This structure must be a vibrational structure as there are no electronic states between \( S_1 \) and \( S_0 \), by definition (excluding triplet states, which, as discussed above, are very weak absorbers and emitters of light). In Figure 1.9, these fluorescence transitions would be indicated by arrows terminating in different vibrational states.

Finally, one should notice that the emission spectrum looks like a mirror image of the absorption spectrum, at least the part of the spectrum attributed to \( S_1 \). Again, Figure 1.9 shows the cause of this. The lowest energy (longest wavelength) transition would excite the \( v = 0 \) (lowest) level of the \( S_1 \) state. As the sample is scanned at
increasing energies (shorter wavelength), the light becomes resonant with higher and higher lying vibrational states in $S_1$. The states that show up most clearly are those associated with any change in geometry of the molecule after electronic excitation. Vibrational relaxation quickly removes this excess vibrational energy. In emission, the highest energy transition is now the transition back down to the $v = 0$ level in $S_0$. Emission to higher and higher vibrational states now corresponds to lower energy photons or longer wavelengths. Therefore the absorption spectrum marches out to the blue with increasing vibrational energy, while the emission spectrum marches out to the red for the same reason.

3. **Excitation Spectra** The excitation spectrum is notionally much the same as the absorption spectrum. While the absorption spectrum measures the amount of light absorbed, the excitation spectrum measures the amount of fluorescence generated following absorption. The excitation spectrum is therefore an indirect measurement of the absorption process. The red-colored excitation spectrum of PSA in Figure 1.12 matches the absorption spectrum from 375 nm down to about 250 nm. Below 250 nm the two spectra deviate. The molecule is clearly absorbing strongly, but this is not showing up in the excitation spectrum. The reason is simple—at high energy (short wavelength) the molecule is not fluorescing any more. We can speculate about the reason; perhaps the molecule is undergoing a photochemical reaction at these energies and the photon is breaking a chemical bond rather than releasing the energy as fluorescence. In technical terms, the absorption and excitation spectra are identical as long as the fluorescence quantum yield remains the same (see Eq. 1.5). If the quantum yield drops off, so does the excitation spectrum compared to the absorption spectrum.

Figure 1.13 shows the absorption and three emission spectra of another fluorescent dye. Again, three different electronic states are evident. The three fluorescence spectra, which are color coded, were generated following excitation of the dye at the three color-coded wavelengths indicated by arrows, that is, excitation into the $S_1$, $S_2$, and

![Figure 1.12](image.png) Absorption, excitation, and emission spectra for pyrenesulfonic acid (structure shown as inset).
Spectroscopy and fluorophores

Figure 1.13 Absorption and fluorescence spectra of 1,1'-diethyl-2,2'-carbocyanine ion (structure shown as inset). Each colored fluorescence spectrum was generated following excitation at the three positions marked by arrow of the same color.

$S_3$ states. It is very clear that the emission spectrum is essentially the same in each case, with only the intensity changing. The difference in intensity is simply related to the difference in absorption coefficient at each wavelength. At first thought, it might seem strange that the emission spectrum does not depend on the excitation wavelength. On second thought, Figure 1.10 provides the explanation. Vavilov’s law or Kasha’s law says that whatever excited energy level is reached through absorption of light, energy loss via VR and IC means that the fluorescence emission always occurs from the lowest vibrational level of the first excited state ($S_1$). Remember here that VR and IC occur on a timescale of $10^{-12}$ s, at least three orders of magnitude faster than the fluorescence emission which typically occurs on a timescale of $10^{-9}$ s.

1.5.2 Basic Properties of Fluorophores

Fluorescence emission generally occurs from organic molecules with conjugated double-bond systems, such as aromatic molecules, which are called fluorochromes or fluorophores. The fluorophores in Figures 1.12 and 1.13 are good examples of this. In the study of biological systems, fluorophores may be naturally occurring molecules such as the amino acid tryptophan (and to a lesser extent tyrosine and phenylalanine), porphyrins, quinine, adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NADH), and other cofactors. In addition, a host of synthetic chemical fluorophores are available for specific labeling of biological systems (e.g., fluorescein, rhodamine, ethidium bromide) as well as fluorescent proteins which can be “genetically” attached to proteins of interest. Such fluorophores will be discussed in later chapters of this book, so only brief mention is made in this introductory chapter. Important considerations in selection of a fluorophore include its absorption coefficient and quantum yield (typically both as high as possible), Stokes shift (typically as large as possible), emission spectrum (and its differentiation from background autofluorescence), solubility, reactivity, and method of labeling. The basis
of a commercial catalogue, *Molecular Probes Handbook of Fluorescent Probes and Research Products* (http://probes.invitrogen.com/), provides a very useful guide to the types and characteristics of synthetic fluorophores.

### 1.6 ENVIRONMENTAL SENSITIVITY OF FLUOROPHORES

It has long been known that the excitation or emission spectra and other parameters such as the lifetime of some fluorophores are sensitive to their local environment. Factors such as polarity and interaction with other molecules may affect the spectra. Various fluorophores are known to have such dramatic changes in fluorescence quantum yield that they effectively fluoresce in some environments but not in others. This has been utilized to advantage in a range of practical techniques. For example, ethidium bromide has a very low quantum yield in aqueous systems, with a high quantum yield when intercalated between the nucleotide bases in double-stranded nucleic acids. Thus, when incorporating ethidium bromide into agarose gels used for electrophoretic separation of DNA, upon illumination with UV light bands of DNA can be identified by their bright fluorescence against a low-fluorescence background. This classical approach in molecular biology is still in use, although newer fluorochromes with less toxicity are becoming more routinely employed. A second example is 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS), which when dissolved in water emits only a very weak, yellow fluorescence with a quantum yield of 0.004. However, upon addition of a solution of protein, an immediate, dramatic increase in fluorescence is noted (with quantum yield of 0.75) as well as a “blue shift” in emission maximum (color change from yellow to blue-green). In similarity to ethidium bromide, the increase of emission reflects movement of ANS from water to a hydrophobic environment provided by the protein. In this case water molecules effectively lower the energy of the ANS excited state (hence the lower energy, yellow emission) and also quench the fluorescence. These features have been applied in numerous studies of protein-folding mechanisms. A further example of useful changes in fluorescence emission is provided by hydrophobic fluorophores that preferentially partition into membranes, for example, 1,6-diphenylhexatriene (DPH) and 6-dodecanoyl-2-dimethylamino naphthalene (laurdan). Since these probes effectively fluoresce in membranes but not in aqueous systems, membranes of whole cells can be analyzed by spectroscopy or microscopy without the complication of background probe fluorescence from aqueous compartments.

In addition to dramatic changes in emission quantum yield, more subtle effects of the environment on fluorophores may be utilized in experimental systems. For example, considering the amino acid tryptophan, upon excitation at an optimal wavelength of around 280 nm, while generally considered to emit at around 340 nm, tryptophan emission is sensitive to the polarity of its environment. This has been used to explore the microenvironment of tryptophan residues in proteins, thereby giving information on protein-folding states and interactions; for example, when buried in the hydrophobic core of a protein, tryptophan emits with maximum around 335 nm, but when the protein is unfolded, the emission “red shifts” to around 365 nm.

In addition to naturally occurring compounds, fluorophores have been produced to have large dipole moments and high sensitivity to their environment. A prime example is the membrane probe laurdan, which was designed and synthesized by Gregorio Weber in the 1970s. The large dipole moment leads to a large Stokes shift as well as
high sensitivity to polarity. In membranes, laurdan shows a 50-nm red shift in emission spectrum over the gel to liquid crystalline phase transition. The spectroscopic property generalized polarization (GP), derived from fluorescence intensities at the red and blue edges of the laurdan emission spectrum, which represent gel (440 nm) and liquid crystalline (490 nm) phases, can be used as a membrane fluidity index. The GP is calculated in an analogous way to the polarization parameter (see Section 1.8) using the intensity at the critical wavelengths instead of polarization orientation. Further examples of environmentally sensitive probes which are analyzed by ratiometric techniques include probes to detect specific ions or indicate pH.

1.6.1 Quenching of Fluorescence

In addition to emission of light and nonradiative processes described above, interaction with a quencher is a further way in which a molecule can lose its excited-state energy. A quencher is a molecule which can deactivate the excited state either through a collisional process or by formation of a nonfluorescent complex. Quenchers that form complexes generally have very specific interactions with particular types of fluorophores. Many fluorophores are based on aromatic ring structures which potentially interact with other aromatic nonfluorescent molecules via weak van der Waals forces, forming nonfluorescent complexes. Quenching of ethidium bromide by caffeine is an example of this type of complexing interaction. Collisional quenchers, on the other hand, tend to be small molecules or ions. Common examples include oxygen (O$_2$), iodide (I$^-$), and heavy metals such as mercury (Hg$^{2+}$). Collisional quenching interactions can be described using the Stern–Volmer equation

\[
\frac{F_0}{F} = 1 + k^*_{Q}[Q] \tau_0 \tag{1.8}
\]

where $F_0$ is the initial fluorescence intensity before addition of quencher, $F$ is the fluorescence in the presence of quencher, $k^*_{Q}$ is the “bimolecular quenching rate constant,” $Q$ is the quencher concentration, and $\tau_0$ is the fluorescence lifetime in the absence of quencher. The plus sign in the bimolecular quenching constant indicates that the process is an interaction between two molecules. Quenching studies can provide direct information on the diffusion of small molecules in solution and in cellular compartments, providing insights into solvent accessibilities and viscosities. However, in some practical situations quenching can be a problem, whereby the intensity of measured fluorescence is reduced by an undesirable component (such as heavy metal contamination) in the system being analyzed. For example, it is not feasible to measure phosphorescence emission in the presence of oxygen, as the latter effectively quenches the emission to a high degree. Therefore, when investigating phosphorescence emission in solutions, it is common practice to deoxygenate the solutions, for example, by utilizing the glucose oxidase reaction.

1.6.2 Photobleaching

As noted above, excited electronic states may have increased chemical reactivity, particularly if triplet states are formed. In their excited state, molecules may react with other molecules such as oxygen or heavy metals, leading to formation of transient or stable complexes that further lead to irreversible deactivation of the excited state.
without the emission of light. Note that, as outlined above, a consequence of loss of excited-state energy by VR leads to heating, which further increases the rate of potentially damaging photochemical reactions. Apart from the specialized case of some green fluorescent proteins, photobleaching is an irreversible process, leading to lowered fluorescence emission intensity as a function of time of illumination of the fluorophore. Practical solutions to this problem have included the use of antifade reagents (containing antioxidants and commonly used for fixed-specimen fluorescence microscopy but incompatible with live cell investigations), development of fluorophores less susceptible to photobleaching, and minimization of illumination intensity or duration. The latter has been achieved in spectroscopy by limiting the illumination wavelength bandwidth and intensity and minimizing the period that illumination shutters are open as well as stirring samples to prevent fluorophores from remaining in the illumination beam for long periods. Approaches in microscopy have included development of confocal laser-scanning microscopy systems which limit the illumination to a cone that is scanned across the specimen (as opposed to whole-specimen illumination by transmission or epifluorescence microscopy) as well as attenuation of the intensity of the illuminating beam. The situation may be improved further by multiphoton laser-scanning microscopy (see Section 1.2.3), which further limits photobleaching by minimizing the focal volume of excitation to a precise point as well as illuminating with wavelengths of half the incident energy.

In some cases, photobleaching has been used to advantage in experimental investigations and in fact is used in the FRAP or fluorescence photobleaching recovery (FPR) technique, as will be described in a later chapter. Laser-based microscopy systems allow high-intensity pulses of illumination to be focused on specific areas of a specimen, causing localized photobleaching. Subsequent observation of the bleached regions over time can elucidate whether fluorescently labeled molecules are able to move into the bleached area, the magnitude of this movement, and the diffusion coefficients of the labeled molecules.

1.6.3 Fluorescence Resonance Energy Transfer

Another process which can lead to nonradiative dissipation of the excited-state energy is transfer of this energy to another “acceptor” molecule in a process known as fluorescence (or Förster) resonance energy transfer. In some cases the acceptor molecule further dissipates this energy by VR mechanisms, although in most cases the transferred energy becomes the excitation of the acceptor molecule which subsequently emits fluorescence. Note this emission would necessarily be of lower energy, that is, of longer wavelength, than would be the emission of the initially excited molecule. The processes and application of FRET will be discussed further in a later chapter.

1.6.4 Solvent Relaxation

As discussed in Section 1.3.1, after excitation, a certain amount of the energy is lost by VR. In some cases, additional energy is lost during the excited-state lifetime by a process known as solvent relaxation, whereby solvent dipoles reorientate around the excited-state dipole. This reorientation favors new dipole–dipole interactions which lower the overall energy of the system. As the resultant “stabilized” $S_1$ level is of
lower energy, the emitted fluorescence will also be of lower energy, that is, longer wavelength. This phenomenon is represented in terms of a Perrin–Jablonski diagram in Figure 1.14.

Figure 1.15 shows a striking visual demonstration of the solvent relaxation phenomenon. Fluorescence emission of the fluorophore laurdan dissolved in glycerol
is seen to red shift as solvent relaxation increases. The tubes are inclined to show the viscosity and flow properties of glycerol solutions at $-70^\circ C$ (solid, bluish fluorescence), $25^\circ C$ (high viscosity, greenish fluorescence), and $80^\circ C$ (low viscosity, high solvent relaxation, yellowish-green fluorescence).

1.7 POLARIZATION OF FLUORESCENCE

As noted above, rotational mobility in biological systems is commonly assessed by measurement of fluorescence emission polarization using instrumentation equipped with linearly polarizing filters or prisms. In this section we will briefly describe concepts underpinning the measurement of fluorescence polarization.

Put in the most simplistic terms, if a molecule is illuminated with polarized light and is able to rotate during the excited-state lifetime, then the fluorescence emission will become depolarized in relation to the incident illumination. The degree of depolarization will relate to the mobility of the emitting species; in other words, fluorescence polarization decreases as rotational mobility increases (in relation to the excited-state lifetime). Thus, the measured polarization of a molecule increases with increasing molecular size, increasing viscosity of the medium, and decreasing temperature, reflecting decreased rotational mobility. To this simplistic picture we need to add that there will be some depolarization of emission due to differences in angle between excitation and emission dipoles, and this can be accounted for in determining theoretical maximum polarization values.

As noted above, for unpolarized light the electric vector could be found at any angle but could be considered as a wave which has an average of half its vibrations in a horizontal plane and half of its vibrations in a vertical plane. This helps provide a rationale for simple measurement of the degree of polarization. Rather than sample all possible angles of polarization, a suitable estimate could be taken by considering the intensity of emission at two orientations perpendicular to each other, commonly denoted as vertical (or $0^\circ$) and horizontal (or $90^\circ$).

The calculated parameters denoted as polarization ($P$) and anisotropy ($r$) are given by the equations below, where $I_{VV}$ is the fluorescence emission intensity measured in the plane parallel to the plane of vertically polarized excitation and $I_{VH}$ is fluorescence emission intensity measured in the plane perpendicular to the plane of vertically polarized excitation (the first subscript refers to the orientation of the excitation polarizer while the second subscript refers to the emission polarizer orientation):

$$P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$ (polarization) (1.9)

$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}$$ (anisotropy) (1.10)

It should be noted that these equations need to be corrected by an instrument grating correction factor to account for differences in the efficiency of monochromators and detectors for the different polarization orientations. This is achieved by multiplying the $I_{VH}$ values by the grating correction factor $G$, which is calculated from measurements taken with the excitation polarizer in the horizontal position (as opposed to the vertical...
orientation for the polarization measurements):

\[ G = \frac{I_{HV}}{I_{HH}} \]  

(1.11)

Anisotropy and polarization are both expressions of the same phenomenon and can be easily interconverted as follows:

\[ r = \frac{2P}{3 - P} \]  

(1.12)

However, the anisotropy expression is generally preferred as anisotropies of individual components are directly additive, making mathematical analyses of multicomponent systems much more straightforward. The anisotropy can be related to the rotational mobility of the fluorophore by the *Perrin equation*:

\[ \frac{r_0}{r} = 1 + \frac{RT\tau}{\eta V} \]  

(1.13)

where \( r_0 \) is the limiting anisotropy (in the absence of any fluorophore rotation), \( r \) is the measured anisotropy, \( \eta \) is the viscosity of the environment, \( V \) is the volume of the rotating molecule, \( R \) is the universal gas constant, \( T \) is the temperature (in degrees Kelvin), and \( \tau \) is the excited-state lifetime. The Perrin equation is often simplified to express the time-dependent decay of anisotropy in terms of the rotational correlation time (\( \theta \), often denoted \( \tau_c \)) of a fluorophore (assuming a spherical molecule rotating isotropically):

\[ \frac{r_0}{r} = 1 + \frac{\tau}{\theta} \]  

(1.14)

where \( \theta = (\eta V)/(RT) \). If the rotational correlation time \( \theta \) is much longer than the lifetime \( \tau \), then the measured anisotropy will approximate \( r_0 \), whereas if \( \theta \) is much shorter than the lifetime \( \tau \), the measured anisotropy will approximate zero. The use of rotational correlation time might be confused with considerations from nuclear magnetic resonance, so an earlier term, the *Debye rotational relaxation time* (\( \phi = 3\theta \)), may be a more suitable term in these expressions.

Another way of expressing the anisotropy–mobility relationship is as follows:

\[ \frac{r_0}{r} = 1 + 6D\tau \]  

(1.15)

where \( D \) is the rotational diffusion coefficient. However, although rotational relaxation times or microviscosities can be calculated, in many studies it is considered that comparison of anisotropy data would be sufficient to estimate and compare relative mobility. It should be noted here that these analyses are simplified, and a full description will take into account the photoselection of fluorophores by polarized illumination, angles between absorption and emission dipoles, and further refinement of calculations to estimate rotational parameters of biological molecules which are nonspherical. An extension of the technique is to incorporate pulsed or modulated excitation sources in the experimental arrangement to determine the temporal decay of the fluorescence anisotropy.
1.8 CONCLUSION

This chapter has provided a basic introduction to fluorescence and has raised in a simplistic manner various issues that will be expanded in the following chapters. More detailed discussion of these issues may be found in the References.

REFERENCES