Introduction to Fluorescence

During the past 20 years there has been a remarkable growth in the use of fluorescence in the biological sciences. Fluorescence spectroscopy and time-resolved fluorescence are considered to be primarily research tools in biochemistry and biophysics. This emphasis has changed, and the use of fluorescence has expanded. Fluorescence is now a dominant methodology used extensively in biotechnology, flow cytometry, medical diagnostics, DNA sequencing, forensics, and genetic analysis, to name a few. Fluorescence detection is highly sensitive, and there is no longer the need for the expense and difficulties of handling radioactive tracers for most biochemical measurements. There has been dramatic growth in the use of fluorescence for cellular and molecular imaging. Fluorescence imaging can reveal the localization and measurements of intracellular molecules, sometimes at the level of single-molecule detection.

Fluorescence technology is used by scientists from many disciplines. This volume describes the principles of fluorescence that underlie its uses in the biological and chemical sciences. Throughout the book we have included examples that illustrate how the principles are used in different applications.

1.1. PHENOMENA OF FLUORESCENCE

Luminescence is the emission of light from any substance, and occurs from electronically excited states. Luminescence is formally divided into two categories—fluorescence and phosphorescence—depending on the nature of the excited state. In excited singlet states, the electron in the excited orbital is paired (by opposite spin) to the second electron in the ground-state orbital. Consequently, return to the ground state is spin allowed and occurs rapidly by emission of a photon. The emission rates of fluorescence are typically 10^8 s^{-1}, so that a typical fluorescence lifetime is near 10 ns (10×10^{-9} s). As will be described in Chapter 4, the lifetime (τ) of a fluorophore is the average time between its excitation and return to the ground state. It is valuable to consider a 1-ns lifetime within the context of the speed of light. Light travels 30 cm, or about one foot, in one nanosecond. Many fluorophores display subnanosecond lifetimes. Because of the short timescale of fluorescence, measurement of the time-resolved emission requires sophisticated optics and electronics. In spite of the added complexity, time-resolved fluorescence is widely used because of the increased information available from the data, as compared with stationary or steady-state measurements. Additionally, advances in technology have made time-resolved measurements easier, even when using microscopes.

Phosphorescence is emission of light from triplet excited states, in which the electron in the excited orbital has the same spin orientation as the ground-state electron. Transitions to the ground state are forbidden and the emission rates are slow (10^3 to 10^0 s^{-1}), so that phosphorescence lifetimes are typically milliseconds to seconds. Even longer lifetimes are possible, as is seen from "glow-in-the-dark" toys. Following exposure to light, the phosphorescence substances glow for several minutes while the excited phosphors slowly return to the ground state. Phosphorescence is usually not seen in fluid solutions at room temperature. This is because there exist many deactivation processes that compete with emission, such as non-radiative decay and quenching processes. It should be noted that the distinction between fluorescence and phosphorescence is not always clear. Transition metal–ligand complexes (MLCs), which contain a metal and one or more organic ligands, display mixed singlet–triplet states. These MLCs display intermediate lifetimes of hundreds of nanoseconds to several microseconds. In this book we will concentrate mainly on the more rapid phenomenon of fluorescence.

Fluorescence typically occurs from aromatic molecules. Some typical fluorescent substances (fluorophores) are shown in Figure 1.1. One widely encountered fluorophore is quinine, which is present in tonic water. If one observes a glass of tonic water that is exposed to sunlight, a faint blue glow is frequently visible at the surface. This glow is most apparent when the glass is observed at a right

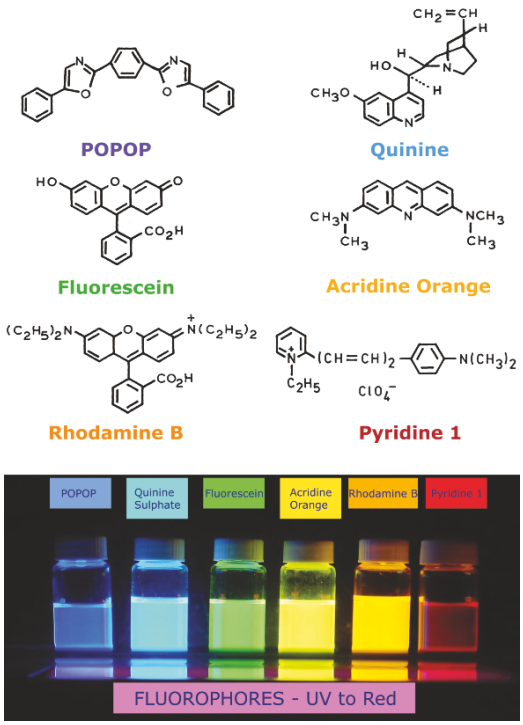

Figure 1.1. Structures of typical fluorescent substances.

Figure 1.2. Sir John Fredrich William Herschel, March 7, 1792 to May 11, 1871. Reproduced courtesy of the Library and Information Centre, Royal Society of Chemistry.

angle relative to the direction of the sunlight, and when the dielectric constant is decreased by adding less polar solvents like alcohols. The quinine in tonic water is excited by the ultraviolet light from the sun. Upon return to the ground state the quinine emits blue light with a wavelength near 450 nm. The first observation of fluorescence from a quinine solution in sunlight was reported by Sir John Frederick William Herschel (Figure 1.2) in 1845.[1] The following is an excerpt from this early report:

> *On a case of superficial colour presented by a homogeneous liquid internally colourless. By Sir John Frederick William Herschel, Philosophical Translation of the Royal Society of London (1845) 135:143–145. Received January 28, 1845 — Read February 13, 1845.*
>
> "The sulphate of quinine is well known to be of extremely sparing solubility in water. It is however easily and copiously soluble in tartaric acid. Equal weights of the sulphate and of crystallised tartaric acid, rubbed up together with addition of a very little water, dissolve entirely and immediately. It is this solution, largely diluted, which exhibits the optical phenomenon in question. Though perfectly transparent and colourless when held between the eye and the light, or a white object, it yet exhibits in certain aspects, and under certain incidences of the light, an extremely vivid and beautiful celestial blue colour, which, from the circumstances of its occurrence, would seem to originate in those strata which the light first penetrates in entering the liquid, and which, if not strictly superficial, at least exert their peculiar power of analysing the incident rays and dispersing those which compose the tint in question, only through a very small depth within the medium.
>
> To see the colour in question to advantage, all that is requisite is to dissolve the two ingredients above mentioned in equal proportions, in about a hundred times their joint weight of water, and having filtered the solution, pour it into a tall narrow cylindrical glass vessel or test tube, which is to be set upright on a dark coloured substance before an open window exposed to strong daylight or sunshine, but with no cross lights, or any strong reflected light from behind. If we look down perpendicularly into the vessel so that the visual ray shall graze the internal surface of the glass through a great part of its depth, the whole of that surface of the liquid on which the light first strikes will appear of a lively blue, ...
>
> If the liquid be poured out into another vessel, the descending stream gleams internally from all

> its undulating inequalities, with the same lively yet delicate blue colour, ... thus clearly demonstrating that contact with a denser medium has no share in producing this singular phenomenon.
>
> The thinnest film of the liquid seems quite as effective in producing this superficial colour as a considerable thickness. For instance, if in pouring it from one glass into another, ... the end of the funnel be made to touch the internal surface of the vessel well moistened, so as to spread the descending stream over an extensive surface, the intensity of the colour is such that it is almost impossible to avoid supposing that we have a highly coloured liquid under our view."

It is evident from this early description that Sir Herschel recognized the presence of an unusual phenomenon that could not be explained by the scientific knowledge of the time. To this day the fluorescence of quinine remains one of the most used and most beautiful examples of fluorescence. Herschel was from a distinguished family of scientists who lived in England but had their roots in Germany.[2] For most of his life Sir Herschel did research in astronomy, publishing only a few papers on fluorescence.

It is interesting to notice that the first known fluorophore, quinine, was responsible for stimulating the development of the first spectrofluorometers that appeared in the 1950s. During World War II, the Department of War was interested in monitoring antimalaria drugs, including quinine. This early drug assay resulted in a subsequent program at the National Institutes of Health to develop the first practical spectrofluorometer.[3]

Many other fluorophores are encountered in daily life. The green or red-orange glow sometimes seen in antifreeze is due to trace quantities of fluorescein or rhodamine, respectively (Figure 1.1). Polynuclear aromatic hydrocarbons, such as anthracene and perylene, are also fluorescent, and the emission from such species is used for environmental monitoring of oil pollution. Some substituted organic compounds are also fluorescent. For example 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) is used in scintillation counting and acridine orange is often used as a DNA stain. Pyridine 1 and rhodamine are frequently used in dye lasers.

Numerous additional examples of probes could be presented. Instead of listing them here, examples will appear throughout the book, with a description of the spectral properties of the individual fluorophores. An overview of fluorophores used for research and fluorescence sensing is presented in Chapter 3. In contrast to aromatic organic molecules, atoms are generally nonfluorescent in condensed phases. One notable exception is the group of elements commonly known as the lanthanides.[4] The fluorescence from europium and terbium ions results from electronic transitions between *f* orbitals. These orbitals are shielded from the solvent by higher filled orbitals. The lanthanides display long decay times because of this shielding and low emission rates because of their small extinction coefficients.

Fluorescence spectral data are generally presented as emission spectra. A fluorescence emission spectrum is a plot of the fluorescence intensity versus wavelength (nanometers) or wavenumber (cm^{-1}). Two typical fluorescence emission spectra are shown in Figure 1.3. Emission spectra vary widely and are dependent upon the chemical structure of the fluorophore and the solvent in which it is dissolved. The spectra of some compounds, such as perylene, show significant structure due to the individual vibrational energy levels of the ground and excited states. Other compounds, such as quinine, show spectra devoid of vibrational structure.

An important feature of fluorescence is high sensitivity detection. The sensitivity of fluorescence was used in 1877 to demonstrate that the rivers Danube and Rhine were connected by underground streams.[5] This connection was demonstrated by placing fluorescein (Figure 1.1) into the Danube. Some sixty hours later its characteristic green fluorescence appeared in a small river that led to the Rhine. Today fluorescein is still used as an emergency marker for locating individuals at sea, as has been seen on the landing of space capsules in the Atlantic Ocean. Readers interested in the history of fluorescence are referred to the excellent summary by Berlman.[5]

1.2. JABLONSKI DIAGRAM

The processes that occur between the absorption and emission of light are usually illustrated by the Jablonski[6] diagram. Jablonski diagrams are often used as the starting point for discussing light absorption and emission. Jablonski diagrams are used in a variety of forms, to illustrate various molecular processes that can occur in excited states. These diagrams are named after Professor Alexander Jablonski (Figure 1.4), who is regarded as the father of fluorescence spectroscopy because of his many accomplishments, including descriptions of concentration depolarization and defining the term "anisotropy" to describe the polarized emission from solutions.[7,8]

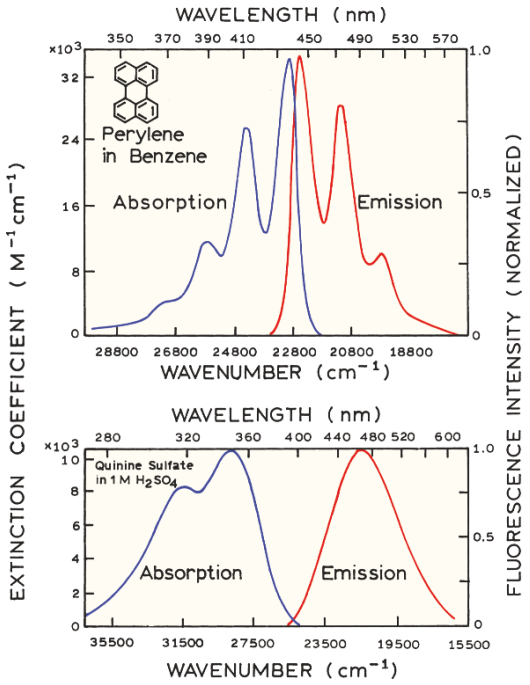

Figure 1.3. Absorption and fluorescence emission spectra of perylene and quinine. Emission spectra cannot be correctly presented on both the wavelength and wavenumber scales. The wavenumber presentation is correct in this instance. Wavelengths are shown for convenience. See Chapter 3. Revised from [5].

Brief History of Alexander Jablonski

Professor Jablonski was born February 26, 1898 in Voskresenovka, Ukraine. In 1916 he began his study of atomic physics at the University of Kharkov, which was interrupted by military service first in the Russian Army and later in the newly organized Polish Army during World War I. At the end of 1918, when an independent Poland was re-created after more than 120 years of occupation by neighboring powers, Jablonski left Kharkov and arrived in Warsaw, where he entered the University of Warsaw to continue his study of physics. His study in Warsaw was again interrupted in 1920 by military service during the Polish-Bolshevik war.

An enthusiastic musician, Jablonski played first violin at the Warsaw Opera from 1921 to 1926 while studying at the university under Stefan Pienkowski. He received his doctorate in 1930 for work "On the influence of the change of wavelengths of excitation light on the fluorescence spectra." Although Jablonski left the opera in 1926 and devoted himself entirely to scientific work, music remained his great passion until the last days of his life.

Figure 1.4. Professor Alexander Jablonski (1898–1980), circa 1935. Courtesy of his daughter, Professor Danuta Frackowiak.

Throughout the 1920s and 30s the Department of Experimental Physics at Warsaw University was an active center for studies on luminescence under S. Pienkowski. During most of this period Jablonski worked both theoretically and experimentally on the fundamental problems of photoluminescence of liquid solutions as well as on the pressure effects on atomic spectral lines in gases. A problem that intrigued Jablonski for many years was the polarization of photoluminescence of solutions. To explain the experimental facts he distinguished the transition moments in absorption and in emission and analyzed various factors responsible for the depolarization of luminescence.

Jablonski's work was interrupted once again by a world war. From 1939 to 1945 Jablonski served in the Polish Army, and spent time as a prisoner of first the German Army and then the Soviet Army. In 1946 he returned to Poland to chair a new Department of Physics in the new Nicholas Copernicus University in Torun. This beginning occurred in the very difficult postwar years in a country totally destroyed by World War II. Despite all these difficulties, Jablonski with great energy organized the Department of Physics at the university, which became a scientific center for studies in atomic and molecular physics.

His work continued beyond his retirement in 1968. Professor Jablonski created a spectroscopic school of thought that persists today through his numerous students, who now occupy positions at universities in Poland and elsewhere. Professor Jablonski died on September 9, 1980. More complete accounts of his accomplishments are given in [7] and [8].

A typical Jablonski diagram is shown in Figure 1.5. The singlet ground, first, and second electronic states are depicted by S_0, S_1, and S_2, respectively. At each of these electronic energy levels the fluorophores can exist in a number of vibrational energy levels, depicted by 0, 1, 2, etc. In this Jablonski diagram we excluded a number of interactions that will be discussed in subsequent chapters, such as quenching, energy transfer, and solvent interactions. The transitions between states are depicted as vertical lines to illustrate the instantaneous nature of light absorption. Transitions occur in about 10^{-15} s, a time too short for significant displacement of nuclei. This is the Franck-Condon principle.

The energy spacing between the various vibrational energy levels is illustrated by the emission spectrum of perylene (Figure 1.3). The individual emission maxima (and hence vibrational energy levels) are about 1500 cm^{-1} apart. At room temperature thermal energy is not adequate to significantly populate the excited vibrational states. Absorption and emission occur mostly from molecules with the lowest vibrational energy. The larger energy difference between the S_0 and S_1 excited states is too large for thermal population of S_1. For this reason we use light and not heat to induce fluorescence.

Following light absorption, several processes usually occur. A fluorophore is usually excited to some higher vibrational level of either S_1 or S_2. With a few rare exceptions, molecules in condensed phases rapidly relax to the lowest vibrational level of S_1. This process is called internal conversion and generally occurs within 10^{-12} s or less. Since fluorescence lifetimes are typically near 10^{-8} s, internal conversion is generally complete prior to emission. Hence, fluorescence emission generally results from a thermally equilibrated excited state, that is, the lowest energy vibrational state of S_1.

Return to the ground state typically occurs to a higher excited vibrational ground state level, which then quickly (10^{-12} s) reaches thermal equilibrium (Figure 1.5). Return to an excited vibrational state at the level of the S_0 state is the reason for the vibrational structure in the emission spectrum of perylene. An interesting consequence of emission to higher vibrational ground states is that the emission spectrum is typically a mirror image of the absorption spectrum of the $S_0 \rightarrow S_1$ transition. This similarity occurs because electronic excitation does not greatly alter the nuclear geometry. Hence the spacing of the vibrational energy levels of the excited states is similar to that of the ground state. As a result, the vibrational structures seen in the absorption and the emission spectra are similar.

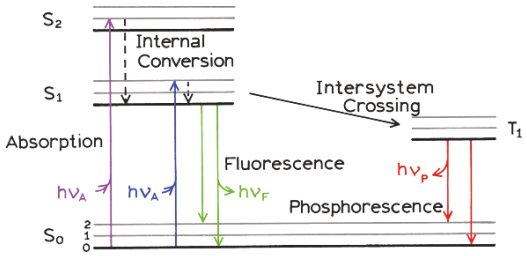

Figure 1.5. One form of a Jablonski diagram.

Molecules in the S_1 state can also undergo a spin conversion to the first triplet state T_1. Emission from T_1 is termed phosphorescence, and is generally shifted to longer wavelengths (lower energy) relative to the fluorescence. Conversion of S_1 to T_1 is called intersystem crossing. Transition from T_1 to the singlet ground state is forbidden, and as a result the rate constants for triplet emission are several orders of magnitude smaller than those for fluorescence. Molecules containing heavy atoms such as bromine and iodine are frequently phosphorescent. The heavy atoms facilitate intersystem crossing and thus enhance phosphorescence quantum yields.

1.3. CHARACTERISTICS OF FLUORESCENCE EMISSION

The phenomenon of fluorescence displays a number of general characteristics. Exceptions are known, but these are infrequent. Generally, if any of the characteristics described in the following sections are not displayed by a given fluorophore, one may infer some special behavior for this compound.

1.3.1. The Stokes Shift

Examination of the Jablonski diagram (Figure 1.5) reveals that the energy of the emission is typically less than that of absorption. Fluorescence typically occurs at lower energies or longer wavelengths. This phenomenon was first observed by Sir. G. G. Stokes in 1852 at the University of Cambridge.[9] These early experiments used relatively simple

instrumentation (Figure 1.6). The source of ultraviolet excitation was provided by sunlight and a blue glass filter, which was part of a stained glass window. This filter selectively transmitted light below 400 nm, which was absorbed by quinine (Figure 1.6). The incident light was prevented from reaching the detector (eye) by a yellow glass (of wine) filter. Quinine fluorescence occurs near 450 nm and is therefore easily visible.

It is interesting to read Sir George's description of the observation. The following is from his report published in 1852:[9]

> *On the Change of Refrangibility of Light. By G. G. Stokes, M.A., F.R.S., Fellow of Pembroke College, and Lucasian Professor of Mathematics in the University of Cambridge. Phil. Trans. Royal Society of London (1852) pp. 463-562. Received May 11, — Read May 27, 1852.*
>
> "The following researches originated in a consideration of the very remarkable phenomenon discovered by Sir John Herschel in a solution of sulphate of quinine, and described by him in two papers printed in the Philosophical Transactions for 1845, entitled "On a Case of Superficial Colour presented by a Homogeneous Liquid internally colourless," and "On the Epipolic Dispersion of Light." The solution of quinine, though it appears to be perfectly transparent and colourless, like water, when viewed by transmitted light, exhibits nevertheless in certain aspects, and under certain incidences of the light, a beautiful celestial blue colour. It appears from the experiments of Sir John Herschel that the blue colour comes only from a stratum of fluid of small but finite thickness adjacent to the surface by which the light enters. After passing through this stratum, the incident light, though not sensibly enfeebled nor coloured, has lost the power of producing the same effect, and therefore may be considered as in some way or other qualitatively different from the original light."

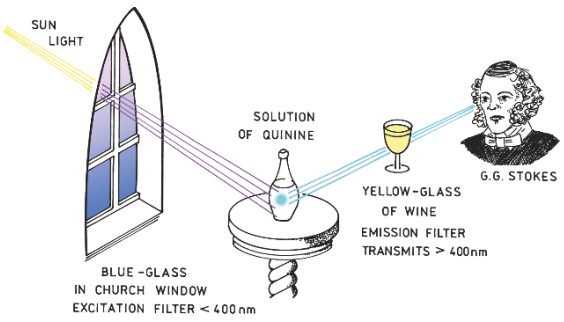

Figure 1.6. Experimental schematic for detection of the Stokes shift.

Careful reading of this paragraph reveals several important characteristics of fluorescent solutions. The quinine solution is colorless because it absorbs in the ultraviolet, which we cannot see. The blue color comes only from a region near the surface. This is because the quinine solution was relatively concentrated and absorbed all of the UV in the first several millimeters. Hence Stokes observed the inner filter effect. After passing through the solution the light was "enfeebled" and no longer capable of causing the blue glow. This occurred because the UV was removed and the "enfeebled" light could no longer excite quinine. However, had Sir George used a second solution of fluorescein, rather than quinine, it would have still been excited because of the longer absorption wavelength of fluorescein.

Energy losses between excitation and emission are observed universally for fluorescent molecules in solution. One common cause of the Stokes shift is the rapid decay to the lowest vibrational level of S_1. Furthermore, fluorophores generally decay to higher vibrational levels of S_0 (Figure 1.5), resulting in further loss of excitation energy by thermalization of the excess vibrational energy. In addition to these effects, fluorophores can display further Stokes shifts due to solvent effects, excited-state reactions, complex formation, and/or energy transfer.

Brief History of Sir. G. G. Stokes:

Professor Stokes was born in Ireland, August 3, 1819 (Figure 1.7). He entered Pembroke College, Cambridge, in 1837, and was elected as a fellow of Pembroke immediately upon his graduation in 1841. In 1849 Stokes became Lucasian Professor at Cambridge, a chair once held by Newton. Because of poor endowment for the chair, he also worked in the Government School of Mines.

Stokes was involved with a wide range of scientific problems, including hydrodynamics, elasticity of solids, and diffraction of light. The wave theory of light was already known when he entered Cambridge. In his classic paper on quinine, he understood that light of a higher "refrangibility" or frequency was responsible for the blue glow of lower refrangibility or frequency. Thus invisible ultraviolet rays were absorbed to produce the blue light at the surface. Stokes later suggested using optical properties such as absorption, colored reflection, and fluorescence, to identify organic substances.

Later in life Stokes was universally honored with degrees and medals. He was knighted in 1889 and became Master of Pembroke College in 1902. After the 1850s, Stokes became involved in administrative matters, and his scientific productivity decreased. Some things never change. Professor Stokes died February 1, 1903.

1.3.2. Emission Spectra Are Typically Independent of the Excitation Wavelength

Another general property of fluorescence is that the same fluorescence emission spectrum is generally observed irrespective of the excitation wavelength. This is known as Kasha's rule,[10] although Vavilov reported in 1926 that quantum yields were generally independent of excitation wavelength.[5] Upon excitation into higher electronic and vibrational levels, the excess energy is quickly dissipated, leaving the fluorophore in the lowest vibrational level of S_1. This relaxation occurs in about 10^{-12} s, and is presumably a result of a strong overlap among numerous states of nearly equal energy. Because of this rapid relaxation, emission spectra are usually independent of the excitation wavelength. Exceptions exist, such as fluorophores that exist in two ionization states, each of which displays distinct absorption and emission spectra. Also, some molecules are known to emit from the S_2 level, but such emission is rare and generally not observed in biological molecules.

It is interesting to ask why perylene follows the mirror-image rule, but quinine emission lacks the two peaks seen in its excitation spectrum at 315 and 340 nm (Figure 1.3). In the case of quinine, the shorter wavelength absorption peak is due to excitation to the second excited state (S_2), which relaxes rapidly to S_1. Emission occurs predominantly from the lowest singlet state (S_1), so emission from S_2 is not observed. The emission spectrum of quinine is the mirror image of the $S_0 \rightarrow S_1$ absorption of quinine, not of its total absorption spectrum. This is true for most fluorophores: the emission is the mirror image of $S_0 \rightarrow S_1$ absorption, not of the total absorption spectrum.

The generally symmetric nature of these spectra is a result of the same transitions being involved in both absorption and emission, and the similar vibrational energy levels of S_0 and S_1. In most fluorophores these energy levels are not significantly altered by the different electronic distributions of S_0 and S_1. Suppose the absorption spectrum of a fluorophore shows distinct peaks due to the vibrational energy levels. Such peaks are seen for anthracene in Figure 1.8. These peaks are due to transitions from the lowest vibrational level of the S_0 state to higher vibrational levels of the S_1 state. Upon return to the S_0 state the fluorophore can return to any of the ground state vibrational levels. These vibrational energy levels have similar spacing to those in the S_1 state. The emission spectrum shows the same vibrational energy spacing as the absorption spectrum. According to the Franck-Condon principle, all electronic transitions are vertical, that is, they occur without change in the position of the nuclei. As a result, if a particular transition probability (Franck-Condon factor) between the 0th and 1st vibrational levels is largest in absorption, the reciprocal transition is also most probable in emission (Figure 1.8).

Figure 1.7. Sir George Gabriel Stokes, 1819–1903, Lucasian Professor at Cambridge. Reproduced courtesy of the Library and Information Centre, Royal Society of Chemistry.

A rigorous test of the mirror-image rule requires that the absorption and emission spectra be presented in appropriate units.[12] The closest symmetry should exist between the modified spectra $\varepsilon(\bar{\nu})/\bar{\nu}$ and $F(\bar{\nu})/\bar{\nu}^3$, where $\varepsilon(\bar{\nu})$ is the extinction coefficient at wavenumber ($\bar{\nu}$), and $F(\bar{\nu})$ is the relative photon flux over a wavenumber increment $\Delta\bar{\nu}$. Agreement between these spectra is generally found for polynuclear aromatic hydrocarbons.

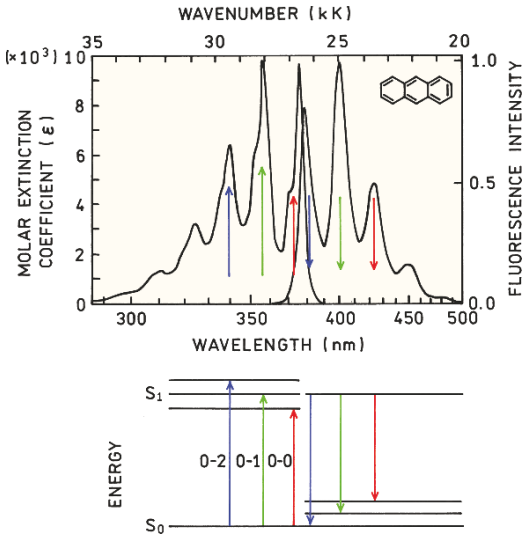

Figure 1.8. Mirror-image rule and Franck-Condon factors. The absorption and emission spectra are for anthracene. The numbers 0, 1, and 2 refer to vibrational energy levels. From [11].

1.3.3. Exceptions to the Mirror-Image Rule

Although often true, many exceptions to the mirror-image rule occur. This is illustrated for the pH-sensitive fluorophore 1-hydroxypyrene-3,6,8-trisulfonate (HPTS) in Figure 1.9. At low pH the hydroxyl group is protonated. The absorption spectrum at low pH shows vibrational structure typical of an aromatic hydrocarbon. The emission spectrum shows a large Stokes shift and none of the vibrational structure seen in the absorption spectrum. The difference between the absorption emission spectra is due to ionization of the hydroxyl group. The dissociation constant (pK_a) of the hydroxyl group decreases in the excited state, and this group becomes ionized. The emission occurs from a different molecular species, and this ionized species displays a broad spectrum. This form of HPTS with an ionized hydroxyl group can be formed at pH 13. The emission spectrum is a mirror image of the absorption of the high pH form of HPTS.

Changes in pK_a in the excited state also occur for biochemical fluorophores. For example, phenol and tyrosine each show two emissions, the long-wavelength emission being favored by a high concentration of proton acceptors. The pK_a of the phenolic hydroxyl group decreases from 11 in the ground state to 4 in the excited state. Following excitation, the phenolic proton is lost to proton acceptors in the solution. Depending upon the concentration of these acceptors, either the phenol or the phenolate emission may dominate the emission spectrum.

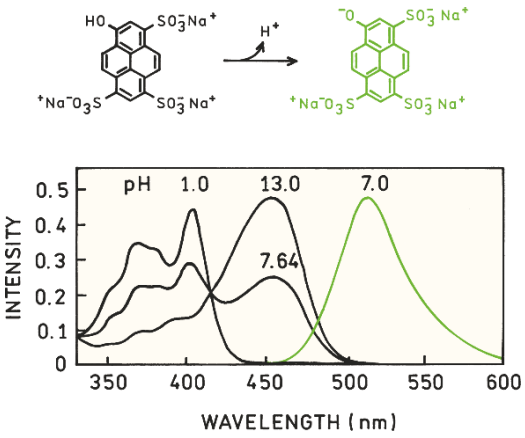

Figure 1.9. Absorption (pH 1, 7.64, and 13) and emission spectra (pH 7) of 1-hydroxypyrene-3,6,8-trisulfonate in water. From [11].

Excited-state reactions other than proton dissociation can also result in deviations from the mirror symmetry rule. One example is shown in Figure 1.10, which shows the emission spectrum of anthracene in the presence of diethylaniline.[13] The structured emission at shorter wavelengths is a mirror image of the absorption spectrum of anthracene. The unstructured emission at longer wavelengths is due to formation of a charge-transfer complex between the excited state of anthracene and diethylaniline. The unstructured emission is from this complex. Many polynuclear aromatic hydrocarbons, such as pyrene and perylene, also form charge-transfer complexes with amines. These excited-state complexes are referred to as exciplexes.

Some fluorophores can also form complexes with themselves. The best known example is pyrene. At low concentrations pyrene displays a highly structured emission (Figure 1.11). At higher concentrations the previously invisible UV emission of pyrene becomes visible at 470 nm. This long-wavelength emission is due to excimer formation. The term "excimer" is an abbreviation for an excited-state dimer.

1.4. FLUORESCENCE LIFETIMES AND QUANTUM YIELDS

The fluorescence lifetime and quantum yield are perhaps the most important characteristics of a fluorophore. Quantum yield is the number of emitted photons relative to the number of absorbed photons. Substances with the largest quantum yields, approaching unity, such as rhodamines, display the brightest emissions. The lifetime is also important, as it determines the time available for the fluorophore

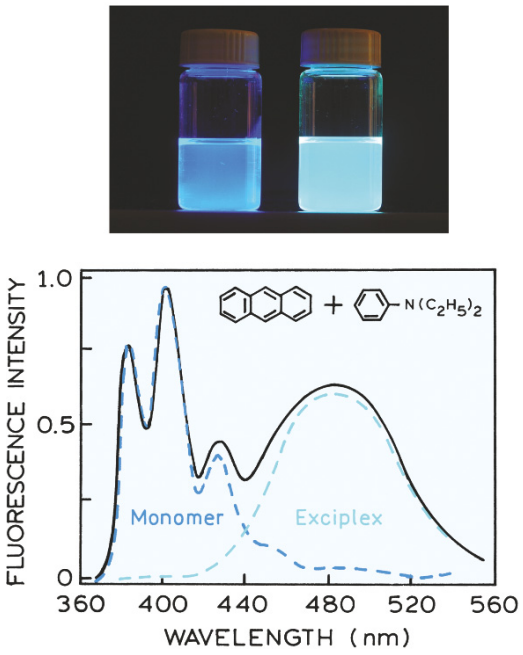

Figure 1.10. Emission spectrum of anthracene in toluene containing 0.2 M diethylaniline. The dashed lines show the emission spectra of anthracene or its exciplex with diethylaniline. Figure revised from [13], photo from [14].

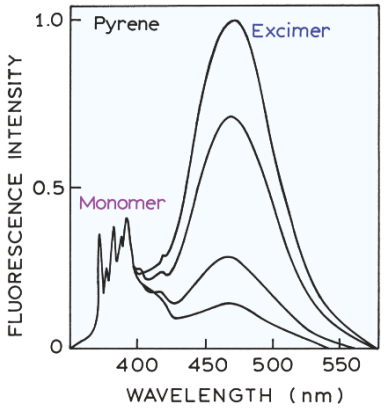

Figure 1.11. Emission spectra of pyrene and its excimer. The relative intensity of the excimer peak (470 nm) decreases as the total concentration of pyrene is decreased from 6 x 10^{-3} M (top) to 0.9 x 10^{-4} M (bottom). Reproduced with permission from John Wiley and Sons Inc. From [12].

to interact with or diffuse in its environment, and hence the information available from its emission.

The meanings of quantum yield and lifetime are best represented by a simplified Jablonski diagram (Figure 1.12). In this diagram we do not explicitly illustrate the individual relaxation processes leading to the relaxed S_1 state. Instead, we focus on those processes responsible for return to the ground state. In particular, we are interested in the emissive rate of the fluorophore (Γ) and its rate of non-radiative decay to S_0 (k_{nr}).

The fluorescence quantum yield is the ratio of the number of photons emitted to the number absorbed. The rate constants Γ and k_{nr} both depopulate the excited state. The fraction of fluorophores that decay through emission, and hence the quantum yield, is given by

$$Q = \frac{\Gamma}{\Gamma + k_{nr}} \tag{1.1}$$

The quantum yield can be close to unity if the radiationless decay rate is much smaller than the rate of radiative decay, that is $k_{nr} < \Gamma$. We note that the energy yield of fluorescence is always less than unity because of Stokes losses. For convenience we have grouped all possible non-radiative decay processes with the single rate constant k_{nr}.

The lifetime of the excited state is defined by the average time the molecule spends in the excited state prior to return to the ground state. Generally, fluorescence lifetimes are near 10 ns. For the fluorophore illustrated in Figure 1.12 the lifetime is

$$\tau = \frac{1}{\Gamma + k_{nr}} \tag{1.2}$$

Fluorescence emission is a random process, and few molecules emit their photons at precisely $t = \tau$. The lifetime is an average value of the time spent in the excited state. For a single exponential decay (eq. 1.13) 63% of the molecules have decayed prior to $t = \tau$ and 37% decay at $t > \tau$.

An example of two similar molecules with different lifetimes and quantum yields is shown in Figure 1.13. The differences in lifetime and quantum yield for eosin and erythrosin B are due to differences in non-radiative decay rates. Eosin and erythrosin B have essentially the same extinction coefficient and the same radiative decay rate (see eq. 1.4). Heavy atoms such as iodine typically result in shorter lifetimes and lower quantum yields.

The lifetime of the fluorophore in the absence of non-radiative processes is called the intrinsic or natural lifetime, and is given by

$$\tau_n = \frac{1}{\Gamma} \tag{1.3}$$

In principle, the natural lifetime τ_n can be calculated from the absorption spectra, extinction coefficient, and

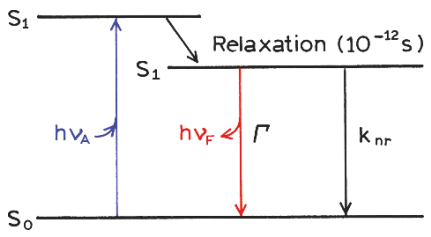

Figure 1.12. A simplified Jablonski diagram to illustrate the meaning of quantum yields and lifetimes.

emission spectra of the fluorophore. The radiative decay rate Γ can be calculated using[15,16]

$$\Gamma \simeq 2.88 \times 10^9 n^2 \frac{\int F(\bar{\nu})\, d\bar{\nu}}{\int F(\bar{\nu})\, d\bar{\nu}/\bar{\nu}^3} \int \frac{\varepsilon(\bar{\nu})}{\bar{\nu}}\, d\bar{\nu}$$

$$= 2.88 \times 10^9 n^2 \langle \bar{\nu}^{-3} \rangle^{-1} \int \frac{\varepsilon(\bar{\nu})\, d\bar{\nu}}{\bar{\nu}} \qquad (1.4)$$

where $F(\bar{\nu})$ is the emission spectrum plotted on the wavenumber (cm^{-1}) scale, $\varepsilon(\bar{\nu})$ is the absorption spectrum, and n is the refractive index of the medium. The integrals are calculated over the $S_0 \leftrightarrow S_1$ absorption and emission spectra. In many cases this expression works rather well, particularly for solutions of polynuclear aromatic hydrocarbons. For instance, the calculated value[15] of Γ for perylene is 1.8 x 10^8 s^{-1}, which yields a natural lifetime of 5.5 ns. This value is close to that observed for perylene, which displays a quantum yield near unity. However, there are numerous reasons why eq. 1.4 can fail. This expression assumes no interaction with the solvent, does not consider changes in the refractive index (n) between the absorption and emission wavelength, and assumes no change in excited-state geometry. A more complete form of eq. 1.4 (not shown) includes a factor $G = g_l/g_u$ on the right-hand side, where g_l and g_u are the degeneracies of the lower and upper states, respectively. For fluorescence transitions $G = 1$, for phosphorescence transitions $G = 1/3$.

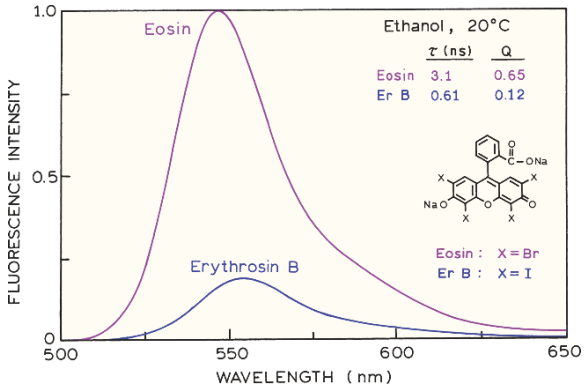

Figure 1.13. Emission spectra of eosin and erythrosin B (ErB).

The natural lifetime can be calculated from the measured lifetime (τ) and quantum yield

$$\tau_n = \tau / Q \qquad (1.5)$$

which can be derived from eqs. 1.2 and 1.3. Many biochemical fluorophores do not behave as predictably as unsubstituted aromatic compounds. Hence, there is often poor agreement between the value of τ_n calculated from eq. 1.5 and that calculated from its absorption and emission spectra (eq. 1.4). These discrepancies occur for a variety of unknown and known reasons, such as a fraction of the fluorophores located next to quenching groups, which sometimes occurs for tryptophan residues in proteins.

The quantum yield and lifetime can be modified by factors that affect either of the rate constants (Γ or k_{nr}). For example, a molecule may be nonfluorescent as a result of a large rate of internal conversion or a slow rate of emission. Scintillators are generally chosen for their high quantum yields. These high yields are a result of large Γ values. Hence, the lifetimes are generally short: near 1 ns. The fluorescence emission of aromatic substances containing $-NO_2$ groups are generally weak, primarily as a result of large k_{nr} values. The quantum yields of phosphorescence are extremely small in fluid solutions at room temperature. The triplet-to-singlet transition is forbidden by symmetry, and the rates of spontaneous emission are about 10^3 s^{-1} or smaller. Since k_{nr} values are near 10^9 s^{-1}, the quantum yields of phosphorescence are small at room temperature. From eq. 1.1 one can predict phosphorescence quantum yields of 10^{-6}.

Comparison of the natural lifetime, measured lifetime, and quantum yield can be informative. For example, in the case of the widely used membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH) the measured lifetime near 10 ns is much longer than that calculated from eq. 1.4, which is near 1.5 ns.[17] In this case the calculation based on the absorption spectrum of DPH is incorrect because the absorption transition is to a state of different electronic symmetry than the emissive state. Such quantum-mechanical effects are rarely seen in more complex fluorophores with heterocyclic atoms.

1.4.1. Fluorescence Quenching

The intensity of fluorescence can be decreased by a wide variety of processes. Such decreases in intensity are called quenching. Quenching can occur by different mechanisms. Collisional quenching occurs when the excited-state fluorophore is deactivated upon contact with some other molecule in solution, which is called the quencher. Collisional quenching is illustrated on the modified Jablonski diagram in Figure 1.14. In this case the fluorophore is returned to the ground state during a diffusive encounter with the quencher. The molecules are not chemically altered in the process. For collisional quenching the decrease in intensity is described by the well-known Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + K[Q] = 1 + k_q\tau_0[Q] \qquad (1.6)$$

In this expression K is the Stern-Volmer quenching constant, k_q is the bimolecular quenching constant, τ_0 is the unquenched lifetime, and $[Q]$ is the quencher concentration. The Stern-Volmer quenching constant K indicates the sensitivity of the fluorophore to a quencher. A fluorophore buried in a macromolecule is usually inaccessible to water-soluble quenchers, so that the value of K is low. Larger values of K are found if the fluorophore is free in solution or on the surface of a biomolecule.

A wide variety of molecules can act as collisional quenchers. Examples include oxygen, halogens, amines, and electron-deficient molecules like acrylamide. The mechanism of quenching varies with the fluorophore–quencher pair. For instance, quenching of indole by acrylamide is probably due to electron transfer from indole to acrylamide, which does not occur in the ground state. Quenching by halogen and heavy atoms occurs due to spin–orbit coupling and intersystem crossing to the triplet state (Figure 1.5).

Aside from collisional quenching, fluorescence quenching can occur by a variety of other processes. Fluorophores can form nonfluorescent complexes with quenchers. This process is referred to as static quenching since it occurs in the ground state and does not rely on diffusion or molecular collisions. Quenching can also occur by a variety of trivial, i.e., non-molecular mechanisms, such as attenuation of the incident light by the fluorophore itself or other absorbing species.

1.4.2. Timescale of Molecular Processes in Solution

The phenomenon of quenching provides a valuable context for understanding the role of the excited-state lifetime in allowing fluorescence measurements to detect dynamic processes in solution or in macromolecules. The basic idea is that absorption is an instantaneous event. According to the Franck-Condon principle, absorption occurs so fast that there is no time for molecular motion during the absorption process. Absorption occurs in the time it takes a photon to travel the length of a photon: in less than 10^{-15} s. As a result, absorption spectroscopy can only yield information on the average ground state of the molecules that absorb light. Only solvent molecules that are immediately adjacent to the absorbing species will affect its absorption spectrum. Absorption spectra are not sensitive to molecular dynamics and can only provide information on the average solvent shell adjacent to the chromophore.

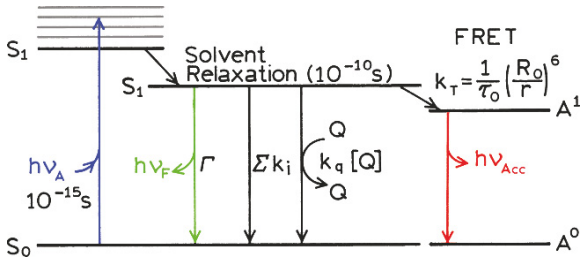

Figure 1.14. Jablonski diagram with collisional quenching and fluorescence resonance energy transfer (FRET). The term Σk_i is used to represent non-radiative paths to the ground state aside from quenching and FRET.

In contrast to absorption, emission occurs over a longer period of time. The length of time fluorescent molecules remain in the excited state provides an opportunity for interactions with other molecules in solution. Collisional quenching of fluorescence by molecular oxygen is an excellent example of the expansion of time and distance provided by the fluorescence lifetime. If a fluorophore in the excited state collides with an oxygen molecule, then the fluorophore returns to the ground state without emission of a photon. The diffusion coefficient (D) of oxygen in water at 25°C is 2.5 x 10^{-5} cm²/s. Suppose a fluorophore has a lifetime of 10 ns. Although 10 ns may appear to be a brief time span, it is in fact quite long relative to the motions of small molecules in fluid solution. The average distance $(\Delta x^2)^{1/2}$ an oxygen molecule can diffuse in 10^{-8} s or 10 ns is given by the Einstein equation:

$$\Delta x^2 = 2D\tau \qquad (1.7)$$

The distance is about 70 Å, which is comparable to the thickness of a biological membrane or the diameter of a protein. Some fluorophores have lifetimes as long as 400