Photosynth Res (2009) 101:181–194
DOI 10.1007/s11120-009-9457-8

REVIEW

Fluorescence lifetimes: fundamentals and interpretations

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Received: 21 January 2009 / Accepted: 8 June 2009 / Published online: 1 July 2009

Abstract Fluorescence measurements have been an established mainstay of photosynthesis experiments for many decades. Because in the photosynthesis literature the basics of excited states and their fates are not usually described, we have presented here an easily understandable text for biology students in the style of a chapter in a text book. In this review we give an educational overview of fundamental physical principles of fluorescence, with emphasis on the temporal response of emission. Escape from the excited state of a molecule is a dynamic event, and the fluorescence emission is in direct kinetic competition with several other pathways of de-excitation. It is essentially through a kinetic competition between all the pathways of de-excitation that we gain information about the fluorescent sample on the molecular scale. A simple probability allegory is presented that illustrates the basic ideas that are important for understanding and interpreting most fluorescence experiments. We also briefly point out challenges that confront the experimenter when interpreting time-resolved fluorescence responses.

Keywords Fluorescence lifetime · Quantum yield · Radiative transition · Non-radiative transitions · Perrin–Jablonski diagram · Excited state · Pathways of de-excitation · Lifetime measurements · Exponential decay · Fundamental fluorescence response

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Abbreviations

FRET	Förster resonance energy transfer
ROS	Reactive oxidation species
S_0	Ground electronic singlet state
S_1	First electronic excited singlet state

Introduction

Fluorescence is a key experimental technique for studying photosynthesis. Fluorescence per se does not play any role in the mechanism of photosynthesis and does not participate in the steps of photosynthesis; however, it is not surprising that light emission from plants has always played a central role in photosynthesis studies. The vital initial step of photosynthesis is the efficient capture of photons via chlorophyll and carotenoids—the usual molecular absorbers for photosynthetic systems. It is common that good absorbers also have a reasonable probability of emitting photons (undergoing fluorescence), and chlorophyll is an efficient fluorophore. To be utilized for photosynthesis, the energy must be transferred rapidly to a *reaction center* (RC) of the photosynthetic system. The energy of the absorbed photons migrates by non-radiative energy transfer to the RC where the excitation energy is converted into a charge separation at the reaction center. This initial charge separated state is accordingly the free energy driver of a series of reactions that synthesize the fundamental chemical components, which form the basis of all life on earth. There is a dynamic competition between the useful migration of the initial energy absorbed from the light by

the chromophores to the reaction center and wasteful energy loss through fluorescence and non-radiative deactivation processes. Thus, the fluorescence lifetimes and the quantum yield of the fluorescence emission of the excited molecules are sensitively influenced by the efficiency and rate of photosynthesis processes, and by the rates of subsequent chemical kinetic steps involved in electron transfer in the RC and other processes competitive with the transfer of energy from the excited fluorophore. Fluorescence is a convenient tool for monitoring many chemical and physical processes involved in photosynthesis because fluorescence can be measured in real time and is relatively easy to measure, and because the probability for an initially excited molecule to emit a fluorescence photon depends on its molecular environment.

The caveat to the expediency for studying photosynthesis by fluorescence measurements is that photosynthetic systems are composed of large, complex, multi-functional, multi-component, compartmentalized, dynamic and highly organized supra-molecular units. Therefore, it is a challenge to devise experimental protocols for fluorescence studies, especially when studying intact photosynthetic systems. Impressive progress has been made studying photosynthesis with fluorescence (Govindjee et al. 1986; Papageorgiou and Govindjee 2005). We refer the reader to these comprehensive treatises and to other literature referenced therein and in the last paragraph of this educational review for expositions of specific fluorescence investigations of photosynthesis, and for descriptions of instrumentation.

This review is a short overview of the fundamental principles of fluorescence and fluorescence lifetimes. The objective is to assist those not so familiar with fluorescence to understand most experiments and discussions of fluorescence lifetimes found in the literature. We will not discuss instrumentation or details of measurements; and we will not present details of analysis methods. We focus on the fundamental physical aspects of fluorescence, concentrating on the dynamics of the fluorescence response (the fluorescence decay). Fluorescence is a widespread technique in all areas of scientific investigations, and it has been a cornerstone of research in many biology fields of research. Measuring dynamic responses of fluorescence signals (related to the fluorescence lifetimes) has provided tremendous insight into the mechanisms and structures of photosynthetic systems.

Units

Because many authors use different units, we present some numerical values of some important parameters (wavelengths and energies) and indicate the conversion between the energy units. Optical spectroscopy involves electromagnetic radiation ($\lambda\nu = c$) throughout the broad range from gamma rays to long radio waves (see Table 1). In this review we only deal with energies from the ultraviolet (UV) to the visible far red; we do not discuss molecular rotations and only consider vibrations in so far as the vibrations lead to broadening of spectra, and enter into the probability of a transition from one state to another (vibrational overlap). The energy of a quantum of light with frequency ν is $E = h\nu = hc/\lambda$ ergs, where Planck's constant is $h = 6.024 \times 10^{-27}$ erg s, and the speed of light is $c = 3 \times 10^{10}$ cm s^{-1}. The frequency ν is in s^{-1}. $\omega = 2\pi\nu$ is called the radial frequency, also in s^{-1}. λ is the wavelength of the light and is in cm. Avogadro's number (the number of molecules in a mole) is $N_A = 6.023 \times 10^{23}$. The definition of an *einstein* is a mole of photons of a certain frequency. This is used in photochemistry because in a photochemical reaction the absorbance of one *einstein* (small "e") can cause the reaction of one mole of absorbing reactant. This is called Einstein's law of photochemical equivalency. Often the unit of frequency of electromagnetic radiation is given as a *wavenumber*, which is the number of waves per unit length (per cm); this is $\nu/c\,(\mathrm{s}^{-1})(\mathrm{cm/s})^{-1} = \lambda^{-1}\,(\mathrm{cm}^{-1}) \equiv \bar{\nu}\,(\mathrm{cm}^{-1})$, where of

Table 1 Approximate sizes of quanta

Radiation	λ (cm) (typical values)	Wavenumber (μm^{-1})	Size of quantum (electron volts)	Size of einstein (kilogram calories)	Absorption or emission of radiation involves
Gamma rays	10^{-10}	10^{6}	1.2×10^{6}	2.9×10^{7}	Nuclear reactions
X-rays	10^{-8}	10^{4}	1.2×10^{4}	2.9×10^{5}	Transitions of inner atomic electrons
Ultraviolet	10^{-5}	10^{1}	1.2×10^{1}	2.9×10^{2}	Transitions of outer atomic electrons
	4×10^{-5}	2.5	3.1	7.1×10^{1}	
Visible	8×10^{-5}	1.25	1.6	3.6×10^{1}	
Infrared	10^{-3}	10^{-1}	1.2×10^{-1}	2.9	Molecular vibrations
Far infrared	10^{-2}	10^{-2}	1.2×10^{-2}	2.9×10^{-1}	Molecular rotations
Radar	10^{1}	10^{-5}	1.2×10^{-5}	2.9×10^{-4}	Oscillations of mobile or free electrons
Long radio waves	10^{5}	10^{-9}	1.2×10^{-9}	2.9×10^{-8}	

course in order to use $\bar{\nu} = \lambda^{-1}$ we have to give the wavelength in cm (cm = 10^7nm).

The following list gives the equivalent values of wavelength, wave number, frequency and quantum energy in various units for a chosen wavelength (1 micrometer) of near infra-red light:

Wavelength λ	10^{-4} cm
	1.000 micrometer (μm)
	1000 nanometer (nm)
Wave number $\bar{\nu}$	10^{-4} cm^{-1}
	1.000 μm^{-1}
Frequency ν	2.998×10^{14} s^{-1}
Energy of one einstein	28.57 kcal mole^{-1}
Energy of one quantum	1.986×10^{-12} erg
	1.240 electron volt
1 einstein s^{-1} =	$1.196\times10^{8}/\lambda$ (λ in nm).

Organization of the paper

Although the fundamental concepts and physical descriptions of optical spectroscopy (especially the dynamic aspects) are firmly based on quantum mechanics, much of the necessary background for understanding and interpreting experiments can be understood from simple principles. This review is organized as follows. (1) We first introduce the formal characteristics of the fluorescence response in order to define the terminology and basic physical principles. (2) Then we present a simple allegorical model (considering the rates of bees escaping from a box) that illustrates further the statistical nature of the fluorescence dynamics; an understanding of this statistical character of the fluorescence response (escape from the excited state through several pathways) will allow the reader to understand the basis of almost all fluorescence experiments. (3) We then conclude with a short discussion of the difficulties and methods of analysis when interpreting multi-component fluorescence decays, which are common when investigating complex biological samples, such as intact photosynthesis systems.

A molecule's sojourn from initial excitation to de-excitation

The following discussion applies mainly to the spectroscopy of multi-atom organic molecules. There are many accessible texts for general theoretical details (Atkins and Friedman 1997; Becker 1969; Birks 1970, 1975; Lakowicz 1999; Stepanov and Gribkovskii 1968; Valeur 2002), as well as reviews targeted for the spectroscopy of photosynthetic systems (Clayton 1965; Rabinowitch and Govindjee 1969).

Electronic and vibrational energy levels, transition dipoles and the vibrational overlap integral

The energy levels of the ground (unexcited) and excited states in a molecule, or atom, can best be depicted as a *Perrin–Jablonski diagram* (Lakowicz 1999; Valeur 2002), Fig. 1. At room temperature, molecules are in the lowest (ground) electronic singlet state (S_0) and usually in the lowest vibrational level of this electronic level. Upon absorption of a photon, the molecules are excited from the S_0 state in $<10^{-15}$ s (femtoseconds) (Fig. 2). This excitation event raises the molecule to many different upper vibrational levels of the first electronic excited singlet state (S_1), while obeying the conservation of energy (the energy of the absorbed photon must exactly equal the energy difference between the energy levels). The actual initially excited vibrational level of the excited state depends on the energy distribution of the excitation light, and the spatial overlap of the nuclei in the ground and excited vibrational states (*vibrational overlap*) (shown later in Fig. 4). The molecules subsequently rapidly lose vibrational/rotational energy through collisions with the environment and internal vibrations (this is a vibrational relaxation, also termed *internal conversion*). This happens in 10^{-12} s (a picosecond) (Fig. 3). If the molecule is initially excited to the higher S_2 electronic singlet state, it will always (excluding a few exceptions) undergo rapid internal conversion from the vibrational levels of the S_2 state to the (overlapping) higher vibrational states of the S_1 state in about 10^{-12} s or less, which then passes in also about 10^{-12} s to the lower vibrational state of S_1 (Fig. 3). From the lowest vibrational level of the S_1 state, the molecule will then exit the excited state (return to the ground state) through one of the many pathways that we will discuss below. If the molecule emits a photon, the molecule will usually drop from the lower vibrational levels of the S_1 state to one of many different higher vibrational levels of the S_0 state, resulting in a broad fluorescence spectrum (Fig. 1). Again this requires the conservation of energy; that is, the energy of the emitted photon must equal the change in the energy levels. Sometimes vibrational levels of the states are evident as peaks in the spectrum; however, the transitions are usually smeared out into rather broad spectra through collisional interactions with the environment. Most spectra are relatively broad, except at very low temperatures.

We can learn a great deal about the environment of a fluorophore by just recording the emission spectrum. The electromagnetic radiation can only exchange as a quantum corresponding to energy differences between the energy levels of the participating energy states. The energy of the molecules can in general be divided up into electronic, E_e, vibrational, E_v, and rotational, E_r, energy (this separation is known as the *Born approximation*). The change in the

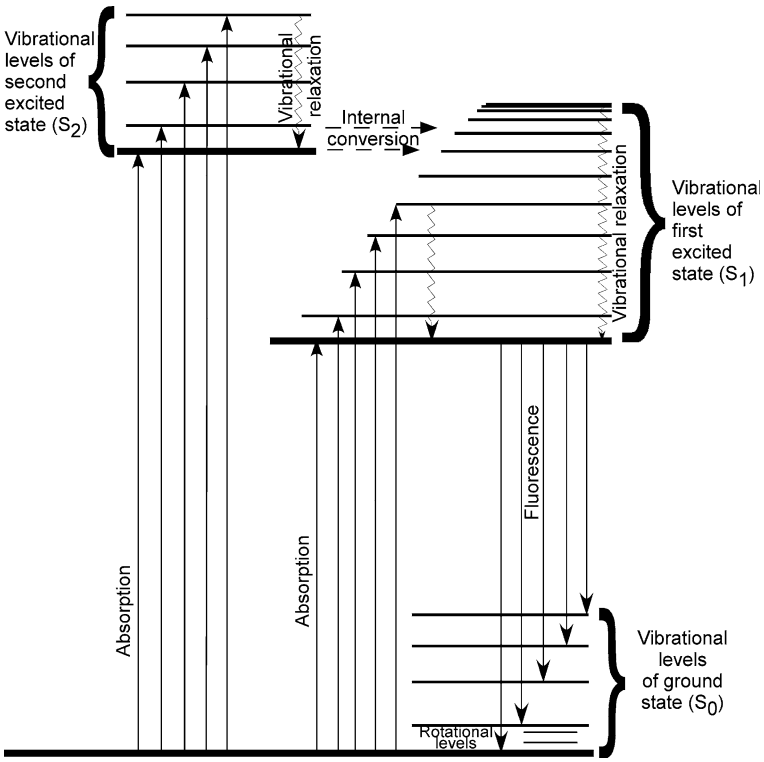

Fig. 1 Perrin–Jablonski diagram showing energy levels and transitions involved in absorption and fluorescence; non-radiative transitions between levels are not shown in this abbreviated diagram

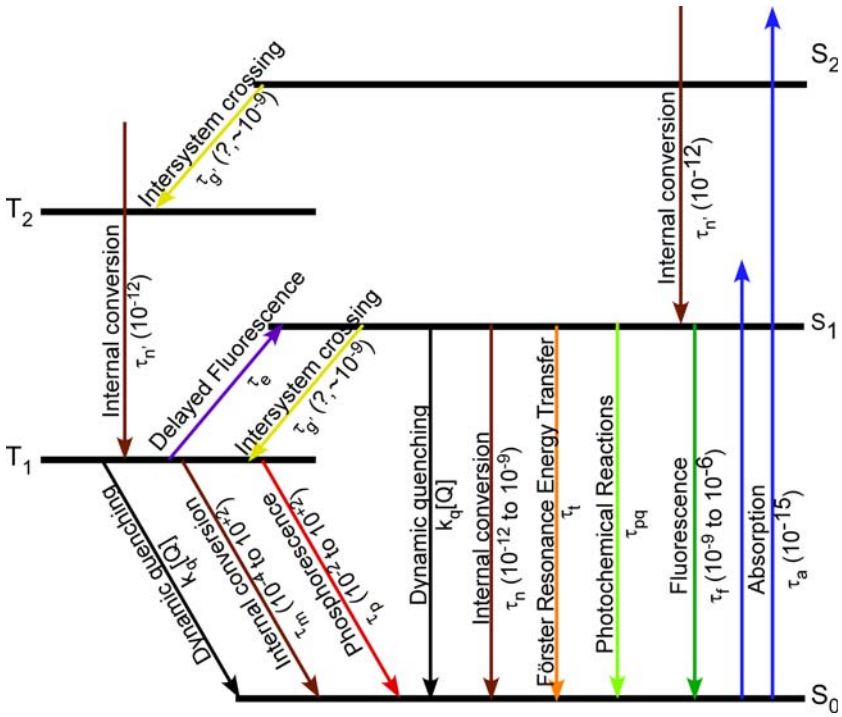

Fig. 2 Typical values of transition times in seconds; the time for intersystem crossing is shown with a "?" because this time is variable and depends on the possibility of an interaction with the spin–orbit interaction with the electrons of a nearby heavy atom

energy of the molecule during a spectroscopic transition (absorption or emission of a quantum of light) must exactly match the energy lost from the electromagnetic field; that is, photon energy $= h\nu = \Delta E_e + \Delta E_v + \Delta E_r$, where the subscripts e, v, and r refer to electronic, vibrational and rotational energy levels. Immediately upon absorbing a quantum of energy from the light field, the configurations of the nuclei in the S_1 state are initially in the same spatial configuration of the S_0 state, because the initial S_0 to S_1 electronic transition involves only a redistribution of the

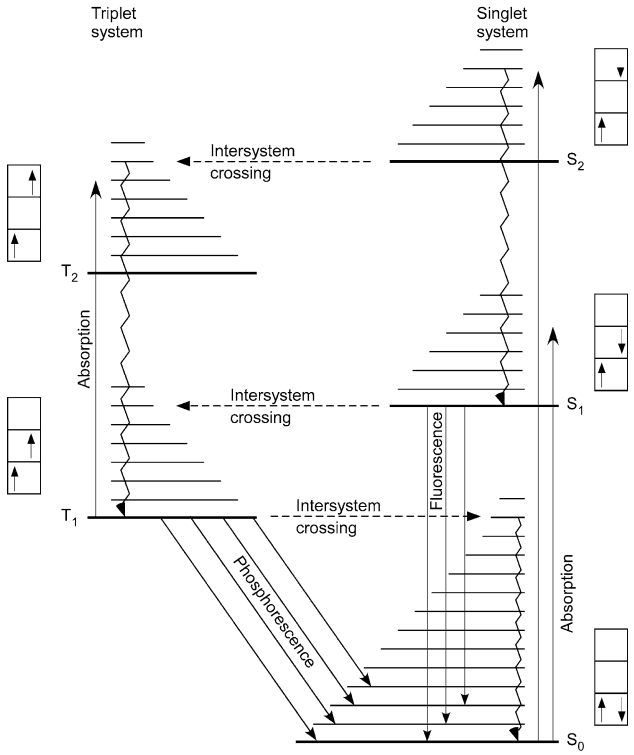

Fig. 3 Energy levels of singlet and triplet states, showing the paired (or opposite) electron spins in singlet states, and unpaired (or parallel) electron spins in the triplet states

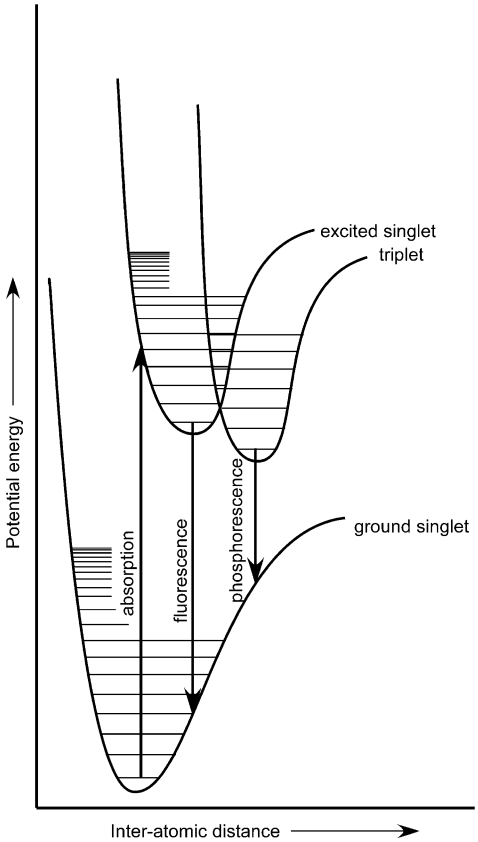

Fig. 4 Potential energy diagram showing the transitions involved in absorption, fluorescence and phosphorescence, and intersystem crossing between excited singlet state (S_1, see Figs. 2 and 3) and the excited triplet state (T_1, see Figs. 2 and 3)

electrons, and the electrons move much faster than the nuclei (see Fig. 4). This initial electronically excited state with the configuration of the nuclei of the S_0 state is called a *Franck–Condon (FC) State* (Lakowicz 1999; Valeur 2002). The Franck–Condon principle is named after James Frank (of Germany/USA) and Edward Uhler Condon (USA).

Apart from exceptional circumstances, the molecule does not emit a photon from the FC-state, but from the lowest S_1 vibrational level *after* the nuclei have relaxed to their equilibrium configurations corresponding to the electron distributions in the excited state (Figs. 3 and 4). The energy level of the relaxed S_1 state depends on molecular interactions with the immediate environment (e.g. as dipole relaxation in a solvent of high polarity, such as water); in other words, the spectrum of the fluorescence emission will depend on the molecular environment of the fluorophore. Thus the fluorescence spectrum is a powerful reporter of the physical molecular environment of the fluorophore as we discuss in the next paragraph.

The intensities of the transitions between the S_0 and S_1 states (both in absorption and emission) depend on the electronic *transition dipoles* and the *vibrational overlap integral*. The electronic *transition dipole* controls the strength of the dipolar coupling of the initial and final electronic states. In absorption, the electric field of the light interacts with the electrons, inducing a transition from S_0 to S_1 if the *change* of the electron configurations of the two states (S_0 and the Franck–Condon S_1 state) can be represented by a dipole. A similar transition dipole between the initial excited state and the ground state is required for emission. The vibrational overlap integral means that the nuclei configurations of the two states must be similar; that is, the vibrational wave functions of the initial and final states must globally overlap when integrated over space. As the initial excited state relaxes to the lowest vibrational level of the S_1 state (prior to the S_1 to S_0 transition), the configuration of the nuclei adjust to be consistent with the lowest energy of the excited electronic state, and this lowers the energy of the S_1 state from the initial Franck–Condon state. Usually, if the chromophore is in a polar environment, and if the excited state has a greater dipole strength than the ground state, the eventual energy of the S_1 state is lowered by solvent relaxation of the polar molecules surrounding the chromophore (the dipoles of the surrounding solvent—often water, which has a large dipole

moment—become oriented around the excited molecule, lowering the energy). The electron distribution within the molecule is often very different in the excited state from that in the ground state; thus the orientations of polar solvent molecules surrounding the molecule are usually quite different in the ground and excited states. The permanent dipole moment or the polarizability of the excited molecule is often more pronounced than that of the ground state (the electrons in the excited state are more spatially extended). Before the excited state emits a photon, the "polar" solvent may have time to reorient, and the solvent molecules will orient such that the energy of the excited molecule is lowered. This is known as *solvent relaxation*. At low temperatures, or high viscosity (high rigidity of the solvent molecules) the solvent molecules do not have time to reorient before emission of a photon. So there is not so much 0–0 splitting in these conditions (the *0–0 splitting* is the frequency difference between the lowest absorption frequency and the highest fluorescence frequency; this is related to the *Stokes shift*, see the next paragraph). Also, if the molecule is in a non-polar environment, there is no solvent dipolar relaxation, and the energy of the excited state is not lowered. If the temperature is higher or the viscosity lower, then the surrounding solvent molecules become rapidly reoriented. There is a maximum 0–0 splitting at some intermediate temperature. There is a useful relationship between the 0–0 band shift, the polarity of the solvent and the dipole moment of the solute upon excitation—this is the *Lippert equation* (Basu 1964; Lippert and Macomber 1995; Seliskar and Brand 1971). These spectral shifts, and the dynamics of the shifts, carry valuable information of the molecular surroundings of the fluorophore.

When the molecule finally arrives at the lowest vibrational level of the S_1 electronic state (after only 10^{-12} s) it is metastable, and usually remains there for at most 10^{-8} s (sometimes more, depending on the molecular species) or less (depending on the rate constants of de-excitation through all the other available pathways for de-excitation). It is from this state that fluorescence is emitted. Fluorescence usually takes place in 10^{-10} to 10^{-8} s, depending on e.g. the environment of the chromophore, the presence of dynamic quenching, or energy transfer. If the molecule fluoresces, it returns to one of many different higher vibrational levels of the S_0 state (Figs. 1, 3 and 4). Therefore, an emitted photon has a lower energy than the excitation photons; this is called the Stokes shift (see Figs. 1 and 4). The Stokes shift is named after Sir George Gabriel Stokes. This is a major reason why the fluorescence spectrum is "*red-shifted*" relative to the absorption spectrum, even though the transitions are both between the S_0 and S_1 states. The measured Stokes shift is even greater in a polar environment because of solvent relaxation around the S_1 state, which lowers the energy level of the S_1 state, as discussed in the previous paragraph.

Fluorescence lifetime experiments exploit the interactions of the fluorophore's S_1 excited state with its molecular environment

There are a number of pathways by which the molecules can leave the excited S_1 state. Some of these pathways are depicted in Fig. 2, along with the fate of molecules that have transferred from the excited singlet state to the excited "triplet" state (Figs. 3 and 4). The passage from the singlet to the triplet state (or back) is termed "*intersystem crossing*".

It might be helpful for some to skim first the sections below of the "bees in a box" allegory before proceeding to the following description of the dynamics of the fluorescence response.

Rate constants of transitions, spins, and singlet and triplet states

The approximate rate constants of conversion from every state to another state are shown in Fig. 2. There is a "rate constant" for each separate pathway for leaving the excited states—e.g. from S_1 to S_0 or from T_1 to S_0. The different pathways of de-excitation compete dynamically with each other in a parallel first order kinetic fashion. The rate constants of the different pathways of de-excitation and the way these pathways are affected by the environment and neighboring molecules dictate the statistical fate of the excited molecules, determine the fraction of molecules progressing through the different pathways out of S_1, and provide a wealth of information about the molecular environment. Other pathways, such as Förster resonance energy transfer (FRET) and photochemical reactions also proceed from S_1 or T_1 states in a parallel kinetic competition with all the other pathways; however, FRET involves a reaction partner (e.g. the acceptor for FRET). FRET is named after Theodor Förster. We are not considering "stimulated" emission, which is when very high intensity light induces emission of a photon from the S_1 state in a manner exactly parallel to the normal absorption of a photon from the S_0 state; this is what takes place in a laser.

The spin of the electrons is important. The electrons of the two highest energy levels of the singlet states (S_0, S_1 and S_2) have opposite spins. The triplet states, T_1 and T_2, (see Figs. 3 and 4) have two electrons with parallel spins. Figure 3 indicates the different spin configurations of the electrons of singlet and triplet states, and shows possible pathways of intersystem crossing (transitions between singlet and triplet states), as well as transitions between states where the spin is conserved. Transitions between pure S

states and pure T states are strictly forbidden. Quantum mechanics forbids a change between states with different total spins. However, transitions between singlet and triplet states can occur if the spin states are not pure; that is, if spin–orbit perturbations mix the S and T character of the states. Then transitions between S and T states become partially allowed by spin–orbit coupling. *Spin–orbit coupling* is a mechanism whereby the orbital angular momentum of outer electrons (in atoms of the same or other molecules) interacts with the spin of the excited electron, assisting a spin flip. Because of the mostly forbidden character, a T to S transition is usually slow (e.g. phosphorescence). In the absence of significant interactions, the triplet state is very long lived (microseconds to seconds). During this extended time, an excited triplet can efficiently interact with the surrounding molecules by simple diffusion. For instance, the triplet state can efficiently react with ground state oxygen, which is a triplet, forming singlet oxygen (and a singlet state of the original triplet state). Singlet oxygen is highly reactive. At normal concentrations of oxygen (1–5 mM), if the molecule has entered the triplet state through intersystem crossing, it is highly likely that it will interact with oxygen by normal diffusion during a small fraction of the isolated triplet lifetime. Therefore, the emission from the triplet is very efficiently quenched, and in solution one does not usually observe phosphorescence. Except for a few exceptional cases, phosphorescence is only observed either in the absence of oxygen or at very low temperatures. Singlet oxygen is a highly reactive substance, and can create destructive *reactive oxygen species* (*ROS*), sometimes leading to the destruction of the chromophore. The ROS are highly damaging to any closely neighboring molecules, and are thought to be a major cause of skin cancer. Because plants cannot avoid intense sunlight, they have developed elaborate and efficient mechanisms to protect themselves from photodestruction (such as the xanthophyll and lutein cycles (Demmig-Adams et al. 2005; Gilmore 1997; Gilmore et al. 1998; Holub et al. 2000, 2007).

Rate constants and competition of the individual pathways of de-excitation

The *efficiency of a specific pathway of de-excitation* is the ratio of the number of times that this pathway event transpires divided by the number of times that the molecule is excited; this is the *quantum yield of that pathway*. All pathways compete dynamically in parallel with fluorescence emission. The rate constants of the different pathways are usually determined indirectly by measuring the rate of fluorescence decay. The kinetic competition between all the different pathways of de-excitation leads us to a simple description of almost all the different ways of measuring rates of any particular pathway. This underlying mechanism is operative in almost every fluorescence experiment. Once the principle and consequence of this simple competitive rate model is appreciated, most fluorescence lifetime experiments can be easily understood. A simple allegory representing this idea is given in the next section. First we discuss shortly the general mechanism in terms of competing transitions.

The following discussion assumes that the different pathways of de-excitation take place incoherently, because the molecules in the excited state are assumed to be in a quasi steady-state equilibrium. This means that by the time different kinetic pathways leading to a de-excitation are operative, the molecule has "forgotten" the excitation event; that is, there is no recollection of the phase, or even of the time, of the electrodynamic perturbation of the excitation light (or rather the moment the molecule entered the excited state through an interaction with a photon). Therefore, we can exploit a simple probability model of de-excitation using a "classical" statistical analysis. The assumption of incoherence may not hold for kinetic activities in the sub-picosecond time range, and this very rapid time range is also important for initial steps in the photosynthetic mechanism. However, for our discussion, we assume that the kinetic processes affecting the fluorescence are incoherent, allowing a statistical kinetic probability approach.

Once a molecule is in the excited state, it can leave this state by any of the available pathways (Fig. 2). The usual operative pathways are photon emission (fluorescence), dynamic quenching, intersystem crossing to the triplet state (and subsequent de-excitation from this state), Förster energy transfer, electron transfer, internal conversion, photo-destruction or other excited state reactions. Each of these pathways has a rate constant, and all pathways act simultaneously in parallel kinetic competition with each other. In some cases, particular rate constants can change within the lifetime of the excited state (such as rapid solvent relaxation); this requires special consideration in the analysis; essentially this means the spectrum and/or the lifetime is changing during the lifetime of the excited state (Birks 1970; Lakowicz 1999; Valeur 2002). We will not explicitly consider this case, but it is important to keep this possibility in mind.

The total rate of leaving the excited state is the sum of the rate constants of all the operative pathways. Because of this direct competition between several pathways of de-excitation, we can observe the effect of the presence or the absence of the different pathways by measuring the rate of the fluorescence decay (and we can also measure the corresponding rate constants of the other pathways by measuring the rate of fluorescence—see below). This simple

statistical, competitive model is the basis of all fluorescence measurements. In effect, we are using the chromophore as a spy and the emitted photons as messengers to report to us the state of affairs of the sample at the position, or in the surroundings, where the fluorophore is located. The excitation does not have to take place only by the absorbance of a photon; any way of entering the excited state leads to the same outcome (such as in bioluminescence, or energy transfer). Because this is such an important concept to understand, and the application of this kinetic competition principle is the basis of almost all fluorescence experiments, we present the following instructive metaphor for the benefit of those not familiar with fluorescence lifetime measurements.

A fluorescence allegory of bees in a box

We present a simple metaphorical example to emphasize the generality of the statistical argument. The following allegorical model is a simple probability calculation of the average time required for a trapped bee X^* (or excited molecule) to escape from a box through different holes in the box, and the holes can be open or closed. It is equivalent to the calculation of the rate of fluorescence. Understanding this allegory is all the background needed to interpret and analyze most fluorescence experiments. See Fig. 5 for a cartoon of the "bee in a box".

We imagine a total of N holes in a box, and the holes can be open or closed (the holes do not change their open or closed state during the duration of the experiment). When open, each hole presents a separate pathway for the bee to escape. Because the box is dark and the bee does not like its confinement, the bee chaotically bumps against the wall in order to find an open hole. The bee also has no inkling where the holes are. You can think of this as an "excited state box". As the bee buzzes around rapidly, but "incoherently", to find an open hole to exit the box, there will be a distinct probability per unit time (rate constant) that the bee will escape through any particular hole. Call this rate constant k_i for passing through the ith hole. We emphasize that this is a rate constant; that is, a probability per unit time. And the rate of escape depends on the bee's search speed and the size of the hole, as well as the size of the box. If the holes have different sizes, the bee will find the larger holes more often than the smaller holes, and therefore the rate constant (or probability per unit time) of escape through any particular hole will be faster for the larger holes. We only observe whether the bee has exited the box through one particular hole, which we will call the fluorescence hole. We cannot see the other holes. We always place the bee in the box at a specific known time. If the bee has exited through any other hole, we will not observe that event. However, if we repeat the experiment with the bee many times, and always keep track of the length of time until the bee escapes through the fluorescence hole, we will be able to tell whether certain other holes are open or closed, and how big the holes are; that is, we can determine the relative rate constant with which the bee can pass out of any other hole, which we cannot observe directly. This simple model comprises the essence of every fluorescence experiment.

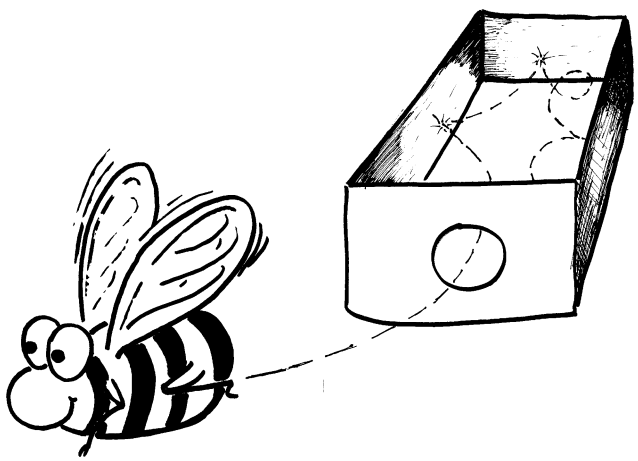

Fig. 5 This cartoon describes the situation of our allegory of a bee in a box, trying to get out, but bumping into the wall in a random search for the hole. The extension to many holes is clear, as well as the situation of many bees simultaneously in the box with one or many holes

The parallel with fluorescence is clear when it is realized that the probability of fluorescence decay from the excited state of a molecule is a random variable; that is, once in the excited state, there is only a probability per unit time that a photon will be emitted; and this probability is a constant. Each hole represents a particular pathway for the bee to leave the box (de-excitation from the excited state), and the probability of exiting through any of the holes (de-excitation pathways) is also constant in time. The usual objective of a fluorescence experiment is to determine the probability of a particular de-excitation pathway by detecting the fluorescence. It is this procedure we want to demonstrate with the following simple model.

A single bee and one open hole in the box

First we consider the experiment with just one open hole; call this hole "f" (which stands for fluorescence). We sit outside of the f-hole, and after we start the experiment (at time T_0), we record whether the bee is still in the box at some later time T. See Fig. 6 for a visual description of the time parameters. This is the same as a photon counting experiment. We divide up our observational times into Δt equal increments (see Fig. 6). The probability that the bee will *still be in the box* at time $T_0 + \Delta t$, if he had started

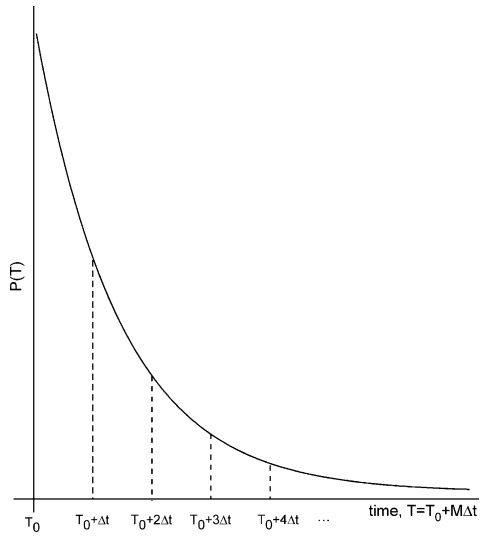

Fig. 6 A plot of the probability that a bee (Fig. 5) will still be in a box after time $T = T_0 + M\ \Delta t$. If we assume that $T_0 = 0$ (starting time), then $P(T) = \exp(-k_f T)$. $P(T)$ is identical to the probability that a single molecule will still be in the excited state after time T has passed if it became excited at time $T_0 = 0$. See the text for the derivation, and for the extension to multiple holes (pathways of de-excitation) and to an ensemble of bees (molecules) in the box (excited state)

there at time T_0, is $P(T_0 + \Delta t) = (1-k_f\,\Delta t)$, where k_f is the *time independent* rate constant (probability per unit time) for finding the "f" hole. We define $T_0 = 0$. The experiment is repeated a large number of times. Each time we record the time $T + \Delta t$ when the bee emerges through the f-hole (this means that the bee was still in the box at time T). For each experiment, the probability that the bee will still be in the box after a time T, which corresponds to M time intervals of Δt, is $P(T) = P(M\Delta t) = \left(1 - k_f \Delta t\right)^M = \left(1 - k_f\left(T/M\right)\right)^M$. The time intervals are divided into smaller and smaller intervals, and we assume the limit $\Delta t \to 0$ and therefore $M \to \infty$. This limit is the definition of an exponential, $P(T) = \exp(-k_f T)$, Fig. 6. So, the probability that the bee emerges at a time T decreases exponentially with time and with a rate constant k_f, which can be written as $P(T) = \exp\left(-k_f T\right) = \exp\left(-T/\tau_f\right)$, where τ_f is the average duration time the bee is in the box (Fig. 6) (remember we carry out the experiment many times to determine this average). For the case of fluorescence, assuming emitting a photon is the only pathway of emission, τ_f represents the intrinsic radiative lifetime (where every excited molecule exits the excited state only by the emission of a photon). k_f is usually a fundamental constant of the molecule, and is independent of the environment of the fluorophore (since with only one exit pathway, there are no other ways of exiting the excited state); it can theoretically be calculated by quantum mechanics (Stepanov and Gribkovskii 1968). The important point to remember is that the rate constant (probability per unit time) of passage of the bee through the fluorescence hole is constant, and that the process of finding the hole is *random*. This leads to the exponential decay, and is true for fluorescence just as it is for radioactivity decay (which was the original model of fluorescence decay (Gaviola 1926, 1927)). Becquerel earlier (before radioactivity was discovered) recognized that the decay of simple luminescence emission (phosphorescence) decays as an exponential (Becquerel 1868, 1871). We have described the situation for a single bee (single molecule) experiment. It is also the way that a single-photon time-correlated fluorescence experiment on single molecules is carried out, because in this case the arrival times of only a single photon from a single molecule is recorded at any time.

An ensemble of bees and one open hole

There is another way we can do the experiment with many, many bees (an ensemble of M_{all} bees in the box at the initial time). We can send them all into the box at time zero, and count how many bees emerge from the f-hole between the time T and $(T + \Delta t)$. This is proportional to the *rate of emergence* (that is, the number of bees coming out of a hole per unit time), which is the "intensity" of emergence. Now we only have to do the experiment once (because we have many bees) but we record the number of bees emerging per Δt at many consecutive times T, and then plot our results. We now get an exponential that decays with the same rate constant as before (k_f); but we measure the *intensity* of bees (the number of bees emerging per unit time at some time T). At time, T only $M_{all}\exp\left(-k_f T\right)$ bees are left in the box. So the *intensity* (or rate) at which bees are emerging at time T is $I_f(T) = k_f M_{all}\exp\left(-k_f T\right)$. We can check if this is correct, by integrating the rate at which the bees are emerging over all times, which should give the total number of bees in the box at T = 0. This is $\int_0^\infty I_f(T)dT = k_f M_{all}\int_0^\infty \exp\left(-k_f T\right) dT = M_{all}$. This is the same as measuring the intensity of fluorescence (number of photons per unit time, which is proportional to the fluorescence intensity) where the only pathway of de-excitation is fluorescence, and where the total number of molecules excited at time zero was M_{all}.

A single bee and two open holes

Now we consider what happens when we open another hole—we call this hole "t". We define k_t as the rate constant (probability per unit time) of going out the t-hole. This is also a constant, but unlike k_f, k_t depends on the experimental design (that is we can open or close this

hole). Now, if the t-hole is open, the probability of leaving the box per unit time (the rate constant) is $k'_f = k_f + k_t$. We still only record the times when the bee comes out the f-hole (we cannot observe the t-hole). The probability the bee is still in the box at time $T = M\,\Delta t$ is now

$$P(T) = \left(1 - \left(k_f + k_t\right)T/M\right)^M = \left(1 - k'_f T \Big/ M\right)^M = \exp\left(-k'_f T\right) = \exp\left(-T \Big/ \tau'_f\right)$$

However, now $k'_f > k_f$, and $\tau'_f < \tau_f$ So, on the average, the bee stays in the box less time. Also, the bee emerges from the f-hole fewer times than we send him into the box. Of course, this is expected, because the probabilities of independent random events add, and we know with two holes $k'_f = k_f + k_t$. Because the fraction of times the bee escapes through any particular hole depends on the relative rate constant for that hole, we can calculate a "quantum yield" for the bee to emerge through the f-hole: $Q_f \equiv k_f \Big/ \left(k_f + k_t\right) = k_f \Big/ k'_f = \tau'_f \Big/ \tau_f$. This is just the definition of the quantum yield as it is defined for fluorescence. The quantum yield of the bee exiting through the t-hole is just $Q_t \equiv k_t \Big/ \left(k_f + k_t\right) = \left(k'_f - k_f\right) \Big/ k'_f = \left(\tau_f - \tau'_f\right) \Big/ \tau_f$. To carry out this experiment, we measure first with the t-hole closed, where $P(T) = \exp\left(-k_f T\right) = \exp\left(-T \Big/ \tau_f\right)$ and then with the t-hole open, where $P(T) = \exp\left(-k'_f T\right) = \exp\left(-T \Big/ \tau'_f\right)$. And then simply take the ratio of the rate constants, or time constants as indicated above. Also note that $k'_f - k_f = k_f + k_t - k_f = k_t$. In other words, we can determine k_t, the rate constant of passing out of the hole "t", by only measuring k_f, and k'_f; that is, we never have to observe directly the bee passing out of the hole "t". This is the quintessence of a fluorescence experiment. For instance, if "t" stands for FRET (Förster energy transfer (Förster 1946, 1948, 1951)), then $Q_t = E =$ *the efficiency of energy transfer*. In general, we only use fluorescence to measure rate constants of other processes besides fluorescence, such as, the efficiency of energy transfer, or the rate of dynamic quenching; that is, we are not usually interested in the physics behind the radiative fluorescence lifetime.

An ensemble of bees and two open holes

The extension to the second method, where we measure the time dependence of the intensity of bees emerging, is straightforward. At time T there are only $M_{all} \exp\left(-\left(k_f + k_t\right)T\right)$ bees left in the box, so the *intensity* of bees emerging at time T *through the f-hole* is $I_f(T) = k_f M_{all} \exp\left(-\left(k_f + k_t\right)T\right) = k_f M_{all} \exp\left(-T\left({}^1\!/\!_{\tau_f} + {}^1\!/\!_{\tau_t}\right)\right) = k_f M_{all} \exp\left(-T\left({}^1\!/\!_{\tau'_f}\right)\right)$, where ${}^1\!/\!_{\tau'_f} = {}^1\!/\!_{\tau_f} + {}^1\!/\!_{\tau_t}$. The quantum yield of bees escaping through the f-hole is $Q_f = \frac{1}{M_{all}} \int_0^\infty I_f(T)dT = \frac{1}{M_{all}} \int_0^\infty k_f M_{all} \exp\left(-\left(k_f + k_t\right)T\right)dT = \frac{k_f}{k_f + k_t}$, where now $M_{all} = \int_0^\infty \left(k_f + k_t\right) M_{all} \exp\left(-\left(k_f + k_t\right)T\right)dT$. This agrees, as it must, with the calculation with a single bee many times. The quantum yield is of course less than the quantum yield when only the f-hole was open, because some of the bees go through the t-hole.

One should note that we can tell whether the t-hole (the t-pathway) is open by measuring either the lifetime τ'_f and comparing to τ_f, or measuring the intensity with the t-hole open and again with the t-hole closed, and then taking the ratio

$$\left[\int_0^\infty I_f(T)dT\right]_{\substack{door \\ open}} \Bigg/ \left[\int_0^\infty I_f(T)dT\right]_{\substack{door \\ closed}} = {}^{k_f}\!/\!_{k_f + k_t} = Q_f.$$

Notice also that the constant k_f is the *same* in the presence or the absence of the other pathway. This trivial observation is important—the measured lifetime of fluorescence decay τ'_f becomes shorter, and the integrated intensity $\int_0^\infty I(T)dT$ becomes less, *only* because we have opened another pathway of escape, *not* because the rate constant of fluorescence has decreased. All the rate constants remain constant during the measurements; we only change the number of open holes. Of course, just as above, measuring the lifetime of fluorescence of an ensemble of molecules, or the quantum yield of fluorescence, and using these simple ideas, we can calculate the rate constant k_t.

Applying the above allegory to fluorescence with an arbitrary number of de-activation pathways

The extension of the "bees in a box" allegory to many open holes (pathways) is obvious, and we no longer refer to the allegory of bees in a box. The important conclusion is: *If we want to measure the presence or rate constant of some pathway "p", we only need to make the above measurements of the rate of fluorescence decay, or the intensity of fluorescence, with and without the availability of pathway "p".*

Once a molecule is in the excited state, it can only depart through one of the allowed exit pathways. All exit events that take place in the excited state have a particular rate constant and they all contribute linearly to the total rate (inverse of the average time) the molecule leaves the excited state. The natural intrinsic radiative rate of fluorescence decay k_f is intrinsic to the molecular structure, and

can be calculated from quantum mechanics if the structure is known for an isolated molecule. The total rate (the probability per unit time) for an excited molecule to leave the excited state region is the sum of all possible rate constants for leaving by any of the possible exit pathways. Some of these individual rate constants can be affected by the molecular environment of the excited molecule (e.g., solvent effects, proximity of dynamic quenchers or energy acceptors). Some of the rate constants can depend on the time (we still call it a rate constant); this can happen if the molecules move or change significantly during the time the molecule is in the excited state. However, if conditions remain constant during the excited state lifetime, then the probability that a particular pathway of de-excitation will be chosen is independent of the time.

With this simple picture, we can determine the rate or the efficiency with which the excited molecule passes through any one of the exit pathways. For instance, in order to measure the efficiency of energy transfer from an excited donor molecule to an acceptor molecule we measure the apparent fluorescence lifetime of the donor in the presence and absence of acceptor. It is important that when these two measurements are carried out, all the other pathways remain identical in the presence and the absence of the acceptor. The rate constant for energy transfer (Förster 1946, 1948, 1951) k_t depends on how close the acceptor is: $k_t = \left(1/\tau_D^0\right)(R_0/r)^6$, where τ_D^0 is the measured lifetime in the absence of acceptor, R_0 is the distance where the measured lifetime is half of τ_D^0 (or equivalently where $1/\tau_0 = k_t$) and r is the distance between the donor and acceptor. Then, as discussed above, the quantum yield of FRET, Q_t, which is the same as the efficiency of FRET (often denoted by E), can be deduced by $Q_t \equiv k_t/\left(k_f + k_t\right) = \left(k_f' - k_f\right)/k_f' = \left(\tau_f - \tau_f'\right)/\tau_f$, where k_f, k_f', τ_f and τ_f' are defined above (the prime and no-prime means measured in the presence and the absence of the acceptor). This shows how convenient it is to estimate the efficiency of energy transfer when lifetimes are measured. However, we emphasize again, the probability per unit time (the rate constant) of passing through the fluorescence exit pathway remains the same, whether the acceptor is present or not; only the total rate changes.

As was mentioned above, the basic physical mechanism does not depend on how the molecule becomes excited. The molecule can get into the excited state by receiving the energy of excitation from another excited molecule (e.g. by energy transfer). Or the molecule could arrive in the excited state through means of a chemical or biochemical reaction—as in bio- and chemical-luminescence. Bio-luminescence has also been used to study energy transfer (Morin and Hastings 1971; Wilson and Hastings 1998). Most experiments are done such that the excitation is accomplished by absorbing a photon. Of course experimental details may depend on how the molecule is excited, but this does not concern our description once the donor is in the excited state. However, the measured time-dependent fluorescence signal may depend on the method of excitation; see the sections below: "The Fundamental and Measured Fluorescence Response" and "Secondary excitation".

The description of the kinetic competition between all possible pathways of de-excitation is very general, and one can of course measure rates and quantum yields of certain pathways without using fluorescence (Clegg 1996). For instance, one can measure the efficiency of FRET by measuring the amount of chemical products formed by photolysis of the donor (in the presence and absence of an acceptor). And the quantification of the photolysed donor product can be done with chromatography—it is not necessary to measure fluorescence. The competition of any two pathways can be used. However, it is in general convenient and easy to measure fluorescence; and the fluorescence measurement is also sensitive.

Where did the simple exponential decays go?

The discussion above assumes that one is observing a single molecule, or an ensemble of *identical* molecules. This is rarely the case. Normally photosynthesis studies are not made at the single molecule level. When fluorophores are in the environment of complex biological samples, the measured dynamic fluorescence response is usually not a simple exponential decay. The response is either a quasi-continuous distribution of exponentials, or several such distributions. This arises because the fluorophores (e.g. chlorophyll) are located in disparate locations, and often there are more than a single fluorophore contributing to the fluorescence signal (e.g. background and other intrinsic chromophores of the sample). This is even true for many "cuvette-type" experiments with relatively pure, but complex biological molecules (James and Ware 1985; James et al. 1985). When we interpret fluorescence experiments, the basis of our understanding on which we construct molecular or biologically relevant models to represent biological mechanisms, it usually involves the simple case of singular individual fluorophores as presented above. However, one must be careful not to draw unqualified conclusions, especially when fitting the measured fluorescence response in terms of single, double or even triple exponential decays; that is, when we try to fit the time course of the fluorescence emission (assuming a very short pulse of excitation) to $Fluorescence(t) = \sum_1^{N=1,2,3} a_i e^{-t/\tau_i}$. This caveat is true even if the chosen

relaxation model fits the data superbly. This difficulty in interpretation arises because real exponentials (that is, exponentials where the exponent is a real number; for instance, $\exp(-t/\tau_i)$) are not orthogonal over any range of time. Orthogonal in this sense refers to the fitting procedure. When fitting data to a sum of real exponentials, varying a fit parameter of one exponential function will affect the parameters of the other exponentials. This means that the fitted values of the real exponential parameters change (and sometimes quite significantly) depending on the number of exponentials that are used in the fitting procedure. This is not true for instance for complex exponentials or sines and cosines, because they are orthogonal over their defined domain (that is, adding more sines or cosines to a sum of sines and cosines defining the fitting function will not change the parameter values of the original sines and cosines used in the earlier fit). Thus, it is mathematically notoriously difficult to separate reliably and robustly multiple real exponential decays, even with very good signal-to-noise. This is an old problem, and has been comprehensively dealt with in the past literature of fluorescence (Cundall and Dale 1983) as well as many other kinetic techniques, such as relaxation kinetics (Bernasconi 1976; Czerlinski 1966). It is not that a high signal-to-noise recorded fluorescence response cannot be fitted very well with multiple exponentials; one can sometimes even show that the fitted parameters are stable with low chi-square values, and the same parameters can be obtained with many different starting conditions of the fit (these tests are at least required to claim a reasonable fit to the data). In case the fit to the data is already very good with a certain number of components, it is usually not worthwhile to add another component. Typically, if the fit is already very good with a certain number of exponential components, including another component to the fitting function (or fitting to a distribution of exponential decays), it will not result in a better fit (same error and residuals); however, the values of the fitted kinetic parameters (the lifetimes and fractional amplitudes) are then usually very different than when considering one fewer component. This can lead to very disparate interpretations of the data in terms of a kinetic model, and determining a consistent and reasonable model is the usual objective of the experiment. This is simply because from only the lifetime measurement, one cannot be sure how many separate molecular species truly underlie the dynamic fluorescence response. One can only determine the minimum number of exponential components that represent well the fluorescence response. The same problem exists with frequency domain measurements when one tries to interpret the frequency domain results in terms of individual (or distributions of) exponential decays. One refers to the frequency domain when the light is modulated repetitively at different frequencies (usually 10–100 megahertz), and one determines the modulation depth and phase of the fluorescence signal relative to the modulation depth and phase of the excitation light (Birks 1970; Clegg and Schneider 1996; Clegg et al. 1996). This caveat does not diminish the value of "fluorescence lifetime" measurements (perhaps this is better called the "dynamic fluorescence response"), but emphasizes the need to integrate and combine the lifetime data with other experimental data and biochemical manipulations (such as added inhibitors, spectral measurements, and mutants). It is prudent to keep these limitations and possibilities for misinterpretations (and over-interpretations) in mind. This is especially pertinent when dealing with complex biological systems, such as intact photosynthetic organisms where the molecular environment is highly heterogeneous, and where the biological system is highly organized. Often the composition of a photosynthetic system changes depending on its "history" (time of day, light exposure, time of dark adaptation, etc.).

The fundamental and measured fluorescence response

Our discussion has so far assumed that the state from which the emission is derived is the same state that is initially excited, and modeling the fluorescence response as a sum of exponentials assumes that the molecule is excited by a very short light pulse. That is, we did not take into consideration how the molecule became excited. That is, the model describes the "*fundamental fluorescence response*" $F_\delta(t')$ (the fluorescence response to a delta-function excitation, where all molecules are excited simultaneously at a defined time). Equation 1 describes how the fundamental fluorescence response is transformed into the measured fluorescence response $F(t)_{meas}$ if the excitation event is not a delta-function excitation event. This is the general, fundamental equation describing all time-resolved fluorescence measurements.

$$F(t)_{meas} = \int_0^t E(t')F_\delta(t-t')dt' \qquad (1)$$

$E(t')$ is the time dependence of the excitation event; for instance, it can be a light pulse of any shape, and can vary from a pure sinusoidal wave or a repetitive square wave to a repetitive series of pulses of short duration. One can think of any form of an excitation pulse $E(t')$ as a series of appropriately weighted delta function pulses $E_\delta(t')$. t' is the time that a very short part of the excitation pulse (essentially a delta function), $E_\delta(t')$, arrives to excite the sample, and t is the time following t' at which the measurement is made (Birks 1970; Clegg and Schneider 1996; Clegg et al. 1996). A delta function pulse is simply a light pulse that is very short compared to any rate of fluorescence decay. If

every fluorescing species is excited directly by a single delta function pulse $E_\delta(t')$, then the decay of every separate component of fluorescence can be described by the *fundamental fluorescence response,* $F_\delta(t-t')_{meas} = F_\delta(t-t') = F_0 \exp(-(t-t')/\tau)$.

Instrumentation imperfections are described by a similar convolution of the fluorescence emission with the instrument response function, which describes the temporal response of the acquisition equipment (Birks 1970; Clegg 1996).

Secondary excitation

If the state in question is excited through an excited state reaction with another initially excited state or molecule, then the measured fluorescence response of the secondarily excited state (or molecule) is described through an equation identical to Eq. 1, except in that case, the temporal form of the de-excitation of the initially excited molecule takes the place of $E(t')$ in Eq. 1. This leads to a different shape of the measured fluorescence response of the secondarily excited molecule. But the fundamental response of the secondarily excited molecule (in this case $F_\delta(t-t')$ of Eq. 1) is described by the same statistical kinetic competition model. This is exactly what happens when one observes the time dependent emission of the acceptor in a FRET experiment (the acceptor is excited through the donor de-excitation event). Of course, in the case of FRET, the excitation of the acceptor does not involve the emission and absorption of a photon, but is caused by a dipole–dipole interaction between the donor and acceptor (Clegg 1996; Förster 1951). The excitation (through intersystem crossing) and emission of the triplet state (phosphorescence) is another example of secondary emission; however, the phosphorescence time decay, when seen, is usually much slower than the fluorescence of the singlet state, and in that case $F(t)_{meas} \approx F_\delta(t)$.

Concluding remarks

We have given an overview of the excitation and de-excitation events and transitions between the different energy states of a fluorophore, and we presented a simple statistical model of dynamically competing pathways. This simple model is the basis of almost all dynamic fluorescence experiments. Expressions describing how the fluorescence response can be used to quantify the rates and efficiencies of all kinetic pathways departing from the excited state can easily be derived from these simple considerations. We note that the discussion of the competitive pathways of de-excitation and Eq. 1 apply to all the different methods of measurement. The methods of measurement and analysis have been described in the context of lifetime-resolved imaging in another manuscript in this special issue ("Fluorescence Lifetime-resolved Imaging", by Y.-C. Chen and R.M. Clegg).

There is an abundant literature on fluorescence of photosynthetic systems, excitation energy transfer and trapping at the reaction centers. For general information, see some reviews (Clegg 2004; Govindjee 2004; Papageorgiou 2004; Sauer and Debreczeny 1996). An entry into the vast literature on excitation energy transfer and trapping of energy in photosynthestic systems may be sought through several reviews (Brody 2005; Frank and Christensen 1995; Koyama and Kakitani 2006; Leupold et al. 2006; Mimuro 2004, 2005; Renger and Holzwarth 2008; Van Stokkum et al. 2008; Van Grondelle and Gobets 2004; Van Grondelle and Novoderezhkin 2008) in the Advances in Photosynthesis and Respiration Series (Springer, Dordrecht).

Acknowledgments We are grateful to John Eichorst for careful reading of the manuscript and suggestions on the text. With gratitude we recognize the many people in the fluorescence community from whom we learned and with whom we have practiced the many aspects of fluorescence spectroscopy. We are indebted to Benjamin F. Clegg for the drawing of the "bee in a box". Both authors thank the Research Board of the University of Illinois for support during the preparation of the manuscript. This manuscript was edited by Govindjee.

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