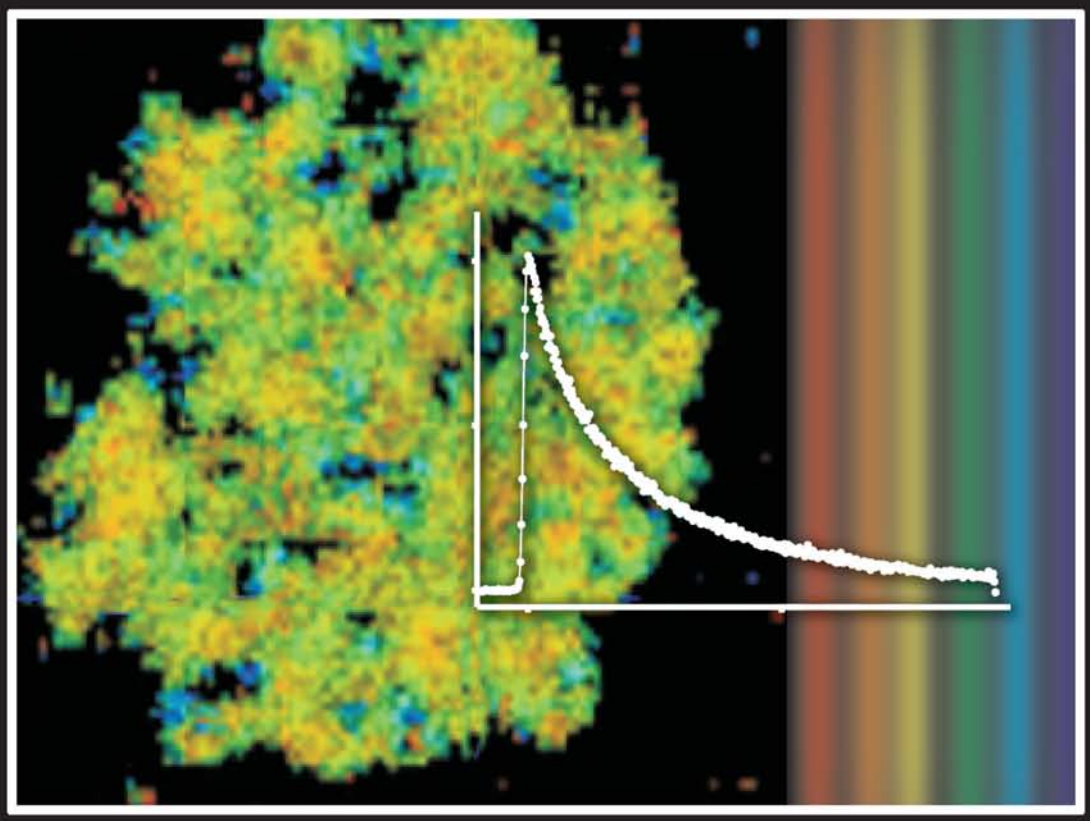
FLIM Microscopy in Biology and Medicine



Edited by
Ammasi Periasamy
Robert M. Clegg

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AMMASI PERIASAMY
ROBERT M. CLEGG

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We dedicate this book to past, present, and future FLIM enthusiasts, including those involved with instrumentation and software development as well as users mainly interested in FLIM applications.

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Preface

Fluorescence microscopy is an established tool for a variety of applications in biology and biomedical research. Recent advances leading to improved contrast and high sensitivity allow for the detection of signals at the single-molecule level. In conjunction with this platform, fluorescence lifetime imaging microscopy (FLIM) provides another dimension of contrast and sensitivity and also offers the additional benefit of independence from fluorophore concentration and excitation intensity. Moreover, the fluorescence lifetime is often sensitive to the physical and chemical environment of the fluorophore; as such, it is an excellent reporter of conformational changes and variations of the molecular surroundings of biological molecules.

These unique advantages of lifetime-resolved fluorescence measurements extend the information that is obtained from measuring only the intensity. The rationale for performing lifetime-resolved measurements in an imaging environment is to acquire, at every pixel of a fluorescence image, the critical information provided by dynamic fluorescence measurements, which has been available for decades from single-channel (cuvette) fluorescence dynamics measurements. The lifetime-resolved fluorescence parameters coupled with the spatial dimension provide valuable insight into the functioning of complex biological systems.

The primary objective of a FLIM investigation is usually quite different from that of single-channel measurements. One is still interested in determining the lifetime-resolved information as accurately, reproducibly, and robustly as possible; however, in FLIM, the structure/morphology of some object (e.g., a structure in a biological cell) under physiological conditions is often the investigation's target of major concern. Thus, the scientific questions asked are analogous to those for normal intensity fluorescence imaging; that is, one is interested in correlating the spectroscopic information with different locations in the imaged object. When lifetime-resolved fluorescence is acquired in addition to the intensity, the identification and quantitative differentiation of fluorophores are considerably improved.

For instance, in the case of FRET, if the lifetime of a donor fluorophore is known in the absence of an acceptor, it is relatively easy for FLIM to differentiate locations in an image with dissimilar lifetime decays; faster lifetimes indicate increased efficiency of energy transfer. Because lifetimes in FLIM are independent of the concentration, complicated control experiments and multiple wavelengths, which may be difficult to align in the image, are not required. FLIM is also an excellent way to discriminate objects that have similar

emission wavelengths but different lifetimes; thus, the image contrast is improved. A common application of FLIM is the elimination of background fluorescence, such as intrinsic fluorescence, or unbound fluorophores, where the bound and unbound fluorophores exhibit different lifetimes. FLIM can be used for many quantitative determinations of ion concentrations, pH, oxygen content, protein–protein interactions, cell motility, and cancer diagnosis. All of these applications are discussed in the various chapters of this book.

It is difficult to say when and where the first FLIM images were observed. The "dawn of FLIM" took place in a few research laboratories with ready expertise in time-resolved fluorescence, fast electronics, and, usually, a strong interest in solving biological problems. The original developments made use of instrumentation already available in cuvette-based spectrofluorometers to acquire the fluorescent decay. The lifetime data were analyzed using on-hand fitting methods.

Once the power and broad applicability of FLIM became evident to the general scientific community, biological investigators' interest in exploring lifetime measurements developed quickly. Both time- and frequency-domain methodologies were rapidly improved and extended in many laboratories. Thanks to the development of various technologies, including optics, electronics, detectors, and the new discovery of visible fluorescent proteins, this development took place at a rapid pace. Not surprisingly, after the introduction of various commercial units, which simplified data acquisition and analysis and provided biological laboratories with ready-made instrumentation, publications covering FLIM microscopy have grown rapidly since 2000.

This book presents the fundamentals of FLIM so that a wider audience can appreciate the rapid advances and increasing applications reported in the literature. In this sense, the goal is pedagogical: In addition to reviewing the latest developments, applications, and approaches to data analysis, we want to convey the exciting future of FLIM and indicate the present state of the art in FLIM imaging as described in the instrumentation section. No measurement method is perfect; the authors have strived to present pros and cons of different methods and to give some indication of where improvements are necessary and desired. Each chapter critically compares FLIM measurements to other techniques.

The book also describes ancillary techniques related to the direct determination of lifetimes, including imaging fluorescence anisotropy for the study of molecular rotations. Moreover, in addition to discussions related directly to FLIM, we also address the fundamentals of dynamic fluorescence measurements and the basic pathways of de-excitation available to electronically excited molecules. An awareness of the diversity of pathways available to an excited fluorophore will assist potential users in recognizing the value of FLIM measurements, as well as inspire innovative experiments using lifetime-resolved imaging.

As time passes, more of the sophisticated methods used in photophysics and photochemistry, as well as new instrumentation, are being incorporated into FLIM. Novel features that apply exclusively to FLIM are being developed, including sophisticated image analysis. Our purpose has been to showcase the broad application of fluorescence lifetime-resolved imaging in biology. We include different aspects of FLIM data acquisition and applications, as well as discussions of FLIM data processing, in a separate section on data processing.

The discussion sections in all the chapters clearly show the challenges for implementing FLIM for various applications. Certain chapters discuss limits on the number of photons required for highly accurate lifetime determinations, as well as the accuracy with which multiple, closely associated lifetime components can reliably be determined. Highly accurate determinations of fluorescence lifetimes are sometimes necessary for answering certain specific, detailed questions concerning some molecular mechanisms. On the other hand, the change in lifetime-related parameters and their location in a cell are of primary concern for many investigations. Such considerations are important for the user when he or she is selecting the most advantageous method of FLIM to use for a particular application. These aspects are discussed in the various chapters. We hope that this book will be useful for experts in FLIM as well as for newcomers to this field.

We realize that the field of FLIM has grown rapidly in the recent past and that it is impossible to do justice to all those who have contributed unique FLIM applications, instrumentation, and data analysis. We acknowledge our indebtedness to all the FLIM enthusiasts who have made this such an exciting field. Most importantly, we wish to thank all the authors who have contributed chapters to this book and have strived to present the fundamentals so that novices can implement FLIM in their research and laboratories, as well as appreciate the uniqueness and usefulness of FLIM in their own research. It has been a great honor to work with them.

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FLIM MICROSCOPY
IN
BIOLOGY AND MEDICINE

1

Introduction, Microscopy, Fluorophores

CHAPTER 1

Fluorescence Lifetime-Resolved Imaging

What, Why, How—A Prologue

Robert M. Clegg*

1.1 INTRODUCTION

Fluorescence lifetime-resolved imaging, FLI, acquires a fluorescence image whereby the dynamic response of the fluorescence decay is temporally resolved at every location (pixel) of the image. When specifically referring to measurements in a light microscope, the acronym is FLIM, where the "M" stands for microscopy. We will use the names interchangeably. FLI measurements are analogous to normal intensity fluorescence imaging measurements and are acquired on the same samples, except that information related to the fluorescence lifetime is recorded in addition to the normal measurement of the fluorescence intensity. One says that in FLI the fluorescence signal is "lifetime resolved" and "spatially resolved." The fluorescence lifetimes (or more often, the apparent fluorescence lifetime) can be determined with the temporal resolution of nanoseconds or less at every pixel of the recorded image. The spectroscopic lifetime-resolved information can be displayed at every pixel in image format. By considering the physical mechanisms that determine the life of an excited fluorophore, insight into the experimental possibilities afforded by FLIM can be better appreciated.

* The author wishes to express his gratitude and appreciation to the community of scientists who have been instrumental in the development of FLI. It is a definite pleasure to work within the "FLI community." Many aspects of FLI are not covered in this chapter, and no details are given of any particular study or instrument. This chapter is not a literature review. Therefore, justice is not afforded to the many innovative contributions of many research groups. The reader can find this information either in the original references that have been given or in the following chapters.

1.2 GOAL OF THIS CHAPTER

One aim of this chapter is to acquaint the reader with the information available by time-resolved fluorescence spectroscopy and to describe how the experimental measurements are related to the fundamental mechanism of fluorescence. Ultimately, an understanding of the different pathways of de-excitation available to a molecule in an excited state is necessary to interpret fluorescence data and leads naturally to an appreciation of the knowledge that can be gained by temporally resolving the emission of a fluorescence signal in FLIM. This is not a review of the many excellent publications and outstanding contributions that have been made in the last decade on different FLI instruments. We discuss the fundamental time-dependent mechanisms that play a role in fluorescence. Many different de-excitation pathways are available to an electronically excited fluorophore, and these independent pathways compete kinetically in parallel. The kinetic rate of each pathway of de-excitation is sensitive to the environment of the fluorophore, each through a different mechanism. The measured rate of fluorescence (which is the inverse of the measured lifetime) is a summation of the rates of all separate available pathways; therefore, the fluorescence lifetime bears witness to the rates of all contributing pathways of de-excitation.

A discussion of this inclusive property of the value of the fluorescence lifetime, which makes the fluorescence lifetime so valuable, will hopefully lead to new, innovative experiments on specific biological systems. This property of the lifetime is one of the major reasons for performing FLIM measurements. We also survey the present methods of FLIM instrumentation and discuss their comparative advantages. Detailed descriptions of FLIM are not the focus of this chapter. The reader should consult the other chapters for in-depth discussions of the different aspects and methods of FLIM.

1.3 WHY MEASURE FLUORESCENCE LIFETIMES?

Upon excitation of a molecule from the ground electronic state to a higher electronic state, a molecule will remain in its primarily excited electronic state only transiently. The residence time in the electronic excited state is usually in the range of picoseconds to tens of nanoseconds. The average time the molecule spends in the electronic excited state is referred to as the "fluorescence lifetime." The primary excitation event that boosts a molecule from its ground electronic state (S_0 state) to an excited state—a vibrationally excited S_1 electronic state—takes place in a femtosecond (fs; 1 fs = 10^{-15} s) or less. The initial vibrationally excited S_1 state rapidly loses its extra vibrational energy to the environment and decays in 10^{-14} to 10^{-12} s to a vibrationally relaxed excited state: the vibrationally relaxed S1 state. Also, through vibrational interactions, the surrounding solvent or other nearby molecules can interact through Coulomb or dipole interactions with the S_1 state, often in a time-dependent manner, and change the electronic energy level (and other properties) of the relaxed S_1 state.

The excited state is normally initiated by the absorption of a photon; but other means of excitation can produce the same relaxed excited state (such as energy transfer from another nearby excited molecule—Förster resonance energy transfer [FRET]—or through a chemical or biochemical reaction). Once in the S_1 vibrationally relaxed state, the molecule

can undergo de-excitation through several different pathways (see the Perrin–Jablonski diagram in Box 1.2):

1. radiative emission of a photon (fluorescence), the *intrinsic* radiative rate, $k_{f,int}$ (this is not the measured rate of fluorescence and is considered to be an intrinsic property of the isolated molecule);
2. intersystem crossing to a triplet state, k_{isc};
3. nonradiative relaxation (usually losing the energy nonradiatively to the environment), which is termed internal conversion, k_{ic}, or nonradiative transitions, k_{nr};
4. dynamic quenching through collisions, k_q;
5. energy transfer to a nearby molecule in its ground state, k_{et};
6. *excited-state reactions* other than quenching, k_{er}, such as charge transfer, molecular isomerizations, and bimolecular reactions; and
7. photolysis (photodestruction of the excited molecule), usually by interaction of its triplet state with triplet oxygen, k_{ph}.

The notation, k_i, refers to the rate constants of the *i*th physical process. We also define k_{nf} as the sum of all the rate constants other than the rate constant for fluorescence, $k_{f,int}$. There are variations of these processes, but the preceding description is the usual case for most fluorophores used in FLIM. All these processes are dynamic, and there is a monomolecular rate constant for each pathway of de-excitation, depending on the conditions. Except for special situations, the "natural intrinsic rate constant," $k_{f,int}$, of radiative emission (fluorescence) is constant (e.g., not dependent on the temperature). $k_{f,int}$ can in principle be calculated from first principles; it is different for each molecule and depends on the details of the excited and ground-state electronic configurations. The rates of all other pathways are often sensitive to the molecular environment. All the separate pathways compete dynamically to first order.

The overall probability per unit time for the molecule to lose its excitation energy and pass to the ground state depends on the sum of the rate constants of all the different pathways (see Box 1.1). If we observe the fluorescence decay, the observed rate of the fluorescence relaxation will equal this sum of the rate constants of all the available pathways. The longest time the molecule can remain in the excited state is set by the rate constant of the fluorescence pathway (the intrinsic radiative lifetime, $\tau_{f,int} = 1/k_{f,int}$); however, as mentioned earlier, this is not the measured rate of fluorescence. The average time a molecule spends in the excited state will decrease as additional pathways become available for de-excitation. In this way, the excited fluorophore acts as a "spectroscopic spy," and the overall average lifetime of the fluorescence emission provides valuable information about the molecular environment of the excited fluorophore. A quantitative interpretation of the rate of emission is given in Box 1.1.

BOX 1.1: INTERDEPENDENCE OF THE PATHWAYS OF DE-EXCITATION AND THE MEASURED FLUORESCENCE LIFETIME OF A FLUOROPHORE, *D*

Discussing fluorescence from the natural point of view of competing kinetic rates emphasizes unequivocally that fluorescence is a convenient and very sensitive method for measuring kinetic mechanisms and molecular configurations on a molecular scale. Assume all the pathways of de-excitation are operative. The total rate of de-excitation from the excited-state D^* to the ground-state D can be depicted as a chemical reaction, $D^* \xrightarrow{\sum_i^{k_i}} D$. That is, the average time that the molecule stays in the excited state (the average lifetime of the excited-state τ_{D^*}) is inversely related to the sum of all the available different pathways of de-excitation, $1/\tau_{D^*} = \sum_i k_i$. The measured lifetime in the absence of pathway j is $(1/\tau_{D^*})_{i\neq j} = \sum_{i\neq j} k_i$. Thus, the rate of deactivation in the presence of pathway j is greater than in its absence; that is, $(1/\tau_{D^*})_{i\neq j} \leq 1/\tau_{D^*}$.

Whenever we allow an additional pathway of excitation, the rate of decay becomes faster. Usually, we choose to measure the rate of fluorescence decay, but the lifetime of D^* can be determined by measuring an experimental variable along *any* of the de-excitation pathways. Define the *measured* parameter to correspond to pathway f (where we have chosen the letter f because we usually measure fluorescence). The rate measured along pathway f in the presence and absence of pathway j will be $1/\tau_{f,meas} = \sum_i k_i$ and $(1/\tau_{f,meas})_{i\neq j} = \sum_{i\neq j} k_i$. Note that $k_{f,int}$ must be a member of both sums (because we are measuring fluorescence, this pathway cannot be the absent parameter). Also, note that $1/\tau_{f,meas}$ is the overall rate at which the excited state is depleted. $1/\tau_{f,meas}$ is *not* the intrinsic rate of de-excitation by way of the fluorescence pathway; that is, $1/\tau_{f,meas} \neq k_{f,int}$.

The intrinsic probability of fluorescence per unit time ($k_{f,int}$) is the same in both sums; however, the total pool of excited molecules becomes depleted faster than $k_{f,int}$ because other pathways for de-excitation are simultaneously actively available for depleting the excited state. The fluorescence decay signal mirrors the total decay of the excited-state population. For instance, in order to determine k_j, we simply subtract the two measured inverse decay times, $1/\tau_{f,meas} - (1/\tau_{f,meas})_{i\neq j} = \sum_i k_i - \sum_{i\neq j} k_i = k_j$.

Note that, in order to determine the rate of a pathway (j), we have measured fluorescence; however, pathway j has nothing to do with fluorescence. The obvious reason for choosing fluorescence to investigate all the other nonemissive pathways is because it is convenient and relatively easy to detect photons on the nanosecond time scale (which is the time window of the measurement). The lifetime of the measured fluorescence relaxation gives us direct insight into and quantitative estimates of the overall molecular dynamics of the nonfluorescence pathways. Through recent developments of FLI instrumentation, the considerable, powerful advantages of time-resolved fluorescence measurements are now available for imaging experiments.

The association between the measured fluorescence lifetime with the sum of all the rates of the competitive pathways of de-excitation is the primary motivation for carrying out fluorescence lifetime experiments. The average lifetime of the excited state, in the presence of all the available pathways of de-excitation, sets a limit on the temporal window of opportunity, during which the excited molecule can explore and report on its surroundings. As stated previously, the excited-state lifetime is usually in the nanosecond time region. Of course, by adding extra pathways for de-excitation from the excited state or by increasing the values of some of the rate constants, the probability per unit time that the molecule

will emit a photon (fluorescence) will decrease; as a result, the intensity of fluorescence will decrease. Thus, the time-averaged fluorescence intensity also carries information on the rates of the different pathways taking place within this time window. However, as we will see, the lifetimes report directly on the molecular dynamics and the temporal information is much richer than simply the time-averaged intensity.

If the excited molecule has transferred from the primary excited singlet state to the triplet state by intersystem crossing (see later discussion), the emission from the triplet state is called phosphorescence. In the absence of triplet quenchers, such as triplet oxygen, the phosphorescence lifetime can be as long as many seconds because the transition from the triplet state to the ground singlet state is not allowed and requires a simultaneous spin flip. To first order, transitions between states with different spin values are forbidden.

There is an extensive history of fluorescence lifetime measurements in single-channel experiments (macroscopic samples in cuvettes) (Birks 1970; Birks and Dawson 1961; Cundall and Dale 1983; Gratton and Limkeman 1983; Grinvald and Steinberg 1974; Lakowicz 1999; Spencer and Weber 1969; Valeur 2002). Lifetime-resolved fluorescence measurements have provided a wealth of invaluable information about biological systems. Of course, directly measured spatial information (imaging) is not available in a cuvette-type spectroscopic measurement. On the other hand, routine fluorescence imaging, whereby one is measuring the time-averaged intensity of fluorescence, has become a familiar measurement in essentially every field of cellular biology and provides an enormous wealth of information in cellular biology. Until more recently, most fluorescence imaging measurements in an optical microscope were limited to spectrally resolved intensity measurements (where wavelengths are usually selected with a simple optical filter). Although normal fluorescence microscopes do not provide nanosecond temporal resolution of the fluorescence signal, sophisticated microscope instrumentation and powerful image analysis algorithms are available that reveal detailed morphological information with high spatial resolution. FLIM aspires to couple both these feature into a single measurement.

1.4 WHY MEASURE LIFETIME-RESOLVED IMAGES?

Due to advances in instrumentation in the last few decades, it has become possible to couple measurements of fluorescence lifetimes with the most common modes of fluorescence microscopy. As the FLI technique emerges and becomes available to more researchers, the enhanced and more refined information content of time-resolved fluorescence measurements will extend significantly the capability of the investigator to reveal physical details on the molecular scale in fluorescence images of biological samples. The dependence on the environment, the kinetic competition between different pathways of de-excitation, and the sensitivity of the measured fluorescence signal to physical events on the scale of microscopic dimensions make fluorescence imaging a valued and highly informative method of measurement. FLIM measures the kinetics of these dynamic processes directly, without "integrating over" the time-dependent information, as when the steady-state intensity is measured.

In addition to the mechanistic and molecular information available by fluorescence lifetime measurements, separating the fluorescence signal into its elementary lifetime components provides a practical way to increase image contrast and distinguish quantitatively

the spatial distribution of multiple fluorophores with different lifetimes. FLIM can also help remove background fluorescence and discriminate intrinsic fluorescence in a biological cell. Reliable measurements can be made of the fraction of fluorophores in selected isomeric states, such as protonated and deprotenated forms in pH measurements (Carlsson et al. 2000; Rink, Tsien, and Pozzan 1982; Szmacinski and Lakowicz 1993). Measuring the fluorescence lifetime is also one of the best and most reliable ways to quantify FRET; this is the most common application of FLIM.

1.5 SPECIFIC FEATURES OF THE DIFFERENT PATHWAYS AND RATES OF DE-EXCITATION

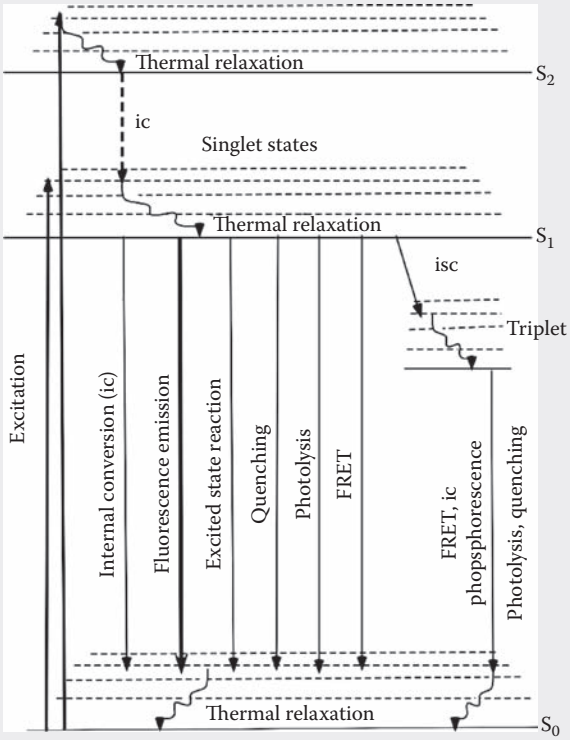
In FLI we usually excite the molecules with light. As has been already mentioned, the total rate of leaving the excited state, which is the reciprocal of the measured excited-state lifetime, is the summation of the rates of all the possible pathways. This is usually depicted in the form of a Jablonski diagram (or perhaps this should also be termed a Perrin–Jablonski diagram; see Box 1.2; Birks 1970; Lakowicz 1999; Nickel 1996, 1997; Valeur 2002). We emphasize again that, when we measure fluorescence lifetimes, we are not measuring the intrinsic rate of emission proceeding only through the fluorescence pathway; on the contrary, we are measuring the total rate of leaving the excited state, which is the *sum* of all the rates for leaving the excited state.

During the time that a molecule is in an excited state, it interacts intimately and dynamically with its molecular environment. Spectroscopists have long taken advantage of the unique molecular information that is available from the emission from a molecule in an excited state when the dynamic decay is resolved by measuring it directly in the time domain (Birks 1970; Cundall and Dale 1983; Lakowicz 1999; Valeur 2002). Although a molecule spends only a very short time in the excited state—picoseconds to nanoseconds—the eventual emission of a photon bears the historical imprint of this sojourn in the excited state. Many events can happen during this short time. Understanding the dynamic characteristics of the major pathways of de-excitation is essential, and it is the starting point for interpreting lifetime measurements.

1.5.1 Intrinsic Rate of Emission (Fluorescence)

The *intrinsic spontaneous rate of fluorescence* defines the longest average time the molecule can stay in the S_1 excited state; it is defined wholly by the quantum mechanical nature of the excited and ground-state electric configurations of an isolated molecule. It can be thought of as the rate constant (that is, the probability per unit time) at which a molecule isolated from all other molecules and with no other pathways of de-excitation will leave the lowest (first) singlet excited state by emitting a photon and returning to its electric ground state. The intrinsic emission rate is the same as the Einstein spontaneous emission rate calculated in most spectroscopy and quantum mechanics textbooks (Atkins and Friedman 1997; Becker 1969; Chen and Kotlarchyk 1997; Craig and Thirunamachandran 1984; Förster 1951; Kauzmann 1957; Lakowicz 1999; Lippert and Macomber 1995; Parker 1968; Schiff 1968; Silfvast 1996). The intrinsic emission rate is also related to the uncertainty in the energy of a system that has a finite lifetime through the relation

BOX 1.2 PERRIN–JABLONSKI DIAGRAM



Perrin–Jablonski Energy Level Diagram
of a Fluorescent Molecule

Energy increases vertically up. The arrows represent the transition of a quantum change. The transitions begin at the lowest vibrational levels of each electronic state (S_0, S_1, and S_2) for both the absorption (upward arrows) and the de-excitation processes (downward arrows). The transition from the excited state to the ground state always happens from the lowest level of S_1 (first singlet excited state). The de-excitation transitions other than the emissive, internal conversion and intersystem crossing transitions are gathered together in a box at the right of the diagram. Via intersystem crossing (isc), the excited molecule passes from the singlet (S_1) to the triplet (T) state.

Emission from the triplet state is phosphorescence. The other de-excitation transitions from the triplet state are similar to those from the singlet state. The electronic transitions usually leave the molecule in an excited vibrational level of the end electronic state. This vibrational excitation energy relaxes thermally very rapidly, within picoseconds, to the lower vibrational levels of the corresponding electronic states. Two possible absorption (excitation) transitions to two different singlet excited states are shown (both contained within the dotted box). If the S_2 state becomes excited, the molecule immediately relaxes by internal conversion to the S_1 state.

$$\Delta E \Delta t = \Delta E \tau_f \geq \hbar$$

where $\hbar = h/2\pi$ and $h \equiv$ Planck's constant. This coupled uncertainty of the values of the energy of a system and the time that the system is in that state is not a true Heisenberg uncertainty relation (Atkins and Friedman 1997; Landau 1997; Schiff 1968).

Nevertheless, the lifetime of the intrinsic emission decay is related to the breadth of the energy of emission. This rate is never measured in FLIM (or any other spectroscopic experiment carried out in solution); neither is the extremely narrow spectral width of the intrinsic fluorescence rate. For this chapter, it is only important to recognize that the intrinsic rate of emission is a basic quantum mechanical property of an isolated molecule and can in principle be calculated from its energy levels and wave functions of its quantum states. The fundamental physical explanation for this intrinsic spontaneous emission is due to the coupling of the excited and ground states through the interaction with what is called fluctuations in the zero-point level of the photon modes of the vacuum radiation field.

Although the density of these photon modes is generally the same in most environments, it is possible that the density can change, and this will change the intrinsic rate of radiation emission. For instance, when a fluorophore is very close to a metal surface with a very sharp curvature, the intrinsic rate can be affected because the density of the photon modes can be increased. We do not discuss this interesting phenomenon, but refer the reader to recent literature where he or she can also find earlier references (Enderlein 2002; Fiuráek et al. 2001; Hamann et al. 2000, 2001; Sánchez, Novotny, and Xie 1999). This phenomenon has until now not been of much use for biological fluorescence measurements, but it is interesting for the future, especially from the point of view of lifetime measurements (it is a way to shorten the intrinsic lifetime). However, for our purposes, it is only necessary to know that this rate of spontaneous emission does not change unless the coupling between the ground and excited electronic structures of the molecule changes (e.g., a change in the positions of the nuclei, changing the molecular conformation).

1.5.2 Thermal Relaxation (Internal Conversion)

The process of internal conversion leads to a nonradiative transition from the excited to the ground state. Thermal interactions with the solvent surrounding the fluorophore or with the immediate surrounding molecular matrix reduce the time a molecule spends in the excited state by providing another pathway (other than the intrinsic photon emission) for leaving the excited state. The excited molecule is coupled to its environment through vibrations and collisions. These interactions are dependent on the temperature and the composition of the solvent. The vibronic coupling considerably broadens the spectrum in solution and reduces the fluorescence lifetime.

The intermolecular coupling of the thermal environment is not the only internal conversion pathway to the ground state. Intramolecular vibrational interactions also facilitate very rapid relaxations from higher vibrational levels of excited molecules to the lowest vibrational states of the first excited state (resulting in a Boltzmann distribution among the lowest vibrational levels of the first electronic excited state). At the normal temperatures of biology, the lowest energy vibrational state is by far the most populated vibrational state. Thermal coupling with

the solvent molecules is the main reason why the energy of emission is always less than the energy of excitation (the Stokes shift: part of the excitation energy is lost to the environment).

This is clear by looking at the Perrin–Jablonski diagram in Box 1.1 and comparing the length of the excitation vector to that of the fluorescence emission vector. This vibrational coupling to the environment also broadens the fluorescence spectrum, so the individual vibrational bands can only rarely be observed. Biological samples are almost always in condensed media and usually measured at ambient temperatures or not far from them. Thus, the intrinsic rate of fluorescence plus the rate of thermal nonradiative deactivations will contribute to the apparent longest experimentally determined decay time observed for fluorophores in biophysical measurements.

1.5.3 Molecular Relaxation of the Solvent or Molecular Matrix Environment

In biophysical measurements, the fluorophores are almost always in a complex condensed matter environment, such as an aqueous environment or in an apolar surrounding such as in lipid membranes. Other biological components can also interact and couple strongly to the excited molecule. Coulomb and dipole interactions with the surrounding environment affect the position and the breadth of the emission. In a polar environment, the solvent molecules (or the neighboring molecular matrix) reorient around the excited molecule during the time window of the excited state (this happens especially in an aqueous solvent due to the very large dipole moment of water, 1.85 debye). This *solvent relaxation* will take place if the dipole moment of the excited molecule differs from the dipole moment of the ground state, changing the energy of the excited electronic state. This can often be observed in a polar aqueous environment and when internal charge transfer takes place after the molecule is excited into the excited state.

These local dipole relaxations, which take place subsequent to the much faster thermal relaxations to the lowest vibrational states, can lead to a further decrease in energy of the excited molecule before emission, shifting the emission toward lower energies—a red shift. The extent of this solvent relaxation is dependent on the temperature and the viscosity of the molecular environment. If the solvent relaxation occurs before or during the time when the molecule exits the excited state, it contributes significantly to the Stokes shift in a highly polar solvent environment; that is, the energy (wavelength) of the emission is less than (longer than) the energy (wavelength) of excitation.

Interestingly, if the "solvent" or "matrix" relaxation occurs on the same time scale of the fluorescence emission, the rate of the relaxation of the molecular matrix can be directly observed in the measured fluorescence lifetime. In this case, if the *measured* dynamic signal shifts out of (or into) the wavelength bandwidth of the emission optical filter, an extra component will enter into the measured signal. This would usually be classified as an artifact; however, it is clear that the time-dependent wavelength shift of the fluorescence emission contains valuable information on the molecular environment. Strong solvent interactions and charge transfer processes can also change the fluorescence lifetime by changing the overlap of the excited and ground-state wave functions.

The spectrum shifts can be observed in a steady-state fluorescence spectrum; spectral shifts of fluorophores have been used extensively in fluorescence imaging without

temporal resolution. However, the emission spectrum can be recorded in a time-resolved mode, giving the shift of the spectrum as a function of the time. Such a time-resolved experiment would record directly the relaxation of the polar "solvent." Detailed measurements utilizing either of the solvent relaxation processes have not yet been extensively employed in FLI; however, considering the heterogeneous environment in a living cell, it is clear that this dynamic information is valuable. Detailed research of the polarity changes and micro-organization in the molecular environment of certain fluorophores—for example, laurydan (which has the very sensitive polarity-sensitive chromophore prodane)—has been carried out in biological membranes (Bagatolli and Gratton 1999; Dietrich et al. 2001).

1.5.4 Quenchers (Dynamic)

Dynamic quenchers collide with the excited molecule by random diffusion (Box 1.3). Because the excited-state lifetime is of the order of nanoseconds, only those quencher molecules very close to the excited molecule will be effective. Triplet oxygen is a major cause of dynamic quenching in normal biological milieu. However, other molecules are effective quenchers, such as Br^-, I^-, and acrylamide (nonpolymerized). Many effective "collisional quenchers" can increase the rate of transfer to the triplet state of the excited molecule (intersystem crossing; see later discussion), thus removing the singlet excited state and thereby decreasing the prompt fluorescence signal. This perturbation is usually accomplished through spin–orbit coupling (where the spins of electrons of the quencher perturb the orbital states of the fluorophore, increasing the rate of transfer from the singlet state to the triplet state of the fluorophore; see Section 1.5.7).

The spin–orbital coupling is especially effective if the collisional quencher has an unpaired, weakly held outer orbital electron (such as I^- and Br^-). Spin–orbit perturbation also takes place with the electrons of the excited molecule itself (see discussion of intersystem crossing in Section 1.5.7). In general, only smaller charged ions are effective quenchers due to their rapid diffusion; however, charged, or highly polarizable, groups on macromolecules can also effectively quench fluorophores that are attached, either covalently or simply bound, to the macromolecules.

For FLI, the principal effect of quenchers is that the fluorescence lifetime is shortened. Often in cellular imaging it is difficult to correlate the intensity of fluorescence with the concentration of the probe because a decrease in intensity could come from a smaller number of fluorescing molecules or from quenching. FLI can easily distinguish these two possibilities by determining the reduction in the quantum yield due to the dynamic quenching, thus allowing an accurate calculation of the concentrations. This is a very powerful application of FLI and is only possible if the fluorescence lifetime can be determined at every pixel of the image.

1.5.5 Excited-State Reactions

If the fluorophore in the excited state reacts with a reaction partner selectively, then lifetime-resolved imaging can be used to map the location of the reactive component. The analysis is the same as that discussed earlier for dynamic quenching (collisional quenching

BOX 1.3

Dynamic quenching involves diffusion of the quencher (and sometimes the diffusion of the fluorophore). We can measure the rate of dynamic quenching by simply measuring the rate of fluorescence decay in the presence and absence of quenching:

$$1/\tau_{f,meas}^{+quencher} - 1/\tau_{f,meas}^{-quencher} = \sum_{i} k_i - \sum_{i \neq quencher} k_i = k_{quencher} = k_q [Q]$$

If we assume that the encounter between the fluorophore and the quencher is diffusion controlled and neglect transient terms (Valeur 2002), then

k_q is the rate constant of diffusional encounter quenching, $k_q = 4\pi N' D$, where the diffusion coefficient, D, is $D = kT/6\pi\eta a$ cm²/s (usually about 10^{-5} cm²/s);
a is the radius of the quencher in centimeters (the distance of closest encounter);
k is Boltzmann's constant;
N' is Avagodro's constant divided by 1,000, and
η is the viscosity.
$[Q]$ is the concentration of the quencher in moles per liter.

This will give the rate constant k_q in liter mole^{-1} s^{-1}, and it usually has a value of about 10^9–10^{10} liter mole^{-1} s^{-1} for ion quenching.

If the quencher concentration is known, we can determine the effective viscosity of the environment. The rate of dynamic quenching is inversely dependent on the viscosity of the environment and representative dimensions of the quencher molecule and the fluorophore. If the quencher is spherical (with radius "a") and only the quencher diffuses, then $k_{quencher} \propto 1/6\pi\eta a$, where.

Thus, the lifetime not only can provide corrections to dynamic quenching, but also can furnish indications of the rigidity (effective viscosity) of the molecular environment of a fluorescence probe through the dynamics of quenching. (Actually, it provides information about the relative mobility of the fluorescence probe and the quencher molecule.) This could be deduced only with great difficulty from steady-state experiments in an image, but it is a simple experiment for FLI.

is essentially an excited-state reaction that does not destroy the fluorophore). For instance, pyrene forms eximers–dimers of pyrene, where one of the reaction partners is a molecule in the excited state and the other bimolecular partner is a pyrene in the ground state. The eximer emission is considerably red, shifted from the emission of the monomolecular species. However, the eximer is an independent chemical species and as such it is the product of an excited-state reaction and will shorten the emission lifetime (and lower the intensity) of the original independent monomolecular excited pyrene molecules. FRET can be considered to be an excited-state reaction.

1.5.6 Förster Resonance Energy Transfer (FRET)

FRET is one of the major applications of FLIM. In FRET the excitation energy of one molecule (called the donor, *D*) is transferred nonradiatively to a nearby molecular chromophore

BOX 1.4

The rate of energy transfer between single donor and acceptor molecules is proportional to $1/R^6$, where R is the distance between the centers of the two chromophores. Förster (1946, 1948, 1951) showed that the rate of energy transfer could be expressed as

$$k_{ET} = \frac{1}{\tau_{F_D^A}} \left(\frac{R_0}{R} \right)^6 \tag{1.1}$$

R_0 is the value of R where the rate of energy transfer, k_{ET}, equals the rate of de-excitation from the excited state in the absence of the acceptor ($1/\tau_{F_D^A}$). R_0 can be calculated from knowledge of the relative orientations between the transition dipoles and the spectral overlap of the emission spectrum of the donor and the absorption spectrum of the acceptor (Förster 1951).

in the ground state (called the acceptor, *A*). The probability of photon emission of *D* is thereby diminished, and *D*'s lifetime in the excited state is shortened. FRET usually takes place over a *D*–*A* separation of 0.5–10 nm. A Coulomb charge–charge interaction (effectively, a dipole–dipole interaction) between the excited *D* molecule and the ground-state *A* molecule takes place through space; no photon is absorbed or emitted. Spectral overlap of the emission and absorption spectra of *D* and *A* is required. The quantum yield of *D* and the absorption coefficient of *A* must be great enough for a significant probability of transfer. FRET can be coupled to a wide variety of biological assays that yield specific information about the environments of the chromophores and distances between *D* and *A*. FRET is probably the major reason why many people want to make FLI measurements (see Chapters 2 and 9).

From the rate of energy transfer (see Box 1.4), we can gain *quantitative* information about the distance between *D* and *A*. We can sometimes learn about the relative orientation between *D* and *A* transition dipoles (the effectiveness of FRET depends on the orientation of the transition dipoles of *D* and *A*).

FLI overcomes difficulties in making reliable FRET measurements in an image using intensities. It is difficult to quantify FRET measurements in imaging experiments (fluorescence microscope) using steady-state fluorescence because standards must be used to calibrate the fluorescence signals; that is, we must compare the fluorescence intensity in the presence and absence of acceptor (Bright et al. 1989; Dunn and Maxfield 1998; Fan et al. 1999; Opitz 1998; Silver 1998). Usually, the variability of concentrations between different biological cells or the distribution within a biological cell is unknown. As can be seen from the equations in Box 1.5, there is no reason to calibrate intensities using FLIM; one only has to be able to measure the lifetimes accurately. This is the great advantage of lifetime measurements. Given that nowadays it does not take too much time to measure a lifetime-resolved image, FLI is the method of choice if one has the instrumentation. We defer further discussion of applications of FLI for FRET measurements until we have considered the methods (see also Chapter 2).

BOX 1.5

The efficiency of energy transfer can also be called the quantum yield of energy transfer because it is the fraction of times that excited molecules follow the ET pathway of de-excitation. Equivalently, it is the ratio of the rate of energy transfer (see Box 1.4) to the total rate (including energy transfer) of de-excitation from the excited state. The efficiency can be determined easily by measuring fluorescence lifetimes. One only has to measure (or know) the fluorescence lifetime of the donor in the absence of the acceptor, $1/\tau_{F_D^{-A}}$, and measure the fluorescence lifetime in the presence of acceptor $1/\tau_{F_D^{+A}}$. The following ratio then determines the efficiency

$$E = \frac{\text{rate of energy transfer}}{\text{total de-excitation rate}} = \frac{\left(1/\tau_{F_D^{+A}}\right) - \left(1/\tau_{F_D^{-A}}\right)}{\left(1/\tau_{F_D^{+A}}\right)}$$

$$= \frac{k_{ET}}{\tau^{-1}_{F_D^{+A}}} = \frac{k_{ET}}{\tau^{-1}_{F_D^{-A}} + k_{ET}} = \frac{1}{\left(k_{ET}\tau_{F_D^{-A}}\right)^{-1} + 1} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} \tag{1.2}$$

1.5.7 Intersystem Crossing and Delayed Emission

The first time-resolved imaging experiments were carried out on samples with delayed emission—phosphorescence and delayed fluorescence (Marriott et al. 1991). The time range of phosphorescence is microseconds to seconds. The ground states of most fluorophores are singlet states, and the first excited state is also a singlet state (the electrons in the highest occupied electronic levels, in the ground and the excited electron configurations, are paired with opposite spins). Thus, the transition from the excited state to the ground state (fluorescence) is "spin allowed" and therefore takes place in the nanosecond time scale. Due to spin–orbit coupling involving the electron that has been elevated to the excited state, there is a probability that the spin of the excited electron will flip, creating a triplet state (where two electrons have parallel spins) with lower energy (intersystem crossing, k_{isc}).

The transition between singlet and triplet states is not highly probable and is only partially allowed (not spin allowed). Therefore, the rate for the triplet molecule to deactivate (e.g., by emission of a photon) to the singlet ground state is much slower than the normal intrinsic rate of fluorescence or the deactivation by internal conversion. If the probability of a singlet–triplet transition (intersystem crossing) is high enough, then the formation of a triplet state will become a viable competitor with the other de-excitation processes of the original singlet state. Intersystem crossing then becomes a competing kinetic pathway, decreasing significantly the measured lifetime of the fluorescence emission from the singlet state. If the triplet can emit a photon, a long-lived emission decay is also observed. The triplet state is very short-lived unless oxygen is removed from solution; therefore, phosphorescence emission is usually not observed.