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RECOMMENDED METHODS FOR FLUORESCENCE DECAY ANALYSIS

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Recommended methods for fluorescence decay analysis

Abstract - This article reviews methods of analysis of fluorescence decay data collected by frequency and time domain techniques. The methods are assessed critically and recommendations are made to assist users to determine the method appropriate for selected analyses. The review covers data collection methods and their pitfalls, data analysis methodolgy and techniques used to judge goodness of fit.

INTRODUCTION

Measurement of the time-resolved fluorescence properties of a molecule is an integral part of the determination of the photophysical parameters of the species. These properties are useful in spectroscopy, kinetics, energy transfer, analytical applications and in characterization of chemical, biological and physical systems which emit light. The purpose of this document is to introduce the concepts of time-correlated fluorescence spectroscopy and the methods of determination and analysis of lifetimes. Two recent texts are devoted to the topic, Demas [1] and O'Connor and Phillips [2]. Detailed examples of applications of lifetime analysis to chemical and biological problems were the topic of a NATO Advanced Study Institute [3].

The topics to be covered include:

- Data Collection Methods
    - Frequency-Domain Methods
    - Time-Domain Methods
- Pitfalls in Data Collection
    - Common to Frequency- and Time-Domain Methods
    - Specific to Time-Domain Methods
- Data Analysis
    - Methods
    - Judgment of Goodness of Fit
- Models in Data Analysis

For a general introduction to photochemical terminology, and to steady state fluorescence spectroscopy and recommended methods and reference materials for calibration of emission spectrometers, see other IUPAC documents [4].

DATA COLLECTION METHODS

Fluorescence is the spontaneous emission of radiation from an excited molecule, forming a low energy state of the molecule in the same spin multiplicity as the emitting excited state. For a steady state absorption-emission system, the important photophysical parameters are the *quantum yield* , ϕ, of fluorescence and the *fluorescence decay time (lifetime), τ*. They are defined by eqns 1 and 2, in which the rate parameters are defined conventionally as listed below.

$$\phi_f = k_f / (k_f + k_{isc} + k_{ic} + k_d) \qquad (1)$$

$$\tau_f = (k_f + k_{isc} + k_{ic} + k_d)^{-1} \qquad (2)$$

Where: k_f is the rate constant of fluorescence,

                k_{isc} is the rate constant of intersystem crossing,

                k_{ic} is the rate constant of radiationless internal conversion, and

                k_d is the rate constant of formation of products

from the emissive state.

Two methods are normally employed to determine fluorescence lifetimes, or, more generally, to recover the parameters characterizing the response of a fluorescent sample to a Dirac-pulse of light (impulse response): time-domain methods (pulse fluorometry), referred to commonly as time-correlated single photon counting (SPC) or single photon timing (SPT) techniques, and frequency-domain methods (phase/modulation fluorometry). The former uses a short exciting pulse of light and gives the impulse response of the sample, convoluted by the instrument response, while the latter uses modulated light at variable frequency and gives the harmonic response of the sample. The harmonic response is the Fourier transform of the impulse response. Therefore the methods are theoretically equivalent. The principles of the instruments are different, but each technique requires an accurate temporal characterization of the instrument response. Each method will be discussed.

Frequency domain methods: phase/modulation techniques

Phase fluorometers using light modulated at one frequency were historically among the first apparatus for lifetime determination. In recent instruments, variable modulation frequency can be achieved from 1 MHz up to 200 MHz by means of a CW laser (or a lamp) together with a Pockels cell, or up to 2 GHz by using the harmonic content of a pulsed laser source.

Upon excitation by a sinusoidally modulated light source, $E(t)$, a fluorescent sample emits light $D(t)$ sinusoidally modulated at the same frequency but delayed in phase and demodulated with respect to the excitation in a manner related to the impulse response $G(t)$ and the angular frequency ω. The time dependence of the excitation can be written as eqn 3 where m_0 is the modulation depth (AC/DC ratio). The angular frequency is defined by eqn 4.

$$E(t) = E_0 \left(1 + m_0\, e^{j\omega t}\right) \tag{3}$$

$$\omega = 2\pi f \tag{4}$$

The system response, $D(t)$ is given by eqn 5 and can be expressed as eqn 6 where m is the modulation depth of the fluorescence and ϕ is the phase shift.

$$D(t) = \int_{-\infty}^{t} E(t')\, G(t\text{-}t')\, dt' \tag{5}$$

$$D(t) = E_0 \left[1 + m\, e^{j(\omega t - \phi)}\right] \tag{6}$$

The modulation ratio, $M = m/m_0$, and the phase shift, ϕ, characterize the harmonic response, eqn 7. The integral represents the Fourier transform of the impulse response. M and ϕ are defined in eqns 8 and 9, respectively. P and Q are the

$$M\, e^{-i\phi} = \int_0^{\infty} G(t)\, e^{-j\omega t}\, dt \tag{7}$$

$$\phi = \tan^{-1}(P/Q) \tag{8}$$

$$M = (P^2 + Q^2)^{1/2} \tag{9}$$

sine and cosine transforms of the impulse response (eqns 10, 11). In the case where

$G(t)$ is a single exponential with lifetime τ, then ϕ and M are expressed by eqns 12 and 13. These relations provide the basis of two independent determinations of the lifetime.

$$P = \frac{\int_0^\infty G(t) \sin (\omega t)\, dt}{\int_0^\infty G(t)\, dt} \qquad (10)$$

$$Q = \frac{\int_0^\infty G(t) \cos (\omega t)\, dt}{\int_0^\infty G(t)\, dt} \qquad (11)$$

$$\phi = \tan^{-1} (\omega \tau) \qquad (12)$$

$$M = (1 + \omega^2 \tau^2)^{-1/2} \qquad (13)$$

When $G(t)$ is multiexponential (eqn 14), then P and Q are given by eqns 15 and 16.

$$G(t) = \sum_{i=1}^{n} a_i e^{-t/\tau_i} \qquad (14)$$

$$P = \frac{\omega \sum_{i=1}^{n} \dfrac{a_i \tau_i^2}{1 + \omega^2 \tau^2}}{\sum_{i=1}^{n} a_i \tau_i} \qquad (15)$$

$$Q = \frac{\sum_{i=1}^{n} \dfrac{a_i \tau_i}{1 + \omega^2 \tau^2}}{\sum_{i=1}^{n} a_i \tau_i} \qquad (16)$$

For very short lifetimes, $D(t)$ is strongly demodulated but only slightly shifted in time, while for long lifetimes it is only slightly demodulated but significantly shifted. Phase modulation techniques are particularly good for short lifetime determinations (to the ps range), and for resolution of spectra into their components on the basis of the lifetimes of these components (phase-resolved spectra).

Instruments which allow a user to measure **both** the phase shift ϕ and the modulation ratio M can enhance the utility and dynamic range of a phase shift apparatus. Determination of lifetimes of several components in a mixture, or analysis of complex $G(t)$, is possible provided that ϕ and M can be monitored over the proper frequency range. Instruments using cross correlation detection offer more accurate measurements of ϕ and M.

Curve fitting of $\phi(\omega)$ and $M(\omega)$ are directly performed by nonlinear least-squares methods and therefore, in contrast to time domain methods, no deconvolution is necessary. The reduced chi-squared value is generally used to judge the quality of the fit.

Commercially available instruments use a Pockels cell with either a laser (He-Cd) or a lamp (Xe). With the He-Cd laser, only two wavelengths can be selected (325 nm and 442 nm). However, since a power of a few milliwatts is generally sufficient, inexpensive, small CW lasers can also be used in the visible region (air-cooled argon ion lasers, tunable He-Ne lasers, etc.). With the xenon lamp source, it is possible to work at wavelengths ranging from 250 nm to 900 nm, but signal to noise decreases, especially with biological samples (or other potential nonhomogeneous samples). Examples of the successful application of phase/modulation techniques to complex systems are discussed by Lakowicz, Gratton and others [5].

Time domain methods: time-correlated single photon counting (timing)

The most popular method of lifetime determination, as judged by number of citations to the method and instruments sold, is time-correlated single photon counting (SPC), or more properly, single photon timing (SPT). We urge the use of the latter name for this method and will follow the practice in this document. The technique employs a short pulse width excitation source (usually a flash lamp, ~1-3 ns pulse width; or a laser, ps-ns duration) which is flashed at relatively modest repetition rates (10^4-10^5 Hz, or, for laser sources, up to the mode-locking frequencies of e.g., 10^8 Hz). A portion of the flash produces a signal as a start impulse for a time-to-amplitude converter (TAC), and the remainder excites the sample. The TAC generates a signal which is directly proportional to the time between the start pulse and a stop pulse generated from the detector side of the instrument by a constant fraction discriminator. The TAC signal is sent to a multichannel analyzer (MCA), used in the pulse height mode. The analog output of the TAC is digitized by an analog-to-digital converter and sent to the MCA. The resulting binary value provides the address of the location (channel) in the memory of the MCA where the content has to be incremented by one. After averaging many flashes, a histogram of the emission decay is developed. At high emission intensities, a pile-up artifact can result, since the TAC is inactivated when the **first** incoming photon is detected. Because the TAC can only provide a signal proportional to the time interval between the start pulse and this first detected photon, an artifact can result. To avoid distortion the number of detected photons per pulse should be kept low (about 1 per 100 excitation pulses or less).

The channel contents of the MCA are independent and strictly Poisson distributed. For sufficiently high numbers of counts per channel, Gaussian statistics are valid. This approximation aids in weighting data in the least squares analysis, see below. Measurement of decay over three to four decades is possible. The method is highly linear throughout the range. A disadvantage of the method, when using flash lamp excitation (nitrogen, hydrogen or deuterium), is that excitation wavelengths are restricted to the 200-400 nm range. Combinations of laser excitation sources can be used, but at the penalty of high cost. Also, when long collection periods are required, errors due to drift in the system can be minimized by measuring the decay of the sample in alteration with collection of the instrument response from a scatter solution or the decay of a reference compound (see below).

Time-resolved spectra can be obtained in two ways. The spectra can be reconstructed from the decays collected at the various emission wavelengths. Then the MCA is used in the pulse height mode. Alternatively, the spectra can be collected directly using time windowing with a single channel analyzer after the TAC and use of a scanning monochromator. The MCA is then used in its multichannel scaling mode.

Data analysis is discussed separately below.

PITFALLS IN DATA COLLECTION

There are several major concerns in collection of emission decay data. They are discussed briefly here to collect them in one place for the reader.

Pitfalls common to both time- and frequency-domain methods

Wavelength-dependent time response of the instrument   Standard PMT tubes have transit times which depend on the wavelength of the incident photon. The effect of wavelength on transit times for microchannel plate detectors is small to negligible. This color sensitivity is greater in end-on tubes than in side-on tubes. Nevertheless, it represents a significant potential problem requiring attention. Wavelength dependence results also from the possible differences in spatial distributions of the sample and scattering solution images on the phototube. The most efficient way to alleviate the wavelength dependence of the instrument response function is to employ a reference compound whose decay is exponential, which absorbs light at the wavelength of excitation of the unknown, and emits at the wavelength where emission is detected. The fluorescence response of this reference can be used for data analysis (see below). In phase/modulation techniques, the phase shift and the modulation ratio can be measured directly against a reference compound. When a scattering solution is used as a reference, the modulation ratio is unaffected by timing errors, whereas correction for phase data is necessary. This correction can be easily made when the timing errors result in a frequency-independent time shift which can be determined independently.

Polarization effects   Monochromators and PMT's have polarization-sensitive through-put and response. If the sample is excited with (partially) polarized light, and if molecular motion occurs on the same time scale as the emission, then distorted signals will result. Since rotational relaxation times of typical organic molecules are on the order of 100 ps in solvents of normal viscosity (0.1-1 cP), polarization effects are rarely a problem when using pulsed-lamp sources. For picosecond laser sources, the problem becomes serious. To remove the distortion, the excitation-pulse polarization must be known in the laboratory frame of reference. For excitation polarized along the vertical to the excitation-emission plane, the light emitted from the sample must be analyzed through a polarizer set at the magic angle (54.7°) with respect to the vertical. Polarization effects will distort the decay profile of a typical short lifetime scintillator or laser dye. One way of testing a system is often to measure the decay profile of a dye known to decay exponentially. In a properly functioning system free of polarization effects, the observed decay should be cleanly single exponential over at least three decades. In phase/modulation techniques, polarization effects result in differences in lifetime measured by phase and modulation, and to a frequency dependence of the lifetime.

Scattering corrections   Light scattering from turbid solutions or from solids is an optical problem which can be minimized by use of filters. When scattering persists its contribution to the data must be removed by an appropriate subtraction algorithm.

Pitfalls specific to time-domain methods

TAC linearity and deadtime   A successful experiment requires linear time response of the TAC. It is probably the most critical aspect of data collection. In the normal SPT configuration, the TAC is activated by the START pulse from a

photodetector and the TAC is occupied either until an emitted photon, detected by the PMT, generates a STOP pulse, or until the TAC completes its sweep. The time during which the TAC is in operation and unable to respond to another signal is known as *deadtime*. Good collection statistics require that only a small fraction of START pulses result in STOP pulses. Data collection in the normal configuration results in substantial deadtime. In the reverse configuration, the PMT signal generates the START pulse and, by use of appropriate delays, the corresponding excitation pulse generates the STOP pulse. Deadtime is minimized and the TAC has a more linear response.

Photon pileup If too large a fraction of excitation pulses (~> 2%) results in the detection of emitted photons, the probability of two photons arriving simultaneously at the detector becomes significant. This phenomenon distorts the counting statistics. Counting efficiency can be increased to ~ 10% if a window discriminator is used to detect the difference in output voltage between one- and two-photon signals.

Counting rates With pulsed-lamp sources, the minimum usable count rate of PMT-detected photons is ~20 counts/sec. Typical noise levels for cooled side-on PMTS are 5-6 counts/sec. Lamp drift is the most serious problem associated with slow count rates when measurement times can stretch over several hours. Changes in room temperature are also a source of concern since the TAC timebase is sensitive to temperature fluctuations. For laser sources, count rates of several thousand counts/sec. are accessible. Counting rates above 1000 counts/sec. often lead to curious problems such as sinusoidal oscillations in the autocorrelation function of the residuals. A more serious problem with laser sources is that the intensity of the excitation pulses can cause sample decomposition.

Data collection Collecting data over 256 channels has become the norm for routine SPT measurements. For many types of analysis models, it is preferable to collect 512, 1024, or more channels of data. This prolongs the time needed for data acquisition, and also places demands on the computer analysis of the data. Matrices which have (256 x k) dimensionallity (where k is the number of adjustable parameters) can be manipulated quickly even on many PC's, but larger arrays present more formidable difficulties.

DATA ANALYSIS
Methods

Deconvolution methods must be used to extract decay parameter(s) from the collected data, $D(t)$, using knowledge of the excitation pulse, $E(t)$. Given the experimental data from the MCA in an SPT experiment, $D(t)$, and the instrumental response function, $P(t)$, obtained at the emission wavelength using a scattering sample, the object of the data analysis is to obtain the decay function, $G(t)$. Often, both the functions $D(t)$ and $P(t)$ are corrected for some background counts from detector noise or light leaks in the system. Wavelength effects on the photomultiplier response (see above) must also be considered in some cases. Such corrections are not included here.

The mathematical statement of the problem is given in eqn 17. For extremely long lifetimes $P(t)$ is not strongly convolved with $G(t)$ and direct methods may be used.

$$D(t) = \int_0^t P(t')G(t-t')\, dt' \qquad (17)$$

Equation 17 treats the histogram data as if it were continuous. Thus, eqn 17 is approximate.

There are many methods to solve eqn 17. The most commonly used methods are reviewed here. The reader is referred to more specific references for details of other methods [1,2].

Nonlinear least squares   Curve fitting using nonlinear least squares techniques is the most widely used method of deconvolution to solve eqn 17. The method usually involves linearization of the fitting function and least squares solution. Because the method is a statistical fitting method, large data sets must be used to insure validity. As in any fitting procedure, the results, that is the parameter(s) derived from it, should be judged against a physically meaningful model. The discussion below follows O'Connor and Phillips [2].

Least squares fitting produces a set of calculated points which describes the experimental set. The calculated values are optimized by minimizing the weighted sum of the squares of deviations of the calculated points, $Y(t)$, from the experimental ones, $y(t)$, according to eqn 18, for all i channels of data. That is, χ^2, is minimized.

$$\chi^2 = \sum_{i=1}^{n} W_i [y(t_i) - Y(t_i)]^2 \qquad (18)$$

In eqn 18, W_i is the weighting factor on the data in the ith channel of n total channels. W_i is usually approximated as the inverse of the measured value of $y(t_i)$ during the fitting procedure. The fitting function, $Y(t)$, is optimum when the condition of eqn 19 is minimized. Strictly, eqn 19 is valid only when the Gaussian approximation is valid and the variance of the calculated i^{th} data point is zero. Thus sufficient counts must be collected in the MCA to provide approximate Gaussian statistics. Various standard methods (simplex, Marquardt, analytical, etc.) may be

$$\chi^2 = \sum_{i=1}^{n} \left\{ [D(t_i) - Y(t_i)]^2 / D(t_i) \right\} \qquad (19)$$

used to seek the function giving the best fit. The Marquardt method is the most common. For multiexponential models, the minimization of χ^2 is done with respect to either the decay parameter itself or both the decay parameter and the preexponential factor. In practice, initial choices for decay parameter(s) τ_i and preexponential(s) a_i are chosen by the user which establishes an initial functional form for the decay function $G(t)$ (eqn 20). Scattered light contributions can be included

$$G(t) = \sum_{i=1}^{n} a_i e^{-t/\tau_i} \qquad (20)$$

and accounted for as a fitting parameter. The initial guesses are used to calculate $Y(t)$, and χ^2 determined. Partial derivatives of the calculated points with respect to the initial guesses are calculated, and they are used in a simple series expansion of the linearized function $Y(t)$ to provide a set of matrix equations which are solved to generate a new set of parameters, incremented from the initial set. In this manner, new values are reconvoluted until the χ^2 value converges to a minimum. Many SPT

instrument makers provide software for data reduction. Both Demas [1] and O'Connor and Phillips [2] give listings for Basic or Fortran programs that can be used to do simple least squares deconvolution. Use of any deconvolution method for more than three component decay ($i = 3$ in eqn 20) is discouraged. Methods such as *global analysis* (see below) are preferred in complex cases, but great care is required.

Method of moments The second most popular deconvolution method is the so-called method of moments. The treatment below follows Demas [1]. For a multiexponential decay function such as that in eqn 20 above, one defines a statistical moment (μ_i, ε_i, eqn 21, 22) for all the *i* parameters for both the decay function $G(t)$ and the excitation function $P(t)$ as defined in eqn 17. When eqn 21 and 22 are

$$\mu_i = \int_0^\infty G_i(t)\, t^i\, dt \tag{21}$$

$$\varepsilon_i = \int_0^\infty P_i(t)\, t^i\, dt \tag{22}$$

substituted into eqn 17, the expression of eqn 23 can be derived where the quantity D_S is as defined in eqn 24 (see reference [1] for some details). This set of 2n linear

$$\mu_i/i! = \sum_{s=1}^{i+1} \left\{ D_s[(\Lambda_{i+1-s})/(i+1-s)]!] \right\} \tag{23}$$

$$D_s = \sum_{i=1}^{n} (a_i \tau_i^s) \tag{24}$$

equations in the *D*'s can be solved using determinant methods to produce roots which are the *n* values of τ in eqn 24. These lifetimes are used to solve the series of linear equations represented by eqn 20 to yield the preexponential factors, *i*.

The method briefly described above is the general method of moments. Variations exist appropriate only for single exponential decays ($i = 1$ in eqn 20). The interested reader should consult Demas [1] and the primary references listed there.

Phase plane method This method is suitable for single exponential decays for which the lifetime is relatively long compared to the excitation pulse. It is a linearization method which, similar to others, directly delivers τ as a slope of a line. The treatment below follows Demas [1] who developed the method with Adamson.

For a single exponential sample decay $G(t) = ae^{-t/\tau}$ the convolution equation (eqn 17) can be written (eqn 25) in simplified form. Both sides of eqn 25 can be integrated in

$$D(t) = ae^{-t/\tau} \int_0^t P(t')\, e^{-t/\tau}\, dt' \tag{25}$$

the second time variable y (y runs from 0 to t) and rearranged to provide eqn 26. The

$$\frac{\int_0^t D(y)\,dy}{\int_0^t P(y)\,dy} = -\tau\frac{D(t)}{P(y)dy} + a\tau \tag{26}$$

integrals are calculated using the trapezoid rule from the observed instrument response $P(t)$ and decay $D(t)$ curves and a simple plot of $Z(t)$ versus $W(t)$ (see eqn 27, 28) produces a straight line of slope $-\tau$: $Z(t) = -\tau W(t) + K\tau$, where K is related to the

$$\frac{\int_0^t D(y)\,dy}{\int_0^t P(y)\,dy} = Z(t) \tag{27}$$

$$\frac{D(t)}{\int_0^t P(y)\,dy} = W(t) \tag{28}$$

preexponential but also contains instrument specific factors. The $Z(t)$ versus $W(t)$ plot is known as a phase plane plot.

Advantages of this method are extreme ease of use (it can be programmed on a hand calculator), and the sensitivity of the plots to nonexponentiallity. However, because of the widespread availability of computers capable of performing least-squares and other more sophisticated methods of analysis at higher precision than the phase plane method, use of this method is discouraged.

Transform methods   The basic convolution equation 17 can be transformed using either Laplace or Fourier formalisms and the transformed equations solved. However, since the transformed equations run over infinite time while the data runs only to finite times, immediate problems arise concerning how to judge convergence. That is, cut off corrections are required. Iterative techniques are employed to judge cut off, and in general suitable convergence can be obtained after five iterations. For multiple exponential decays where the lifetimes are similar ($\tau_1/\tau_2 < 2$), errors accumulate rapidly. We do not recommend this method and the reader is referred to discussions in Demas [1] or O'Connor and Phillips [2] and the original literature cited there for further details.

Global analysis   The simultaneous analysis of several related experimental sets of data is referred to as *global analysis*. Brand and coworkers [6] have developed and popularized this method of deconvolution and applied it to several complex analysis problems.

Target analysis In this method [7], one analyzes the decay directly in terms of discrete physical models, rather than relying on the empirical nature of the pre-exponential factors and relaxation times derived from conventional deconvolution. A *global target analysis* combines the two approaches into a very powerful method of analysis. It is the recommended methodology for solving complex problems involving linked emission decay experiments, e.g., those sets of experiments involving series of quencher concentration variations, or temperatures, or some other variable that leads to data sets that together constitute a functional block that needs to be solved cohesively. This method is amenable to solution using transform formalisms also.

In a global analysis, related experiments are simultaneously analyzed by considering the total collected fluorescence decay surface at once. This approach leads to a better parameter recovery with less uncertainty on the estimates, and to a better model discrimination power. A decay data surface is obtained by collecting decay samples across the wavelength range of an emission spectrum, for example, or along any other experimental axis. The total decay surface is then analyzed for all the different parameters, to reveal those parameters which are linked. It is not necessary that the set of decay experiments be analyzed for the same set of relaxation times (nor necessary that it be the set of relaxation times which are the linked parameters). In some cases the simultaneous analysis can only be realized by the assumption of an explicit model (the *target*, see section on kinetic models) in which the empirical parameters are the pre-exponential factors and the relaxation times. It is more common to reanalyze the first-obtained parameters (a_i and τ_i) in terms of the model parameters of true interest to the investigator, that is, the rate constants. This method is generally applicable to problems of direct interest to photochemists.

Deconvolution against a reference Improved ease and accuracy of fitting can often be obtained by using the experimental decay profile of a reference emitter in place of the instrument response function obtained with a scattering solution. This method is equally valid for both SPT and phase-shift instruments. The reference emitter can either emit at the same wavelength as the sample under study, or it may be a component of the emission to be fitted, e.g., the reference could be the localized ("monomer") emission of an aromatic unit used to help deconvolute an excimer (exciplex) rise-and-fall data set. The virtue of this method is that instrument response functions as well as complex kinetic components can be accommodated within one reference set of data.

Other methods Several other methods deserve mention, though they are not treated in detail here. Ware and coworkers [8] have developed an *exponential series* method reviewed by O'Connor and Phillips [2]. It is the objective of this method to fit the decay curve accurately rather than recover individual values of a_i and τ_i. Up to 30 exponentials are included. The drawback of this method is that the parameters returned have no physical meaning.

Valeur and coworkers [9] have developed a *modulating function* approach to deconvolution which is also described by O'Connor and Phillips [2]. The method is based on differential forms of the decay, instrument and impulse functions $G(t)$, $P(t)$ and $D(t)$, which can be easily calculated numerically. The differential equation is multiplied across by a modulating function, which is a function with the property that both the

function and its first derivative are zero at times t = 0 and t = T. The cross multiplied function is integrated from t = 0 to t = T where T is the time at which the data is truncated. The method allows for direct solution of the decay parameters using a set of n modulating functions, where n is the number of parameters to be solved for (i.e., four for a two component decay). The method would appear to have promise. It has successfully deconvoluted two and three component decays [2].

Maximum entropy methods (MEM) [10] are now being applied to decay analysis [11]. MEM promises to have great utility for complex systems since it is capable of recovering lifetime distributions over broad lifetime ranges. In the MEM, one does not analyze for a discrete set of multiexponential functions to fit the decay, but rather probes for a distributed set of N decay functions logarithmically spaced across the time spectrum. Decay function amplitudes are reconstructed from an entropy-like function with the imposed constraint that χ^2 must be near unity. The calculated spectrum of decay functions is known to be unique, and artefacts which introduce correlations between calculated parameters are eliminated.

Judgment of goodness of fit

This section addresses problems in data analysis and in presentation of data in such a way that the quality of fit can be assessed by others. This is a serious problem because all SPT measurements produce data distorted by the instrument time response function. These data are analyzed in the context of a model in which the instrument response function is convoluted into the decay profile predicted by the model. There are as yet no reasonable means for publishing raw data in a way that can be reanalyzed by other laboratories. Until such means are available, it is critical that at least data presentation in graphical form be effective and useful to the reader as a visual means of examining experimental data. Since the true value of the SPT technique is to provide decay profiles over two to four decades of decay (99.99%), published decay profiles should only be presented in logarithmic form, never in simple I vs t format. For complex decays, deviations in fits often only become obvious in the second or third decade of decay. While statistical parameters are important criteria for judging goodness of fit, we urge that plots of weighted residuals and autocorrelation of residuals versus channel number be routinely published along with the I vs log(t) data to aid visual inspection of data and fit.

For data reduction by least squares methods, statistical tests of the quality of the fitted decay function must be employed in order to judge the convergence criteria of the fit. The methods commonly used include the (reduced) chi squared value, plots of calculated residuals and the autocorrelation function, the runs test, and the Durbin-Watson parameter, as well as several less popular tests (Z-test, T-test). Additionally, two qualitative guides should be routinely used in practice: the effect of initial guesses on the fit parameters, and the effect of the fitting range on the fit. We will discuss several of these methods so the reader can begin to judge when SPT data in the literature are meaningful. For the user, experience is an invaluable guide.

It is useful to distinguish between *graphical* and *numerical* methods for judging the goodness of fit. The analysis of residuals and autocorrelation functions is *inherently graphical in its presentation and use, while chi- squared and runs tests are*

numerical in nature. Each has advantages and disadvantages and, again, experience is an invaluable guide.

Chi squared　　Chi squared (χ^2), as defined by eqn 19, can be modified in its definition to include truncation of the data set. In the fitting of the data for an actual SPT experiment the beginning channel (n_1) and the ending channel (n_2) for data reduction can be set by the user. Then χ^2 is properly defined as in eqn 29, which is strictly valid only when the ith data point $Y(t_i)$ is zero. A new, *reduced $(\chi_r)^2$* can be defined as in eqn 30 in which the n's have the meaning above and p is the number of variables in the fitting function. The reduced chi squared value is a

$$\chi^2 = \sum_{i=n_1}^{n_2} \left\{ [D(t_i) - Y(t_i)]^2 / D(t_i) \right\} \tag{29}$$

$$\chi_r^2 = \frac{\chi^2}{n_2 - n_1 + 1 - p} \tag{30}$$

convenient statistical tool to judge the quality of the fit since it is directly related to the convergence parameter. This quantity also has a precise interpretaion statistically. Acceptable values tend to be near 1 for ideal Poisson distributed data. Values too low (~0.75 or lower) are symptoms of too small a data set for a meaningful fit, while high values (>1.5) indicate significant deviation from the degree of exponentiallity fitted. "Ideal" values are 0.8 - 1.3. Fits with other values should be carefully examined to seek the source of the divergence. Since χ^2 is dependent on the number of degrees of freedom in the data, it is possible to calculate the normal deviation Z_{χ^2} (eqn 31, 32) corresponding to χ^2. Z_{χ^2} is normally distributed, so that normal distribution tables can be used to reliably judge the goodness of fit. From published tables of the χ^2 distribution in terms of the number of degrees of freedom (v, see eqn 32) of the system, one can determine the probability α that χ^2 exceeds some level ($\chi^2_{v,\alpha}$) for systems in which the number of degrees of freedom is less than about 100 (the limit of most tabulations).

$$Z_{\chi^2} = \frac{v(\chi_v^2 - 1)}{\sqrt{2v}} \tag{31}$$

$$v = n_2 - n_1 + 1 - p \tag{32}$$

Residuals　　For each channel of data, the residual value in that channel is the discrepancy between the actual datum and the calculated value, $R_i = D(t_i) - Y(t_i)$. The *weighted residual* $r(t_i)$ is given by eqn 33 in which $D(t_i)$ are the observed data, $Y(t_i)$ the fitted data and W_i is a weighting factor. The weighting factor most normally used

$$r(t_i) = W_i [D(t_i) - Y(t_i)] \tag{33}$$

corresponds to the standard deviations in the R_i. Good fits should yield randomly distributed residuals centered about zero. Weighted residuals are more sensitive at long times than unweighted residuals.

Autocorrelation The autocorrelation function (eqn 34) also provides a useful visual check of the quality of the fit. Random distribution about the zero point is

$$C_j = \frac{\frac{1}{m}\sum_{i=1}^{m} R_i R_{i+j}}{\frac{1}{N}\sum_{i=1}^{N} R_i^2} \qquad (34)$$

desired. The presence of any pattern in the distribution of either the residuals or the autocorrelation function is an indication that the model used is inadequate to describe the data. The autocorrelation function C_j is a measure of the correlation between the residual in channel j and channel $i + j$, summed over a selected number of the total channels (N). In eqn 34, $m = N\text{-}j$. In some cases the autocorrelation function is a more sensitive function than the residual plot and thus a more critical test for random noise present in high quality fits. Bad fits provide low frequency periodicity in an autocorrelation plot which can be easily detected.

Runs test Just as plots of residuals and the autocorrelation function signal bad fits by the presence of patterns in the plots, runs tests also seek repeating patterns, but they provide a numerical value that can be quoted rather than a qualitative picture. The most common runs test is the *ordinary runs test*, to be discussed here. Demas [1] also describes the *runs up/down test*, which is categorically not recommended for SPT data.

The ordinary runs test looks for patterns in binary data (yes, no; heads, tails; or positive, negative deviations) only; the magnitude of the deviation is not considered. For long sequences of data of total number N and number V of one type and T of the other ($N = T + V$), R is the total number of runs, where a run is defined as an unbroken sequence of a single value. For a normal distribution there exists an expected number of runs R_{exp} for truly randomly distributed data, given by eqn 35. The standard distribution σ in the number of runs is given by eqn 36 [1]. In practice, one

$$R_{exp} = 1 + 2VT/N \qquad (35)$$

$$\sigma = [2VT(2VT - N)]/[N^2(N - 1)] \qquad (36)$$

determines the number of runs of each type in the data, sums them to determine the total number of experimental runs, and compares it to the expected number of runs for some degree of confidence desired. If the numbers are similar, the model passes the runs test.

Durbin–Watson parameter A numerical parameter indicative of fitting quality for multilinear models is the Durbin-Watson (*DW*) parameter (eqn 37).

$$DW = \frac{\sum_{i=n_1+1}^{n_2} [r(t_i) - r(t_{i-1})]^2}{\sum_{i=n_1}^{n_2} [r(t_i)]^2} \qquad (37)$$

Its use in nonlinear fitting is premature and is not recommended for this application.

O'Connor and Phillips [2] urge the use of the *DW* parameter, which is similar functionally to the autocorrelation equation (eqn 34), but they argue that the *DW* parameter provides a statistically significant numerical value that can be quoted. For SPT data, *DW* values higher than 1.7-1.8 indicate acceptable quality fits for 256 or 512 data channels, using single, double or triple exponential models, but numbers lower than those imply an incorrect fitting function or skewed data [2]. This assertion is based on numerical simulation and extrapolation of the original statistical tabulations by Durbin and Watson, and they should not be taken as proved.

Parameter correlation One of the most neglected problems in time-correlated SPT is that of parameter correlation. After an appropriate model is chosen for data analysis, the computer uses a minimization method to obtain an "optimum" value of the fitting parameters. It often happens that a family of fits of comparable quality can be obtained with changes in one parameter compensated for by variation in another. By calculating the correlation matrix, one can determine the extent to which the values of the various fitting parameters are correlated with one another and thus establish confidence in the uniqueness of the fit.

MODELS IN DATA ANALYSIS

Time-correlated SPT is most valuable as a technique for measuring non-exponential decay profiles. Because of the instrumental response function, one needs a model to predict a decay form, which one then uses to fit the data. The kinetic models themselves generate mathematical functions which are conveniently grouped into two classifications. The first are mathematical functions for which the first and second derivatives can be obtained analytically. These derivatives are used in the fitting procedure which minimizes the differences between the raw data and the fitted function. Expressions containing sums and differences of exponential terms fall into this category.

A second class of function does not have derivatives which can be evaluated analytically. Computer programs for analysis must calculate the derivatives numerically. This can be time consuming. Following are several examples (not meant to be exhaustive) of kinetic models encountered in photochemical situations which lead to mathematical expressions which require fitting with numerical differentiation.

Distribution of sites   Emitting species located at different physical sites in a system emit with exponential decay times characteristic of each type of site, and the resulting overall decay profile is a superposition of all these emissions. If the number of sites is sufficiently large, the system can be viewed as a continuum of environments, characterized by an emission decay profile of the form in eqns 38 and 39.

$$k = \bar{k} \, \exp(\gamma x) \tag{38}$$

$$I(t) = \frac{I_0}{\pi^{1/2}} \int_{-\infty}^{\infty} \exp(-\chi^2) \, \exp[-\tau_0 \exp(\gamma x)] \, dx \tag{39}$$

Here the parameter γ characterizes the width of the distribution and τ_0 represents the unquenched (exponential) lifetime.

Energy transfer in rigid media   Energy transfer by the dipole coupling mechanism represents a special case of the system excited-dye/quencher pairs in a rigid matrix whose interaction rate $k(r)$ is distance dependent. In the specific case of dipole energy transfer in three dimensions, the energy transfer rate takes the form $k(r) = ar^{-6}$ and one averages over all pairs of orientations, eqns 40 and 41, where N'_A is

$$I(t) = I_0 \exp\left[\frac{t}{\tau_0} - P\left(\frac{t}{\tau_0}\right)^{0.5}\right] \tag{40}$$

$$P = 4\pi^{1.5} N'_A R_0^3 [A] \tag{41}$$

Avagodro's number per millimole, $[A]$ is the bulk concentration of the acceptor, **A** (in mole/liter), and R_0 is the critical energy transfer distance. This concept has been extended to direct (one-step) transfer on fractal surfaces and to cases of restricted geometry [12]. Under these conditions the decay profile takes the form of eqn 42 with $\beta = d/s$. Here d is the effective dimension of the system and s is the exponent in the expression $k(r) = ar^{-s}$. P' is related to the

$$I(t) = I_0 \exp\left[\frac{-t}{\tau_0} - P\left(\frac{t}{\tau_0}\right)^{\beta}\right] \tag{42}$$

concentration of acceptors within the distance R_0.

Energy transfer by the Dexter mechanism requires orbital overlap between donor and acceptor. The corresponding rate constant varies as $k(r) = ae^{-br}$ and leads to decay profiles of the form of eqn 43 where the function g is as given in eqn 44 and the

$$I(t) = I_0 \exp\left[\frac{-t}{\tau_0} - \alpha g(\beta t)\right] \tag{43}$$

$$g(z) = 6z \sum_{M=0}^{\infty} (-z)^M / [M!(M+1)^4] \tag{44}$$

terms α and β can be related to macroscopic constants. By expanding the $g(z)$ term, the decay reduces to the exponential-logarithmic expression of eqn 45, useful to describe a number of processes such as electron scavenging, electron-hole recombination and hole-burning phenomena in glasses [10]. Further complications

$$ I(t) = I_0 [-t/\tau_0 - \exp B \ln{}^\alpha (\frac{t}{\tau_0})] \qquad (45) $$

arise when the orientational distribution of the D-A pairs must be considered. Improvements in fluorescence data collection and analysis methods may be required to distinguish among multiparameter models which can describe these phenomena.

Transient effects in diffusion controlled processes   Rate coefficients for diffusion controlled reactions are time-dependent, reflecting the time evolution of the distribution of surviving reactants. In three dimensions, at times not too close to zero, $k(t)$ has the form originally derived by Smoluchowski, $k(t) = a(1 + bt^{0.5})$. This leads to fluorescence quenching decays as shown in eqn 46. Deviations from exponential decay occur at short times. They can be very difficult to detect. In systems where the product of the reaction generates new emission, as in excimer or exciplex formation or energy transfer, a convolution relationship connects the donor ($I_M(t)$) and the product ($I_E(t)$), as

$$ I(t) = I_0 [-At - 2B (\frac{t}{\tau_0})^{0.5}] \qquad (46) $$

$$ I_E(t) = I_M(t) \cdot k(t) \otimes \exp [\frac{-t}{\tau_E}] \qquad (47) $$

in eqn 47. Global analysis of both data sets (I_M and I_E) in terms of (46) is extremely sensitive.

Micelles   Fluorescence quenching in micelles is treated in terms of a model which assumes a Poisson distribution of fluorophores and quenchers. Analysis leads to decay profiles such as eqn 48 where B provides information from which the aggregation

$$ I(t) = I_0 \exp \{ -At - B[1 - \exp (-ct)]\} \qquad (48) $$

number of the micelle can be inferred.

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