A SYSTEMS-THEORY APPROACH TO THE ANALYSIS OF MULTIEXPONENTIAL FLUORESCENCE DECAY

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ABSTRACT A mathematical model of the fluorescence decay experiment based on linear systems theory is presented. The model suggests an experimental technique that increases the probability of correctly determining the decay constants of a multicomponent system. The use of moment methods for data analysis improves accuracy by combining information obtained from several discrete experiments. Examples are presented to show that the analysis of a three component system composed of known standards is improved as the number of experimental determinations is increased from one to four. The discrete measurements are made by changing the excitation and emission wavelengths.

INTRODUCTION

The analysis of multiexponential fluorescence decay curves is a problem that has attracted much attention in recent years, resulting in several mathematical methods of parameter estimation (1–4). The object of this paper is to consider the problem from the point of view of systems theory (5). This approach has suggested a new method of analysis that allows one to make use of observed data from several experiments. Examples are given demonstrating that the fluorescence lifetimes of pyrene, carbazole, and 2,5–diphenyloxazole (PPO) in dimethylformamide can be determined with improved accuracy if more than one experiment is used for the analysis. The systems-theory description also has the advantage of modeling the problem in a way that includes parameters related to the excitation and emission processes. In particular, the model indicates how more information can be gained by systematically varying these processes.

A SYSTEMS-THEORY MODEL

The fluorescence decay experiment is usually modeled as a convolution process:

$$f(t) = \int_0^t I(t-s)e(s)\mathrm{d}s + \xi e(t). \qquad (1)$$

In the somewhat traditional notation, $f(t)$ is the observed fluorescence, $e(t)$ is the excitation, ξ is the scatter coefficient, and $I(t)$ is the impulse response function modeled as an exponential sum:

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp(-\lambda_i t). \qquad (2)$$

The problem is to estimate the decay rates $\lambda_1, \lambda_2, \ldots, \lambda_n$ or equivalently the time constants $\tau_i = 1/\lambda_i, i = 1, 2, \ldots, n$.

Regardless of the method of analysis used, if the data [i.e. the discrete values of $f(t)$ and $e(t)$] do not adequately express each unknown decay rate, it is unreasonable to expect a computer program to produce satisfactory results. More precisely, difficulty may be caused by a particular amplitude, α_i, which may be too small to permit the detection of its associated decay rate, λ_i. We propose a method to help alleviate this type of difficulty.

Since the fluorescence decay experiment is a single input/single output process involving n distinct compartments or responses, it may be expressed in terms of the standard systems-theory equations (5, 6):

$$\dot{\mathbf{F}}(\mathbf{t}) = A\mathbf{F}(\mathbf{t}) + Be(t), \quad \mathbf{F}(0) = \mathbf{0}, \tag{3}$$

$$f(t) = C\mathbf{F}(\mathbf{t}) + De(t), \tag{4}$$

where A is an $n \times n$ matrix, B is an $n \times 1$ matrix, C is a $1 \times n$ matrix, D is a scalar, $\dot{F}(t)$ is the state vector, and $e(t)$ and $f(t)$ are the functions appearing in Eq. 1. It remains to interpret these parameters in terms of the fluorescence decay experiment. We denote the matrices in terms of their elements:

$$A = \{a_{ij}\}, \quad B = (\beta_1, \beta_2, \ldots, \beta_n)^T, \quad C = (\gamma_1, \gamma_2, \ldots, \gamma_n), \tag{5}$$

where T denotes transpose. The elements a_{ij} are called the transfer coefficients (from compartment j to compartment i). Our $a_{ij} = 0$ when $i \neq j$. Thus A is a diagonal matrix. Moreover, it is known from systems theory that the decay rates λ_i associated with the impulse response function $I(t)$ are necessarily eigenvalues of the matrix $-A$. Since A is diagonal it follows that:

$$A = \mathrm{diag}\,(-\lambda_1, -\lambda_2, \ldots, -\lambda_n). \tag{6}$$

To find, B, C, and D, we first integrate in Eq. 3 to obtain $\mathbf{F}(\mathbf{t}) = \int_0^t \exp[(t - s)A]Be(s)\mathrm{d}s$. We next multiply by C and substitute the resulting expression into Eq. 4 to obtain:

$$f(t) = \int_0^t \phi(t - s)e(s)\mathrm{d}s + De(t). \tag{7}$$

From Eq. 7 we see that $\phi(t)$ is the representation of the impulse response function $I(t)$ in terms of the "realization" (A, B, C):

$$\phi(t) = C\exp(tA)B. \tag{8}$$

In Eq. 5 we notice that $\exp(tA) = \mathrm{diag}\,[\exp(-\lambda_1 t), \exp(-\lambda_2 t), \ldots, \exp(-\lambda_n t)]$ and so $\phi(t) = \sum_{i=1}^{n} \gamma_i\beta_i \exp(-\lambda_i t)$. Since $\phi(t) = I(t)$,

$$\alpha_i = \beta_i\gamma_i, \quad (i = 1, 2, \ldots, n). \tag{9}$$

A comparison of Eqs. 1 and 7 shows that D is the scatter coefficient, ξ. We are thus led to the system of equations: $f_i(t) = 0, i = 1, 2, \ldots, n$

$$\begin{pmatrix} \dot{f}_1(t) \\ \dot{f}_2(t) \\ \vdots \\ \dot{f}_n(t) \end{pmatrix} = \begin{pmatrix} -\lambda_1 & 0 & \cdots & 0 \\ 0 & -\lambda_2 & \cdots & 0 \\ \vdots & \vdots & & \vdots \\ 0 & 0 & \cdots & -\lambda_n \end{pmatrix} \begin{pmatrix} f_1(t) \\ f_2(t) \\ \vdots \\ f_n(t) \end{pmatrix} + \begin{pmatrix} \beta_1 \\ \beta_2 \\ \vdots \\ \beta_n \end{pmatrix} e(t), \qquad (3')$$

and

$$f(t) = (\gamma_1, \gamma_2, \ldots, \gamma_n) \begin{pmatrix} f_1(t) \\ f_2(t) \\ \vdots \\ f_n(t) \end{pmatrix} + \xi e(t). \qquad (4')$$

From Eq. 3′ we see that

$$f_i(t) = \int_0^t e^{-\lambda_i(t-s)} \beta_i e(s) \mathrm{d}s, \quad (i = 1, 2, \ldots, n), \qquad (10)$$

which states that $f_i(t)$ is the fluorescence response resulting from the excitation $\beta_i e(t)$ in the i^{th} compartment. If the excitation $e(t)$ is regarded as monochromatic (through the excitation filter), then the β_i may be interpreted as the relative absorption, cross-section constants for each compartment. The matrix B may then be viewed as the excitation processor.

From Eq. 4′ we see that the contribution to the observed fluorescence, $f(t)$, from the i^{th} compartment is $\gamma_i f_i(t)$. Thus the γ_i may be interpreted as the relative emission intensity (at the wavelength selected by the emission filter) from compartment i, resulting from excitation $\beta_i e(t)$. The matrix C may then be regarded as the emission processor. A schematic representation of the system of equations is presented in Fig. 1.

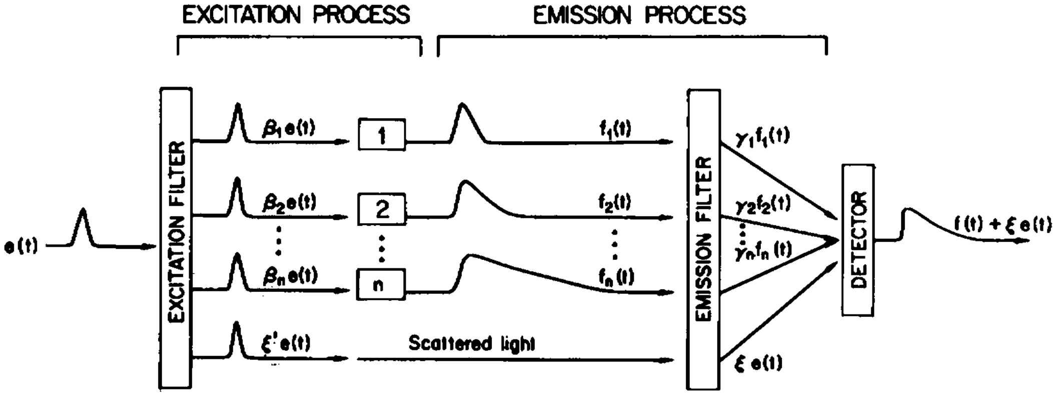

FIGURE 1 Schematic representation of the systems-theory description of the fluorescence decay experiment. The β_i are the elements of matrix B and the γ_i are the elements of matrix C in Eq. 8. Each compartment $1, 2, \ldots, n$ emits fluorescence $f_i(t)$ in response to excitation $\beta_i e(t)$.

It is known that the decay rates λ_i and all other information identifiable from the input/output experiment are contained in the so-called Markov parameters, $M_k = CA^kB$, $(k = 0, \pm 1, \pm 2, \ldots)$. In fact, these parameters are related by the recursion formula:

$$M_{n+k} + c_{n-1+k} + \ldots c_0 M_k = 0, \tag{11}$$

where the c_i are the coefficients of the monic polynomial $P(s)$, whose roots are $-\lambda_1, -\lambda_2, \ldots, -\lambda_n$, i.e.,

$$P(s) \equiv \prod_{i=1}^{n} (s + \lambda_i) = s^n + c_{n-1}s^{n-1} + \ldots + c_0. \tag{12}$$

Clearly if we have n linearly independent equations like Eq. 11, then we may invert the system of equations for the coefficients c_k and then estimate the $-\lambda_i$ as the roots of $P(s)$.

The Isenberg method of moments (1) (IMOM) is based on this principle. IMOM estimates moments G_k as,

$$G_k = (-1)^k M_{-k} = \sum_{i=1}^{n} \alpha_i \lambda_i^{-k}, \quad (k = MD + 1, MD + 2, \ldots). \tag{13}$$

The moment displacement index MD is chosen to be ≥ 1 to avoid scatter and other nonrandom errors (7–9). With $2n$ successive parameters M_{-k}, we obtain an equation of the form of Eq. 11, which we invert to estimate the coefficients c_k.

IMOM often yields very satisfying results and has been observed to compare favorably with competing nonlinear-least-squares methods (10). There are cases, however, for which the method may fail (11). In most of these cases, the failure can be traced to one or more basic problems. As mentioned previously, part of the problem may be due to the presence of very small amplitudes. Another reason may be traced to the cutoff error in estimating the moment integrals, an error which has a greater effect on G_k as k increases. IMOM provides an iterative procedure to help correct the cutoff errors. Other difficulties may arise because the higher moments favor the longer lifetimes. In other words, as k increases, the term $\alpha_i \tau_i^k$ in Eq. 13 increases faster for larger time constants ($\tau_i = 1/\lambda_i$) than for shorter ones.

To help remedy some of these difficulties, we suggest performing several experiments. In each experiment we vary the amplitudes $\alpha_i = \beta_i \gamma_i$. This may be achieved previously in such a way that each mode, $\exp(-\lambda_i t)$, is sufficiently expressed in at least one of the impulse responses,

$$I_q(t) = \sum_{i=1}^{n} \alpha(q, i) \exp(-\lambda_i t), \tag{14}$$

corresponding to the q^{th} experiment.

In the following discussion, p will denote the number of experiments from which data is being utilized for the estimation procedure.

Case: $p = n$ (Number of Experiments = Number of Time Constants)

In each of the experiments, q, we compute (say with IMOM) $n + 1$ consecutive moments, starting with the MD index. In terms of the time constants, τ_i, these moments may be defined by

$$m(q,p) \equiv m(q, k - \mathrm{MD}_q) \equiv G(q,k) = \sum_{i=1}^{n} \alpha(q,i)\tau_i^k,$$

$$(k = \mathrm{MD}_q + 1, \mathrm{MD}_q + 2, \ldots, \mathrm{MD}_q + n + 1), \qquad (i = 1, 2, \ldots, n). \quad (15)$$

In most instances we would choose $\mathrm{MD}_q = 1$. Since we are now discussing the problem in terms of the time constants instead of the decay rates, we have a related but different monic polynomial for estimating the τ_i:

$$Q(s) \equiv \prod_{i=1}^{n} (s - \tau_i) = s^n + d_{n+1}s^{n+1} + \cdots + d_0. \quad (16)$$

The coefficients d_i may be estimated by inverting the system:

$$\begin{pmatrix} m(1,1) & m(1,2) & \cdots & m(1,n) \\ m(2,1) & m(2,2) & \cdots & m(2,n) \\ \vdots & \vdots & & \vdots \\ m(n,1) & m(n,2) & \cdots & m(n,n) \end{pmatrix} \begin{pmatrix} d_0 \\ d_1 \\ \vdots \\ d_{n-1} \end{pmatrix} = - \begin{pmatrix} m(1,n+1) \\ m(2,n+1) \\ \vdots \\ m(n,n+1) \end{pmatrix} \quad (17)$$

Once these coefficients are estimated, the time constants are obtained as the roots of $Q(s)$. Let $M = \{m(q,p)\}$ denote the matrix of the system of equations in Eq. 17. To invert the system, we must have

$$\det M \neq 0. \quad (18)$$

The condition in Eq. 18 expresses the fact that the n rows of M, each obtained from a separate experiment, must be linearly independent. Notice that for the condition in Eq. 18 to be satisfied, it is necessary that each mode be observable in at least one experiment. On the other hand, it is possible that all modes are sufficiently expressed but, as luck would have it, the rows of M are still linearly dependent (or det M is relatively small). This situation may be improved by changing some of the MD_q.

Case: $\rho < n$

From the q^{th} experiment we determine $n + \sigma_q + 1$ consecutive moments, beginning with the MD index MD_q, where the integers σ_q (the number of additional moments required from the q^{th} experiment sum to $n - \rho$. From the first sequence of $n + \sigma_1 + 1$ consecutive moments, we form $\sigma_1 + 1$ distinct rows $[m(i,1), m(i,2), \ldots, m(i, n+1)]$, $(i = 1, 2, \ldots, \sigma_1 + 1)$, where each row is constructed from $n + 1$ consecutive moments. With the second sequence of $n + \sigma_2 + 1$ consecutive moments, we form $\sigma_2 + 1$ distinct rows $[m(i,1), \ldots, m(i, n+1)]$, $i = \sigma_1 + 2, \ldots, \sigma_1 + \sigma_2 + 2$. We continue in this fashion until we have n rows $[m(i,1), \ldots m(i, n-1)]$, $(i = 1, 2, \ldots, n)$, from which we form the system of equations in Eq. 17. As before, the time constants are the roots of the polynomial, $Q(s)$, whose coefficients are determined by inverting the system of Eq. 17. In the extreme case for which $\rho = 1$, the algorithm reduces to IMOM.

Case: $p > n$

The procedure required in this case is similar to the case $p = n$, except that the dimension of matrix M is $p \times n$. The condition of Eq. 18 must be replaced by rank $(M) = n$. To solve for the polynomial coefficients we apply least squares:

$$(d_0, d_1, \ldots d_{n-1}) = -[m(1, n+1), \ldots, m(n, n+1)]M(M^TM)^{-1}. \qquad (19)$$

The preceding algorithm for estimating the time constants has two nice features. First, we use all available data from each of the p experiments. Second, since we have more data available, we may avoid the use of troublesome higher moments. Examples are presented in following sections.

We close this presentation with the remark that Eqs. 16 and 17 may be written as a single equation:

$$\det \begin{pmatrix} 1 & \tau & \cdots & \tau^n \\ m(1,1) & m(1,2) & \cdots & m(1, n+1) \\ \vdots & \vdots & & \vdots \\ m(n,1) & m(n,2) & \cdots & m(n, n+1) \end{pmatrix} = 0. \qquad (20)$$

The time constants are the roots of Eq. 20, but it is more computationally advantageous to use Eqs. 16 and 17.

MATERIALS AND METHODS

Three fluorescent molecules were used to form a test system: pyrene, carbazole, and PPO. The compounds were dissolved in dimethylformamide (DMF), and the decay times of each were measured independently at concentrations of 1.0×10^{-6}M for the pyrene, 10.5×10^{-6}M for the carbazole, and 2.0×10^{-6}M for the PPO. Pyrene (99+%) and carbazole (99+%) were purchased from the Aldrich Chemical Co. (Milwaukee, Wis.). PPO (scintillation grade) was obtained from Research Products International Corp. (Elk Grove Village, Ill.). Excitation and emission wavelengths were selected by Baird Atomic bandpass filters (Baird-Atomic Corp., Bedford, Mass.) with peak transmissions at 340 and 400 mm, respectively. Values of the individual lifetimes for each compound are given in Table II as expected values.

A three component system was made by mixing pyrene, carbazole, and PPO. Final concentrations were [pyrene] = 1.6×10^{-6}M, [carbazole] = 6.4×10^{-6}M, and [PPO] = 1.6×10^{-6}M. Four separate lifetime measurements were then made at excitation/emission wavelength pairs of 340/400 nm ≡ R1, 320/360 nm ≡ R2, 280/360 nm ≡ R3, and 320/380+ nm ≡ R4. All wavelengths were selected by Baird Atomic bandpass filters except for the fourth emission wavelength, which was selected by a Baird sharp cut-on filter with 50% transmission at 380 nm.

Uncorrected excitation and emission spectra were recorded on a Farrand mark V spectrofluorometer with a resolution of 2.5 nm (Farrand Optical Co., Inc., Valhalla, N.Y.). Lifetime measurements were made with an Ortec 9200 system using an Ortec 6240B multichannel analyzer (Ortec Inc., EG&G, Inc., Oak Ridge, Tenn.). The excitation pulse $e(t)$ from a free-running air gap pulser was obtained by scattering light at the excitation wavelength through a dilute solution of Ludox.

Data analysis was performed with IMOM by using a DEC-10 computer (Digital Equipment Corp., Maynard, Mass.). An exponential depression parameter (EDP) of 0.0185 was selected to reduce cutoff error and an MD of 1 was used to reduce nonrandom errors (7). The corrected moments at convergence

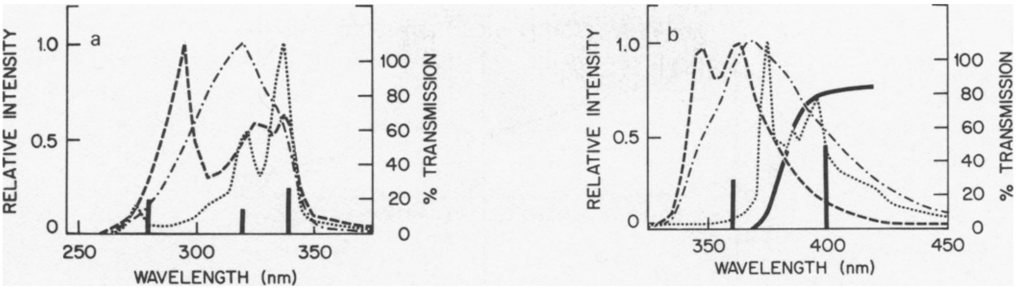

FIGURE 2 (*a*) Uncorrected excitation spectra of pyrene (. . .), carbazole (- - -), and PPO (- . -). Emission is at 400 nm in DMF, 25°C. Solid vertical lines (——) represent the transmission characteristics of the filters used. (*b*) Uncorrected emission spectra of pyrene (. . .), carbazole (- - -), and PPO (- . -). Excitation is at 320 nm in DMF, 25°C. Solid vertical lines (——) represent the transmission characteristics of the filters used.

were printed by the program and were used to form the system of equations in 17. A Texas Instruments programmable 58 calculator (Texas Instruments, Inc., Digital Systems, Houston, Tex.) was used to invert 17 and obtain the coefficients d_k of polynomial $Q(s)$ in Eq. 16. The same calculator was used to extract the roots of $Q(s)$.

Linearly independent measurements of the three-component system were made by varying the wavelengths of excitation and emission. This procedure corresponds to changing the parameters β_i and/or γ_i in Fig. 1 and Eqs. 8, 3′, and 4′. The excitation and emission filters can thus be used to change the excitation and emission processors B and C. In practice, we are changing the relative contributions of the three components to the observed fluorescence decay. This method of changing β_i and γ_i is illustrated in Figs. 2 *a* and 2 *b* for the pyrene-carbazole-PPO system. In each figure the vertical lines represent the transmission characteristics of the filters used. Clearly, changing the excitation filter from 280 nm to 320 nm to 340 nm with emission at 400 nm will change the β_i of matrix B and thus the relative contribution of each component to the fluorescence at 400 nm. A complementary situation occurs when the emission filter is changed.

In our test system, the excitation wavelengths 280, 320, and 340 nm were selected to change the fraction of $e(t)$ absorbed by each compound. The emission filters 380+ and 400 nm mix the relative emission intensities of the three compounds, while the 360 nm filter emphasizes PPO and carbazole, effectively removing the pyrene component. The fluorescence decay profiles measured at each of the four wavelength pairs are shown in Fig. 3 (*a*–*d*). Each channel corresponds to 0.4225 ns. In a qualitative way, it can be seen that the three decay times of 1.5, 10.3, and 39.3 ns are present in different amounts in the four determinations. The second $n + 2$ moments (MD = 1), as analyzed by IMOM for each experiment R1, R2, R3, R4, are presented in Table I.

PARAMETER ESTIMATION

Case: $p = 3$

For each of the three experiments R1, R2, R3, we computed four consecutive moments (Table I) starting with a moment displacement index MD = 1. These moments G_{i2}, G_{i3}, G_{i4}, G_{i5} ($i = 1, 2, 3$) were used to form Eq. 17:

$$\begin{pmatrix} 8.983 & 222.643 & 7110.939 \\ 48.242 & 542.553 & 8184.709 \\ 38.031 & 577.419 & 9681.490 \end{pmatrix} \begin{pmatrix} d_0 \\ d_1 \\ d_2 \end{pmatrix} = - \begin{pmatrix} 2.375 \times 10^5 \\ 1.324 \times 10^5 \\ 1.657 \times 10^5 \end{pmatrix}.$$

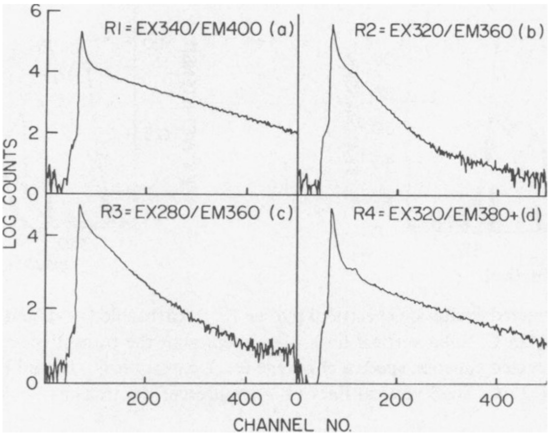

FIGURE 3 Semilogarithmic plots of fluorescence intensity vs. time (t) for the individual experiments R_i. Each channel corresponds to 0.4225 ns. Visual comparison of the four curves (*a-d*) reveals that the three components in the test system are present in different amounts. These curves were obtained by changing the wavelengths of excitation and emission as indicated in each frame.

Using triangular decomposition with partial pivoting (12), we computed the coefficients d_k and formed the polynomial $Q(s) = s^3 - (55.01)s^2 + (776.90)s - 2148.50 = 0$. We used the half-interval method (12) to estimate the depressed roots τ_i'. The roots are rescaled using the relation $\tau_i = \tau_i'/(1 - \tau_i'\lambda)$, where $\lambda = 0.0185$ is the exponential depression parameter used. The τ_i are presented in Table II under R1 + R2 + R3. The total average relative error (TARE),

$$\text{TARE} = \frac{1}{n}\sum_{i=1}^{n}\left|\frac{(\text{expected } \tau_i - \text{estimated } \tau_i)}{\text{expected } \tau_i}\right|, \tag{21}$$

has the relatively low value of 5.4% for this example.

Case: $\rho = 2$

Starting with MD = 1, we use five moments from experiment R1 and four moments from experiment R2. From R1 we form two rows of four consecutive moments, $(G_{12}, G_{13}, G_{14}, G_{15})$

TABLE I
MOMENTS CALCULATED BY IMOM FOR THE INDIVIDUAL EXPERIMENTS R_i

i	G_{i2}	G_{i3}	G_{i4}	G_{i5}	G_{i6}
1	8.98298	2.22643×10^2	7.11094×10^3	2.37476×10^5	8.03664×10^6
2	48.24202	5.42553×10^2	8.18471×10^3	1.32351×10^5	2.20538×10^6
3	38.03121	5.77419×10^2	9.68149×10^3	1.65656×10^5	2.87778×10^6
4	40.7010	5.24899×10^2	1.36500×10^4	4.24380×10^5	1.38415×10^7

Moments corresponding to experiment R_i with $MD = 1$ and $EDP = 0.0185$ in each sequence.

TABLE II
SUMMARY OF LIFETIME VALUES OBTAINED BY USING VARIOUS COMBINATIONS OF EXPERIMENTS R_i

Description	τ_1	τ_2	τ_3	Tare (%)	Case
Expected values	1.50	10.30	39.30	0	
R1 + R2 + R3	1.65	10.76	38.65	5.4	$\rho = 3$
2R1 + R2	1.25	9.86	39.19	7.1	
2R1 + R3	1.31	10.56	39.29	5.1	$\rho = 2$
2R2 + R3	2.46	10.83	—	—	
R1	0.73	6.64	38.80	29.4	
R2	1.30	9.96	—	—	
R3	1.44	10.64	—	—	$\rho = 1$
R4	1.05	6.42	36.85	24.6	
R1 + R2 + R3 + R4	1.50	10.56	37.35	2.5	$\rho = 4$

Timebase for experiments R_i was 0.4225 ns/channel. Values are given in nanoseconds.

and $(G_{13}, G_{14}, G_{15}, G_{16})$. A third row of four consecutive moments was formed from R2, $(G_{22}, G_{23}, G_{24}, G_{25})$ and the system of equations in Eq. 17 was:

$$\begin{pmatrix} 8.983 & 222.643 & 7116.939 \\ 222.643 & 7110.939 & 2.375 \times 10^5 \\ 48.242 & 542.553 & 8184.709 \end{pmatrix} \begin{pmatrix} d_0 \\ d_1 \\ d_2 \end{pmatrix} = - \begin{pmatrix} 2.375 \times 10^5 \\ 8.037 \times 10^6 \\ 1.324 \times 10^5 \end{pmatrix}$$

As in the previous example, this system was inverted for the coefficients of $Q(s)$. The roots of $Q(s)$ are tabular in Table II under 2R1 + R2. A TARE of 7.1% in this case is not quite as good as the case using three experiments. The result of a second determination with $\rho = 2$ is listed in Table II under 2R1 + R3. In this example, the use of two experiments resulted in a slightly better estimation (TARE = 5.1%) than that obtained for R1 + R2 + R3. The third example of the case $\rho = 2$ is 2R2 + R3 in Table II. This combination does not yield values for all three components because neither R2 nor R3 contains enough information about the longest lifetime component.

Case: $\rho = 1$

As indicated earlier, this case reduces to IMOM. The results of individual analyses of R1, R2, R3, and R4 are listed in Table II. We note that experiments R2 and R3 do not converge for three components but do yield two lifetimes that reasonably approximate the two smallest expected values. Experiments R1 and R4 converge for three components but with poor TARE's of 29.4 and 24.6%, respectively. Apparently none of the individual experiments contain enough information about each component to give a good analysis.

Case: $\rho = 4$

In this example we use experiments R1, R2, R3, and R4. The dimension of matrix M is 4 × 3 and we use the least squares algorithm in Eq. 19 to evaluate the coefficients of $Q(s)$. Eq. 17 becomes

$$\begin{pmatrix} 8.983 & 222.643 & 7110.939 \\ 48.292 & 542.553 & 8184.209 \\ 38.0312 & 577.419 & 9681.490 \\ 40.706 & 524.900 & 13650.020 \end{pmatrix} \begin{pmatrix} d_0 \\ d_1 \\ d_2 \end{pmatrix} = - \begin{pmatrix} 2.375 \times 10^5 \\ 1.324 \times 10^5 \\ 1.657 \times 10^5 \\ 4.244 \times 10^5 \end{pmatrix} \equiv V,$$

where the matrix $(d_0, d_1, d_2) = V^T M (M^T M)^{-1}$. The polynomial $Q(s)$ of Eq. 16 is $s^3 - (54.05)s + (744.24)s - 1919.54 = 0$. As seen in Table II, the roots τ_i using R1 + R2 + R3 + R4 have the lowest TARE of 2.5%.

DISCUSSION

The preceding examples demonstrate that the parameter estimation method based on the systems-theory model provides greater accuracy in determining the decay times of a multicomponent system. This result is obtained because the method utilizes information from several experiments to determine the decay parameters. Since multiple experiments are combined to perform the analysis, conditions can often be chosen to observe different decay modes in each measurement. For systems like that in our example, where each component has distinctive excitation and emission spectra, the observed decay modes can be easily changed by variations in the wavelengths of excitation and emission. This simple technique of changing matrices B and C is also expected to apply to situations where structural and environmental factors produce different lifetimes and spectral shifts for a single fluorophore in multiple binding sites.

Generally, we have found that more experiments will enhance accuracy. In fact, we note the poor analyses from individual experiments R1, R2, R3, and R4 (Table II). The combining of 2R1 + R2 or 2R1 + R3 leads to a substantial improvement in our ability to correctly determine the values of the three time constants. The combination of R1 + R2 + R3 further improves the analysis and R1 + R2 + R3 + R4 resulted in the lowest TARE of 2.5%. Perhaps equally important to the experimentalist is the presence of some consistency in the estimated decay parameters obtained from the multiple experiments listed in Table II. The method appears to allow for greater confidence in the analysis than would be possible for any single experiment alone.

The determination 2R2 + R3 in Table II is presented as an example of a combination that fails. In this case, both R2 and R3 contain little information about the longest decay time of 39.3 ns from the pyrene (Figs. 3 *b* and 3 *c*.). Individually, these two data sets give a reasonable answer for the two shorter time constants but no value for the longest (Table II). Combining them does not improve a situation where little information representing a particular decay model is available. The results of individual analyses of the multiple experiments and the qualitative appearance of the decay curves must be used as guidelines in deciding which measurements to combine.

If the amplitudes $\alpha(q, i)$ related to the q^{th} experiment are desired, they may be computed by inverting the system of equations in 15 with $p = 1, 2, \ldots, n$. This method for computing amplitudes is the same as that used in IMOM (1).

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