RESOLUTION OF MIXTURES OF FLUOROPHORES USING VARIABLE-FREQUENCY PHASE AND MODULATION DATA

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ABSTRACT We measured fluorescence phase shift and modulation data for one-, two- and, three-component mixtures of fluorophores at modulation frequencies ranging from 1 to 140 MHz. These data were analyzed using the least-squares procedure described in the preceding paper (Lakowicz, J. R., G. Laczko, M. Cherek, E. Gratton, and M. Limkeman, 1984, *Biophys. J.*, 46:463–477). Using data obtained at a single emission bandpass, the lifetimes and preexponential factors of two-component mixtures could be easily resolved if the lifetimes differed by a factor of 2. With currently available instrumental stability, three-component mixtures could be resolved when the overall range of decay times was 10-fold, (e.g., 1.3, 4.4, and 12 ns). Measurement of phase and modulation data at several emission wavelengths, where the ratio of the preexponential factors varied, enhanced our ability to resolve closely spaced two and three-component decays. Two-component mixtures could then be resolved if the lifetimes differed by 30% (4.4 and 6.2 ns). Also, the multiple-wavelength data allowed the lifetimes and emission spectra of the three-components of a mixture to be resolved. These results demonstrated that resolution of multiexponential decay laws was possible using frequency-domain phase-modulation fluorometry.

INTRODUCTION

Fluorescence spectroscopic methods are widely used in chemical and biochemical research. Although considerable information is available from steady state measurements, it is usually informative to examine the time-resolved decays of fluorescence intensity. If a sample contains several distinct fluorophores, then the decay law is expected to be a sum of exponentials. Alternatively, because the decay time of a single fluorophore is often sensitive to its surrounding environment, the time-resolved data can indicate whether this fluorophore is present in one or more environments, whether the fluorophore undergoes an excited-state reaction, or whether the dynamic properties of the environment result in time-dependent emission spectra (1–3).

Time-resolved information may be obtained using either pulsed or harmonic methods. The pulse method entails using brief pulses of light for the excitation, followed by measuring the time-dependent decay of intensity, and determining the impulse response function of the sample, $I(t)$ (2, 4). In the harmonic method, the sample is excited with sinusoidally modulated light. The impulse response function of the fluorescence determines the frequency-dependent phase angle and modulation of the emission, both of which are measured relative to the modulated excitation. In principle, if a wide range of modulation frequencies are available, the information contained in the phase and modulation values is equivalent to that obtained from the more direct pulse measurements (5–6). In practice such equivalence has not been realized because only two or three modulation frequencies are available with most phase fluorometers (7). Resolution of multiple lifetimes using two or three fixed frequencies has been difficult, although a few successful resolutions have been accomplished (8–11). Instruments are now available to measure the phase and modulation data over a wide range of modulation frequencies (12). In the previous paper we presented a detailed description of a nonlinear least-squares procedure for the analysis of these data (13). In this paper we present measurements of samples containing one, two, or three fluorophores. We demonstrate that one may reliably determine the lifetime and proportion of each component in such mixtures and that the resolving power is enhanced by measurements at several emission wavelengths if the preexponential factors vary with emission wavelength. The use of multiple emission wavelengths was initially suggested by Knutson et al. (14) to increase the resolution obtained by the pulse method.

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THEORY

The frequency-dependent phase and modulation data were analyzed using a nonlinear least-squares procedure that minimizes the squared deviations between the observed and expected phase and modulation values (13). The expected values at each modulation frequency can be predicted for the assumed impulse-response function (5). For a mixture of n fluorophores, each of which displays a single lifetime independent of emission wavelength, the impulse response function ($I[\lambda,t)]$) at each emission wavelength λ and time t is a sum of exponential decays, such that

$$I(\lambda,t) = \sum_i^n \alpha_i(\lambda)\, e^{-t/\tau_i}. \tag{1}$$

In this expression τ_i is the decay time of the ith component and $\alpha_i(\lambda)$ is the preexponential factor of this same component at wavelength λ. The fractional contribution of each component to the steady state emission spectrum is given by

$$f_i(\lambda) = \frac{\alpha_i(\lambda)\,\tau_i}{\sum_j \alpha_j(\lambda)\,\tau_j}. \tag{2}$$

The values of $\alpha_i(\lambda)$ also depend on the excitation wavelength. This dependence is not shown explicitly, because the excitation wavelength was constant in all our measurements.

The calculated (c) values of the phase $\phi_{c\omega}(\lambda)$ and modulation $m_{c\omega}(\lambda)$ data at each modulation frequency ω and each emission wavelength are given by

$$\phi_{c\omega}(\lambda) = \arctan\,[N_\omega(\lambda)/D_\omega(\lambda)] \tag{3}$$

$$m_{c\omega}(\lambda) = [N_\omega(\lambda)^2 + D_\omega(\lambda)^2]^{1/2}, \tag{4}$$

where

$$N_\omega(\lambda) \cdot J(\lambda) = \sum_i \frac{\alpha_i(\lambda)\,\omega\,\tau_i^2}{1 + \omega^2\,\tau_i^2} \tag{5}$$

$$D_\omega(\lambda) \cdot J(\lambda) = \sum_i \frac{\alpha_i(\lambda)\,\tau_i}{1 + \omega^2\,\tau_i^2} \tag{6}$$

and

$$J(\lambda) = \sum_i \alpha_i(\lambda)\tau_i. \tag{7}$$

In many of our experiments the emission was observed through an emission filter that essentially transmitted the total emission. In these cases there was only one value of α_i and the wavelength indicator could be deleted. Then, the value of χ^2 is given by

$$\chi^2 = \sum_\omega \frac{1}{\sigma_{\phi\omega}^2}(\phi_\omega - \phi_{c\omega})^2 + \sum_\omega \frac{1}{\sigma_{m\omega}^2}(m_\omega - m_{c\omega})^2. \tag{8}$$

In this expression ϕ_ω and m_ω refer to the measured phase and modulation values at the indicated frequency, respectively. $\sigma_{\phi\omega}$ and $\sigma_{m\omega}$ are the random errors of the measured phase and modulation values under the chosen experimental conditions.

Phase and modulation values were also measured at multiple emission wavelengths. In the fitting procedure the values of τ_i are restricted to be independent of wavelength, but the $\alpha_i(\lambda)$ values are of course dependent on emission wavelength (13). The value of χ^2 is given by

$$\chi^2 = \sum_\lambda \sum_\omega \frac{1}{\sigma_{\phi\omega}^2}[\phi_\omega(\lambda) - \phi_{c\omega}(\lambda)]^2 + \sum_\lambda \sum_\omega \frac{1}{\sigma_{m\omega}^2}[m_\omega(\lambda) - m_{c\omega}(\lambda)]^2. \tag{9}$$

In this expression we assume that the random errors in $\phi_\omega(\lambda)$ and $m_\omega(\lambda)$ are not dependent upon emission wavelength.

The goodness-of-fit may be judged by the value of reduced χ^2,

$$\chi_R^2 = \frac{\chi^2}{\nu} = \frac{\chi^2}{2Nq - p}, \tag{10}$$

where ν, the number of degrees of freedom, is given by $2Nq - p$. N is the number of modulation frequencies, q is the number of emission wavelengths, and p is the number of floating parameters. For example, assume phase and modulation data are measured at 10 frequencies and 10 emission wavelengths. For a two-component fit the number of floating parameters is twelve (two lifetimes and one value of $\alpha_i(\lambda)$ at each wavelength), and $\nu = 188$.

EXPERIMENTAL PROCEDURES

Fluorescence phase shift and modulation data were measured at modulation frequencies ranging from 1 to 140 MHz. The instrument was designed and constructed by E. Gratton (12) and uses cross-correlation detection (7). The excitation source was an argon ion laser (model 164/9; Spectra-Physics Inc., Mountain View, CA), which was tuned for output at 351 nm. Broadband modulation was obtained using a transverse field electro-optic modulator (model LMA1; Lasermetrics Inc., Englewood, NJ. The modulation and cross-correlation detection frequencies were obtained using individual frequency synthesizers. The detection photomultiplier was from Hamamatsu Corp., Middlesex, NJ (model R928). We noticed a minor systematic increase in phase angle at the longer emission wavelengths and at modulation frequencies >90 MHz, but the origin of this effect is not known. These small systematic errors increased the values of χ_R^2 but did not significantly alter the calculated values of the decay times. Some measurements were performed on an instrument constructed by Lakowicz and Maliwal (24). This instrument is similar to that described in reference 12, except the light source is a He-Cd laser from Liconix (Sunnyvale, CA) (325 nm), the electro-optic modulator is from Inrad (model 102-020; Northvale, NJ), and the frequency synthesizers are from Programmed Test Sources Inc., Acton, MA (model PTS 500).

9,10-diphenylanthracene (DPA), 9-methylanthracene (9-MA), and 9-cyanoanthracene (9-CA) were from Aldrich Chemical Co., Inc., Milwaukee, WI, and p-bis[2-(5-phenyloxazolyl)]benze (POPOP) was from Eastman Laboratory and Speciality Chemicals, Rochester, NY. All fluorophores were dissolved in ethanol and maintained at 25°C. Samples were equilibrated with the atmosphere and were not purged with inert gas to remove dissolved oxygen. The excitation was polarized 35° from the vertical and emission was observed without polarizers. The emission was sometimes observed through a monochromator and its transmission efficiency varied for each polarized component of the emission. These are conditions that do not eliminate the effects of Brownian rotation on the apparent decay times (15). However, these rotation effects are not significant because of the low viscosity of our samples.

Phase and modulation measurements were obtained relative to either a glycogen scatterer or a reference fluorophore solution (16). A scatterer was used for most measurements at a single emission bandpass. For measurements at multiple emission wavelengths the reference solutions was POPOP in ethanol (400 nm) with a reference lifetime of 1.32 ns. This reference solution was also used when measurements were obtained using the instrument built by Lakowicz (24).

Unless indicated otherwise the entire emission was observed through a liquid filter consisting of a 2-mm thickness of 2M $NaNO_2$. For measurements at multiple emission wavelengths we used a Jarrell-Ash Div. (Fisher Scientific Co., Waltham. MA) 1/4-meter monochromator and an emission bandpass of 6.6 nm. The concentration of each component in the mixtures was adjusted to the desired fractional intensity. In all cases the total optical density at 351 or 325 nm was 0.2 or smaller.

Each phase and modulation value is the average of several measurements, each being the average of 100 phase or modulation readings. The data were analyzed using the least-squares programs described in the previous paper (13). The uncertainties are those estimated from the diagonal elements of the error matrix (17), using the average random errors observed over a period of 1 y. The uncertainties therefore reflect the long-term uncertainties in the parameter estimates (see below).

RESULTS AND DISCUSSION

Estimation of the Experimental Uncertainties in ϕ_ω and m_ω

In the least-squares estimation of parameters it is essential to properly weight the deviations between the measured and calculated values of ϕ and m (Eqs. 8 and 9). We estimated these errors by averaging the deviations between the measured and calculated values for the samples containing one or two fluorophores (Fig. 1). The deviations shown in Fig. 1 are averaged values for data collected over a period of 1 y on Gratton's instrument (12) for a variety of samples and using a variety of experimental conditions. This approach is different from that in which a direct estimate of the standard deviation is obtained from multiple determinations of ϕ_ω and m_ω. In our opinion, by using a diverse group of experiments, we obtained an estimate of errors that applies generally to all our measurements. The uncertainty in any phase or modulation measurement could be decreased to very small levels by averaging for minutes. However, over hours or days, the differences between repeated measurements on the same sample were comparable with those shown in Fig. 1. It is not clear whether such longer term fluctuations should be regarded as random or systematic. Clearly, the errors were systematic over a period of minutes, but unpredictable over longer periods of time. Because the origin of the fluctuation of the measured quantities is unknown, we regard these differences as random deviations. Therefore, we believe all the phase or modulation values that were averaged on a single day to represent a single phase or modulation measurement. It was estimated that the uncertainty in this measurement is given by the measured deviations over a long period of time (Fig. 1).

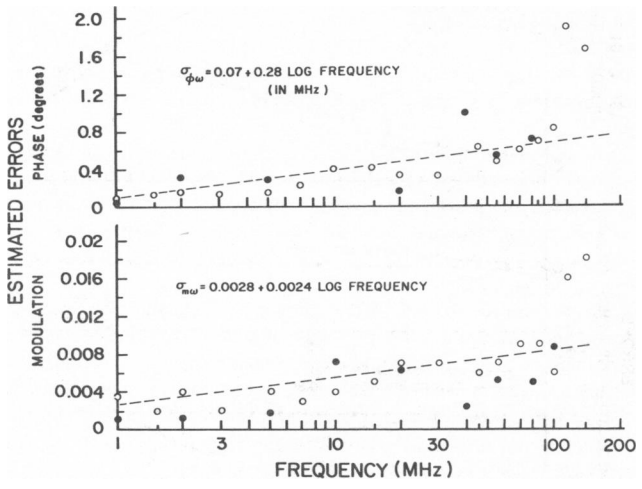

FIGURE 1 Experimental deviations between the measured and calculated phase and modulation values are shown. The deviations (absolute values of the difference between the measured and calculated values) shown are for data obtained for a number of one- and two-component mixtures. The open and closed symbols indicate the averaged deviations found for a number of samples, measured ~1-y apart.

Note that these deviations increase gradually with frequency up to 100 MHz. Above 100 MHz somewhat greater deviations were observed. Because these deviations were systematically positive at higher frequencies, we suspect a larger systematic error at these frequencies rather than an increase in the smaller unpredictable noise level of the measurements seen at lower frequencies. For simplicity we used a linear relationship between the estimated errors and log ω. These relationships are $\sigma_{\phi\omega} = 0.07 + 0.28 \log f$ and $\sigma_{m\omega} = 0.0028 + 0.0024 \log f$, where f is expressed MHz, ($f = \omega/2\pi$). Because these expressions underestimate the errors at high modulation frequencies, we expect the values of χ^2_R to reflect these systematic errors and exceed the value expected for purely random errors (approximately unity). However, we found that the estimated parameters do not significantly depend on the values of $\sigma_{\phi\omega}$ and $\sigma_{m\omega}$ (13). In our judgement, the best apparent fits were obtained using the frequency-dependent weighting factors shown in Fig. 1.

We stress that selection of the estimated errors ($\sigma_{\phi\omega}$ and $\sigma_{m\omega}$) determines the estimated uncertainties in the parameters (13). Because we use values for the errors that were averaged over all the measurements, the uncertainties in the parameters are the averaged uncertainty expected based on the error matrix (13). On any given day the measurements may be better or worse than the average. Then, the actual errors will be smaller or larger than the estimated values, respectively. Nonetheless, we feel averaged uncertainty gives a dependable estimate of the uncertainties in the parameters due to random errors. The uncertainties in the individual parameters calculated from the error (covariance) matrix, however, are based on the assumption that the linearized form of the model is valid in the region of the final parameter estimates.

Analysis of One-Component Solutions

Phase and modulation data for several one-component solutions are presented in Fig. 2. Longer lifetimes result in larger phase shifts and smaller modulations. The solid lines indicate the best one-component fits to these data. The experimental deviations are given in the lower panels. It is

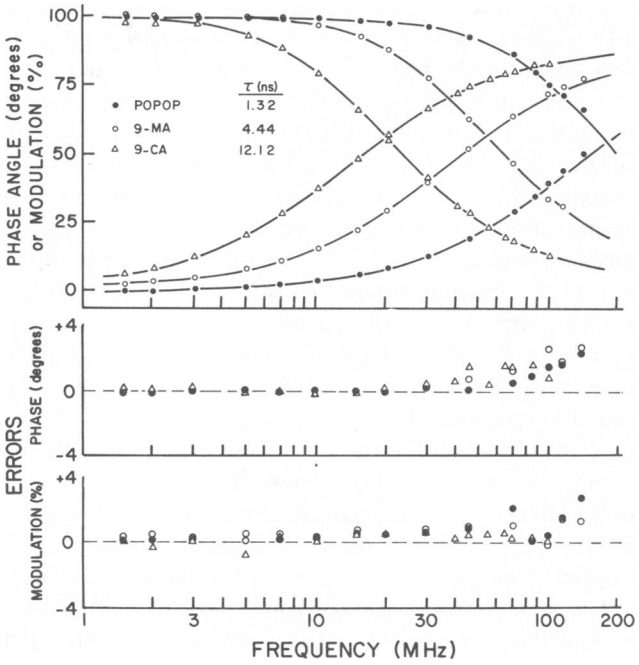

FIGURE 2 Phase and modulation data for solutions containing a single fluorophore are shown.

evident that these deviations are small, although somewhat larger deviations are seen at frequencies >100 MHz. The lifetimes and uncertainties of four single-component solutions are summarized in Table I. Each lifetime is similar to that expected from previous measurements (16, 18, 19). When the entire range of modulation frequencies is used (1–140 MHz), the values of χ^2_R are larger than unity as a result of systematic errors at high frequencies (>100 MHz). When the higher frequency (> 100 MHz) data are eliminated, the χ^2_R values approach unity (Table I), but the calculated lifetimes are not substantially altered. These results indicate that the weighting factors derived from Fig. 1 are appropriate for our experimental conditions and that χ^2_R for these data can be interpreted as usual. Specifically, values of χ^2_R greater than unity may indicate the presence of either systematic errors or an inappropriate model.

TABLE I
LIFETIMES OF ONE-COMPONENT SAMPLES

Compound	Frequency range	τ*	χ^2_R
	MHz	*ns*	
POPOP	1–140	1.32 (0.01)	2.0‡
9-MA	1–140	4.44 (0.04)	2.2
DPA	1–140	6.18 (0.07)	4.7
9-CA	1–140	12.12 (0.05)	1.3
POPOP	1–80	1.31 (0.01)	1.1
DPA	1–100	6.16 (0.03)	1.0

*The values in brackets are the experimental uncertainties.
‡The χ^2_R values obtained using the 1–140 MHz data are somewhat larger than expected because of larger systematic errors in the high frequency (>100 MHz) measurements.

Analysis of Two-Component Mixtures

We examined a number of two-component solutions, with equal fractional intensities ($f_1 = f_2 = 0.5$), but with varying differences between the two decay times. The samples were chosen to reveal the range of lifetimes that can be resolved using variable-frequency data. Data for the most widely spaced lifetimes in a mixture are shown in Fig. 3. The lifetimes of POPOP and 9-CA were found individually to be 1.32 and 12.12 ns. The presence of two components in the decay is clearly evident from the frequency dependence of the phase and modulation values, as may be seen by comparing Figs. 2 and 3. The dashed line shows the best one-component fit to these data. This fit and the value of χ^2_R (421) are obviously unacceptable. The deviations between the measured and calculated phase angles are as large as 20° for the one-component fit, and these deviations are systematically dependent on the frequency. Similarly, large (20%) and systematic deviations are seen for the modulation values. Including a second component in the fitting algorithm dramatically decreases χ^2_R to 0.80. Additionally, the phase and modulation deviations for the two-component fit are random and of a magnitude comparable with the experimental random errors. The lifetimes calculated from this two-component fit (1.36 and 12.05 ns) are in excellent agreement with the expected values of 1.32 and 12.12 ns (Table I).

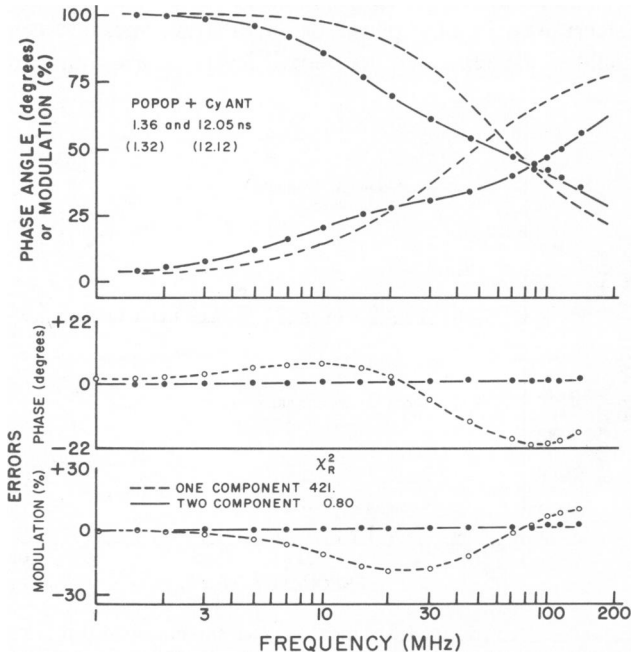

FIGURE 3 Phase and modulation data for a mixture of POPOP and 9-cyanoanthracene are shown. The dashed line indicates the best one-component fit, and the solid line, the best two-component fit. The values that are not in parentheses are the experimental values and those in parentheses are from measurement of the pure compounds.

Data from a two-component mixture with more closely spaced decay times are shown in Fig. 4. This mixture contained POPOP and 9,10-DPA. Once again, the large value of χ^2_R (87) and the systematic deviations between the measured and calculated phase angles clearly indicate the one-component fit is inadequate. A two-component fit yields χ^2_R = 1.27 and the expected values for the two lifetimes are 1.42 and 6.14 ns. By comparing Figs. 3 and 4, one notices that the deviations between the data and the one-component fit become smaller as the lifetimes become more closely spaced. (Note that the scales differ.)

We examined a series of two-component mixtures in which the individual decay times were progressively more closely spaced (Table II) in order to determine the difference in relative lifetimes needed to obtain a successful resolution. When the lifetimes differed by twofold or more, the lifetimes and fractional intensities could be determined quite reliable. Two lifetimes that differed by 30% (9-MA and DPA) could not be resolved from our data. The difficulty in resolving closely spaced lifetimes is evident from the similar χ^2_R values for the one- and two-component fits. For widely spaced lifetimes, the values of χ^2_R for the two-component fits are substantially smaller than these for the one-component fits.

In the mixtures described above the fractional intensities of the components were equal. Frequently, the researcher needs to determine the lifetime of a minor component in the emission. To model this, we analyzed mixtures of 9-MA (4.44 ns) and 9-CA (12.12 ns) in various proportions (Table III). In these mixtures the fractional intensity of 9-MA was varied from 90 to 10%. As the fractional intensity of 9-MA decreased to 10%, the calculated lifetime did not diverge from the expected value. The uncertainty in this lifetime was increased slightly. Similarily, as the fractional intensity of 9-CA increased, its lifetime was determined with decreased uncertainty. From the one- and two-component values of χ^2_R it is evident that the presence of a 10% component of either 9-MA or 9-CA can be easily detected. Also, the lifetimes can be accurately determined even at these low fractional intensities.

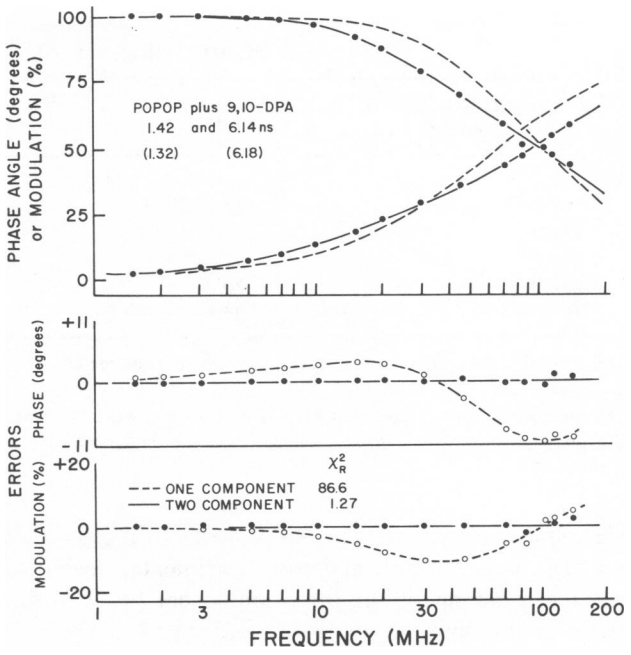

FIGURE 4 Phase and modulation data for a mixture of POPOP and 9,10-diphenylanthracene are presented. The values that are not in parentheses are the experimental values and those in parentheses are from separate measurements of the pure compounds.

Analysis of a Three-Component Mixture of POPOP, 9-MA, and 9-CA

The resolution of a three-component decay is substantially more difficult than a two-component decay law (4, 13). Data for this three-component mixture of POPOP (1.32 ns), 9-MA (4.44 ns), and 9-CA (12.12 ns) are shown in Fig. 5. The fractional intensities of the three components were approximately equal. The three-component fit is

TABLE II
ANALYSIS OF TWO-COMPONENT MIXTURES ($f_1 = f_2$)

Compounds	One-component χ^2_R	Two-component χ^2_R	τ_1	τ_2	f_1^*
			ns	*ns*	
POPOP/9-CA	442	0.77	1.36 (0.02)‡	12.05 (0.14)	0.47
POPOP/DPA	87	1.22	1.42 (0.04)	6.14 (0.12)	0.48
POPOP/9-MA	30	0.55	1.47 (0.05)	4.48 (0.14)	0.52
9-MA/9-CA‖	98	0.27	4.58 (0.05)	11.88 (0.16)	0.52
DPA/9-CA‖	24	0.47	6.36 (0.18)	11.92 (0.32)	0.46
9-MA/DPA	1.3	—	5.16 (0.03)§	—	—

*$f_1 + f_2 = 1.0$

‡The numbers in the brackets are the uncertainties in the estimated lifetimes.

§This mixture could not be resolved using data obtained at one emission bandpass. This mixture could be resolved using data obtained at multiple emission wavelengths (See Table V and the related discussion).

‖These results were obtained using the instrument constructed by Lakowicz and Maliwal (24). We used σ_p = 0.3° and σ_m = 0.0003, independent of modulation frequency. The frequencies ranged from 4 to 100 MHz.

TABLE III
ANALYSIS OF MIXTURES OF 9-MA AND 9-CA IN VARYING PROPORTIONS*

Fractional intensities 9-MA/9-CA‡	One-component χ_R^2	Two-component χ_R^2	τ_1§	τ_2	f_1‖
			ns	*ns*	
90/10	11.1	0.50	4.49 (0.04)	12.13 (1.0)	0.90
75/25	42.1	0.44	4.60 (0.04)	12.18 (0.47)	0.78
50/50	97.8	0.27	4.58 (0.05)	11.88 (0.16)	0.52
25/75	80.6	0.26	4.59 (0.07)	12.10 (0.10)	0.29
10/90	23.7	0.17	4.66 (0.13)	12.00 (0.06)	0.12

*These results were obtained using the instrument constructed by Lakowicz and Maliwal (24). The assumed uncertainties were σ_p = 0.3° and σ_m = 0.003.
‡The lifetimes measured separately for 9-MA and 9-CA were 4.44 and 12.12 ns, respectively.
§The numbers in the brackets are the uncertainties in the lifetimes.
‖$f_1 + f_2 = 1.0$.

substantially better than the two-component fit ($\chi_R^2 = 0.26$ and 5.52, respectively), and the experimental deviations are more random for the three-component fit. Also, the estimated lifetimes (1.29, 4.43, and 11.87 ns) are in excellent agreement with the expected values. These results are encouraging in light of the difficulties inherent to a three-component analysis. This degree of resolution agrees well with that predicted using simulated three-component decays (13). In particular, with phase angles accurate to ±0.2° and modulation values accurate to ±0.004, it was predicated that a three-component decay could be resolved if the total range of lifetimes was fivefold or greater (see reference 13, Fig. 11).

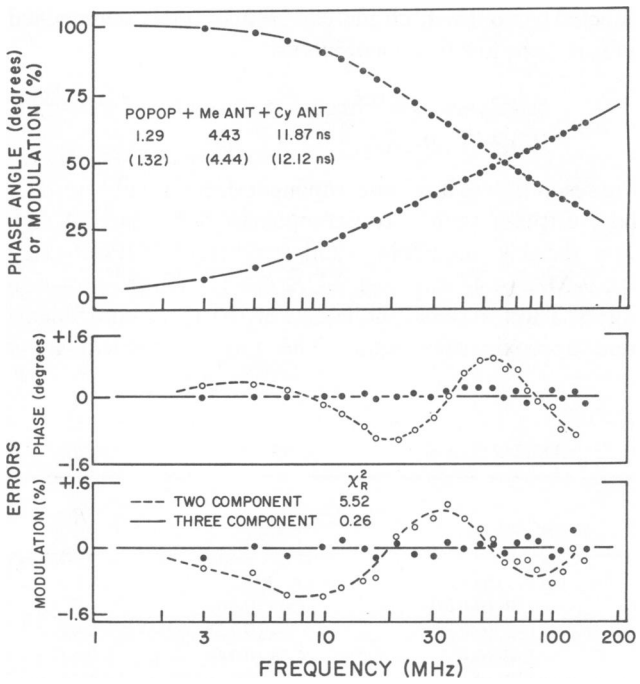

FIGURE 5 Phase and modulation data for a three-component mixture of POPOP, 9-methylanthracene and 9-cyanoanthracene are presented. The values that are not in parentheses are from the three-component fits and those in parentheses are from separate measurement of the pure compounds. The fractional intensities of the three components were equal ($f_1 = f_2 = f_3 = 1/3$). The excitation wavelength was 325 nm (24) the emission filter was model 0-52 from Corning Medical and Scientific, Medfield, MA, and the measurements were performed relative to a POPOP reference solution (16). σ_p = 0.3° and σ_m = 0.003.

Analysis of Multiexponential Decays Using Data at Multiple Emission Wavelengths

Frequently, the emission spectrum of each component in a mixture is distinct, and by varying the emission wavelength one also varies the preexponential factor of each component, that is, the values of $\alpha_i(\lambda)$. Data at multiple emission wavelengths are expected to yield improved resolution when either pulse (14) or phase-modulation data are used (13). Brand has called this method Global Analysis (14). This enhanced resolution is illustrated for the three-component mixture of POPOP, 9-MA, and 9-CA (Table IV). Phase and modulation data were measured at 13 emission wavelengths from 380 to 500 nm. These data were fitted using Eq. 9. The derived lifetimes with those determined individually for each fluorophore. Additionally, the uncer-

TABLE IV
MULTIPLE-WAVELENGTH ANALYSIS OF A THREE-COMPONENT MIXTURE OF POPOP, 9-MA, AND 9-CA*

Emission wavelengths	τ_1‡	τ_2	τ_3
	ns	*ns*	*ns*
380–500 nm§	1.36 (0.04)	4.86 (0.27)	12.76 (0.19)
380, 420, 460, and 500	1.33 (0.09)	4.24 (0.42)	12.71 (0.29)
400, 430, and 460	1.41 (0.07)	5.55 (0.92)	13.19 (0.69)
410 and 450	1.37 (0.10)	5.21 (0.92)	12.85 (0.80)
410	1.26 (0.17)	5.16 (1.4)	14.85 (5.4)
Entire emission‡	1.57 (0.06)	5.67 (0.65)	14.04 (1.6)
Expected values	1.32 —	4.44 —	12.12 —

*Gratton's instrument (12).
‡The numbers in brackets are the uncertainties in the estimated decay times.
§At 10-nm intervals.
‖These data are different from those in Fig. 5 because they were obtained on a different instrument (12).

TABLE V
MULTIPLE-WAVELENGTH ANALYSIS OF A MIXTURE OF 9-METHYLANTHRACENE AND 9,10-DIPHENYLANTHRACENE

Emission wavelengths	τ_1	τ_2
nm	*ns*	*ns*
390–460‡	4.56 (0.11)	5.66 (0.07)
400–460	4.56 (0.18)	5.70 (0.02)
400, 420, 440, and 460	5.00 (0.25)	5.70 (0.23)
400, 430, and 450	4.84 (0.43)	5.73 (0.22)
400 and 440	4.40 (1.0)	5.49 (0.22)
420	4.91 (3.4)	5.77 (4.7)
Expected values	4.44	6.18

*The numbers in brackets are the uncertainties in the estimated decay times. The frequency range was 2–85 MHz.
‡At 10-nm intervals.

tainties in these values (Table IV) are considerably smaller than those found using data for a single emission bandpass or single emission wavelength (410 nm).

To find the number of emission wavelengths that are necessary to reliably determine the decay times of the three-component mixture, the number of emission wavelengths used in the analysis was decreased (Table IV). As the number of wavelengths decreases to three or four, the decay times become less precise and less reliable. The results obtained when a single emission wavelength was used are similar to those found when the total emission is observed. Fortunately, a successful multiple-wavelength analysis does not require an unreasonable number of wavelengths. The results obtained using four, three, or even just two emission wavelengths are comparable to those obtained with data measured at 13 emission wavelengths.

We also used multiple-wavelength data to resolve closely spaced lifetimes (Table V). This is illustrated by the results obtained for a mixture of 9-MA (τ = 4.44 ns) and DPA (τ = 6.18 ns). The decay times of this mixture could not be resolved using data measured at a single emission bandwidth (Table II). In contrast, using phase and modulation data measured at eight emission wavelengths, we obtained reasonable values for the two decay times (4.56 and 5.66 ns). Importantly, the χ^2_R values for the one- and two-component fits are 8.26 and 0.98, respectively, which clearly indicate that it is valid to include the second component. For data measured at a single emission wavelength, the values of χ^2_R are similar to those for the one- and two-component fits. We note that the estimated uncertainties are underestimates because our procedure does not account for correlation between the parameters.

The inclusion of data measured at 390 nm for the 9-MA/DPA mixture may be artificial because at this wavelength the emission was dominated (90%) by that of 9-MA. Nonetheless, exclusion of the data obtained at 390 nm did not substantially alter the resolution. In this method it is essential to select wavelengths at which the preexponential factors $\alpha_i(\lambda)$ are substantially different. In these analyses we used data obtained from 2 to 85 MHz. Apparently, for these closely spaced decay times, the systematic errors seen at frequencies >90 MHz (Fig. 1) made it more difficult to resolve the lifetimes of 9-MA and DPA.

CONCLUSIONS

Our results demonstrate that the measurement of phase and modulation data over a range of modulation frequencies can be used to resolve multiexponential decays of fluorescence. Our resolution of multiexponential decays appears to be comparable with the resolution that can be obtained using pulse fluorometry. In addition, more recent results indicate that the frequency-domain data can also be used for the more complex task of computing time-resolved emission spectra (20–22) and time-resolved decays of anisotropy (23). The latter have been obtained for asymmetric and/or hindered fluorophores in solvents and lipid bilayers. In summary, these results indicate that frequency-domain measurements can provide the results usually obtained from time-resolved measurements.

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