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A Method for On-Line Background Subtraction in Frequency Domain Fluorometry

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Time-resolved fluorescence determinations on biochemical samples are often complicated by contributions from background. In the time-correlated single-photon counting method, background subtraction is a routine procedure. A limitation of frequency domain fluorometry, however, arises from the difficulty of performing this operation. This limitation has become increasingly significant as frequency domain methods are being applied to evermore complex biological systems using the higher-frequency capabilities of modern instrumentation. We have devised a method for such a correction in the frequency domain, regardless of the complexity of the background decay, based on measurement of the background phasor and subsequent subtraction from the sample phasor. This method is applicable to both lifetime and dynamic polarization measurements, and it can be readily implemented on commercially available frequency domain fluorometers. Decay curves may be accurately recovered from samples containing background contributions ranging from less than 5% to greater than 90% of the total signal intensity.

KEY WORDS: Frequency domain; background; lifetime; dynamic polarization.

INTRODUCTION

Phase fluorometry has become a widely utilized technique for performing time-resolved fluorescence measurements. As this technique becomes increasingly applied to complex samples at low concentrations, notably biological samples, unavoidable background fluorescence has come to significantly limit its overall sensitivity. Fluorescent contaminants in reagents, parasitic light, and scattered light (Rayleigh and Raman) are among the most common sources of background. If background is attributable to a single exponential decay, then a multicomponent analysis, in which one component can be clearly assigned to the background, is adequate to recover the sample's time-resolved parameters. Usually background contributions are more complex, though, rendering this approach inappropriate.

A method to correct frequency domain data for background signals, whether they originate from contaminant fluorescence or scattered light, has been reported by Lakowicz *et al.* [1]. In their method, the fraction of the total steady-state signal attributable to the background must be determined in a separate experiment under identical instrument conditions. This measurement is necessitated by the fact that, in their method, the signal acquisition is based on phase and modulation data alone, so that the AC contributions of the background must be calculated *a posteriori* from the independently determined DC values. Moreover, in their approach a rigorous error propagation analysis cannot be achieved during data acquisition but must be performed sepa-

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rately. Consequently the method is not a true on-line procedure.

Another approach to blank correction was recently proposed by Swift and Mitchell [2] specifically for the case of parallel data acquisition. In parallel phase fluorometry, a method ideally suited for relatively intensely emitting samples, a low-frequency waveform replica of the high-frequency signal is generated [3]. This waveform is digitized and stored in computer memory. Background subtraction has been implemented [2] by acquiring both sample and background waveforms followed by direct subtraction in a manner completely analogous to time-domain methodologies. This approach, which requires a waveform digitizer, does not, however, address the problem of error propagation, a nontrivial operation after Fourier transformation. In addition, it is applicable only to the minority of experimental situations that are amenable to parallel acquisition and for which appropriate instrumentation is available.

The biggest drawback associated with both of these approaches is the fact that the acquisitions of the sample and blank frequency spectra are performed at different times. This feature requires that the system, both instrument and sample, be stable over time. If this were to be the case, these methods should produce the desired correction. In reality, however, such stability is rarely achieved, and even more rarely evaluated. Variations in laser and/or lamp intensity, electronic drift in detector sensitivity, temperature variation, sample instability, etc., will all lead to technical inaccuracy in performing the blank correction.

Here we describe a different approach which circumvents the difficulties inherent in the previous methods and allows for true on-line[7] background correction. The method corrects for any type of fluorescence background, regardless of the complexity of the decay, by directly subtracting the measured background phasor from that produced by the composite sample. The correction is performed entirely in software and, hence, can be implemented with any phase fluorometer which provides separate phase, AC and DC signals to a computer, i.e., waveform digitization is not required. We have made accurate measurements of fluorescence lifetime and dynamic polarization in cases where background fluorescence contributes up to 90% of the total signal, thus substantially increasing the effective dynamic range of frequency domain fluorometry when applied to complex samples.

Of course in this method, as in any spectroscopic measurement, background correction requires the ability to prepare a blank sample that exhibits only those properties properly attributed to the background in the composite sample. Whether or not this is feasible in specific instances relies on issues of experimental design beyond the scope of the following discussion.

METHOD FOR BACKGROUND SUBTRACTION: BASIC EQUATIONS

In every kind of spectroscopy it is important to recover the signal in the presence of background. When the quantity measured is an intensity, background correction can be achieved by simple arithmetic subtraction of the value of an independently determined background from the measured overall signal. In frequency domain fluorometry, however, the basic signal is not characterized by a single intensity value. Instead this signal, at each modulation frequency, is a sinusoidal wave characterized by an amplitude (or AC value), a phase angle (measured with respect to a reference signal), and a constant DC value. The subtraction of the DC value due to background is easily obtained by a simple arithmetic operation. The subtraction of the sinusoidal signal (i.e., AC and phase) due to background can also be obtained using a more complex procedure as outlined below.

At each modulation frequency the phase, ϕ, and AC components of the fluorescence can be represented as a phasor [4]. The phasor is a vector which depicts a sinusoidal wave at a given frequency. The length of the vector is proportional to the AC intensity at that frequency and its direction is given by the phase angle of the sinusoidal wave with respect to the reference signal (see Fig. 1A).

In the following discussion we do not explicitly consider the effect of the background on the DC signal since its contribution can be subtracted separately. If the signal measured at a given frequency is the sum of two independent signals, i.e., sample + background, then the measured phasor is the vector addition of the two phasors corresponding to the sample and to the background. If the background phasor can be independently determined, then the corrected sample phase and the AC signals can be obtained using simple vector algebra to subtract the background contribution (see Fig. 1B). The equations corresponding to this operation are reported below using the phasor projection on the x–y axis.

[7] We use the term "on-line" to convey the idea that the method we describe provides for the determination of the contribution to phase and modulation made by the background at each frequency as these quantities are being measured. This stands in contrast to the aforementioned approaches in which the entire frequency spectrum of both sample and blank must be determined separately before a correction is made.

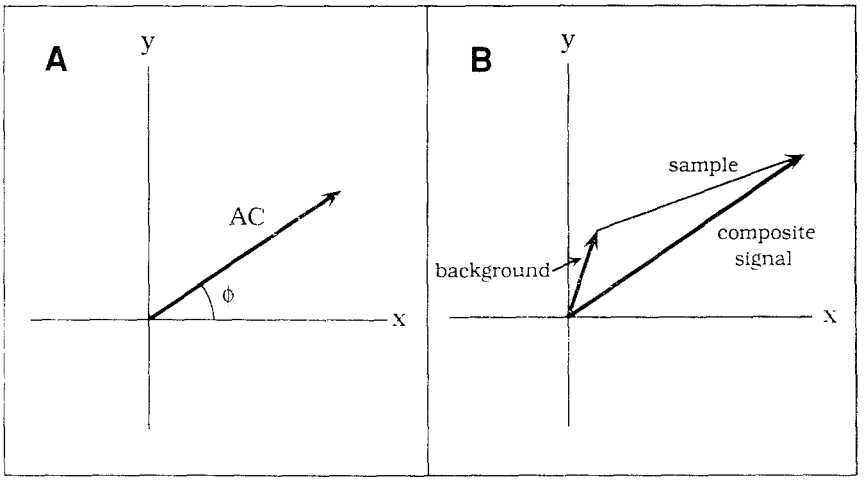

Fig. 1. (A) Phasor representation of the AC part of the fluorescence signal. The length of the phasor is proportional to the AC value, and the angle is equal to the phase angle as indicated. (B) Vector composition of background and sample signal to give the overall measured signal.

Measured Quantities

The data output by the fluorometer at each frequency is the AC, DC, and phase (ϕ) of the signal from the composite sample and the blank sample:

Composite signal:	$(AC)_C$	$(DC)_C$	ϕ_C
Background signal:	$(AC)_B$	$(DC)_B$	ϕ_B

Calculated Quantities

X-coordinate of composite phasor:	$C_x = (AC)_C \cos \phi_C$
Y-coordinate of composite phasor:	$C_y = (AC)_C \sin \phi_C$
X-coordinate of background phasor:	$B_x = (AC)_B \cos \phi_B$
Y-coordinate of composite phasor:	$B_y = (AC)_B \sin \phi_B$
X- and Y-coordinates of phasor difference:	$S_x = C_x - B_x$ $S_y = C_y - B_y$
Signals of sample corrected for background:	$(AC)_S = \sqrt{S_x^2 + S_y^2}$ $(DC)_S = (DC)_C - (DC)_B$ $\phi_S = \tan^{-1}\left(\frac{S_y}{S_x}\right)$

Implementation

These equations are used by our computer program to recover the sample signal in the presence of background at each frequency during data acquisition. We note that these equations apply to every type of sinusoidally varying signal including lifetime and dynamic polarization data as shown below as well as nonoptical signals such as radiofrequency pickup noise.

EVALUATION OF THE MAGNITUDE OF THE EFFECT OF BACKGROUND ON SAMPLE SIGNAL

Lifetime Simulations

In phase fluorometry lifetimes are determined by the phase and modulation (AC/DC) values at several modulation frequencies [4]. The effect of a background, contributing 10% to the DC levels, on the phase and modulation of the measured signals as a function of the phasor angle between signal and background, is shown in Fig. 2. We now consider two limiting cases to illustrate how the phase and modulation values are individually affected by background contribution. Since the phase values are determined only using AC signals, their background correction requires only the phasor equations. For the modulation values (AC/DC) we must instead separately evaluate the effects on the AC component of the phasor and on the DC sample signal.

(1) If the background phasor is almost in phase with the sample signal, the AC values rather than the phase will be affected (Fig. 2). The DC signal will also be modified by a similar amount, so the total effect on the measured modulation can be small. Of course, the signal

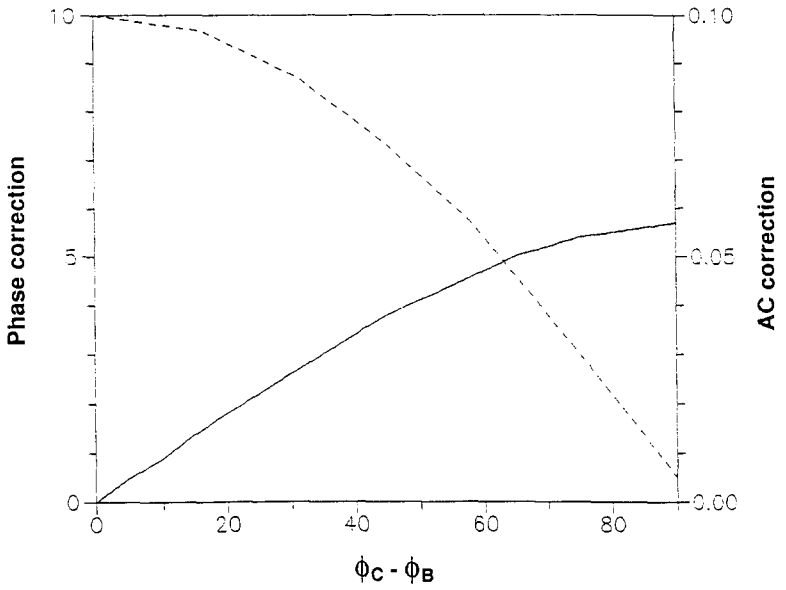

Fig. 2. Calculated phase (—) and AC (---) corrections as a function of the difference between the composite phase angle (ϕ_c) and the background phase angle (ϕ_B). This simulation is based on an AC background equal to 10% of the composite AC signal. AC correction is given as the fraction of the total AC signal attributable to the background when calculating the modulation ratio (AC/DC). The phase correction is given in degrees.

and the background will not be in-phase at all frequencies, and the contribution of the background to the AC part will be frequency dependent, while the contribution to the DC part will be frequency independent. Therefore the extent of the background correction must be assessed at each particular frequency. If the background phasor has the same direction as the sample at all frequencies, then the correction will have no effect on the recovered lifetime because the sample and background have the same lifetime.

(2) If the background phasor is at 90° with respect to the signal, the phase value can be affected to a large extent (Fig. 2). The AC value itself will have only a small correction. Despite this fact, the measured modulation can still be affected substantially since the background signal is also added to the DC part. This situation often occurs at a high frequency if there is a small amount of scatter or Raman signal contributing to the total signal. Even 0.5% of Raman contribution to the steady-state emission can greatly affect the values of the phase angles and modulation at frequencies above 1000 MHz since the Raman or scattering will exhibit a 0° phase angle, while the sample signal, originating from a decay component in the nanosecond range, will exhibit a large phase angle.

Dynamic Polarization Simulations

In dynamic polarization measurements the phase delay and relative demodulation of the AC parts of the parallel and perpendicularly polarized components of the emission are determined [5,6]. Background signal is often polarized, especially that due to Rayleigh or Raman scattering, and hence can seriously affect the measurement since the background contribution to the parallel polarized component will be much greater than its contribution to the perpendicularly polarized component. Furthermore, in dynamic polarization measurements, the phase differences can be very small. At very high frequencies, where signals are dominated by fast rotations, a small amount of scattering component in the signal can have a large effect. For example, assuming that we are interested in measuring a rotational component of a protein in the subnanosecond range and that the associated lifetime is 3 ns (a typical value for tryptophan or tyrosine residues), the rotational motion can be reliably evaluated at frequencies in the range 500–1000 MHz. At those frequencies the absolute modulation of the sample due to the demodulation effect of a 3-ns lifetime is about 0.05. Studies of the intrinsic tryptophan fluorescence of proteins are often carried out at 300-nm excitation to excite the electronic transitions associated with the highest polarization values [7], and a small contribution from the Raman scattering, appearing at 334 nm, is not unusual when cut-on filters are used to view the emission. The phase correction for this case can be estimated from Fig. 2 to be as large as 5 or 6° since at high frequencies

the phase angle of the scattered (or Raman) signal, with respect to the fluorescence, can approach 90°. If uncorrected, the background contribution will give the appearance of an ultrafast rotation. Hence, for dynamic polarization measurements of fast rotations, even a fraction of a percentage of a scattering contribution can give rise to a large artifact as shown in Fig. 3.

The modulation ratio in dynamic polarization is the ratio of the AC signals of the perpendicular and parallel polarized components (as opposed to the modulation ratio in lifetime determinations which is the AC/DC ratio). Since only parallel polarized components are affected by scattering, the modulation ratio in dynamic polarization measurements will be affected differently than in lifetime determinations. Therefore, at high modulation frequencies, particular attention should be paid to the Raman or Rayleigh scattering signal. Our background correction method operates separately on the parallel and perpendicular polarized components for the background signal and can fully correct for these effects.

EXPERIMENTAL

Instrumentation

Frequency domain measurements were performed at the Laboratory for Fluorescence Dynamics (Urbana, IL) using a home-built multifrequency fluorometer [8]. This instrument uses the harmonic content of a modelocked Neodynium-Yag laser [9]. The output is frequency doubled to produce 536-nm pulses which are used to pump synchronously a Rhodamine 6G dye laser. The output of the dye laser is cavity dumped at 2 MHz and frequency doubled to produce output in the 280- to 310-nm region. The detector system uses the cross-correlation method [10].

Sample Preparation

N-Acetyl-tryptophanamide (NATA) was purchased from Chemical Dynamics Corporation. Fructose 6-phosphate, potassium salt, and 3-(*N*-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma. Gold label grade indole was obtained from Aldrich and p-terphenyl was obtained from Kodak. Isooctane, high purity, was purchased from American Burdick & Jackson, Muskegon, MI, and absolute ethanol was obtained from USI Chemical Company.

Human serum albumin (HSA), essentially fatty acid free, was purchased from Sigma and dissolved in 20 m*M* Tris–HCl, pH 7. The protein solution was chromatographed using a Superose 12 column on an FPLC apparatus (Pharmacia) to remove possible dimers. The chromatogram of HSA showed three well resolved peaks; only the last protein-containing fraction (monomer) was collected.

Sodium bis(2-ethylhexyl)sulfosuccinate (AOT) was

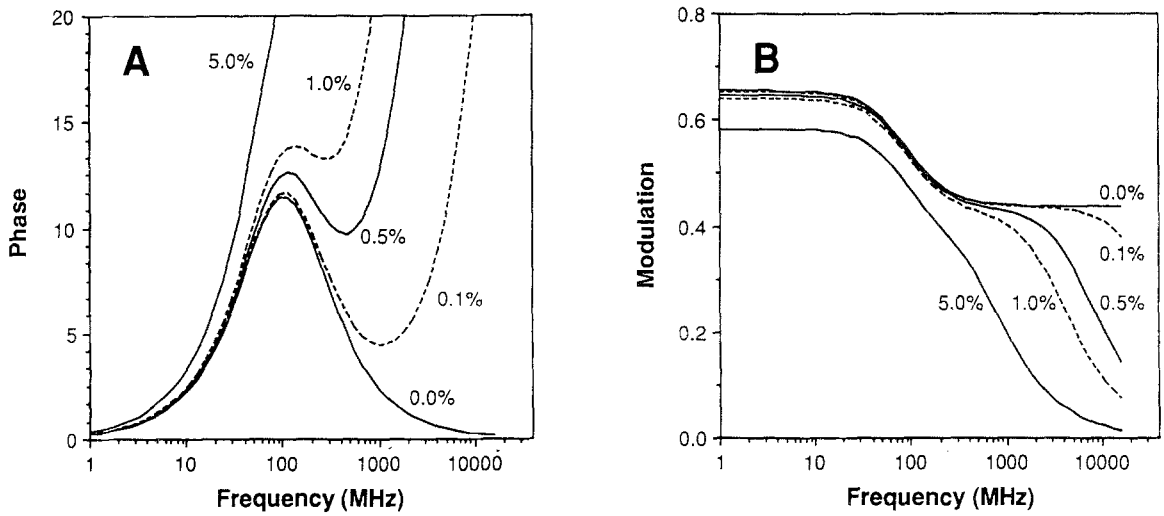

Fig. 3. Simulated dynamic phase (A) and modulation (B) polarization data with the indicated contribution of scatter signal to steady-state emission intensity. Curves were calculated assuming a lifetime of 3 ns and an isotropic rotational correlation time of 3 ns with a limited anisotropy of 0.3. Scatter contributes only to the parallel component as discussed in the text.

obtained from Sigma and purified according to the procedure described by Menger and Yamada [11]. However, even after the purification, the AOT suspension showed a small fluorescence background upon excitation at 300 nm.

Reverse micellar suspensions were prepared by dissolving AOT in isooctane at a final concentration of 0.11 *M*. Concentrated stock solutions of HSA in buffer (20 m*M* Tris–HCl, pH 7) were added to the micellar suspension with a Hamilton microsyringe. Protein concentration in aqueous solution was adjusted to give an absorbance of 0.1 at 295 nm. The absorbance in reverse micelles was 0.07 for HSA.

Data Acquisition and Analysis

Acquisition software from I.S.S. Inc. was modified to incorporate the blank acquisition procedure. Data analysis was performed using the Globals Unlimited software from the Laboratory for Fluorescence Dynamics, University of Illinois.

RESULTS

To illustrate the method, both lifetime and dynamic polarization measurements were performed on several fluorophore/background systems. Our goal in these studies was not to present an exhaustive list of situations which the fluorescence practitioner is likely to encounter. Rather we have chosen a few specific examples with the purpose of illustrating the method, verifying its validity, comparing the on-line method with *a posteriori* data analysis, and demonstrating that such *a posteriori* data analysis is fundamentally inapplicable in some cases.

Lifetime Measurements

(1) The validity of the technique was initially verified using a simple binary system in which the sample and background both exhibit single exponential decays. Specifically, we have utilized indole and ρ-terphenyl in ethanol as the sample and background, respectively. The measurements on these fluorophores were carried out at 25°C; phase and modulation data over the frequency range of approximately 7 to 300 MHz were obtained. Data were then collected on a mixture containing approximately 30% ρ-terphenyl and 70% indole; a pure ρ-terphenyl solution containing the same concentration of fluorophore present in the mixture was utilized as the blank. Phase and modulation data for the pure indole and the mixture after blank subtraction are superimposible as shown in Fig. 4. The lifetime recovered for indole from both pure solution and the mixture, the latter utilizing the blank subtraction procedures just described, are the same within experimental error and equal to 3.4 nsec. We note that an *a posteriori* two-component analysis of the mixture also properly identifies the contribution of the background and provides the correct lifetime for indole. However, the success of such a two-component analysis depends upon the ratio of the lifetimes as well as the precision of the data as has been discussed previously [4,6]. Our background subtraction procedure obviates the necessity of such an *a posteriori* analysis with its inherent limitations.

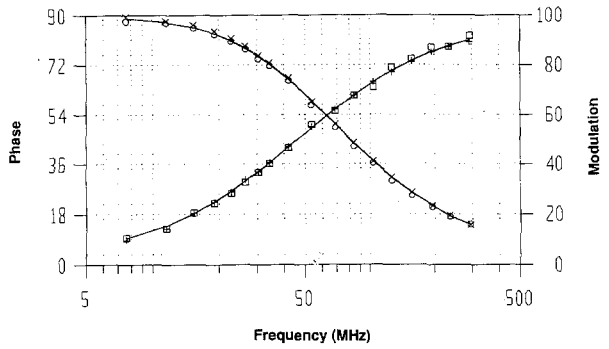

Fig. 4. Phase and modulation, respectively, of pure indole in ethanol (+, ×) and indole recovered from a binary mixture in ethanol after implementing the blank correction procedure described in the text (□, ○). The binary mixture contained an approximate 30% contribution from added ρ-terphenyl, and the blank consisted of an identical concentration of ρ-terphenyl alone. λ_{ex} = 295 nm, and emission was observed through a Hoya UV32 filter.

(2) We next considered the case of a sample exhibiting a single exponential decay but with a background which has a complex decay pattern. The choice of this particular system was motivated by our interest in evaluating the effects of ligand binding on native protein fluorescence. In such cases contaminant fluorescence associated with commercial preparations of the ligand of interest is often observed. The on-line correction of phase and modulation data for the contributions made by the contaminant fluorescence provides an alternative to the often difficult and time-consuming task of further purifying the ligand. We examined the case of one such ligand, fructose 6-phosphate, in combination with NATA, with which it has no specific interaction. Commercial preparations of fructose 6-phosphate contain substantial fluorescence impurities. The background associated with these impurities is complex; i.e., it cannot be fit to a single- or double-exponential decay and hence provides

a suitable case for assessing the validity of our approach in more complex cases.

Phase and modulation data for 6 μ*M* NATA (in an aqueous solution also containing 20 m*M* MOPS-KOH, pH 7, 40 m*M* KCl, and 2 m*M* $MgCl_2$) were collected in the presence and absence of 20 m*M* fructose 6-phosphate (Fig. 5). Phase and modulation data for 20 m*M* fructose 6-phosphate in the same buffer, but without NATA, are also presented in Fig. 5. The composite signal contains contributions from both NATA and background. The contribution of the impurities to the total steady-state (DC) fluorescence of this solution was approximately 80%.

The phase and modulation data for NATA alone, NATA plus fructose 6-phosphate, and the blank subtracted values are well described by a simple decay scheme consisting of 1 lorentzian distribution [12] and one discrete component, and the results of these analyses are presented by the curves in Fig. 5 and the numerical results summarized in Table I. The contaminant fluorescence cannot be well described by this scheme, as indicated by the relatively large value for χ^2 as shown in Table I. Other simple decay schemes, involving up to three components, also failed to describe the phase and modulation behavior of the contaminant fluorescence (data not shown).

By applying our method, we can recover the true phase and modulation data corresponding to NATA alone. These data are virtually superimposable with data obtained directly for NATA alone and analyze to virtually the same lifetime with comparable χ^2. Attempts to analyze the composite data by providing for an extra blank component does not fit the data any better or yield an accurate lifetime for NATA. This failure is likely due to the ill-defined and complex nature of the background decay characteristics.

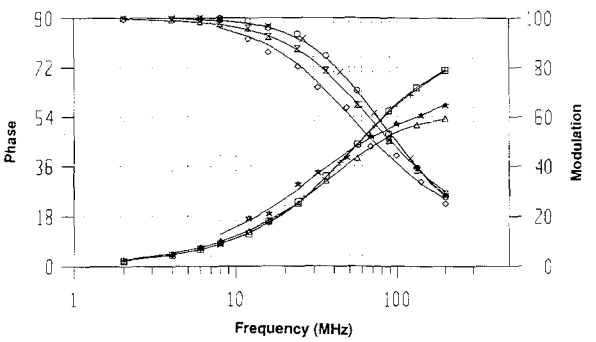

Fig. 5. Phase and modulation, respectively, of 6 μ*M* NATA in the presence (Δ, ⧖) and absence (+, ×) of 20 m*M* fructose 6-phosphate and phase and modulation of 20 m*M* fructose 6-phosphate alone at 20°C (★, ◇). Also shown are phase (□) and modulation (○) of the sample after subtracting the background fructose 6-phosphate contribution as described in the text. Curves correspond to the fits reported in Table I. λ_{ex} = 295nm, and emission was observed through a Hoya UV32 filter.

Table I. Analysis of Phase and Modulation Data Presented in Fig. 5[a]

	C_1 (ns)	W_1 (ns)	f_1	C_2 (ns)	f_2	χ^2
Composite[b]	3.185	1.146	0.887	0.217	0.113	1.743
Blank[c]	4.304	1.631	0.855	0.583	0.145	57.56
Sample (with blank subtracted)	2.861	0.050	0.980	0.325	0.020	2.590
NATA alone	2.878	0.050	0.965	0.590	0.035	1.063

[a]All data were fit to a two-component model in which the first component conformed to a lorentzian distribution (12) about a central lifetime value (C_1) with the indicated width (W_1) and fractional contribution to total fluorescence intensity (f_1). The second component was a discrete exponential decay with indicated lifetime (C_2) and fractional contribution (f_2). χ^2 values were calculated as described previously (4).

[b]NATA, 6 μ*M*, and fructose 6-phosphate, 20 m*M*, in pH 7 buffer.

[c]Fructose 6-phosphate, 20 m*M*, in pH 7 buffer. Steady-state (DC) fluorescence intensity was equal to approximately 80% of the emission of the composite.

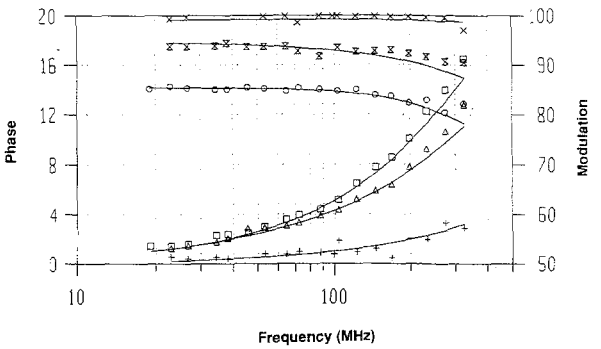

Fig. 6. Dynamic phase and modulation, respectively, of indole plus ρ-terphenyl (Δ, ⧖), ρ-terphenyl alone (□, ○), and phase and modulation resulting from blank correction (+, ×). The latter conforms to the dynamic phase and modulation values expected for pure indole. Details of the analysis results are presented in Table II. Samples were as described in Fig. 4. λ_{ex} = 295 nm, and emission was observed through a Hoya UV32 filter.

Dynamic Polarization Measurements

(3) The indole and ρ-terphenyl solutions used for lifetime measurements (part 1) were also employed to demonstrate the background subtraction method in dynamic polarization measurements. The data on the ρ-terphenyl blank, the composite signal, and the signal with the background subtracted are shown in Fig. 6.

Data were analyzed using a single rotational component attributable to indole and/or ρ-terphenyl as appropriate, and the recovered parameters are shown in Table II. We note that the recovered rotational correlation time for indole, 30 ps, is very close to the range of values reported in the literature for different solvent and temperature conditions [13]. We did not succeed in recovering reasonable rotational parameters attributable to the mixture components solely by analysis of the mixture data assuming two rotational species; this data set in fact fit best to a single rotational rate which represented a value weighted more toward the ρ-terphenyl (background) value (data not shown). The correct solution was obtained only by linking the analysis of the mixture to the analysis of the blank and blank-subtracted data (see Table II). Considering that this system is inherently simple we can predict, therefore, that an *a posteriori* analysis approach to the analysis of a mixture would be totally inapplicable in most cases.

(4) Finally, we considered a case wherein the background is very complex. In Fig. 7 we present dynamic polarization data collected from HSA in reverse micelles of AOT. Reverse micelles prepared as described in methods contain substantial fluorescent contaminants. In this case the steady-state fluorescence contribution of the contaminants equals 5%. In experiments described elsewhere, the lifetime of this sample was corrected for this blank contribution [14]. Dynamic polarization data were corrected for the blank contribution as shown in Fig. 7. The rotation analysis of these data are summarized in Table III. Note the substantial effect of the 5% contaminant fluorescence on the differential phase, at high frequencies in particular.

Table II. Analysis of Dynamic Polarization Data Presented in Fig. 6[a]

	Φ_1 (ns)	f_1	$(r_0)_1$	Φ_2 (ns)	f_2	$(r_0)_2$	χ^2
Composite[b] (indole + ρ-terphenyl)	0.030	0.691	0.310	0.173	0.309	0.390	6.48
Blank[c] (ρ-terphenyl)	—	—	—	0.173	1.000	0.390	7.66
Indole[d] (with blank subtracted)	0.030	1.000	0.310	—	—	—	8.86

[a]Lifetimes were determined in a separate experiment to be equal to 3.6 ns for indole and 1.08 ns for ρ-terphenyl at 20°C. $(r_0)_1$ is the limiting anisotropy in the absence of rotation for indole, and $(r_0)_2$ is the corresponding parameter for ρ-terphenyl. Values were determined in separate experiments (data not shown) and fixed in this analysis. All data were fit to two rotational correlation times (Φ_1 and Φ_2) linked across all data sets. f_1 and f_2 equal the fractional contribution of each rotational component to the total depolarization of the sample signal. χ^2 values were calculated as described previously (4).

[b][Indole]/[ρ-terphenyl] ≈ 70/30.

[c]100% ρ-terphenyl. f_1 fixed and set equal to 0.

[d]f_2 fixed and set equal to 0.

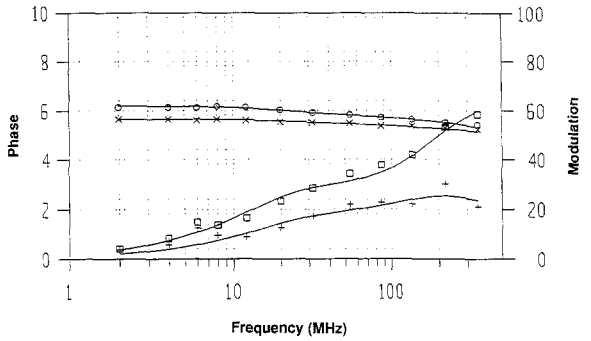

Fig. 7. Dynamic phase (□) and modulation (○) polarization of HSA in reverse micelles. Corresponding values after blank subtraction (+, ×) are also shown. Curves correspond to the fits reported in Table III. λ_{ex} = 300 nm, and emission was observed through Hoya UV32 and UV330 filters.

Table III. Analysis of Dynamic Polarization Data Presented in Fig. 7[a]

	Φ_1 (ns)	f_1	Φ_2 (ns)	f_2	r_0	χ^2
Composite[b]	30.2	0.687	0.386	0.313	0.276	1.14
Sample[c] (with blank subtracted)	47.9	0.854	0.730	0.146	0.250	1.45

[a]All data were fit to two rotational correlation times (Φ_1 and Φ_2). f_1 and f_2 equal the fractional contribution of each rotational component to the total rotation. r_0 is the limiting anisotropy in the absence of rotation and was allowed to vary in the analysis. χ^2 values were calculated as described previously (4).

[b]Lifetime was determined in a separate experiment to be best fit by a single gaussian distribution with a center at 2.87 ns and a width of 2.74 ns.

[c]Lifetime was determined in a separate experiment to be best fit by a single gaussian distribution with a center at 2.96 ns and a width of 1.85 ns.

DISCUSSION

The procedure outlined above for background correction represents a true on-line method that can be routinely implemented during frequency domain data acquisition. Once incorporated into the acquisition software, the correction is automatic. Complete automation in the case of lifetime determinations requires at least a three-position cuvette holder (one position for sample, one for background, and one for a reference lifetime solution), whereas dynamic polarization measurements require only a two-position holder (only sample and background are required).

Our background correction method works if the background phasor can be accurately determined. We have successfully performed measurements with back-

ground contributions ranging from 5 to 90% of the overall composite signal intensity. We note that in the case of small contributions (<10%) the phase and modulation corrections presented in Fig. 2 will vary proportionally to the percentage background contribution. Hence, for example, a 1% background at 90° will give a phase correction of only 0.5°. Therefore this figure provides the complete description of the effect of small blank contributions.

If the background AC signal is too small to be reliably detected, i.e., too small to trigger the zero-crossing detector type of phase meter, the correction will not work properly. This observation suggests that in some circumstances, it should be preferable to "add" more background signal to the sample to facilitate subtraction. This consideration will be largely mitigated when digital waveform acquisition, which improves signal to noise 10-fold, becomes widely utilized [3].

Although a small, e.g., 0.5%, contribution cannot and usually need not be directly subtracted in the lifetime mode, in the case of dynamic polarization it can be critically important to perform this correction as discussed previously. The basis of our method is the proper determination of the AC part of the signal. In general, the contribution of background to the AC part of the signal is frequency dependent. For the determination of fast rotational rates, where measurements must be performed at a high frequency (e.g., >300 MHz), the fluorescence signal usually is very demodulated (i.e., the AC signal is very small), and consequently the background AC will be proportionally larger (Fig. 3). Consequently the correction becomes not only efficient but also necessary, since as shown earlier, failure to correct for this background gives the appearance of a fast rotation. This error would have significant consequences when comparing experimental data to theoretical molecular dynamics predictions [15].

The difference in the consequences of low background contribution in lifetime versus dynamic polarization measurements arises because the polarization measurements have to be performed at frequencies appropriate to the rate of rotation which can be removed from the frequencies giving substantive modulation, whereas for lifetime determinations the frequencies are chosen to yield measurable amounts of modulation. We note that one cannot overcome this problem by reducing the lifetime to the proper frequency range for rotational measurements by using quenching agents because the contribution of the background scatter will then be increased in relative magnitude.

One of the difficulties shared by the background correction methods described by Lakowicz *et al.* [1] and Swift and Mitchell [2] is proper determination of the uncertainty associated with the phase and modulation recovered after correction. Since our procedure operates on-line by alternating between sample and blank cuvettes, and hence performs multiple determinations of the corrected signals at each frequency, the standard errors of the corrected signals are directly observed. One can therefore continue acquisition until the standard error of the corrected signal falls within acceptable limits (which conforms to the customary way frequency domain data are currently collected). In other words, the user does not have to enter into an *a posteriori* evaluation of the error as required by the method described by Lakowicz *et al.* [1].

To summarize, we have developed an automated method applicable to frequency domain fluorometry to obtain effective background subtraction during data acquisition. The basic assumption of the method is that the background signal can be measured independently and that the measured overall signal is the superposition of the sample and the background signals. If there is interaction between sample and background, for example, in the case of molecular complex formation, this correction method will provide evidence of just such an interaction by yielding an altered lifetime for the sample.

It is worth noting that, in the four systems we have presented, effective background correction using an *a posteriori* data analysis can be realized only for the lifetime data on the binary mixture of pure fluorophores. Hence, the on-line background subtraction method described is not merely a convenience which can be substituted by routine analysis after data acquisition but rather is fundamentally required for proper handling of time-resolved frequency domain data.

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