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Measurement of the Intensity of Long-Lifetime Luminophores in the Presence of Background Signals Using Phase-Modulation Fluorometry

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Abstract

We describe a method to correct for background fluorescence and to measure the intensity of long-lifetime probes using phase-modulation fluorometry. The theoretically predicted and simulated data were supported by two experiments. The fractional contribution of background fluorescence in the sample was determined by measurement of phase angle and/or modulation at single modulation frequency. In certain experimental situations, where the mean decay times of the background and the long lifetime probe are widely separated, determination of background signals in the sample does not require a blank sample or information about the nature of intensity decay of the background. Hence, phase-modulation fluorometry can be used to directly determine the intensity of the long-lifetime probe in the presence of an unknown short-lifetime background. We also discuss the effects of ambient light (indefinitely long lifetime) and scattered excitation light (zero lifetime) on phase-modulation measurements.

Keywords

Fluorescence; Sensing; Phase modulation; Frequency domain; Background; Autofluorescence; Metal–ligand complex

INTRODUCTION

At present, there is considerable interest and research activity in the field of chemical sensing. Rapid and continuous monitoring of many analytes (pH, pCO₂, O₂, metal ions, etc.) is required in many areas of science, including analytical chemistry, biochemistry, environmental sensing, clinical chemistry, and industrial applications.^{1–4} Fluorescence sensing is one promising approach because of its sensitivity, as well as the increasing number of sensitive and specific fluorescent probes for a variety of analytes (e.g., Molecular Probe Catalog) and their fabrication into practical sensors.

Currently, most types of fluorescence sensing are based on the intensity. In these measurements, the intensity of the probe molecule changes in response to the analyte. While

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intensity measurements are simple and accurate in the laboratory, they are often inadequate in real-world situations. This is because the sample may be turbid, the optical surfaces may be imprecise and become dirty, and optical alignment may vary from sample to sample. A significant disadvantage of intensity-based sensing is the problem of referencing the intensity measurements. Fluorescence intensity measurements depend on the intensity of exciting light, the optical density at the excitation and emission wavelengths, the light losses in the optical path, the detector sensitivity, and the concentration of the fluorophore. These difficulties with intensity-based sensing result in the need for frequent recalibrations and appear to limit the more widespread use of fluorescence for quantitative chemical sensing.

Recent advances in optoelectronics have now made possible a new type of fluorescence sensing. Instead of fluorescence intensities, it is possible to measure fluorescence lifetimes, particularly by the phase-modulation method, by using simple light sources and electronics. Lifetime-based sensing is possible for several molecular mechanisms of analyte-induced changes in the lifetime, as reviewed elsewhere in detail.⁵ The preferred lifetime-based sensing technique is phase-modulation fluorometry, where analyte-induced changes in a lifetime of the probe are measured from phase angle and modulation at a single light modulation frequency.

In any spectroscopic measurement it is important to recover the desired signal independent of background signals in the sample. When the quantity measured is intensity, correction for background components is straightforward and can be accomplished by simple arithmetical subtraction of the signal from the blank solution from that of the sample. In time-domain measurements, time-dependent data for the blank can be subtracted point by point from that of the sample. The components can also be separated by using a fast excitation pulse and selectively gating the detection system. The time-gating method is especially useful if the lifetime of the probe is significantly longer than that of the background fluorescence. The background signal may be due to scattered light with a zero decay time, due to impurities with finite lifetimes, or a combination of scattered light and auto-fluorescence.

In the frequency-domain measurement, phase angles and modulation factors cannot be simply corrected for the values measured for the blank. Methods for background correction have been described for the frequency-domain technique.^{6,7} At each modulation frequency the phase angle, ϕ , and modulation factor, m , are a complex weighted average of the desired and the unwanted signals. Lakowicz et al.⁶ used a frequency-domain data file of the blank sample to correct the phase angles and modulations of the sample. Reinhart et al.⁷ described a method similar to that of Lakowicz, but phase and modulation corrections are performed at each modulation frequency, avoiding a problem associated with sample and instrument instabilities over the time of collection of the frequency-domain data.

The methods for background correction described above require a blank sample that exhibits only those properties properly attributed to the background in the composite sample. In chemical sensing, the clinical and biological samples are usually complex, and it is not feasible to obtain a reliable blank sample. Also, the background correction is unsuitable in cases when background signals are intrinsic to the sample, or it is unstable.

Here we describe a different approach which circumvents the difficulties inherent in obtaining a reliable blank sample and allows for background correction using phase-modulation fluorometry. This method relies on large lifetime differences between the probe and the background, as is schematically illustrated in the Fig. 1. In the present report we describe measurements of the short lifetime background contribution in samples with long-lifetime probes using frequency-domain fluorometry. The results of analysis of frequency-domain data have been compared with those expected from steady-state measurements and with results obtained by using a single modulation frequency.

This approach is of interest because of the increasing number of applications using long-lifetime fluorophores such as the chelated lanthanides and transition metal–ligand complexes.^{8–10} There is continuing effort in several laboratories to develop the functional long-lifetime probes in the range above 100 ns.^{11–15} The lanthanides have been already widely used in time-gated immunoassays.¹⁶ To the best of our knowledge, the frequency-domain analog of time-gated method has not been published. When long-lifetime probes are used, the sensing can be carried out at a low modulation frequency in the range of 10–1000 kHz, and the phase-modulation instrument can be designed with inexpensive components.

THEORY

In general, the total intensity decay of fluorescence is often described by a sum of exponentials

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

where the α_i are the pre-exponential factors and τ_i the decay times. The fractional intensity of each component is proportional to the pre-exponential factor and decay time,

$$f_i = \alpha_i \tau_i / \sum_j \alpha_j \tau_j. \quad (2)$$

In frequency-domain fluorometry, the sample is excited with light modulated with circular frequency ω . This procedure results in fluorescence that is also modulated with frequency ω and is phase shifted and demodulated relative to the excitation. The frequency-dependent phase angle ϕ_ω and modulation m_ω values can be calculated from the sine (N_ω) and cosine (D_ω) transforms of the impulse response $I(t)$. The necessary transforms are

$$N_\omega = \frac{\int I(t) \sin \omega t \, dt}{\int I(t) \, dt} \quad (3)$$

$$D_\omega = \frac{\int I(t) \cos \omega t \, dt}{\int I(t) \, dt}. \quad (4)$$

The analytical expressions of these transforms for a multiexponential decay are given elsewhere.^{17,18} The phase and modulation can be calculated from N_ω and D_ω and are given by

$$\phi_{\omega} = \tan^{-1}(N_{\omega}/D_{\omega}) \quad (5)$$

$$m_{\omega} = (N_{\omega}^2 + D_{\omega}^2)^{1/2}. \quad (6)$$

Assume that total emission of the sample (I_M) consists of *background* fluorescence with a short intensity decay (τ_S) and the *probe fluorescence* with a long lifetime (τ_L). The total intensity of the sample consists of the fractional intensity of the short-lifetime component, f_S , and that of the long-lifetime component, f_L , where $f_L + f_S = 1$. At each frequency the total emission can be described by¹⁹

$$N_M = f_S m_S \sin \phi_S + f_L m_L \sin \phi_L$$

$$D_M = f_S m_S \cos \phi_S + f_L m_L \cos \phi_L \quad (7)$$

where $\phi_i = \tan^{-1}(\omega\tau_i)$ and $m_i = (1 + \omega^2\tau_i^2)^{-1/2}$. These equations for the phase angle and modulation can be derived by inserting Eqs. 7 into Eqs. 5 and 6. In this report, we are interested in cases where the ratio of a long lifetime to short lifetime, τ_L/τ_S , is on the order of 50 or larger.

Figure 2 shows simulated frequency-domain data for a sample with a long lifetime of 500 ns and increasing contributions of fluorescence with a short lifetime of 1 ns. The data in Fig. 2 clearly indicate that phase angles and modulations of a sample with a long-lifetime probe are very sensitive to the presence of a short-lifetime background. The phase angle and modulation differences between those of a sample and those of a long-lifetime probe depend strongly on the modulation frequency. Let us consider the frequency range where we expect changes due to the long-lifetime probe, in this case 500 ns—i.e., not higher than 10 MHz (Fig. 1). For that modulation frequency range, the phase angle and modulation values for a short-lifetime background are $\phi_S \approx 0$ and $m_S \approx 1.0$, respectively. In this limit, the analytical expressions for the phase angle and modulation of a sample can be reduced to the forms

$$\phi_M = \tan^{-1} \frac{\omega\tau_L}{1 + \frac{f_S}{1-f_S}(1 + \omega^2\tau_L^2)} \quad (8)$$

$$M_M = \sqrt{f_S^2 + m_L^2(1 - f_S^2)}. \quad (9)$$

Equations 8 and 9 indicate that, at low modulation frequency, the fractional intensity of a short-lived background can be determined by measuring the phase angle and/or modulation of a sample at a single modulation frequency without knowing the value of short lifetime. One can rewrite Eqs. 8 and 9 with the fractional intensity of a long-lifetime probe in the sample by applying $f_L = 1 - f_S$. Hence, the fluorescence intensity of a long-lifetime probe in the sample in the presence of a short lifetime background can be determined from

measurements of the phase angle and/or modulation of total fluorescence signal. One can imagine a number of situations where the signal from the sample is affected by variation of the probe concentration and variation of the background signal. In order to determine the intensity of the probe in the sample with phase-modulation measurements at a single modulation frequency, there are certain conditions: (1) The lifetime of the probe (τ_L) needs to be known. (2) The lifetime of the probe needs to be substantially longer than that of background signals (τ_S). (3) The phase-modulation measurements must be performed at low frequency where $\phi_S \approx 0$ and $m_S \approx 1.0$. And (4) the steady-state signal of a sample must be measured.

It should be noted that the value of a short-lifetime background signal is not needed to determine the intensity of the long-lifetime probe. However, if the ratio of lifetimes τ_L/τ_S becomes smaller, the practical single modulation frequency range for phase-modulation measurements becomes lower. This case is illustrated in Fig. 3, where the intensity decay data were simulated for background lifetimes from 0.5 to 10 ns.

Background correction using phase-modulation measurements can be extended to the short lifetime probes in samples where scattered light with a zero lifetime can be regarded as a background signal. In such cases, the phase-modulation measurements need to be performed at high modulation frequency, which is appropriate to the lifetime of the probe.

Besides the background fluorescence and scattered excitation light, one can imagine a contribution from ambient light. Ambient light, in contrast to background fluorescence and scattered excitation light, is not intensity modulated. More precisely, ambient light is not coherent with the excitation light. Assuming that the lifetime of ambient light can be regarded as indefinitely long, with a modulation equal to zero, one can easily determine its effect on the phase angle and modulation values (Eqs. 5–7). The phase angle will be independent on the ambient light, allowing sensing in illuminated environments. In contrast, the modulation value of the sample fluorescence will be reduced to $(1 - f_a) m_L$, where f_a is the fractional intensity of an ambient light in the total signal.

EXPERIMENTAL

In many applications it is necessary to measure very low fluorescence probe concentrations. Such measurements are often complicated by the background signals. To simulate such a situation, we used a long-lifetime fluorophore, (bis-2,2'-bipyridine)₂(dicarboxy-2,2'-bipyridine)ruthenium(II) complex, $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$, with a lifetime of 1060 ns in glycerol. The glycerol (from Fluka) displayed a short-lifetime (less than 4 ns) background fluorescence that overlaps with the emission of the Ru(II) complex. An increasing contribution of background fluorescence from the solvent was obtained by the dilutions of the Ru(II) complex in glycerol. Figure 4 shows the emission spectra of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$ with decreased concentrations and background fluorescence from glycerol. The fractional intensity of glycerol calculated by integrating the spectra varied from 0.066 to 0.878 with dilution of the ruthenium complex from 750 to 1.2 nM.

These samples were used for the time-resolved measurements. Frequency-domain data were acquired over a wide range of modulation frequencies in order to analyze the intensity decays using the multiexponential model (Figs. 5 and 6). The phase and modulation data were analyzed together with the use of a multiexponential model and a standard least-squares fitting procedure.^{17,18} The fitted functions are plotted as solid lines in Figs. 5 and 6. The recovered parameters such as the lifetime of the ruthenium, τ_L , and the fractional intensity of the glycerol fluorescence (background), f_S , determined from the intensity decays are summarized in Table I. The intensity decay of glycerol can be described by two decay times of 4.2 and 0.7 ns. The short decay times of glycerol compared to the lifetime of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$ allow determination of the fractional intensity of the background by using the phase angle and/or modulation at a single modulation frequency. The values of f_S were calculated from phase and modulation data at each modulation frequency with the use of Eqs. 8 and 9. The lifetime value of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$ was kept constant at 1069 ns. The fractional intensities determined from the phase angle and modulation were compared with those determined from multifrequency data analysis in Figs. 7 and 8. At low modulation frequencies, there is excellent agreement between values of f_S determined from multifrequency data analysis (dashed lines in Fig. 7) and those calculated on the basis of a single modulation frequency. More accurate results can be obtained by using Eqs. 8 and 9 to fit the phase and modulation data for several low-modulation frequencies. Then the value of the probe intensity can be determined and the lifetime of the probe can be confirmed. These results are summarized in Table I. It should be stressed again that a blank sample is not needed, and there is no separate measurement of the background intensity.

For this specific probe/background system, which is characterized by $\tau_L/\tau_S \sim 350$, the following conclusions can be stated: (1) Low intensities of long-lifetime probes can be determined from phase and modulation values. (2) The intensity of the probe can be accurately determined from the phase angle for frequencies lower than 0.8 MHz (Fig. 7). (3) The intensity of the probe can be accurately determined from the modulation for frequencies lower than 8 MHz (Fig. 8). These results confirm that the background intensity can be determined by measurement phase and/or modulation measurement at single modulation frequency without the need for a blank sample. A background contribution of more than 95% of the total signal still allows accurate determination of the probe intensity.

CONCLUSION

It has been shown that under certain experimental conditions, measurements of the phase angle and/or the modulation at single light modulation frequency allow one to determine the absolute intensity of the probe in the presence of background fluorescence without the need for a blank sample. Thus, phase-modulation measurements may allow determination of the concentration of a probe that displays a long lifetime in the presence of autofluorescence or scattered excitation light.

References

1. Janata J, Josovicz M, Vanysek P, and DeVaney DM, *Anal. Chem* 70, 179 (1998).
2. Arnold M, *Anal. Chem* 64, 1015 (1992).

3. Anderson DJ, Guo B, Xu Y, Ng LM, Kricka LJ, Skogerboe KJ, Hage DS, Schoeff L, Wang J, Sokoll LJ, Chan DW, Ward KM, and Davis KA, *Anal. Chem* 69, 165R (1997).
4. *Fiber Optic Chemical Sensors and Biosensors*, Vols. I and II, Wolfbeis OS, Ed. (CRC Press, Boca Raton, Florida, 1991).
5. Szmazinski H and Lakowicz JR, in *Topics in Fluorescence Spectroscopy*, Lakowicz JR, Ed. (Plenum Press, New York, 1994), Vol. 4, pp. 295–334.
6. Lakowicz JR, Jayaweera R, Joshi N, and Gryczynski I, *Anal. Biochem* 160, 471 (1987). [PubMed: 3578773]
7. Reinhart GD, Marzola P, Jameson DM, and Gratton E, *J. Fluoresc* 1(3), 153 (1991). [PubMed: 24242993]
8. Hemmilä I, Dakubu S, Mikkala V-M, Siitari H, and Lövgren T, *Anal. Biochem* 137, 335 (1984). [PubMed: 6375455]
9. Lakowicz JR, Terpetschnig E, Murtaza Z, and Szmazinski H, *J. Fluoresc* 7(1), 17 (1997).
10. Selvin PR and Hearst JE, *Proc. Natl. Acad. Sci. USA* 9, 10024 (1994).
11. Li L, Szmazinski H and Lakowicz JR, *Anal. Biochem* 244, 80 (1997). [PubMed: 9025912]
12. Friedman AE, Chambron J-C, Sauvage JP, Turro NJ, and Barton JK, *J. Am. Chem. Soc* 112, 4960 (1990).
13. Murtaza Z, Chang Q, Rao G, Lin H, and Lakowicz JR, *Anal. Biochem* 247, 216 (1997). [PubMed: 9177680]
14. Price JM, Xu W, Demas JN, and DeGraff BA, *Anal. Chem* 70, 265 (1998).
15. Shen Y and Sullivan BP, *J. Chem. Ed* 74(6), 685 (1997).
16. Diamandis EP, *Clin. Biochem* 21, 139 (1988). [PubMed: 3292080]
17. Lakowicz JR, Laczko G, Cherek H, Gratton E, and Limkeman M, *Biophys. J* 46, 463 (1984). [PubMed: 6498264]
18. Gratton E, Limkeman M, Lakowicz JR, Maliwal BP, Cherek H, and Laczko G, *Biophys. J* 46, 479 (1984). [PubMed: 6498265]
19. Lakowicz JR and Balter A, *Biophys. Chem*, 16, 99 (1982); *idem*, 16, 117 (1982). [PubMed: 7139052]

FIG. 1. Time-domain (top) and frequency-domain (bottom) intensity decays for a short and long lifetime.

FIG. 2. Simulated frequency-domain data for various sample intensity compositions with two fluorescence components with decay times of 1 and 500 ns.

FIG. 3. Simulated frequency-domain data for several values of a short lifetime component with a constant long-lifetime component.

FIG. 4. Emission spectra of $\text{Ru}(\text{bpy})_2(\text{dcbpy})$ in glycerol at various concentrations (solid lines). Background fluorescence from glycerol is indicated with a dashed line.

FIG. 5. Frequency-domain phase angles for Ru(bpy)₂(dcbpy) in glycerol at various concentrations. Results of analysis are in Table I.

FIG. 6.
Frequency-domain modulations for $\text{Ru}(\text{bpy})_2(\text{dcbpy})$ in glycerol at various concentrations.
Results of analysis are in Table I.

FIG. 7.

Fractional intensities of a background signal (f_s) determined from phase angles (—○—) using Eq. 8. Dashed lines indicate f_s values determined from entire frequency-domain data analysis (see Figs. 5–6 and Table I).

FIG. 8.

Fractional intensities of a background signal (f_s) determined from modulations (—○—) using Eq. 9. Dashed lines indicate f_s values determined from entire frequency-domain data analysis (see Figs. 5–6 and Table I).

TABLE I.

Fractional intensity of the background of the glycerol fluorescence and the long-lifetime probe Ru(bpy)₂(dcbpy) recovered from the steady state, from the fluorescence intensity decay (F-D) analysis, from the phase (Eq. 8), and from the modulation (Eq. 9).

[Ru(bpy) ₂ (dcbpy)] (nM)	Steady state f_2	F-D data		Equation 8		Equation 9	
		f_s	τ_L (ns)	f_s	τ_L (ns)	f_s	τ_L (ns)
37 nM	N/D ^a	0.001	1072	0.001	1071 (7) ^b	0.012	1079 (7)
3700	0.018	0.020	1066	0.021	1068 (4)	0.028	1073 (15)
750	0.066	0.060	1063	0.063	1032 (2)	0.062	1115 (32)
150	0.313	0.288	968	0.290	927 (2)	0.280	1013 (11)
30	0.636	0.681	971	0.682	926 (2)	0.680	940 (9)
6	0.789	0.889	1010	0.875	1008 (1)	0.891	940 (7)
1.2	0.878	0.960	843	0.943	956 (0.7)	0.972	970 (5)

^aN/D = not determined.

^bUpper modulation frequency in MHz that was used for data analysis.