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Elimination of Fluorescence and Scattering Backgrounds in Luminescence Lifetime Measurements Using Gated-Phase Fluorometry

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A new gated form of phase fluorometry for measuring lifetimes is presented. The technique uses a square-wave excitation and gates the detector on only during the off period of the excitation. Using a long-lived sample, this eliminates or reduces errors from scattered light and short-lived fluorescences. Using a square-wave modulated excitation source with a 50% duty cycle, traditional data treatment can be used after, at most, a simple $\pi/2$ phase adjustment. A combination of theory and experimental results demonstrates the validity of this new gated method and its utility for eliminating or reducing background. The results are precise, accurate, eliminate scattering errors, and greatly reduce errors due to short-lived fluorescence impurities. Errors from fluorescence bleed-through into the detection period or a slow excitation source turn off can be mitigated by using an offset time prior to gating the detector on.

Luminescent probes are widely used in both fundamental measurements and in analysis of biological, biochemical, and clinical samples.[1] The intensity or the excited-state lifetime (τ) of the probe can be monitored to reveal changes in the level of a desired analyte. Lifetime measurements are preferred, since they are not as susceptible to errors from fluctuations of the excitation source, concentration changes in the sensor molecule, and photodecomposition as are intensity measurements.[2] The common ways of measuring lifetimes are by pulsed and modulation techniques.

Pulsed measurements typically consist of recording the complete sample decay following excitation with a short duration optical pulse; the decay is then fit to a single or multiple exponential decay model using a nonlinear least-squares method. This approach requires expensive, complex equipment and time. A major advantage is that it inherently discriminates against interfering scattered light and luminescent impurities that are short-lived relative to the sample, but it is unsuitable for real time analysis, such as that required in HPLC.

In phase-modulation measurements, the sample is excited with a modulated excitation source. The phase shift (ϕ) or demodulation of the sample emission relative to the excitation waveform is used to calculate the lifetime. This method can be both precise and low-cost, but in a single frequency format, it gives no discrimination against short-lived contaminants and scattered interferences. These contributions produce spurious phase shifts or demodulations, yielding erroneous lifetimes. One solution to this problem consists of multifrequency phase shift/demodulation measurements and fitting multifrequency data to a multicomponent decay.[3−5] Short-lived components are reduced, but speed, computational and instrumental simplicity, precision, and accuracy are sacrificed.

For low-cost instrumentation, the phase shift method is the preferred technique. It is much simpler and lower cost than equivalent pulse technology. Further, it provides directly a single number, the phase shift, which is generally monotonically related to the parameter of interest. Especially for long-lived emitters, such as the new inorganic luminophores, the instrumentation is simple, low-cost and can use LEDs as the excitation sources.

Although gating has been used in pulsed measurements, until recently, it has not been used in phase fluorometry. Using a pulsed excitation source and gated detection, a phase-shift fluorometer has been described that avoids scatter and errors from short-lived fluorescences.[6] As proposed, it uses only short duration excitation sources and is not suitable for low-cost modulatable light sources, such as LEDs, in which the intensity is not available in a delta or near delta function pulse.

We describe the theory and application of a new gated phase-modulation decay method that provides all of the phase information of conventional phase measurement but eliminates the short-lived and scattering interfering agents that distort conventional ungated modulation schemes. We use a square-wave modulated excitation source and gate the detection system on only during the off period of the excitation. Both theoretical and experimental data demonstrate the effectiveness and advantages of our gated scheme. This method readily lends itself to uses with low-cost

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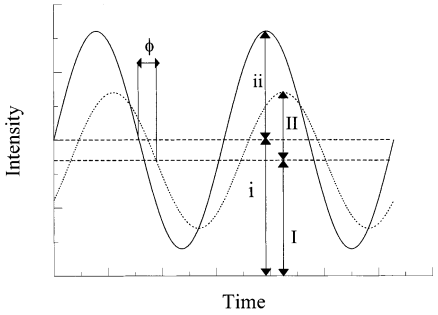

Figure 1. Basic phase fluorometry schematic representation showing the excitation from a sinusoidal source(solid line), corresponding sample emission (dotted line), the modulated amplitudes of the excitation source (ii) and sample emission(II), the average intensities of the excitation source (i) and sample emission (I) with corresponding horizontal dashed lines, and the phase shift between the excitation source and the sample emission.

LEDs or diode lasers. Although we use Fourier analysis for demonstrating the theory and limits of the method, the preferred implementation would be with conventional ac signal processing, such as with lock-in amplifiers.

THEORY

Figure 1 shows a representative waveform in conventional-phase fluorometry. The finite sample lifetime causes the emission to lag or be phase-shifted relative to the excitation and reduces the depth of modulation. The emission delay or phase shift (ϕ) relative to the excitation source is given by

$$\tan(\phi) = \omega\tau_\phi \tag{1}$$

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

where ϕ is the phase shift (the difference between the phase angles for the excitation and emission waveforms), τ_ϕ is the phase shift lifetime, and f is the excitation frequency. ϕ can span 0 to 90°. The modulation factor and modulation lifetime are given by

$$m = \frac{\mathrm{II}/I}{\mathrm{ii}/i} \tag{3}$$

$$m = (1 + \omega^2\tau_\mathrm{m}^2)^{-1/2} \tag{4}$$

where m is the degree of demodulation of the emission, II is the modulated amplitude of the emission light, I is its average intensity, ii is the modulated amplitude of the excitation source, i is the average intensity of the excitation source, and τ_m is the modulation lifetime. For a single exponential decay, both the phase shift and demodulation factors give the same lifetime. Since phase measurements are generally simpler than modulation measurements, they are the preferred methods on low-cost instruments. For more complex decays, the two will differ, and the differences will depend on the detailed decay kinetics and the modulation frequency.[2] For sensor analysis, a reproducible apparent lifetime versus analyte concentration is required, even if it is not a true single exponential decay. Unfortunately, the apparent lifetimes of conventional phase fluorometry are highly sensitive to minor and variable amounts of impurity fluorescence or scattering contributions. These errors reduce the attractiveness of conventional fluorometry in many applications.

The method discussed will use more complex excitation waveforms. For the analysis of these waveforms, Fourier analysis can be used. This is a rich field, and to realize it experimentally can require considerable attention to instrumentation and data analysis.[7−9] The analysis is based on the waveform after it has passed the transient start up and becomes periodic. The excitation and emission waveforms can be represented as a sum of sine and cosine functions

$$F(t) = \frac{A_0}{2} + \sum_{j=1}^{N}[A_j\cos(jt) + B_j\sin(jt)] \tag{5}$$

where $F(t)$ is the approximated waveform, A_0 is the DC constant or offset, and A_j and B_j are the cosine and sine Fourier coefficients, respectively, and j is the order of the term and ranges from the fundamental ($j = 1$) to the harmonics ($j > 1$). The lifetime can also be calculated from the phase shifts and demodulation factors of the sample relative to the excitation source for each frequency present.

The Fourier coefficients of the fundamental and higher harmonics can be used to calculate the amplitudes and phase angles for each frequency

$$\mathrm{Amp}_j = \sqrt{A_j^2 + B_j^2} \tag{6}$$

$$\tan\left(\frac{\Phi_j}{j}\right) = \frac{-B_j}{A_j} \tag{7}$$

where Amp_j is the amplitude, Φ_j is the phase angle, and j is the corresponding harmonic.

The excitation and emission waveforms can be decomposed into their Fourier components. For each frequency, using the difference between the phase angles of the sample emission and excitation ($\phi = \Phi_{\mathrm{sample}} - \Phi_{\mathrm{excitation}}$), the sample lifetime can be calculated using

$$\tan(\phi_j) = j\omega\tau_\phi \tag{8}$$

In contrast to conventional phase fluorometry (Figure 1), our gated method uses square-wave excitation. Figure 2a shows the emission of a typical single component emitting species excited by a 50% duty cycle square-wave excitation. Figure 2b shows the same experiment but with the emission contaminated with a scatter/fluorescent contribution. The contaminations show up as discontinuities on the emission at the turn on and turn off of the excitation source. We can analyze these excitation and emission waveforms directly using eqs 5−8. We will refer to this as the ungated emission analysis. As is clearly visible when comparing parts a and b of Figure 2, the emission waveforms are quite different, and the lifetimes extracted from the two data sets by the ungated analysis will not agree, because there is no discrimi-

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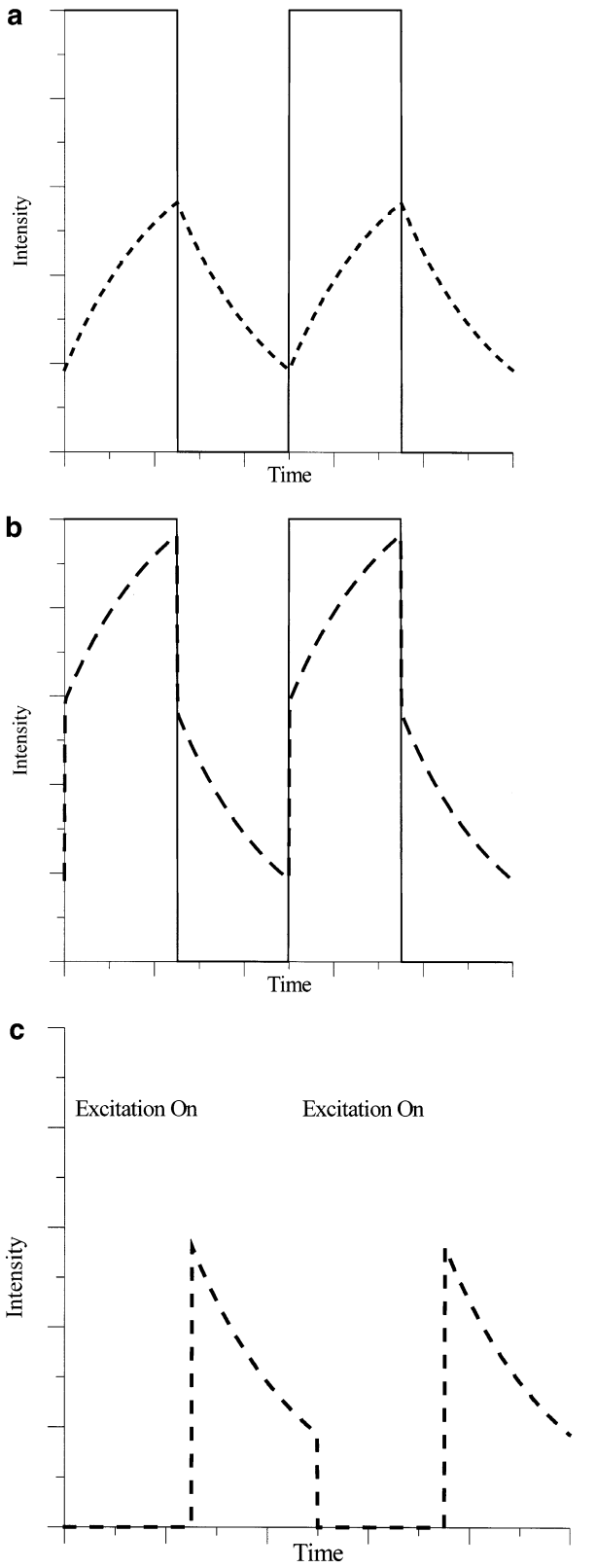

Figure 2. (a) Square-wave excitation (solid line) and ungated emission waveform (dashed line). (b) Square-wave excitation (solid line) and ungated emission waveform with short-lived luminescence/scattered light contaminants (dashed line). (c) Gated emission waveform with short-lived luminescence/scattered light contaminants (dashed line) to be collected by detector.

nation against scattered light or short-lived fluorescence in the sample emission.

For the gated method, the detector is gated on only during the off cycle of the excitation source. The gated waveform from Figure 2b is shown in Figure 2c. This is the waveform that would be processed by subsequent electronics and is exactly the same waveform as would be obtained for gating of the uncontaminated emission of Figure 2a. We assume that the impurity fluorescence lifetime is very short, as compared to the sample lifetime. Clearly, there is no contribution from impurity fluorescences/scatter to the gated waveform. The excitation and the emission waveforms are decomposed using Fourier analysis into their frequency components, and the phase angles, amplitudes, and phase shifts of the sample relative to the excitation source are computed using eqs 5−7. Because we are no longer using the entire emission decay, eq 8 no longer accurately describes the lifetimes.

For the square-wave excitation with gating, there is an even/odd harmonic dependence on the relationship between the phase shift and the calculated lifetime. The lifetimes are given by

$$\tan(\phi_{\text{gated}} + (\pi/2)) = n\omega\tau_\phi \qquad j = 1, 3, 5, 7... \qquad (9a)$$

$$\tan(\phi_{\text{gated}}) = n\omega\tau_\phi \qquad j = 2, 4, 6, 8... \qquad (9b)$$

The fundamental and odd harmonics of the gated sample waveform are phase-shifted 90° ($\pi/2$) behind the fundamental and odd harmonics of the ungated sample waveform. After this adjustment in eq 9a, the sample lifetimes for the fundamental and odd harmonics can be calculated. Equation 9b can be used to calculate the sample lifetimes for the even harmonics.

As long as the lifetimes of any luminescence impurities are short, as compared to the off period, these will decay promptly and not be observed by the detector in gated detection. These short-lived components and stray light will be present only during the excitation and will have minimal contribution to the gated signal. Thus, our gated phase fluorometry has inherent rejection of short-lived or scattering interfering agents.

A number of variations of the basic gated-phase lifetime measurement are possible. If the interfering agents are sufficiently long-lived to persist into the monitoring time or the shut-off of the light or gate circuit is not crisp enough, the on excitation period can be reduced to less than a 50% duty cycle while leaving the emission detection gate at 50%. This provides a delay or offset between the excitation and detection periods during which the interfering agent's emission or scattering will decay to minimal levels. The phase angle and lifetime calculations for the offset remain identical to that given above for the standard 50% duty cycle, although the usable intensity during the off period will be reduced somewhat.

In addition, the excitation pulse period can be much less or greater than 50%, and the monitoring gate can be adjusted to fill the off period. This modification can actually reduce the available emission intensity and increase the timing complexity, so in general, a 50% duty cycle is probably optimum.

Another advantage of our gated method is the larger number of harmonics with usable amplitudes. Ungated phase-shift measurements using a 50% duty cycle square-wave excitation have a very rapid falloff in the amplitude of the higher harmonics, and only the odd harmonics have appreciable intensity. Therefore, the

standard method with square-wave excitation is limited at most to a few harmonics. The gated phase-shift lifetime measurements have a slow falloff in the amplitude of both the even and odd harmonics. Thus, in gated phase-shift lifetime measurements, simultaneous measurements are possible at a number of frequencies using the calculated phase angles for the fundamental and the harmonics of the excitation and decay waveforms.

Mathematical Simulations. Simulations on the effect of fluorescence bleed-through were carried out by generating steady-state ac waveforms for specific lifetimes and modulation frequencies. This was done for a long-lived analyte and a short-lived fluorescent interfering agent. For contaminated data, the two waveforms were summed with the proper weights to give varying degrees of contamination. For different gating schemes, the waveforms had a digital gate applied to the waveform (0 when off, unattenuated when on). The resultant waveforms were then Fourier-analyzed. In those cases in which there was no closed form solution, computations were carried out numerically. All computations were performed in Mathcad 8 (MathSoft Engineering & Education, Inc., Cambridge, MA).

EXPERIMENTAL SECTION

Materials and Sample Preparation. $Na_3[Tb(dpa)_3]$ (dpa = 2,6-pyridinedicarboxylic acid) was the sample used in earlier measurements.[10] The $[Tb]^{3+}$ luminophore was chosen for its insensitivity to oxygen quenching and relative insensitivity to temperature changes near room temperature (<0.01%°C).

Pulsed Luminescence Lifetime Instrumentation. A pulsed VSL-337 nitrogen laser (337 nm) decay system was used to measure lifetime data.[10,11] Transients were recorded using a Tektronix TDS-540 digital oscilloscope. Emission decay curves were monitored at 500 nm. Each decay curve was the average of 400 sample decays. All data were taken at room temperature (22 ± 2°) in air-saturated water.

Phase Lifetime Instrumentation. The instrumentation has been described earlier.[12] A 40 mW CW Ion Technology Laser (Frankfort, IL) argon ion laser (488 nm) was the excitation source. The laser beam was modulated with an IntraAction (Bellwood, IL) acousto-optic light modulator (AOM) model AOM-40 using an IntraAction signal processor model ME-40 rf driver. The ME-40 required an analogue input signal generated by a Stanford Research System DS340 digitally controlled signal generator that also served as the trigger for the photon counter.

The laser beam monochromaticity was improved with a 488-nm narrow band-pass interference filter to eliminate the plasma emission. Three cobalt blue filters were used to attenuate the intensity. The sample emission was filtered with a 488-nm blocking notch filter and light orange and two yellow filters that further reduced 488-nm scatter and attenuated the intensity.

The emission was detected with a low dark count cooled R928 photomultiplier tube. The single photon pulses were counted with the Stanford Research System SR430 (Palo Alto, CA) multichannel averager/scaler. The bin width used for the acquisition was 2560 ns with 6144 bins (ca. 16 ms). The scaler was controlled by computer software written in Labview 5.1.1 (National Instruments, Austin, TX) that allowed direct downloading of the waveforms into the computer and Fourier analysis.

Exact timing of the excitation waveform relative to the emission was established by recording a reference transient of the square wave using a solvent blank in place of the $[Tb^{3+}]$ complex with only cobalt blue filters to attenuate the emission intensity. This combination produces an intense, easily measured scatter signal for exact timing of the excitation relative to the sample transients. The reference transient allows the software to automatically determine the starting and ending points of the excitation profile. Using these points, the excitation portion of the profile can be gated out of the sample emission transient. In a hardware implementation of this system, gating could be controlled directly from the driver.

Data Analysis. Because of the imperfections in the modulator, there was an artifact in the data that needed to be corrected. The modulator did not have a perfect off period, and there was a small constant background value of 12−15 counts/bin during the off period relative to ∼2700 counts at the peak. To determine this background, we fit the decay during the off period to

$$I(t) = e^{-t/\tau} + c \tag{10}$$

where $I(t)$ is the emission intensity as a function of time, and c is the constant, which is subtracted from the transients to create corrected data sets. These corrected data sets were then used for the Fourier analysis. In an LED-based instrument, there would be no correction of this type.

The waveforms were analyzed using Labview's Discrete Fourier Transform (DFT) function on the corrected transients for the excitation reference, the ungated sample, and the gated sample to obtain the phase angles. The function returns several thousand sets of Fourier coefficients for the fundamental and harmonics; however, only a relatively small number of the lower frequencies contained useful information for the current study. The coefficients include real and imaginary or complex numbers in the form of real + imaginary i. The phase angles are calculated from

$$\phi = \arctan\left(\frac{\text{imaginary}}{\text{real}}\right) \tag{11}$$

To calculate the lifetimes for the transients at a fixed modulation frequency, the reference phase angles for the excitation fundamental and harmonics were subtracted from the corresponding phase angles for the sample to obtain the phase shifts. For the ungated waveforms, the lifetimes were calculated from eq 8. For the gated transients, eqs 9a and 9b were used to calculate the lifetimes.

The $[Tb]^{3+}$ complex lifetime from the pulsed system was determined using nonlinear least-squares fitting. The decay fit confirmed the expected lifetime of 1.970 ms with a standard deviation of 0.004 ms.[12]

RESULTS AND DISCUSSION

Typical ungated and gated transients for the $[Tb]^{3+}$ sample are shown in Figure 3a and b. Lifetimes were calculated from these waveforms by means of Fourier analysis and eqs 8−9.

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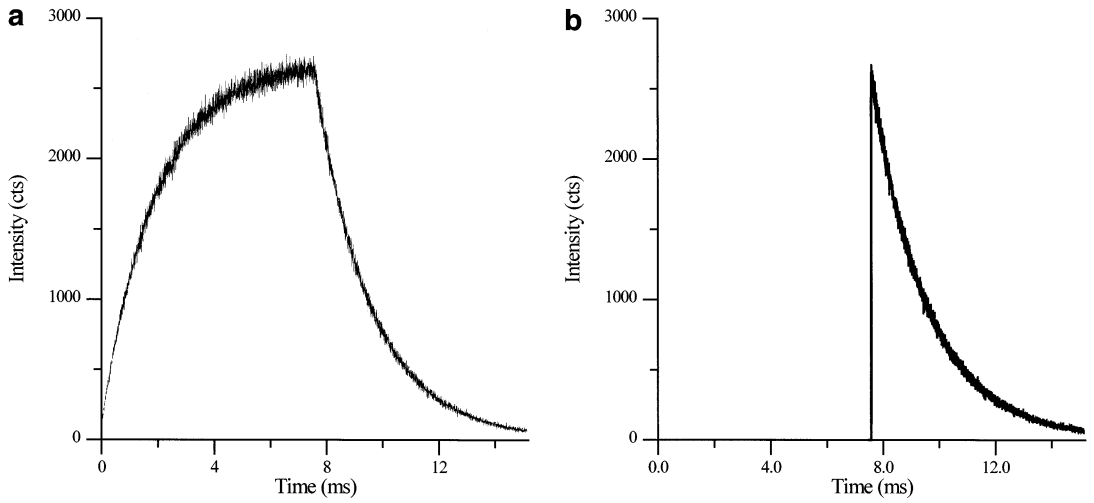

Figure 3. (a) Digitized waveform of a typical ungated transient. (b) Digitized waveform of a typical gated transient. The values-before-emission portion of the 50% duty cycle are replaced with zeros.

Table 1. Results of the Ungated and Gated DFT Lifetime Average Calculation and Standard Deviation for the Fundamental and First 17 Harmonics of Three $[Tb]^{3+}$ Experiments

harmonic	ungated av τ (ms)	gated av τ (ms)
expected	1.970 ± 0.004	
fundamental	1.909 ± 0.002	1.963 ± 0.004
2	2.3 ± 0.8	1.963 ± 0.004
3	1.781 ± 0.014	1.956 ± 0.007
4	1.5 ± 1.2	1.964 ± 0.004
5	1.51 ± 0.06	1.966 ± 0.010
6	1.5 ± 0.9	1.939 ± 0.009
7	1.27 ± 0.05	1.94 ± 0.03
8	0.7 ± 0.7	1.93 ± 0.07
9	1.07 ± 0.01	1.93 ± 0.15
10	0.30 ± 0.12	1.93 ± 0.14
11	0.71 ± 0.07	1.86 ± 0.08
12	2 ± 3	1.76 ± 0.05
13	0.8 ± 0.5	1.74 ± 0.015
14	0.4 ± 0.4	1.71 ± 0.07
15	0.61 ± 0.11	1.71 ± 0.09
16	0.13 ± 0.05	1.79 ± 0.07
17	0.45 ± 0.04	1.74 ± 0.17

Lifetime Results. The average ungated and gated transient lifetimes and the standard deviation for the fundamental through the 17th harmonic of three $[Tb]^{3+}$ experiments are listed in Table 1.

For the ungated transient, the fundamental and, less accurately, the third harmonic produce the only accurate lifetime measurements. In this case, the accuracy is not as good as the gated data. This may be a consequence of small amounts of uncorrected fluorescence in the on period. It would require an uncorrected fluorescence contribution of only 1.5% to the continuous excitation intensity (CI) to produce this error, and it would not show up visually on the experimental data. The even harmonics of the ungated transient are consistently inaccurate and imprecise, as expected from their zero amplitude in a noise-free Fourier analysis. However, the odd harmonics have a rapid falloff in amplitude, which also gives very poor precision and accuracy. What is amazing is that the even harmonics actually do yield estimates of the lifetime. This is probably a consequence of the noise generating nonzero components for the even harmonics.

Gated detection was more accurate for the fundamental and the higher harmonics, which is due to slower falloff of the amplitude. The results of the gated detection were reliable and yielded lifetime values within 1% of the actual lifetime for the fundamental and first four harmonics. Although the 17th harmonic is within 12% of the actual sample lifetime, the 12th is the first one that is not within experimental error of the correct lifetime. Thus, it would be prudent to not use data beyond the 11th harmonic.

Effect of Finite Fluorescence Lifetime. To explore the sensitivity of the gated method to bleed-through of a finite lifetime fluorescent impurity into the detection period, we modeled two systems consisting of a short-lived contaminant (5 ns) and a longer-lived sample (1000 or 200 ns) as a function of the degree of impurity contribution. For the degree of sample contamination, we used the percentage of impurity emission under conditions of continuous illumination (CI). We chose 5 ns for the contamination, since many contaminants have lifetimes on the order of 5 ns or shorter. The longer lifetimes are similar to those of inorganic complex sensor molecules. For the 1000-ns lifetime component, we used 200 kHz modulation, and for the 200-ns lifetime, we used 1 MHz. These are slightly higher than the optimum frequencies that give a 45° phase shift.

Figure 4 shows the apparent calculated ungated and gated lifetimes for the 1000-ns sample with increasing fluorescence contamination (0−83% CI). We show the fundamental and third harmonic for the ungated data and through the third harmonic for the gated case. For comparison, we include the gated data for the third harmonic using a 25-ns offset of the excitation source; this gives the impurity fluorescence time to decay before the detector is turned on. For the ungated data, our results confirm the well-known sensitivity of conventional phase fluorometry to even small amounts of contamination from short-lived components. When 5% of CI intensity is due to the contaminant, the ungated calculated lifetime is off by more than 11%. In comparison, the gated technique produces astoundingly accurate results. When the contaminant produces 83% of the CI intensity, the gated lifetime (fundamental) is off by only 6%, as compared to 92% for the ungated case. Thus, under many circumstances, a short-lived

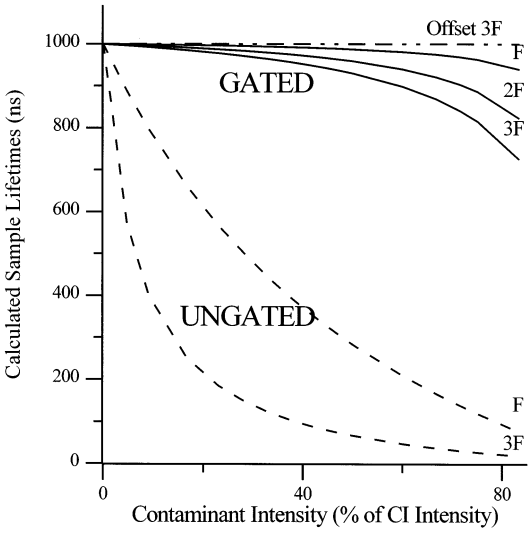

Figure 4. Ungated (dashed lines), gated (solid lines), and offset gated (dashed−dotted line) calculated lifetimes for the 1000-ns sample with increasing contaminant intensity, where *F* is the frequency for the fundamental (F) and corresponding higher harmonics (2F, 3F).

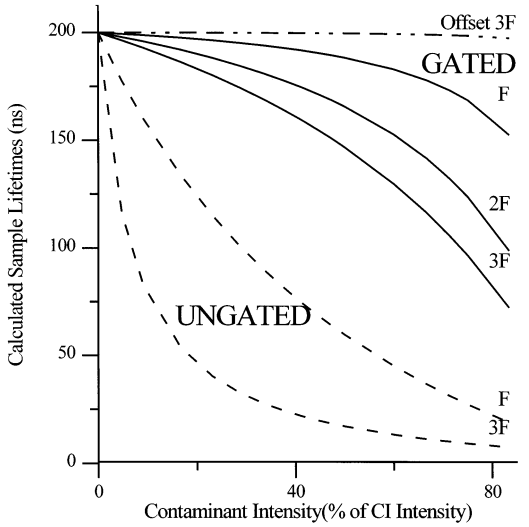

Figure 5. Ungated (dashed lines), gated (solid lines), and offset gated (dashed−dotted line) calculated lifetimes for the 200-ns sample with increasing contaminant intensity, where *F* is the frequency for the fundamental (F) and corresponding higher harmonics (2F, 3F).

component will produce only minor degradation of accuracy with the gated method, even without accounting for fluorescence bleed-through into the detection period. As expected, the errors in both gated and ungated methods rise with the higher-order harmonics, because they give increasingly greater weights to the faster components.

However, if the errors from bleed-through are unacceptably large with the gated detection, we can reduce the errors substantially by using offset gating. By setting the offset to five lifetimes of the fluorescence impurity, its contribution will be reduced by 2 orders of magnitude before the detector is gated on. The measured gated lifetimes using this offset are also shown in Figure 4 for the third harmonic, where the errors are much larger than for the fundamental. Even for the third harmonic, the calculated gated lifetime with 83% of the CI intensity due to contaminant is within 0.3% of the correct value, which is a negligible error.

Figure 5 shows analogous data using 1 MHz modulation with a 200-ns sample and 5-ns impurity. Because the lifetime of the contaminant and the analyte are much closer, this is a more demanding resolution problem. However, even here, gated fluorometry has reasonable discrimination against the short-lived fluorescence impurity. When 5% of the CI intensity is due to the contaminant, the ungated calculated lifetime for the fundamental is again off by more than 11%. The gated technique produces significantly more accurate results. The 6% error mark for the fundamental is reached when the contaminant produces 50% of the CI intensity. At 83% CI, the gated lifetime calculated is off by 24%. However, accuracy is recovered by using an offset gate equal to 5 times the contaminant lifetime (25 ns); the calculated lifetime is within 0.3% and 1.4% of the correct value for the fundamental and third harmonic, respectively.

The offset can also be used to reduce errors from a finite turn-off time for the excitation sources or the gating circuit. If the excitation source, such as an LED, does not turn off instantly, it can be treated as an impurity. The 25-ns offset of excitation does cause loss of gated emission intensity, but this effect is small for the 200-ns case and negligible for the 1000-ns case.

If bleed-through were significant, it would be possible to use a modification of the Jameson-Weber method to further improve the data. They presented ungated equations that allow the resolution of two components using phase and modulation measurements at only two frequencies.[13] These equations would have to be modified for the gated case.

Implementation. Although our method of data collection using the single photon counter is effective and demonstrates the substantial advantages of gated phase fluorometry, it is not the only gating scheme possible. The detection can be gated either mechanically or electrooptically before the detector. The detector can be turned on only during the detection (e.g., pulsed high voltage on the PMT dynode strings or a gating grid on the PMT). The output signal can be gated either by analogue or by digital techniques, depending on whether the signal is a single photon-counting pulse train or an analogue signal. Alternatively, in-line digital filters can be used. Phase-angle measurements would be simplified by the use of a lock-in amplifier, which averages the signal and gives a better S/N ratio. Using traditional lock-in amplifier signal processing, only the fundamental in the Fourier analysis would be used.

CONCLUSIONS

The combination of theory, modeling, and experiment demonstrate the validity of this new gated phase-modulation decay method and its utility for reducing or eliminating background. Using a 50% duty cycle square-wave excitation, traditional data treatment can be utilized and requires at most only a phase adjustment. The gated approach yields results that are precise, accurate, eliminate scattering errors, and greatly reduce errors from short-lived fluorescence impurities. Using only a single frequency excitation, the gated method provides usable detection

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intensities for much higher harmonics than conventional fluorometry. With gating, both even and odd harmonics can be used, whereas in conventional fluorometry, only the fundamental and odd harmonics have usable intensity. Errors from fluorescence bleed-through into the detection period or a slow excitation source turn off can be effectively eliminated by introducing an offset time after the excitation is turned off but before the detector is turned on. With an offset of five times the fluorescence impurity lifetime or the excitation turn off time constant, more precise, accurate, contaminant-free lifetime measurements are possible for several harmonics in addition to the fundamental.

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