Published in final edited form as:

Anal Biochem. 2002 October 01; 309(1): 19–26. doi:10.1016/s0003-2697(02)00213-0.

Real-time background suppression during frequency domain lifetime measurements

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Abstract

We describe real time background suppression of autofluorescence from biological samples during frequency domain or phase modulation measurements of intensity decays. For these measurements the samples were excited with a train of light pulses with widths below 1 ps. The detector was gated off for a short time period of 10 to 40 ns during and shortly after the excitation pulse. The reference signal needed for the frequency domain measurement was provided by a long-lifetime reference fluorophore which continues to emit following the off-gating pulse. Both the sample and the reference were measured under identical optical and electronic conditions avoiding the need for correction of the photomultiplier tube signal for the gating sequence. We demonstrate frequency domain background suppression using a mixture of short- and long-lifetime probes and for a long-lifetime probe in human plasma with significant autofluorescence. © 2002 Elsevier Science (USA). All rights reserved.

Fluorescence measurements of biological samples are frequently compromised by the presence of background or autofluorescence from the samples. Tissues often display autofluorescence from collagen, NADH, and other components [1–7]. In some instances the autofluorescence is the desired signal. However, there are many other cases in which the desired signal is from an added or extrinsic fluorophore, so it is desirable to subtract or suppress the background emission. For steady state measurements one may subtract the emission observed from the control sample to obtain the background corrected emission spectra. Similarly, in time domain measurements one can subtract the time-dependent signal from the unlabeled sample to obtain the background correction time-dependent decay of the added label.

At present, frequency domain FD² or phase modulation measurements are widely used for time-resolved spectroscopy of biopolymers [8], fluorescence sensing [9,10], and lifetime imaging [11–14]. In the FD measurements the background emission contributes to the phase angle and modulation measurements at each light modulation frequency. Simple subtraction procedures are not adequate for correction of the FD data. Procedures to correct the FD data have been developed [15,16]. These procedures are general and work for any values of the

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²Abbreviations used: bpy, 2,2'-bipyridine; dc bpy, 4,4'-dicarboxy-2,2'-bipyridine; FD, frequency domain; MLC, multiligand complex; PG, propylene glycol; PMT, photomultiplier tube; TR, Texas red.

sample and background lifetime. However, the methods are complex and require multifrequency phase and modulation measurements of the background signal which are then used to calculate the corrected phase and modulation data.

The autofluorescence from most biological samples is known to be on the nanosecond timescale [1,2]. These lifetimes are much shorter than those of some commonly used probes, such as the lanthanides with millisecond decay times [17] or the transition metal–ligand complexes (MLCs) with microsecond lifetimes [18,19]. In fact, lanthanides [20–22] and MLCs [23–26] have been developed for medical assays because of their long decay times and opportunities for background rejection.

In recent papers (e.g., [27]) we proposed a theoretical approach to the suppression of autofluorescence during frequency domain measurements. This approach depends on the use of pulse train excitation, off-gating of the detector during and shortly after the excitation pulse, and a long lifetime reference fluorophore. We now demonstrate experimentally that this approach in fact provides rejection of short-lived autofluorescence during the FD measurements. Suppression was demonstrated in mixtures of short- and long-lifetime fluorophores and during the measuring of microsecond decay times from human plasma.

Theory

Prior to describing the FD measurements with background suppression we will describe the theory and principles of the method. Suppose the intensity decay is multiexponential

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

where α_i are the amplitudes of each decay time component τ_i and $\alpha_i = 1.0$. The contribution of each decay time component to the steady state or total emission is given by

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

Recovery of the α_i and τ_i values requires phase angle (Θ) and modulation (m) measurement over a wide range of light-modulated frequencies [28–30]. An amplitude-modulated light source is often used to obtain approximate sine wave-modulated light. An amplitude-modulated light source would not allow background suppression because the background contributes to the signal whenever the sample is illuminated. That is, the desired signal and the autofluorescence contribute to the phase and modulation values at all modulation frequencies. However, FD measurements can also be accomplished using a train of light pulses [31–33]. In this approach the measurements rely on the harmonic content of the light pulses. Fortunately, such a pulse train is ideal for background suppression during the frequency domain measurements.

Assume that the intensity decay displays two lifetimes, an unwanted background emission with $\tau_B = 10$ ns and the desired signal from the sample $\tau_S = 1$ μ s. If this sample is excited by a train of light pulses, each excitation pulse initiates the 10-ns and 1- μ s decays of Fig. 1 (top). Now, suppose that emission is observed using a detector which is gated off during the light pulse. The gating signal will be a repetitive square wave (Fig. 1, middle) which turns on shortly after an excitation pulse and remains on until most of the signal has decayed. If the sample is examined with such a gated detector, the only signal will be from the 1- μ s component which continues to emit when the detector is turned on.

Frequency domain measurements are performed using the emission from a reference sample which either scatters light or displays a known lifetime [34,35]. Scattered light displays an apparent zero lifetime. Lifetime reference fluorophores typically have nanosecond lifetimes [34,35]. Neither scattered light nor nanosecond fluorophores can be used with the gating scheme shown in Fig. 1 because there will be no detectable emission when the detector is gated on approximately 100 ns after the excitation pulse. This difficulty can be circumvented by the use of reference fluorophores with a lifetime exceeding 100 ns. Suppose the reference displays a lifetime of 500 ns (dotted line). Then its emission will persist in the time window when the detector is activated (Fig. 1, bottom). When the signals from the sample and the references are compared electronically, it appears that the emission begins when the detector is turned on, just as if the detector was on at all times. That is, the electronic signal appears when the detector is turned on, just like the signal appears during the excitation pulse when the detector is always on. This means that the data can be analyzed directly as if gating was not being used, except for two simple considerations. First, as is always needed with reference fluorophores, the phase and modulation measurements relative to the reference fluorophore ϕ_{obs} and m_{obs} need to be corrected to the values which would have been observed for a zero decay time scattering reference. These correction phase and modulation values are given by

$$\phi = \phi_{obs} + \phi_R \quad \text{and} \quad (3)$$

$$m = m_{obs} m_R, \quad (4)$$

where ϕ_R and m_R are determined by the lifetime (τ_R) of the reference fluorophore and the modulation frequencies in radians per second (ω) by

$$\phi_R = \tan^{-1}(\omega\tau_R) \quad \text{and} \quad (5)$$

$$m_R = (1 + \omega^2\tau_R^2)^{-1/2}. \quad (6)$$

Correction for τ_R is a standard part of most FD analysis software. For a single exponential decay no further corrections are needed.

Suppose the intensity decay of the longer-lived emission is multiexponential. The correct decay times will be recovered from the analysis described above. However, there will be some distortion of the preexponential factors. Suppose these samples display a nanosecond background lifetime τ_B , plus two longer decay times τ_1 and τ_2 . The amplitudes recovered with gating represent the observed values of α_i^{Obs} at the time that the detection is turned on [27]. These are different from the true time-zero values because the decay times are different and each component has decayed as different amounts between the excitation pulse and the turn-on time of the gate (t_{ON}). It is straightforward to calculate the α_i values at $t = 0$ (α_i^0) using the recovered lifetimes and amplitudes. For a double exponential decay these amplitudes are proportional to

$$\alpha_1^{Obs} = k\alpha_1^0 [\exp(-t_{ON}/\tau_1) - \exp(-t_{OFF}/\tau_1)], \quad (7)$$

where k is the proportionality constant. The ratio of α_1^0 to α_2^0 can be calculated using

$$\frac{\alpha_1^0}{\alpha_2^0} = \frac{\alpha_1^{Obs} [\exp(-t_{ON}/\tau_2) - \exp(-t_{OFF}/\tau_2)]}{\alpha_2^{Obs} [\exp(-t_{ON}/\tau_1) - \exp(-t_{OFF}/\tau_1)]}. \quad (8)$$

The normalized values of α_1^0 and α_2^0 are calculated by recalling that $\alpha_1^0 + \alpha_2^0 = 1.0$.

Materials and methods

A block diagram for the FD instrument with gated detection is shown in Fig. 2. The FD measurement electronics were based on an ISS, Inc. K2 instrument. Excitation at 400 nm with a 100-kHz repetition rate was obtained after doubling the 800-nm output of a regeneratively amplified Ti:sapphire laser, RegA-OPA system from Coherent, Inc. The emission was detected through a OG 550-nm long-pass filter. The synchronization for gating was provided by a small fraction of the incident light directed onto an avalanche photodiode with the output amplified by an EG&G Ortec 9306 1-GHz preamplifier. The output of this amplifier was shaped by an EG&G Ortec 9307 pico-timing discriminator delayed by an Ortec 425A passive delay line, and then used to trigger a Model 8114A high-voltage programmable pulse generator from Agilent Technologies. This device provides an adjustable DC offset, pulse amplitude, pulse width, delay, and polarity.

The gating signal from the pulse generator was DC-coupled to the ninth dynode of a Hamamatsu R928 photomultiplier tube (Fig. 3). By using the last dynode we were able to take advantage of the DC offset of the high-voltage pulse generator. Use of the ninth dynode also appeared to minimize cross talk and interference between gating and modulation that we found when using the third dynode for both gating and heterodyne modulation of the PMT. Typically we applied a -50 -V DC offset to the last dynode. Gating was accomplished using positive 55-V pulses with a duration of 130 ns. The effective gate width was determined by the overlap of this pulse width and the fluorescence decay. The overlap was

adjusted by time delay of the gating pulse relative to the excitation pulse. For most experiments we used a 40-ns effective gate width of the detector. Scattered light from a dilute solution of Ludox was used to set the timing of the gating electronics. We found that the prompt signal from scattered light was suppressed 95 to 98% by gating. Heterodyne gain modulation was accomplished by standard methods [28,29] using a Marconi 2022D frequency synthesizer and an ENI 403 LA power amplifier. Data acquisition was accomplished as usual using the ISS, Inc. software. As a lifetime standard we used Ru(bpy)(mcbpy)₂ in water with a decay time of 269 ns. Gated and ungated emission spectra were obtained using the same instrument with a monochromator inserted into the emission light path.

Results

As a test of our gated FD instrument we used a mixture of Ru(bpy)(mcbpy)₂ and Texas red (TR) in propylene glycol. When measured individually these probes display single exponential decay times of 557 and 4.45 ns, respectively (Fig. 4, bottom and Table 1). Without gating, a complex frequency response was observed from a mixture of these two fluorophores (Fig. 4, top, •). With gating, the frequency response become similar to that found for Ru(bpy)(mcbpy)₂ alone, as can be seen from the larger phase angle near 2 MHz (Fig. 4, top, ○). Without gating, the mixture displayed roughly equal fractional intensities (f_i) from each probe (Table 1). With gating, the recovered decay was dominantly (98%) due to the long-lifetime MLC. The gating was not complete as seen from the 2% contribution of TR and the slight downturn of the phase angles at frequencies above 2 MHz. Since this was a proof-of-principle experiment we did not attempt to maximize the off gating. However, it is well known that cutoff ratios larger than 7×10^5 can be obtained with side window PMTs [36], so we believe that complete suppression of scattered light can be readily accomplished.

As a further test of FD gating we examined human plasma which contained 10 μ M Ru(bpy)(dcbpy)₂. Under our experimental conditions about half of the steady state emission was due to autofluorescence from the plasma centered at 510 nm and the remainder from the MLC centered at 630 nm (Fig. 5). Emission spectra taken with gated detection showed strong suppression of the plasma autofluorescence, but an approximate 5% component remained near 510 nm (Fig. 5).

Frequency domain data from plasma, Ru(bpy)(dcbpy)₂, and plasma containing Ru(bpy)(dcbpy)₂ are shown in Fig. 6. Plasma displayed a complex decay with decay times ranging from 0.1 to 13.7 ns. The frequency response of the mixture without gating shows a complex response (Fig. 6) with about half of the emission due to decay times of 14 ns or shorter (Table 2). The use of gating suppresses these short decay times so that the frequency response becomes characteristic of the MLC, and 94 to 97% of the decay was due to a 410-ns component (Table 2). In plasma, the MLC displayed a nearly single exponential decay of 387 ns (Table 2). The usefulness of gating is further shown in the time-dependent decays reconstructed from the FD data (Fig. 7).

In the absence of gating, there is a dominant nanosecond component with a total amplitude (α_i) of 99.9% for components with decay times less than 10 ns. With gating, the short component is greatly reduced to 3% or less (Table 2).

Discussion

Frequency domain lifetime measurements with real-time background suppression can have numerous applications in fluorescence spectroscopy and imaging. Long-lifetime species are becoming widely used in biomedical research. For example, sensors based on transition metal-ligand complexes [37–40] and on luminescent lanthanides [41–43] have been developed. Luminescent lanthanides have also been developed as MRI contrast agents to provide both magnetic resonance and optical signals [44–46]. Since the microsecond to millisecond lifetimes of the sensors are much longer than tissue autofluorescence, one can imagine lifetime-based sensing in tissues with gated FD measurements. The use of MLCs or lanthanides with gated detection may be of use with resonance energy transfer (RET). In these cases RET results in acceptor decays with long lifetimes which are specific for the donor-acceptor pairs [47–51]. Gated FD measurements are also likely to be used in fluorescence lifetime imaging microscopy (FLIM) [52,53] and with DNA arrays or gene chips [54–56]. In both cases sensitivity can be limited by autofluorescence from the sample or the optical components. The use of gated FLIM would allow lifetime imaging of long-lived probes in cellular imaging or gene expression.

Acknowledgments

The authors thank Agilent Technologies for the loan of the high-voltage pulse generator. This work was supported by the NIH, National Center for Research Resource (RR-08119).

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Fig. 1.

Intuitive description of frequency domain measurements with gated detection. The parameter values used for the simulations are $\tau_B = 10 \text{ ns}$, $\alpha_B = 1000$, $\tau_S = 1000 \text{ ns}$ (solid line), $\alpha_S = 10$, $\tau_R = 500 \text{ ns}$ (dotted line), $\alpha_R = 10$, and a pulse repetition rate of 50 kHz. The gain of the detection **PMT** was turned on at the time of $t_{ON} = 100 \text{ ns}$ after the excitation pulse and turned off at top $t_{OFF} = 5000 \text{ ns}$. The on/off gain ratio was set to the value of 10^5 .

Fig. 2.
Block diagram of the gated FD fluorometer.

Fig. 3.
Electrical scheme of the **PMT** socket for gated frequency domain fluorescence experiments.

Fig. 4. Frequency responses of a mixture of Texas red and Ru(bpy)(mcbpy)₂ in propylene glycol (PG) with and without PMT gating (top) and the individual frequency responses (bottom). The solid lines are for the fitted parameters in Table 1.

Fig. 5. Spectra of Ru(bpy)(dcbpy)₂ in plasma acquired with and without gating. For comparison the ungated spectra of the individual components are shown.

Fig. 6. Frequency responses of $\text{Ru}(\text{bpy})(\text{dcbpy})_2$ in plasma without gating and with 10- and 40-ns gate widths (top). Fluorescence decays of the individual fluorescent components are shown at the bottom. The solid lines are for the fitted parameters in Table 2.

Fig. 7. Reconstructed time domain fluorescent decays of $\text{Ru}(\text{bpy})(\text{dcbpy})_2$ in plasma without gating and with 40-ns gate width. These decays are based on values from Table 1.

Table 1

Effect of gating on the fluorescence decay of a mixture of Ru(bpy)(dc bpy)₂ and Texas red in propylene glycol

Compound	τ (ns)	α_i	f_i^a	$\langle \tau \rangle$ (ns) ^b	χ_R^{2c}
Ru(bpy)(mc bpy) ₂ + TR	4.13	0.993	0.511		
No gate ^d	560	0.007	0.489	275.8	3.8
Ru(bpy)(mc bpy) ₂ + TR	4.13	0.695	0.017		
40-ns gate ^d width	560	0.305	0.983	550.8	3.8
Ru(bpy)(mc bpy) ₂	15	0.05	0.001		
No gate	557	0.95	0.999	556.2	1.3
TR	0.1	0.54	0.026		
No gate	4.45	0.46	0.974	4.3	1.1

^aFractional fluorescence intensity.

^bMean lifetime $\langle \tau \rangle = \sum f_i \tau_i$

^cFor experimental uncertainties $\phi = 0.3$ deg and $m = 0.008$.

^dGlobal analysis.

Table 2
Effect of gating on the fluorescence decay of a mixture of Ru(bpy)(dc bpy)₂ in plasma

Compound	τ_i (ns)	α_i	f_i^a	$\langle \tau \rangle$ (ns) ^b	χ_R^{2c}
Ru(bpy)(dc bpy) ₂ in plasma	0.01	0.954	0.07	207.0	2.8 ^d
Without gate ^d	0.52	0.035	0.13		
	2.7	0.009	0.17		
	14	0.001	0.12		
	410	0.0002	0.51		
10-ns gate ^d width	0.01	0.927	0.002	385.0	
	0.52	0.000	0		
	2.7	0.055	0.03		
	14	0.008	0.02		
	410	0.010	0.94		
40-ns gate ^d width	0.01	0.982	0.003	398.0	
	0.52	0.000	0		
	2.7	0.000	0.003		
	14	0.008	0.03		
	410	0.010	0.97		
Plasma without gate	0.1	0.77	0.18	4.5	2.7
	0.6	0.16	0.22		
	2.7	0.05	0.35		
	13.7	0.008	0.25		
Ru(bpy)(dc bpy) ₂ in H ₂ O	387	1.0	1.0	387.0	1.4

^aFractional fluorescence intensity.

^bMean lifetime.

^cFor experimental uncertainties $\phi = 0.3$ deg and $m = 0.008$.

^dGlobal analysis.