Improved sensitivity for two-photon frequency-domain lifetime measurement

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Abstract: We demonstrate a method to improve the measurement sensitivity of two-photon frequency-domain lifetime measurements in poor signal to background conditions. This technique uses sinusoidal modulation of the two-photon excitation source and detection of the second harmonic of the modulation frequency that appears in the emission. Additionally, we present the mathematical model which describes how the observed phase shift and amplitude demodulation factor of two-photon phosphorescence emission are related to the phosphorescence lifetime and modulation frequency. We demonstrate the validity of the model by showing the existence of new frequency terms in the phosphorescence emission generated from the quadratic nature of two-photon absorption and by showing that the phase shift and demodulation match theory for all frequency components.

OCIS codes: (170.3650) Lifetime-based sensing; (190.4180) Multiphoton processes.

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1. Introduction

Fluorescence lifetime measurement techniques can be used to probe energy transfer processes between a fluorophore and its local environment. Because lifetime-based measurements are generally independent of fluorescence intensity, they are better suited for absolute quantitative measurements. This is particularly true for measurements in turbid samples such as biological tissues because intensity levels are strongly affected by the inhomogeneous nature of tissue, which leads to unpredictable dye concentrations, light absorption and scattering. Through the use of various fluorescent, phosphorescent and luminescent probe molecules, these techniques have proven to be very effective for quantifying a wide range of phenomena of interest to medical and biological researchers [1–3]. Due to their long excited-state lifetimes, phosphorescent probe molecules have been used for measuring local environmental concentrations of quenching agents. Other long lifetime probes such as ruthenium and lanthanide complexes have also gained considerable interest due to their sensitivities to local environment and long luminescence lifetimes. For example, ruthenium based and porphyrin based complexes have been used to measure pH and pO_2 respectively [4–7]. The desire to make these measurements depth-resolved has led to the combining of two-photon excitation with time-domain and frequency-domain lifetime determination methods [8,9]

Frequency-domain lifetime determination relies on using an intensity modulated excitation source and measuring the phase shift and demodulation factor of the emitted light. Well-established mathematical models relate the phase shift and demodulation factor to the lifetime [10]. For short lifetime samples (i.e. fluorescent samples), the most common method used for two-photon, frequency-domain lifetime measurement is multifrequency phase fluorometry based on the harmonic content of the pulse train from a mode locked Ti:Sapphire laser [11–14]. The phase shift and/or demodulation factor at each harmonic can be measured, and these values can be used to calculate the lifetime. Since the repetition rate of a Ti:Sapphire laser is generally ~70 - 100 MHz, this method is typically used for measuring lifetimes shorter than ~10 ns. To measure longer lifetimes when doing two-photon, frequency-domain measurements, the pulse train must be modulated such that the period of the modulation is of the same order of magnitude as the lifetime. The merits of various modulation functions have been evaluated [15]. In general, a low duty-cycle square wave that approaches the idealized pulse train used in multifrequency phase fluorometry produces optimal results [15].

Previously, others have reported the use of modulation at a single frequency to improve on the sensitivity of two-photon absorption measurements in high background conditions [16,17]. The basic principle is to modulate the two-photon source with a pure sinusoid and then analyze the resulting power loss at the second harmonic. The presence of a signal at the second harmonic is a result of the quadratic nature of the two-photon absorption process. Since the incident light does not contain the second harmonic, this signal can be distinguished from linear scattering processes (Rayleigh, Mie, etc…) in the frequency domain.

In this paper we show that this concept can be used to improve the sensitivity of two-photon luminescence lifetime measurements of long lifetime probes under low signal to background conditions. These conditions might be encountered when the fluorophore or phosphor concentration, or the two-photon action cross-section are low. We present a mathematical expression that relates the phase shift and demodulation factor of the emitted

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light to the lifetime. Furthermore, we demonstrate experimentally that by examining the generated second harmonic, measurement sensitivity can be improved.

2. Phase and demodulation in two-photon luminescence

In this section we outline the mathematical expressions that relate the measured phase shift and demodulation of the emission to the excitation light in the case of sinusoidal modulation of the laser pulse train. The derivation of these expressions follows an approach similar to that of one photon excitation [10], except that the quadratic nature of the excitation process is considered. The results show mathematically that when the excitation signal is sinusoidally modulated, the resulting two photon emission contains signal at the second harmonic of the modulation frequency. As demonstrated below in Section 3, this signal can then be used to determine the lifetime of the emission, which is particularly useful in low signal to background conditions.

Fluorescence and phosphorescence can be treated as an excitation process, which occurs on the femtosecond time scale, followed by an emission process which occurs from the picosecond to millisecond time scale. Furthermore, the intensity of the emission, as a function of time, is proportional to the product of the number of excited state molecules $N_e(t)$, and the quantum efficiency. Immediately after an excitation pulse, the number of excited molecules begins to decay according to the following homogeneous, linear differential equation:

$$\frac{dN_e(t)}{dt} + \frac{1}{\tau} N_e(t) = 0 \tag{1}$$

The solution to Eq. (1) is $N_e(t) = N_0 e^{-t/\tau}$. This exponential decay in $N_e(t)$ can be thought of as the impulse response of the emission process. In the simplest case, frequency-domain lifetime determination employs a sinusoidal excitation signal of the form $L(t) = a + b \cdot \cos(\omega t)$. Under two-photon absorption, $N_e(t)$ is quadratically related to $L(t)$, such that $L(t)^2$ becomes a source term for Eq. (1):

$$\frac{dN_e(t)}{dt} + \frac{1}{\tau} N_e(t) = cL(t)^2 \tag{2}$$

Although the excitation process is non-linear under two-photon absorption, the emission process is still governed by a linear differential equation. Consequently, the behavior of the system can be determined by convolving the source term with the impulse response of the emission process [10]

$$\begin{aligned} N_e(t) &= cN_0 \left[a + b\cos(\omega t)\right]^2 \otimes e^{-t/\tau} \\ &= \frac{cN_0}{2}\left[2a^2 + b^2 + 4ab\cos(\omega t) + b^2\cos(2\omega t)\right] \otimes e^{-t/\tau} \\ &= \frac{cN_0\tau}{2}\left[2a^2 + b^2 + \frac{4ab}{\sqrt{1+\omega^2\tau^2}}\cos(\omega t - \phi_1) + \frac{b^2}{\sqrt{1+4\omega^2\tau^2}}\cos(2\omega t - \phi_2)\right] \end{aligned} \tag{3}$$

Equation (3) represents the analytical expression for the fluorescence or phosphorescence when the excitation light is sinusoidally modulated and under two-photon absorption. This expression contains an offset term, a term that varies at the same frequency as the excitation (1*f* term) and a term that varies at twice the frequency of the excitation light (2*f* term). The phase shift of the 1*f* term is then given by $\phi_1 = \tan^{-1}(\omega\tau)$ and the phase shift of the 2*f* term is given by $\phi_2 = \tan^{-1}(2\omega\tau)$.

The concept of the demodulation factor must be considered more carefully in the two-photon case because of the generation of new frequency components. To generalize this concept so that it still has meaning for two-photon frequency-domain measurements we define the demodulation factor to be the ratio of the modulation depth of the fluorescence to the

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modulation depth of the number of excited state molecules, $N_e(t)$. For one-photon excitation, $N_e(t)$ is linearly related to the excitation function. Therefore, the modulation depth of the excitation function is an accurate substitute for the modulation depth of $N_e(t)$. Under our new definition, the demodulation factor can be calculated in the usual way as the ratio of the modulation depth of the emission over the modulation depth of the excitation. However under two-photon excitation, $N_e(t)$ varies as the linear combination of an offset term, a 1*f* term and a 2*f* term (Eq. (3)). The demodulation factors for the 1*f* and 2*f* terms, $M_1(\omega, \tau)$ and $M_2(\omega, \tau)$, respectively, should be the ratio of the modulation depth of the *1f* and *2f* terms of the emission to the modulation depth of the 1*f* and 2*f* terms in the source term of $N_e(t)$. For the 1*f* term we get:

$$M_1(\omega,\tau) = \frac{\dfrac{4ab}{(2a^2+b^2)\sqrt{1+\omega^2\tau^2}}}{\dfrac{4ab}{2a^2+b^2}} = \frac{1}{\sqrt{1+\omega^2\tau^2}} \tag{4}$$

Similarly, for the 2*f* term we get:

$$M_2(\omega,\tau) = \frac{\dfrac{b^2}{(2a^2+b^2)\sqrt{1+4\omega^2\tau^2}}}{\dfrac{b^2}{2a^2+b^2}} = \frac{1}{\sqrt{1+4\omega^2\tau^2}} \tag{5}$$

3. Results

To verify these expressions we first determined the lifetime of sample solutions using a standard time-domain approach with a two-photon excitation source. We then performed frequency-domain tests to compare the measured demodulation factor and phase shift of the 1*f* and 2*f* components to that predicted by the model given this measured lifetime. Figure 1(a) depicts our experimental setup. The plot in Fig. 1(b) illustrates the appearance of the 2*f* term in the two-photon fluorescence signal under sinusoidal excitation. In this illustration we modulated the intensity of the 76 MHz pulse train at 300 Hz, and measured the fluorescence emission from a cuvette of rhodamine, which is proportional to $N_e(t)$. When the resulting signal is fit to the standard one-photon expression of $A + B\cos(\omega t - \phi_1)$, the residual clearly shows *t*he existence of the 2f component as expected. Deviations of the residuals from a pure sinusoid at 2*f* are caused by small non-linearities in the response of the acousto-optic modulator (AOM).

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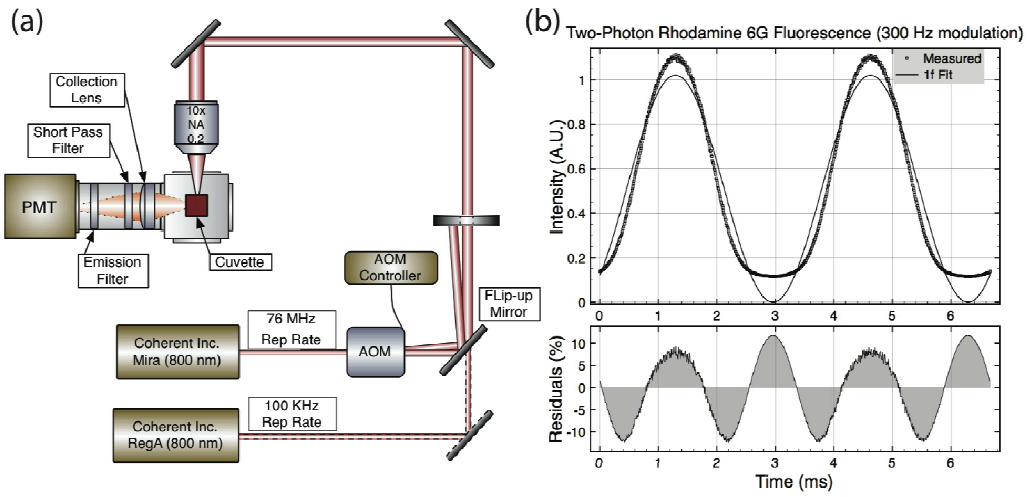

Fig. 1. (a) Experimental setup. (b) Rhodamine 6G fluorescence excited by a two-photon excitation source modulated at 300 Hz. The residuals from the best 1*f* fit clearly demonstrate the presence of the 2*f* term.

3.1 Time-domain measurements

We used Tris (2,2'-Bipyridyl) Ruthenium(II) Chloride Hexahydrate (CAS # 50525-27-4) as our luminophore because its long lifetime (~600 ns) obviated the need for more sophisticated detection methods such as cross-correlation detection. Time-domain experiments were done using a 4 ml cuvette containing 200 μM of ruthenium complex in physiologic saline. Because Tris (2,2'-Bipyridl) Ruthenium(II) Chloride Hexahydrate is readily quenched by oxygen, the dissolved oxygen was removed from the solution by adding 100 mg of glucose and 10 mg of glucose oxidase and sealing the cuvette. A Ti:Sapphire regenerative amplifier (RegA 9000, Coherent Inc.) was used as our two-photon excitation source for time-domain lifetime measurements. The repetition period of the RegA (4 μs) was well suited for the ruthenium complex's lifetime and its short pulse width (~200 fs) produced and ideal impulse of excitation. The ruthenium complex's emission was collected using an optical band-pass filter (HQ610/75M, Chroma Technology Corp.), an optical low-pass filter (< 750 nm, FF01-750/SP-25, Semrock) and photomultiplier tube (H7422P-40, Hamamtsu Corp). The photomultiplier tube (PMT) signal was fed to a transimpedance amplifier (HFAC-26, Becker and Hickl) and then to a photon-counting lifetime measurement board (DPC-230, Becker and Hickl).

Figure 2(a) shows the normalized fluorescence decay and optimal non-linear least squares fit to a single exponential decay. The best fit decay lifetime was found to be 604 ns, which agrees well with the published decay time for this ruthenium complex [18]. Although the residuals demonstrate that the luminescence decay curve does not perfectly follow a single exponential decay, fitting to multi-exponential decay similarly failed to produce a perfect fit and did not produce significantly improved chi-squared values. Furthermore, the magnitude of the residuals is sufficiently small (< 0.5%) using the single exponential decay model that any errors caused by assuming this model are negligible.

3.2 Frequency-domain measurements

The same setup used for the time-domain tests was used for the frequency-domain tests, except that a different excitation source was used. A Ti:Sapphire laser source (Mira 900, Coherent Inc) with a repetition rate of 76 MHz was intensity modulated using an acousto-optic modulator (NEOS Technologies 23080-2-LTD) to produce a pulse train inside of a sinusoidal envelope. Part of the modulated excitation light was directed to a photodiode and the signal was analyzed for any 2*f* content which may have been produced by non-linearities

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in the AOM. It was necessary to minimize the 2*f* content of the excitation light so that any residual scattered excitation light transmitted by the filters would not interfere with our measurement of the 2*f* fluorescence generated by the non-linear excitation process. The function generator was adjusted such that the 2*f* content in the excitation was at least 50 dB smaller than the 1*f* component.

We used a cuvette of fluorescein as a reference fluorophore so that we could characterize any instrumentation phase shifts at the various frequencies used. Because fluorescein's lifetime is short (<6 ns) compared to ruthenium's (~600 ns), no demodulation or phase shifting occurs at the modulation frequencies used (100 – 800 kHz) to test the ruthenium complex. Therefore, fluorescein was suitable as a zero phase shift reference fluorophore. Fluorescein data was acquired in an analogous fashion to ruthenium, except that a different band pass filter was used (HQ510/80M-2P, Chroma Technology Corp.). The fast Fourier transform (FFT) of the normalized signal was used to determine the phase shift and modulation depth for the 1*f* and 2*f* components. The offset term (DC component) of the resulting fluorescence was determined by taking the magnitude of the zero frequency component of the FFT. The magnitudes of the 1*f* and 2*f* components were similarly determined from the FFT. The phase shifts were then calculated by taking the difference of the ruthenium complex phases and the fluorescein phases for each frequency. To calculate the demodulation factors for the 1*f* and 2*f* components, the modulation depths of the 1*f* and 2*f* components within $L(t)^2$ *were needed. Although this could be calculated from the FFT of the photodiode signal* used to minimize the 2f content of the excitation light, we chose to use the fluorescein modulation depths as more accurate indicators of these values.

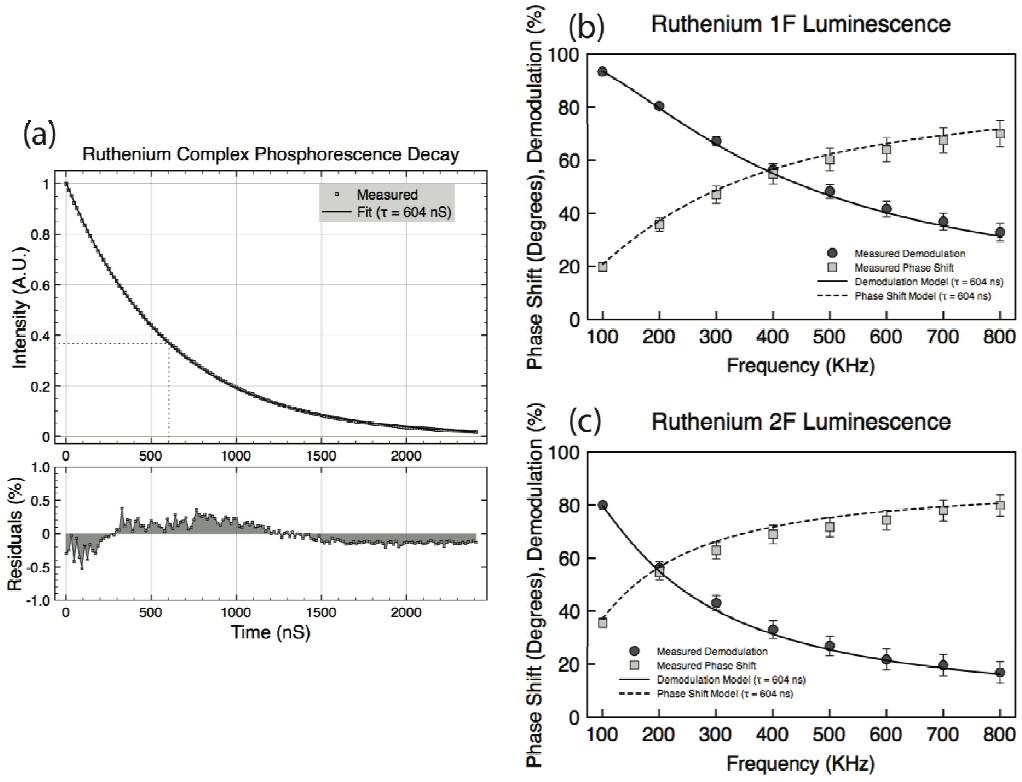

Fig. 2. Lifetime measurements of ruthenium solution. (a) Time-domain lifetime measurement. (b) Phase shift and demodulation data of 1*f* component of detected luminescence signal. (c) Phase shift and demodulation of 2*f* component of detected luminescence signal.

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Figure 2(b), 2(c) show the measured phase shift and demodulation of the 1*f* and 2*f* components. The trend lines in Fig. 2(b), 2(c) depict the values predicted by the model given the apparent lifetime determined from the time-domain measurement. The measured data agree with the model to within experimental error. The slight systematic discrepancy between the data and the predicted values can be attributed to the fact that the decay function of the intensity does not strictly follow a single exponential decay [Fig. 2(a)].

The existence of a 2F component, whose phase shift and demodulation factor follow the mathematical model derived above, is confirmation of the validity of the model.

4. Using the model to improve measurement sensitivity

To demonstrate the improvement in sensitivity we performed phase shift and demodulation factor measurements for 1*f* and 2*f* components under poor signal to background conditions. We used a solution of a palladium porphyrin complex as our sample since the complex is known to have a long lifetime and a small two-photon action cross-section [19]. The higher excitation powers needed to get appreciable phosphorescence from this complex results in greater leakage from scatter through our emission filters and therefore a poor signal to background measurement. The specific type of palladium porphyrin complex used for this experiment was a commercial molecular probe used for measuring dissolved oxygen concentration called Oxyphor R2 (Oxygen Enterprises) [20,21]. We used a 200 μM solution dissolved in saline. The same experimental setup and data analysis described above were used. Dissolved oxygen was removed from the solution by adding glucose and glucose oxidase to the cuvette just before sealing as described above. The emission filter used in this case was centered at 700 nm (HQ700/75M, Chroma Technology Corp.) to match the emission peak of Oxyphor R2.

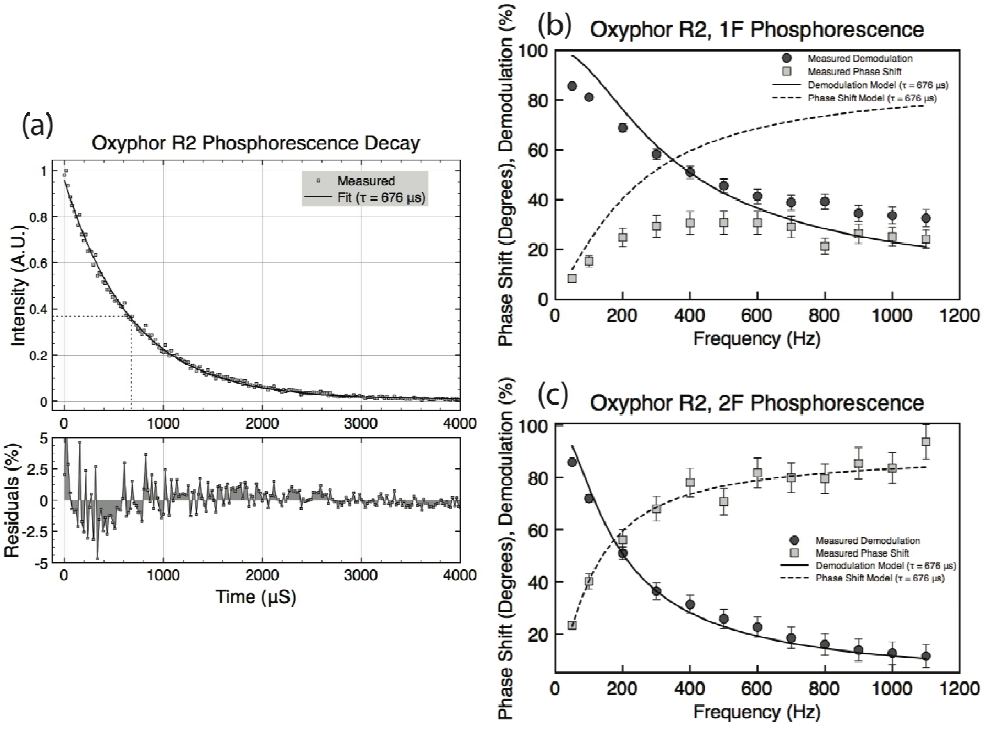

Fig. 3. Lifetime measurements of porphyrin phosphorescence. (a) Time-domain lifetime measurement. (b) Phase shift and demodulation of 1*f* component of detected phosphorescence. (c) Phase shift and demodulation of 2*f* component of detected phosphorescence.

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Using a time-domain measurement, we determined the lifetime of the solution to be 676 μs [Fig. 3(a)]. This agreed well with the published value of 686 μs [20]. The trend lines in Fig. 3(b), 3(c) depict the values predicted by the model given the apparent lifetime determined from the time-domain measurement. The frequency-domain data show that 1*f* measurements do not follow the model and that the discrepancy is worse for higher frequencies than lower frequencies. This is due to the demodulation factor producing lower phosphorescence signal as the frequency increases in the presence of a constant magnitude background signal. Determining the phosphorescence lifetime from the best fit (curve not shown) of the 1*f* data to the model results in a lifetime of 360 μs. Unlike the 1*f* data, the 2*f* results agree with the model throughout the range of frequencies tested. Since the background light varies at 1*f*, the 2*f* phosphorescence signal can easily be separated in the frequency domain. Determining the lifetime by fitting the 2*f* data to the model (curve not shown) results in a lifetime of 669 μs, which agrees well with the lifetime determined by the time-domain technique.

5. Discussion

We have demonstrated that when a pure sinusoid is used to modulate a two-photon excitation source, the 2*f* component of the resulting emission can be used to improve lifetime measurement sensitivity in poor signal to background ratio conditions. As shown in Fig. 3(b), the phase shift and demodulation of the emission light at 1*f* do not yield accurate measurements of the lifetime under these conditions due to contamination by background signal. However, the 2*f* component of the emission light yields the correct lifetime even under poor signal to background [Fig. 3(c)]. This is due to the fact that only the emission light contains the 2*f* component. Therefore, our approach is similar to that of others that have taken advantage of this for two photon absorption measurements [16,17] We also note that this approach is not limited to modulation at a single frequency. When multiple modulation frequencies are used, only the emission light will contain components at all of the sum and difference frequencies. Therefore, these frequency components as well as the harmonics can all be used to determine the emission lifetime.

Interestingly, if a virtual infinite sum of harmonics is used, as in the case of the multifrequency phase fluorometry, the sum and difference frequencies are all still harmonics. The weighting of the harmonics in the resultant emission is unchanged by the nonlinear absorption process. Consequently, the well-established one-photon mathematical model that describes frequency-domain lifetime measurements can also accurately describe two-photon frequency-domain lifetime measurements if an infinite pulse train is used as the excitation source. In general, if a finite number of frequency components are used in the excitation, the one-photon model will only give an incomplete description of the resultant emission, as it will fail to account for the existence of the sum and difference frequencies.

6. Conclusions

We presented an approach that takes advantage of the nonlinear absorption process of two photon luminescence measurements to determine the emission lifetime. This approach is particularly advantageous in poor signal to background conditions. A mathematical model, which relates the excited state lifetime to the phase shift and demodulation factor of the 1*f* and 2*f* terms is also presented. Furthermore, we extend the concept of the demodulation factor so that is has meaning for frequency components which exist in the emission but not the excitation. Although we have described the case where the emission process can be described by a single exponential decay, more complex decay patterns can be similarly analyzed by making the impulse response of the emission process be a multi-exponential decay.

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