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Digitally synthesized beat frequency-multiplexed fluorescence lifetime spectroscopy

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Abstract: Frequency domain fluorescence lifetime imaging is a powerful technique that enables the observation of subtle changes in the molecular environment of a fluorescent probe. This technique works by measuring the phase delay between the optical emission and excitation of fluorophores as a function of modulation frequency. However, high-resolution measurements are time consuming, as the excitation modulation frequency must be swept, and faster low-resolution measurements at a single frequency are prone to large errors. Here, we present a low cost optical system for applications in real-time confocal lifetime imaging, which measures the phase vs. frequency spectrum without sweeping. Deemed Lifetime Imaging using Frequency-multiplexed Excitation (LIFE), this technique uses a digitally-synthesized radio frequency comb to drive an acousto-optic deflector, operated in a cat's-eye configuration, to produce a single laser excitation beam modulated at multiple beat frequencies. We demonstrate simultaneous fluorescence lifetime measurements at 10 frequencies over a bandwidth of 48 MHz, enabling high speed frequency domain lifetime analysis of single- and multi-component sample mixtures.

OCIS codes: (170.3650) Lifetime-based sensing; (170.2520) Fluorescence microscopy; (170.1065) Acousto-optics; (170.1790) Confocal microscopy.

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1. Introduction

Fluorescence lifetime spectroscopy is an optical technique which provides additional chemical information to the fluorescence intensity given its sensitivity to inter-molecular chemical environments and intra-molecular biochemical interactions. Because of this, fluorescence lifetime imaging microscopy (FLIM) is routinely used in applications such as lifetime-based fluorescence resonance energy transfer imaging and assays [1–3], DNA sequencing [4–6], and tumor margin detection [7–9].

Fluorescence lifetime is an established fluorescence imaging parameter and can be measured using time-domain [1–5,7,10–14] or frequency-domain techniques [6,8,15–19]. In the frequency domain approach, an intensity modulated excitation beam illuminates a fluorescent sample and generates a phase-delayed fluorescence emission signal at the same modulation frequency. Although phase measurements at a single RF frequency are theoretically sufficient to resolve the lifetime, systematic errors [20] or the presence of extra lifetime components [21] often limit their accuracy. Since these systematic errors become prohibitively large when measuring samples involving multi-exponential decays or mixed lifetime samples, multiple RF frequencies are typically necessary in lifetime imaging techniques to accurately resolve the lifetime at each pixel in the image. While multi-frequency measurements can be made by sweeping the excitation frequency, this approach is too slow to image dynamic samples over a large field of view [21].

Wide-field lifetime imaging techniques exist which enable higher speed lifetime imaging [15,16], but provide limited depth resolution. To improve the depth-sectioning capabilities without sacrificing speed, confocal spinning disk modules can be employed in combination with wide-field techniques [22,23]. Time-domain lifetime imaging techniques, such as time-correlated single photon counting (TCSPC) [1–4,13,14] combined with beam scanning, provide better depth resolution at high sensitivities, at the cost of limited data acquisition rates. If the count rate is too low, such as for the case of low sample emission intensities, the acquisition time increases; conversely, photon pile-up occurs if the count rate is too high. Currently, detectors with count rates of 76 MHz are available (LaVision TCSPC X16 [24]), which implies a pixel rate of 760 kHz (assuming 100 photons/pixel [25]). Ultimately, the lifetime resolution of time-domain methods are limited by Poissonian photon statistics [26,27].

Here we present a frequency domain multiplexing technique, termed Lifetime Imaging using Frequency-multiplexed Excitation (LIFE), as a low cost, tunable means to increase the pixel acquisition rate of confocal fluorescence lifetime imaging. LIFE uses a digitally-synthesized frequency comb to drive a single acousto-optic deflector (AOD) to generate multiple RF beat frequency modulations on a single excitation laser beam. The phase delay of the detected fluorescence signal is analyzed at each RF frequency in the digital domain and compared to a reference signal to recover the lifetime. Furthermore, the AOD is used in a cat's-eye configuration [28], which enables full digital control of the RF frequency comb without altering the alignment of excitation beam. By multiplexing the beat frequencies, accurate determination of the sample lifetime can be obtained at high speed without frequency sweeping, such that the imaging speed of this technique is limited instead by the maximum scanning speed of the focal point across the sample and the SNR of the detected signal.

By using heterodyne interference and frequency shifting with an AOD, LIFE benefits from its unique, highly linear modulation scheme, enabling a multi-octave fluorescence

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lifetime characterization tool. LIFE avoids the generation of higher order harmonics, which can contribute unwanted interference in a multi-octave system, and can reduce the system dynamic range. However, in some frequency-domain approaches to fluorescence lifetime spectroscopy, the harmonic content of modulated lasers or mode-locked pulse trains can be exploited to perform multi-frequency lifetime analysis [29,30]. Performing frequency down-conversion by gain modulation in a PMT enables measurement of the phase delay of a fluorescent sample over a bandwidth limited by the detection electronics. While both LIFE and multi-harmonic approaches to fluorescence lifetime spectroscopy collect similar information, LIFE exclusively uses digital signal processing to recover the fluorescence lifetime, in contrast to the analog cross-correlation methods used in the multi-harmonic approaches.

2. Experimental

To generate a fluorescence excitation source modulated with multiple RF frequencies, a 532-nm CW laser is directed into an AOD at the + 1-order Bragg angle. The AOD is driven by a frequency comb generated by an arbitrary waveform generator. Each digitally-synthesized RF tone in the comb generates a single + 1-order optical beam that is shifted by a precise radio frequency. Since each + 1 order beam is also diffracted at a unique Bragg angle, a cat's-eye configuration is employed to direct each beam back into the AOD, which diffracts all + 1-order beams back onto the optical axis. As a result, the beam that exits the AOD on the second pass is collinear with the incident beam, but is frequency-shifted by twice the amount of the original RF signal frequency. A quarter-wave plate placed in the path of the + 1 order beams rotates the polarization such that the double-diffracted beams can be separated by a polarizing beam-splitter. The combination of the AOD with the cat's-eye configuration ensures that the second pass beam is always collinear with the incident beam, regardless of the frequencies used to drive the AOD. A 10-μm pinhole ensures that only the doubly-shifted beams are used for sample excitation. This beam is then passed through a 30%R:70%T beam-splitter, and the reflected beam is detected by a fiber-coupled high speed photodiode. This photodiode output signal is used as a phase reference during digital analysis of the fluorescence lifetime.

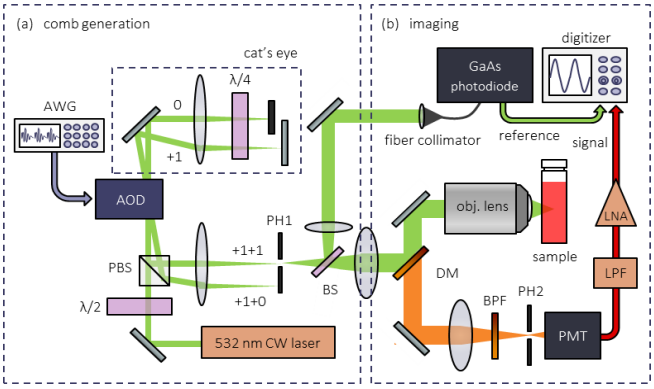

Fig. 1. Schematic of (a) comb generation and (b) imaging setup. The laser beam is frequency-shifted at multiple beat frequencies using the AOD (Crystal Technology, AOMO 3200-125) in a cat's-eye configuration, driven by a frequency comb from an arbitrary waveform generator (AWG). The pinhole (PH) selects the doubly-diffracted beam from the AOD. The single beam from the comb generation setup is focused by a 10x, 0.3-NA objective lens onto the sample. Fluorescence is collected in the epi-direction and filtered by a 532 nm long pass dichroic mirror (DM) and a 580/20 band pass filter (BPF), before focusing through a 40-μm pinhole in a confocal configuration. Finally, the fluorescence signal is collected by a photomultipler tube (PMT). The electronic output from the PMT is low-pass filtered (LPF) and amplified (LNA) before being digitized along with the reference photodiode signal. (PBS: polarizing beam splitter)

#217042 - $15.00 USD Received 23 Sep 2014; revised 24 Sep 2014; accepted 25 Oct 2014; published 26 Nov 2014

The transmitted beam is collimated and focused onto the sample using a 10x, 0.3-NA objective lens. The fluorescence emission is epi-collected and filtered, using a 532-nm long-pass dichroic mirror and 580/20 bandpass filter. A side-on PMT (Hamamatsu R3896) detects the fluorescence emission, and the output is sent to a 225-MHz low pass filter before being amplified by a 1-GHz bandwidth low-noise amplifier. The amplified PMT output as well as the photodiode reference signal are digitized at a sampling rate of 2.5 GS/s. Both the digitizer and the arbitrary waveform generator are clocked from the same 10-MHz reference signal. A diagram of the setup is shown in Fig. 1.

It can be shown [31] for frequency domain lifetime measurements using a single RF-modulated beam, that the lifetime can be extracted from the modulation depth decrease m_τ or from the phase delay φ_τ by

$$m_\tau = \frac{1}{\sqrt{1+\omega^2\tau^2}} \tag{1}$$

$$\varphi_\tau = \tan^{-1}(\omega\tau) = \cos^{-1}\left(\frac{1}{\sqrt{1+\omega^2\tau^2}}\right), \tag{2}$$

where ω is the RF modulation frequency and τ is the lifetime.

For the multiple RF-frequency case, the excitation beam intensity can be written in the time domain as

$$I(t) = \sum_{j=1}^{n}\left|E_j \exp i\left[\left(\omega_0 - 2\omega_j\right)t + \varphi_j\right]\right|^2 = I_0 + 2\sum_{j=1}^{n-1}\sum_{k=j+1}^{n} I_{jk}\cos\left(2\Omega_{jk}t - \Phi_{jk}\right), \tag{3}$$

where $I_0 = \sum_j E_j^2$ is the DC intensity signal, $I_{jk} = E_j E_k$ is the intensity amplitude of the given modulation beat frequency $\Omega_{jk} = \omega_j - \omega_k$ between fields j and k, and $\Phi_{jk} = \varphi_j - \varphi_k$ is the phase shift at the given modulation beat frequency. Let $I(t)$ be the measured intensity at the reference photodiode. Assuming negligible time delay between the reference measurement at the photodiode and the PMT detector, the fluorescence intensity $F(t)$ can be derived by convolving $I(t)$ with a decay function of half-life τ in the time-domain as

$$F(t) = I_0 + 2\sum_{j=1}^{n-1}\sum_{k=j+1}^{n}\frac{I_{jk}}{\sqrt{1+\Omega_{jk}^2\tau^2}}\cos\left(2\Omega_{jk}t - \Phi_{jk} - \varphi_{\tau,jk}\right) \tag{4}$$

where $\varphi_{\tau,jk} = \cos^{-1}\left[\left(1+\Omega_{jk}^2\tau^2\right)^{-1/2}\right]$ is analogous to Eq. (2) above.

From the analytical expression of $F(t)$, we see that both the magnitude and the phase can both be recovered for each beat frequency Ω_{jk}. However, in practice the fitting sensitivity of modulation depth measurements is lower than that of phase delay [32]. Moreover, for our system, the modulation depth is also more sensitive to losses due to the detector frequency response and AOD diffraction efficiency, and thus less accurate than phase measurements. We therefore discard the modulation depth in favor of phase delay.

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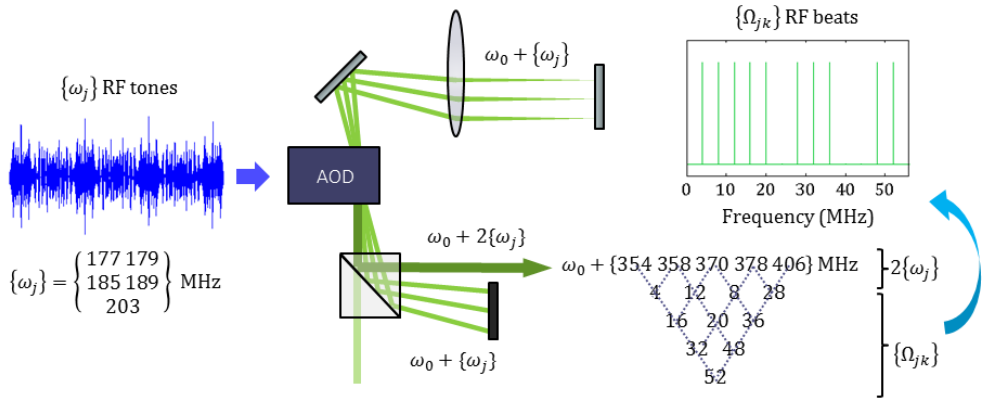

Fig. 2. Generation of multiple unique beat frequencies using the AOD and cat's-eye configuration. (a) Time domain signal of RF tones $\{\omega_j\}$. (b) The signal in (a) drives the AOD in the cat's-eye configuration to produce an optical beam containing frequency shifts of twice the input RF tone frequencies. (c) These RF tones are engineered to avoid beat frequency overlap, which causes phase ambiguity.

To avoid complications from multiple phase contributions at a single RF frequency, the frequency comb must be engineered such that each beat frequency is unique. Figure 2 demonstrates the arrangement of 5 RF tones that are centered around 200 MHz are used to produce 10 distinct beats Ω_{jk} ranging from 4 MHz to 52 MHz, with a minimum spacing of 4 MHz.

3. Results

To demonstrate the capabilities of our lifetime measurement system, we measured the fluorescence lifetime of several Rhodamine dyes in a variety of solvents. We also prepared samples of Rhodamine B and Rhodamine 6G mixed at different concentrations to probe the ability of our system to analyze multi-component mixtures. Dye cells, consisting of a 0.12-mm-thick adhesive silicone spacer sandwiched between a cover slip and a microscope slide, are filled with 10-µl volumes of fresh sample for each measurement. To avoid photobleaching in the small volume custom-made dye cells, we increased the dye concentration from the traditional range of 1-10 uM to 0.5 mM. This concentration is just low enough to avoid quenching effects resulting from monomer aggregate absorption and re-emission [33]. Similar to [34], we also assume that oyxgen quenching effects are negligible in these experiments.

The custom-made dye cells have a small enough volume to avoid lifetime measurement errors due to the effects of absorption and reemission [34]. The power of the 532-nm laser excitation was 15 µW, as measured before the objective. Based on the AOD's specification for single-tone, single-pass diffraction efficiency, it should be possible to achieve up to ~70% optical throughput for the cat's eye portion of the system (before the pinhole). However, in this demonstration, driving the AOD in the cat's eye with a 24-MHz bandwidth signal results in a significantly lower optical throughput (approximately 1%), due to diffraction efficiency roll-off in the AOD. The PMT photocathode voltage was set to −800 V for all measurements.

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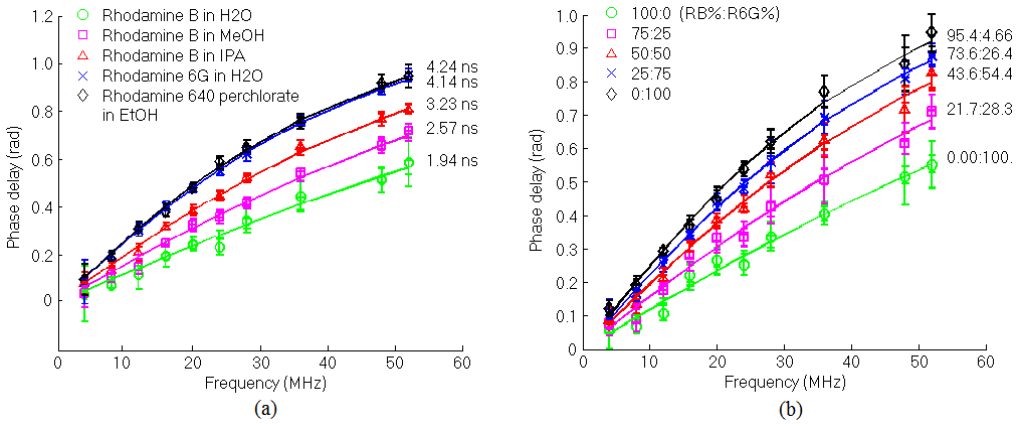

Fig. 3. (a) Selected fluorescence lifetime measurements. (b) Lifetime measurements of Rhodamine B / Rhodamine 6G mixtures at various ratios in water. The error bars denote the 95% confidence interval of the signal windows at each beat frequency. A non-linear fitting of Eq. (2) was applied to the data to extract the fluorescence lifetime values.

Since the beat frequencies used in the experiments are known exactly from the reference photodiode signal, we used a digital lock-in amplifier (LIA) algorithm implemented in MATLAB to extract the relative phase delay from the fluorescence signals. We extract mean and standard deviation values of the relative phase at each beat frequency using ten values from each 40-µs window within the record length of 400 µs. From the LIA outputs, the modulation depth and relative phase can be extracted at each excitation frequency. The phase vs. frequency lifetime results for single and multi-component Rhodamine dye samples are shown in Fig. 3(a).

To recover the lifetime, we apply non-linear fitting of Eq. (2) to the data. The lifetime measurements of various dye solutions, along with their corresponding literature values, are shown in Table 1. To calibrate the background phase delay of the system between the reference and fluorescence signals, we used the literature lifetime values of Rhodamine 6G in H_2O as a standard during the curve fitting procedure. The Rhodamine 6G sample is used to generate two calibration parameters: $\varphi_{\tau,jk}$, as well as a delay factor $2\pi f \tau_0$, to compensate for experimental deviations. The first parameter is used to calibrate the intensity amplitudes I_{jk}, which are setup-dependent (i.e. relative to the output signal intensity, I_{jk}). The second parameter is used to calibrate the non-negligible delay from the path difference between the reference and the signal arms, largely due to electronic delays (such as the electron transit time of the PMT). These calibration parameters were generated by sweeping the parameter space for Rhodamine 6G in H2O by iterative non-linear fitting to locate the parameter pair which minimizes the regression residual. Using this fitting approach, our experimentally determined lifetime values for Rhodamine 6G agree with the literature value to within experimental error.

Table 1. Calculated lifetimes of selected fluorescent dye solutions, with literature values

Measured solution	Solvent	Lifetime (ns)	Literature value (ns)	Reference
Rhodamine B	H_2O	1.94 ± 0.13	1.74 ± 0.02	[35]
			1.68 ± 0.1	[34]
	MeOH	2.57 ± 0.07	2.46 ± 0.1	[34]
	IPA	3.23 ± 0.10	3.26 ± 0.1	[34]
Rhodamine 6G	H_2O	4.14 ± 0.08	4.08 ± 0.1	[34]
Rhodamine 640 perchlorate	EtOH	4.24 ± 0.11	4.25 ± 0.2	[36]

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To demonstrate the ability of the LIFE technique to measure the lifetime of multi-component samples, we measured the frequency response from two-component dye mixtures, using the single-component data for calibration. Lifetime measurements were employed to resolve the relative fraction of each of the two different Rhodamine dyes using a multi-exponential non-linear fitting procedure similar to that described in [31]. Rhodamine B and 6G were selected as they were well-characterized lifetime standards and are stable in bulk solution. The results are shown in Fig. 3(b) and Table 2. Figure 4 shows the tabulated data in graphical form.

Table 2. Calculated lifetimes and component ratios of RB:R6G two-component mixtures

Ratio of RB:R6G (%:%)	Lifetime (ns)	Measured ratio (%:%)
100: 0	1.90 ± 0.12	95.4: 4.66
75: 25	2.51 ± 0.14	73.6: 26.4
50: 50	3.14 ± 0.15	43.6: 54.4
25: 75	3.59 ± 0.10	21.7: 78.3
0: 100	4.03 ± 0.15	0.00: 100.

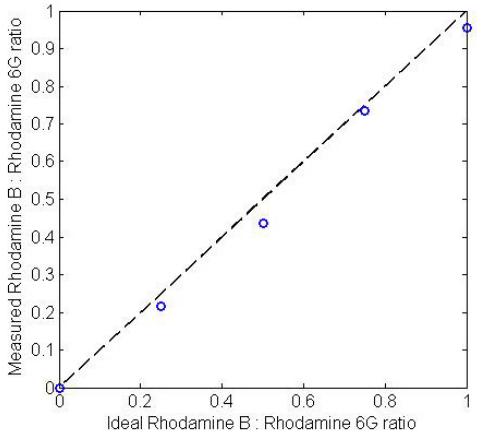

Fig. 4. Measured RB:R6G two-component mixtures at different component ratios (circles), as compared to the ideal case (dashed line).

4. Discussion

As mentioned, techniques exist which perform cross-correlations in the analog domain between the harmonics of the optical pulse train and the modulated detector gain for frequency-domain fluorescence lifetime measurements. While these techniques offer the convenient advantages of analog cross-correlation and low-bandwidth digitization requirements, the speed of this technique is ultimately limited by the small resultant frequency spacings, since the signals are digitized after down-conversion. If we consider replacing the cross-correlation detection with direct digitization of the signal and reference beams, performance similar to that of LIFE should be achievable. However, these multi-harmonic approaches typically require either pulsed excitation using modelocked lasers, which usually have fixed repetition rates in the 50-100 MHz range, or employ CW lasers modulated with a Pockels cell, which requires a costly MHz-frequency, high-voltage driver.

The measurement error of the values reported here is similar to, but larger than that of literature values. This discrepancy can mainly be attributed to the use of a side-on PMT, which has a higher transit time spread than the head-on micro-channel plate (MCP) detectors used in [13,26,31]. The smaller the lifetime value to be measured, the more the accuracy will be affected by the PMT transit time spread. Another source of error is the range of beat frequencies used, which is limited by the RF bandwidth of the AOD. Using a wider range of

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beat frequencies will increase the accuracy of the non-linear fitting technique. Given a sufficient photodetector bandwidth, LIFE can address a larger frequency range by using a wider AOD bandwidth (greater than 1 GHz [37]) than the 50-MHz bandwidth used here. Due to these factors, the current implementation of the LIFE technique is well suited for measuring lifetimes in the range of 1.2-10 ns.

Given the collimated excitation beam at output of the cat's-eye AOD system (see Fig. 1(a)), the beat-frequency multiplexing technique can be easily integrated with a laser-scanning microscope for depth-resolved confocal lifetime imaging. In this demonstration, the pixel dwell time is 40 μs, which corresponds to a pixel rate of 25 kHz. Using beat frequencies with greater separation can increase this rate. The per-pixel recording speed in this implementation is currently limited by the measurement uncertainty introduced by the transit time spread (TTS, 1.2 ns) of the PMT. We record for a sufficient time window to minimize the effect of this parameter on our measurements, as our digital lock-in algorithm assumes a constant electron transit time – that is, a negligible TTS relative to the lifetime – as part of the background delay that is removed in the calibration routine. As a result of the 1.2-ns TTS, a longer recording time is needed to reduce its effect on the measurements when the sample lifetime approaches the value of the TTS. By Using an MCP-PMT with a sub-nanosecond TTS [13] for example, the per-pixel record time of LIFE can ultimately be reduced to the Fourier transform resolution limit, which implies a pixel rate equal to the minimum frequency comb spacing (4 MHz in this demonstration) [38]. Given a sufficiently large RF bandwidth and small photomultiplier TTS, LIFE offers performance suitable for confocal fluorescence lifetime imaging applications where both optical sectioning and high-speed, high-resolution measurements are desired.

5. Conclusions

We have demonstrated proof-of-concept frequency-multiplexed phase-resolved single-point fluorescence lifetime measurements of various pure and mixed dye samples. By using an AOD in a cat's-eye configuration, we multiplex beat-frequency modulations on a single laser beam, avoiding the need to sweep the modulation frequency to measure the frequency response of a sample. The fluorescence lifetime is then resolved using non-linear fitting of the phase delay spectrum. Future integration of this technique with a laser-scanning microscope will yield a fluorescence lifetime imaging system.

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