Published in final edited form as:

Opt Lett. 2010 August 1; 35(15): 2558–2560.

High-speed multispectral fluorescence lifetime imaging implementation for *in vivo* applications

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Abstract

Fluorescence lifetime imaging microscopy (FLIM) offers a noninvasive approach for characterizing the biochemical composition of biological tissue. In recent years, there has been an increasing interest in the application of multispectral FLIM for medical diagnosis. Central to the clinical translation of FLIM technology is the development of robust, fast, and cost-effective FLIM instrumentation suitable for *in vivo* tissue imaging. Unfortunately, the predominant multispectral FLIM approaches suffer from limitations that impede the development of high-speed instruments for *in vivo* applications. We present a cost-effective scanning multispectral FLIM implementation capable of achieving pixel rates on the order of tens of kilohertz, which will facilitate the evaluation of FLIM for *in vivo* applications.

In recent years there has been increasing interest in evaluating the potential of multispectral fluorescence lifetime imaging microscopy (FLIM) to characterize autofluorescence for medical diagnosis, with several studies showing promising results [1,2]. A central issue for the translation of FLIM into the clinical arena is the development of robust, fast and cost-effective FLIM instrumentation suitable for *in vivo* tissue imaging. However, most current FLIM implementations, including those summarized below, are relatively slow, expensive, and susceptible to motion artifact, thus providing a barrier to translation.

In wide-field time-domain FLIM, a sequence of fluorescence intensity images are acquired at specific delay times relative to the excitation pulse. The time-resolved fluorescence decay at each pixel is then reconstructed by assembling the images in order of increasing delay. The main advantage of wide-field FLIM for clinical application is its fairly straightforward implementation for endoscopy [1]. This advantage, however, is mitigated by a number of disadvantages. First, it is extremely sensitive to motion artifact because the reconstruction of the time-resolved fluorescence decay at each pixel requires accurate registration of all the intensity images. Second, the excitation pulse energy is distributed across the whole field of view; hence, averaging of several intensity images is usually needed to achieve adequate signal levels resulting in long acquisition times that can be prohibitive for *in vivo* imaging. Other disadvantages include poor time resolution and slow multispectral imaging implementation.

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A second approach uses time-correlated single-photon counting (TCSPC) in order to measure the fluorescence temporal decay [3]. In TCSPC, the time of the first detected photon after pulse excitation is recorded and the temporal decay is built up as a histogram of measured counts over a specified time interval. The excitation is scanned across the sample, and a histogram is recorded at each spatial position in order to construct an image. The main advantages of TCSPC include low required pulse energy, low average power, and high temporal resolution ($\sim 25\text{--}30$ ps). The main limitation of TCSPC for *in vivo* imaging is its slow speed (typically a few kilohertz pixel rate), which is determined by the photon counting rate (typically a few megahertz) and the total photon count per decay (at least $10^3\text{--}10^5$). Another disadvantage is the high cost of implementation, which usually requires expensive mode-locked pulsed lasers with megahertz repetition rates and specialized TCSPC electronic cards. Finally, multispectral FLIM based on TCSPC cannot be realized without significantly increasing both imaging time and cost.

The most straightforward FLIM approach is the direct measure of the fluorescence temporal decay using high-speed detectors and electronics. Using this strategy, a single laser pulse can generate a temporal decay; hence, the pixel rate is equal to the laser repetition rate. As shown below, it is also possible to implement limited spectral resolution without compromising the imaging speed. The major advantages of this approach are the high speed and the lower implementation cost compared to other time-domain FLIM approaches. The main disadvantage is the need for relatively high pulse energy. The high pulse energy is only a problem for *in vivo* imaging if it exceeds the tissue damage threshold. Because both exposure time and pulse energy contribute to the tissue damage threshold, a high imaging speed enables higher average power than could typically be tolerated with the slower approaches noted above.

We have implemented a high-speed multispectral FLIM design based upon this final approach. The imaging system is shown schematically in Fig. 1. A frequency-tripled (355 nm) Q-switched Nd:YAG laser (Elforlight, England) with a pulse width of 1 ns was used as the excitation source. The maximum pixel rate using this laser system was limited by the maximum laser repetition rate of 30 kHz. A multimode fiber (core diameter $50\text{ }\mu\text{m}$) delivered the excitation light to the laser scanning optics. Laser scanning was accomplished using a pair of galvanometer-mounted mirrors placed approximately in the backfocal plane of a microscope objective (Zeiss $5\times$, 0.16 NA ECPlan-Neofluar), which provided a maximum field of view of 4 mm. The lateral beam profile was measured with a beam profiler showing a near top profile with FWHM of $100\text{ }\mu\text{m}$. The lateral resolution was verified using a 1951 U.S. Air Force resolution target placed over paper. The excitation and emission optical paths were separated by a dichroic mirror ($T > 95\%$ 388–900 nm), before the emission was launched into a multimode fiber (core diameter $200\text{ }\mu\text{m}$).

The multimode fiber delivered the emission to a multispectral detection system previously implemented for time-resolved fluorescence spectroscopy, which allows recording multiple fluorescence decays at different emission bands using a single excitation pulse [4]. Following this strategy, the emission was separated into three bands using a set of dichroic mirrors and filters. The specific bands are customizable based upon the target fluorophores. We selected the following set, 390 ± 20 nm, 452 ± 22.5 nm, 550 ± 20 nm, in order to

optimally distinguish emission from collagen, reduced nicotinamide adenine dinucleotide (NADH), and flavin adenine dinucleotide (FAD). Each band was launched into a multimode fiber with different lengths (1 m, 10 m, and 19 m, respectively) chosen to provide ~45 ns intervals between each emission band decay. The three emission bands were detected on a multichannel plate photomultiplier tube (rise time: 150 ps) (R3809U-50, Hamamatsu). The detector signal passed through a preamplifier before being digitized by a wide bandwidth (1.5 GHz, 4 GS/s per channel) high-speed (476 kHz rearm rate) A/D PC card (CobraMax, Gage). The Nyquist limited temporal resolution is then 500 ps. Custom LabVIEW software controlled the laser scanning, data collection, and storage.

The multispectral FLIM data consisted of three fluorescence decays per pixel (one per emission band). The relatively long excitation pulse width required the temporal deconvolution of the instrument response from the measured fluorescence decay to obtain accurate estimation of the intrinsic fluorescence decays. The measured instrument response had an FWHM of 1 ns. After deconvolution, six images were generated to quantify the fluorescence emission of the samples: normalized fluorescence intensity and average lifetime maps for each of the three emission bands. Time deconvolution was performed using a Laguerre expansion technique algorithm capable of near real-time lifetime map estimation [5]. The normalized intensity images were calculated by $I_1/(I_1 + I_2 + I_3)$, $I_2/(I_1 + I_2 + I_3)$, and $I_3/(I_1 + I_2 + I_3)$, where I_1 , I_2 and I_3 are the absolute intensity values at corresponding pixels in the three intensity images. The average lifetime (τ_{avg}) images were calculated via $\tau_{\text{avg}} = \sum t h(t) / \sum h(t)$, where $h(t)$ is the deconvolved temporal decay.

The FLIM instrument was first validated with a set of three quartz capillary tubes loaded with 1 mM solutions of (1,4-bis(5-phenyloxazol-2-yl)benzene)–POPOP–(in ethanol), NADH, and FAD (in phosphate buffered saline, PBS). The peak emission after excitation at 355 nm for POPOP, NADH, and FAD are approximately 420 nm, 450 nm, and 520 nm, respectively [2]. A multispectral FLIM data set of the three capillary tubes was acquired in 360 ms, at a pixel rate of 10 kHz and a pulse energy of 240 nJ. The normalized fluorescence intensity images from the three spectral channels are displayed in Fig. 2(a). As was expected, strong emission from POPOP was visible in both channel 1 and 2, from NADH in channel 2, and from FAD in channel 3. The known average lifetime for POPOP, NADH, and FAD are approximately 1.3 ns, 0.5 ns, and 2.5 ns, respectively [2]. The average fluorescence lifetime images from the three spectral channels are displayed in Fig. 2(b). The time-resolved fluorescence spectrum for each of the same three solutions, shown in Fig. 2(c), were also measured using a calibrated spectrometer [5]. The results from the three FLIM channels were in good agreement with both the literature values for the three fluorophores and the spectrometer measurements. Similar results were obtained at a pixel rate of 20 kHz, resulting in an acquisition time of 180 ms; however, since the pulse energy at this rate was reduced to <100 nJ, the lifetime maps showed higher variability, indicating less accuracy in the lifetime value estimation.

We have also recorded *in vivo* images of a cheek pouch in a hamster model of oral cancer [6]. Images of a visible lesion (diagnosed by histology as squamous cell carcinoma) were acquired over a 2×2 mm field of view with an average power of ~3 mW on the sample and a laser repetition rate of 10 kHz. To improve the signal-to-noise ratio, 20 fluorescence

profiles from each pixel were averaged. The image acquisition time was ~ 7.2 s. Multispectral FLIM images of the oral lesion surrounded by healthy tissue are displayed in Fig. 3. The normalized fluorescence intensity images showed stronger fluorescence at 390 nm for the surrounding healthy tissue relative to the lesion area, while at 450 nm and 550 nm, the fluorescence was stronger in the lesion area than in the healthy tissue. The average fluorescence lifetime images showed longer values at 390 nm for the surrounding healthy tissue (~ 2 – 2.5 ns) relative to the lesion area (~ 1.4 – 1.6 ns), while at 450 nm both the lesion and healthy tissue showed similar lifetime values (~ 1.4 – 1.7 ns). The fluorescence of the healthy tissue resembles collagen fluorescence, while the emission of the lesion area resembles NADH fluorescence. These results are consistent with the biochemical composition of normal and cancerous epithelial tissue [7].

As noted earlier, the goal of this work was to develop and execute an approach that would enable high-speed *in vivo* multispectral FLIM imaging. In the limit that a single laser pulse generates sufficient fluorescence, the pixel rate is equal to the laser repetition rate. While we have achieved this paradigm for the validation experiments, the available maximum pulse energy (240 nJ at 10 kHz) prevented single-pulse pixel acquisition in the biological sample. However, there are a number of relatively low-cost commercial laser systems that are capable of delivering >10 μJ /pulse at repetition rates of >100 kHz. The ultimate limit to the pulse energy and repetition rate is the tissue damage threshold. The primary application of this technology will be imaging the mucosa; hence, the American National Standards Institute (ANSI) standards [8] for the safe use of lasers on skin can serve as a rough guide to the upper limit. The single-pulse limit for a 1 ns pulse exceeds 300 μJ in the limiting aperture ($\phi = 3.5$ mm); hence, we will be limited by the total energy deposited over the dwell time. We will consider any pulse that is incident in an area enclosed by the limiting aperture a single exposure; hence, the dwell time is equal to the time it would take to image the area of the limiting aperture, or s/f , where s is the number of samples and f is the sampling frequency. The maximum permissible pulse energy is then $Ep(J) = 5.39 \times 10^{-2} (s/f)^{0.25}$. In imaging the hamster cheek pouch, we used 20 averages with 240 nJ pulses to generate sufficient signal to noise. If we are near the shot-noise limit, the improvement due to averaging is $n^{1/2}$, where n is the number of samples averaged; hence, a single pulse of 1.1 μJ would be sufficient for single-pulse acquisition. If we assume that 4 μJ /pulse would be sufficient to image any tissue with a single pulse/pixel, at 100 kHz and 50 μm spatial sampling, we would be more than 20% below the ANSI limit. Such performance is readily available in relatively inexpensive commercially available laser systems.

In conclusion, we have developed an approach and instrument capable of high-speed scanning multispectral fluorescence lifetime imaging (tens of kilohertz pixel rate). The combination of spectral and lifetime information provides high-fidelity fluorophore discrimination. The instrument was validated using well-characterized fluorophores and demonstrated *in vivo* with a hamster model of oral cancer. We believe that the proposed scanning multispectral FLIM implementation represents a valuable alternative to TCSPC for applications in which temporal resolution can be sacrificed at the expense of speed and cost.

Acknowledgments

The authors would like to acknowledge Qiujie Wan for her help in setting up the optical system, and Irma Gimenez-Conti, Jimi Brandon, Elsie Ponce, Noemy Ponce, and Aditi Dabir for their assistance with the hamster protocol. This research work was supported by the National Institutes of Health (NIH) via research grant 1R21CA132433.

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Fig. 1.

(Color online) Schematic of the FLIM system. The same lens collects the emission. Dichroic mirrors guide the laser beam to the sample and the emission beam to the FLIM collection system: BP, bandpass filter; DM, dichroic mirror.

Fig. 2.

(Color online) (a) Normalized fluorescence intensity maps of the three fluorophores: emissions of POPOP peaks at ~ 420 nm (20 dB SNR at 390 nm), NADH at ~ 450 nm (20 dB SNR at 450 nm), and FAD at ~ 520 nm (19 dB SNR at 550 nm). (b) Lifetime maps. Color scale in nanoseconds. (c) Time-resolved spectroscopic measurements of the same fluorophore solutions taken using a calibrated spectrometer.

Fig. 3.

(Color online) Multispectral FLIM images of an oral lesion in a hamster cheek pouch. (a) Normalized intensity maps at various emission bands. (b) Corresponding average lifetime maps. Color scale in nanoseconds. The result shows clear contrast between the lesion (arrows) and surrounding healthy tissue.