Fluorescence lifetime imaging with picosecond resolution for biomedical applications

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We describe a novel whole-field fluorescence lifetime imaging system, based on a time-gated image intensifier and a solid-state laser oscillator–amplifier, that images lifetime differences of less than 10 ps. This system was successfully applied to discrimination between biological tissue constituents.

Recent advances in all-solid-state laser technology offer the potential to develop chemically specific (i.e., functional) biomedical imaging modalities based on nonhazardous, low-cost optical instrumentation. Fluorescence imaging can provide a map of tissue type, or one can use it to probe the local chemical or structural environment through its effects on the nonradiative decay rate, and therefore on the quantum efficiency, of a fluorophore. Contrast (spectral or temporal) between different types of tissue can be improved by the use of exogenous fluorophores.

In principle, quantum efficiency can be determined by absolute intensity measurements, but the heterogeneity of tissue and background autofluorescence usually makes these measurements extremely difficult to quantify. Spectrally resolved measurements that exploit wavelength-ratiometric probes can improve contrast where they are available. Alternatively, one can determine the characteristic fluorescence lifetime of any fluorophore, which also depends on the nonradiative decay rate, using only relative intensity measurements. Imaging the fluorescence lifetime can thus provide spatially resolved information concerning the local tissue environment of a distributed fluorophore.

Fluorescence lifetime probes have been demonstrated for biologically significant analytes, including \([\text{Ca}^{2+}], [\text{O}_2] \), and pH.

Fluorescence lifetime can be measured in either the frequency domain (e.g., Ref. 2) or the time domain (e.g., Ref. 3). Most previously reported fluorescence lifetime imaging (FLIM) systems were limited to temporal responses of a few nanoseconds by the available sources and detectors. We report a FLIM system, depicted in Fig. 1, that uses a time-gated optical intensifier to provide a temporal response of \(<100 \text{ ps} \) and that is able to image lifetime differences as small as 10 ps.

For the experiments reported here, fluorescent samples were typically excited at 415 nm by pulses of \(\sim 10\)-ps duration, with energies as great as \(\sim 1 \mu\text{J} \) at a repetition rate of 5 kHz. These pulses were derived from a commercial Ti:sapphire laser (Spectra-Physics Tsunami) and amplified in a home-built Cr:LiSAF regenerative amplifier that is tunable from 800 to 880 nm. Frequency doubling in a \(\beta\)-barium borate crystal provided pulses from 400 to 440 nm. We note that this laser system could be replaced with the diode-pumped oscillator–amplifier system reported by Mellish et al.\(^6\)

Using a 10-cm focal-length singlet lens, we imaged the sample fluorescence onto a time-gated image intensifier (Kentech Instruments Ltd. gated optical imager (GOI)). We measured the spatial resolution by imaging a U.S. Air Force test chart and determined that there were 13 line pairs per millimeter, with an 18-mm field of view. The system has a temporal response of \(\sim 90 \text{ ps} \), including timing jitter (determined by measurement of the apparent width of the 10-ps excitation pulses that were imaged directly onto the GOI). The overall timing jitter between the GOI and the laser system of 20 ps was achieved with a Kentech Instruments Model JE1, which synchronized the GOI trigger with the laser oscillator, and a precision computer-controlled delay generator (Kentech Instruments Model PPDG).
DASPI is sensitive to solvent viscosity, which one can resolve both temporally and spectrally from multiple samples in a single acquisition by use of a FLIM instrument to the study of autofluorescence of different (healthy) tissue constituents taken from the same rat. These whole-tissue samples were stored in phosphate-buffered saline before being mounted upon a glass slide. Collagen (primarily type I) was taken from rat tail, and single fibers were cleared of adherent connective tissue before the sample was mounted. We prepared the elastin by boiling strips of aorta in 0.1-M NaOH for 30 min. Following hydrolysis of other tissue constituents, the resulting elastin was washed thoroughly in phosphate-buffered saline. We measured the auto-fluorescence of samples of pure elastin and collagen. For excitation at 415 nm, we observed a double exponential fluorescence decay for both tissues, as illustrated in Fig. 4, which we attribute to the cross linkage of the proteins.

The excellent temporal dynamic range of this FLIM system is illustrated in Fig. 2, which shows a FLIM map of three samples of Coumarin 314 interlaced with pipettes of the saturable absorber dye DASPI (80 μM dissolved in a 50:50 mixture of ethanol:glycerol). FLIM maps that distinguish between these fluorophores could be acquired and displayed in near real time, with an update rate of 0.3 Hz. Both dyes exhibited monoexponential decays, and their lifetimes ± standard deviation were determined to be 3.46 ± 0.02 ns (Ref. 7) and 143 ± 5 ps, as shown in Fig. 2. These lifetimes were independently measured with a photon-counting system and a streak camera to be 3.45 ± 0.02 ns and 145.7 ± 0.4 ps, respectively. We note that the imaging capability makes it possible to resolve both temporally and spectrally fluorescence from multiple samples in a single acquisition by use of a monochromator before the GOI.

The fluorescence lifetime of a fluorophore such as DASPI is sensitive to solvent viscosity, which one can adjust by preparing solutions in different mixtures of ethanol and glycerol. To test the system’s temporal discrimination and repeatability, we prepared two pairs of 40-μM solutions of DASPI in solvent mixtures of 70:30 and 64:34 ethanol:glycerol, with measured viscosities of 8.0 and 8.8 CP, respectively. Over a period of 1.6 h, 14 FLIM maps of this sample were recorded, of which Fig. 3(a) is a typical example. Figure 3(b) shows how the spatially averaged value of the fluorescence lifetime for each pipette varied over this time. Averaging these values over 1.6 h gives mean lifetimes of 107.6 ± 2.0 and 107.8 ± 1.5 ps for the 70:30 solvent mixture and 115.0 ± 1.8 and 114.6 ± 1.9 ps for the 66:34 mixture. These results demonstrate a sub-10-ps temporal discrimination, which is comparable with the discrimination of more-expensive nonimaging detectors, such as streak cameras and single-photon-counting instruments.

For biomedical applications of FLIM it is important to investigate the autofluorescence of biological tissues. We have begun applying our FLIM instrument to the study of autofluorescence of different healthy tissue constituents taken from the same rat. These whole-tissue samples were stored in phosphate-buffered saline before being mounted upon a glass slide. Collagen (primarily type I) was taken from rat tail, and single fibers were cleared of adherent connective tissue before the sample was mounted. We prepared the elastin by boiling strips of aorta in 0.1-M NaOH for 30 min. Following hydrolysis of other tissue constituents, the resulting elastin was washed thoroughly in phosphate-buffered saline. We measured the auto-fluorescence of samples of pure elastin and collagen. For excitation at 415 nm, we observed a double exponential fluorescence decay for both tissues, as illustrated in Fig. 4, which we attribute to the cross linkage of the proteins. Figure 5 shows a FLIM map of a field of view containing two samples each of elastin and aorta and one of collagen. The figure shows a consistent contrast among the different types of tissue. Table 1 summarizes the different measured fluorescence lifetimes. In general the autofluorescence intensity was ∼25× weaker than that of

![Fig. 2. Top, FLIM map of three samples of Coumarin 314 interlaced with samples of DASPI (note the nonlinear gray scale). Bottom, cross section through the FLIM map.](Image 323x336 to 551x486)

![Fig. 3. (a) Fluorescence lifetime map of samples of DASPI in solutions of, from left to right, 70:30, 66:34, 70:30, and 66:34 ethanol:glycerol. (b) Variation of spatially averaged lifetimes over time (the error bars represent the standard deviation of spatial variations across samples).](Image 339x105 to 534x288)
intensifier and a solid-state regenerative amplifier laser system, which exhibits both excellent temporal dynamic range and temporal resolution, successfully imaging lifetime differences of <10 ps. To our knowledge, this is the highest-resolution FLIM system yet reported. We demonstrated the capability of our FLIM system to image local fluorophore environment (i.e., viscosity). We applied this FLIM system to characterization of the fluorescence of tissue constituents, and we showed, for the first time to our knowledge, that FLIM can discriminate between collagen and elastin, even though their fluorescence spectra for 415-nm excitation are indistinguishable. The laser source for this system could be replaced by an all-solid-state diode-pumped oscillator–amplifier system, potentially yielding a compact, portable, and commercially feasible instrument for biomedical and other applications, such as studies of combustion or photoluminescence.

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Table 1. Observed Mean Lifetimes and Relative Amplitude $|I_1|/|I_2|$ of Decay Components for the Samples Defined in Fig. 5

| Sample | $\tau_1$ (ps) | $\tau_2$ (ps) | $|I_1|/|I_2|$ |
|--------|---------------|---------------|----------------|
| e1     | 258 ± 23      | 1963 ± 77     | 41/59          |
| a1     | 293 ± 37      | 2077 ± 112    | 35/65          |
| e2     | 274 ± 26      | 1942 ± 93     | 40/60          |
| c1     | 291 ± 43      | 1680 ± 146    | 39/61          |
| a2     | 293 ± 38      | 2072 ± 133    | 38/62          |

Fig. 4. Fluorescence decay profiles of (a) collagen and (b) elastin. The top plots show the measured intensity values and the best-fitted single and double exponential decays. The lower plots show the residuals of the fits.

Fig. 5. (a) Time-gated fluorescence image recorded immediately following excitation, along with FLIM maps of (b) the short-lived ($\tau_1$) and (c) long-lived ($\tau_2$) components of fluorescence decay. The samples are elastin (e1 and e2), aorta (a1 and a2), and collagen (c1).

Coumarin 314, and typical integration times were ~100 s.

In conclusion, we have demonstrated a time-domain fluorescence lifetime imaging system, based on a GOI...

References

7. The shorter fluorescence lifetime that we reported in Ref. 5 for Coumarin 314 was measured with a tightly focused pump beam that resulted in significant stimulated emission. For this measurement we reduced the pump intensity and eliminated this artifact. We are unaware of any published value for this lifetime.