Whole-Field Fluorescence Lifetime Imaging with Picosecond Resolution Using Ultrafast 10-kHz Solid-State Amplifier Technology


Abstract—We report the development of a high temporal resolution whole-field fluorescence lifetime imaging system based on an ultrafast solid-state laser system and a time-gated image intensifier operating at up to 10 kHz. The temporal instrument response is ~110 ps and we have imaged (environmentally perturbed) differences in fluorescence lifetime as small as 20 ps. Fluorophores exhibiting single- or double-exponential fluorescence decay profiles are routinely imaged and a near real-time update time of 3 s for the fluorescence lifetime map has been demonstrated using a modest personal computer. We also present provisional fluorescence lifetime images of tissue constituents. This fluorescence lifetime imaging technology is applicable to almost any optical instrument configuration and, when coupled with existing all-solid-state diode-pumped ultrafast laser technology, may yield a potentially inexpensive instrument for in vitro and in vivo biomedical imaging.

Index Terms—Biomedical imaging, biomedical microscopy, biomedical optical imaging, fluorescence, laser applications, laser biomedical applications.

I. INTRODUCTION

In recent years, the advances in all-solid-state laser technology have prompted the investigation of optical imaging in the visible/near-infrared (NIR) spectral region as an alternative to traditional medical diagnostic techniques. The potential benefits of spectroscopic resolution (discrimination), low hazard and low cost make biomedical optical techniques increasingly attractive. Biomedical optical imaging is limited in its efficacy, however by the structural and chemical heterogeneity of biological tissue and the strong scattering which limits the ability to quantify variations in the optical properties of the tissue under investigation. A method commonly employed to increase the optical contrast between tissue types is to use fluorescent marker dyes (fluorophores) which can be designed such that they are selectively absorbed in the specific area of tissue under investigation. In medical diagnostic techniques, the presence of a particular tissue type (e.g., cancerous tissue) can be established by detecting the emission-wavelength signature of the fluorophore (which will only be seen if the tissue of interest is present).

An alternative approach is fluorescence lifetime imaging (FLIM), in which the temporal decay of the fluorescence signal, rather than just its intensity, is measured. Fluorescence lifetime is a signature of a fluorophore that is relatively unaffected by the heterogeneous absorption properties of tissue,
making it possible for measurements to be made through
greater tissue depths using visible or near-infrared (NIR)
excitation. Fluorescence lifetime measurements may also be
spectrally resolved to provide more information about the
fluorophores and to aid discrimination between them.

In biochemical applications of fluorescence imaging, the
effect of environment on the process of fluorescence may be
used to map chemical or physical changes within a sample.
The quantum efficiency of fluorescence is a function of the
radiative and nonradiative decay rates. The radiative decay
rate is considered constant for a given fluorophore, while the
nonradiative decay rate can vary with environment. Unfortu-
nately the quantum efficiency is not easy to determine as it
is difficult to measure the exact quantity of fluorophore in
a particular region, and to quantify how much pump-light is
absorbed. Fluorescence lifetime, however, is also a function of
fluorophore environment [1] and, since it may be determined
using only relative intensity measurements, its determination
does not require knowledge of the fluorophore concentration
or excitation flux in the sample. Imaging fluorescence lifetime
may therefore provide spatially resolved chemically specific
(functional) data about a tissue sample under investigation.
Fluorescence lifetime probes already exist for the measurement
of, e.g., Ca$^{2+}$ concentration, O$_2$ concentration and pH. Non-
biomedical applications of FLIM have also been demonstrated,
including determination of impurities in metal samples for nu-
clear process control [2], and in combustion related studies [3].

In the following sections, we describe the operation of our
FLIM system and its performance is evaluated using conven-
tient laser dye fluorophores. We then discuss its application
to laser induced autofluorescence in biological tissue constituents
and then briefly outline the scope for future developments of
this technology.

II. MEASUREMENT OF FLUORESCENCE LIFETIME

Fluorescence lifetime may be measured in the frequency
domain or the time domain. The most common technique
is in the frequency domain where the sample is illuminated
with a sinusoidally modulated continuous-wave laser and
the fluorescence lifetime determined from the phase change
between the excitation and measured fluorescence modulation
[4]. This work concerns the time domain in which an ultrashort
light pulse is used to excite the fluorophore and the intensity
of the fluorescence is then measured as a function of time.
Previous demonstrations of this technique have used detectors
which have exhibited temporal resolutions of a few nanosec-
onds e.g., [5]–[7]. We report a fluorescence lifetime imaging
system based on a novel time-gated optical intensifier [8], [9]
which allows simultaneous measurement of the fluorescence
lifetime at all pixels in the field of view with a demonstrated
temporal resolution significantly better than alternative FLIM
techniques. The detector technology operates up to 10-kHz
repetition rate and is well matched to our solid-state ultrafast oscillator-amplifier technology based on Cr:LiSAF [10]. The pulse energies required (>1 μJ) have already been demonstrated from an all-solid-state diode-pumped version of this laser system [11] and so this technology promises to provide a more versatile, practical and considerably cheaper alternative to large-frame ultrafast lasers and streak cameras.

Using the apparatus shown in Fig. 1, fluorescent samples were illuminated by ~10-ps pulses of up to 1 μJ energy at 415 nm, at a repetition rate of 5 kHz. These were derived from a commercial ultrafast Ti:sapphire laser (Spectra-Physics Tsunami) and amplified in a home-built Cr:LiSAF regenerative amplifier whose output was tuneable from 800 to 880 nm (400–440 nm in the second-harmonic signal). An image of the fluorescent sample was relayed onto the cathode of the time-gated image intensifier (Kentec Instruments Ltd. Gated Optical Imager), for which the gate width was measured to be 110 ps (this measurement includes triggering jitter). The intensifier was triggered by the switching of the regenerative amplifier Pockels cell via an electronic delay (Stanford Research Systems DG535) to set its position relative to the arrival time of the excitation light. By recording images of the fluorescence at different delays after excitation, a temporal profile of the fluorescence intensity was obtained simultaneously for each point in the field of view. Exponentials were fitted to the decay profiles on a pixel by pixel basis, and the spatial distribution of decay time constants (lifetimes) displayed—a FLIM map. In principle, since we record the complete temporal history of the fluorescence, we can fit (using a least-squares algorithm) any order of exponential decay to the fluorescence profiles. We have so far demonstrated FLIM imaging of fluorophores exhibiting single and double exponential decays. The data was acquired, via an intensified charged-coupled device (CCD) camera, to a personal computer (Cyrix P200+ processor). The total time required to acquire data and calculate and display a FLIM map depended on the number of samples and typically took several minutes. For single exponential, and with lower spatial and temporal resolution, it was possible to record FLIM maps with an update time of only three seconds. Further development of the processing algorithms and computer hardware should yield FLIM update rates of less than one second.

Fig. 2 shows a typical FLIM map, assuming single exponential decay profiles, of a fluorescent phantom consisting of three pipettes containing a Coumarin 314 solution in ethanol, separated by pipettes containing a DASPI solution in an ethanol/ethylene glycol mixture. The fluorescence lifetime of a solution of 10^-6 M Coumarin 314 in ethanol was independently measured to be 3.45 ns using time-correlated photon counting. If we spatially average over a 1-mm² area (~2000 pixels) of the Coumarin 314 portion of our FLIM maps to derive a characteristic lifetime and average these values obtained for two measurements, each of two different samples of 10^-6 M Coumarin 314 in ethanol, we obtain a fluorescence lifetime of 3.47 ns with a standard deviation of 0.02 ns. The fluorescence lifetime of DASPI is highly sensitive to the solvent properties, making calibration difficult, but an independent measurement of a pure ethanol solution of (10^-3 M) DASPI using a streak camera has yielded a lifetime of ~70 ps.

Fig. 2 illustrates the excellent dynamic range of our FLIM system. The upper limit of the lifetimes that can be measured is constrained only by the repetition rate of the system, while the lower limit is determined by the instrument response function. Note that the system can detect and image differences in fluorescence lifetime shorter than ~10 ps, provided that the fluorescence decays are measured over sufficient dynamic
range [12], although absolute lifetime measurements are not presently determined to better than \( \sim 100 \) ps. This latter precision could be improved by using more sophisticated deconvolution techniques.

### III. Temporal Discrimination

To demonstrate the temporal resolution of the FLIM imaging system presented here, DASPI, a laser dye whose lifetime is a sensitive function of its solvent viscosity [13], was used to provide a fluorescence phantom with an adjustable lifetime. It was dissolved in ethanol mixed with different amounts of glycerol to provide solutions of variable viscosity. FLIM lifetime maps were thus made of a range of samples prepared with different viscosities. As can be seen in the FLIM maps shown in Fig. 3, the measured lifetimes varied from 155 ps, for the least viscous phantom (lhs), to 320 ps for the most viscous solution (rhs). Further improvements in resolution could be made by reducing the system response time. This has recently been reduced to \( \sim 90 \) ps by triggering the detection system from the Tsunami oscillator output and thereby reducing the jitter.

### IV. Imaging of Tissue Constituents

Any practical biomedical application of FLIM, either in vivo or in vitro, must obviously take account of the autofluorescence of tissue itself. Indeed, it is possible to exploit endogenous fluorescence as a means to detect and study metabolic function and disease, e.g., [14]. To this end we have begun to characterize the fluorescence of biological tissue, in vitro, using the apparatus reported here. Fig. 4(a) shows a conventional fluorescence microscope image of a sample of collagen located between two samples of elastin (excited at 450–490 nm), which are from calcaneous tendon and aorta of rat. Fig. 4(b) shows the time-gated fluorescence intensity image (horizontal illumination) recorded directly after excitation and Fig. 4(c) and (d) shows the FLIM maps obtained by fitting a double exponential decay to the data, giving two lifetimes, \( \tau_1 \) and \( \tau_2 \), for each region of interest.

Fig. 5 shows the fluorescence decay curves of (a) elastin and (b) collagen, derived from the FLIM data of Fig. 4. These decay curves clearly fit a double exponential profile as shown by the plots of the residuals to single- and double-exponential fits, Fig. 5. Although this preliminary data has not yet been fully analyzed and so should be treated with caution, it appears that the FLIM profiles do provide a means to discriminate between different tissue constituents. Future work will try to establish how different techniques to fix and preserve tissue samples influence their fluorescence lifetime signatures. Before these measurements were made, these samples had been frozen for \( \sim \)five weeks since their preparation, during which time some dehydration may have occurred. We are currently investigating the impact of different modes of sample preparation and sample history on the FLIM signature of biological tissue. Ultimately, this work will be directed toward FLIM investigations of diseased and healthy tissue in vivo in the hope of achieving a useful contrast.

![Fig. 5](image_url)

Fig. 5. Fluorescence decay profiles (spatially averaged over samples) of (a) elastin and (b) collagen extracted from freshly excised rat tissue. The upper graphs are fluorescence decay profiles with the calculated exponential fits. The dashed line is a fit to a single-exponential decay, with \( \tau = 1.08 \) ns for elastin and 0.74 ns for collagen. The solid line is a fit to a double exponential decay, with \( \tau_1 = 285 \) ps, \( \tau_2 = 2.16 \) ns for elastin and for collagen \( \tau_1 = 257 \) ps, \( \tau_2 = 1.71 \) ns. The lower traces show the residuals to each of the fits, again the dashed line represents the residuals from a single exponential fit (the initial value has been cropped to show detail) while the solid line is for a double exponential fit.
V. CONCLUSION AND FUTURE WORK

We have demonstrated a fluorescence lifetime imaging system, with an excellent temporal dynamic range, based on a time-gated image intensifier and a solid-state regenerative oscillator/amplifier laser system. We have measured an instrument temporal response of 110 ps and have demonstrated the ability to detect (and image) fluorescence lifetime differences of ~20 ps. The laser source for this FLIM system may be replaced by an all-solid-state diode-pumped oscillator amplifier system (already demonstrated [11]), potentially leading to a commercially viable instrument. It is hoped that this may be useful for in vitro and in vivo imaging of fluorophore location and environment for biomedical and other applications. Recently, we have also started to characterize the temporal signatures of the endogenous fluorophores of biological tissue and have imaged double exponential fluorescence decays for collagen and elastin. In the near future the all-solid-state diode-pumped oscillator amplifier system will be developed such that the apparatus may be operated from a conventional electricity supply with little or no water-cooling. We have already established that this system requires a total of only three pump diodes providing 500-mW power at 670 nm from 100-μm-wide stripe facets. Anticipated reductions in cost and size compared to existing laser and time-resolved detection systems will permit this technology to be located outside laser laboratories and in hospitals/medical research centers. It should be understood that this whole-field time-gated detection system is applicable to almost any optical instrumentation. We intend to investigate operation in conjunction with both microscope and endoscope configurations.

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REFERENCES


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