

# Analysis of histology specimens using lifetime multiphoton microscopy

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**Abstract.** Observations of cells or tissues with fluorescence microscopy can provide unique insights into cellular physiology and structure. Such information may reveal the pathological state of a tissue to the physician or information on cytoskeletal dynamics to the research scientist. However, problems of overlapping spectra, low signal, and light scatter impose serious limitations on what can be achieved in practice with fluorescence microscopy. These problems can be addressed in part by the development of new imaging modalities that make maximum use of the information present in the fluorescence signal. We describe the application of a new technology to the study of standard histological pathology specimens: a multiphoton excitation fluorescence microscope that incorporates a novel, photon-counting detector that measures the excited-state lifetimes of fluorescent probes. In initial investigations, we have applied this system to the observation of *C. elegans* embryos and primate histology specimens, with the objective of identifying potentially diagnostic signatures. Our findings demonstrate that lifetime multiphoton microscopy has considerable potential as a diagnostic tool for pathological investigations. © 2003 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.1584053]

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## 1 Introduction

Traditionally, pathologists have relied on mechanically sectioned samples that are stained and then observed using brightfield microscopy. However, optical sectioning techniques may offer an attractive alternative, since they allow 3-D structures to be visualized within a specimen without the need for mechanical sectioning. Fluorescence microscopy has become the method of choice for biomedical research because this technique offers a high signal-to-background ratio and the ability to spectrally discriminate among multiple fluorophores. Recently developed techniques for fluorescence optical sectioning microscopy such as confocal<sup>1</sup> or multiphoton<sup>2</sup> imaging allow stacks of images at different focal depths to be obtained. These stacks of images may be collected at regular time intervals in order to reveal the dynamics of three-dimensional structures in living tissue.<sup>3</sup> Fortunately, many classic histological stains are fluorescent and therefore can be directly viewed with fluorescence microscopy.

A fluorescence signal contains more information than just intensity and color. The lifetime of the excited state, which gives rise to the fluorescence signal, provides an extra dimension of information that is diagnostic of the fluorophore and also of its microenvironment.<sup>4</sup> Factors such as ionic strength, hydrophobicity, oxygen concentration, binding to macromolecules, and the proximity of molecules that can deplete the excited state by resonance energy transfer can all modify the lifetime of a fluorophore. Measurements of lifetimes can

therefore be used as indicators of these parameters.<sup>4</sup> Fluorescence lifetime measurements are generally absolute, being independent of the concentration of the fluorophore. Furthermore, lifetime properties may be particularly useful in identifying fluorophores with significantly overlapping spectral properties. Fluorescence lifetime imaging (FLIM) combines the advantages of lifetime measurements with fluorescence microscopy by revealing the spatial distribution of a fluorescent molecule together with information about its microenvironment.<sup>5–11</sup> In this way an extra dimension of information is obtained. This extra dimension can be used to discriminate among multiple labels or to glean information about the molecular microenvironment of the fluorophores.

Multiphoton microscopy<sup>2</sup> is a fluorescence optical sectioning technique that offers unprecedented ability to obtain optical sections from deep within biological tissue.<sup>12</sup> Multiphoton microscopes have recently become commercially available. However, current-generation instruments have no facility for lifetime measurements. We have developed a multiphoton imaging system that incorporates a lifetime detector. We have used this system for the characterization of fluorescent probes in the *C. elegans* embryo model and in thick (200  $\mu$ ) histological sections. We have found that multiphoton lifetime data can yield more information than traditional imaging of fluorescence intensities. We anticipate that similar systems could open up a whole new realm of possibilities for pathological imaging by their improved ability to discriminate probe signals from within different compartments of a cell or tissue.

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Subtle changes of fluorophore lifetimes could be used in the identification of pathological tissue.

## 2 Methods

We have set up an FLIM system that incorporates a 1047-nm mode-locked Nd:YLF laser as an excitation source (Coherent Biolight 1000), a modified MRC 600 confocal microscope scanning head, a photon-counting detector, and a very high-speed (5 MHz), TCSPC-based lifetime measurement system (Becker & Hickl model SPC-730). This system has been employed to image fluorescence lifetimes of probes in the *C. elegans* embryo and in histologically stained sections of the primate liver, uterus, and kidney. The system description follows.

### 2.1 Scanning System

A modified MRC 600 confocal microscope is used as the laser-scanning system. The derived  $x$  and  $y$  scan synchronizing pulses together with a pixel clock signal from this system are used to synchronize data collection in the SPC-730 board with laser scanning. Laser pulses are detected by a high-speed PIN photodiode and used by the SCP-730 board to determine the detection time of a photon (an anode pulse from the photomultiplier tube PMT) relative to the laser pulses. The measurement system used requires that the timer (a time-to-amplitude converter, TAC) only be activated on receipt of a detected photon rather than at every laser pulse. The Becker & Hickl SPC-730 system starts timing on the receipt of a detected photon and measures the time interval until the next laser pulse. A fluorescence decay histogram of photon emission times relative to the laser excitation pulse is generated from the distribution of interpulse intervals at each pixel of the image. The lifetime of the excited state is deduced from an exponential curve fit to the photon time distribution histograms by a separate offline program supplied by Becker & Hickl. These data are displayed as a pseudocolored image, with color representing lifetime.

### 2.2 Laser and Optical Detector

The pulse repetition frequency of the laser is 120 MHz and the dwell time per pixel is approximately 10  $\mu$ s, resulting in 1200 laser pulses per pixel interval. Most of the fluorophores that we have been using had lifetimes of about 2 ns and so the relatively short (8.3 ns) interpulse interval of this laser allowed collection over four lifetimes, which was sufficient to obtain good exponential curve fits (Fig. 1) (see Color Plate 1). However, if considerably longer lifetimes are to be measured, either a pulse picker or a laser with a lower pulse repetition rate would have to be used. The signal from each pixel is detected using a single-channel, photon-counting PMT (Becker & Hickl model PMH-100) close to the objective lens to maximize collection of the scattered emission signal in order to obtain images from deep optical sections.<sup>12</sup> The system was calibrated using a second-harmonic crystal, which gives an instantaneous signal coincident with the laser pulse. The measured pulse width was 150 ps, which corresponds to the listed time response of the PMT. This measurement indicated that the jitter in the laser pulse interval is less than the time response of the detector. We found that the brightest fluorescent signals from a well-stained specimen gave a count rate of

approximately 100 counts per pixel interval at each scan, which would give a count rate of 10 MHz. The photon counting system had a maximum count rate of 5 MHz, so we reduced the excitation power on the brightest specimens so that the peak count rate was around 2 Mz to minimize lost counts caused by dead time. The system dead time while detecting and measuring a photon is 125 ns. Because only about 1 in 60 laser pulses produces a photon at peak signals, there is a low probability of two photons being produced by a single laser pulse, a situation that could underrepresent late photons when determining lifetimes because the second pulse would be lost in the detector's dead time. We would typically scan the specimen for 80 s of data collection (about 80 scans) in order to accumulate sufficient counts in each time interval to facilitate lifetime estimates by exponential curve fitting.

### 2.3 Data Acquisition and Analysis Software

Becker & Hickl supply two programs, one for capturing image data and a separate program for displaying a lifetime image. Photon time distribution histograms are assembled into 256, 16-bit channels at each pixel (we typically use about 128 channels). The imaging program determines the best exponential fit to the histograms at each pixel and displays lifetime data utilizing a color-mapping scheme (Fig. 1).

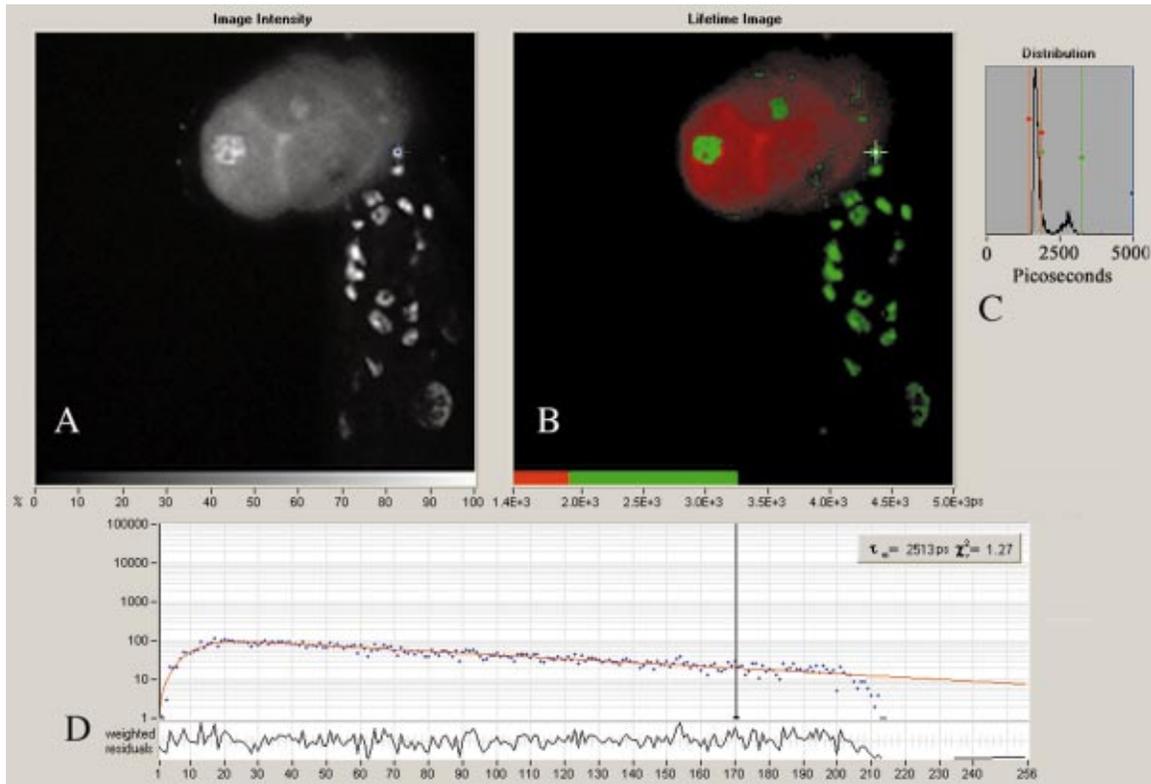
## 3 Results

We first studied *C. elegans* embryos as a model system in which we used two separate fluorophores of different lifetimes, which we knew localized to different regions of the cell. We used (DAPI) to stain the nuclei and a Cy-3 labeled antibody probe that binds a cytoplasmic component (septins). Although no spectral separation was used, we found that we could easily discriminate these two signals solely on the basis of lifetime (Fig. 1).

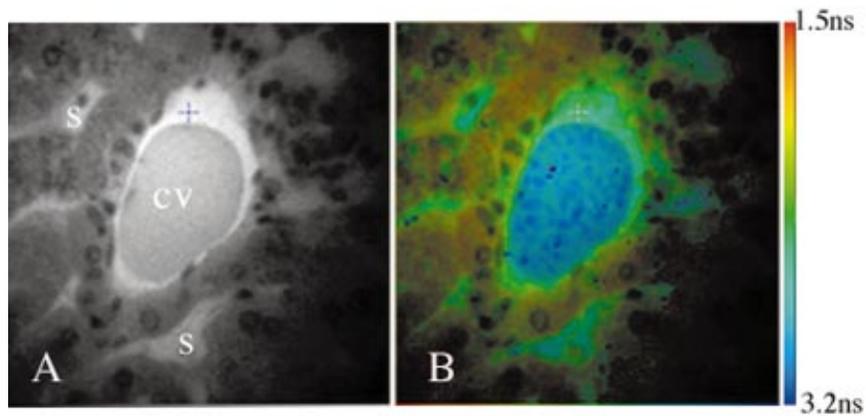
We next looked at 200- $\mu$  thick histological specimens stained with a single probe. In most of the specimens we studied, we observed discrete lifetime distributions that correspond to signals emanating from different structures (Fig. 2, Fig. 3, Fig. 4) (see Color Plates 1 and 2). These lifetime differences most likely arise from the varying microenvironments in different regions of the cell or tissue.

Figure 2 shows a central vein surrounded by sheets of hepatocytes and sinusoids within a methyl green-stained primate liver sample. The lifetime on the fluorophore bound to the blood cells within the vein is strikingly longer than in the hepatocytes surrounding the vein. Figure 3 shows brightfield (A), multiphoton (B), and FLIM (C) images of a methyl green-stained primate kidney medulla. In Fig. 3(C), nuclei are seen to have shorter lifetimes than that of the surrounding cytoplasm, while the cells of the descending thin limbs exhibit longer lifetimes than the surrounding structures. Note that in Fig. 3(C) there are structures revealed by short lifetimes that cannot be observed in the multiphoton image of Fig. 3(B). Possibly the acidic microenvironment of the nucleus, owing to the high concentration of DNA, might be the cause of the observed shortening of the lifetime signal.

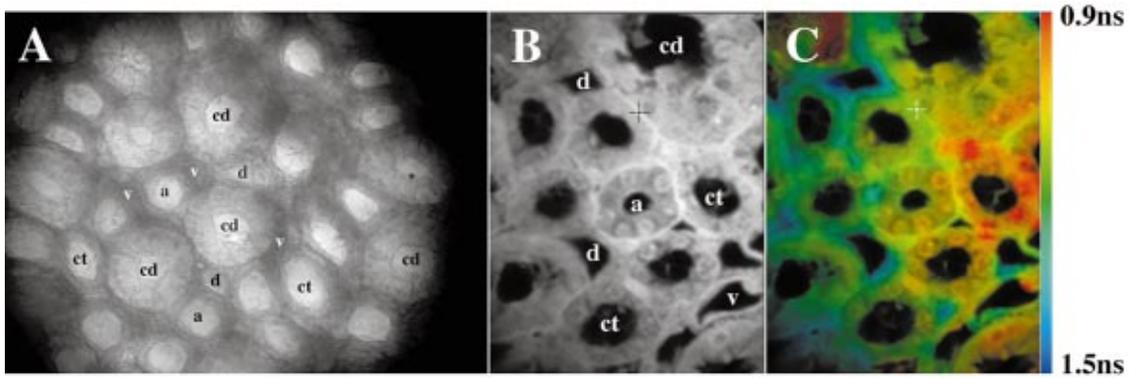
Figure 4 compares brightfield (A), multiphoton (B), and FLIM (C) images of an acridine orange-stained primate uterus endometrium. The lifetime of the fluorophore bound to the



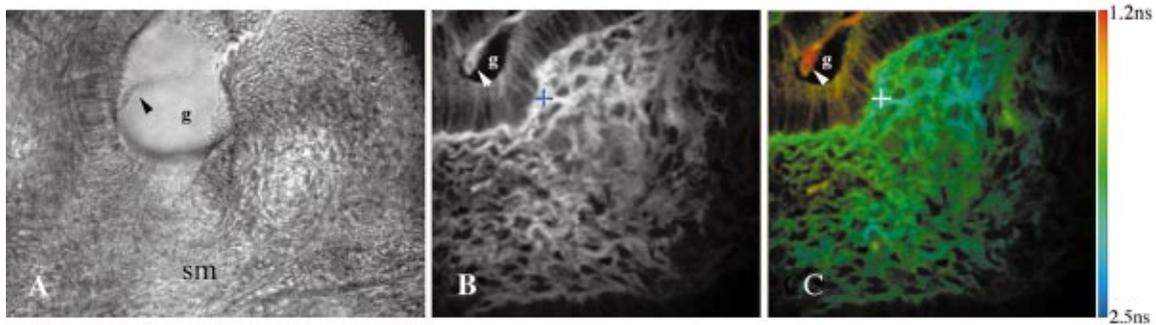
**Fig. 1** (A) Multiphoton and (B) FLIM images of a fixed *C. elegans* embryo; 1047-nm excitation (60× PlanApo 1.4 NA lens). The plot (D) shows the fitted exponential and the residuals for a single pixel indicated by a cross in the images. The distribution of lifetimes is shown in (C) with peaks at 2.4 ns (Cy-3) and 2.7 ns (DAPI). DAPI binds to DNA in the nuclei and a Cy-3 labeled antibody binds to septin localized in the cytoplasm. No spectral separation is used. As can be seen, these two fluorophores are easily distinguished by lifetime.



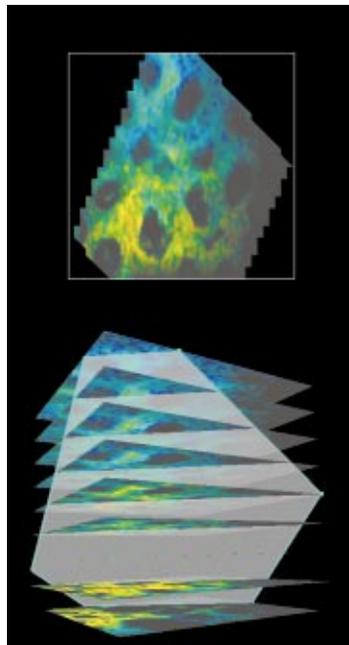
**Fig. 2** (A) Multiphoton and (B) FLIM images (60× PlanApo 1.4 NA lens) of a transverse methyl green-stained section through a liver lobule of a cynomolgus monkey showing the central vein (cv) surrounded by the liver parenchyma (sheets of hepatocytes) and sinusoids (s). The lifetime of the fluorophore bound to the blood cells within the vein is strikingly longer than in the hepatocytes surrounding the vein.



**Fig. 3** (A) Brightfield, (B) multiphoton, and (C) FLIM images ( $60\times$  PlanApo 1.4 NA lens) of a transverse methyl green-stained section through the medulla of a kidney from a cynomolgus monkey. The images show collecting ducts (cd), which are recognized by their columnar epithelium, collecting tubules (ct), which have a cuboidal epithelium with a diameter that is wider and less regular than the ascending thick limbs (a) of the loop of Henle, the descending thin limbs of the loop of Henle (d), and the vasa recta (v) (capillaries filled with erythrocytes). In the lifetime image (c), nuclei in the collecting duct, tubule, and ascending thick limb can be seen to have shorter lifetimes than that of the surrounding cytoplasm. The cells of descending thin limbs exhibit longer lifetimes than the surrounding structures.



**Fig. 4** (A) Brightfield, (B) Multiphoton, and (C) FLIM images ( $60\times$  Plan Apo 1.4 NA lens) of a section through the endometrium of a cynomolgus monkey uterus stained with acridine orange. The images show a portion of an endometrial gland (g) with secretion (arrow) entering the lumen. Underlying the gland are strands of smooth muscle (sm) at the edge of the myometrium. In (B) and (C) an endometrial gland (g) can be seen surrounded by connective tissue stroma. The gland is secreting a thick, glycogen-rich fluid (arrow). The lifetime on the fluorophore bound to the secretion within the gland is strikingly shorter than in the columnar gland epithelium. The lifetime on the fluorophore bound to the blood cells within the capillaries of the stroma is longer than the surrounding connective tissue cells.



**Fig. 5** Arbitrary slice through a 3-D dataset of a cynomolgus monkey kidney tissue sample stained by acridine orange in which color corresponds to fluorescence lifetime.

gland and its luminal secretion is strikingly shorter than that of the fluorophore bound to the surrounding tissue.

By utilizing histological slides that were cut thicker than traditional pathological specimens, we were able to obtain three-dimensional information by taking stacks of optical sections through the 200- $\mu$  depth. The upper part of Fig. 5 (see Color Plate 2) shows an arbitrary slice through a stack of FLIM images of acridine orange stained kidney. The ability to visualize three-dimensional structures could provide useful additional information over and above what can be seen within a single thin section and obviate the need for mechanical sectioning.

#### 4 Discussion

The FLIM system described is being developed for the identification and measurement of fluorescent probes in cells or tissues and for the characterization of lifetime properties of endogenous fluorophores that could be diagnostic of human pre-cancers and cancers. In the initial investigations described here, we imaged fixed histology specimens. Experience gained in the course of these studies will be used to direct and refine future experiments with models of neoplasia. Our multiphoton FLIM system is being optimized for *in vivo* studies, allowing the maximum use to be made of all the information present in weak signals from fluorescent probes and endogenous fluorophores.

In summary, we believe that multiphoton lifetime imaging systems have considerable potential as a diagnostic method for pathological investigations. While much work needs to be done in this area, the deep sectioning and high signal-to-background imaging capabilities of the multi-photon imaging system when combined with the extra dimension of lifetime information could be used to identify and track the changes in fluorescence signals of standard histological stains in diverse microenvironments. This could lead to possible identification of lifetime signatures of fluorescent probes and / or endogenous fluorophores that are specific to diseased states.

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