Whole-field optically sectioned fluorescence lifetime imaging

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We describe a novel three-dimensional fluorescence lifetime imaging microscope that exploits structured illumination to achieve whole-field sectioned fluorescence lifetime images with a spatial resolution of a few micrometers. © 2000 Optical Society of America

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Functional optical microscopy can now provide a wealth of spectroscopic information, and submicrometer spatial resolution is routinely achieved in three dimensions by use of confocal scanning microscopy or two-photon microscopy. For many spectroscopic modalities, however, the acquisition time required for obtaining functional information can lead to extremely low image frame rates when convolved with the need to scan the object pixel by pixel (see, e.g., Ref. 1). It is therefore highly desirable to realize three-dimensional (3-D) functional imaging with parallel pixel acquisition, particularly for *in vivo* biomedical applications. As well as affecting acquisition time, whole-field imaging can also improve the efficiency of light collection. One such approach is that of structured illumination, proposed by Neil *et al.*,[2] which was recently applied to fluorescence microscopy.[3] We report what we believe to be the first microscope in which structured illumination is employed to realize whole-field 3-D fluorescence lifetime imaging microscopy (FLIM).

Functional information can readily be derived from the fluorescence lifetime because of its dependence on fluorophore radiative and nonradiative decay rates. FLIM provides contrast between specific fluorophores (with characteristic radiative decay rates) and monitoring of local environmental perturbations (which affect the nonradiative decay rate).[4] Fluorescence lifetime probes have been demonstrated for many biologically significant analytes, including $[Ca^{2+}]$, $[PO_2]$, and pH. In well-controlled microscopy experiments, FLIM can provide quantitative functional information concerning fluorophore distributions.

Fluorescence lifetime can be measured in either the frequency domain (see, e.g., Refs. 5 and 6) or the time domain (see, e.g., Ref. 7). Although it is straightforward to combine fluorescence lifetime measurement with a scanning confocal microscopy that exploits single- or multiphonon excitation (see, e.g., Ref. 8), whole-field 3-D FLIM has so far proved more challenging. A frequency-domain FLIM microscope was recently reported[9] that employs image-restoration techniques to improve the sectioning performance beyond that of a conventional microscope. In the time domain, an elegant pseudo-whole-field multifocal multiphoton microscope that uses ~25 beams scanning in parallel was applied to 3-D FLIM.[10] Although this is a step toward full parallel pixel acquisition, the image-acquisition times are still longer than is desirable for many applications, and the whole-field detector is not used at its full efficiency. Moreover, this approach is applicable only to multiphoton excitation.

Our time-domain whole-field FLIM system, which can image lifetime differences from less than 10 ps to tens of microseconds, was described in Ref. 11. Its ability to contrast different tissue constituents and states of tissue by use of autofluorescence[12] suggests many potential biomedical applications, and we are working toward developing a near-real-time 3-D FLIM instrument that can be applied to depth-resolved functional imaging *in vivo*. We demonstrated the use of all-solid-state diode-pumped laser technology for FLIM,[13] illustrating the potential for compact and portable instrumentation, and here we address the extension of this technique to rapid 3-D imaging. In fact, as was pointed out in Ref. 9, 3-D sectioning is necessary because the out-of-focus light that blurs the conventional whole-field microscope image also degrades the fluorescence lifetime image and can affect both spatial and temporal resolution.

A schematic of the optical arrangement that we used to realize whole-field 3-D FLIM is illustrated in Fig. 1. Even in a conventional microscope, it is only the zero spatial frequency component of the transmitted light that does not attenuate with defocus. Thus, by use of a structured illumination of the sample, it is possible to obtain a sectioned image from the spatially modulated component of the collected light.[2] This is achieved with a suitable computer algorithm that

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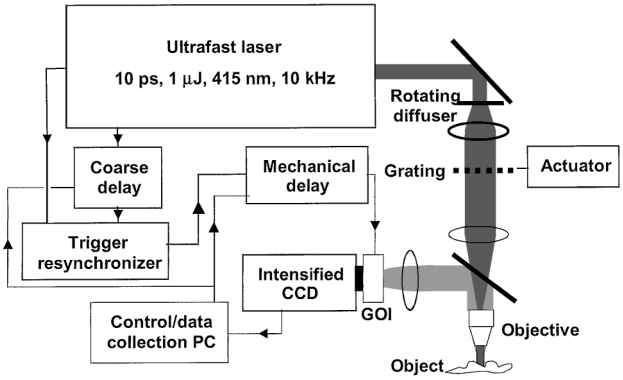

Fig. 1. Experimental setup for whole-field 3-D fluorescence lifetime imaging by use of structured light. GOI, gated optical intensifier.

provides both a sectioned and a conventional image of the sample. Following the procedure discussed in Ref. 2, we sequentially projected a grating onto the sample at three different transverse positions (corresponding to spatial phase changes of 0, $2\pi/3$, and $4\pi/3$) and acquired a set of time-gated fluorescence images at each position. By combining the spatially modulated images corresponding to each sampled time delay, we then obtained a series of time-gated sectioned and conventional fluorescence images from which we calculated a sectioned and a conventional FLIM map.

Our first test sample consisted of cotton wool fibers stained with a solutions of Coumarin 314 and DASPI. In this first set of experiments we projected a grating of 50 lines/in. (and unity mark-space ratio) onto the sample, using a 10×, 0.25-N.A. microscope objective. Figures 2(a) and 2(e) show conventional fluorescence intensity images obtained at two positions separated by 315 μm in the z direction. Both images are significantly blurred by out-of-focus light. Figures 2(b) and 2(f) show the corresponding FLIM maps, in which out-of-focus blur is clearly a problem, although the longer lifetime of Coumarin 314 is contrasted with the shorter lifetime of DASPI. As noted by Squire and Bastiaens,[9] the fluorescence intensity recorded at each point in the image is a weighted sum of intensities from all the neighboring points within the 3-D point-spread function of the optical system. Since the fluorescence lifetime is determined from a series of such images, the calculated lifetime maps will suffer from a significant loss of temporal resolution. Figures 2(c) and 2(g) show the sectioned intensity images. The out-of-focus blur has been eliminated, and in the corresponding sectioned FLIM images [Figs. 2(d) and 2(h)] the fluorescence lifetime contrast has been preserved, with a dramatic improvement in localization. For this selection of grating pitch and microscope objective, the sectioning strength is calculated to be 71.6 μm.[2]

We applied the 3-D FLIM microscope to a sample of polystyrene microspheres coated with fluorescent dye (Molecular Probes FluoroSpheres; 15-μm diameter, blue-green). In this case we used a 50-line/in. grating with a 60×, 0.8-N.A. objective to improve the sectioning, which we calculated to be 3.1 μm.[2]

Figures 3(a) and 3(b) show conventional and sectioned time-gated intensity images, respectively, of a large 3-D cluster of microspheres at an arbitrary plane in the sample ($z = 0$ μm). It can be seen that the out-of-focus light clearly degrades the conventional image but is almost eliminated in the sectioned image. This improvement is even more dramatic when we compare the corresponding FLIM maps in Figs. 3(c) and 3(d). The individual microspheres are well resolved only in the sectioned FLIM map. Figure 3 also shows FLIM maps acquired at different planes in the sample ($z = 11$, 19 μm). The conventional FLIM maps do not exhibit any significant sectioning, owing to the

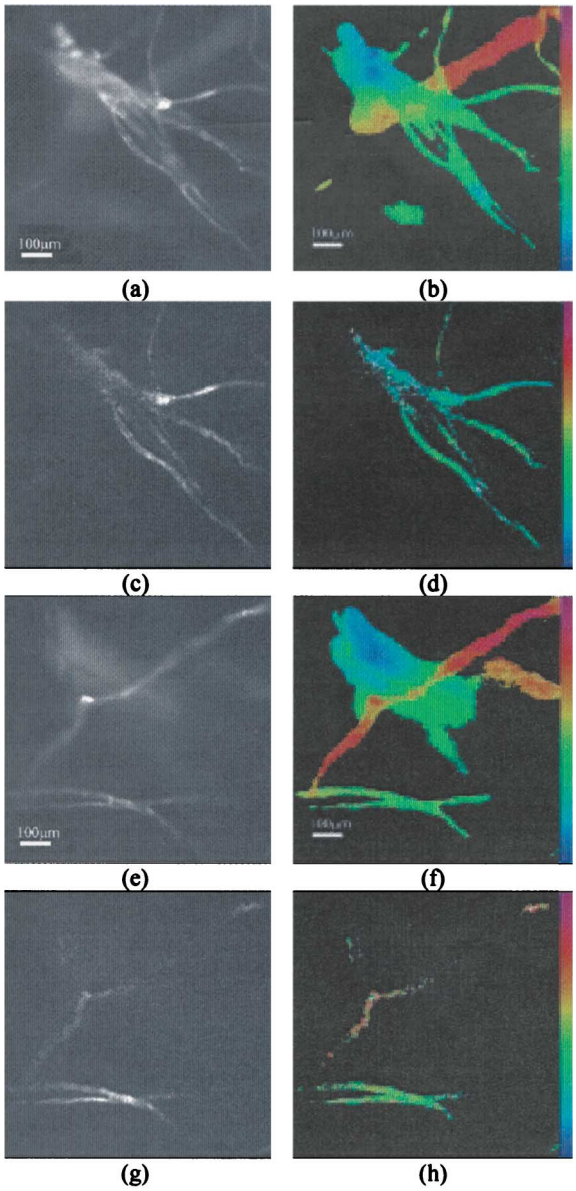

Fig. 2. Microscope images (x, y plane) of cotton wool stained with Coumarin 314 and DASPI for two different z positions separated by 315 μm. (a), (e) Conventional fluorescence intensity images. (b), (f) Corresponding conventional FLIM maps. (c), (g) Sectioned fluorescence intensity images. (d), (h) Sectioned FLIM maps. All lifetime false-color scales span from 700 ps (blue) to 2.6 ns (pink).

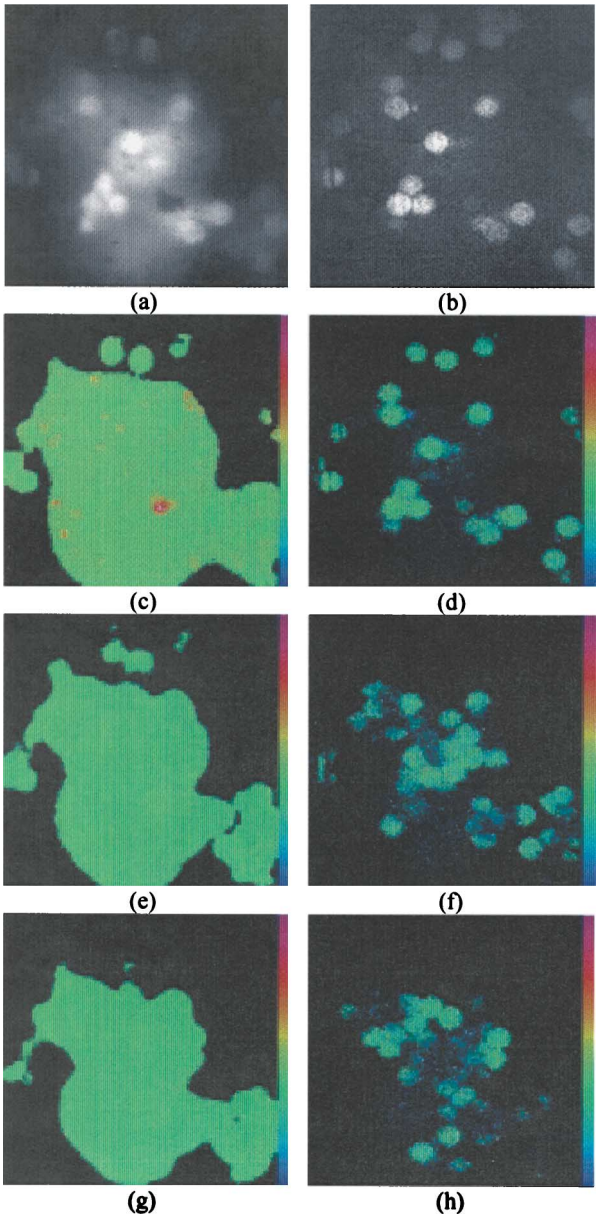

Fig. 3. (a) Conventional and (b) sectioned intensity images (x, y plane) of 15-μm-diameter fluorescent microspheres at $z = 0$ μm. The corresponding (c), (e), (g) conventional and (d), (f), (h) sectioned FLIM images at $z = 0$, 11, 19 μm are also shown. The fluorescence lifetime false-color scale spans from 0.2 ns (blue) to 7 ns (pink) in each case. The field of view is 170 μm $\times$ 170 μm.

out-of-focus fluorescence, whereas the sectioned FLIM maps clearly resolve and separate individual microspheres located at different depth planes.

We note that any intensity fluctuations or movement of the excitation beam across the field of view during acquisition will result in field-dependent intensity variations among the three grating images. As a result, the sectioned intensity images may exhibit some residual spatial modulation. This effect has been minimized by normalization of the grating images on a pixel by pixel basis. Currently our images are acquired with an intensified 8-bit CCD camera. To improve the sectioned image quality further we hope to use a 12-bit cooled CCD camera. Since improving the depth resolution results in a reduction of the sectioned image signal relative to that of the conventional blurred image, increasing the dynamic range (and hence facilitating detection of the reduced sectioned signal) will also enhance the potential sectioning strength that is achievable.

In summary, we have combined FLIM with an optical sectioning technique to achieve whole-field 3-D FLIM. Further refinement of these results should permit FLIM with sub-10-ps lifetime discrimination,[11] combined with submicrometer sectioning strength[2] in a compact, portable system.[13] Other extensions of this research will include 3-D imaging of biological samples and endoscope application.

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