Fluorescence lifetime three-dimensional microscopy with picosecond precision using a multifocal multiphoton microscope

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The combination of pulsed-mode excitation multifocal multiphoton microscopy with a high-repetition, time-gated intensified CCD camera enables efficient three-dimensional (3D) fluorescence lifetime imaging. With a 200-ps gate opening at 76 MHz repetition rate, fluorescence decay can be traced in a sequence of images with varying delays between pulse and gate. Fluorophore lifetimes are measured with a precision of a few picoseconds. As an application we show that, upon two-photon excitation at 800 nm, certain pollen samples feature a multiexponential fluorescence relaxation. Our results indicate that efficient four-dimensional microscopy with hundreds of nanometer spatial and tens of picoseconds temporal resolution is within reach. © *1998 American Institute of Physics.* [S0003-6951(98)01139-5]

Three-dimensional (3D) fluorescence microscopy is a key method for biological and medical research. Multiphoton absorption greatly facilitates the realization of 3D fluorescence microscopes because nonlinearity confines the fluorescence emission to the focal region. Thus the absorption of two or three infrared photons enables three-dimensional scanning microscopy without the requirement of a confocal pinhole. Standard multiphoton microscopy, however, relies on the galvanometric scanning of a single beam and requires recording times of the order of seconds. The maximum usable power is in the range of 5–30 mW due to nonlinear limiting effects and saturation.

By rapidly scanning an array of high aperture foci (typically 5×5) across the focal plane, the recently introduced multifocal multiphoton microscope[1] (MMM) parallelizes[1,2] multiphoton imaging.[3,4] The MMM is able to use all the power emitted by a Ti:sapphire laser of typically 500–2000 mW and delivers multiphoton video-rate imaging at high spatial resolution.[1] The nonlinear nature of excitation allows the view of planar sections of (live) samples in real time.[1] In fact, by allowing observation of the sample with the eye, the MMM enables the operator to work with a two-photon microscope as intuitively as with a conventional microscope.

Another important development in fluorescence microscopy is lifetime imaging of the fluorescent state.[5–11] Fluorescence lifetimes are sensitive to the fluorophore environment and can be used to distinguish fluorophores with overlapping emission spectra. Two-photon lifetime microscopy has been demonstrated both in the frequency domain[12,13] and in the time domain.[14] Both methods reveal good time-resolved 3D images. However, they are limited by the limited efficiency of galvanometer beam scanning. The recent advent of gated, intensified CCD cameras with a gate of 200 ps and gating repetition rates of up to 100 MHz opens up new opportunities for 3D lifetime microscopy. By synergistically combining this camera system with the MMM, we are now able to report efficient, technically uncomplicated, high-resolution 3D lifetime microscopy (4D microscopy) with 200 ps temporal resolution.

The experimental setup is sketched in Fig. 1. The light source was a mode-locked Ti:sapphire laser emitting pulses of 200 fs duration at a repetition rate of 76 MHz (Mira 900F, Coherent, Palo Alto). The two-photon excitation wavelength was 790 nm. Residual emission in the visible was removed by an OG 550-Schott color glass filter. The beam (TEM_{00} mode) was expanded by a telescope (L_1, L_2) and its edges were cut off by a beam stop. By impinging on a rotating microlens disk, the beam was divided into ~25 beamlets, which, after having passed the intermediate optics of an inverted microscope, were transformed in ~25 high aperture foci of ~1–10 mW. Rotating the disk ensured a dense and uniform scan in the focal plane with 375 scanned frames per second.

Fluorescence was filtered out by a dichroic mirror and

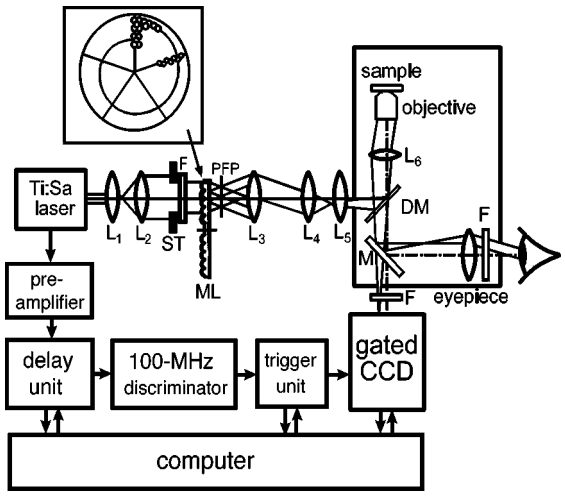

FIG. 1. Experimental setup of the time-resolved multifocal multiphoton microscope. The femtosecond pulses of the Ti:sapphire laser pass a rotating microlens disk and are projected onto the sample as an array of foci. Fluorescence light is imaged onto a gated intensified CCD camera which opens the gate in synchronism with the laser pulses.

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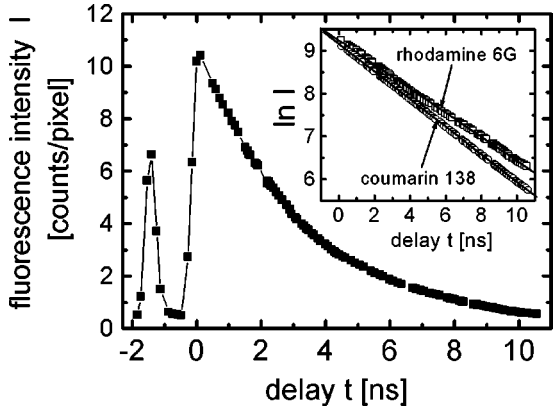

FIG. 2. Exponential decay of the fluorescence of rhodamine 6G. The spike at $t=-1.5$ ns before the onset of fluorescence at $t=0$ is due to a reflection of laser light from the tube lens L_6. The logarithmic plot (inset) reveals lifetimes of 3554 ± 10 and 3096 ± 2 ps for rhodamine 6G in water and coumarin 138 in immersion oil, respectively.

imaged onto the camera. Remaining infrared light was absorbed by 2 mm BG 39 Schott color glass. The gated camera (PicoStar HR, La Vision, Göttingen, Germany) featured a S20 photocathode whose photocurrent was amplified by a microchannel plate intensifier (Kentech Instruments Ltd., South Moreton, UK). The amplified signal was accumulated by a Peltier cooled CCD chip with 384×576 pixels. The gate was triggered by the pulses of the mode-locked laser. For this purpose, the signal of a photodiode was amplified and, after having passed a programmable delay unit, was used to trigger the gate. A full-field 2D image was then taken at a certain delay and the lifetime was determined by comparing the signal for various delays. The delay unit was calibrated with a 1 GHz oscilloscope. Delay steps as small as 100 ps could be programmed, and the delay could be extended throughout the 12.5 ns time gap between the laser pulses.

A 3D stack of *XY* image data is taken by subsequently moving the sample along the optic axis. As the gate width of 200 ps is small as compared to usual fluorescence decay times ($\tau\approx0.5-10$ ns), no further deconvolution with respect to the instrument function is required for the determination of the lifetime. In addition, due to the short rise time, the gate can be switched on immediately after excitation. Conversely, narrow gating inevitably rejects the light that is not emitted during the offered time span which leads to a reduction of overall signal. The advantage of the MMM is that the ~25 fold increase in imaging speed can also be used to narrow the gate and to scan the fluorescence decay more densely.

To verify the function of the system as well as to calibrate it, a highly concentrated aqueous solution of rhodamine 6G was initially imaged. Figure 2 depicts the lifetime measurement based on the oscilloscope calibration. As shown by the logarithmic plot in the inset, fluorescence decays monoexponentially with a decay time of $\tau=3554\pm10$ ps. By using appropriate filters, we could establish that the first spike from left ($t=-1.5$ ns) before the onset of fluorescence ($t=0$) is caused by a partial reflection of the laser pulse from the tube lens (L_6). As the laser pulse is only ~200 fs, this peak at $t=-1.5$ ns is a direct measure of the instrument function of the experimental setup.

As the next step, we imaged a solution of coumarin 138

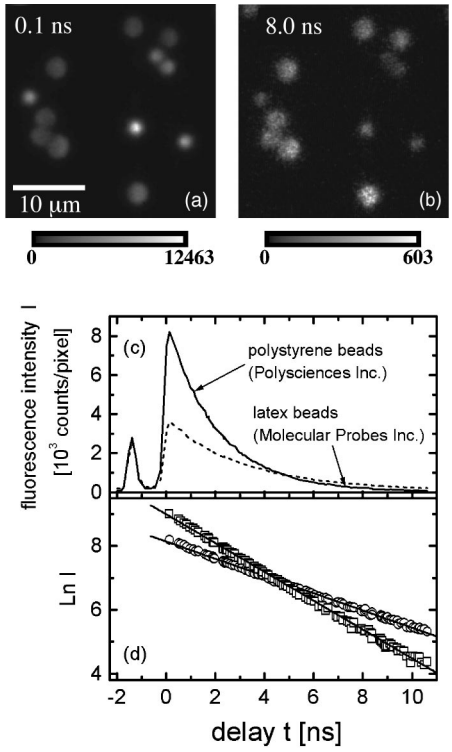

FIG. 3. Images of randomly dispersed fluorescent polystyrene and latex beads immediately (a) after the onset of fluorescence and (b) after 8.0 ns. While the fluorescence of the smaller polystyrene beads has a shorter lifetime, the latex beads dominate at a longer delay. Panels (c) and (d) depict the complete, densely measured time dependence.

in immersion oil. Careful evaluation of the data revealed a minor systematic deviation from the exponential decay, in the order of a few percent in both fluorophore solutions. Therefore we calibrated the delay with the single-exponential relaxation of rhodamine 6G. As a result, the fluorescence relaxation of coumarin 138 in immersion oil (inset of Fig. 2) also exhibited an ideally monoexponential relaxation with a decay time of 3096 ± 2 ps.

Figure 3 shows *XY* images of two different species of randomly dispersed beads with different lifetimes and diameters. In Fig. 3 the fluorescence is collected immediately after the excitation at (a) $t\leqslant0.1$ ns and at a delay of (b) $t=8.0$ ns. In (a) the smaller beads (Polysciences Inc., Warrington, PA) fluoresce more brightly than the larger ones (Molecular Probes Inc., Eugene, OR). At delays $t\geqslant8$ ns, the larger beads clearly dominate the image. The full temporal dependence of the fluorescence signals is depicted both (c) linearly and (d) logarithmically in the lower panels. Again, a single-exponential decay with appreciably different lifetimes is found, namely, 2.2 and 3.7 ns for the smaller and larger beads, respectively.

Biological samples can exhibit more complex lifetime patterns. Figure 4 shows three *XY* images of a sequence of 125 taken from the same planar section in the interior of a pollen grain (Carolina Biological Supply Company, Burlington, NC). Figure 4(a) displays the *XY* section shortly after the two-photon excitation, (b) after 3.5 ns, and (c) after 7.0 ns. Figure 4(d) shows the fluorescence decay measured in steps of 100 ps for a region at the center and the edge of the pollen grain. The fluorescence decay features a very fast component but comparatively large components are observed, too. The

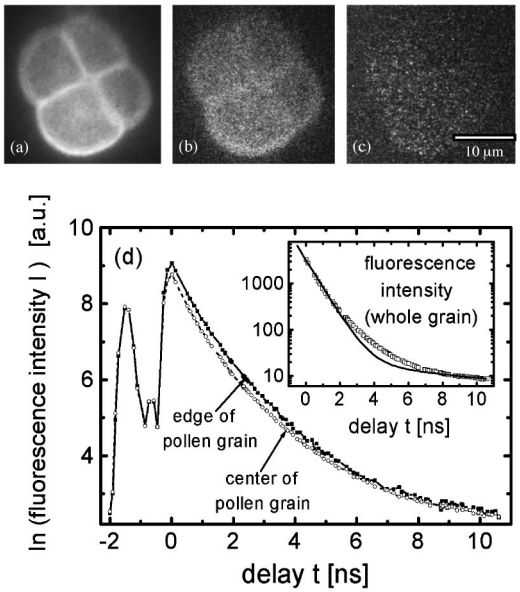

FIG. 4. Time-resolved *XY* section inside a pollen grain obtained by two-photon excitation: (a) shortly after the excitation, (b) after 3.5 ns, and (c) after 7 ns. As shown in (d) the fluorescence relaxation is multiexponential and varies in space. The logarithmic curve in the inset in (d) also displays a double-exponential fit (solid line) of the signal of the whole grain. In the fluorescence decay of the pollen grain at least three different lifetimes are involved.

logarithmic display (inset of Fig. 4) shows that the fluorophores relax multiexponentially, with decay times, τ, ranging from 0.7 ns up to more than 10 ns. A double-exponential fit does not match the decay as indicated by the solid line in the inset. The detailed recording of 125 images (of 200 ps gate time) took approximately 7 min. Within this time frame, fluorescence bleaching by 8% was observed so that we corrected the data of Fig. 4(d) for bleaching.

It is straightforward to enlarge the gate and also to reduce the number of delay times in the sequence. This measure increases the signal or the imaging speed. In general, the speed is determined either by the available total photon flux, or by the read-out time of the CCD camera. In our case, the speed was determined by the total of 500 ms needed to set the next delay. In any case, the MMM is faster than any high-resolution single-beam multiphoton excitation system due to the multifocal arrangement.

We further note that by changing the electronics, our system consisting of MMM and intensified camera can also be adapted to operate in the frequency domain mode which, by increasing the duty cycle, might be valuable for other applications. The gated camera can also be combined with Nipkow-type confocal disk scanners[15] which are expected to achieve good performance whenever single-photon excitation is required.

In summary, by combining multifocal multiphoton microscopy with a high-repetition rate, gated CCD camera, it is possible to efficiently perform lifetime 3D microscopy, i.e., 4D microscopy. At high apertures, the MMM features a spatial resolution of 250–300 and 850–1500 nm in the lateral and axial direction, respectively. The time gate of 200 ps is already sufficient for most fluorophores and enables us to measure the lifetime of fluorophores with picosecond precision. Picosecond precision in 2D fluorescence lifetime imaging using a gated camera has recently been demonstrated.[16] Evidently the gate width could still be further reduced through the use of faster shutter electronics. Therefore, 3D microscopy with temporal resolution in tens of picoseconds appears to be realistic. Simplicity, life cell imaging ability, high temporal and spatial resolution render our microscope promising for a large number of (biological) applications. To this end, the gated camera MMM represents an efficient 3D lifetime microscope with a spatial resolution of several hundreds of nanometers and a precision of a few picoseconds.

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