Pulsed laser fluorophore deposition: 
a method for measuring the axial resolution in two-photon fluorescence microscopy

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We report the generation of thin (< 50 nm) fluorescence layers on a glass substrate in the focal region of a high-numerical-aperture lens by employing subpicosecond pulses of 0.75-TW/cm² peak intensity. The conditions for generating the fluorescence layers are described. We find that the fluorescence molecules at the glass interface are less affected by bleaching than those in the surrounding area. The fluorescence layers are suited for measuring and monitoring the axial resolution of two-photon fluorescence microscopes.

Key words: two-photon fluorescence microscopy, axial resolution, 4Pi confocal microscopy.

1. INTRODUCTION

Two-photon-excitation scanning microscopy is a promising method for the generation of three-dimensional images. The advantages of two-photon-excitation microscopy are the inherent axial sectioning capability and the confinement of bleaching to the closest vicinity of the focus. This was first realized by Denk et al., who used a colliding-pulse, mode-locked dye laser providing a stream of 100 fs pulses at a repetition rate of 80 MHz.¹ The advent of easy-to-operate Ti:sapphire lasers facilitated the implementation of two-photon excitation in a standard scanning fluorescence microscope.² A promising technique for high-axial-resolution imaging is 4Pi confocal microscopy.³ The 4Pi confocal microscope features two high-numerical-aperture lenses placed opposite to each other. In 4Pi confocal fluorescence microscopy of type A, coherent wavefronts are focused to the same object point, thus enlarging the illumination aperture in the axial direction. The focal intensity distribution of a 4Pi confocal microscope is governed by the interference pattern exhibiting a main maximum and a sidelobe above and below the focal plane for the case of constructive interference. Two-photon excitation is particularly useful in 4Pi confocal fluorescence microscopy, because one can apply a smart method to suppress the axial lobes by using the large-illumination point-spread function in combination with a narrow-detection point-spread function.⁴ ⁵ The narrow-detection point-spread function probes the inner region of the illumination focus, thus leaving a narrow focal maximum of ~140-nm axial width and lobes of 30–50%.

Axial discrimination is one of the key properties of a two-photon fluorescence microscope. One usually performs the measurement of the axial resolution by axially scanning a layer of fluorophore dissolved in immersion oil through the focus of the microscope and recording the edge of the layer to the cover glass.³–⁶ The edge response [Fig. 1(a)] and its derivative are a measure of the axial resolution of the microscope. In this Communication, however, we describe an effect that we incidentally observed when axially scanning fluorescence layers. We demonstrate that this effect can be used for determining and monitoring the axial resolution in a two-photon-excitation microscope when one is working with subpicosecond pulses.

2. MATERIALS AND METHODS

We prepared a fluorescent layer by dissolving 0.5 mg of Rhodamine 6G (Kodak Optical Products, Rochester, N.Y.) in 0.6 ml of ethanol. A drop of dyed ethanol was added to 0.3 ml of immersion oil (Zeiss, Oberkochen, Germany). Ethanol was used because the fluorophore dissolves faster in ethanol than in the rather viscous immersion oil. A drop of fluorescent oil was placed between two cover slips, forming a thin layer. We experienced that the thickness of such layers varies between 5 and 15 μm. Immersion oil was employed to ensure refractive-index matching with the cover glasses.

Figure 1(a) shows an axial response of the cover-glass–oil interface when the interface is scanned along the optical axis of a two-photon-excitation scanning microscope. The light of a mode-locked Ti:sapphire laser (Coherent, Mira 911F) was used for excitation. The wavelength of the laser was 750 nm, and the pulse length was ~220 fs in the focal region.⁷ The repetition rate of the pulses was 3.8 MHz. The pulse rate could be varied by an acousto-optical beam deflector (Coherent, 9200 Pulse Picker). The scanning stage (Physik Instrumente, Waldbronn, Germany) provided a piezoelectrically driven three-dimensional movement.

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with an accuracy of 10 nm. The opening of the detector was large compared with the geometrical image of the focal spot in the detector plane, thus ensuring that no additional spatial filtering in the detector plane (confocal effect) could take place. The effective numerical aperture of the objective lens was 1.35 (Leitz Planachromat, 100 X, 0.7–1.4). For imaging, peak intensities of 0.15 TW cm\(^{-2}\) were used. The corresponding continuous power of the excitation light was \(P_{cw} = 0.4\) mW. We obtained this power by attenuating the laser beam with a metallic density filter.

3. PULSED LASER FLUOROPHORE DEPOSITION

In a typical experiment we first removed the attenuation filter and exposed the layer to pulses of 0.74 TW/cm\(^2\), which is approximately five times higher than the signal used for imaging. After a few seconds we replaced the attenuation filter and continued to observe the fluorescence signal with the lower intensity. We found that the two-photon-excitation fluorescence signal had dramatically increased at the glass–oil interface close to the objective lens [Fig. 1(b)]. One of the experiments is tracked in Fig. 1(c), where we displayed the recorded intensity versus the elapsed time. The stage was scanned with a speed of 10 \(\mu\)m/s over a range of 15 \(\mu\)m throughout the recording. The comparatively low speed of scanning ensured a high precision of measurement. The thickness of the fluorescent layer was \(\approx 7.5\) \(\mu\)m. We performed a full axial scan through the sample. The first edge in Fig. 1(c) appeared when the cover-glass–oil interface entered the focus. The second edge appeared when the rear cover-glass–oil interface left the focus. As shown in Fig. 1(c), we first performed three scans at the lower intensity of 0.15 TW/cm\(^2\). Then we exposed the layer to the high-intensity beam of 0.74 TW/cm\(^2\) for \(\approx 5\) s. During this period the layer was scanned twice through the beam. After reducing the peak intensity to the initial value of 0.15 TW/cm\(^2\), we observed that the fluorescence signal was dramatically increased at the edge directed toward the objective. Figure 1(c) shows that the fluorescence signal at the edge increased by a factor of 4. The increased edge signal was maintained over very long periods of time, up to 1 h. Once generated, the signal remained stable during scanning.

To understand the origin of the edge signal, we stopped the stage from axial scanning and recorded lateral and axial images of the exposed region. Figure 2(a) shows an image of the interface. One notes a bright spot at the
place where the scanning beam passed the oil–glass interface. The spot is approximately 1.5 μm in diameter. Figure 2(b) shows an axial image (axial section) of the same site. Figure 2(b) reveals that the fluorescently labeled oil region behind the bright fluorophore spot is darkened, indicating that the dye was removed or bleached in this region and possibly transported to the glass interface. Apparently, the dye molecules attached to the cover glass are less affected by bleaching than those in the surrounding area. We concluded that the strong fluorescence signal is due to a deposition of the fluorophore molecules onto the glass surface and termed this effect pulsed laser fluorophore deposition (PLFD).

As a next step, we varied the power of the beam to find a threshold at which PLFD occurs. First, we decreased the continuous power of the beam by decreasing the repetition rate of the pulses. At the peak intensity of 0.74 TW/cm², PLFD was less pronounced and virtually did not occur for pulse repetition rates lower than 110 kHz. For a repetition rate of 3.8 MHz, the effect decreased with decreasing peak intensities. No indications of PLFD could be found for focal peak intensities lower than 0.05 TW/cm².

We examined several fluorophore–solvent combinations. We found that PLFD occurs with Rhodamine 6G, Rhodamine B, and Coumarin 138 immersed in immersion oil. Glycerol was also suited for Rhodamine B and 6G but not for Coumarin 138. Rhodamine 6G produced the most pronounced effect, which is probably due to the high photostability of this compound. We observed PLFD also at a Mercoglass (Merck, Darmstadt, Germany)–immersion-oil interface, but the signal from the interface was low. For Coumarin 138 the response was lower than the fluorescence signal stemming from the unexposed fluorescence layer.

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Figure 3 shows another example of a PLFD-generated Rhodamine 6G layer response. In contrast to the previous recordings, the solid curve of Fig. 3 was produced with pulses of a repetition rate of 76 MHz, which is the standard pulse repetition rate of our Ti:sapphire laser. Furthermore, we generated the layer by exposing the interface to the high peak intensities for ~15 s. Further-
more, we placed a suitable pinhole in front of the detector and made the two-photon-excitation microscope confocal. We found a full width at half-maximum (FWHM) of the peak of 690 nm.

In a further experiment we recorded the response of a 4Pi confocal microscope. It is interesting to record axial responses of a two-photon-excitation 4Pi confocal microscope because of the high axial resolution of this system. Having a theoretical FWHM of ~145 nm and knowing the numerical aperture and wavelengths, one can employ the 4Pi confocal response for assessment of the thickness of the PLFD-generated layer. Figure 3(b) shows a 4Pi confocal response generated by the PLFD technique in a Rhodamine-6G–oil solution. The interface was exposed to the high peak intensity for ~30 s before recording. The measurement shown was recorded approximately 4 min after the exposure. The interface response was lower than that of the unexposed environment. We observed this to be the case for exposures of the same place at the interface that last longer than ~10 s. The FWHM of the main maximum of the 4Pi confocal response is 140 nm, whereas the two axial lobes have a relative height of ~28%. We could observe the changes of the relative phase of the interfering wave fronts when we were monitoring the signal. Figure 3(b) shows a response for constructive interference in the 4Pi confocal microscope. The recordings of Fig. 3 were generated by a single scan along the optical axis.

4. DISCUSSION

The assumption that the observed effect is due to a thin layer of fluorophore generated by PLFD explains consistently the pronounced peaks in Figs. 1 and 3. We found that the PLFD-generated layers can be optically very thin, so that one can measure the axial resolution of a two-photon fluorescence microscope directly simply by measuring the PLFD curve. The solid curve in Fig. 3(a) resembles an axial response of a two-photon-excitation confocal microscope to a thin fluorescence plane. One can note also a slight asymmetry around the peak value, which is typical for a focus suffering from spherical aberration. The pronounced responses of Fig. 3 indicate that the thickness of the layer can be as thin as 50 nm or thinner.

The axial response to an infinitely thin fluorescence plane in an aberration-free two-photon-excitation confocal microscope is defined by

$$I(z) = \int \int |h_{exc}(x, y, z)|^2 h_{det}(x, y, z) dx dy.$$  \hspace{1cm} (1)

The function

$$h_{exc, det}(x, y, z) = |(e_x, e_y, e_z)|^2$$  \hspace{1cm} (2)

is the focal intensity distribution around the geometric focus \((x = 0, y = 0, z = 0)\) calculated for the excitation and the detected wavelength, respectively. In a vectorial theory the electric field is given by

$$(e_x, e_y, e_z) = -i[I_0 + I_2 \cos(2\varphi), I_2 \sin(2\varphi), -2I_1 \cos \varphi],$$  \hspace{1cm} (3)

with \(I_0, I_1, I_2\) being integrals defined over the lens aperture. The axial response for a numerical aperture of 1.35 (oil) is displayed in Fig. 3(a) as a bold dashed curve. A comparison of the two curves shows that the curve generated by PLFD is very similar to the theoretical response. The FWHM of the theoretical curve is 460 nm. This is 35% narrower than its experimental counterpart (solid curve), indicating that the PLFD-generated layer of Fig. 3(a) might not be optically thin. However, it is known that theoretical axial responses are generally narrower than their experimental counterparts.\(^6\)\(^,\)\(^7\) Another test of the thickness of the PLFD-generated layers is the comparison with the response gathered by the standard method of measuring the responses to an axial edge and calculating the derivative. The latter is equivalent to the response to an infinitely thin layer.

We determined the axial response to an edge by consecutively recording 50 axial images at low peak intensities. We added the responses to improve the signal-to-noise ratio and performed a least-square fit. The fitted curve was differentiated, and the derivative is shown as a thin dashed curve in Fig. 3(a). A comparison of the two curves shows that the PLFD-generated axial response is very close to the response gained from the axial edge. Therefore we can conclude that the PLFD-generated layer in Fig. 3(a) is optically thin compared with the axial resolution of the microscope.

The fact that the experimental responses are broader than the theoretical counterparts is known but not fully investigated. Possible reasons are that the manufactured lenses do not fully obey the sine condition and are apodized.\(^6\) The axial resolution depends strongly on the amplitude and phase of the focused wave fronts, so the deviations could be due to aberration of the lens–object system. Another possible reason is the orientation of the emitting fluorophore molecules. Only the molecules with a dipole orientation parallel to the incident electric field are excited.\(^10\) However, we would like to emphasize that the agreement between the PLFD responses and the responses gained from the differentiation of the fluorescence edge proves the validity of the PLFD approach to the determination of axial resolution.

Our experiments have shown that PLFD requires both a certain level of peak intensity but also a high enough repetition rate, which is equivalent to a certain level of quasi-continuous power. Our interpretation of the effect is that the high intensities cause the fluorescent liquid to circulate in the region close to the focus. This flow is inhibited at the glass–oil interface, thus generating a dense and stable accumulation of dye molecules at this site.

The recordings shown in Fig. 3 were made with a single scan; the signal was not obtained by averaging. Thus PLFD-generated layers should be well suited for real-time monitoring of the axial resolution of a two-photon-excitation microscope. In addition to allowing for online determination of the axial resolution of two-photon-excitation microscopes, PLFD could be of practical interest to other applications, the discussion of which is beyond the scope of this initial report. As they are not subject to strong bleaching, PLFD-coated nanometer-scale particles could possibly act as a local probe for near-field imaging inside a specimen.\(^11\)\(^,\)\(^12\) Another application could be the
coating of invisible surfaces immersed in fluorescently labeled media for subsequent evaluation of the topography of the surface.

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