Effect of pulse duration on two-photon excited fluorescence and second harmonic generation in nonlinear optical microscopy

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Abstract. We have developed a multiphoton microscopy (MPM) system using a 12-fs Ti:sapphire laser with adjustable dispersion precompensation in order to examine the impact of pulse duration on nonlinear optical signals. The efficiencies of two-photon-excited fluorescence (TPEF) and second harmonic generation (SHG) were studied for various pulse durations, measured at the sample, ranging from ~400 fs to sub-20 fs. Both TPEF and SHG increased proportionally to the inverse of the pulse duration for the entire tested range. Because of improved signal-to-noise ratio, sub-20-fs pulses were used to enhance MPM imaging depth by approximately 160%, compared to 120-fs pulses, in human skin. © 2006 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2177676]

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In this paper, we report the development of a multiphoton microscopy system using a 12-fs titanium sapphire (Ti:sapphire) laser. Sub-20-fs pulses measured at the focal plane of the objective are achieved using a prism-pair dispersion compensator. The TPEF and SHG signals are found to increase inversely with pulse duration ($\tau_p$) when $\tau_p$ is reduced from 400 fs to below 20 fs. Approximately a 160% improvement in imaging depth for ex vivo human skin specimens is demonstrated for sub-20-fs versus 120-fs pulses (260 μm versus 160 μm, respectively).

The experimental setup is shown in Fig. 1. A 12-fs Ti:sapphire laser (Femtolasers) provides the excitation source. The center wavelength of the laser is 800 nm with a bandwidth of ~100 nm. The average laser output power is 500 mW at a 75-MHz repetition rate. The Ti:sapphire laser is pumped by a neodymium doped yttrium vanadate (Nd:YVO$_4$) laser (Coherent). The laser output from the Ti:sapphire first passes through a pair of fused silica Brewster prisms. The prism pair introduces negative dispersion to later compensate the positive material dispersion from the objective lens and other optics in the beam delivery path. The pulse duration is adjusted by varying the apex separation between the prisms. The dispersion precompensated beam from the prism pair is reflected backward but angled slightly to be separated from the input beam. Afterward, the laser beam is sent to two galvanometer mirrors for raster scanning. The scanned laser beam is ex-
panded by two lenses before entering the back entrance of the objective. A tightly focused laser beam is delivered to the sample by the objective.

The emitted TPEF and SHG signals from the sample are collected by the same objective lens in a backward direction. The emitted signals are separated from the excitation with a dichroic mirror (675DCSCP, Chroma). TPEF and SHG are further separated by a second dichroic mirror (475DCLP, Chroma) and selected by suitable band-pass filters (optical density larger than 6 to block excitation). In imaging mode, the separated TPEF and SHG signals are detected by two photomultiplier tubes, respectively. In spectral mode, the emitted signals are collected by a fiber bundle after the photomultiplier tubes. In imaging mode, the emitted TPEF and SHG signals from the sample are directly separated by a second dichroic mirror (675DCSCP, Chroma). TPEF and SHG are further separated by a second dichroic mirror (475DCLP, Chroma) and selected by suitable band-pass filters (optical density larger than 6 to block excitation). In imaging mode, the separated TPEF and SHG signals are detected by two photomultiplier tubes, respectively. In spectral mode, the emitted signals are collected by a fiber bundle after the photomultiplier tubes.

The emitted TPEF and SHG signals from rat-tail tendon in forward and backward detections, from a dilute fluorescein solution. The circles and triangles are for SHG signals obtained with the 400 fs and 40× objective. The squares are for TPEF signals and a freshly excised rat-tail tendon was used to generate SHG. Two Zeiss objective lenses, a 10× Plan-Neofluar (0.3 NA) and a water immersion 40× Achroplan (0.8 NA) were studied.

Figure 2 shows the intensities of the TPEF and SHG signals excited from samples with laser pulses of different durations from 400 fs to sub-20 fs measured by intensity autocorrelation. When the pulse duration was varied, the average laser power was maintained at a constant level. The SHG intensities from the rat-tail tendon were measured in both forward and backward propagation directions for the interest of studying the directionality of SHG generation. The SHG signals reported here are generally detected in backward direction if not otherwise specified.

Figure 2(a) shows the TPEF and SHG signals obtained with the 10× objective. As the pulse duration is shortened from 400 fs to below 20 fs, we see that the intensities of TPEF and SHG increase according to $\tau_p^{-0.85}$. TPEF and SHG measurements (both forward and backward SHG) showed very similar trends. Figure 2(b) shows that multiphoton signals obtained with the 40× objective are similar to those obtained with the 10× objective. These results demonstrate that significant improvement in excitation efficiency can be achieved by shortening the laser pulses. Such improvement continues as the pulse duration is shortened to the sub-20-fs regime. This result holds true for both high and low NA objectives. Therefore, theoretically the two-photon signals should be proportional to $\tau_p^{-1}$ if the average laser intensity is constant. Our experimental result ($\tau_p^{-0.85}$) agrees well with the theoretical prediction.

The significant increase in TPEF and SHG signal intensities using ultrashort laser pulse excitation has important im-

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**Fig. 1** Schematics of the experimental setup. The block in the dashed lines is inserted when interferometric autocorrelation is measured. BS: beam splitter; DM: dichroic mirror; F: filter; L: objective; P: prism; PMT: photomultiplier tube.

**Fig. 2** Intensities of TPEF and SHG versus pulse duration obtained with (a) 10× and (b) 40× objectives. The squares are for TPEF signals from dilute fluorescein solution. The circles and triangles are for SHG signals from rat-tail tendon in forward and backward detections, respectively.
applications for nonlinear microscopy. As the excitation efficiency is improved, we can potentially image deeper into thick tissues and increase frame rates.

In order to demonstrate this concept, we compared SHG and TPEF signals obtained from sub-20-fs and 120-fs excitation laser pulses in excised human skin. The specimen was kept frozen until the time of imaging. The intrinsic autofluorescence and SHG signals were captured with a spectrophotograph. The recorded spectra are shown in Fig. 3(a) when sub-20-fs excitation pulses are used. The solid, dashed, and dotted lines represent spectra obtained at the depths of 40, 80, and 120 μm, respectively, under the surface of the skin. The 10× objective was used in this experiment and multiphoton signals were obtained with 20-mW average excitation power on the specimen with 0.5-s integration time. TPEF and SHG signals are spectrally resolved with the TPEF emission centered around 500 nm and the SHG around 400 nm. Peak values of the TPEF and SHG spectra are plotted as a function of penetration depth in Fig. 3(c). The black and white bars represent the values of SHG and TPEF signals respectively. TPEF intensity peaks around 40 μm below the surface and is mainly derived from the epidermal cellular layer. This observation is confirmed by the TPEF image of tightly packed epithelial cells. SHG signals peak slightly deeper inside the tissue due to the fact that they are mainly derived from the collagen matrix in the dermis. This observation is supported by the corresponding SHG image of fibrous collagen connective tissue. The images are acquired with a 63× high magnification objective in the MPM system. For comparison, the experiment was repeated with the excitation pulse stretched to 120 fs. The corresponding results are reported in Figs. 3(b) and 3(d). Signal levels are much lower for the 120-fs source. Sub-20-fs pulses are able to acquire SHG and TPEF signals up to approximately 260 μm, nearly 160% deeper than 120-fs pulses.

When using ultrashort pulses to improve MPM efficiency by increasing the peak intensity, the influence from the broad bandwidth also needs to be considered. In TPEF generation, broad bandwidth would eventually reduce the efficiency of two-photon absorption if the laser spectrum becomes broader than the absorption window of the fluorophore. For SHG, broad bandwidth could make it difficult to phase-match all the spectral components. However, in practical terms, the phase matching condition is not critical for SHG interactions over the microscopy length scale (i.e., focal depths <20 μm). The main limitation to the use of increasingly short pulses is probably the efficiency of TPEF generation.

In conclusion, we have studied the effects of pulse duration on the generation of TPEF and SHG signals at the tight focusing of objectives in nonlinear optical microscopy. Both the TPEF and SHG efficiencies are found to increase proportionally to the inverse of the pulse duration even in the sub-20-fs regime. Practical advantages of using sub-20-fs pulses for multiphoton microscopy are demonstrated by increased tissue penetration and reduced integration times.

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References