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. [DOI: 10.1117/1.2177676]

Keywords: multiphoton microscopy; two-photon excited fluorescence; second harmonic generation; ultrashort pulse; femtosecond laser.

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Multiphoton microscopy, combining two-photon excited fluorescence (TPEF) and second harmonic generation (SHG), has become an important tool for high-resolution, noninvasive imaging of thick biological tissues.[1–3] However, the efficiencies of TPEF and SHG excitation are very low in biological tissues because they are both nonlinear processes. Tissues are also highly scattering, a phenomenon that causes light intensity to drop off rapidly with excitation depth. Therefore, it is of great interest to increase MPM imaging depth in tissues by optimizing the spatial, spectral, and temporal properties of laser sources.

Generally, femtosecond pulsed lasers are required in order to increase the peak intensity of excitation. The excitation efficiencies of TPEF and SHG both depend quadratically on the peak intensity of the excitation laser beam. Increasing the average laser intensity can improve the signal levels of TPEF and SHG. However, this can result in greater tissue damage and loss of cell viability. An alternate approach involves increasing peak laser power by reducing pulse duration while maintaining average intensity. McConnell and Riis observed a sevenfold increase in TPEF yield when laser pulses were compressed from 250 to 35 fs.[4] Xu and Webb examined the impact of pulse duration on TPEF and observed an inverse relationship for pulses longer than 90 fs.[5] For shorter pulses, they found significant deviation from the inverse relationship because the pulses were measured before the objective, which could not accurately represent the pulses at the focal plane due to the dispersion from the objective. For the same reason, the inverse dependence of SHG on pulse duration was demonstrated only for pulses longer than 500 fs.[5] Therefore, dispersion from beam delivery optics, especially objectives, is a major concern when using ultrashort pulses in multiphoton microscopy. Such dispersion can be precompensated by a pair of prisms or gratings. In Müller et al.,[6] optical pulses of 15-fs duration were achieved at the focal plane of high numerical aperture (NA) objectives and a significant increase in TPEF was observed.

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 (τ_p) when τ_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-

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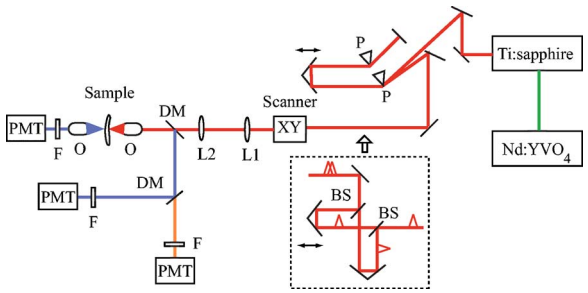

Fig. 1 Schematics of the experimental setup. The block in the dashed lines is inserted when interferometric autocorrelation is measured. BS: beam splitter; DM: dichroic mirror; F: filter; L: lens; O: objective; P: prism; PMT: photomultiplier tube.

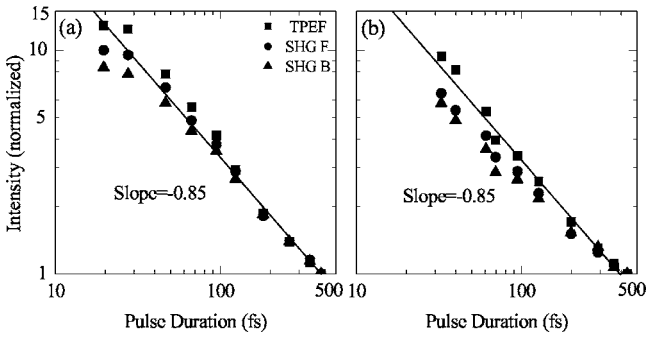

Fig. 2 Intensities of TPEF and SHG versus pulse duration obtained with (a) 10× and (b) 40× objectives. The squares are for TPEF signals from dilute fluorescein solution. The circles and triangles are for SHG signals from rat-tail tendon in forward and backward detections, respectively.

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 by a dichroic mirror (675DCSP, 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 675DCSP dichroic mirror and sent to a spectrograph (SpectraPro-150, Acton Research) with a grating of 300 grooves blazed at 500 nm, followed by a cooled CCD camera (NTE/CCD-512-EBFT, Princeton Instruments). In the setup, there is also an objective, which collects the forward SHG signal. The forward SHG is detected by a PMT after an SHG filter. The average laser power is measured with an 1835-C multifunction optical meter (Newport).

In order to understand the impact of pulse duration on MPM excitation, it was essential to accurately determine pulse duration at the sample location. We employed two approaches, based on autocorrelation methods, to measure laser pulse width. Because standard autocorrelators do not work using focused beams, we adapted a previously reported approach that can measure pulse width at the focus of a high NA, short working distance objective.[7] We compared this method to a second technique that we devised using a standard autocorrelator on the recollimated beam after the objective.

In the first approach, the delay lines for autocorrelation are inserted into the beam path before the objective where the beam is collimated. A nonlinear material (such as an SHG crystal) is placed at the focal plane and the 2nd order nonlinear signal (e.g., SHG) is detected by the PMT in the MPM system. Thus interferometric autocorrelation can be measured and pulse width characterized. However, interferometric autocorrelation is sensitive to chirping, which can introduce greater complexity in the interpretation of the measurement.[8] Consequently, we used a second method, based on intensity autocorrelation, by relocating lens 1 (L1) in Fig. 1 to a position after the objective so that the laser beam was recollimated and introduced into a standard autocorrelator (Femtolasers). No new optical components are introduced in this arrangement: L1 is simply relocated. As a result, this approach characterizes the impact of total system dispersion on pulse width at the sample. We found a close match between pulse durations measured with the interferometric and intensity autocorrelations when the dispersion was well compensated. However, for severely dispersed pulses that do not have standard pulse shapes and phase information, the intensity autocorrelation measurement was more reliable.

For the study of excitation efficiency versus pulse duration, a dilute fluorescein solution was used to generate 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 autocorrelations. 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 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 τ_p^{-1} if the average laser intensity is constant.[9] 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-

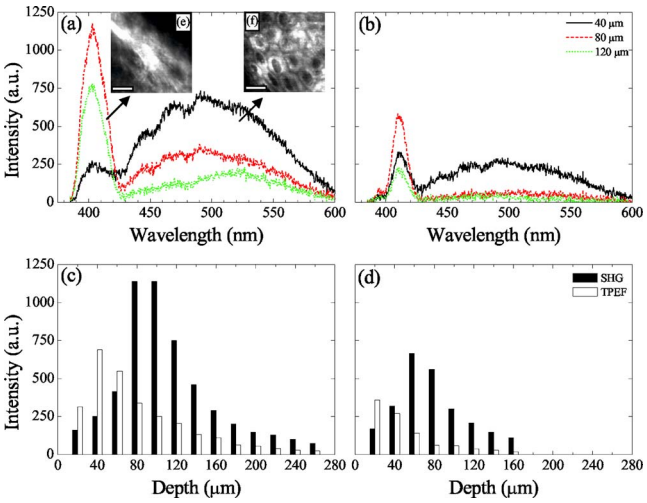

Fig. 3 TPEF and SHG spectra from human skin obtained at various depths excited with (a) sub-20-fs and (b) 120-fs pulses, respectively. Peak values of the TPEF and SHG spectra versus penetration depth obtained with corresponding (c) sub-20-fs and (d) 120-fs pulses. The insertions show the (f) TPEF and (e) SHG images of cellular and fibrous collagen structures at ~40-μm and ~80-μm depths, respectively. The scale bars are 10 μm.

plications 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 spectrograph. 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.

Acknowledgments

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