High-throughput multiphoton-induced three-dimensional ablation and imaging for biotissues

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Abstract: In this study, a temporal focusing-based high-throughput multiphoton-induced ablation system with axially-resolved widefield multiphoton excitation has been successfully applied to rapidly disrupt biotissues. Experimental results demonstrate that this technique features high efficiency for achieving large-area laser ablation without causing serious photothermal damage in non-ablated regions. Furthermore, the rate of tissue processing can reach around $1.6 \times 10^6 \mu m^3/s$ in chicken tendon. Moreover, the temporal focusing-based multiphoton system can be efficiently utilized in optical imaging through iterating high-throughput multiphoton-induced ablation machining followed by widefield optical sectioning; hence, it has the potential to obtain molecular images for a whole bio-specimen.

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OCIS codes: (140.3390) Laser materials processing; (170.1020) Ablation of tissue; (190.4180) Multiphoton processes.

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1. Introduction

Multiphoton excitation (MPE) fluorescence microscopy has become a widespread tool for biological imaging applications [1]. Additionally, second harmonic generation (SHG) can be employed to directly obtain non-centrosymmetric contour information in specimens, such as collagen within tissue [2]. Due to deeper penetration depth, significantly reduced off-focus photobleaching, and minimum invasion, MPE is not only effective in nonlinear optical imaging, but is also very useful in fabrication [3–5] and machining [6–21]. A short pulse width indicates a higher peak power at lower average energy, and can easily confine the reaction region in the focal spot via a high numerical aperture (NA) objective [13]. Therefore, this approach is suitable for biomedical micro/nano-processing ablation of biological materials and tissues [9–21]. In femtosecond laser machining of bio-tissues, the underlying mechanism can be classified into photothermal reaction and multiphoton-induced ablation. Generally speaking, the material simultaneously absorbs several photons to induce a nonlinear process under the femtosecond laser pulse excitation. This phenomenon is achieved by plasma-induced ablation. However, the photothermal reaction also damages adjacent regions, and generated heat in the surrounding areas must be controlled. Reducing thermal accumulation can be accomplished by decreasing the repetition rate [8] or enhancing the efficiency of multiphoton-induced ionization by the use of a shorter wavelength and pulse width ultrafast laser to improve machining quality [14]. Nevertheless, some disadvantages of the conventional multiphoton-induced ablation machining based on focal spot beam scanning still exist, including slow machining throughput at low peak power, and filamentation and self-focusing-induced focal spot shift at high peak power that affect precision in defining the machining area [22,23].

Recent studies have shown that temporal focusing techniques can provide widefield and axially-resolved multiphoton excited imaging [24–32]. The combination microscope typically consists of a diffraction grating, a collimating lens, and a high NA objective [24]. When the laser pulse impinges on the grating, spatial separation of the pulse spectrum occurs, which is collected by the collimating lens. The pulse spectrum then propagates along the optical axis, and is focused into the sample from different angles by an objective. Only in the focal plane do the different frequency components overlap in phase and produce a shorter, higher peak power pulse, allowing effective multiphoton excitation to occur [24,25,27–32]. Compared to conventional beam scanning multiphoton excitation microscopy, widefield multiphoton excitation microscopy with the temporal focusing technique detects the overall nonlinear optical signal of the entire illumination area, with detection controllable via the laser beam size and the magnification of the microscope [26]. One advantage of widefield microscopy is that less time is required to capture one frame, enabling a fast frame rate for capturing dynamic events [32]. Moreover, this technique can ablate bio-tissues without inducing the self-focusing problem [22,23]. In our previous study, a temporal focusing-based widefield multiphoton microscope was successfully applied to rapidly fabricate freeform polymer microstructures [33]. Herein, we also adopt the temporal focusing technique featuring widefield multiphoton excitation to achieve rapid disruption of bio-tissues. Experimental results of machining chicken tendon show that large-area ablation machining without dramatic photothermal damage in non-machining regions can be efficiently achieved with a machining speed of approximately $1.6 \times 10^6$ $\mu$m$^3$/s. This means that the ablation rate is 4 times faster than that performed by conventional focusing multiphoton-induced ablation machining [21]. Further, we acquired SHG images by the temporal focusing technique. Preliminary results demonstrate that the temporal focusing technique is suitable for all-optical histology via alternatively iterating high-throughput ablation machining and imaging to obtain optical images within the biological specimen.
2. Optical setup of high-throughput multiphoton-induced ablation system

2.1. Overall setup

Figure 1 illustrates a schematic of the developed temporal focusing-based high-throughput multiphoton-induced ablation micromachining system featuring widefield multiphoton excitation [32,33]. Key components include an ultrafast amplifier (Spitfire Pro., Newport, USA), an ultrafast oscillator (Tsunami, Spectra-Physics, USA) as the seed beam of the amplifier, an upright optical microscope (Axio imager 2, Carl Zeiss, Germany), a triple-axis sample positioning stage (H101A ProScan™, Prior, UK), an EMCCD camera (iXon 885 EMCCD, Andor, UK), and a data acquisition (DAQ) card with a field-programmable gate array (FPGA) module (PCI-7831R, National Instruments, USA). The ultrafast amplifier has a peak power of 400 μJ/pulse, a pulse width of ~100 fs, and a repetition rate of 10 kHz. The center wavelength can be adjusted from 750 to 850 nm and the output beam diameter is about 12 mm. A half-wave plate and a polarizer were used to adjust the polarization and power of the amplifier. The beam is spatially dispersed via a blazed grating with a groove density of 1200 lines/mm, the incident angle of which is adjusted to ensure the central frequency follows the optical axis and propagates through the 4f setup, comprised of the collimating lens and objective lens (Plan-APOCHROMAT 20X/ NA 0.8, Carl Zeiss, Germany). In addition, the system is equipped with both bright field and widefield multiphoton excitation optics, so bright-field and nonlinear optical images can be acquired during the micromachining process. For nonlinear imaging onto the EMCCD camera, the collected signal passes through a dichroic mirror and a short-pass filter to remove the excitation light. By controlling the motorized stage in the z-axis via the FPGA, sequential sectioning images at different depths can be obtained, and then assembled to reconstruct a three-dimensional (3D) image. To measure the pulse width of the laser beam on the sample, the incident beam was guided into a Michelson-interferometry-based autocorrelator by one flip mirror; then, the interference beam was reflected onto the sample by a second flip mirror [27].

![Fig. 1. Optical setup of the temporal focusing-based high-throughput multiphoton-induced ablation micromachining system.](image)

2.2. System calibration

Two key parameters were calibrated: the pulse width of the laser beam on the sample and the excitation volume. To verify both key parameters, a R6G fluorescence thin film with a thickness of less than 200 nm was employed. A shorter pulse width on the sample provides superior efficiency of multiphoton-induced ablation. The pulse width is primarily broadened
by the group velocity dispersion of the ultrafast amplifier due to the objective lens, collimating lens, and other refractive elements, as well as the sample itself [34]. To correct this effect, the amplifier’s built-in prism pair can be adjusted to the optimal pulse width by measuring the pulse width of the amplifier on the R6G fluorescence thin film. The experimental results show that a minimum pulse width of approximately 113 fs can be obtained for the spectral bandwidth of ~8.5 nm at the central wavelength of 750 nm (USB2000+, Ocean Optics, USA). The time-bandwidth product was 0.51, which is near the 0.44 Gaussian pulse transform limit with the same spectral bandwidth corresponding to a 97 fs pulse width. Consequently, the pulse width of the system is near the optimum.

Based on the current geometry and magnification of the system, the illuminated spot was an ellipse with a major axis of 60 μm and minor axis of 40 μm, as observed by the two-photon excitation fluorescence (TPEF) image of the fluorescent thin film. The temporal focusing with axial-resolved capability can provide optical sectioning for high-throughput multiphoton machining and widefield imaging. To measure the excitation volume in the axial direction (or axial spatial resolution), the fluorescent thin film was axially scanned with a range of 40 μm at a step size of 1.0 μm by controlling the motorized stage. The TPEF intensity as a function of the scanning depth has a maximum intensity in the focal plane. The axial excitation volume in full width at half maximum (FWHM) was around 3.0 μm under the current system setup. In our previous study, the lateral and axial spatial resolutions of the temporal focusing were improved through second-order nonlinear structured-illumination microscopy (NSIM) from 397 nm to 168 nm (2.4-fold) and from 2.33 μm to 1.22 μm (1.9-fold), respectively, in FWHM [35]. A higher NA and magnification oil-immersed objective (EC Plan-NEOFLUAR, 40X/NA 1.3, Carl Zeiss, Germany) were also used in this present study with similar pulse width and axial resolution.

3. Experimental results and discussions

3.1. High-throughput multiphoton-induced ablation machining parameters

Plasma-induced ablation is beneficial for the removal of bio-tissue without photothermal damage. In order to induce ablation, the photon energy must be greater than the chemical bonding energy to directly break the chemical bonds of machined samples, thereby generating plasma. For this use of multiphoton-induced ablation, machined samples absorb photons that break chemical bonds; consequently, it could be predicted that machining efficiency at shorter wavelengths would be better than that at longer wavelengths. In comparing the excitation wavelengths of 750 and 830 nm under the same machining conditions, results indicated that the average energy fluence per pulse at the 750 nm excitation wavelength was lower. As observed from the TPEF image of thin fluorescent film, the intensity profile was that of a Gaussian distribution without any beam shaping. This could lead to Gaussian-like machining efficiency in the illuminated area. Therefore, it is clear that the machining efficiency in the central illuminated area is higher than that at the edges.

To investigate the high-throughput ablation effect in this study’s setup, the surface of a ~100 μm thick chicken tendon was placed on the focal plane of the objective lens. The average energy fluence per pulse was set to 1.2 J/cm² according to our previous experience [3] and the machining direction was along the minor axis of the pulsing laser spot with a scanning speed of 3.0 mm/sec. The SHG images of the machined chicken tendon were captured by a home-built, point-scanning multiphoton microscope with a laser source of the ultrafast oscillator [3]. The excitation wavelength of the SHG signal was 750 nm with relatively low energy fluence per pulse of 28.4 mJ/cm² to avoid ablating non-targeted regions. The image size was 150 × 150 μm², which corresponds to 256 × 256 pixels with a scan rate of 20 kHz and layer-by-layer interval of 1.0 μm. After ablation, SHG images of the machined chicken tendon were acquired by axial scanning from the machining surface to the sample’s interior, at 1.0 μm interval. Figure 2(a) shows the SHG images at the depths of 0, 15, 45, and 75 μm.
(left to right) from the machined surface. Further, shown in Fig. 2(b) is the cross-sectional view of the 3D rendered SHG image at the layer-by-layer interval of 1.0 μm. As can be seen, the top of the image shows that an elliptical cut was produced by the Gaussian-like machining efficiency, with a central machined depth of around 15μm. However, the SHG signal below the elliptical cut is missing, indicating the fact that the sample below the machining region may be damaged by the self-focusing effect at high peak power or that the SHG signal could not be excited or detected. To confirm this observation, the sample was overturned so that whatever had been preventing SHG signal collection beneath the machined region could be decreased or eliminated. Figure 2(c) shows the SHG images at the same defined depths of 0, 15, 45, and 75 μm (left to right) as those of Fig. 2(a). Figure 2(d) shows the cross-section of the 3D rendered SHG image at the layer-by-layer interval of 1.0 μm, corresponding to Fig. 2(c). In Fig. 2(d), the machining depth at the center area can be verified as being approximately 15 μm; hence, the self-focusing photothermal damage was prevented occur in undesired machining regions. A comparison of the SHG images in Figs. 2(b) and 2(d) indicates that the main difference is found in the region below the machining area. A possible explanation for the missing SHG signal below the machined region in Figs. 2(a) and 2(b) could be due to the fact that the refractive index mismatch in the machined region blocked the laser energy from penetrating the area below the machined curve and exciting the SHG signal. In Fig. 2(e), the only imaging condition that differed from Fig. 2(a) was that index matching oil was injected into the machining area to reduce the index mismatch effect. After this correction, the chicken tendon was turned over and imaged again. Figure 2(f) shows the cross-section of the 3D rendered SHG image corresponding to Fig. 2(e). The SHG signal below the machined region in Figs. 2(e) and 2(f) can now be observed. Accordingly, the experimental results demonstrate that the high-throughput multiphoton-induced ablation machining system can provide high efficiency to rapidly disrupt bio-samples without inducing photothermal damage outside the targeted region. The machined volume rate can be estimated from the 3D rendered SHG image to be $1.6 \times 10^6 \mu m^3/s$ based.

![Fig. 2.](image_url)
As a point of interest, the machining width and depth were also estimated, with the width being around 50 μm, which is close to the square root of 2/3 of the illuminated spot with the major axis of 60 μm. In other words, the effective spot of three-photon excitation was about 49 μm, which implies that the high-throughput multiphoton-induced ablation machining can be attributed to three-photon absorption. Furthermore, the machining depth was deeper than the excitation volume in the axial-direction by around 3.0 μm. However, based on three-photon absorption, the machining depth was predicted to be around 2.5 μm. Plasma-induced ablation occurs at a power density of between 10^{11} and 10^{13} W/cm^2 [36]; and in this setup, the power density was around 10^{13} W/cm^2 for a pulse width of 120 fs and 10^{12} W/cm^2 for pulse width of 500 fs. Therefore, the machining depth could be attributed to the pulse width. In other words, the pulse width is broadened as the defocus distance in the temporal focusing technique is increased. More specifically, the pulse width at a defocus of 20 μm was measured at around 500 fs by the Michelson-interferometry-based autocorrelator in Fig. 1. Moreover, the current machining depth is acceptable and the high-throughput multiphoton-induced ablation machining is attributable to the plasma-induced ablation effect. Consequently, the machined line has a 50 μm width and 15 μm depth.

3.2. Large-area ablation machining

As discussed above, the experimental results demonstrate that the high-throughput multiphoton-induced ablation micromachining system can rapidly disrupt bio-tissue without causing serious photothermal damage in non-machining regions. In this section, chicken tendon with SHG signal is examined again. Herein, the average energy fluence per pulse was increased to 1.6 J/cm^2 and the scanning speed reduced to 0.5 mm/sec. The line-to-line interval was initially 30.0 μm. In order to obtain superior roughness and uniformity on the machined surface for further imaging, each ablation region was machined three times. The imaging conditions were the same as those in Figs. 2(e) and 2(f) with injected index matching oil, but the image size was extended to 500 × 500 μm^2. The layer-by-layer imaging interval was also 1.0 μm. Figure 3(a) shows the acquired SHG images at the depths of 10, 20, 40, 60, and 70 μm from left to right, while Fig. 3(b) shows the cross-section of the 3D rendered SHG image from Media 1. Through observation of the SHG image at 20 μm from Fig. 3(b), a periodic stripe on the machined surface and shadow below the machined region was observed. Moreover, these SHG images reveal that the machined surface was not smooth, and that the periodic stripe was due to the discordant energy fluence of the machining laser beam. The machining laser beam had a Gaussian-like shape with an effective machining width of around 50 μm. Therefore, to improve the machining uniformity, the line-to-line interval was reduced to smaller than half of the effective machining width, yielding a line-to-line interval of 20.0 μm. Experimental results are given in Figs. 3(c) and 3(d), with the latter offering the cross-section of the 3D rendered SHG image from Media 2. Clearly, the periodic stripe was eliminated and the shadow below the machined region appears only at the edges corresponding to the whole machined area. Figure 3(d) demonstrates that the collagen fibrils of the chicken tendon after ablation can still be observed from the SHG image, and that a smooth machined surface is produced. Furthermore, some non-photothermal modifications, such as mechanical deformation resulting from shockwaves, are likely involved in plasma-induced ablation processing. However, such non-photothermal modifications cannot be clearly isolated in the current system setup. Nevertheless, the experimental results validate that the high-throughput multiphoton-induced ablation micromachining system is suitable for large-area ablation machining without causing photothermal damage outside the targeted region.
3.3. Widefield SHG images of machined chicken tendon

Temporal focusing-based multiphoton-induced ablation with sectioning capability has been successfully applied to rapidly disrupt large-area bio-tissues. The temporal focusing technique can also quickly acquire widefield nonlinear optical images [32]. Here, the SHG images of machined chicken tendon were acquired by illuminating the ultrafast amplifier at the same wavelength of 750 nm at the high frame rate of 50 frames/sec, as shown in Fig. 1. The excited SHG signals were collected by an EMCCD camera via a dichroic mirror and a 680 nm short-pass filter. It should be noted that the EMCCD chip was cooled to $-90 \degree C$ to reduce the noise level. Figure 4 shows that a chicken tendon after surface laser ablation can be imaged down to the energy fluence per pulse of $30.0 \text{ mJ/cm}^2$, i.e. $<3\%$ of the machining energy fluence. The illumination area was approximately $60 \times 40 \mu m^2$, which relates to $190 \times 108$ pixels. The sample was then axially scanned with a range of $40 \mu m$ and a step size of $1.0 \mu m$ by controlling the piezo-motorized stage, yielding an overall acquisition time for the 3D rendered SHG image of less than 1 second. Figure 4(a) shows SHG images at the depths of 10, 15, 20, and $25 \mu m$ (left to right) without injected index matching oil, while Fig. 4(b) offers the cross-section of the 3D rendered SHG image from Media 3. Although the machined features can be recognized by employing temporal focusing-based widefield microscopy, compared with the SHG images by scanning multiphoton microscopy, the widefield image is blurred. Because the spatial resolution and the correction of the emission nonlinear signal for the widefield image encountered serious interference generated by the scattering effect via the turbid bio-sample and then further degradation under the two-dimensional area camera approach [37], the submicron-level collagen fibrils of the chicken tendon were too blurry to be observed by the fast, one-shot widefield SHG imaging approach [32]. In our previous studies, the lateral
and axial spatial resolutions and the imaging depth of temporal focusing were improved through NSIM [35] and wavefront sensorless adaptive optics (AO) [34], respectively. However, the frame rate under these two enhancement approaches is dramatically slowed. Furthermore, it is clear that the increased SHG signal appears at the ablation border. We consider that this phenomenon is the same as that in Fig. 2(b). The refractive index mismatch at the ablation border induced a strong spherical aberration to reduce the excitation efficiency at the region below the ablation border and resulted in a relatively bright SHG signal at the ablation border. Although the temporal focusing technique for widefield imaging has some drawbacks, its inherent high-throughput capability could be efficiently utilized in optical histological imaging by iterating multiphoton-induced ablation and imaging to obtain whole optical molecular images for thick bio-specimens.

Fig. 4. SHG images of chicken tendon via temporal focusing-based widefield multiphoton microscopy. (a) Depths are 10, 15, 20 and 25 μm from left to right, (b) projection image from the 3D reconstructed image of (a) (Media 3).

4. Conclusion

A temporal focusing-based high-throughput multiphoton-induced ablation machining system offering axially-resolved widefield multiphoton excitation has been successfully applied to rapidly disrupt chicken tendon and provide corresponding SHG images. High efficiency, large-area laser ablation machining of chicken tendon without inducing serious photothermal damage in non-targeted regions was achieved with a machined volume rate of approximately $1.6 \times 10^6 \mu m^3/s$. Although the submicron-level collagen fibrils of chicken tendon were too blurry to be observed by fast one-shot widefield SHG imaging, imaging improvement approaches such as NSIM and wavefront sensorless AO could be integrated with the system in the future. Moreover, the temporal focusing technique with inherent high-throughput capability could be efficiently utilized in all-optical histology by iterating multiphoton-induced ablation and imaging; hence, the temporal focusing technique has the potential to obtain optical images for an entire bio-specimen.

Acknowledgments

Shean-Jen Chen acknowledges support from MOST 101-2221-E-006-212-MY3 and MOST 101-2221-E-006-213-MY3.