Implementation of spatial overlap modulation nonlinear optical microscopy using an electro-optic deflector

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Abstract: A spatial overlap modulation (SPOM) technique is a nonlinear optical microscopy technique which enhances the three-dimensional spatial resolution and rejects the out-of-focus background limiting the imaging depth inside a highly scattering sample. Here, we report on the implementation of SPOM in which beam pointing modulation is achieved by an electro-optic deflector. The modulation and demodulation frequencies are enhanced to 200 kHz and 400 kHz, respectively, resulting in a 200-fold enhancement compared with the previously reported system. The resolution enhancement and suppression of the out-of-focus background are demonstrated by sum-frequency-generation imaging of pounded granulated sugar and deep imaging of fluorescent beads in a tissue-like phantom, respectively.

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1. Introduction
Nonlinear optical microscopy based on a variety of nonlinear optical techniques, such as two-photon excitation fluorescence (TPEF) [1–3], second-harmonic generation (SHG) [4], third-harmonic generation [5, 6], four-wave mixing [7–10], two-photon absorption (TPA) [11, 12], stimulated Raman scattering (SRS) [13–15] and cross-phase modulation [16, 17], has been developed for applications in physics, chemistry, and biology. Nonlinear optical microscopy offers several advantages over linear optical microscopy, which include three-dimensional resolution without a confocal pinhole, high penetration depth with near-infrared light excitation, less out-of-focus photon-induced damage and photobleaching. In particular, multi-photon excited fluorescence microscopy benefits from the ability to image deeper inside samples than confocal fluorescence microscopy when near-IR excitation that allows maximum optical transparency in biological systems is employed [18–22]. Nonetheless, deep imaging is intrinsically difficult because the excitation light is attenuated by scattering and absorption in the sample. To maintain sufficient excitation intensity at the focus at significant depths in scattering media, the energy of the excitation pulse must be increased. A regenerative amplifier producing 150-fs pulses at the μJ level has been used to image green-
fluorescent-protein-labelled neurons at a depth of up to 1 mm within the neocortex [18]. However, increasing the pulse energy causes an increase in background signals, which include fluorescent signals generated in the out-of-focus regions. This background limits the maximum imaging depth inside a highly scattering sample [19].

Extensive efforts have been made to suppress out-of-focus signals [20–29]. A decrease in scattering of the excitation light in the sample, which was achieved by using TPEF at 1280 nm [20, 21] and three-photon excited fluorescence at 1700 nm [22], has extended the maximum imaging depth. Temporal focusing suppresses out-of-focus signals in wide-field TPEF microscopy [23, 24]. Structured illumination microscopy [25], focal modulation microscopy [26] and differential aberration imaging techniques [27], which employ spatial intensity modulation near the focal point, provide out-of-focus background rejection. Adaptive optics can be used to recover diffraction-limited performance by compensating for wavefront distortion and have been applied to TPEF imaging [28]. The suppression of the background fluorescence in TPEF microscopy has been also achieved by utilizing photoactivatable fluorophores, which remain in a non-fluorescent state until optically triggered [29]. This technique is similar to cyclic-sequential-multiphoton excitation microscopy using reversible photoswitchable fluorophores [30].

Recently, we have developed a spatial overlap modulation (SPOM) technique which enables the rejection of the out-of-focus signals and enhances the three-dimensional spatial resolution [31]. In SPOM nonlinear optical microscopy (SPOM-NOM), the spatial overlap between two color pulses is temporally modulated by means of beam pointing modulation or wavefront modulation, and nonlinear optical processes excited by a combination of two-color pulses are used. The generated nonlinear signal is temporally modulated by the SPOM and the modulation depth of the spatial overlap in the focal region is much greater than those in out-of-focus regions. Thus, by extracting the modulated nonlinear signals selectively, the out-of-focus background is well rejected. In addition, the frequency dependence of the nonlinear signal varies from the center to the edge of the irradiated region. The frequency at the center is an even-multiple of the modulation frequency of the SPOM whereas at the edge is an odd-multiple of the modulation frequency. Therefore, the spatial resolution is enhanced by demodulating the even-multiple components of the modulation frequency through a lock-in amplifier. However, the image acquisition time of the previous reported system was 1000 times longer than that of typical TPEF microscopy because the modulation frequency of SPOM was limited to 1 kHz by the frequency response of the galvano-scanner for beam pointing modulation. In this article, we describe the implementation of SPOM in which beam pointing is modulated at a frequency of 200 kHz by an electro-optic deflector (EOD) and nonlinear signals are demodulated at a frequency of 400 kHz.

2. SPOM-NOM using an EOD

A system implemented for improving the image acquisition time is illustrated in Fig. 1. We used a custom-built nonlinear microscope with a mode-locked Ti:sapphire oscillator (Coherent Mira-900F) and an optical parametric oscillator (OPO, Coherent Mira-OPO-IR-FAN) operating at a repetition rate of 76 MHz. The center wavelength of the Ti:sapphire oscillator and the OPO were 830 nm and 1100 nm, respectively. To compensate for the dispersion of all the optical components before the focal point of the microscope objective (OB), the pulses passed through an SF10 prism pair (PC1) after the Ti:sapphire oscillator, and an S-TIH6 prism pair and six-bounce chirped mirror (PC2) after the OPO. The two color pulses from the oscillators were overlapped in time using an optical delay line and overlapped in space by the first dichroic mirror (DM1). The pulses were focused into the samples by a silicone immersion objective lens (OB, Olympus UPLSAPO30 × S having a numerical aperture of NA = 1.05) for TPEF imaging or an objective lens (Olympus, UPLSAPO40 × , NA 0.9) for sum-frequency generation (SFG) imaging. The spatial overlap between the two-color pulses was modulated at a frequency of 200 kHz by modulating the beam pointing of the OPO pulse using a potassium, tantalum, niobium, and oxygen (KTN) crystal electro-optic deflector (EOD, NTT Advanced Technology KSCT00RK00-00). The generated nonlinear
signals were collected by the objective lens and were separated from the two excitation pulses by using the second DM (DM2, Semrock FF705-Di02-25x36). The nonlinear signals were detected with a photomultiplier tube (PMT) (Hamamatsu Photonics H7422A-40) through a lock-in amplifier (Stanford Research Systems SR844) at a demodulation frequency of 400 kHz. In order to compare signal-to-noise ratio (SNR) at a demodulation frequency of 400 kHz with that at 2 kHz, which corresponds to the demodulation frequency of the previous setup [31], the spatial overlap between the two-color pulses was modulated at a frequency of 1 kHz by using the EOD and the signal was detected through a lock-in amplifier (Stanford Research Systems SR830) at a demodulation frequency of 2 kHz. By scanning the laser beams in the lateral (x, y) directions with a two-axis galvano scanner (Cambridge Technology 6210H) and scanning the sample in the axial (z) direction with a stepping-motor-driven stage, three-dimensional images were obtained with a pixel dwell time of 30 μs or 100 μs. The EOD plane was projected onto the galvano scanner whose plane in turn was projected onto the back aperture of the objective lens. To exclude the residual excitation pulses, short-pass filters (SPF, Semrock FF01-720/SP-25 and Semrock FF01-770/SP-25) were placed in front of the PMT. Band-pass filters (BPF, Semrock FF01-520/35-25 for TPEF; Semrock FF01-475/35-25 for SFG) were also placed in front of the PMT to select the detection wavelength.

Fig. 1. SPOM-NOM setup. PBS: polarizing beam splitter, OPO: optical parametric oscillator, PC: prechirper, EOD: electro-optic deflector, DM: dichroic mirror, OB: objective lens, SPF: shortpass filter, BPF: band-pass filter, PMT: photomultiplier tube.

3. Sample preparation

To demonstrate the background suppression by SPOM-NOM, we prepared a tissue-like phantom as a highly scattering sample. We added 45 μL of a yellow-green (505/515) fluorescent polystyrene bead solution (Molecular Probes F8877) with a concentration of 4.55 × 10⁹ beads/mL to 160 μL of low-melting-point agarose gel, then pipetted it onto a 35-mm Petri dish with a cover glass bottom and covered it with another cover glass. The phantom contained fluorescent polystyrene beads with a diameter of 2 μm at a concentration of 1.0 × 10⁸ beads/mL as scatterers and tracers. To demonstrate the resolution enhancement by SPOM-NOM, we also prepared samples for SFG imaging. We pounded granulated sugar in a mortar and sandwiched it between a glass slide and a cover glass.

4. Results and discussion

We investigated SNRs at demodulation frequencies of 400 kHz and 2 kHz by changing the generated signal intensity. As the signal, we used the SFG signal from a 10-μm-thick β-barium borate (BBO) crystal. The SHG signal generated only by either the Ti:sapphire laser pulse or the OPO pulse was not removed by the BPF but was employed as the background. The integration time of the lock-in amplifier was set to 3 ms. As shown in Fig. 2, we can see that SNR at 400 kHz is improved at the higher signal level by 10 dB compared with that at 2 kHz. At the higher signal level and the lower frequency, the laser 1/f noise dominates over other noise sources. The improved SNR is attributed to the reduction of the laser 1/f noise because of the increase of the demodulation frequency. By increasing the demodulation frequency to the MHz range, the SNR could be more improved.
We applied SPOM-NOM to deep TPEF imaging of fluorescent beads in the tissue-like phantom. Figures 3(a) and 3(b) respectively show the images observed by conventional TPEF microscopy and SPOM-NOM with a maximum focus displacement of 400 nm in the lateral (y) direction, which corresponds to the modulation depth of 55%. The left panels in Fig. 3 show the three-dimensional TPEF images, which were reconstructed from 431 and 571 xy images (128 × 128 pixels) obtained at depth increments of 1 μm by conventional TPEF microscopy and SPOM-NOM, respectively. The center and right panels in Fig. 3 show a maximum-intensity y projection of the image stacks and the xy images at various penetration depths, respectively. This result is similar to that obtained with the previous setup at a demodulation frequency of 2 kHz [31]. Compared with images obtained by the conventional technique, we found that the background TPEF was dramatically suppressed by the SPOM technique. Thus, the imaging depth could be extended by the SPOM technique. However, since we could not maintain sufficient excitation intensity at the focus when the penetration depth was beyond 530 μm, signals from this depth or more were insufficient. The input power at each penetration depth for conventional TPEF microscopy and SPOM-NOM are depicted in Figs. 4(a) and 4(b), respectively. The input power from the Ti:sapphire laser and the OPO was the same. The input power from the Ti:sapphire oscillator and the OPO at the sample was 100 μW. Since the laser power at the focal point for deep TPEF imaging decays as \( \exp(-\alpha z) \) due to absorption and scattering, the laser power was increased by \( \exp(\alpha z) \) to keep a constant TPEF intensity at the focal point. Here \( z \) is the penetration depth. The compensation coefficient \( \alpha \) for the losses of the laser power was set to 0.013 μm\(^{-1}\). The input power from the Ti:sapphire oscillator and the OPO at the penetration depth of 530 μm reached the maximum power that can be available. Therefore, the input power could not be increased at the penetration depth beyond 530 μm.

To quantify the background suppression, the TPEF intensity of the beads in the focal plane (signal) and the background TPEF intensity are plotted as a function of penetration depth in Figs. 4(a) and 4(b), and the contrast ratio between the signal and background intensities is plotted as a function of penetration depth in Fig. 4(c). We obtained the signal and background intensities by taking the averages of TPEF intensities at 49-neighboring pixels (7 × 7 pixels) around the center of a bead, and at 49-neighboring pixels (7 × 7 pixels) around a dark pixel in each image, respectively. We found that even though the penetration depth is increased, the signal intensity in the focal region can be maintained by compensating for the reduced excitation intensity at the focus by increasing the excitation power at the sample surface. On the other hand, the background TPEF intensity increased with increasing
Fig. 3. TPEF images of tissue-like phantom obtained by (a) conventional TPEF microscopy and (b) SPOM-NOM.
penetration depth. As a result, the signal-to-background ratio (SBR) decreased with increasing penetration depth by a factor of $e^{-az}$. However, the rate of increase of the background TPEF intensity is reduced by the SPOM technique. Thus, the rate of decrease of the SBR is lower using the SPOM technique. We performed a least squares fit to the SBR, $S/B$, using the function $\log(S/B) = -az + b$. We obtained $a = 0.00597 \, \mu m^{-1}$ and $b = 2.41$ for conventional TPEF microscopy, and $a = 0.00291 \, \mu m^{-1}$ and $b = 2.86$ for SPOM-NOM. This result represents that the rate of decrease of the SBR of the SPOM-NOM can be improved by a factor of 2.1. The demodulated signal intensity in SPOM-NOM is proportional to the square of the maximum focus displacement [31]. Therefore, by increasing the maximum focus displacement, the SBR can be more improved.

To demonstrate the resolution enhancement by SPOM-NOM, we acquired SFG images of pounded sugar. The input power from the Ti:sapphire oscillator and the OPO was 1 mW. The full results for the stack of images in the $yz$ plane can be seen in an online movie (Media 1) in the supplementary material. Figures 5(a) and 5(b) respectively show single frames from Media 1 for conventional SFG microscopy and SPOM-NOM with a maximum focus displacement of 200 nm in the lateral ($y$) direction, which corresponds to the modulation depth of 21%. Because we can achieve a sufficiently high enhancement of the spatial resolution by setting the maximum focus displacement to half the radius of the focal spot [31], we adjusted the maximum focus displacement to 200 nm. The movie was reconstructed from 41 $xy$ images ($128 \times 128$ pixels) obtained at depth increments of 0.5 $\mu m$. Figures 5(c)-5(g) illustrate the normalized signal profiles along the lateral ($y$) and axial ($z$) directions indicated by blue, green, red, yellow, and pink arrows in Figs. 5(a) and 5(b). From the profiles in Figs. 5(c)-5(g), we can observe that smaller structures are clearly visible and distinctive from each other in the SPOM-NOM image, and that the background is significantly reduced. This result indicates that our SPOM-NOM system can provide better lateral and axial resolutions, and higher image contrast.
5. Conclusions

We have developed and experimentally demonstrated SPOM-NOM implemented with an EOD to provide the enhancement of the spatial resolution and a suppression of the out-of-focus background. SPOM-NOM extends the achievable imaging depth inside a highly scattering sample. Compared with our previous setup, the imaging speed for SPOM-NOM implemented with the EOD has been improved by 200 times. Further improvements are still necessary, especially in shortening the pixel dwell time. In the present study, the pixel dwell time was limited to 30 μs/pixel because the modulation frequency was limited by the frequency response of the power-supply circuit controlling the EOD. The KTN crystal used in the EOD has a length of 4 mm and a capacitance as large as 2 nF, which requires currents of 1 A for 1-MHz, 100-V operation and 10 A for 10-MHz, 100-V operation. Since the applied voltage and the modulation frequency are high, the required current becomes too high. The beam deflection angle is proportional to the product of the interaction length in the KTN crystal and the applied voltage [32]. Thus, by increasing the interaction length in the KTN crystal, a sufficient amount of beam deflection could be achieved using a lower applied voltage. In practice, multi-pass scheme tends to provide a superior result to that obtained using a longer crystal [33]. By employing a 5-pass EOD, the applied voltage can be reduced by a factor of 5, which corresponds to the required current of 0.2 A for 1-MHz, 20-V operation and 2 A for 10-MHz, 20-V operation. These currents can be supplied with a commercial power-supply circuit. This improvement would allow real-time imaging of thick biological tissues in vivo.

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