Simultaneous imaging of two-photon absorption and stimulated Raman scattering by spatial overlap modulation nonlinear optical microscopy

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Abstract: Imaging of simultaneous two-photon absorption and stimulated Raman scattering is accomplished by detecting the intensity changes of the two-color pulses simultaneously and the mathematical operations of addition and subtraction. The stimulated Raman scattering is quantitatively separated from the two-photon absorption, generated in a mixed solution in which a glycerin solution is miscible in various proportions with a quantum dot solution. Our technique is applied to simultaneous two-photon absorption and stimulated Raman scattering imaging.

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1. Introduction

A variety of nonlinear optical microscopy technologies have been developed for applications in physics, chemistry, and biology [1–15]. In nonlinear optical microscopy based on generation of photons at a new frequency, induced by two-photon excitation fluorescence (TPEF) [1–3], second-harmonic generation [4], third-harmonic generation [5, 6], or four-wave mixing [7, 8], the signal wavelengths vary according to the nonlinear optical processes. One signal can therefore be easily separated from the other signals by selecting the detection wavelengths [16]. On the other hand, for nonlinear optical microscopy based on the detection of intensity changes of excitation pulses induced by two-photon absorption (TPA) [9, 10], stimulated Raman scattering (SRS) [11–13], or cross-phase modulation (XPM) [14, 15], the signal wavelengths do not depend on the nonlinear processes. In the case of a small intensity change in the excitation pulse, the loss or gain in TPA, SRS, or XPM has to be discriminated from the laser 1/f noise. That noise can be reduced by modulating the intensity of one of the two-color pulses at a high frequency and measuring the modulation transfer to the other pulse [17]. By adjusting the time delay between the two-color pulses to zero, the XPM signal can be suppressed [15]. For phase sensitive detection using a lock-in amplifier, the loss and gain in SRS for vibrational modes respectively produce a positive signal in loss imaging and a positive signal in gain imaging, whereas the losses in TPA from purely absorptive samples give a positive signal in loss imaging and a negative signal in gain imaging. Therefore, the loss in TPA from absorptive samples can be discriminated from the gain in SRS from vibrational samples by phase-sensitive detection, but it is difficult to separate the loss in TPA from that in SRS at the same phase. Furthermore, in the case of samples where absorptive molecules are mixed in with vibrational molecules, it is hard to separate the loss in TPA from the gain in SRS. One signal may be the background when imaging the other signal. When the magnitude of the intensity change by TPA is equal to that by SRS, the overall change is zero due to the destructive superposition of the loss in TPA and the gain in SRS. In Raman imaging of biological samples, the molecules are identified from a comparison of the Raman and fluorescence images of the samples labeled by fluorophores [18]. Extensive efforts have been made to separate SRS from TPA [19, 20]. Differences in spectral response [19] or temporal response [20] can be used for this purpose. By measuring the dependence of the intensity change on the excitation wavelength and unmixing their spectra, SRS can be distinguished from TPA [19]. On the other hand, by measuring the dependence of the intensity change on the time delay between the two-color pulses and using a phasor analysis,
which is a powerful approach in fluorescence lifetime imaging microscopy [21–23], the complex loss or gain signals can be determined [20].

Here we propose a new concept to separate TPA from SRS. It only requires detecting the intensity changes of the two-color pulses simultaneously and the mathematical operations of addition and subtraction. By modulating the excitation intensities of the two-color pulses at different frequencies, the intensity changes of the two pulses induced by TPA and SRS can be simultaneously demodulated at the sum and difference frequencies. Instead of the intensity modulation technique, the spatial overlap modulation (SPOM) technique, recently developed to suppress the out-of-focus background and enhance the spatial resolution in nonlinear optical microscopy [24], can be used. The intensity changes of the two-color pulses induced by TPA and SRS can be simultaneously modulated by varying the spatial overlap between the pulses. The resulting intensity changes of the two-color pulses can be simultaneously measured by two detectors. The operations of addition and subtraction of the two intensity changes enable us to separate the TPA signals from the SRS signals quantitatively. In this article, we describe simultaneous TPA and SRS imaging by SPOM nonlinear optical microscopy (SPOM-NOM) and the resolution enhancement in SRS imaging by SPOM-NOM.

2. Separation of TPA and SRS signals

Figures 1(a) and 1(b) show simplified energy diagrams for TPA and SRS, respectively. Two-photon absorption results in the losses of the two-color pulses (ω₁ and ω₂), while SRS leads to the loss of the pump light (ω₁) and the amplification (gain) of the Stokes light (ω₂). Using SPOM, as shown in Fig. 1(c), the focal position of the first excitation pulse at ω₁ is fixed whereas that of the second excitation pulse at ω₂ is temporally modulated with displacement δ at modulation frequency f. The intensity changes of the two-color pulses induced by TPA and SRS are modulated by SPOM as depicted in Fig. 1(d). The intensity changes of the two-color pulses are proportional to the product of the intensities of the excitation pulses at ω₁ and ω₂. Thus, the intensity changes ΔI_{TPA1}(r, t) and ΔI_{TPA2}(r, t) of the two-color pulses in TPA are temporally modulated according to

\[ \Delta I_{TPA1}(r, t) \propto -C_{ER} \text{Im} \chi^{(3)}_{ER} I_1(r) I_2(r - \delta \cos 2\pi f t), \]  

and

\[ \Delta I_{TPA2}(r, t) \propto -C_{ER} \text{Im} \chi^{(3)}_{ER} I_1(r) I_2(r - \delta \cos 2\pi f t), \]

where \( I_1(r) \) and \( I_2(r - \delta \cos 2\pi f t) \) are respectively the intensities of the excitation pulses at ω₁ and ω₂, \( \text{Im} \chi^{(3)}_{ER} \) is the imaginary part of the resonant electronic contribution of the third-order susceptibility, and \( C_{ER} \) is the concentration of absorptive molecules. On the other hand, the intensity changes ΔI_{SRS1}(r, t) and ΔI_{SRS2}(r, t) of the pulses in SRS are modulated as

\[ \Delta I_{SRS1}(r, t) \propto -C_{RR} \text{Im} \chi^{(3)}_{RR} I_1(r) I_2(r - \delta \cos 2\pi f t), \]

and

\[ \Delta I_{SRS2}(r, t) \propto C_{RR} \text{Im} \chi^{(3)}_{RR} I_1(r) I_2(r - \delta \cos 2\pi f t), \]

where \( \text{Im} \chi^{(3)}_{RR} \) is the imaginary part of the resonant Raman contribution of the third-order susceptibility and \( C_{RR} \) is the concentration of vibrational molecules. As illustrated in Figs. 1(e) and 1(f), the intensity changes of the two-color pulses at \( r = 0 \) are modulated at a frequency of 2f by TPA and SRS, respectively. By increasing the SPOM frequency f and measuring the 2f components in the intensity changes of the two-color pulses, the laser 1/f noise can be suppressed as demonstrated by the modulation transfer technique [17]. Thus, the two intensity changes ΔI₁ and ΔI₂ can be simultaneously measured at a frequency of 2f by lock-in detection using two detectors even if the excitation intensities are not temporally...
modulated. According to Eqs. (1)–(4), by adding the intensity changes of the two-color pulses the TPA signal is doubled, whereas the SRS signal is removed so that

\[ \Delta I_1(r,t) + \Delta I_2(r,t) = 2\Delta I_{\text{TPA}}(r,t) \approx -2C_{\text{ER}} \text{Im}\chi^{(3)}_{\text{ER}} I_1(r)I_2(r - \delta \cos 2\pi ft). \]  

(5)

In contrast, by subtracting the intensity change of the pump light (\(\omega_1\)) from that of the Stokes light (\(\omega_2\)), the SRS signal is doubled and the TPA signal is removed,

\[ \Delta I_1(r,t) - \Delta I_2(r,t) = 2\Delta I_{\text{SRS1}}(r,t) \approx -2C_{\text{RR}} \text{Im}\chi^{(3)}_{\text{RR}} I_1(r)I_2(r - \delta \cos 2\pi ft). \]  

(6)

Therefore, SRS can be separated from TPA without measuring the dependence of the intensity change on the excitation wavelength or on the time delay between the two-color pulses.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{energy_diagram.png}
\caption{Simplified energy diagrams describing the processes of (a) TPA and (b) SRS. (c) Intensity distributions of the two pulses in SPOM. (d) Excitation intensities for TPA or SRS detection. (e) Intensity changes by TPA. (f) Intensity changes by SRS.}
\end{figure}

3. Experiments

3.1 Experimental setup

The experimental setup is sketched in Fig. 2. 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 (\(\omega_1\)) and 1100 nm (\(\omega_2\)), respectively. The frequency difference between the two-color pulses of 2957 cm\(^{-1}\) was identified by CH stretching modes. To compensate for the dispersion in the optical components prior to microscope objective OB1, 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 using a dichroic mirror (DM1). The pulses were focused into the samples by a silicone immersion objective lens (OB1, Olympus UPLSAPO30 \times S having a numerical aperture of NA = 1.05). At the focal point, the pulse durations of the 830-nm and 1100-nm pulses were 140 and 142 fs, respectively. The TPEF signal was separated from the two excitation pulses by using the second DM (DM2, Semrock FF705-Di02-25x36) and was detected with a photomultiplier tube (PMT) (Hamamatsu Photonics H7422A-40). The signals at 830 nm and 1100 nm, and the coherent anti-Stokes Raman scattering (CARS) signal were collected with the water-immersion objective (OB2, Olympus LUMFLN 60 × W with NA = 1.1) in the forward direction. By using the third DM (DM3, Semrock FF705-Di02-25x36), the CARS signal was separated from the signal light at 830 nm and 1100 nm and was detected with the second PMT (Hamamatsu Photonics H7422A-40). The signal light at 830 nm and 1100 nm was divided into two excitation wavelength bands using DM4. The signals at 830 nm and 1100 nm were detected by a Si photodiode (PD, Thorlabs DET36A) and an InGaAs PD (Thorlabs DET20C), respectively. To select the detection wavelength, a bandpass filter (BPF, Semrock FF01-855/210-25) and a longpass filter (LPF, Semrock BLP01-980R-25) were placed in front of PD1 and PD2. By scanning the laser beams in the lateral (x, y) directions with a two-axis galvano scanner (Cambridge Technology 6210H), the image in the xy plane was obtained with a pixel dwell time of 1 ms. By scanning the sample in the axial (z) direction by a stepping motor-driven stage, the signal distribution in the axial direction was measured with a pixel dwell time of 10 ms. The galvano scanner plane was imaged onto the back aperture of the first objective lens, OB1, whereas the back aperture of the second objective lens, OB2, was imaged onto the PDs. The spatial overlap between the two-color pulses was modulated at a frequency $f_S$ of 200 kHz with a maximum focus displacement of 700 nm for simultaneous TPA and SRS imaging or a maximum focus displacement of 200 nm for high resolution SRS imaging in the lateral ($y$) direction 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) whose plane was imaged onto the galvano scanner. The intensity changes of the two-color laser pulses were modulated at a frequency $2f_S$ of 400 kHz by TPA and SRS. However, the small intensity of the OPO pulse was directly modulated at 400 kHz due to the loss of the beam edge in the objective lens. In order to separate the intensity changes by TPA and SRS from the direct intensity change by SPOM, the intensity of the Ti:sapphire pulse was modulated using an optical chopper at a frequency $f_I$ of 1 kHz. By the modulation of the intensity of the Ti:sapphire pulse, the intensity changes by TPA and SRS are modulated as a function of $\cos 2\pi f_S t \cos 2\pi f_I t = \cos 2\pi (2f_S + f_I) t + \cos 2\pi (2f_S - f_I) t / 2$, while the direct intensity modulation by SPOM is not related to the modulation of the intensity of the Ti:sapphire pulse and is observed at a frequency of 400 kHz. Thus, by measuring the intensity changes at the frequencies of $2f_S + f_I$ of 401 kHz or $2f_S - f_I$ of 399 kHz, the influence of the direct intensity change by SPOM can be alleviated. In this study, the intensity change at the frequency $2f_S + f_I$ of 401 kHz was measured by a lock-in amplifier (Stanford Research Systems SR844). Because the detector sensitivity at 830 nm was different from that at 1100 nm, the detection system was calibrated so that the sum of the intensity losses at 830 nm and 1100 nm was zero when the SRS signal from a silicon immersion oil (Olympus, SIL300CS-30SC) was measured. In order to compare the axial resolution of the SPOM technique with that of the intensity modulation technique, the beam pointing modulator using the EOD was modified to achieve the intensity modulation by inserting a pinhole with a diameter of 0.8 mm into the focal point of the scan lens.

3.2 Sample preparation

In this study, as a demonstration of the quantitative separation of TPA from SRS by SPOM, a mixed solution was prepared. A quantum dot (QD) solution (Qdot 625 conjugates, Molecular Probes) at a concentration of 8 μM was miscible in various proportions with a glycerin solution at a concentration of 99%. The one-photon excitation spectrum of the QD ranges from UV to 620 nm. The solution was in a glass container having an optical pathlength of 70
μm. The sum frequency excitation of 830 nm and 1100 nm induced TPA from the QD solution, while the difference frequency excitation of 830 nm and 1100 nm resulted in SRS from the glycerin solution.

To compare the dependence of the XPM signal on the time delay between the two-color pulses with that of TPEF, we also prepared a solution of green-fluorescent protein (GFP) with a concentration of 10 mg/cc in HEPES NaOH buffer (50 mM, pH 7.4), which was set in a glass container with an optical path length of 70 μm.

To demonstrate simultaneous TPA and SRS imaging, polystyrene beads were suspended in a QD solution. A solution of polystyrene beads (Polysciences) with a diameter of 10 μm was drop cast onto a glass slide and covered with a slip. A QD solution was sandwiched between the glass slide and the cover slip.

To demonstrate the resolution enhancement by SPOM-NOM, we also prepared samples for SRS imaging. A solution of polystyrene beads (Polysciences) with a diameter of 200 nm was drop cast onto a glass slide and covered with a slip.

3.3 Results

We measured the dependence of the XPM signal from the cover slip and the TPEF signal from the GFP solution on the time delay between the two-color pulses. The input powers at 830 nm and 1100 nm were 1.5 mW. As shown in Fig. 3, the XPM signal is almost zero at a time delay of zero, while the peak for the TPEF signal is located at around a time delay of zero. Thus, by adjusting the time delay to zero, the XPM signal can be suppressed [15].

![Fig. 3. Intensity change at 830 nm by XPM and the TPEF intensity in the cover glass and the GFP solution as a function of the time delay between the two-color pulses, respectively.](image)

The line profiles of the intensity losses at 830 and 1100 nm in the axial direction were measured near the region of the silicon-oil objective lens, cover slip, and mixed solution of QD and glycerin. Figure 4 graphs those profiles, together with the sum and difference of the two losses for the mixed solution in various proportions, averaging over five measurements. A negative value of the intensity loss represents a gain. In the silicon oil and in the glycerin solution, the loss at 830 nm is positive, while that at 1100 nm is negative. Because the magnitudes of these intensity changes are equal, the sum of the losses is zero but the difference equals double the loss. This result indicates that the intensity change in the silicon oil and in the glycerin solution was induced by SRS. On the other hand, in the QD solution, the losses at both 830 and 1100 nm are positive and their magnitudes are equal. Thus, the sum gives double the loss, while the difference is zero. This result suggests that the intensity change in the QD solution was induced by TPA. In a mixture of 75% QD and 25% glycerin, the loss at 830 nm is high whereas that at 1100 nm is almost zero. This result implies that the loss in TPA was equal to the gain in SRS. Even under this condition, the sum and difference of the two losses enables the TPA and SRS to be separated. The XPM signals were not observed in the glass, because the time delay between the two-color pulses was zero.
Fig. 4. Line profiles of the intensity losses at 830 and 1100 nm, and the sum (TPA) and difference (SRS) of the two losses in the axial direction near the silicon-oil objective lens, cover slip, and mixed solution of quantum dot (QD) and glycerin (Gly) in various proportions.

To measure the separation of the TPA and SRS signals, the intensity losses at 830 and 1100 nm in the mixed solution, and the sum and difference of the losses are plotted as functions of the concentrations of QD and glycerin in Fig. 5. The sum is proportional to the concentration of QD, and the difference is proportional to that of glycerin. These results agree with Eqs. (5) and (6). Therefore, the TPA signal can be quantitatively separated from the SRS signal.

The dependence of the intensity losses at 830 and 1100 nm on the excitation power were also measured, along with the sum and difference of the two losses in the glycerin and QD solutions. The excitation power of one pulse was varied, while that of the other pulse was kept fixed. As graphed in Figs. 6(a) and 6(b), the difference of the two losses in the glycerin solution is proportional to the product of the excitation powers of the 830-nm and 1100-nm pulses, whereas the sum does not depend on the powers. In contrast, the sum of the two losses in the QD solution is proportional to the product of the excitation powers of the pulses but the difference does not depend on them. These results again agree with Eqs. (5) and (6), indicating that the intensity change in the glycerin solution is induced by SRS, while the intensity change in the QD solution is due to TPA.

Fig. 5. Intensity losses at 830 and 1100 nm, and the sum and difference of the two losses as a function of the concentrations of QD and glycerin in the mixed solution.
Next, simultaneous TPA and SRS imaging of polystyrene beads in a QD solution were performed. The input powers at 830 nm and 1100 nm were 1.5 mW. Figure 7 presents loss images at 830 and 1100 nm, and their sum and difference. In Figs. 7(a) and 7(b), the positive value (red-yellow) in the color scale indicates a loss, and the negative value (blue-cyan) represents a gain. On the other hand, in Figs. 7(c) and 7(d), the positive value (red-yellow) in the color scale indicates the signal intensity of TPA and SRS, and the negative value (blue-cyan) implies the degree of crosstalk of TPA and SRS signals. The loss signal by SRS is mixed with that by TPA in Fig. 7(a), while the gain signal by SRS appears to be distinguished from the loss signal by TPA by means of the attribute of being positive or negative in Fig. 7(b). However, there are no evidence that the gain signal by SRS is not mixed with the loss signal by TPA because the negative signal can be also formed as a result of the subtraction of the loss signal by TPA from the gain signal by SRS. In contrast, after addition and subtraction of the two images in Figs. 7(c) and 7(d), the SRS signals from the polystyrene beads and the TPA signals from the QD solution are successfully distinguished. From the sign of the signals, we found no crosstalk. Therefore, the operations of addition and subtraction can be applied to simultaneous SRS and TPA imaging without crosstalk.

Fig. 7. Loss images of polystyrene beads in a QD solution at (a) 830 nm and (b) 1100 nm. Images of the (c) sum and (d) difference of these two images.

In order to demonstrate the improvement in spatial resolution by the SPOM technique, we obtained an SRS image of 200-nm polystyrene beads. The input powers at 830 nm and 1111 nm were 1.5 mW. Figure 8(a) shows the SRS image for SPOM-NOM with a maximum focus displacement of 200 nm in the lateral (y) direction, which allows the resolution enhancement in the y and z directions [24]. We obtained lateral full width at half maximums (FWHMs) of
0.55 ± 0.018 \mu m and 0.29 ± 0.03 \mu m in the x and y directions, respectively. As shown in Fig. 8(b), we also obtained the CARS image for conventional CARS microscopy. In CARS microscopy, the FWHMs in the x and y directions are 0.43 ± 0.017 \mu m and 0.47 ± 0.023 \mu m, respectively. The FWHM in the x direction for CARS microscopy is smaller than that for SPOM-NOM. This is because the CARS intensity is proportional to the product of the square of the excitation intensity at \omega_1 and the excitation intensity at \omega_2, while the SRS intensity is proportional to the product of the excitation intensities at \omega_1 and \omega_2. In contrast, the FWHM in the y direction for SPOM-NOM is smaller than that for CARS microscopy. Because the FWHM in the x direction is comparable to that in the y direction in CARS microscopy, the resolution enhancement in SRS imaging by the SPOM technique was evaluated by comparing the FWHM for SPOM-NOM in the y direction with that in the x direction. We found that the lateral (y) resolution in SRS imaging by the SPOM technique was enhanced by a factor of 1.9.

![Fig. 8. Enhancement of the spatial resolution in SRS imaging by SPOM-NOM.](image)

We also investigated the resolution enhancement in the axial (z) direction. Figure 8(e) shows SRS signal distributions in the axial direction near the interface between a cover slip and a glycerin solution obtained by SPOM-NOM with a maximum displacement of the focus of 200 nm and SRS microscopy based on the modulation transfer technique. The response of SPOM-NOM is steeper than that of SRS microscopy using the modulation transfer technique. By differentiating the intensity distributions along the axial direction, the axial resolutions of SPOM-NOM and SRS microscopy using the modulation transfer technique were estimated to be 1.0 \mu m and 1.9 \mu m, respectively. Therefore, the axial resolution of SPOM-NOM was enhanced by a factor of 1.9.

4. Discussion and conclusions

Using SPOM-NOM, we have demonstrated quantitative separation of TPA and SRS signals, and its application to simultaneous TPA and SRS imaging, and resolution enhancement in SRS imaging by a factor of 1.9. However, artifacts at the edges of the polystyrene beads are apparent in Fig. 7. If the artifact is produced by a combination of the two-color pulses, it
should appear both in the loss images at 830 nm and 1100 nm. However, the artifact appeared only in the loss image at 1100 nm. In addition, this artifact did not depend on the time delay between the two-color pulses. This result indicates that the artifact was produced only by the 1100-nm pulse. The reason is considered as follows. Scattering at the interface between the polystyrene beads and the QD solution results in an increase in the direct intensity change of the 1100-nm pulse by SPOM at a frequency of 400 kHz, which corresponds to the doubled frequency of the pointing modulation. In order to separate the intensity changes by TPA and SRS from the direct intensity change by SPOM, the modulation frequency of the intensity changes by TPA and SRS was shifted to 401 kHz by using the intensity modulation of the 830-nm pulse and the intensity changes by TPA and SRS was demodulated at 401 kHz. However, the difference between the demodulation frequency and the doubled frequency of the pointing modulation was not large enough to separate the intensity changes by TPA and SRS from the direct intensity change at the edges of the polystyrene beads by SPOM. By increasing the difference between the demodulation frequency and the doubled frequency of the pointing modulation, and the difference between the demodulation frequency and the intensity modulation frequency, the artifact due to the direct intensity loss at 1100 nm can be eliminated. The sum frequency of the intensity modulation frequency at 830 nm (ω1) and the doubled frequency of the beam pointing modulation at 1100 nm (ω2) can be used as the demodulation frequency of the two-color pulses. Thus, this goal can be achieved by increasing the intensity modulation frequency at 830 nm to the order of MHz using a high-speed modulator such as an electro-optic or acousto-optic modulator. This improvement would also allow shortening the pixel dwell time to the order of μs. In the present study, in order to suppress the direct intensity change by SPOM, not only the SPOM based on pointing modulation but also the intensity modulation were employed. However, the direct intensity change by SPOM could be also suppressed without intensity modulation by using the SPOM based on wavefront modulation or reducing the beam diameter at the back aperture of the objective lens. Thus, the use of the intensity modulation would not be essential for quantitative separation of TPA and SRS signals.

The spectral unmixing method [19] and the phasor analysis method [20] are powerful techniques not only for the separation of TPA and SRS signals, but also for the discrimination of the SRS signal due to one species of vibrational molecules from that of other species. In the spectral unmixing technique, TPA and SRS spectra are first obtained from single species of absorbive molecules and vibrational molecules, to serve as references during the unmixing [19]. Similarly, in the phase analysis method, delay traces must be obtained from single species of absorbive molecules and vibrational molecules [20]. However, the spectra and delay traces can depend on the local environment of the target molecules and on the binding states of the target molecules to other molecules such as lipids, proteins, and DNA. In such a case, errors in the separation may be generated. Synchronization timing jitter and laser spectral drift during the measurement may also cause errors in the separation [19]. If the two excitation intensities are temporally modulated at different frequencies, the intensity changes of the two pulses can be simultaneously demodulated at the sum and difference frequencies. The quantitative separation of TPA and SRS using the double modulation technique is comparable to that by combining SPOM with intensity modulation. Our proposed method based on the operations of addition and subtraction can be combined with the spectral unmixing method and the phase analysis method to reduce these errors. However, it is difficult to apply our proposed method to deep imaging of a highly scattering sample. This is because as follows. At the focal point, the magnitude of the intensity change by TPA or SRS at a frequency of ω1 is equal to that at ω2. However, because the loss in the propagation of the generated signal inside the highly scattering sample depends on the frequency, the magnitudes of the intensity changes by TPA or SRS at frequencies of ω1 and ω2 are different on the detectors. If we know at least one reference point at which either TPA or SRS is induced, we could calibrate the difference of the losses in the propagation.

Finally, note that the intensity changes of the two-color pulses, which can be used to separate SRS from TPA by the operations of addition and subtraction, can be simultaneously
detected by implementing modulation of the spatial overlap and/or of the excitation intensity, or double modulation of the excitation intensities at different frequencies. In order to achieve label-free imaging of biological samples including organic molecules (such as DNA, RNA, proteins and lipids), intrinsic absorbers (such as hemoglobin and melanin), and endogenous fluorophores, (such as nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD) and tryptophan) by using SRS and TPA, SRS signals from the organic molecules must be separated from TPA signals from the intrinsic absorbers and the endogenous fluorophores. Consequently, the present method to separate TPA from SRS may become an important tool for label-free imaging based on TPA and SRS.

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