Improving resolution of second harmonic generation microscopy via scanning structured illumination

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Abstract: Second harmonic generation microscopy (SHGM) is a well-known technique for examining the noncentrosymmetric structures in biomedical research. However, without real-state transitions, fluorescence-based superresolution methods cannot be applied. To improve the resolution, fringe-scanning SHGM (FS-SHGM), which combines SHGM with structured illumination based on point-scanning, is introduced in this paper. The scanning path was modulated to generate illumination patterns. For the coherent parts of SHG signals, a mathematical model of image formation and reconstruction was established. Both simulations and experiments showed a resolution improvement factor of ~1.4 in the lateral and 1.56 in the axial directions for chicken tendons and mouse skin.

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1. Introduction

Second harmonic generation microscopy (SHGM), which has an inherent optical sectioning power and ability to reveal the nonlinearity of materials, has been widely applied in biomedical research [1–3]. Bio-tissues which are non-centro-symmetric, such as collagen fibers and nerve fibers, can easily be observed by SHGM without staining [2,3]. Based on the optical sectioning power, SHG images can even be obtained in thick tissue with high axial contrast. The lateral resolution of SHGM is about 400-1000 nm, depending on the excitation wavelength. Although SHGM seems to provide higher resolution under the same excitation wavelength, the resolution is still limited by the diffraction limit. To further improve the resolution, various superresolution techniques have been developed since the 1990s, such as stimulated emission microscopy (STED) [4], structured illumination microscopy (SIM) [5], stochastic optical reconstruction microscopy (STORM) [6] and so forth. These superresolution techniques were originally designed for fluorescence-based microscopy, because fluorescence can easily be switched and modulated. However, scattering or higher harmonic generation signals cannot directly fit in with the original superresolution modalities. To overcome this limitation, superresolution modalities based on scattering signals [7] and higher harmonic generation signals [8–11] have been proposed. Among these methods, subtractive SHGM [8] and second harmonic superresolution microscopy (SHaSM) [9] were introduced to achieve superresolution SHG imaging, based upon the concepts of STED and STORM, respectively. In both techniques, different methods have been developed to achieve modulation of the SHG signals.

In SHaSM, a single-molecule localization approach is employed, which is the main core of STORM. The probes used in STORM can be switched on/off by two different excitation sources. The randomly-activated probes are localized to map the molecules’ positions. An overall image can be reconstructed by repeating the localization process for thousands (or more) cycles [6]. In STORM the probes are fluorophores or fluorophore pairs and the
excitation sources are two lasers with different wavelengths. Alternatively, in SHaSM, nanosized barium titanium oxide (BTO) is used as a photoswitchable probe which can be switched on/off by controlling the excitation polarization [9]. Alternatively, subtractive SHGM makes use of the concept of STED to reduce the effective size of the point spread function (PSF). In STED, an excited PSF is overlaid by a donut-shaped beam. Within the overlapping area, the fluorescence is de-excited, so that the effective size of the PSF can be reduced [4]. In subtractive SHGM, a Gaussian beam with circular polarization is used to obtain one conventional image. A donut-shaped beam with apodized vortex phase modulated circular polarization is used to obtain the other image. Finally, the subtractive image is reconstructed by subtracting these two images [8]. Comparing these two methods, SHaSM relies on special contrast agents to achieve single-molecule localization, while subtractive SHGM requires two beams with different modes and polarization states to reduce the effective PSF. There is a lower resolution improvement in subtractive SHGM than SHaSM, but the higher acquisition rate makes it more suitable than SHaSM for dynamic observation.

In addition to STORM and STED, SIM is another well-known superresolution method. SIM can be performed without using specialized contrast agents or excitation sources. The only requirement for SIM is the production of applicable structured illumination patterns with precise phase shifts. In conventional SIM (cSIM), the fluorescent sample is subjected to wide-field illumination by sinusoidal patterns. Fluorescent signals with a higher spatial frequency (HSF) can then be diffracted into the imaging system and recorded. This indicates that the HSF components are shifted into the passband of the system optical transfer function (OTF) [12]. With these HSF components, a superresolution image with an effectively-extended OTF passband can be reconstructed. A variety of fluorescence-based methods such as 3D SIM [13], optical sectioning SIM [14], light sheet SIM [15] and two-photon fluorescence SIM (TPF-SIM) [10,16] have been introduced to improve the poor axial resolution due to the missing cone problem which occurs under wide-field illumination [12]. In authors’ previous work [16], a laser scanning two-photon structured illumination microscope (LSTP-SIM) was developed based on point-scanning geometry, due to the extremely high intensity requirement for two-photon excitation. The illumination pattern is generated by temporally modulating the excitation intensity along with spatial scanning. Taking advantage of similar process of two-photon excitation, SHG can be integrated with SIM in the same way. To investigate optically-thick tissues, an epi-detection geometry should be applied to SHGM. Unlike TPF, backward SHG signals are known to be partially contributed by the direct backward SHG, which is coherent, and partially contributed by the scattered forward SHG, which is incoherent [17]. The degree of coherence depends on the depth into the tissues. Near the tissue surface more coherent components can be observed. The image formation and reconstruction of the incoherent part can be described by the theory developed in the previous work [16]. For the coherent part, the image formation and reconstruction based on coherent signals has to be developed.

To show the possibility of combining SHG with SIM, a fringe-scanning SHGM (FS-SHG) system was established and demonstrated in this work. A structured illumination pattern was generated by modulating the laser scanning path, while a 2D sCMOS camera was used as the detector to obtain the information for the system’s PSF. Given the coherent nature of SHG signals, we built a mathematical model of the processes of image formation and reconstruction. Simulations were carried out to prove the validity of the theoretical model. Experiments were performed using unsectioned chicken tendon and mouse skin samples to show the resolution improvement. After acquiring 6 pattern images for reconstruction, the FS-SHG images showed a factor of improvement in the resolution of ~1.4 in the highly-scattering bio-tissues, in contrast to the conventional camera-based SHGM (C-SHG).
2. Principles

SHG is a nonlinear optical process which exhibits a square dependence on the excitation intensity [18]. Ultrafast laser pulses and tight focusing are required for SHG excitation. Therefore, a point-scanning geometry is commonly used in SHGM. Figure 1 shows the configuration of illumination pattern generation based on a point-scanning geometry and quasi-comb scanning. SHG signals arising from each excitation spot will form an image on the sCMOS camera. During the scanning period of a frame, the excitation spot is scanned zig-zag over a rectangular area. The SHG images of every excitation spot are integrated and recorded by the camera to obtain one SHG image. In the case of C-SHGM, adjacent fast scan lines are very close to each other, so the illumination is regarded as uniform. Simply increasing the separation between two fast scan lines can produce a structured illumination pattern for FS-SHGM. In contrast to the temporal intensity modulating used in the previous study of LSTP-SIM [16], the quasi-comb scanning method needs no additional intensity modulation element so that the system complexity can be reduced. The period and duty cycle of the illumination patterns can be easily adjusted by assigning a new scanning path. This makes it more convenient to integrate SIM with a conventional SHGM. Moreover, since quasi-comb scanning can provide 0-to-1 dynamic range of intensity modulation, the contrast of the illumination pattern can be increased as well.

For a single excitation spot, the excited SHG field on the sample is proportional to the square of the excitation PSF (Ex-PSF), $P_{ex}^2(r)$, and the $\chi^{(2)}$ distribution of the sample, $S(r)$, where $r$ represents the location vector. The SHG intensity distribution captured by the camera for the coherent parts, corresponding to an excitation spot centered at $r_n$, can be described as

$$I_n(u) = \int |P_{2p}(r-r_n)S(r)P_{em}(u-r)dr|^2,$$

where $P_{2p}(r)$, standing for $P_{ex}^2(r)$, is the two-photon excitation PSF (TP-PSF); and $P_{em}$ denotes the amplitude PSF of the imaging system (Em-PSF). Due to the coherent nature, the convolution in Eq. (1) represents the SHG field at the imaging plane. The image captured by the camera is the square of the absolute value of the SHG field [19]. Scanning the excitation spot, a complete C-SHGM image is constructed by integrating $I_n(u)$ spot by spot, which yields

$$I_C(u) = \int \int |P_{2p}(r-r_n)S(r)P_{em}(u-r)dr|^2 \, dr_n.$$

By modulating the scanning path, and spatial modulation of the excitation intensity, $M_m(r)$, can be generated. With scan lines equally-spaced, the modulation function can be described by a square wave function.
\[ M_m(r) = \frac{1}{a} \text{comb} \left( \frac{r - r_m}{a} \right) \otimes \text{rect} \left( \frac{r}{b} \right), \]  

where \( a \) and \( D = b/a \) denote the period and duty cycle, respectively; and \( r_m \) controls the phase shift. With this modulation function, the patterned SHG image is given by

\[
I_p^m(u) = \int \left| \int M_m(r) P_{2p}(r - r_m) S(r) P_{\text{en}}(u - r) \, dr \right|^2 \, dr_m
= \int M_m(r) \left[ \int P_{2p}(r - r_m) S(r) P_{\text{en}}(u - r) \, dr \right] \left[ \int \bar{P}_{2p}(r - r_m) \bar{S}(r) \bar{P}_{\text{en}}(u - r) \, dr \right] \, dr_m
= \int M_m(r) \left( \tau - r_m P_{2p} \otimes P_{\text{en}} \right)(u) \left( \tau - r_m \bar{P}_{2p} \otimes \bar{P}_{\text{en}} \right)(u) \, dr_m, \tag{4}
\]

where \( \tau - r_m P_{2p} \) denotes the \( P_{2p} \) shifted by \( r_m \); and \( \bar{S} \) denotes the conjugate of \( S \). Since \( M_m(r) \) is a square wave function ranging from 0 to 1, \( |M_m(r)|^2 \) is equal to \( M_m(r) \) and can be rewritten as a complex Fourier series

\[
|M_m(r)|^2 = M_m(r) = D \left[ 1 + \sum_{N=1}^{\infty} \sin c(N\pi D) \left[ e^{i(N\pi u - \pi N)} + e^{-i(N\pi u - \pi N)} \right] \right], \tag{5}
\]

where \( \omega = 2\pi / a \) and \( \phi_m = -2\pi r_m / a \) denote the fundamental frequency and the phase shift. Replacing \( |M_m(r)|^2 \) by Eq. (5), the Fourier transform of Eq. (4) is given by

\[
\hat{I}_p^m(k) = D \left[ 1 + \sum_{N=1}^{\infty} \sin c(N\pi D) \left[ e^{i(N\pi u - \pi N)} + e^{-i(N\pi u - \pi N)} \right] \right] \left[ \hat{r}_{\text{en}} S \otimes \hat{S} \right](k) \left[ \hat{r}_{\text{en}} \bar{S} \otimes \bar{S} \right](k) \left[ e^{-i(\omega \phi - \pi N)} + e^{i(\omega \phi - \pi N)} \right] \hat{r}(k) \, dk,
\]

where \( \hat{r}(k) \) denotes the autocorrelation operator; \( M = \sin c(\pi D) \) is the modulation depth; \( h_{2p}(k) \) is the two-photon excitation OTF (TP-OTF); and \( h_{\text{en}}(k) \) is the OTF of the imaging system (Em-OTF). The first three terms of Eq. (6) can be rewritten as

\[
\hat{I}_p^m(k) = D \left[ \hat{h}_{2p}(k) e^{i\omega \phi} \otimes \hat{S}(k) \hat{h}_{\text{en}}(k) \right] \left[ \hat{h}_{2p}(k) e^{-i\omega \phi} \otimes \hat{S}(k) \hat{h}_{\text{en}}(k) \right] \, dk,
\]

In Eq. (8), \( \ast \) denotes the cross-correlation operator. The spectrum of the patterned SHG image is given by

\[
\int h_{2p}(k) \hat{r}_{\text{en}}(k) \, dk,
\]

where \( h_{2p}(k) \) is the two-photon excitation OTF (TP-OTF); and \( h_{\text{en}}(k) \) is the OTF of the imaging system (Em-OTF). The first three terms of Eq. (6) can be rewritten as

\[
\hat{I}_p^m(k) = D \left[ \hat{h}_{2p}(k) e^{i\omega \phi} \otimes \hat{S}(k) \hat{h}_{\text{en}}(k) \right] \left[ \hat{h}_{2p}(k) e^{-i\omega \phi} \otimes \hat{S}(k) \hat{h}_{\text{en}}(k) \right] \, dk,
\]

where \( \hat{r}(k) \) denotes the autocorrelation operator; \( M = \sin c(\pi D) \) is the modulation depth; \( h_{2p}(k) \) is the two-photon excitation OTF (TP-OTF); and \( h_{\text{en}}(k) \) is the OTF of the imaging system (Em-ATF). The first three terms of Eq. (6) can be rewritten as

\[
\hat{I}_p^m(k) = D \left[ \hat{h}_{2p}(k) e^{i\omega \phi} \otimes \hat{S}(k) \hat{h}_{\text{en}}(k) \right] \left[ \hat{h}_{2p}(k) e^{-i\omega \phi} \otimes \hat{S}(k) \hat{h}_{\text{en}}(k) \right] \, dk,
\]
The first term represents the spectrum of the SHG image without modulation, referred to as the “dc term” in the following paragraph. The last two terms give the spectrum of the modulation parts of the SHG image, referred to as the “modulation terms” in the following paragraph.

In fact, the square wave modulation function contains an infinite series of harmonic components. However, according to Eq. (8), as \( \omega \) is set close to \( 2\omega_{c,em} \), twice the cut-off frequency of \( h_{em}(k) \), the higher harmonic terms will be blocked from the system and can thus be ignored. Comparing Eq. (7) and Eq. (8), \( \tau_{p}\tilde{S}h_{em} \ast \tau_{\rho,em}\tilde{S}h_{em} \) can bring higher frequency components of \( \tilde{S}(k) \) than can \( \tau_{p}\tilde{S}h_{em} \ast \tau_{\rho,em}\tilde{S}h_{em} \). The larger the \( \omega \) is, the higher the frequency of the components which can be recorded. However, as the \( \omega \) increases, the coefficient \( h_{2p}(k)h_{2p}(k) \) becomes smaller and the contrast of the modulation terms decreases as well. There has to be a compromise for the selection of \( \omega \) has and the contrast. Besides \( \omega \), the duty cycle, \( D \), is also a key factor of the modulation contrast. The modulation depth is described as \( M = \text{sinc}(\pi D) \). As \( D \) increases from 0 to 1, \( M \) descends from 1 to 0. It seems that the best contrast can be obtained by keeping \( D \) as small as possible. However, besides the contrast, \( D \) also affects the total signal intensity. To retain a sufficient signal-to-noise ratio (SNR), the compromised \( D \) is around 0.3.

3. Simulation

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Fig. 2. Simulation FS-SHGM images of the USAF resolution: (a) 1951 USAF resolution target; (b) profiles of \( h_{2p}(k) \) and \( h_{em}(k) \) along the \( k_x \) direction; (c) and (d) are the C-SHGM image and its spectrum; (e) and (f) show the patterned SHG image and its spectrum; (g) and (h) show the reconstructed FS-SHGM image and its spectrum. To reveal the details with low contrast, contrast enhancement was applied to (c), (e) and (g).

To demonstrate the principles for the coherent part of SHG signals, a resolution target (1951 USAF; Fig. 2(a)) was used as a sample in the simulation. Figure 2(b) shows the profiles of \( h_{2p}(k) \) and \( h_{em}(k) \) along the \( k_x \) axis. The cut-off frequencies of \( h_{2p}(k) \) and \( h_{em}(k) \), \( \omega_{c,2p} \) and \( \omega_{c,em} \), are equal to \( 2\pi \left( 2N A / \lambda_{ex} \right) \) and \( 2\pi \left( N A / \lambda_{SHG} \right) \) [19], where \( \lambda_{ex} \) and \( \lambda_{SHG} \) are set to 1064 nm and 532 nm, respectively, and NA is 1.2. Since \( \lambda_{ex} = 2\lambda_{SHG} \), \( \omega_{c,2p} \) is equal to \( \omega_{c,em} \). The C-SHGM image and its corresponding spectrum are shown in Fig. 2(c) and Fig. 2(d). According
to Eq. (7), the cut-off frequency in Fig. 2(d) would be $2\omega_{c,em}$. The smallest resolvable line pair based on the Rayleigh criterion is Group 11, Elements 4, which corresponds to resolutions of 345 nm. Applying a modulation pattern with $\omega = 0.81\omega_{c,em}$ along the x direction, the patterned SHG image and its corresponding spectrum are shown as Fig. 2(e) and Fig. 2(f). Figure 2(e) shows a modulated pattern. While in the Fourier domain (Fig. 2(f)), the dc term and two modulation terms with peaks at $\pm 0.81\omega_{c,em}$ can be seen. To solve three unknown terms, three images with different modulation phases of $\phi_m = 0, 2\pi/3, \text{and } 4\pi/3$ are required [12]. After solving the three terms and shifting them back to the right positions, the apodization function is applied to perform weighted averaging of the reassembled spectrum [20]. The reconstructed spectrum and the reconstructed FS-SHGM image are shown in Fig. 2(g) and 2(h), respectively. Due to the modulation frequency being $0.81\omega_{c,em}$, the cut-off frequency of the reconstructed spectrum is expanded to $2.81\omega_{c,em}$. In Fig. 2(h), the smallest resolvable line pair is Group 12, Elements 1 with corresponding resolutions of 244 nm. A resolution improvement ratio of around 1.41 is observed. For the incoherent part of SHG signals, the theoretical model in the previous work of LSTP-SIM [16] can be applied. With the same settings of wavelength, objective and modulation frequency, the resolution improvement factor of the incoherent part is also around 1.41. Since the improving factors of the coherent part and incoherent part are almost the same, this indicates the degree of coherence of the backward SHG signals would not affect the resolution improvement of FS-SHGM.

4. System configuration

The FS-SHGM’s system configuration is shown in Fig. 3(a). For SHG excitation, a 1064-nm femto-second laser (FPL-03UFF0 with Calmar pulse compressor) was utilized as the light source. A 2D galvo-mirror (GVSM002/M, Thorlabs) was controlled to create the scanning pattern. After being scanned by the galvo-mirror, the laser beam was expanded to fill the back aperture of the objective (UPLSAPO 60XW, NA 1.2, Olympus) and was then tightly focused onto the sample. The excited SHG signals were backward collected by the same objective and imaged onto a sCMOS camera (ORCA Flash 4.0 LT, Hamamatsu) point by point. The image magnification of this system was 116X with one pixel on the sCMOS camera corresponding to $55.7 \times 55.7 \text{ nm}^2$ on the sample. A motorized-stage with a step resolution of 0.2 $\mu$m moved the sample along z-axis so that 3D image could be obtained. Figure 3(b) represents the time sequences of the control signals for the 2D galvo mirrors and sCMOS camera. These components are triggered simultaneously. The image acquisition cycle is started by the sending of a trigger signal, T. Within a frame time, the fast-axis galvo mirror is actuated by using a triangle wave voltage to scan the laser beam back and forth. As defined in Eq. (3), $b/a$ is the duty cycle of the illumination pattern. To acquire an image, the sCMOS exposure time, $E$, should be set to a multiple of the frame time. During the sCMOS readout time, $R$, the 2D galvo mirrors are reset. After the readout process, the trigger signal is sent again to start the next image acquisition cycle.

Fig. 3. (a) The system configuration of FS-SHGM. L: lens; M: mirror; DBS: dichroic beamsplitter; Obj.: objective; TS: motorized translation stage; (b) time sequences of the control signals for the fast-axis and slow-axis of the galvo mirror and sCMOS camera. T: trigger signal; $E$: exposure time; $R$: readout time.
5. Results and discussion

To demonstrate the resolution improvement, an unsectioned chicken tendon sample without staining was utilized. The chicken tendon samples were excised from chicken wings and immersed in a phosphate-buffered saline (PBS) solution. The excitation power at the sample was 30 mW. Figure 4(a) and 4(b) show C-SHGM images of the chicken tendon aligned along x-direction and y-direction, respectively. Figure 4(c) and 4(d) show the FS-SHGM images corresponding to Fig. 4(a) and 4(b). For each FS-SHGM image, 6 patterned images (3 with x-direction patterns and 3 with y-direction patterns) were acquired. The period of the scanning pattern as 874 nm and the duty cycle was 0.3. To get a patterned image with an SNR higher than 3, the exposure time for one patterned image was 1.9 s. After applying SIM, there is an apparent improvement in the contrast of the FS-SHGM images compared to the C-SHGM images. Figure 4(e) shows the normalized intensity profiles along the yellow lines in the enlarged boxed regions in Fig. 4(a) and 4(c), while Fig. 4(f) shows the normalized intensity profiles for Fig. 4(b) and 4(d). The lateral resolution of C-SHGM and FS-SHGM was determined by fitting these curves with Gaussian functions. The full width at half maximum (FWHM) of the fitting curves in Fig. 4(e) was measured to be 345 nm for C-SHGM and 249 nm for FS-SHGM. The FWHMs of the fitting curves in Fig. 4(f) were measured to be 329 nm for C-SHGM and 231 nm for FS-SHGM. The resolution improvement factors in Fig. 4(e) and 4(f) are 1.39 and 1.42, respectively. These improvement factors match the theoretical value of 1.41.

In traditional SHGM, photomultiplier (PMT) is used to integrate SHG signals from the focal area, including both direct backward SHG and scattered forward SHG. Using the sCMOS camera instead as the detector, SHG signals scattered by the surrounding tissues will result in background noises in the C-SHGM image. As shown in Fig. 5(a) and 5(b), the PMT-based SHGM image presents better contrast than the C-SHGM image. Besides, the fibrillar structures revealed by PMT-based SHGM is more continuous than that revealed by C-SHGM. By applying SIM to C-SHGM, not only the resolution can be improved but the image contrast can be enhanced through the optical sectioning property of SIM [21,22]. As the intensity profiles shown in Fig. 5(c), the lateral resolution of PMT-based SHGM is larger than that of
The resolution ratio of PMT-based SHGM to C-SHGM is about 1.14 while the ratio of PMT-based SHGM to FS-SHGM can achieve ~1.6.

Fig. 5. (a) PMT-based SHGM, (b) C-SHGM and (c) FS-SHGM images of chicken tendons. (d) The normalized intensity profiles along the yellow lines in (a), (b) and (c).

The nonlinearity of SHG gives this system its inherent optical sectioning power. To demonstrate the FS-SHGM’s capability for 3D imaging in highly scattering bio-tissues, unsectioned mouse skin was used as the sample. The mouse skin was fixed in 4% paraformaldehyde. Then C-SHGM and FS-SHGM 3D image stacks of the dermis layer were obtained by acquiring 90 \( x \)-\( y \) images along the \( z \)-direction with a 0.2-\( \mu \)m step size. Figure 6(a) and 6(b) show each 3D image stack in an image set, consisting of \( x \)-\( y \), \( y \)-\( z \) and \( x \)-\( z \) sections. The normalized intensity profiles of the fibrils in the \( x \)-\( y \), \( y \)-\( z \) and \( x \)-\( z \) sections are shown in Fig. 6(c)-6(e), respectively. From the \( z \) sections of the C-SHGM image, the SHG’s inherent optical sectioning power can already be observed. However, the off-focus SHG signals which came from the scattered on-focus SHG signals resulted in strong background noise and thus reduced the axial resolution. After performing SIM to each \( x \)-\( y \) section, a remarkable improvement in the axial contrast can be seen in the FS-SHGM \( z \) sections. This axial resolution improvement was achieved by removing the off-focus SHG signals through the structured illumination [21,22]. In Fig. 6(c), the lateral profiles of the fibrils were fitted with a Gaussian function to define the FWHM for the lateral resolution. By measuring the FWHMs for 10 lateral profiles, the average lateral resolutions for C-SHGM and FS-SHGM are 329 and 235 nm. The resolution improvement factor is 1.4. In Fig. 6(d), the same analysis was applied to the axial profiles of the fibrils. The average axial resolutions for C-SHGM and FS-SHGM are 1084 nm and 693 nm and the improvement factor is 1.56. In Fig. 6(e), the profile of the FS-SHGM \( x \)-\( z \) section shows a much higher contrast than does the C-SHGM profile. Due to the sectioning power of SHGM, the three-dimensional OTF of C-SHGM has no missing-cone problem [12], which provides the original axial contrast in the C-SHGM image. By applying SIM, not only the lateral spectrum, but also the axial spectrum corresponding to high lateral frequencies can be extended, which results in the improvement in the axial contrast in the FS-SHGM image.
6. Conclusion

The FS-SHGM system introduced above is demonstrated to successfully perform superresolution SHG imaging inside highly scattering bio-tissues, based on the point-scanning geometry and quasi-comb scanning. This FS-SHGM system can be easily integrated into conventional SHGM systems. The frequency, phase, duty cycle and orientation of the illumination patterns can be simply controlled by electrical signals. Without the use of moving elements we obtained greatly enhanced accuracy and stability during pattern generation. For the coherent part of the backward SHG signals, a theoretical model of image formation and image reconstruction was established. In simulations, a 1.41-fold resolution improvement could be obtained for the coherent part of SHG. Based on the theoretical model of LSTP-SIM, the same resolution improvement of the incoherent part of SHG can be obtained with the same system parameters. In the experiments, we obtained an improvement in the resolution in contrast to that obtained with C-SHGM for highly scattering bio-tissues. The lateral improvement factors were ~1.4 for both chicken tendon and mouse skin samples, while the axial improvement factor was 1.56 for mouse skin. Due to the enhancement in axial contrast during image reconstruction, there was an efficient improvement in the lateral resolution which was higher than the theoretical prediction. Since FS-SHGM needs no special contrast agent and no excitation polarization modulation, this system can be easily adapted to the process of third harmonic generation microscopy.

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Disclosures

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