Spectroscopic second and third harmonic generation microscopy using a femtosecond laser source in the third near-infrared (NIR-III) optical window

YUSUKE MURAKAMI,1,2 MINORI MASAKI,1,2 SHINICHI MIYAZAKI,1,2 RYOSUKE OKETANI,3 YU HAYASHI,1,2,4 MASASHI YANAGISAWA,1,2 SAKIKO HONJOH,1,2 AND HIDEAKI KANO1,3,*

1Ph.D. Program in Humanities, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
2International Institute for Integrative Sleep Medicine (WPI-IIIS), 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
3Department of Chemistry, Faculty of Science, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan
4Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 603-8363, Japan
*hkano@chem.kyushu-univ.jp

Abstract: In this study, second harmonic generation (SHG) and third harmonic generation (THG) spectroscopic imaging were performed on biological samples using a femtosecond laser source in the third near-infrared (NIR) optical window (NIR-III). Using a visible-NIR spectrometer, the SHG and THG signals were simultaneously detected and were extracted using spectral analysis. Visualization of biological samples such as cultured cells (HEK293T), mouse brain slices, and the nematode Caenorhabditis elegans was performed in a label-free manner. In particular, in an SHG image of an entire coronal brain section (8 × 6 mm²), we observed mesh-like and filamentous structures in the arachnoid mater and wall of the cerebral ventricle, probably corresponding to the collagen fibers, cilia, and rootlet. Moreover, the THG images clearly depicted the densely packed axons in the white matter and cell nuclei at the cortex of the mouse brain slice sample and lipid-rich granules such as lipid droplets inside the nematode. The observations and conclusions drawn from this technique confirm that it can be utilized for various biological applications, including in vivo label-free imaging of living animals.

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1. Introduction

Second harmonic generation (SHG) is a second-order nonlinear optical process that was discovered by Franken et al. [1] approximately 60 years ago. Following the development of short-pulse laser sources, SHG has been applied widely as an effective method of wavelength conversion in ultrafast science. Shortly after its application in wavelength conversion, SHG emerged in biological research fields such as SHG microscopy [2–4]. SHG activities due to biological harmonophores have been found in biomolecules and their assemblies in living cells and tissues and have been compiled in textbooks [4]. These well-known harmonophores include biomolecules such as collagen (e.g., that found in mouse tail tendons) [3–5], muscle myosin [6], special types of tubulins such as spindle fibers and nerve axons [7], and spider silk [8]. In terms of protein filaments, collagen, myosin, tubulin, and fibroin have been studied widely and intensively [4]. However, despite these widespread studies, SHG imaging has been applied to limited harmonophores thus far [8–10]. Recently, we previously reported that ciliary rootlets are SHG-active owing to their non-centrosymmetric structures [10]. One of the characteristic features of these rootlets is that the SHG signal intensity is approximately 1/1000 times weaker than that of collagen at...
the sclera. Inspired by this observation, we believe that other biologically intrinsic asymmetric structures with weak SHG activity exist as well. In order to explore these weak SHG signals in non-transparent tissues, however, it is desired to use laser pulses with high peak power and large penetration depths into the samples.

As optical windows are suitable for deep tissue imaging, the NIR-I (650–950 nm), NIR-II (1000–1350 nm), and NIR-III (1550–1850 nm) optical windows [11–15] and various types of laser sources have been developed in these zones. To date, the direct outputs of mode-locked femtosecond laser sources such as Ti:sapphire [5], Cr:forsterite [16,17], and Yb$^{3+}$-doped fiber laser sources [18–21] have played important roles in developing an excitation light source for SHG microscopy. Although these laser sources have exhibited satisfactory performance and proven themselves to be useful, these typical laser systems are bulky, and their emission wavelengths are restricted to the range of 700–1350 nm, corresponding to the NIR-I and NIR-II optical windows. In contrast, the longer attenuation length in the NIR-III window [22] indicates the great potential of this optical window. In addition, a laser wavelength of 800 or 1064 nm should be avoided while performing simultaneous detection of third harmonic generation (THG) signals and SHG signals as the wavelength of the THG signal corresponds to 266 or 355 nm. Therefore, it may be absorbed by tissue samples or microscope objectives. Furthermore, two- or three-photon excitation processes can cause unwanted auto-fluorescence.

Recently, laser sources in the NIR-III optical window were utilized to develop SHG, THG, and multiphoton excitation fluorescence microscopy [11–15]. As its excitation wavelength (1550 nm) is longer compared with those of the NIR-I and NIR-II windows, the microscopic system offers great advantages in increasing the penetration depth, owing to reduced scattering loss [22–24] and photodamage suppression. However, despite these advantages, the biological applications of NIR-III are focused primarily on well-known targets such as collagen and myosin [13,25,26].

In this study, we developed an SHG and THG microscope using a compact Er$^{3+}$-doped fiber laser oscillator. As demonstrated in the pioneering works [13–15], a laser wavelength of 1550 nm provided SHG and THG signals at 775 and 517 nm, respectively, which are perfectly suitable for standard optics and detectors with near-maximum efficiency. We built the spectroscopic SHG and THG microscopy systems by combining a microscope with a spectrometer. This modality was reported in our previous reports on sub-nanosecond [10] and 100 ps [27] laser sources for CARS microspectroscopic setups as well. We simultaneously detected SHG and THG signals in the visible and NIR regions using an ultraviolet–visible–NIR spectrometer with a coverage range of 365–930 nm. Based on the spectroscopic analysis, we recognized clear SHG and THG signals and distinguished them from the background or baseline artifacts. Therefore, the proposed method exhibited great potential to reveal weak and buried SHG signals that were not clearly observed in living cells and tissues.

2. Methods

2.1. Spectroscopic SHG and THG imaging system

The experimental setup is illustrated in Fig. 1(a). The laser source was an Er$^{3+}$-doped fiber laser source (CNT-1550-TK-A, KPHOTONICS, Az, USA). The output wavelength, repetition rate, typical pulse duration, and average power were 1550 nm, 40 MHz, 150 fs, and 100 mW, respectively. The laser pulses were guided by an optical fiber and collimated using a fiber collimator (F280APC-1550, Thorlabs Inc., NJ, USA). As the THG of the fundamental was observed at the output of the laser source, only the NIR-III spectral components were selected by an optical filter (IR80, Kenko-optics, Tokyo, Japan). The laser pulses were introduced into a custom-made inverted microscope (TE2000, Nikon Corporation, Tokyo, Japan) and were focused on the sample through a microscope objective (CFI Plan Apo 60x NA 1.27, water immersion, Nikon, Tokyo, Japan). The sample was placed on a motorized stage (Micro Stage (2-axis), Mad City Labs, WI, USA) with a maximum scanning range of 25 × 25 mm$^2$ combined with a piezo
stage (MCLS03542, Mad City Labs) in the axial direction. The visible and NIR signals were collected using a second objective lens (Plan S Fluor 40× NA 0.6, dry, Nikon, Tokyo, Japan). The respective SHG and THG signals at 775 and 517 nm were detected using a spectrometer (SpectraPro-300i, Princeton Instruments, NJ, USA) equipped with a charge-coupled device (CCD) camera (PIXIS 100 BR, Princeton Instruments, NJ, USA). The SHG and THG signals were extracted by fitting each spectral profile using a Gaussian function, and their respective amplitudes for each spatial position were mapped out.

Fig. 1. Experimental setup of the spectroscopic SHG and THG imaging system. Inset: spectral profile of the fundamental laser pulses.

2.2. Sample preparation

A starch granule is considered to be a model biological sample for SHG microscopy due to its helical amylose structure [28,29]. Therefore, we utilized a potato starch granule to evaluate the performance of the microscope. The potato starch was purchased from a local supermarket. The evaluation was conducted by placing dry starch granules without further purification between a cover glass and glass slide and then sealed with nail lacquer.

HEK293 T cells (RCB2202) were obtained from the RIKEN BRC Cell Bank. The cells were cultured in Dulbecco’s modified Eagle’s medium (Wako, Japan) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. The HEK293 T cells were grown to ~80% confluency in a 35 mm glass bottom dish (Matsunami
Glass, Japan). The culture dish was removed from the incubator immediately before measurement and recording was performed without changing the medium.

Coronal brain sections of mouse were used as samples for wide-area (8 × 6 mm²) imaging. A wild type (C57BL/6 N) male mouse at p118 was deeply anesthetized with 5% isoflurane and an i.p. administration of somnopentyl (32.4 mg/kg). Thereafter, the mouse was transcardially perfused with 0.1 M phosphate buffered saline (PBS) with heparin for 1 min, followed by being transcardially perfused with 4% paraformaldehyde for 10 min. The brain was removed and immersed in the same post-fix buffer at 4 °C for 24 h. The removed brain was washed three times with PBS (5 min each, shaking in a shaker) and subsequently sectioned into 50 µm thick sections using a vibratome (Leica). The resulting brain sections were used for label-free SHG and THG imaging.

Concerning depth-resolved measurement and immunostaining, we prepared the sample in the following manner. The protocol was the same as that described before removing the brain and also used a wild type (C57BL/6 N) male mouse at p84. The brains was then postfixed in 4% PFA at 4 °C overnight, cryoprotected in 30% sucrose in PBS for 2 days, embedded in OCT compound (Sakura Finetech), and stored at −80 °C until use. The brain was cryosectioned coronally at thicknesses of 40 and 300 µm by Cryostat (Leica). These sliced sections were subsequently mounted on glass slides with Mowiol. The samples were stored at 4 °C for 24 h. Optical images were obtained using a stereoscopic microscope (Zeiss AXIO Zoom.V16, Carl Zeiss GmbH, Jena, Germany).

We used anti-neuronal nuclei (NeuN) antibody to visualize neuron cell nuclei and anti-glial fibrillary acidic protein (GFAP) antibody to visualize astrocyte. After sectioning the brain as described above, the 40-µm sections were rinsed with PBS and incubated in 0.4% Block Ace (Snow Brand Milk Products) in PBS with 0.1% Tween20 (0.1% PBST) for 2 h at room temperature. This procedure was followed by overnight incubation with mouse-NeuN antibodies (1:1000; Millipore MAB377) and rabbit anti-GFAP (1:1000; Dako # Z0334) antibodies in 0.2% Block Ace in 0.1% PBST overnight at 4 °C. The sections were rinsed with PBS and then incubated with AlexaFluor488-conjugated donkey anti-mouse IgG (1:1000; Thermo Fisher # A-10667) and AlexaFluor594-conjugated goat anti-rabbit IgG (1:1000; Thermo Fisher # A-11012) antibodies in 0.2% Block Ace in 0.1% PBST for 2 h at room temperature. The sections were rinsed with PBS and incubated with trihydrochloride (Hoechst 33342) (10 µg/ml; Thermo Fisher # H1399) for 10 min at room temperature, then rinsed again and mounted with Vectashield mounting medium (Vector Lab, #H-1700). Fluorescence imaging was conducted using an LSM 800 confocal microscope system (Carl Zeiss, Germany) and NanoZoomer XR (Hamamatsu Photonics, Japan).

Wild-type strain N2 of Caenorhabditis elegans was obtained from the Caenorhabditis Genetics Center (CGC) and was cultured as described previously [30] on agar plates containing a nematode growth medium with OP50 Escherichia coli at 20 °C. A gravid hermaphrodite was mounted on a fresh 2% agar pad with M9 buffer before the measurements.

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Tsukuba. All animals were maintained in accordance with the institutional guidelines of the animal facilities of the Laboratory of Animal Resource Center.

3. Results and discussion

3.1. SHG and THG imaging of a starch granule

To evaluate the performance of the proposed system, we measured a starch granule. Figure 2 depicts the (a) conventional wide field, (b) SHG, and (c) THG images of the starch granule. The image and step sizes were 50 × 100 µm² (51 × 101 pixels) and 1 µm/pixel, respectively. The exposure time at each spatial position was 50 ms. The laser power at the sample position was approximately 20 mW. The spectral profiles of the SHG and THG signals are displayed in Fig. 2(d). As described in Section 2.1, we fitted the spectral profiles using a Gaussian function to
extract their amplitudes as the signal intensity. As demonstrated in Fig. 2(b), the SHG image displays high image contrast in some granule areas. The spectral profile of the SHG signal at the cross of position (i) in Fig. 2(b) is shown in Fig. 2(d), indicating multiple peaks. The spectral feature of the SHG signal is reflected by that of the fundamental laser spectrum (inset in Fig. 1). On the other hand, a strong THG signal is observed outside the granule. The spectral profiles of the THG signal at the crosses of positions (ii) and (iii) in Fig. 2(b) are shown in Fig. 2(d). The THG signal originates from the interface between the air and underlying cover glass, providing a uniform contrast except in the granule area [31]. The THG image visualized the granule, whose peripheral area gave relatively high image contrast as well, which could be due to the refractive index difference between the granule and surrounding air. Similar results were also obtained by using a multimodal nonlinear optical microscope with a 1064-nm laser source [32], which is shown in Fig. S1 in the Supplement 1.

Fig. 2. (a) Conventional wide-field, (b) SHG, and (c) THG images of a potato starch granule. The image and step sizes were 50 × 100 µm² (51 × 101 pixels) and 1 µm/pixel, respectively. The spectral profiles of the SHG and THG signals were obtained at crosses (i), (ii), and (iii) in (b) and (c); inset in (d): zoomed-in view of the THG spectra. The black curves correspond to the results fitted using a Gaussian function.
We evaluated the limit of detection (LOD) using the incident laser power dependence of the SHG signal of a starch granule. The details are described in the Supporting Materials (Fig. S2). With decreasing incident laser power, the SHG signal firstly showed quadratic dependence on the incident laser power, followed by linear dependence, as shown in Fig. S2. Based on this result, the LOD was estimated to be roughly in the order of 10 CCD counts in the present study.

3.2. SHG and THG imaging of cultured cells

For biological applications, we firstly visualized the cultured cells (HEK293 T). Figures 3(a) and (b) display the SHG and THG images, respectively. The image and step sizes were 100×100 µm² (201×201 pixels) and 0.5 µm/pixel, respectively. The exposure time at each pixel was 100 ms. We found clear bright spots in the SHG image, whereas the THG image displayed several large dark and round areas. These dark and round areas were predicted by cell nuclei [33], which are encircled by solid lines (Fig. 3(c)). The merged image of the SHG and THG signals (Fig. 3(c)) indicates that the SHG spots were likely to coexist with the cell nuclei. In our previous study [10], we observed similar SHG spots for cultured cells (COS7) and assigned them as Rootletin filaments that are the linker proteins of centrioles [10,34,35]. Therefore, it was determined that the SHG spots corresponded to Rootletin [10]. Although the SHG spots are weakly observed, the signal is clearly evident in the spectral domain, which is manifested in Fig. 3(d).

We evaluated the upper limit of the lateral spatial resolution using the following procedures. By fitting the spatial profile of the small SHG spots (i) observed in Fig. 3(a) using a Gaussian function, the upper limit of the lateral spatial resolution of SHG imaging was estimated to be 0.8 ± 0.1 µm. Similarly, using the sharp edge indicated by the red line in Fig. 3(b), the upper limit of the lateral spatial resolution of THG imaging was estimated to be 0.8 ± 0.1 µm. Concerning the axial spatial resolution, we evaluated the depth-resolved THG signal at the interface between the glass and air and estimated 1.9 ± 0.1 µm to be the upper limit of the axial spatial resolution for both THG and SHG signals. These values are larger than the numerical simulation results, which are 0.5 (SHG) and 0.4 (THG) µm for the lateral direction and 1.1 (SHG) and 0.9 (THG) µm for the axial direction, probably because the microscope objective was not optimized for 1.55 µm.

3.3. SHG and THG imaging of a mouse brain slice

We performed wide-field SHG and THG imaging to visualize the entire mouse brain slice sample. Figure 4 presents the obtained macroscopic images for the label-free (no staining) (a) and immunostaining (b, c) samples. The macroscopic fluorescent images obtained by Hoechst, GFAP, and NeuN and the correspondences of the regions in (i)–(iv) are provided in Fig. S4 in the Supplement 1. The image and step size used to acquire the SHG and THG images in Fig. 4(a) were 8×6 mm² (801×601 pixels) and 10 µm/pixel, respectively. The exposure time for each pixel was 200 ms. The inset in Fig. 4(a) illustrates a bright-field image of the entire region of the slice sample. We state again for emphasis that the SHG and THG images were obtained simultaneously. The SHG image in Fig. 4(a) (left) displays high contrast at the meninges on the brain surface (indicated by (i)) and the meninges between the hippocampus and thalamus (indicated by triangles). These results were reproduced well using the immunostaining sample, as shown in Fig. 4(b), where the image and step sizes for the SHG image were 4.1×6.4 mm² (411×641 pixels) and 10 µm/pixel, respectively. The exposure time for each pixel was 200 ms. It should be noted that the SHG image in Fig. 4(a) exhibits medium contrast in the areas indicated by (ii) and (iii), corresponding to the cerebral ventricle and cerebral white matter, which will be discussed later with the microscopic images. On the other hand, the THG image in Fig. 4(a) (right) displays image contrast different from that of the bright-field image (inset in Fig. 4(a)), indicating a characteristic contrast enhancement. One of the prominent features is the high contrast in the cerebral white matter, as indicated by area (iii) and two spots denoted by magenta triangles. It should be noted that the THG spots indicated by the magenta triangles were not observed in the
Fig. 3. (a) SHG and (b) THG images of cultured cells (HEK293 T). The image and step sizes were 100 $\times$ 100 $\mu$m$^2$ (201 $\times$ 201 pixels) and 0.5 $\mu$m/pixel, respectively. (c) Merged image of (a) and (b). The broken lines indicate cytoplasm identified by the bright filed. The round areas indicated by the solid lines correspond to the nuclei. (d) Spectral profiles (red) at the SHG spots indicated as (i)–(iii) in (a) and fitted results (black) obtained using a Gaussian function. Note that all spectral profiles were acquired at single pixels, not spatially averaged. The spatial resolution of the THG signal was evaluated at the boundary of the cell and medium indicated by the red line in (b).

bright-field image (inset in Fig. 4(a)). In the cerebral white matter, axons are tightly bundled. The areas indicated by the two triangles contain bundled axons oriented perpendicular to the THG image as well. As both the areas indicated by the cerebral white matter (iii) and the triangles were composed of the same organelles (bundled axons), we assumed that the THG image contrast originated from the densely packed axons that provide a refractive index different from those of the peripheral areas. Moreover, at the cortex indicated by region (iv), the microscopic THG image showed a large number of spots. It will be discussed later.

In the SHG images, several structures extending vertically from the brain surface are evident. One of them is observed in Fig. 4(c), which corresponds to the box indicated by (v) in the merged image (right) in Fig. 4(b). In Fig. 4(c) (middle), GFAP, a marker for astrocytes, is visualized. Astrocytes are known to form the blood–brain barrier, and the GFAP-positive areas in Fig. 4(c) indicate astrocyte-covered blood vessels. In the merged image in Fig. 4(c), the straightened structure with high contrast in SHG is consistent with GFAP, corresponding to a blood vessel.
Fig. 4. (a) Wide-area macroscopic images of the SHG (left) and THG (right) signals of the no-staining mouse brain slice. The image size and step size were $8 \times 6 \text{ mm}^2$ (801 $\times$ 601 pixels) and 10 $\mu$m/pixel, respectively. Inset in (a): conventional wide field; (b) cerebral hemisphere images of immunostaining brain sample. Left: SHG, middle: Hoechst, right: merge (SHG: yellow, Hoechst: cyan); In Figs (a) and (b), the scale bars correspond to 1 mm. (c) zoomed-in view of the blood vessels near the brain surface indicated by the box in (b). Top: SHG, middle: GFAP, bottom: merge (SHG: yellow, GFAP: gray). In Fig (c), the scale bars correspond to 50 $\mu$m.

Next, we performed microscopic imaging of the brain slice samples. Figure 5 summarizes the images obtained around the brain surface for the label-free (no staining) (a) and immunostaining (b) samples. The SHG images exhibit mesh-like structures. In Fig. 5(b), the SHG imaging results are reproduced by the immunostaining sample. In particular, the merged image (SHG, THG, and GFAP) and zoomed-in view in Fig. 5(b) reveal that the SHG signal extends outside the astrocyte end-feet (indicated by GFAP). In a previous study, SHG was reported to visualize the pia arachnoid complex [36]. However, the position of the pia mater is found just above the astrocyte end-feet, indicating that the mesh-like structures are not pia mater, but rather subarachnoid space and/or arachnoid mater. Since Collagen-Ia1-positive inner arachnoid fibroblasts [37] exists at this area, we consider the collagen fibril structure produced by fibroblasts [38] are visualized by SHG. The mesh-like structures were partly visualized in the THG image in Fig. 5(a). This tendency is also clearly observed in the merged image in Fig. 5(a); the mesh-like structures in both the SHG and THG images overlap well. The collagen fibers in arachnoid mater additionally exist at the meninges between the hippocampus and thalamus [37] (indicated by the white triangles in Fig. 4(a) (left)), at which the SHG signal is observed.
Fig. 5. (a) Microscopic SHG and THG images of the no-staining mouse brain slice and their combination (SHG: yellow; THG: magenta) at the arachnoid mater (i). The image size and step size were 200 × 200 µm² (201 × 201 pixels) and 1 µm/pixel, respectively; (b) SHG, THG, and fluorescence images of the immunostaining brain slice and their combination (SHG: yellow; THG: magenta; GFAP: cyan). The image size and step size were 200 × 200 µm² (201 × 201 pixels) and 1 µm/pixel, respectively. The scale bars correspond to 50 µm. The dimensions of the zoomed-in image in (b) are 75 × 75 µm². Schematic of arachnoid cells, pia mater cells, and astrocyte in the merge diagram. SAS: subarachnoid space

Moreover, bright THG spots were sparsely observed in the cortex section that were previously reported and assigned as cell nuclei [39]. The cell nuclei were more clearly observed in area (iv), which corresponded to the deeper position of the cortex where cell bodies densely exist. Therefore, our results are compatible to those obtained using previously reported THG images [39].

Secondly, Fig. 6 depicts the microscopic imaging results around the cerebral ventricle indicated by (ii) in Fig. 4(a) for the label-free (no staining) (a) and immunostaining (b) samples. The SHG image in Fig. 6(a) reveals filamentous structures (i) and underlying straightened structure (ii), which are indicated by arrows in the merged image. The spectral profiles of the SHG signals at filamentous structures are shown in Fig. S3(b). The THG image indicated by (ii) in Fig. 4(a) displays the image contrast at the ventricle walls. Therefore, as depicted in the merged image in Fig. 6(a), these structures in the SHG image are on the ventricle walls.

The overall tendency is reproduced well by the results obtained using the immunostaining sample, and the SHG image in Fig. 6(b) displays high contrast on the ventricle walls. As clearly shown in the zoomed-in views of the merged (SHG (yellow) and Hoechst (cyan)) images, the straightened structure in area (ii) is observed on the ventricular side of each nucleus of the ventricular cells. The filamentous structures indicated by (i) spread out on the ventricular side from area (ii). According to the literature [40], harmonophores can be assigned to the cilia lining the inner walls of the ventricles. However, it is known that ependymal cells, underneath the cilia, have SHG-active Rootletin [10], a component of the rootlet that supports cilia. Moreover, the rootlet appears to be wrapped around the nuclei in ependymal cells [41]. Therefore, we believe that not only the cilia (i), but also the rootlets (ii) of the ependymal cells are observed in the SHG image in Fig. 6.

Thirdly, we investigated the area (iii) in Fig. 4(a) in more detail for the label-free (no staining) (a) and immunostaining (b) samples in Fig. 7. The microscopic THG image in area (iii) depicts the cerebral white matter, where axon bundles exist provides image contrast due to the spatial heterogeneity of the white matter. In addition, the microscopic SHG image in this area reveals numerous distinct SHG spots. The spectral profiles of the SHG signals at several spots are shown in Fig. S3(d). Although the axonal microtubules are SHG-active, their morphologies
are not filamentous. Moreover, fixation is known to lead to a significant loss of SHG signal [42]. Therefore, the SHG spots in area (iii) could not be ascribed to the axonal microtubules. This finding is validated by the results obtained using the immunostaining sample, as shown in Fig. 7(b). SHG spots are not observed for the immunostaining sample, probably because the immunostaining procedures makes the organelles SHG-inactive. Based on the immunostaining results, the cell nuclei (Hoechst) are concentrated in area (iii), where dark THG spots are observed in Fig. 7(b) (bottom row left). On the other hand, the SHG image shows bright spots in label-free samples as Fig. 7(a) (combined). Because neuronal cell bodies at this site were confirmed to be absent by NeuN immunostaining, these nuclei can probably be assigned as derived neuroglial support cells such as oligodendroglia and astrocytes [43]. The presence of astrocyte cell bodies in this region is also confirmed by GFAP in Fig. 7(b) (bottom row right). Therefore, we determined
Fig. 8. (a) Microscopic SHG and THG images of the no-staining mouse brain slice and their combination (SHG: yellow; THG: magenta) at the cortex (iv). The image size and step size were 200×100 µm² (201×101 pixels) and 1 µm/pixel, respectively; (b) SHG and fluorescence images of the immunostaining brain slice and their combination (SHG: yellow; NeuN: cyan). The image size and step size were 400×200 µm² (401×201 pixels) and 1 µm/pixel, respectively. The scale bars correspond to 50 µm.

Fig. 9. Depth-resolved SHG (a) (yellow) and THG (magenta) (b) images. The image size and step size were 100×100 µm² (101×101 pixels) and 1 µm/pixel, respectively. (c) Spectral profiles of the SHG and THG signals at the positions indicated as (i)-(iv) in (a).
that the SHG spots originated from organelles in the neuroglial support cells. Similar spots are also observed at the periventricular (indicated by the arrow) in the SHG image in Fig. 6(a), as confirmed by the spectral profile in Fig. S3(a).

Finally, we will focus on the area of the cortex (iv). The results are summarized in Fig. 8. The macroscopic SHG image indicated by area (iv) in Fig. 4(a) provided negligible visual evidence of the existence of a signal. However, the microscopic SHG image in this area (Fig. 8(a)) exhibits weak but distinct spots. These weak SHG spots are also confirmed by the spectral profiles of the SHG signal in corresponding areas, as shown in Fig. S3(f). At the cortex, numerous cell bodies exist. Area (iv) in the THG image clearly exhibits bright spots, as discussed regarding area (i). These spots originated from the cell nuclei in the cortex [39]. The SHG and THG signals were combined to investigate the origin of the SHG signal in Fig. 8(a), and the immunostaining sample was used to confirm the location of the neuronal nuclei in Fig. 8(b). The label-free sample shows that some of the SHG spots appear close to the cell nuclei. In addition, the immunostaining sample demonstrates that most of the SHG spots are in contact with the NeuN. Therefore, the organelles close to the cell nuclei, such as centrioles, were among the candidates for the SHG source. The centrioles, which are components of the centrosome, are connected to each other by a linker protein called Rootletin [34,35] that is SHG-active [10]. In addition, similar small SHG spots are observed in cultured cells (HEK293) with the same experimental setup (Fig. 3).

![Fig. 10](image-url)

**Fig. 10.** (a) Conventional wide-field, (b) SHG, and (c) THG images of *Caenorhabditis elegans*. The image size and step size were 400 × 100 μm² (401 × 101 pixels) and 1 μm/pixel, respectively.
To evaluate the deep penetration length obtained with 1.55 \( \mu \)m excitation, we performed depth 
(z)-resolved SHG and THG imaging. Figure 9 presents the z-stacking results for the SHG and 
THG images of the cortex with a 10 \( \mu \)m difference in depth position, as well as the spectral 
profiles. The sample thickness was 300 \( \mu \)m. The results demonstrate that image contrast is not 
significantly degraded at deeper positions. As shown at the upper images in Fig. 9(a), the SHG 
signal also displays vascular structures due to collagen. Moreover, the lower images exhibit weak 
SHG spots, as discussed in regard to Fig. 8. These structures are also confirmed by the spectral 
profiles shown in Fig. 9(c). On the other hand, the THG images in Fig. 9(a) depict cell nuclei, 
whose contrast is different at different depth positions.

3.4. In vivo SHG and THG imaging of Caenorhabditis elegans

Next, we performed in vivo SHG and THG imaging of living nematode Caenorhabditis elegans, 
as they are frequently chosen as model animals. Figure 10(a) provides an optical image of the 
worm, and Figs. 10(b) and (c) display the corresponding SHG and THG images, respectively. 
The exposure time at each spatial position was 50 ms. The image and step sizes were 400 \times 100 
\( \mu \)m\(^2\) and 1 \( \mu \)m/pixel, respectively. The SHG image in Fig. 10(b) displayed a high image contrast 
around the pharyngeal and body-wall muscles. These tissues express high levels of muscle 
myosin, which is known to be SHG-active. This finding is consistent with the literature [44,45]. 
In contrast, the THG image in Fig. 10(c) displays numerous bright spots in the intestinal cells, as 
indicated by arrow A. According to the literature [46], several types of granules, such as lipid 
droplets, yolks, endosomes, and other granules, are plausible candidates for the THG spots.

4. Conclusion

In conclusion, we developed a label-free bio-imaging platform using a femtosecond laser source 
in NIR-III (1550 nm). We validated the usefulness and efficiency of the system by measuring 
cultured cells, mouse brain slices, and a living nematode. In particular, the SHG image of the 
brain slice displayed unique microscopic structures such as mesh-like structures due to collagen 
in the arachnoid mater and meninges, filamentous structures due to cilia and rootlet at the cerebral 
ventricle, and spot-like patterns that we assumed were due to neuroglial support cells in the 
white matter. Some of the features observed in label-free SHG and THG images are validated 
by comparing them with immunostaining images. Owing to the large penetration depth of the 
proposed imaging platform due to deep NIR excitation, this technique will pave the way for in 
vivo label-free imaging of living animals.

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Supplemental document. See Supplement 1 for supporting content.

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