Ex vivo and in vivo imaging of myelin fibers in mouse brain by coherent anti-Stokes Raman scattering microscopy

Yan Fu¹, T. Brandon Huff², Han-Wei Wang¹, Haifeng Wang¹, and Ji-Xin Cheng¹, ², *
¹ Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN, 47907, USA
² Department of Chemistry, Purdue University, West Lafayette, IN, 47907, USA

Abstract

Coherent anti-Stokes Raman scattering (CARS) microscopy was applied to image myelinated fibers in different regions of a mouse brain. The CARS signal from the CH₂ symmetric stretching vibration allows label-free imaging of myelin sheath with 3D sub-micron resolution. Compared with two-photon excited fluorescence imaging with lipophilic dye labeling, CARS microscopy provides sharper contrast and avoids photobleaching. The CARS signal exhibits excitation polarization dependence which can be eliminated by reconstruction of two complementary images with perpendicular excitation polarizations. The capability of imaging myelinated fibers without exogenous labeling was used to map the whole brain white matter in brain slices and to analyze the microstructural anatomy of brain axons. Quantitative information about fiber volume%, myelin density, and fiber orientations was derived. Combining CARS with two-photon excited fluorescence allowed multimodal imaging of myelinated axons and other cells. Furthermore, in vivo CARS imaging on an upright microscope clearly identified fiber bundles in brain subcortex white matter. These advances open up new opportunities for the study of brain connectivity and neurological disorders.

1. Introduction

The brain is a massively interconnected neuronal network comprising numerous fiber tracts. The fiber tracts, commonly wrapped by a multiple-layer membrane known as myelin sheath, play crucial roles in reliable and efficient signal transmission. Morphological connectivity in the brain white matter is related to the normal brain function. Irreversible injury to the white matter leads to severe functional loss in many neurological diseases such as stroke and multiple sclerosis [1]. The connectivity of brain fiber tracts and their structural changes in diseases have been studied by several imaging methods, each with their own merits and limitations. Electron microscopy provides ultrastructural information of brain, however, the complicated sample preparation such as fixation and dehydration precludes the possibility of in vivo imaging of brain structure and disease progression. White matter can also be mapped by optical microscopy using luxol-fast blue [2] or lipophilic dyes [3] for myelin staining, but nonspecific binding and inefficient diffusion of dye obscure the data analysis. Positron emission tomography (PET) and magnetic resonance imaging (MRI) have provided a noninvasive way for whole brain imaging to measure white matter properties and diagnose neurodegenerative diseases. PET relies on an exogenous radioactive labeling agent which may induce the potential toxicity to the body and also impair data analysis due to nonspecific labeling [4]. Diffusion

*Corresponding author: E-mail: jcheng@purdue.edu.

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tensor imaging (DTI), an MRI-based method, is able to characterize the diffusion orientation of water molecules within the brain. Analysis of DTI data with magnetic resonance tractography has provided exceptional information on white matter architecture within the human brain [5,6]. Although DTI is becoming an important tool for the diagnosis of various white matter abnormalities, the accuracy of the mapped connectivity is restricted in spatial resolution at the level of millimeter and impaired by the lack of specificity for estimating the fiber orientation.

In this paper, we map the myelin fibers in mouse brain using coherent anti-Stokes Raman scattering (CARS) microscopy that utilizes the intrinsic molecular vibration to produce microscopic images [7–9]. CARS is a four-wave mixing process in which the interaction of a pump field $E_p(\omega_p)$ and a Stokes field $E_s(\omega_s)$ with a sample generates an anti-Stokes field $E_{as}$ at frequency $2\omega_p-\omega_s$ [10]. The CARS signal is significantly enhanced when $\omega_p-\omega_s$ is tuned to a Raman band, creating the vibrational contrast. Compared to the weak spontaneous Raman scattering, the coherent addition of CARS fields from the vibrational oscillators in the focal volume results in a large signal, permitting molecular imaging with a high speed. Moreover, the nonlinear nature of the CARS process ensures that the signal is only generated from the small focal volume, which provides an intrinsic 3D sub-micron resolution. With applications to a broad range of tissue samples including spinal cord [11], arteries [12], atherosclerotic lesions [13], and mammary tumor [14], CARS microscopy is becoming an essential tool for tissue biology. Furthermore, with label-free and high-speed imaging capabilities, CARS microscopy has been applied to intravital imaging of mouse skin [15], mouse sciatic nerve [16], and lipid accumulation in C. elegans [17].

In the central nervous system, the myelin is formed by oligodendroglial cell membranes and contains about 70% lipid and 30% protein by weight [18]. The myelin membrane contains a high density of ordered CH$_2$ groups which produce a large CARS signal. By CARS imaging of myelin in fresh guinea pig spinal tissue with lateral resolution of 0.28 $\mu$m and axial resolution of 0.70 $\mu$m [11], a Ca$^{2+}$-dependent pathway in lyso-PtdCho induced demyelination has been revealed [19]. Recently, CARS microscopy has been used to image brain tumor in fresh and unstained tissues with chemical selectivity, showing the promise of CARS microscopy as a clinical tool for neuropathological diagnosis [20]. However, so far CARS imaging of single myelinated axons in brain has not been achieved. In this paper, laser-scanning CARS microscopy was used to depict the detailed structures of myelin fibers in different regions of a mouse brain. Our data signify the advantages of CARS microscopy for brain white matter research --- label-free, high spatial resolution to resolve single myelinated axons, multimodal NLO imaging of different brain components, and the capability of mapping microscopic structures of brain myelin in vivo.

2. Materials and methods

2.1 Preparation of brain slices

BALB/c mice were anesthetized with 0.4–0.6 mL of a mixture containing 0.625 mg/mL xylazine and 6.25 mg/mL ketamine. During deep anesthesia, they were perfused transcardially with 10 mL of cold phosphate buffer solution (PBS) (pH=7.4), and then with 10 mL of PBS containing 4% paraformaldehyde for in vivo fixation. The whole brain was immediately excised and kept in 4% paraformaldehyde for an additional period of 3 days. The whole brain was then transferred to PBS and sectioned into 300 $\mu$m horizontal slices using a tissue slicer (OTS-4000, Electron Microscopy Sciences, Hatfield, PA). The slices were stored in PBS prior to imaging. For myelin staining, the fixed brain slices were incubated in PBS supplemented with 4 $\mu M$ lipophilic dye DiOC$_6$(3) (Molecular Probes, Inc., Eugene, OR) for 4 h and washed with PBS prior to imaging. For nuclei staining, the fixed brain slices were incubated in PBS supplemented with 0.4 ng/mL Hoechst dye for 2 h and washed with PBS prior to imaging.
2.2 Animal preparation for in vivo imaging

BALB/c mice were anesthetized with 0.4–0.6 mL of a mixture containing 0.625 mg/mL xylazine and 6.25 mg/mL ketamine and held in a stereotaxic apparatus. A dental drill was used to create a circular craniotomy (~3 mm in diameter) above the parietal cortex centered at 2 mm posterior and 2 mm to the right of bregma. The dura beneath was removed carefully. To access the brain white matter, blunt dissection and aspiration were used to remove the grey matter in the cortex and expose the subcortex white matter. A custom-made metal plate with an imaging window (5 mm in diameter) was glued to the skull with dental acrylic cement. The metal plate was fixed to an animal stage to reduce the head movement. PBS was used to cover the exposed area during imaging. After imaging experiments, animals were euthanized by pentobarbital overdose.

2.3 CARS microscopy

The schematic of our CARS microscope was described previously [21]. The pump and Stokes beams are generated from two Ti:sapphire oscillators (Mira 900, Coherent Inc., Santa Clara, CA). Both lasers are tunable from 700 nm to 1000 nm, where water absorption is minimized. One laser operating at 706 nm served as the master and provided the clock for synchronization with the other laser. The frequency difference of two laser beams was tuned to 2840 cm$^{-1}$ corresponding to CH$_2$ symmetric stretching mode. The pulse durations of both lasers were 2.5 ps. The corresponding spectral width matches the line width of the CH$_2$ symmetric stretch Raman band. The two lasers were parallel-polarized and tightly synchronized (Sync-Lock, Coherent Inc) with an average timing jitter of 100 fs. A Pockels cell (Model 350-160, Conoptics) was used to reduce the repetition rate from 78 MHz to 3.9 MHz. This pulse picking method reduces the average power to 5 mW but maintains a high peak power (about 500 W) at the sample. The collinearly combined beams were directed into the scanning unit (FV300) of a confocal microscope (IX70, Olympus America Inc., Center Valley, PA) and focused into a sample through a 60X water immersion objective lens with a numerical aperture (NA) of 1.2 or a 20X air objective lens with an NA=0.75. Polarization of both laser beams was controlled by a half-wave plate. Images were acquired by scanning a pair of galvanometer mirrors in FV300. The epi-detected CARS (E-CARS) signal was collected by the same objective and detected by an external PMT (H7422, Hamamatsu, Japan). The same picosecond laser beams were also used for two-photon excited fluorescence (TPEF) imaging. The epi-detected TPEF signal was spectrally separated from the E-CARS signal by a dichroic splitter, and detected with the same type of PMT. The dwell time for each pixel was 2 μs. The analysis of fiber volumes and inclination angles from the image plane (parallel with the surface of brain horizontal sections) was performed using Image J software.

For in vivo imaging, the same pump and Stokes beams were directed into an upright laser-scanning confocal microscope (FV300/BX51WI, Olympus America Inc.). This microscope was equipped with a dipping mode 60X water immersion objective lens (LUMFL60XW-SP, NA=1.1, Olympus) which focused the beams into a sample. The height of the sample stage can be adjusted to accommodate the animal of different sizes between the stage and the objective. In order to maximize the signal collection efficiency, a special turret (U-IT140, U-DP) was adopted to minimize the distance between the sample and the external PMT detector (H7422, Hamamatsu) at the back port. The dwell time for each pixel was 2 μs. All the imaging experiments were conducted at the optics lab temperature, 22 °C.

2.4 Polarization property of CARS signal from myelin membrane

The CARS signal is dependent on the angle between excitation polarization and the dipole of molecular vibration for molecules with ordered orientation [22]. In our myelin imaging experiment, the pump and Stokes fields are polarized along the same direction and the CARS signal arises from the symmetrical CH$_2$ stretch vibration. When the pump and Stokes excitation

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polarizations \((E_p \text{ and } E_s)\) are in the same direction and have an \(\alpha\) angle with the symmetry axis of \(\text{CH}_2\) groups, we obtain

\[
I(\alpha) = |I_{\text{axial}}|^2 = |3 \chi^{(3)}_{1111} E_p^2 E_s^2 \cos^3 \alpha|^2.
\] (1)

Where \(\chi^{(3)}_{1111}\) is a component of the third-order susceptibility tensor and \(I(\alpha)\) is the CARS intensity. We have demonstrated the dependence of CARS signal on excitation polarization for the parallel myelin in spinal cord white matter [11]. When the excitation polarization and the symmetry axis of \(\text{CH}_2\) groups are parallel along the length of nerve fiber, the CARS signal \((I_{\parallel})\) is maximized. When they are perpendicular to each other, the CARS signal \((I_{\perp})\) is minimized. The higher ratio of \(I_{\parallel}/I_{\perp}\) represents the higher ordering degree of \(\text{CH}_2\) groups in myelin membrane. In the diseased state, myelin disruption could destroy the myelin structure and lose the CARS polarization dependence leading to a 1:1 ratio of \(I_{\parallel}/I_{\perp}\) [18].

For CARS imaging of brain white matter, the polarization dependence needs to be calibrated due to the different orientation of axons (i.e., different values of \(\alpha\)) in one image plane. To minimize the polarization effect, we rotate the excitation polarization by \(90^\circ\) and acquire a second image at the angle of \(\alpha + \pi/2\). A new image is then reconstructed based on the following model,

\[
I = [I(\alpha)^{1/3} + I(\alpha + \pi/2)^{1/3}]^3 = |3 \chi^{(3)}_{1111} E_p^2 E_s^2|^2.
\] (2)

Equation (2) shows that in the reconstructed image, the CARS intensity \((I)\) is independent on angle \(\alpha\) and only relates to the third order susceptibility and excitation fields. Therefore we can reconstruct the two complementary images using Matlab to eliminate the influence of polarization dependence of CARS signals.

### 2.5 Whole brain imaging on a dual-scanning CARS microscope

We constructed a dual-scanning CARS microscope based on a modified confocal breadboard (Lucid Inc., Henrietta, NY). The laser system employed to integrate CARS microscopy on this system has been described previously [12]. The collinearly combined beams were directed onto a multifaceted polygon mirror which provided fast laser scanning along the x-axis [23]. The scanned beams were then passed through a telescope to be focused onto a plane-galvanometer which provided the y-axis scan. The beams were then expanded by a second telescope to fill the back aperture of a 20X air objective (UPlanSApo, Olympus, NA=0.75). Using this system, a 1000 × 1000 pixel image of 250 μm × 250 μm was acquired at a rate of 1 frame per 330 ms. Total power at the sample was ~40 mW. The signal was collected in the backward direction by the objective and separated from the excitation beams by a dichroic mirror (670 DCXR, Chroma Technology Corp., Rockingham, VT), spectrally filtered by band-pass filters (600/65, Ealing Catalog) and detected by a PMT (H7422-40, Hamamatsu Corp.) mounted at the back of the microscope.

In order to enable large-area mapping, a sample stage driven by two stepper motors was utilized. These motors drove the stage along the x- and y-axes in a manner similar to sample scanning. To create a map of a sample, a series of images were obtained by moving the stage in a snake-like pattern, pausing 500 ms at each new position to allow sample vibration from the stage movement to subside before image acquisition. To create a map in which the edges of each image align with those of the adjoining images the stage should not be moved farther than one
full field-of-view between successive images. In CARS microscopy, the field of view can vary with the frequency difference between the pump and Stokes beams [24]. For this reason, the field of view in CARS imaging did not fill the 250 μm × 250 μm area scanned by the system. To compensate for this, a 200 × 200 pixel region where the CARS signal was most uniform was defined as the region of interest and the system was then configured such that the steppers would move the stage a distance of 200 pixels laterally before acquiring each image in the sequence. The result of this operation was that each sequential image partially overlaps with adjacent images. This issue was resolved by clipping the overlapping regions of each image during image stitching.

3. Results

3.1 Validating the CARS signal from brain white matter

To image mouse brain with preserved cellular structures, we performed in vivo perfusion/fxation of BALB/c mouse. The brain was explanted and sectioned into horizontal slices of 300 μm thick after post-fixation. The myelin-rich corpus callosum region was used to evaluate the quality of CARS images. Figure 1(a) shows an epi-detected CARS image of corpus callosum acquired with a 60X water objective. With ωp–ωs tuned to 2840 cm⁻¹ corresponding to CH₂ symmetric stretching vibration, the bundles of myelinated axons displayed a clear CARS contrast. It should be noted that CARS microscopy allows vibrational imaging of fresh tissues in a fixation- and label-free manner [11, 20]. The reason for performing in vivo fixation was to avoid trauma damage of axons during sectioning of fresh brain (see supplemental material Fig. S1).

Due to chromatic aberration during laser scanning, we observed a stronger signal at the center of a CARS image. Such effect is especially obvious when the scanning angle is large or when the two laser wavelengths have a large separation [24]. To minimize such effect, we normalized Fig. 1(a) with a non-resonant CARS image of coverglass acquired with the same imaging parameters. The normalized image was shown in Fig. 1b which illustrated more uniform brightness over the entire field of view. For other images acquired in a smaller field of view such as Fig. 1(c), the dark edge effect was not obvious and the normalization procedure was not performed.

To compare CARS imaging with commonly used fluorescence imaging in which myelin is labeled by lipophilic dyes [25], we incubated fixed brain sections with lipophilic dye DiOC₆(3). After washing, we first acquired an epi-detected TPEF image of labeled myelin (Fig. 1(d)) and then an epi-detected CARS image (Fig. 1(b)) in the same position. The CARS and TPEF signals (Fig. 1(b) and 1(d)) were well colocalized and both displayed densely packed nerve fibers. However, the CARS image showed higher resolution than the TPEF image. This difference became more obvious when a smaller area of the brain slice was imaged at higher magnification (Fig. 1(c) and 1(e)). Blurred fibers were observed in the TPEF image (Fig. 1(e)) while the CARS image (Fig. 1(c)) gave clear contrast of single myelinated nerve fibers. Similar results were obtained in fresh brain slices (Supplemental Fig. S2). The different signal generation mechanism between CARS and TPEF could account for the better spatial resolution in the CARS images. First, the CARS signal directly comes from the myelin lipid whereas the TPEF signal comes from the fluorescent dye DiOC₆(3). The non-specific binding of the labels to structures other than myelin may obscure the contrast. Second, CARS is a third order process with a quadratic dependence on the pump field’s intensity and a linear dependence on the Stokes field’s intensity, whereas TPEF is a second order process and only has a quadratic dependence on the excitation field’s intensity. Therefore, CARS has a smaller excitation volume under the tight-focusing condition. Third, CARS is a coherent process in which the signal is proportional to the square of the number of oscillators in the focal volume. On the other hand, the TPEF signal is linearly proportional to the number of dye molecules in the focal
volume. A combination of these factors result in a much sharper contrast in CARS images. An additional advantage of CARS over fluorescence is of no photobleaching. After laser scanning in the center of the image in Fig. 1(d), the TPEF signal became much weaker than the surrounding area (Fig. 1(f)). Such effect, however, did not occur in CARS imaging because the CARS signal directly comes from intrinsic molecular vibration.

We have further studied the polarization effect in CARS imaging of myelin. Fig. 2(a) shows transverse-sectioning nerve fibers in a horizontal brain slice in which myelin displays a ring shape. It was observed that CARS signal was strongest along the excitation polarization direction (Fig. 2(a) and 2(b)). The ratio of $I_\parallel/I_\perp$ was measured to be 2.71±0.38. Through reconstruction with Eq. (2), CARS signal is nearly uniform along the ring (Fig. 2c). In the CARS images with sectioning along the nerve fibers (Fig. 2(d) and 2(e)), the polarization effect on CARS images became weaker and the ratio of $I_\parallel/I_\perp$ was reduced to 1.58±0.16. This can be explained by the orientation relationship between excitation polarization and dipoles of CH\_2 groups in myelin lipid. The diameters of fiber in mouse brain are mostly near 1 μm which is close to the axial width of the focal volume. The CH\_2 groups in myelin lipid on the top and bottom surface of the fiber contribute to CARS signal equally no matter the excitation polarization is parallel with or perpendicular to the nerve fiber. Thus only CH\_2 groups on two sides of the fiber display the dependence of CARS signal on excitation polarization. However in the transverse-sectioning, all the CH\_2 groups in the ring-shaped fiber contribute to the polarization effect. Therefore, the CARS signal in transverse-sectioning fibers is more dependent on the excitation polarization than that in longitude-sectioning fibers. In the grey matter where myelinated axons orient randomly (Fig. 2(g) and 2(h)), the polarization effect was even smaller and the ratio of $I_\parallel/I_\perp$ was measured to be 1.30±0.11. As a result, little difference between Fig. 2(g)–2(h) and the reconstructed image (Fig. 2(i)) was observed. In the following study of mapping brain structure in a large scale where most fiber bundles are along the horizontal direction, we used horizontally polarized excitation beams for the best contrast without further correction of the polarization effect.

### 3.2 CARS imaging of whole brain

With defined imaging properties and a dual scanning method, CARS microscopy can be used to map the whole mouse brain to illustrate the myelinated fibers in different regions. Figure 3 shows a mosaic CARS image of a horizontal mouse brain section acquired on our dual-scanning CARS microscope. This mosaic was obtained by computer-controlled stitching of 9579 partially overlapped CARS images acquired with a 20X air objective. The mosaic image depicts the gross anatomy of mouse brain. In comparison with the CARS image of a coronal section reported by Evans et al. [20], the horizontal section allowed us to visualize the white matter structure in the cerebellum region (see Fig. 3). The intense CARS signal mainly arose from white matter tracts associated with fiber bundles in the genu of corpus callosum (indicated by ‘gcc’) and fimbria hippocampus (indicated by ‘fi’). Connected with the genu of corpus callosum, cingulum and the following external capsule (indicated by ‘ec’) were able to be identified. In the caudate putamen (indicated by ‘CPu’ and ‘CPu’*), a lot of axonal bundles constituted ‘white islands’ surrounded by grey matter connecting with lateral ventricle (indicated by ‘LV’). The fimbria hippocampus in the left and right sides merged in the midline of the brain, forming dorsal fornix (‘dfi’) where fiber bundles have an orientation perpendicular to the horizontal excitation polarization and thus show relatively weak CARS contrast. Cerebellar white matter also produced a strong CARS signal forming a bright band in the whole brain image. It should be noted that, besides white matter, grey matter such as cortex and thalamic nuclei also produced a relatively strong CARS signal. In such areas, the CARS contrast could arise from CH\_2 groups in cellular membrane, intracellular lipid droplets [26], and mitochondria [7] in accumulated neurons, astrocytes, and other cells. Meanwhile, the myelin in grey matter as a multiple-layer membrane generates stronger CARS signal than other...
single-layer membrane structures. Therefore we observed single myelin fibers but no cell membrane in the grey matter.

### 3.3 Quantitation of fiber volume, density, and orientation

To obtain quantitative information about the volume, density, and orientation of myelinated fibers in a specific brain location, serial CARS images with different depths were stacked together to form 3D reconstructed images. Figures 4(a) and 4(b) illustrate 3D fiber organization in the genu of corpus callosum (‘gcc’) and cortex near the external capsule marked in Fig. 3, respectively. Myelinated axons observed in single frames at selected depths were also shown. The CARS images at each depth were normalized with the non-resonant CARS image of coverglass acquired with the same imaging parameters and then subtracted with a background measured as the average CARS intensity from areas surrounding myelin fibers. The fiber area % and myelin intensity as a function of depth are shown in Fig. 4(c) and 4(d), respectively. In Fig. 4(c) and 4(d), all curves went up first to reach a peak and then dropped. This is because the brain slice was not flat and only some parts of slice were in focus and detected by CARS microscopy at the first few depths. When the entire slice was at the focus, both fiber area% and CARS intensity reach the highest level. The CARS intensity then dropped with increasing depth because of the scattering of incident beams by the white matter. Notably, the fiber area % also dropped with the depth. This is because the decrease of CARS intensity with depth made some myelin fibers at increased depth indiscernible from the surrounding background.

The fiber volume% defined as the percentage of fiber volume in the total imaging volume was obtained by integrating the curve of area% over the depth. With the same imaging volume, higher fiber volume% represents more myelinated fibers. The myelin density defined as the number of lipids in a unit fiber volume and was calculated as the square root of CARS intensity divided by the total fiber volume in the whole imaging cube. A larger myelin density represents that fibers possess higher degree of myelination or myelin with more ordered lipids. The fiber volume% and myelin density in the white matter (‘gcc’) and grey matter (cortex) were calculated and shown in Fig. 4(e). In the equal imaging volume the white matter at ‘gcc’ holds more than 2 times the number of fibers than that of grey matter at the cortex near the external capsule, although their myelin density is similar.

Fiber orientations can also be derived from 3D reconstruction of Z-stack CARS images. In the ‘CPu’ (Fig. 3), each island of white matter tracts constitutes one fiber bundle and CARS images were used to delineate their inclinations with respect to the image plane. Figure 5(a) shows that the fiber bundles are almost parallel to the image plane. One such bundle was enlarged in Fig. 5(a)’ where individual fibers running through the image plane were clearly resolved. On the contrary, the fiber bundles in the ‘CPu*’ (Fig. 5(b)) are nearly perpendicular to the image plane as illustrated in the view from another angle (Fig. 5(b)’). The white matter tracts in the ‘ec’ (Fig. 5(c)) wrap the putamen and connect the cortex, and a 3D reconstruction displays an inclination of 48.4° of fibers in ‘ec’ from the image plane (Fig. 5(c)’). As one of the prominent band of white matter, the ‘fi’ shows strong and sharp CARS contrast (Fig. 3) displaying fiber bundles along the image plane (data not shown). While fiber bundles in ‘dfi’ (Fig. 5(d)) made up by fimbria in two hemispheres incline into the image plane with an angle of 55.2° (Fig. 5(d)’). The cerebellar white matter showing a band with strong CARS signal in Fig. 3 consists of fiber bundles both parallel with and perpendicular to image plane (Fig. 5(e) and 5(e)’).

### 3.4 Multimodal NLO imaging of multiple brain components

For study of complex tissue samples, it is beneficial to incorporate other NLO imaging modalities into a CARS microscope [21,26]. In this study, simultaneous CARS and TPEF imaging were carried out to illustrate the organization between cells and axonal tracts. We used the TPEF signal from the Hoechst dye to locate the cellular nuclei and the CARS signal from myelin to locate the axons. As shown in Fig. 6a, cell nuclei in the cortex seated in the space
among the axon-knitted network were observed in different depths. The stack image of 16-
μm layers with a depth of 1 μm depicts the random cell distribution among the axonal networks. These cell nuclei can be assigned to neurons and astrocytes [18]. Differently, cell nuclei in the white matter are lined in the clefts between axonal bundles illustrated in a stack image of 12 μm layers (Fig. 6b), consistent with the organization of oligodendrocytes in the white matter [18].

3.5 In vivo CARS imaging of mouse brain

With the demonstration of imaging single axons in brain slices on an inverted CARS microscope, we have further tested the capability of CARS microscopy in imaging brain in vivo. To image the brain in live animals, we used an upright microscope with a dipping mode water objective (Fig. 7(a)). The in vivo E-CARS signal is generated both at the interface between the myelin sheath and the intra- and extra- axonal medium [16] and from the back-reflection of forward-CARS signal [15]. In the field of craniotomy at the parietal part of brain, only a few myelinated axons with random orientations were observed in the cortex (Fig. 7(b)). Because CARS microscopy has limited optical penetration depth into the brain tissue, approximately 30 μm in both white matter and grey matter (Fig. 4(c)), we aspirated the grey matter layer in the cortex to access the white matter. The exposed subcortex white matter was then imaged with CARS microscopy. The bundled myelinated axons along one direction (Fig. 7(c)) were clearly observed without any labeling.

4. Discussion

We have demonstrated high-quality CARS imaging of brain white matter based on the signal from CH2 symmetric vibration. Compared with fluorescence imaging of stained myelin, CARS microscopy avoids photobleaching and non-specific binding and provides sharper contrast (Fig. 1). The capability of imaging myelin without any labeling simplifies sample preparations so that white matter in fixed slices, fresh tissues, and live animals can all be investigated. Because the CARS signal directly arises from the myelinated axons, the mosaic CARS image of whole brain provides superior specificity of fiber bundles over other whole brain imaging techniques such as DTI that maps water diffusion routes through the white matter of the brain [5]. Therefore, CARS imaging of whole brain slices could provide complementary information for the study of connectivity of human brain white matter [6]. Moreover, with sub-micron resolution, CARS microscopy can be used to map the organization of cerebral fiber tracts at specific brain locations. Visualization of these intermingled fiber bundles could help elucidate brain functions [27].

As for quantitative analysis, the volume of myelinated white matter can be acquired by volumetric MRI where the white matter volume is obtained by applying the segmentation threshold method to MRI images of brain [28]. In addition, the mean optical density of the myelin stain with Luxol Fast Blue can be used as a histopathological measure of myelin density [29]. Compared to these methods, the CARS signal from myelin sheath allows direct measurement of the volume%, density, and orientation of myelin (Figs. 4 and 5). The quantitative information derived from CARS microscopy could be complementary to other techniques for the analysis of diseases related to myelin sheath. For example, DTI study showed reduced white matter connectivity in corpus callosum of children with Tourette syndrome [30]. Whether fewer interhemispheric fibers or reduced axonal myelination in corpus callosum causes connectivity reduction could be potentially determined by CARS imaging of individual axons followed by analysis of fiber volume% and myelin density. It is anticipated that myelin density should decrease due to myelin degradation in the demyelination diseased brain tissue. Therefore, quantitative information about fiber orientation derived from analysis of z-stack CARS images is useful for construction of brain microstructural anatomy and quantitative

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information about fiber volume% and myelin density are helpful for diagnosis of demyelination diseases.

The current work has shown the feasibility of imaging brain in vivo with epi-detected CARS (Fig. 7). TPEF microscopy with in vivo fluorescence labeling has enabled the investigation of cellular functions in brain grey matter of living animals [31]. However, labeling myelin sheath in white matter in vivo is challenging even using genetic techniques [32]. Using an upright microscope, we have demonstrated in vivo CARS imaging of brain subcortex white matter without any labeling. A major concern for in vivo imaging is the limited optical penetration depth. In this work, aspiration of grey matter was necessary for CARS microscopy to reach subcortex white matter. The optical penetration depth can be increased by using lasers of longer wavelengths to reduce tissue scattering [20] or adaptive optics to compensate wavefront aberration of incident lights [33,34]. Alternatively, CARS imaging with a microprobe objective lens with a 1.3-mm diameter tip allowed in situ imaging of axons in spinal cord with minimal surgery to the vertebra [35]. With a smaller probe with diameter of 350 μm to 1000 μm, fluorescence microendoscopy has provided cellular resolution in deep brain area [36,37]. Coupling CARS modality with these fiber-based microendoscopy techniques promises a way for CARS imaging of deep brain tissues.

Due to the limited imaging depth, invasiveness, and small field of view, CARS microscopy or endoscopy is not yet a competitive tool for clinical diagnosis. Nonetheless, CARS microscopy permits label-free and real-time imaging of myelin structures with 3D submicron resolution. These capabilities are important for monitoring demyelination and remyelination occurring at single myelin fibers and special structure like the node of Ranvier. Additionally, combining CARS with second harmonic generation that monitors astrocyte processes [21] and axonal microtubules [38] and TPEF that captures fluorophore-labeled objects, one could study the communication between myelin and other components in the CNS under physiological conditions [39]. These technological advances provide new opportunities for white matter studies using animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Comparison of CARS imaging with TPEF imaging of myelin labeled by DiOC₆(3). (a) and (b) are raw and normalized CARS images of brain white matter. (d) is TPEF image of the same brain white matter labeled with DiOC₆(3). (c) and (e) are magnified CARS and TPEF images at the center of (a) and (d), respectively. After magnified imaging, photobleaching of fluorescent probes was clearly observed (f), while the CARS image did not show any photobleaching effect.
Fig. 2. 
Reconstruction of CARS images to eliminate the dependence of CARS intensity on excitation polarization. (a), (d), and (g) are CARS images with vertically polarized excitations showing stronger CARS signal from vertically oriented membranes and fibers. (b), (e), and (h) are CARS images with horizontally polarized excitations showing stronger CARS signal from horizontally oriented membranes and fibers. (c), (f), and (i) are reconstructed CARS images showing independence of CARS signal on excitation polarization.
Fig. 3.
Mosaic CARS image of a horizontal mouse brain slice. The whole image contains about 9579 partially overlapped images acquired with a 20X objective. The magnified images at the positions indicated by yellow and black letters are shown in Fig. 4 and Fig. 5. gcc: genu of corpus callosum; CPu: caudate putamen; ec: external capsule; dfi: dorsal fornix; fi: fimbria hippocampus; LV: lateral ventricle.
Fig. 4.
Quantitation of fiber volume% and myelin density using the magnified CARS images at different depths. (a) At the genu of corpus callosum (‘gcc’) with a thickness of 31 μm and an imaging depth step of 0.5 μm. (b) At the cortex with a thickness of 30 μm and an imaging depth step of 0.5 μm. These images were taken by a 60X water objective. (c) Analysis of fiber area % (percentage of fiber area in the whole image area) at each depth. (d) Distribution of CARS intensity at each depth. (e) Comparison of fiber volume% (percentage of fiber volume in the whole image volume) and myelin density (the number of lipids in a unit fiber volume) in ‘gcc’ and cortex.
Fig. 5.
Magnified CARS images showing the fiber orientation with respect to the image plane. (a-a') Magnified fiber bundles at ‘CPu’ position in the Fig. 3 where fiber bundles orient along the image plane. (b-b') Magnified fiber bundles at ‘CPu*’ marked at Fig. 3 showing that fiber bundles go through the image plane perpendicularly. (c-c') Magnified image of ‘ec’ marked at Fig. 3 showing an inclination of 48.4° of fibers through the image plane. (d-d') Magnified image of ‘dfi’ marked at Fig. 3 showing that fiber bundles incline into the image plane with an angle of 55.2°. (e-e') Magnified image of ‘cerebellar white matter (WM)’ marked at Fig. 3 showing the perpendicular fiber bundles.
Fig. 6.
Simultaneous CARS imaging of axons and TPEF imaging of nuclei in slices of grey matter and white matter. Red: CARS signal from myelin sheath. Green: TPEF imaging of Hoechst labeled cell nuclei. (a) 3D imaging of a 16 μm-thick layer in grey matter with a depth of 1 μm. Different layers show different cell nuclei. A Z-stack image shows irregular cell distribution in the axonal networks. (b) 3D imaging of a 12 μm-thick layer in white matter with a depth of 1 μm. A Z-stack image shows cell locating along lines between the axonal bundles. YZ and XZ images along the corresponding lines indicated by arrows show the nerve fiber orientation and other views of cell nuclei.
Fig. 7.

In vivo CARS imaging of mouse brain using an upright microscope with a dipping mode water objective. (a) A schematic of the in vivo CARS microscope. D: dichroic mirror. (b) CARS image of the parietal cortex. (c) CARS image of bundles of myelinated fibers in the subcortex white matter.