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EDGE ARTICLE

Label-free chemical imaging *in vivo*: three-dimensional non-invasive microscopic observation of amphioxus notochord through stimulated Raman scattering (SRS)[†]

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Notochord is a rod-shaped axial supporting structure unique only to chordates. In this study, we use cephalochordate amphioxus (*Branchiostroma belcheri*, a living basal chordate) and zebrafish (*Danio rerio*), a vertebrate, as model animals and employ stimulated Raman microscopy (SRS), a newly developed label-free technique, to investigate notochord structure and chemical composition in live animals. Coherent anti-Stokes Raman scattering (CARS) images have also been acquired for comparison. Both CARS and SRS images can construct the detailed three-dimensional structure of the notochord with resolution better than 1 μ m. Label-free live imaging allows us to obtain the whole animal's intact internal morphology, which is difficult to obtain through other mechanical or optical sectioning methods. Intrinsically, chemical sensitive SRS images, with a simple data processing procedure, show that the amphioxus notochord is protein-rich. Our result agrees well with that drawn from the labeling and cryo-sectioning observation, verifying that the coherent Raman scattering techniques are useful to examine the structure and chemical composition of live animals without labels in a fast, simple and accurate way.

Introduction

The development of optical microscopes has allowed investigations of the micro-scale world with non-invasive optical detection at sub-micron spatial resolution. It has always been of great interest to directly observe the chemical distribution in a specimen, especially living organisms.^{1,2} The distribution and the quantity of specific chemical components can help us to decipher detailed mechanisms of living processes. Many chromophoric chemical reactions have been developed for centuries to specifically label certain chemicals.^{3–5} By tagging or generating⁶ fluorescent probes for molecules of interest, or genetically coding fluorescent probes,^{7–9} many systems can be studied at the single molecule level.^{10,11} However, many staining processes may terminate the normal life processes or create many side effects to perturb the living organisms.^{12,13} Moreover, many staining protocols for fixed samples are not suitable for living animal studies because the labels may be accumulated in the metabolic process, leading to misleading results.¹⁴ Labeling is also difficult for many molecules, especially small molecules, due to the lack of specificity.^{15,16} Although many newly developed labeling techniques have shown promising applications, label-free chemical imaging is still highly desired for investigating living biological species.

Both second harmonic generation (SHG)^{17,18} and third harmonic generation (THG)^{19,20} microscopies are able to provide label-free images with diffraction-limited spatial resolution. SHG is highly chemically selective, but the targets are limited by the requirement of optical nonlinearities of specific molecules or their orientations, while THG is poor in chemical specificity. In infrared microscopy, the spatial resolution is limited²¹ and the water absorption makes IR microscopy unsuitable for live imaging. Spontaneous Raman has improved spatial resolution by using visible light and three-dimensional (3D) images can be obtained with a confocal laser Raman microscope. However, due to the low cross-section of spontaneous Raman scattering, the signal is extremely weak. Usually for an image with appreciable contrast, both high power excitation lasers and a long image acquisition time are needed.²²

Coherent Raman scattering, including coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS), can strongly enhance the Raman signals^{23–26} and push the

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detection sensitivity close to the shot-noise limit.²⁵ With increased sensitivity, the image acquisition time is significantly shortened, offering the possibility to capture dynamic behavior in vivo.^{27,28} Both approaches require two laser beams, the pump and the Stokes, which are colinearly aligned and tightly focused on the sample.^{15,29,30} Similar to other multi-photon microscopy techniques, the nonlinear process of CARS and SRS only happens at the focus, providing intrinsic 3D sectioning capability.25 CARS offers label-free morphological images,31,32 but it faces difficulties in providing quantitative assessment due to the non-resonant background,³³ the nonlinear concentration dependence³⁴ and the distorted spectra.^{23,24,35,36} SRS, which has almost identical spectra to spontaneous Raman scattering^{15,26} and the absence of the non-resonant background, 15,26,28,37 is much more convenient for quantitative chemical imaging. SRS signal intensity is linearly proportional to the concentration, making quantitative imaging reliable in transparent samples. For samples that are not particularly transparent, the influence of scattering and absorption should be considered deep in tissue. Amphioxus lies at the evolutionary base of chordates and is

a closely related ancestor of vertebrates. The notochord is the first example of an axial skeleton and persists in chordates. In vertebrates, however, the notochord is present only in the embryo state. The notochord in amphioxus consists of musclelike cells that are arranged in a striped pattern, while the notochordal cells in vertebrates are vacuolated and transform gradually from mainly epithelial type to the mesenchymal (connective) cells.

In this study, we use cephalochordate amphioxus (Branchiostroma belcheri) and the vertebrate, zebrafish (Danio rerio) as model organisms and applied label-free SRS and CARS microscopy to perform 3D chemical imaging in vivo. Both SRS and CARS microscopy generate appreciable chemical contrast on these biological samples and the SRS images show substantially fewer artifacts. Distributions of different chemical components in the sample are measured with SRS microscopy. The results verify that the notochord of amphioxus is a proteinrich organ rather than a lipid-rich organ,³⁸⁻⁴¹ which agrees with the results of conventional staining methods using cryo-sectioned samples. Amphioxus larvae and zebrafish embryos were imaged in vivo using SRS and both the morphological and chemical differences in notochord structure are clearly represented. Our results demonstrate that SRS microscopy is an ideal tool for quantitative in vivo label-free chemical imaging. 3D reconstruction from SRS images also provides the whole animal's intact internal morphology, which is difficult to obtain through other mechanical or optical sectioning methods.

Experimental section

Sample preparation

Adults and larvae of amphioxus were collected from the South China Sea (Beihai, Guangxi, China) at an average depth of 20 m. 1 and 2 days post-fertilization (dpf), wild type zebrafish embryos were selected for SRS imaging of notochord. 1-phenyl-2-thiourea (PTU) was added to prevent pigment formation. The animals were anesthetized by tricaine $(0.08\% \text{ W V}^{-1})$ for *in vivo* imaging, or were snap-frozen in liquid nitrogen and cryosectioned in sagittal and transverse directions. Each section had a thickness of 30 $\mu m.$

Spontaneous Raman spectroscopy

The spontaneous Raman spectra were acquired using a confocal Raman spectrometer (Labram HR800, Horiba Jobin Yvon Inc., Japan). A 10 mW (at focal plane) 532-nm laser was used to excite the sample through a 50X, 0.75NA objective (MPlan N, Olympus). The data acquisition took 1s and was repeated 5 times. The data processing including background correction was performed with the LabSpec software.

Staining of lipid and protein in sectioned samples

For bright field observation of sectioned samples, lipid was stained with Oil Red O (ORO). 300 mg of ORO was dissolved in 100 mL isopropanol (IPA) to make the stock solution. The staining solution was made of 3 parts of stock solution mixed with 2 parts of water, and filtered before use. The sagittal sections of amphioxus notochord were fixed with neutral formalin and immersed in ORO staining solution for 30 min. The excess dye was washed away by 60% IPA solution in water. Protein was stained with alcoholic mercury-bromphenol blue reagent (Hg-BPB). 10 g of HgCl₂ and 100 mg of bromphenol blue were dissolved in 100 mL 95% ethanol to form the staining solution. The samples were immersed in the staining solution for 15 min and rinsed for 20 min in 0.5% acetic acid, then immersed in water for 3 min to convert into the blue alkaline form. Stained samples were visualized using an upright microscope (BX51, Olympus) with a 10× objective (NA 0.30, UPLFL 10X). ORO and Hg-BPB images were obtained with a cooled digital color camera (DP72, Olympus).

Label-free SRS/CARS microscopy

A high-power 1064 nm picosecond Nd:YVO₄ laser (picoTRAIN, HighQ, Austria), which produces 8 ps pulses at a 76-MHz repetition rate, was used as the laser source. A portion of the output was frequency-doubled to 532nm ($\sim 4W$) to pump an optical parameter oscillator (OPO) (Levante Emerald, APE-Berlin, Germany). The OPO produced a signal beam, tunable between 680-1010 nm, which was used as the pump beam in CARS and SRS microscopy. A band-pass filter (CARS 890/ 220m, Chroma Technology, USA) was placed in the output of the signal beam. Another portion of the 1064 nm beam was used as the Stokes beam, modulated by an Electro-Optic Modulator (EOM) (EO-AM-NR-C1, Thorlabs, USA) at 9.78 MHz. The modulation frequency was provided by an arbitrary function generator (AFG310, Tektronix, USA). Both pump and Stokes beams were divided into two beams by polarization beam splitters (PBS) (Dayoptics, China), making the simultaneous use of two microscope systems possible. Each Stokes beam had a manual delay line to adjust the temporal overlap of laser pulse trains between pump beam and Stokes beam, which were combined through a dichroic mirror (1064DCRB, Chroma). A $\lambda/4$ waveplate was added between EOM and PBS for Stokes beam and a $\lambda/2$ waveplate was added before PBS for the pump beam, making it easy to distribute power between two microscopes. Each pair of the two spatially and temporally overlapped

laser beams was directed into a microscope. Two laser scanning microscopes, an up-right one (DM6000B/TCS-SP5 II, Leica Microsystems, Germany) and an inverted one (IX81/FV1000, Olympus, Japan), were used for SRS/CARS microscopy. For the Leica system, a $20 \times$ dry objective (NA 0.70, HC PL APO/CS) was used for imaging sectioned samples. The transmitted light was collected with an air condenser (NA 0.90, POL 0.90 S 1). A 20× water immersion objective (NA 1.00, HCX APO L W) and an oil immersion condenser (NA 1.40, P 1.40 OIL S 1) were used for imaging live samples. To acquire the high-magnification images, we used an Olympus system with a $60 \times$ water immersion objective (NA 1.20, UPLSAPO 60XW) and an oil immersion condenser (NA 1.4). The condensers were aligned at the position for Koehler illumination. Forward SRS signal was collected from the condensers and the Stokes beam was filtered out by a bandpass filter (CARS 890/220m, Chroma). A large-area photodiode (FDS1010, Thorlabs) with 60V reverse bias was used for signal detection. The output of the photodiode was filtered by an electronic bandpass filter (9.5-11.5 MHz, BBP-10.7, Mini-Circuits, USA) and then demodulated by a home-built lock-inamplifier (by Brian Saar).28 The influence of laser power fluctuation can be removed by high frequency modulation of the incident beam and demodulation of the signal.¹⁵ For the Leica system, a scan speed of 400 lines/s was used without zoom and 700 lines/s was used when zoom was enabled. For the Leica system, the average powers of the pump and the Stokes at the focus were 60 mW and 50 mW for C-H bond imaging. For the Olympus system with acquisition rate at 2 µs/pixel, the average powers of the pump and the Stokes at the focus were 15 mW and 20 mW for C-H bond imaging. The relatively low average power of excitation at the near-infrared wavelengths reduced the photodamage in the samples. For SHG microscopy, the excitation wavelength was set at 890nm (40 mW at the focus) and the signal was acquired by forward NDD using the Leica system. Since both the SRS and SHG signal were acquired in the forward direction, they cannot be performed at the same time. Images were acquired using the Leica Application Suite and Olympus FluoView software. The raw data were processed using MAT-LAB (Mathworks, USA). 3D images were reconstructed with Amira (Visage Image, Austria).

Results and discussion

Chemical analysis of tissue inside the living animal body is not trivial since most existing methods are harmful to living organisms. Notochord is an organ that plays a critical role in pattern formation during development.⁴² Notochord, a stiffening axial rod in the body, distinguishes chordates from other animals.42,43 Notochord has been found in the early development of all chordates, but in most chordates it disappears after the embryo period and is replaced by the vertebral column. Amphioxus, also known as lancelet or as cephalochordates (Fig. 1(a)), is the only organism that keeps the notochord as a skeletal support throughout the whole life.41,43 Based on its remarkable morphology, amphioxus has long been considered the most basal chordate. This conclusion has been recently confirmed by the draft genome sequence.44,45 Splitting from vertebrates more than 520 million years ago,45 amphioxus and its unique notochord structure are one of the key components to help understanding

the evolution and origin of vertebrates. Many studies have been done to reveal the structure of the amphioxus notochord, but few have been done with live animals.^{39–41}

The structure of notochord in adult amphioxus is distinctive from its counterparts in other chordate embryos. The amphioxus notochord, a solid rod of tissue that lies between the dorsal wall of the gut and the ventral wall of the nerve cord, 43,46 is made up of a cylinder of cells that are vertically stacked together (Fig. 1(b)). It is flexible, but rigid enough as a supporting structure for the whole body, extending from posterior end to the anterior end.41,46 A transmission laser scanning microscopic image of a living amphioxus is shown in Fig. 1(c). The image is blurred due to the light absorption and scattering through muscle tissues. In the sagittal section, we observed numerous parallel-aligned notochordal plates attached to the notochordal sheath, as shown in Fig. 1(d). The notochordal plates contain muscle and connective tissue, while the notochordal sheath is pure connective tissue, probably made of collagen fibers.⁴¹ We took a transverse section (Fig. 1(e)) of amphioxus and measured the spontaneous Raman spectra (Fig. 1(f)) at several locations: notochordal plate (np), notochordal sheath (ns), neural tube (nt) and muscle. The result shows that all of the tissues have strong peaks at CH₃ stretching band (2950 cm^{-1}) and Amide I band (1650 cm^{-1}), indicating the dominant existence of protein. The neural tube shows higher intensity of C-H stretching from CH₂ at 2845 cm⁻¹, indicating that it has a greater lipid composition. Staining the lipid with Oil Red O (ORO) and protein with alcoholic mercury-bromphenol blue (Hg-BPB) supported this conclusion (Fig. S1[†]). Live animal 3D chemical imaging is optimal for morphological studies since the cryo-sectioning may destroy details of the sample, especially the integrity of notochordal structure in amphioxus. Although the spontaneous Raman spectroscopy can identify the chemical distribution from the cryo-sectioned samples, it cannot be applied to live amphioxus. Spontaneous Raman takes ~1h to acquire an image with acceptable resolution, which is futile for live animal imaging. The SRS setup is shown in Fig. S2⁺.⁴⁷ One beam is the pump beam at frequency $\omega_{\rm p}$ and the other is the Stokes beam at $\omega_{\rm S}$. When the frequency difference $(\omega_{\rm p} - \omega_{\rm S})$ matches the Raman frequency $Q_{\rm vib}$ of the specific chemical component of interest, the intensity of the Stokes beam experiences a gain called stimulated Raman gain (SRG) while the intensity of the pump beam experiences a loss called stimulated Raman loss (SRL). Although SRS intensity is orders of magnitude higher than spontaneous Raman intensity, it is still small compared to the incident laser intensity. The intensity change due to either SRG or SRL, typically less than 10⁻⁴, is much smaller than the laser intensity fluctuation. To pick up this weak signal, we modulate the Stokes beam at a high frequency (~10 MHz) at which the 1/f noise of laser intensity and the sample heterogeneity effects can be removed through lock-in detection.¹⁵ We used a bandpass filter to block the Stokes beam in front of the detector and detect the SRL of the pump beam.

Another related nonlinear process, CARS, occurs simultaneously when SRS is generated. CARS is a parametric process, during which a new frequency of light is created at $\omega_{aS} = 2\omega_p - \omega_S = \omega_p + \Omega_{vib}$. In our system, we detected SRS signal in the forward direction, while the backward CARS signal is collected simultaneously through photomultiplier tubes built inside the non-descan-detector units of microscopes.



Fig. 1 Notochord in amphioxus. (a) Bright field macro-images of an amphioxus head. Inset is a transmitted polarizing dark field image of a larval amphioxus. (b) The structure of the notochord; *np*: notochordal plate; *ns*: notochordal sheath; *nt*: neural tube. (c) A transmission microscopic image of a living larval amphioxus, Scale bar = $100 \,\mu\text{m}$. (d) A sagittal section of amphioxus notochord, scale bar = $200 \,\mu\text{m}$. (e) A transverse section of amphioxus notochord, scale bar = $200 \,\mu\text{m}$. (e) Spontaneous Raman spectra of amphioxus notochord, measuring at different locations marked in (e). The neural tube (red cross) shows higher intensity at 2845cm^{-1} , indicating it has more lipid component.

To quantitatively analyze the different chemical species in biological samples, we need to identify the proper Raman frequencies for image acquisition. We took the SRS images of pure lipid (glycerol tristearate, GT) and protein (bovine serum albumin, BSA) samples and measured their spontaneous Raman spectra, as shown in Fig. 2. The protein was first dissolved in water and then dried on a glass slide to form a thin film for measurement. SRS images are taken at both 2845 cm⁻¹ (CH₂ stretching) and 2950 cm⁻¹ (CH₃ stretching). Lipid has stronger signal at 2845 cm⁻¹ than at 2950 cm⁻¹, while protein has the opposite trend. This is not surprising since the CH₂/CH₃ ratio is much higher in lipid than in protein. From the image acquired at both wavenumbers, we can derive the quantities of lipid and protein. The average pixel intensities of Fig. 2(a–d) can be recorded as I_{L2950} , I_{L2845} , I_{P2950} , and I_{P2845} , respectively. For any pixel in the sample images, the intensities i_{2950} and i_{2845} can be written as

$$\begin{bmatrix} i_{2950} \\ i_{2845} \end{bmatrix} = \begin{bmatrix} I_{\text{L}2950} & I_{\text{P2950}} \\ I_{\text{L}2845} & I_{\text{P2845}} \end{bmatrix} \begin{bmatrix} C_{\text{L}} \\ C_{\text{P}} \end{bmatrix},$$

where the $C_{\rm L}$ and $C_{\rm P}$ are concentrations of lipid and protein at each pixel. They can be easily calculated as



Fig. 2 Coherent Raman signal strength of lipid and protein. (a–d) SRS images of glycerol tristearate and bovine serum albumin (BSA) at the CH_3 stretching vibration (2950 cm⁻¹) and the CH_2 stretching vibration (2845 cm⁻¹). Protein has a stronger SRS signal at 2950 cm⁻¹, while lipid has a stronger signal at 2845 cm⁻¹. (e) Spontaneous Raman spectra of glycerol tristearate and BSA.

$$C_{\rm L} = \frac{i_{2950} \cdot I_{\rm P2845} - i_{2845} \cdot I_{\rm P2950}}{I_{\rm L2950} \cdot I_{\rm P2845} - I_{\rm L2845} \cdot I_{\rm P2950}},$$

$$C_{\rm P} = \frac{I_{2845} \cdot I_{\rm L2950} - I_{2950} \cdot I_{\rm L2845}}{I_{\rm L2950} \cdot I_{\rm P2845} - I_{\rm L2845} \cdot I_{\rm P2950}}$$

With C_{L} and C_{P} , we can map the distribution of lipid and protein in our samples. Before the processing, all of the images need to be re-normalized by the intensity of the pump and Stokes laser beams. This model presented is a rough estimate, since in animals there are many types of lipids and the tristearate is just one of them. More accurate results can be obtained if the composition of the lipids has been obtained beforehand.

Using this method, we captured SRS images of the cryosections of amphioxus and observed the chemical distribution of both lipid and protein through paired images at 2950 cm⁻¹ and 2845 cm⁻¹. We chose the cryo-sectioning samples to verify our method because the sections could be reproducibly examined using multiple wavelengths at different locations and with different magnifications. We also compared our label-free images with conventional staining approaches, which could not be performed using live animals. Figs 3 and 4 show the results from sagittal and transverse sections, respectively. All of the images show the clear morphological structure of notochord, including the well-aligned notochordal plates - the characteristic feature of amphioxus. For all of the samples, the SRS signal at 2950 cm⁻¹ is much stronger than that at 2845 cm⁻¹, indicating that the major chemical component of notochord and other surrounding tissues is protein. This observation is also verified through direct imaging of the protein's amide I band at 1650 cm^{-1} , which shows identical morphological features as those observed at 2950 cm⁻¹. However, although the amide signal may be more chemically specific for protein, the CH₃ signal is much stronger, providing better contrast and signal-to-noise ratio.

Fig. 3(p) shows the mosaic image stitched from 84 highmagnification SRS images at 2950 cm⁻¹ using a 60× objective. Protein dominates the whole notochord structure. The concentration of lipid is low and the weak CH₂ signal from lipid is obscured in the signal from protein. Although direct observation of lipid is impractical in our case, with the method presented above we can unveil the lipid distribution quantitatively, as shown in Fig. 3(q-s) and Fig. 4(h-i). SRS images of the transverse sections show that both notochordal plates and notochordal sheath are protein-rich tissues with fibroid structures. The thin fibers in the notochord plates were mostly myofilaments that have been observed by electron microscopy.⁴¹ With the $60 \times$ water immersion objective we achieved the lateral resolution close to the diffraction limit (Fig. 4(g)). These fibers, mostly collagen, which is confirmed by SHG microscopy images (Fig. S3[†]), are not perfectly aligned in the sheath. This characteristic feature is identical to the previous report using electron microscopes.^{38,41} Fig. 4(h-j) show the protein and lipid distributions along a transverse section of amphioxus. There is an area adjacent to the notochord with a noticeably higher concentration of lipid. This organ is the neural tube and the imaging result matches the indication from microscopic spontaneous Raman spectra in Fig. 1(f).

Since the CARS images are obtained simultaneously with SRS detection, we can compare the images taken by these two



Fig. 3 SRS and CARS microscopic images of sagittal sections of amphioxus notochord. (a–e) $20 \times$ objective, scale bar = $100 \ \mu\text{m}$. (f–j) $20 \times$ objective with $1.7 \times \text{zoom}$, scale bar = $50 \ \mu\text{m}$. (k–o) $20 \times$ objective with $2.5 \times \text{zoom}$, scale bar = $50 \ \mu\text{m}$. SRS images at 2950 cm⁻¹ (CH₃ stretching vibration) and 2845 cm⁻¹ (CH₂ stretching vibration) show drastic differences in signal intensity, while CARS images are very similar at these two wavenumbers. SRS images at 1650 cm⁻¹ show the contrast from protein amide I band. (p) A mosaic SRS image of notochord at the CH₃ stretching vibration. Scale bar = $500 \ \mu\text{m}$. (q–s) Lipid and protein distribution in notochord processed from images (a) and (b). Scale bar = $100 \ \mu\text{m}$.

technologies under identical conditions. CARS images of amphioxus notochord at both 2950 cm⁻¹ and 2845 cm⁻¹ are shown in Figs 3 and 5. The intensity at 2950 cm⁻¹ is slightly higher than that at 2845 cm⁻¹. The non-resonant background of CARS not only limits the sensitivity of detection, it also seriously distorts the CARS spectrum through constructive and destructive interference with the resonant vibrational modes at both

sides of the peak.^{23,24,36} This distortion generates inevitable difficulties for identifying the chemical composition through either the images or the spectra. The intensity of the signal in CARS is not linear to the concentration of molecules, making quantitative studies complicated.³⁴ Several approaches have been developed to do quantitative CARS measurements, such as Multiplex-CARS^{48,49} and heterodyne detection.^{36,50} As shown in



Fig. 4 SRS microscopic images of transverse sections of amphioxus notochord. (a–c), $10 \times$ objective, scale bar = 200 µm. (d–f), $20 \times$ objective with $1.7 \times$ zoom, scale bar = 50 µm. The images show similar information to that we observed from Fig. 3(g). A high-resolution SRS image of the notochordal sheath at 2950 cm⁻¹ (CH₃ stretching vibration). Scale bar = 5 µm. (h–j) Lipid and protein distribution in a notochordal plate processed from images (a) and (b). The neural tube contains more lipid than other organs. Scale bar = 200 µm.

the images, conventional CARS does offer great contrast to observe the morphology without labeling and all of the details of notochordal plates and fibers are also shown with similar resolution to SRS images. However, with the conventional CARS images taken at 2845 cm⁻¹ and 2950 cm⁻¹, it is challenging to separate the protein from lipid. SRS, with almost identical spectra to spontaneous Raman, is more suitable to provide the quantitative chemical images.

Amphioxus notochord, full of myofilaments and collagen fibers, enables the organ to support the body and also serves as a mechanical swimming organ.41,46 Amphioxus notochord cells act like muscle cells,46 which is different from all other chordates, including all vertebrates. To see this difference chemically, we applied SRS microscopy to study the notochord in both living amphioxus and living embryonic zebrafish. During development, the central cells of the zebrafish notochord acquire large vacuoles, which eventually lead to tissue degeneration.⁵¹ The proteinrich fibrous muscle cells are clearly represented in the SRS images of zebrafish (Fig. 5), while the notochord has a much weaker signal than the surrounding muscles. Only membranes between the vacuolar cells generate a faint SRS signal at 2950 cm⁻¹, 2845 cm⁻¹ and 1650 cm⁻¹, coming from both protein and lipid. The notochordal cells in zebrafish, as well as in other vertebrates, will gradually transform from predominantly epithelial type to the more mesenchymal type of cells, which is not only

morphologically different, but also chemically different from the notochordal cells in amphioxus.

SRS microscopy, with great penetration length and intrinsic 3D sectioning ability, is perfectly suitable for whole animal imaging of live amphioxus. We took the z-scan of the living amphioxus head at 2950 cm⁻¹ to visualize the unique 3D structure of the protein-rich notochord. The notochord is embedded 200-300 µm deep inside the body head part. We take 45 sectioning images, with 2 μ m in between, to reconstruct the 3D SRS image (Fig. 5(o-r)). The characteristic notochordal plates are clearly shown in the images, with a more uniform and ordered structure than that observed in cryo-sectioned samples. That is because the integrity and the structural details of tissue are partly destroyed by blade cutting. The strain of the functional muscle fibers and other structural components in the notochord will also release when the whole structure is broken, altering the structure of the organs. From the sagittal section of the 3D reconstructed structure of notochord (Fig. 5(r)), we obtained the images with resolution better than 1 µm and measured the average thickness of each stacked notochordal cell to be 20 µm. During the measurement there was no observable photo-damage to the sample. This chemical specific imaging technology can be of great potential to observe the dynamic and detailed structural information from living organisms with no need for labeling.



Fig. 5 SRS and CARS images of living zebrafish and amphioxus. (a–j) SRS and CARS images of living zebrafish larvae tail. Scale bar = $50 \ \mu m$. (k–n) SRS and CARS images of living amphioxus larvae head. CARS images suffer from the strong non-resonant background of water. Scale bar = $100 \ \mu m$. (o–r) A 3D reconstruction SRS image of a living amphioxus larvae head at 2950 cm⁻¹. Scale bar = $100 \ \mu m$.

Conclusions

We use SRS microscopy as a label-free imaging technique to identify the chemical components in amphioxus notochord. By selectively visualizing specific molecular vibrations at 2845 cm⁻¹ and 2950 cm⁻¹, images with appreciable contrast from proteins and lipids were obtained through image processing. The results confirmed results from conventional staining methods. Unlike CARS microscopy, SRS microscopy does not suffer from non-resonant background and displays a clean and undistorted spectrum, making quantitative analysis possible. The intrinsic 3D sectioning ability also makes SRS microscopy suitable for imaging live animals. We reconstructed the 3D structure of the amphioxus notochord based on the SRS images. SRS

microscopy has shown to be a powerful tool to perform noninvasive label-free imaging on biological samples with high resolution and chemical specificity.

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Supporting Information



Fig. S1. Conventional staining of lipid (a) and protein (b) using amphioxus sections. Lipid was stained with Oil Red O, and protein is stained with Hg – bromophenol blue. Scale bars = $200 \mu m$.



Fig. S2 The stimulated Raman scattering microscopy setup. A high repetition rate picosecond laser is used to provide Stokes beam at 1064 nm. The second harmonic (532 nm) is employed to drive the optical parameter oscillator (OPO) to generate the pump beam. EOM: electro-optical modulator, PBS: polarizing beamsplitter, DL: delay line, DM: dichroic mirror, GM: galvo mirror, O: objective, S: sample, C: condenser, M: flat mirror, L: lens, F: filter, PD: photodiode



Fig. S3. Collagen fibers in notochordal sheath. (a) Transmission confocal image of transverse section of amphioxus notochord. *np*: notochordal plate; *ns*: notochordal sheath; *nt*: neural tube. (b) SHG microscopy image of collagen fibers in notochordal sheath. Scale bars = 50μ m. (c) The zoomed image of transverse section of amphioxus notochord. (d) High resolution SHG microscopy image of collagen fibres in notochordal sheath. Scale bars = 5μ m.



Fig. S4. SRS and CARS images of living zebrafish larvae at 2950 cm⁻¹. SRS image has better contrast and CARS image shows artifacts from the non-resonant background of water. Scale bar = $50 \mu m$.