Video Article

Spectral Reflectometric Microscopy on Myelinated Axons *In Situ*

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Abstract

In a mammalian nervous system, myelin provides an electrical insulation by enwrapping the axon fibers in a multilayered spiral. Inspired by its highly-organized subcellular architecture, we recently developed a new imaging modality, named spectral reflectometry (SpeRe), which enables unprecedented label-free nanoscale imaging of the live myelinated axons *in situ*. The underlying principle is to obtain nanostructural information by analyzing the reflectance spectrum of the multilayered subcellular structure. In this article, we describe a detailed step-by-step protocol for performing a basic SpeRe imaging of the nervous tissues using a commercial confocal microscopic system, equipped with a white-light laser and a tunable filter. We cover the procedures of sample preparation, acquisition of spectral data, and image processing for obtaining nanostructural information.

Video Link

The video component of this article can be found at https://www.jove.com/video/57965/

Introduction

In the mammalian nervous system, myelin provides rapid nerve conduction and axonal integrity by enwrapping the axon fibers with multilayered membranous sheaths. Its multilayered structure is composed of alternating nanoscale thin-films composed of plasma membranes (~5 nm), cytosol (~3 nm), and extracellular spaces (~7 nm)[1,2]. Optical microscopy, including the recent super-resolution microscopy, are not suitable for observing the nanoscale myelin dynamics due to their insufficient resolution due to optical diffraction[3,4,5]. Although electron microscopy can provide fine details of the myelin nanostructure, it is not compatible with the living biological systems due to highly invasive sample preparations involving chemical fixation and ultrasectioning[6,7]. Until recently, there has been no technique applicable to observe nanoscale dynamics of myelinated axons *in situ*.

Schain *et al*. previously reported that myelinated axons exhibit colorful light reflectance[8]. By adopting the spectroscopic analysis on the reflected light, we have devised a new imaging modality for nanoscale imaging of myelinated axons, named spectral reflectometry (SpeRe)[9]. SpeRe is based on the thin-film interference occurring in the multi-layered structure of the myelin sheath (**Figure 1**). By optic simulation on various axons, we have revealed that the reflectance spectrum is a periodic function of wavenumber and its periodicity ($\tilde{\nu}_p$) is inversely proportional to the axon diameter (d). This simple relationship ($d = 0.27/\tilde{\nu}_p$) offers facile quantification of axon diameter from the SpeRe data. Utilizing this, we revealed the prevalent axon bulging under mild traumatic brain injury in our prior report.

The SpeRe system is based on confocal microscopy and consists of a specialized laser source and filters (**Figure 2**). The input source is a white-light laser, providing broadband spectral output visible to infrared regions. For the spectral scan, the system is equipped with two acousto-optic devices: an acousto-optic tunable filter (AOTF) for delivering a selected wavelength from the input broadband source and an acousto-optic beam splitter (AOBS) for guiding the selected reflected wavelength to the detector. The software for hyperspectral confocal microscopy (see **Table of Materials**) provides a customizable spectral scan option to sequentially acquire the reflectance images at various input wavelengths. In addition, chromatic aberration can critically interfere in the spectral measurement; therefore, use of an apochromat objective lens is recommended.

Of note, white-light lasers produce an uneven spectral output and the optical components also affect the spectral profile. Therefore, the acquired spectra need to be calibrated for the subsequent quantitative analysis. A protected silver mirror is typically used as a reference, which provides a nearly constant reflectance (> 97%) over the full visible region. The acquired spectra are then divided by the reference spectra from the mirror.

The spectral step size for the spectral scan determines the acquisition speed; thus, it needs to be optimized. As a larger axon has a higher spectral period, it requires finer spectral sampling. For example, an axon with a diameter of 10 μm, one of the largest physiologic axons, has a spectral period of ~8 nm. By applying Nyquist sampling criteria, we employed the spectral sampling interval of 4 nm to cover all the physiologic axons in the mouse nervous tissues. This approach typically takes over several seconds for a full spectral scan and thus is not suitable for *in vivo*

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applications, where physiologic motion (*e.g.* respiration and heartbeat) interferes stable spectral acquisition. We previously solved this issue by instrumenting a customized upright microscope, designed to acquire the full spectrum for each point using an array spectrometer (acquisition speed ≈ 30 ms per pixel).

In this report, we describe a detailed protocol on the SpeRe imaging on a fixed brain slice, which can be performed in a commercial hyperspectral microscope (see **Table of Materials**). Thus, the protocol can be completed by experimenters without expertise in optical instrumentation. We also cover the potential issues and troubleshooting for acquisition and analysis of SpeRe data.

Protocol

All surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Sungkyunkwan University.

1. Sample Preparation

Note: Autoclave all surgical instruments before animal handling. Conduct all surgical performance in a room dedicated to surgical procedures. Sterile surgical gowns and gloves should be worn by all personnel in the surgical room at all times.

1. **Tissue Fixation**
    1. Prepare two 10 mL syringes each filled with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) in PBS.
    2. Anesthetize a 7-12-week-old mouse, (C57BL/6J or any mouse lines with either gender) by administering a mixture of zoletil and xylazine (1:1, 20 mg/kg each) or an alternative suitable anesthesia regime (*e.g.*, mixture of 80 mg/kg ketamine and 10 mg/kg xylazine) with an intraperitoneal injection.
    3. Once the mouse has reached a surgical plane of anesthesia (use the toe pinch-response method), expose the heart by cutting off the skin and the rib cage with scissors.
    4. Snip the right atrium with small scissors to drain the blood and perfuse the PBS and PFA solution sequentially through the left ventricle with a needle (perfusion rate = 4 mL/min, volume = 10 mL for each solution).
    Note: The mouse becomes stiff if the procedure is successfully completed. The use of eye ointment or sterilization is not necessary as the procedure is terminal. The fixation procedure may be skipped. However, this step is recommended to minimize structural deformations including mechanical tissue damage and ischemic axonal swelling. For further details on tissue fixation, refer to the article by Gage, G. J. *et al.*[10]

2. **Preparation of the Brain Slice**
    1. Decapitate the mouse with surgical scissors through the ventral thorax and abdomen.
    2. Remove the scalp and periosteum using a suitably small scissors until fully exposing the cranium.
    3. Break the frontal bone and cut the cranium along the sagittal suture from the occipital bone using small scissors with care not to damage the brain.
    4. Gently remove the cranium and the dura mater sequentially using fine forceps.
    5. Extract the brain using a soft plastic spoon, taking care not to damage the tissue.
    6. Rinse and soak the extracted brain in a 4% PFA solution for 30 min at 4 °C.
    7. Slice the fixed brain into 100 – 150 µm sections using a vibratome.
    Note: For further details on the tissue preparation, refer to the article by Segev *et al.*[11]. For subsequent procedures, the tissue should be sliced into sections thinner than the thickness of the spacer.

3. **Mounting of the Brain Slice**
    1. Prepare 1 glass slide and 2 square cover glasses (22 × 22 × 0.17 mm) for each tissue slice.
    2. Cut one of the square cover glasses into half (two rectangular pieces) by using a glass cutter.
    3. Attach the two of the halved cover glasses using a super glue on the slide glass.
    Note: The space between the two halved glasses should be slightly larger than the size of the tissue slice.
    4. Harvest the brain slice using a brush or a pipette and lay the tissue on the slide glass between the spacer. Take care not to fold the tissue.
    5. Dispense 100 µL of PBS on the tissue surface.
    6. Place the other square cover glass on top of the tissue. Avoid inclusion of air bubbles during this stage.
    7. Seal around the cover glass using a nail polish (or alternatively suitable adhesives) to prevent evaporation of PBS and contamination by dust during the subsequent imaging session.
    Note: The experimenter may pause at this stage.

2. Calibration

1. Switch on the microscope at least 1 h before the imaging to allow thermal stabilization of the laser source (see **Table of Materials**). Then, turn on the software for spectral scanning (see **Table of Materials**) and click the **Acquire** button on the top of GUI (**Figure 3a**).
2. Turn on the software shutter for the white-light laser (WLL) and photomultiplier tube (PMT) (**Figure 3a**).
3. Select the **xyΛ mode** on the drop-down list in **Acquisition Mode**, then, the laser input mode is automatically changed from **Constant percent** to **Constant Power**. Uncheck the check-box of **Automatic SP Movement**. And then, set the spectral window of the input laser to 470–670 nm and the spectral step size to 4 nm on **Λ-Excitation Lambda Scan Settings** (**Figure 3b**).
4. SET the spectral range of PMT to 450-690 by double-clicking or moving the adjustment bar for spectral range (**Figure 3c**).
Note: This spectral range should include the complete bandwidth of the input source.

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5. Select a water-immersion objective lens, suitably with a high numerical aperture (NA > 0.7) and give any value to PMT and laser power to activate **AOBS configuration** and **Live Scan** button. Switch the optical path by checking reflection in the **AOBS Configuration** (**Figure 3d**).
6. Mount a reference mirror (see **Table of Materials**) on the microscope stage. The mirror surface should be faced against the objective lens. If it is not easy to put the mirror on the microscope stage, bond a mirror onto the flat plate (*e.g.* slide glass).
7. Control the microscope stage to align the focal plane to the mirror surface.
8. Adjust the PMT gain and the laser power considering the dynamic range of the detector and then change the laser input mode from **Constant Percent** to **Constant Power**.
   Note: As is typical, a PMT gain of 500 (V) and a relative laser power of 0.1% are used at 570 nm.
9. Confirm in a pseudo-color mode to check that there is no saturation throughout the wavelength range. If saturation is observed, lower the laser power (**Figure 3a**).
10. Run the **Lambda Scan** acquisition.
11. Remove the mirror from the stage and repeat the same acquisition without a sample in order to obtain the dark reference (*i.e.*, dark offset).
12. Save the data in a **Multistacked TIF** format.

3. SpeRe Image Acquisition

1. Place the mounted tissue on the microscope stage. To roughly align the tissue to the focal plane of the objective lens, use the wide-field fluorescence mode through an eye-piece.
2. With the **Live Scan** on, control the microscope stage to align the focal plane to the region of interest in the tissue. To avoid the background noise from the cover slip, select the target region of at least 15 µm depth from the glass-tissue interface.
3. Acquire the spectral image stack for the target region using the same procedure as described in steps 2.1–2.10.
4. Save the data for the tissue and the dark offset in **Multistacked TIF** format.
   Note: The experimenter may pause at this stage.

4. Image Processing and Analysis

1. Open the spectral data (multistacked TIF) for the reference mirror and the brain tissue in ImageJ.
2. Select the ROIs (regions of interest) for the opened image stacks—the central area for the reference mirror and the segment of an axon fiber for the brain tissue.
3. Acquire the raw spectra for the selected ROIs by running **Image** → **Stacks** → **Plot Z-axis profile** on the ImageJ menu.
4. Open the dark offset data, one taken for the reference mirror and the other taken for the brain tissue.
5. Acquire the spectra for the dark offsets by running **Image** → **Stacks** → **Plot Z-axis profile** on the ImageJ menu.
6. Save all the acquired spectra by using the **Copy and Paste** options.
   Note: For SpeRe imaging, use of the central imaging field to minimize off-axis optical aberrations is recommended. The axon fibers may be structurally heterogeneous along their length. Hence, selecting the ROI on a small axon segment, typically < 5 µm, to minimize partial-volume artifact is recommended.

5. Baseline Correction and SpeRe Signal Analysis

1. Subtract the offset spectra from the spectra of the reference mirror and the brain tissues.
2. Normalize each spectrum by dividing the maximum intensity of the spectrum.
3. Divide the normalized spectrum of the axon by the normalized spectrum of the reference mirror and subtract DC offset from the normalized spectrum.
4. Measure the wavenumber frequency by fitting the acquired spectrum to a sinusoidal curve (see **Table of Materials**), and then convert the acquired wavenumber to periodicity by taking reciprocal.
5. Convert the wavenumber periodicity to the axon diameter using the equation in **Figure 4c**.

Representative Results

According to the protocol, a fixed brain slice was prepared with exogenous staining, a myelin-targeting fluorophore (see **Table of Materials**). SpeRe imaging was performed on the brain slice using a commercial hyperspectral confocal microscope in conjunction with confocal fluorescence imaging (**Figure 4a**). For SpeRe, input optical intensity was set to 5 µW/µm^2 with a pixel dwell time of ~1 µs. This light dose is over an order-of-magnitude lower than conventional fluorescence confocal microscopy[12]. It takes ~17 s for spectral scanning over 470-670 nm at an interval of 4 nm (51 images).

The SpeRe signal was localized along the center of the myelinated axons as expected by the geometry of the optical reflection. From the reflectance spectrum of an axon segment, the wavenumber periodicity was obtained, which was subsequently converted to axon diameter (**Figure 4b, c**). The diameter measured by SpeRe was found to be in good agreement with the fluorescence-based measurement (**Figure 4d**). The residual minor error could have originated either from the diffraction-limited resolution of the fluorescence-based method or the incomplete geometrical model for SpeRe.

The SpeRe imaging is based on optical reflection, thus a silica-based coverslip can introduce a significant background noise. In our optic setup, the background noise was considerable when the imaging depth from the coverslip is less than 5 µm but was avoided when the imaging depth is greater than 15 µm (**Figure 5**).

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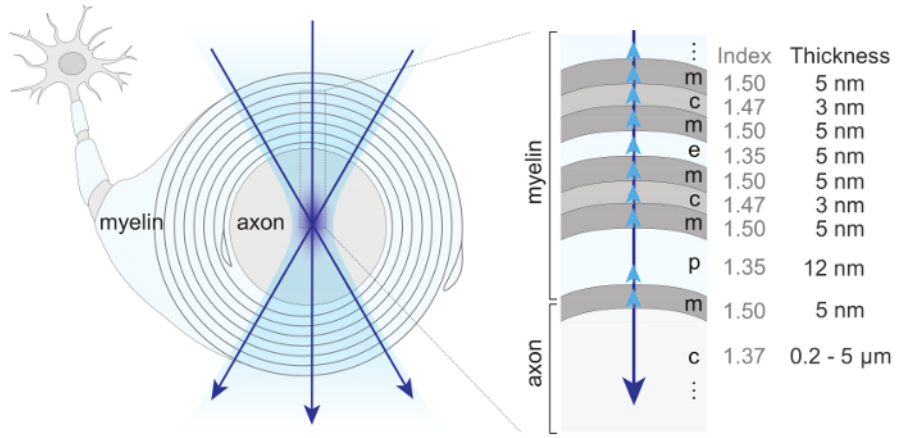

Figure 1: Principle of SpeRe. Myelinated axon has a multilayered thin-film structure. The incident light is partially reflected off at the interfaces, as described by Fresnel's Law. These reflected light waves interfere with each other; therefore, the resulting reflected light encodes the nanostructural information. Spectral Reflectometry (SpeRe) obtains the nanostructural information by decoding the reflected light from the myelinated axon. This Figure has been reprinted from Kwon, J. *et al.*[9] Please click here to view a larger version of this figure.

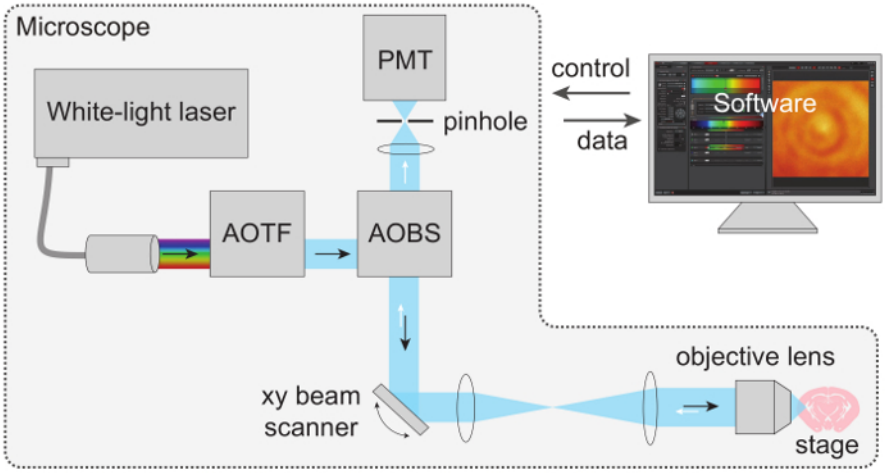

Figure 2: Layout of the SpeRe system. The SpeRe system is based on a confocal reflectance microscope. For spectral scanning, three additional components are needed along the excitation path: (1) a supercontinuum laser, (2) acousto-optic tunable filter (AOTF), and (3) acousto-optic beam splitter (AOBS). The broadband laser source is spectrally filtered by AOTF to transmit a selected wavelength with a narrow bandwidth. AOBS functions as a beam splitter for the selected wavelength to guide the excitation light to the sample. The light is then incident on the sample through a galvanometric scanner and an objective lens. The reflected light from the sample is descanned, spatially filtered by a confocal pinhole, and collected by the photomultiplier tube (PMT). Please click here to view a larger version of this figure.

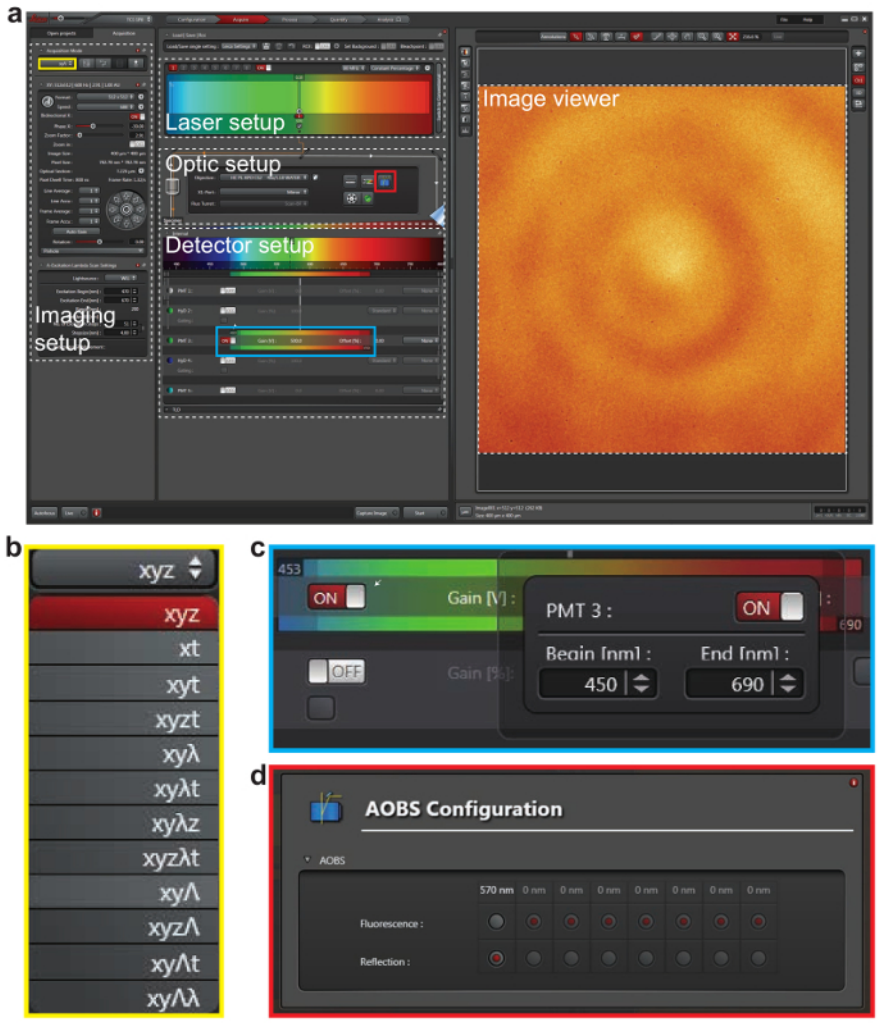

Figure 3: Software setup. (**a**) The graphical user interface (GUI). In the main window, there are mainly five sub-panels: imaging setup, laser setup, optic setup, detector setup, and image viewer. (**b**) The drop-down menu to select the imaging mode. For SpeRe, xyλ is selected. (**c**) The panel for adjusting the spectral window for the detector. (**d**) The panel for setting up the acousto-optic beam splitter (AOBS). The 'reflectance' is selected to guide the reflected light to the detector. Please click here to view a larger version of this figure.

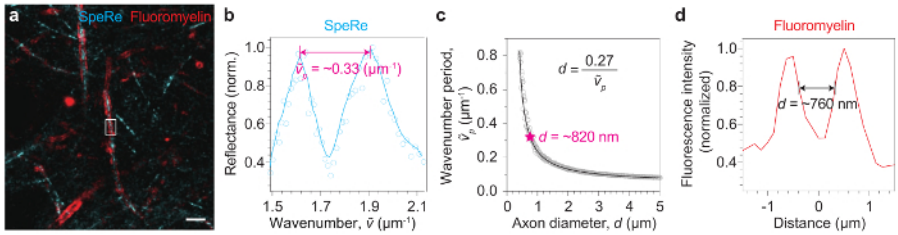

Figure 4: SpeRe on a brain tissue. (**a**) A SpeRe image of a murine brain tissue stained with a fluorescent counterstaining of the myelin (fluoromyelin). (**b**) A representative reflectance spectrum obtained from the white dashed box in (a). (**c**) A simulated reference curve to estimate the axon diameter from spectral periodicity. Blue asterisk is the data point obtained from (b). (**d**) Transversal profile of fluorescence intensity from the boxed region in (a). The myelin external diameter estimated by using the fluorescence signal is ~760 nm, which agrees well with the SpeRe measurement. The residual error is conceivably from diffraction error of the fluorescence-based measurement. Scalebar, 5 μm. Please click here to view a larger version of this figure.

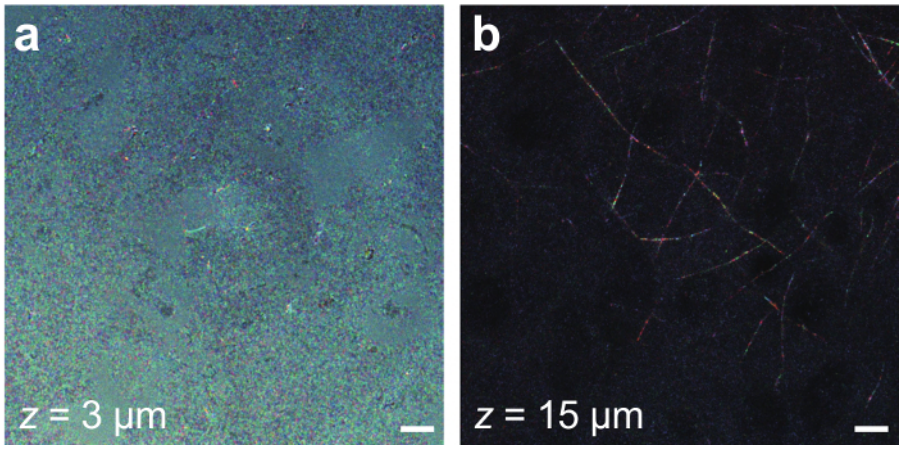

Figure 5: Effect of a cover slip. (**a**, **b**) Reflectance images of a brain tissue at the same lateral position are acquired at different depths from the tissue-coverslip interface: at 3 μm (a) and 15 μm (b). Scalebar, 10 μm. Please click here to view a larger version of this figure.

Discussion

SpeRe is a new label-free imaging modality based on spectral interferometry, which for the first time, offers the nanoscale information in live myelinated axons. In the current acquisition protocol, the spatial resolution for the axon diameter is of the order of 10 nm. Moreover, SpeRe utilizes orders-of-magnitude lower light dose compared to other super-resolution microscopies; thus, it is free from phototoxicity and photobleaching. SpeRe would provide a new avenue for studying nanoscale dynamics of the myelinated axons.

The protocol described herein is based on spectral scanning mediated by acousto-optics and a point detector (*i.e.*, PMT). This approach is advantageous for acquiring spectral images of the full field-of-view. However, the time resolution is limited by the spectral scanning, which is typically > 15 s in our current configuration. As an alternative, a high-speed spectroscope can be incorporated to enable real-time observation of a small field-of-view[9]. This system can be simply constructed from a conventional confocal system by switching the PMT by a spectroscope and by adding a white-light laser. For acquisition software, the galvanometer position needs to be synchronized with the running of the spectroscope. In case of a single point measurement, the temporal resolution is determined by the frame rate of the spectroscope, which can be > 10 kHz for recent ultrafast spectroscopes. This sub-millisecond temporal resolution should be enough for observing most of the physiological dynamics of the myelinated axons *in vivo*. SpeRe is based on light reflection; therefore, the detected light has the same wavelength as the input light. On the contrary, fluorescence involves spectral shift (*i.e.*, Stokes shift); thus, the detected light is spectrally separable from the input light. This feature is helpful for simultaneous acquisition of SpeRe and fluorescence on the same specimen (as demonstrated in **Figure 4**). Absorption of the input light by a fluorophore can affect the SpeRe measurement, but the absorption from typical fluorescent staining is negligibly low (< 1%).

Of note, SpeRe has a limited angular detection range-only the axons nearly parallel to the imaging plane can be detected. This angular limit of SpeRe is around ± 13.5° [9]. To acquire specific orientation of interest, the sample may be tilted. In case of transparent specimens such as zebrafish embryo, the full volumetric acquisition may be possible by rotating the specimen. An additional practical limitation is that a coverslip mounted on a sample produces strong back-reflection leading to the generation of high background signal as shown in **Figure 5**; it is recommended that this superficial region should be avoided. SpeRe based on visible light has the 1/e attenuation length of ~20 μm in the brain tissues. The typical tissue penetration depth for acquiring reliable spectral information is limited to ~100 μm. Deeper imaging may be possible if a longer input source is used. In the end, the validation of SpeRe is shown by comparing it with diffraction-limited fluorescence microscopy. This has apparently been insufficient for evaluating the nanoscale precision (**Figure 4**). Recent techniques, such as correlative light-electron microscopy and expansion microscopy, would offer a way to validate the SpeRe at nanoscale regime[13,14].

Disclosures

The authors declare competing financial interests: J. Kwon and M. Choi are inventors of the patent-pending technology described in this Article.

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