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Yuxiang Wu
Tao Sui
Xiaojian Cao
Xiaohua Lv
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Peng Sun

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Yuxiang Wu,[a,c,*] Tao Sui,[b,*] Xiaojian Cao,[b] Xiaohua Lv,[a,c] Shaoqun Zeng,[a,c] and Peng Sun[d]

[a]Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics-Huazhong University of Science and Technology, Wuhan 430074, China

[b]First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China

[c]Key Laboratory of Biomedical Photonics of Ministry of Education, Huazhong University of Science and Technology, Wuhan 430074, China

[d]Nanjing Medical University, Department of Physiology, Nanjing 210029, China

Abstract. Peripheral nerve injury repair is one of the most challenging problems in neurosurgery, partially due to lack of knowledge of three-dimensional (3-D) fine structure and organization of peripheral nerves. In this paper, we explored the structures of nerve fibers in ventral and dorsal nerves with a laser scanning confocal microscopy. Thick tissue staining results suggested that nerve fibers have a different 3-D structure in ventral and dorsal nerves, and reconstruction from serial sectioning images showed that in ventral nerves the nerve fibers travel in a winding form, while in dorsal nerves, the nerve fibers form in a parallel cable pattern. These structural differences could help surgeons to differentiate ventral and dorsal nerves in peripheral nerve injury repair, and also facilitate scientists to get a deeper understanding about nerve fiber organization. [DOI: 10.1117/1.3575167]

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In microsurgical repair of nerve injuries, as the fine structure of peripheral nerve is not clear, the nerve ends are sometimes mistakenly connected. Exploring the microstructure and organization of peripheral nerves is of great importance in helping to understand their neurophysiology and improving the repair surgery.[1–3] In this paper, nerve fibers in dorsal and ventral nerves are finely imaged by the laser scanning confocal microscopy to acquire the intrinsic structure of nerves. As the peripheral nerve is a complicated matter with high scattering, the traditional imaging method is bothered by image blurring. In this study nerve fibers in spinal nerves are imaged with confocal microscopy to provide higher spatial resolution and to eliminate image blurring due to the high scattering nature of the peripheral nerve.

Twelve adult beagle dogs were anesthetized with sodium pentobarbital and killed; then both ventral and dorsal roots were excised and cut into 2 to 3-mm long segments, fixed in 4% paraformaldehyde for 24 h, washed for 2 h, and then dehydrated with graded ethanol and vitrified by xylene. Transverse sections of nerves were cut by a paraffin slicing machine (Leica RM 2135) at a the thickness of 5 and 20 μm. Paraffin slicing slides were heated in an oven at 60°C for 20 min, deparaffinized and washed, then microwaved in boiling 10 mm sodium citrate buffer. After washing the slides with PBS and adding 1% BSA, the sections were immersed in 200 μm primary antibody neurofilament 200 (NF-200) goat serum/PBS for 8 h. Finally, the sections were washed with PBS and added with goat anti-mouse Alexa 555 (Ex/Em = 555 nm/565 nm) for 90 min at room temperature (Invitrogen, USA).[4–7]

A typical laser scanning confocal microscope (FV1000, Olympus, Japan) was used to acquire images of a transverse section of spinal nerve roots. 10 and 40× water immersion objectives (Olympus, Japan) were used. Images were obtained by the software FLUOVIEW (Olympus, Japan) image analysis and reconstruction was done using Image J (NIH, USA).

As we all know, a thin cross section is good for staining and conventional imaging, so commonly the thickness of a cross section for staining is less than 5 μm. In our experiment, thick section staining is applied and two section thicknesses are considered.[8,9]

Confocal fluorescence images were collected at 1-μm z-intervals, then serial cross section images were projected along the z-axis, we can see the details of the 3-D structure throughout the length of the nerve at the thickness of 5 μm. Bright dots could be seen in both dorsal and ventral nerves [see Figs. 1(b) and 1(f)], which represent the transection of individual nerve fibers, there seemed to be no structural differences. For the serial section of the same nerve, if the section thickness increased to 20 μm, the projection images from dorsal and ventral nerves show an obvious difference [see Figs. 1(d) and 1(h)]. For the 20-μm thick ventral nerve sections, stripes pattern toward different directions is quite evident, while this stripe pattern cannot be observed from 5-μm thick ventral nerve sections. For the dorsal nerve, the increased section thickness did not make an obvious difference.

In Figs. 1(a) and 1(c), each nerve fiber throughout the dorsal nerve root is formed as a round shape, so it is easy to infer nerve fibers in dorsal nerves are arranged like a cable pattern in realistic physiological conditions, while nerve fibers in the ventral nerves are distributed in complicated winding pattern [see Fig. 1(g)]. The phenomenon suggests a different 3-D fiber structure between the two kinds of nerves.

To further quantify the similarity and difference, correlation coefficients between the thin and thick section images for the same type of nerve were calculated. In Fig. 2, the bar graph shows correlation coefficients of 0.73 ± 0.08 and 0.43 ± 0.06 for dorsal and ventral pairs, respectively (mean $\pm$SE, $P<0.05$, $n = 12$), dots of (○) and (●) represent the calculated correlation coefficients.

*These authors contributed equally to this work.

Address all correspondence to : Xiaohua Lv, Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics-Huazhong University of Science and Technology, Wuhan 430074, China. Tel: 86 27 877 92033; E-mail: xhlv@mail.hust.edu.cn.

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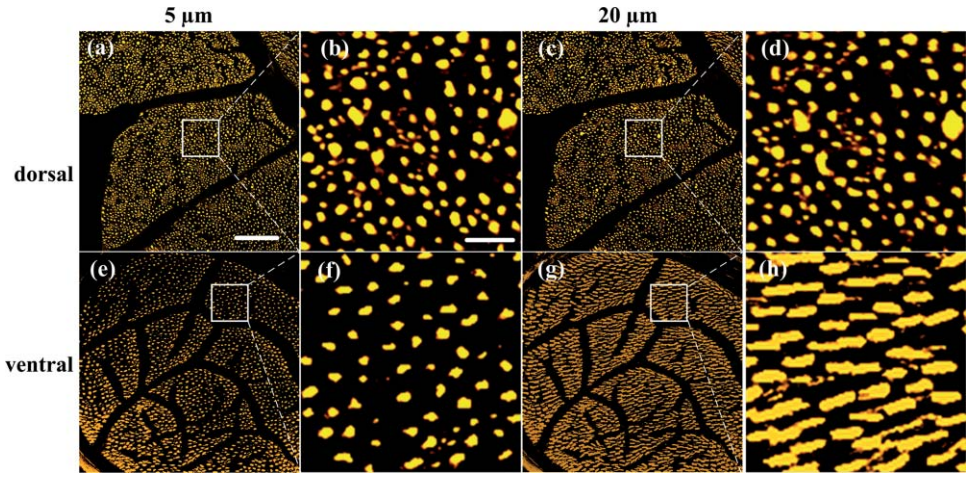

Fig. 1 Serial cross section projections of the dorsal (a)–(d) and ventral (e)–(h) nerves immunostained with NF-200. Scale bar: 200 μm for the regular images, and 40 μm for the close-ups.

Correlation coefficient r was calculated as follows:

$$r = \frac{\sum_m \sum_n (A_{mn} - \bar{A})(B_{mn} - \bar{B})}{\sqrt{\left(\sum_m \sum_n (A_{mn} - \bar{A})^2\right)\left(\sum_m \sum_n (B_{mn} - \bar{B})^2\right)}}, \quad (1)$$

where A_{mn} and B_{mn} represent 5 and 20 μm images. $\bar{A}$ and $\bar{B}$ are mean values of A_{mn} and B_{mn}.

Based on the above results, distinct microstructural patterns of the two kinds of nerves were demonstrated. As shown in Fig. 3, nerve fibers in the ventral nerve were inferred to form a complicated winding pattern, while nerve fibers in dorsal nerves formed a parallel cable pattern. Based on this inference, it can be easily understood that for a complicated winding pattern, the transverse section image projection can easily show a stripe pattern for a thicker section, while the cable pattern makes no significant difference for both thin and thick section projection.

Further, 3-D reconstructions through serial cross sections were investigated to confirm the structure difference between ventral and dorsal nerve roots.[10] Confocal fluorescence images were collected at 1-μm z-intervals, then serial cross section images were projected along the z-axis, and the details of the 3-D structure throughout the length of the nerve could be observed. From the axial direction, stripe-like nerve fibers in ventral nerve and individual dots in dorsal nerve were observed [Figs. 4(b) and 4(e)]. When sliced parallel to the x-z plane [the slice positions are indicated with lines in Figs. 4(a) and 4(d)], complex and disordered nerve fibers in ventral nerve and parallel distributed fibers in dorsal nerve were seen in the slice image [Figs. 4(c) and 4(f)]. This confirmed that nerve fibers are arranged orderly like cables in the dorsal root, while winding in a complex form along the ventral root.

In previous research, such structural differences were not found as a result of imaging technique and slicing thickness restriction. To explain in detail, the studies on nerve roots with optical microscopy often depended on thin sections (<5 μm) to achieve good staining results, and the classical structure of neurofilaments in nerves obtained by electron microscope is limited to be smaller than 1 μm. These methods lead to the fact that the 3-D structure of nerve fibers cannot be directly observed and understood,[11–13] whereas in this study, in order to observe the nerve fiber structure and organization in a larger axial

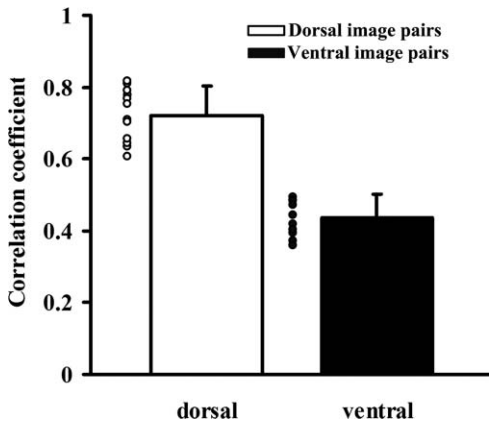

Fig. 2 Correlation coefficients of dorsal image pairs and ventral image pairs. Images with thickness of 5 and 20 μm from the same sample are treated as an image pair. Dots of (○) and (●) are the calculated correlation coefficients from image pairs.

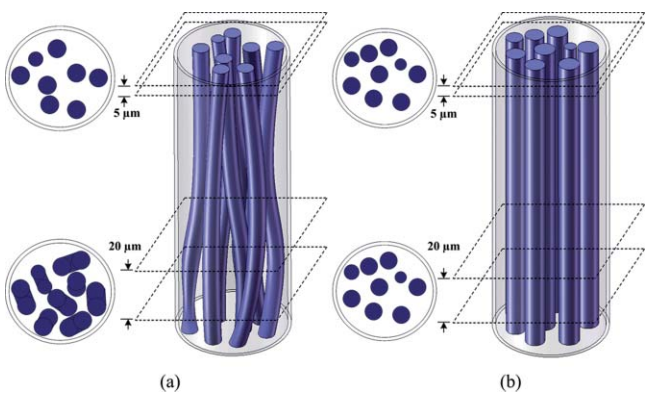

Fig. 3 The model of ventral (a) and dorsal nerve (b). Note the difference in images of cross sections with the thickness of 5 and 20 μm.

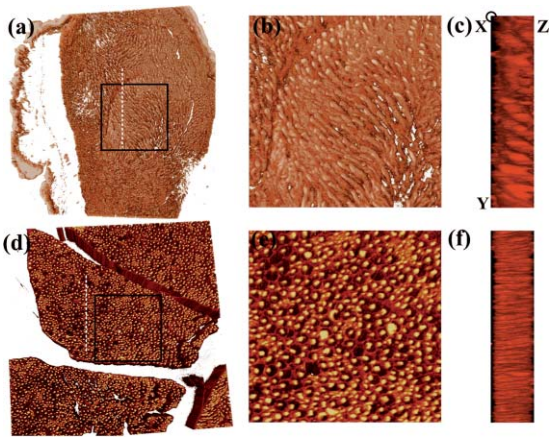

Fig. 4 The 3-D reconstruction from serial cross sections, (a) Ventral and (d) dorsal nerve roots. Section thickness: 20 μm. (b) and (e) are magnified images of the square in (a) and (d). Multistripes can be seen in (a) and individual nerve fibers can be clearly seen in (b). (c) and (f) are *x*-*z* projections of the region of the dotted lines in (a) and (d).

range, 20 μm thick nerve tissues were stained. High resolution images of nerves were then collected by laser scanning confocal microscopy, and 3-D reconstruction results showed that nerve fibers of ventral nerves were formed in a winding pattern, while nerve fibers of dorsal nerves were formed in a parallel cable pattern.

In summary, a 3-D subtle structure of spinal nerves were obtained using laser scanning confocal microscopy in combination with the thick tissue staining technique. The structure differences between ventral and dorsal nerves revealed in this study not only provide helpful information for precise microsurgery, but also facilitate a deeper understanding about nerve fiber organization.

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