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Fluoro-ruby retrograde tracing and three-dimensional visualization of the corticospinal tract in the guinea pig☆

Xiao Han[1], Lulian Xu[2], Yue Wu[2], Hua Xun[2], Jinxiu Pan[2], Yingying Huang[2], Dafeng Ji[1], Huiqun Wu[3], Guangming Lv[1], Lemin Tang[4]

1*Department of Human Anatomy, Institute of Neurobiology, Jiangsu Key Laboratory of Neuroregeneration, Medical School, Nantong University, Nantong 226001, Jiangsu Province, China*
2*Medical School, Nantong University, Nantong 226001, Jiangsu Province, China*
3*Department of Medical Informatics, Medical School, Nantong University, Nantong 226001, Jiangsu Province, China*
4*Department of Imaging Engineering, Medical School, Nantong University, Nantong 226001, Jiangsu Province, China*

Abstract

Fluoro-ruby was injected into the posterior funiculus of the spinal cord in the cervical (C_5–T_2) and lumbar (L_{3-6}) segments of adult guinea pigs. The spinal cord was cut into serial frozen sections. The Fluoro-ruby labeling was clearly delineated from the surrounding structure. The labeling traversed the cervical, thoracic and lumbar segments, and was located on the ventral portion of the posterior funiculus on the injected side, proximal to the intermediate zone of the dorsal gray matter. The fluorescence area narrowed rostro-caudally. The spinal cord, spinal cord gray matter and corticospinal tract were reconstructed using 3D-DOCTOR 4.0 software, resulting in a robust three-dimensional profile. Using functionality provided by the reconstruction software, free multi-angle observation and sectioning could be conducted on the spinal cord and corticospinal tract. Our experimental findings indicate that the Fluoro-ruby retrograde fluorescent tracing technique can accurately display the anatomical location of corticospinal tract in the guinea pig and that three-dimensional reconstruction software can be used to provide a three-dimensional image of the corticospinal tract.

Key Words: corticospinal tract; Fluoro-ruby; retrograde fluorescent tracing technique; three-dimensional reconstruction; guinea pig

Xiao Han☆, M.D., Lecturer, Department of Human Anatomy, Institute of Neurobiology, Jiangsu Key Laboratory of Neuroregeneration, Medical School, Nantong University, Nantong 226001, Jiangsu Province, China

Corresponding author: Guangming Lv, Ph.D., Professor, Master's supervisor, Department of Human Anatomy, Institute of Neurobiology, Jiangsu Key Laboratory of Neuroregeneration, Medical School, Nantong University, Nantong 226001, Jiangsu Province, China; Lemin Tang, Professor, Master's supervisor, Department of Imaging Engineering, Medical School, Nantong University, Nantong 226001, Jiangsu Province, China
gmlu@ntu.edu.cn; lemintang@ntu.edu.cn

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INTRODUCTION

Various methods have been used to visualize the normal anatomical location and spatial orientation of the corticospinal tract (CST) in the spinal cord. For example, Liu *et al* [1] conducted immunolocalization of the rat CST using a specific marker for the CST, protein kinase Cγ, and achieved good results. In addition, medical imaging technologies, such as diffusion tensor imaging magnetic resonance tractography and Mn^{2+}-enhanced magnetic resonance imaging, have also been used to study CST morphology[2-3]. Although these technologies have been able to appropriately display the positioning of the CST in the brain and upper cervical spinal cord, they have difficulty in exhibiting the CST in the lower spinal cord. Recently, biotinylated dextran amine and horseradish peroxidase have successfully been used as neural tracers to visualize the CST in rodents[4-9]. Compared with nerve tracing methods based on immunohistochemistry, fluorescent tracers, such as Fluoro-gold and Fluoro-ruby (FR), have been commanding increasing attention because of their simple color development process, and the results are clearer than those obtained using traditional immunohistochemical methods and less background interference is observed[10-12]. Due to the dispersed distribution of motor neurons in the guinea pig cerebral cortex, the CST cannot be easily traced using anterograde tracing methods. Therefore, in this study, the three-dimensional topography of the CST was reconstructed using three-dimensional reconstruction software, which provides spatial information that traditional two-dimensional sections cannot.

RESULTS

Quantitative analysis of experimental animals

Twelve healthy adult guinea pigs were initially included in the experiment and all of them were included in the final analysis.

Visualization of the CST in the spinal cord of the guinea pig using FR retrograde fluorescent tracing

The posterior funiculus of the spinal cord of

guinea pigs showed labeled FR-positive axons, a clearly marked red fluorescence signal, and a clear boundary between the labeled area and surrounding structures using FR fluorescent staining (Figure 1). In transverse sections of the guinea pig spinal cord, FR-positive signals located on the ventral side of the posterior funiculus of the spinal cord were visible on the injected side, proximal to the intermediate zone of the dorsal gray matter, and no FR-positive signal was present on the contralateral side (Figure 1A). Figure 1B shows longitudinal sections of the guinea pig spinal cord. FR-positive signals traverse the posterior funiculus along the long axis of the spinal cord, through cervical segment 1 to lumbar segment 6.

Reconstruction of the spinal cord white matter, gray matter and CST of guinea pigs

The spinal cord, spinal cord gray matter and the CST of guinea pigs were reconstructed using 3D-DOCTOR 4.0 software, to obtain a robust three-dimensional profile. The reconstruction results show that the guinea pig CST is positioned on the ventral side of the posterior funiculus in cervical segment 1 to lumbar segment 6 of the spinal cord, proximal to the intermediate zone of the dorsal gray matter, tapering in the descending segment caudally. Using functionality provided by the reconstruction software, free multi-angle observation and sectioning could be conducted on the guinea pig spinal cord and CST (Figure 2).

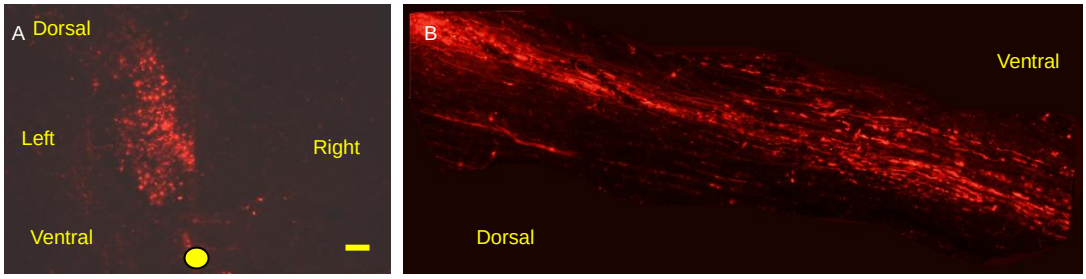

Figure 1 Fluoro-ruby fluorescent staining of the corticospinal tract (red) in the spinal cord of the guinea pig (scale bar: 1 mm).
Fluoro-ruby fluorescent staining of transverse (A) and longitudinal (B) sections of the corticospinal tract (◯ = central canal).

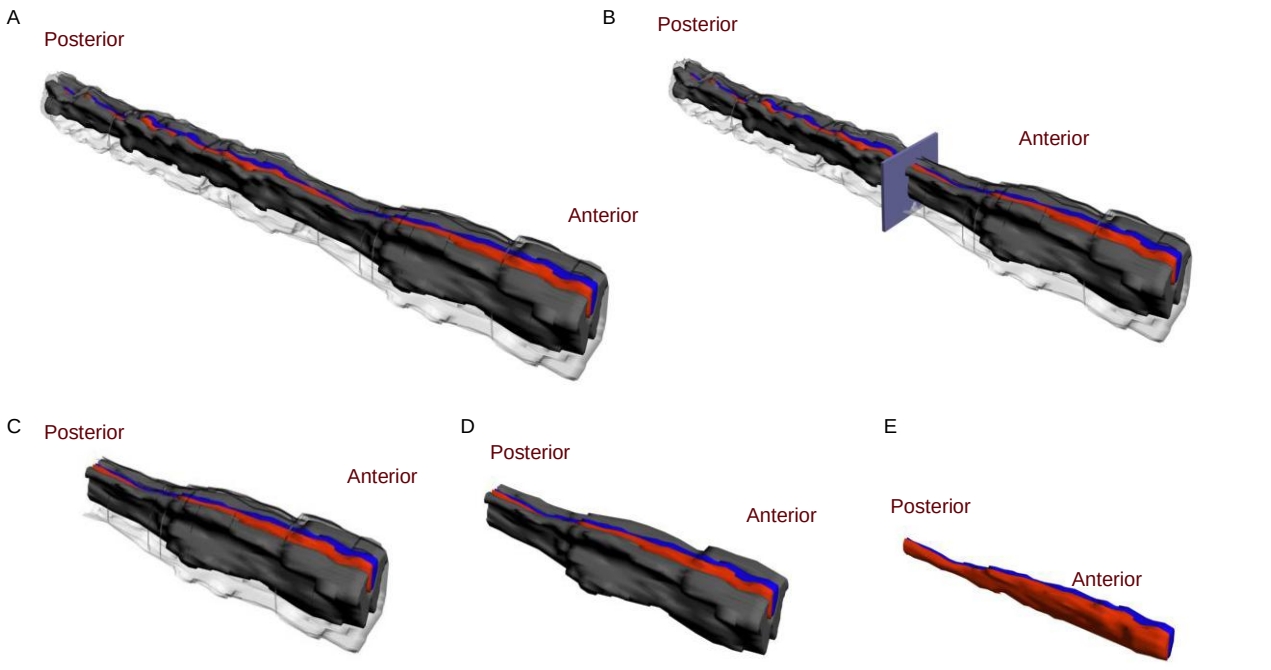

Figure 1 The reconstructed white matter, gray matter and corticospinal tract (CST) of the guinea pig spinal cord. White matter is white and transparent, gray matter is black, the left CST is blue, and the right CST is red.

(A) The reconstructed guinea pig spinal cord (X −22°; Y −51°; Z 0°).

(B) The reconstructed guinea pig spinal cord (X −22°; Y −51°; Z 0°) was dissected by arbitrary sectioning. The section was gray and between $T_{4\text{-}5}$ segments.

(C) The residual guinea pig spinal cord.

(D) The residual guinea pig gray matter and CSTs.

(E) The residual guinea pig CSTs.

DISCUSSION

Compared with nerve tracers, such as biotinylated dextran amine and horseradish peroxidase, fluorescent FR retrograde tracing does not require complex immunohistochemical procedures and does not demand close observation during the color reaction process. The results of this study demonstrate that positive staining of CST fibers of guinea pigs on the injection side is visible as red fluorescence, with a clearly demarcated signal that is located on the ventral side of the posterior funiculus of the spinal cord, and proximal to the intermediate zone of the gray matter. On the contralateral side, no FR-positive signal is seen. Longitudinal sections of the posterior funiculus of the spinal cord exhibit FR-positive labeling present as a red stripe that traverses from cervical segment 1 to lumbar segment 6. No positive staining is seen in either the lateral or anterior funiculus of the bilateral spinal cord, which is in accordance with the observations of Rapisarda *et al* [5] and Brown[13].

Based on FR labeling, a study on virtual visualization was conducted on the guinea pig spinal cord and CST by our group. With the development of information and digital technology, researchers have been able to virtually reconstruct specific organs and tissues, such as blood vessels and liver, using tissue slices, to obtain a macroscopic image[14-16]. However, it is impossible to obtain effective reconstruction of fine structures, such as the CST, because reconstruction is hindered by factors such as limited imaging technology and lack of resolution. In contrast, *in vivo* small animal imaging technology has achieved great progress, and reconstruction has been applied to numerous tissues and organs. For example, in imaging experiments of rat brain, detailed structures of ventricles and the major nuclei have been obtained[17-19]. However, for the internal anatomical structure of the spinal cord, there are only a few *in vivo* imaging studies in rodents. Therefore, scholars are more inclined to use microscopic serial tissue sections for three-dimensional reconstruction of various tracts[20-22].

Researchers have employed numerous methods to increase the fidelity of the reconstruction process. For example, lead sulfide is used as a colorant for the staining of embryo paraffin sections to distinguish different tissue densities, which is then followed by three-dimensional reconstruction[23-24]. But as an embedding medium, paraffin will dehydrate tissue samples, inevitably resulting in a reduction in size of the reconstructed structure. Therefore, serial spinal cord tissue sections are used for the three-dimensional reconstruction of the rat CST[25]. In this procedure, as used in this study, frozen sections are directly attached to the slide using a direct slide-attaching method and are immediately observed with microscopy and radiography. This procedure better maintains the shape of the spinal cord and avoids the structural deformation caused by tissue dehydration. Consequently, a more satisfactory reconstruction is obtained. In addition, different angles of observation and cross-sectional viewing can be conducted on the overall structure, as well as on the gray and white matter structures of the virtual spinal cord. Furthermore, measurement of topographical features, such as surface area and volume, using functionality provided by the software, is useful for obtaining information on the three-dimensional shape of the spinal cord, which traditional two-dimensional tissue sections cannot provide. Thus, the technique provides a simple and feasible solution for digital reconstruction based on macroscopic sections.

In this study, a three-dimensional model of the CST in guinea pigs was successfully constructed using FR tracing of the neural tract with three-dimensional reconstruction software. However, such reconstruction methods based on serial tissue sections have difficulties, such as complex section preparation and long experimental procedures. Furthermore, they only display the tissue in a static state, without the ability to resolve physiological dynamics. If *in vivo* biological tissues can be reconstructed using a combination of neural tracing and imaging methods, more effective research methodology will be possible, and will enhance our ability to study the CST and other internal fine structures of the spinal cord.

MATERIALS AND METHODS

Design

A three-dimensional neuroanatomical reconstruction.

Time and setting

The experiment was performed at the Department of Human Anatomy, Institute of Neurobiology, Jiangsu Key Laboratory of Neuroregeneration, Medical School, Nantong University, Jiangsu Province, China, from January 2009 to March 2011.

Materials

Twelve healthy, clean, adult guinea pigs, male and female, aged 1.5 years, weighing 350–400 g, were provided by the Experimental Animal Center of Nantong University, China (license No. SYXK (Su) 2007-0021). All animals were kept in an environment with a 12-hour light/dark cycle and caged in an approved facility, with free access to food and water. All animal experiments were conducted according to protocols approved by the *United States National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Methods

FR retrograde tracing

Guinea pigs were anesthetized using the anesthetic Chlorpent (0.2 mL/100 g) *via* intraperitoneal injection. Animals were placed in the prone position after being anesthetized. Their heads were fixed on a Kiangwan type II locator (Kiangwan, Shanghai, China) while limbs were fixed. This was followed by disinfection and skin

preparation. During the surgery, the vertebral lamina was opened and the spinal cord was exposed. 10% FR solution[26] (Invitrogen, Carlsbad, CA, USA) was injected into the posterior funiculus of the cervical thoracic segment (C_5–T_2) and lumbar segment (L_{3-6}) by pressure injection (a dose of 0.2 μL in each of four sites, at an average depth of 1 mm).

Serial section preparation

On day 3.5 after injection with FR, animals were perfused through the heart and fixed for 1 hour with phosphate buffered saline (pH 7.4, 4°C) containing 4% paraformaldehyde after being anesthetized. The brain and the spinal cord were removed, fixed for 24 hours, and placed in successive 20% and 30% sucrose solutions. After the tissues sank, the cervical, thoracic and lumbar segments of the spinal cord were selected for transverse and longitudinal sectioning. The section thickness was 30 μm.

CST image acquisition

Sections were kept in the dark, dried at room temperature, mounted with 20% glycerol and observed under a Leica DMR upright fluorescence microscope (Leica, Wetzlar, Germany). The excitation wavelength was 540 nm. Photographs were taken under a 10 × objective lens and using Olympus DP2-BSW software (Olympus, Tokyo, Japan). It was difficult to obtain a complete image of the spinal cord section samples under the 10 × objective lens because of their large size. In order to show the exact position of the CST in the central nervous system, the step shooting method was used in this study to obtain images of transverse and longitudinal sections of the spinal cord. Each spinal cord section required about 35–70 frames. The focal length and aperture of all specimen images were kept constant. Each image was saved in JPG format, and the resolution was 2 272 × 1 704 pixels.

Images were reduced to 25% of the original image size using Photoshop 7.0 software (Adobe Systems, San Jose, CA, USA) to reduce the computer memory used by image processing to improve processing speed. The final resolution of each image was 568 × 426 pixels, and then photos for each plane were spliced to obtain complete medulla oblongata and spinal cord fluorescence staining images. Serial transverse sections of the spinal cord were selected for cutting, and the resolution of each image was set at 1 278 × 1 158 pixels. Batch processes such as light curve, auto contrast adjustment, conversion into 8-bit grayscale images, image reversal and adjustment of image size to 50% of the original image were conducted successively on the cut images in Photoshop 7.0 software, and images were renamed and saved as “0.jpg”, “1.jpg” ... “3 553.jpg”. A total of 3 554 effective slices were obtained.

Designating the central canal of the spinal cord as the center, a line connecting the anterior median fissure and the posterior median sulcus was used as the axis, and image alignment was conducted on acquired images using Photoshop 7.0 software[27-29].

CST image reconstruction

3D-DOCTOR 4.0 (SN-4.0.061212) software (Lexington, MA, USA) was run on an ordinary PC (Pentium (R) 4, 3.06 GHz/3.07 GHz, 40 GB, 512 MB) (Acer, Taipei Taiwan). Reconstruction was conducted in five main steps. First, a “New Stack” was created, into which, all continuous images were selected and imported. It was saved as “spinal cord. Stack”. Second, the “Auto alignment” software was used for automatic picture arrangement. Third, calibration was conducted. The parameters for X pixel, Y pixel, Z thickness and Unit resolution were set to “3.5”, “3.5”, “30” and “μm”, respectively. Fourth, edge extraction was conducted. To differentiate reconstructed structures, white matter was displayed as “milk white”, gray matter as “black”, left CST as “blue”, and mirror edge extraction was conducted on the right side, referencing the shape of the left CST with red color. Fifth, three-dimensional reconstruction and observation were performed. The function Fast Complex Surface Rendering provided by the software was used for fast reconstruction. The property of X, Y and Z was set in 3D Display Setting for observing different angles of the spinal cord. “White” was selected as the background color, and in the 3D Object Setting window, the transparent properties of “white matter” and “gray matter” were set to On and Off, respectively to achieve a translucent display of the white matter. Axes was selected to add X, Y and Z axes to the image. The measurement tools on the toolbar could be chosen to measure segment distance, angle, histograms and other data of the reconstructed spinal segments. In order to section the spinal segment for observation, the *Tools* menu on the menu bar was chosen, Cutting plane in the drop-down menu was selected to adjust the cut surface appearance, and finally, Split object was selected to section the spinal cord. In this study, a total of three sets of serial sections of the spinal cord were reconstructed, and results were consistent. The results from the group with the best reconstruction were selected.

Author contributions: Xiao Han completed the majority of the anterograde tracing experiments, wrote the manuscript, and took the fluorescence images. Huiqun Wu and Dafeng Ji performed part of the experiments and processed the images. Guangming Lv and Lemin Tang were responsible for the study proposal and design. Lulian Xu, Yue Wu, Hua Xun, Jinxiu Pan and Yingying Huang were responsible for the animal experiment, image acquisition and processing.

Conflicts of interest: None declared.

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Ethical approval: All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Nantong University in China.

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