Video Article

Imaging Serotonergic Fibers in the Mouse Spinal Cord Using the CLARITY/CUBIC Technique

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Abstract

Long descending fibers to the spinal cord are essential for locomotion, pain perception, and other behaviors. The fiber termination pattern in the spinal cord of the majority of these fiber systems have not been thoroughly investigated in any species. Serotonergic fibers, which project to the spinal cord, have been studied in rats and opossums on histological sections and their functional significance has been deduced based on their fiber termination pattern in the spinal cord. With the development of CLARITY and CUBIC techniques, it is possible to investigate this fiber system and its distribution in the spinal cord, which is likely to reveal previously unknown features of serotonergic supraspinal pathways. Here, we provide a detailed protocol for imaging the serotonergic fibers in the mouse spinal cord using the combined CLARITY and CUBIC techniques. The method involves perfusion of a mouse with a hydrogel solution and clarification of the tissue with a combination of clearing reagents. Spinal cord tissue was cleared in just under two weeks, and the subsequent immunofluorescent staining against serotonin was completed in less than ten days. With a multi-photon fluorescent microscope, the tissue was scanned and a 3D image was reconstructed using Osirix software.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53673/>

Introduction

Supraspinal projections are responsible for the modulation of diverse behaviors such as pain perception. One of the projections carrying nociceptive information contains serotonergic fibers, which originate from the hindbrain raphe and adjacent reticular nuclei^{1,2}. Physiological and pharmacological studies have demonstrated an increased release of serotonin in the dorsal horn of the spinal cord after electrical stimulation of the raphe nuclei in the hindbrain³⁻⁵. In the rat and opossum, serotonergic raphespinal fibers have dense terminals, not only in the dorsal horn⁶⁻⁸, but also in the intermediate zone^{7,9,10}, the ventral horn^{7,11}, and even lamina 10^{12,13}. There are no similar studies in the mouse. The present study aimed to map the termination pattern of serotonergic fibers arising from the hindbrain raphe nuclei and their adjacent reticular nuclei in the mouse spinal cord using the recently published CLARITY¹⁴ method and its modification - CUBIC¹⁵.

Conventional fluorescence or peroxidase immunohistochemistry of the spinal cord clearly shows the distribution of serotonergic fibers in the gray matter of the spinal cord in 30-40 µm thick cross-sections. However, this approach does not show the continuity of the serotonergic fiber tracts in the white matter and their collaterals in the gray matter. Although the 3D reconstruction of histological sections has advanced our knowledge of fiber tracts, it remains a challenge for histologists and anatomists to follow a single tract due to small distortions in the tissue caused by cutting. To circumvent this obstacle a number of researchers have developed various protocols for making the whole tissue structure transparent, and collecting an image of unaltered tissue in a single video file¹⁷⁻²¹. So far, the clear, lipid-exchanged, acrylamide-hybridized rigid, imaging/ immunostaining compatible, tissue hydrogel (CLARITY) technique, developed by Deisseroth's group^{14,15}, as well as CUBIC, developed by Susaki et al¹⁶ are the most successful. Since the publication of the protocols, many researchers have started using these techniques to investigate various aspects of biological tissues, including, not only the brain²²⁻²⁵, but also the heart, kidneys, intestine, and the lungs^{26,27}.

By fixing the mouse spinal cord with the hydrogel solution (CLARITY) and clearing with the CUBIC reagents (which is a much faster method than that described by the original CLARITY protocol^{14,15}), a spinal cord tissue block of 2-3 mm long was cleared within two weeks and immunofluorescence staining for serotonin completed in eight days. With just a combination of chemical agents, conventional immunohistochemistry can be used to create an image of individual fiber tracts in a 3D video file in approximately one month.

Protocol

Ethics Statement: All procedures involving animal subjects follow the guidelines of the Animal Care and Ethics Committee (ACEC) at The University of New South Wales (the approved ACEC number is 14/94A).

1. Preparation of the Transparent Mouse Spinal Cord

1. Preparation of Ice Cold Hydrogel Solution
 1. Preparation of 16% paraformaldehyde solution (PFA)
 1. Add 16 g paraformaldehyde powder into 70 ml pre-warmed distilled water (50-55 °C) and stir on a heated magnetic stirrer until paraformaldehyde is dissolved. Note: Do not allow the solution to heat over 55 °C and be aware that paraformaldehyde is toxic.
 2. Transfer the paraformaldehyde solution to a cylinder and add distilled water to 100 ml. Cool the paraformaldehyde solution using a 4 °C refrigerator.
 2. Dissolve 125 mg VA-044 initiator in 26.25 ml distilled water and cool the solution in a 4 °C fridge.
 3. Cool 5 ml acrylamide solution (40%), 1.25 ml Bis solution (2%) (CAUTION: Bis and acrylamide solutions are toxic, may cause genetic defects or cancer), 5 ml 10x PBS, 12.5 ml 16% PFA solution, and the VA-044 initiator solution on ice.
 4. Mix the above solutions on ice.
Note: the total volume is 50 ml.
2. Perfusion and Collection of the Mouse Spinal Cord
 1. Anaesthetize a mouse with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) diluted in 0.9% normal saline. The dosage for mouse can be lower than that for rats.
 2. On a suitable plastic surface in a fume hood, fix the limbs of the mouse away from the body (sticky tape is effective) once the mouse does not respond to a pinch to its skin of the foot.
 3. Cut the mouse skin over the chest and then the bone chest to expose the heart with a scissor.
 4. Using a peristaltic pump, with a 25 G needle attached to the end of the tubing, insert the needle into the left ventricle of the heart, snip the right atrium to create an exit point for the blood and then start washing with 40 ml 0.9% normal saline at a rate of approximately 10 ml/min.
 5. When complete, perfuse the mouse with 35 ml ice cold hydrogel solution.
Note: perfuse at a speed of 10 ml/min to avoid swelling of the nervous tissue.
 6. Incise the skin over the back with a blade and then remove the muscles next to the vertebrate. After cutting the vertebral arches on one side and flipping them to the other side, take the spinal cord out of the vertebral column with a fine scissor.
3. Clearing the Mouse Spinal Cord
 1. Place the spinal cord in 15 ml of hydrogel solution overnight at 4 °C.
 2. Remove 10 ml of the hydrogel solution and pour the rest of the solution with the spinal cord segments to a 5 ml tube. Fill the 5 ml tube with the hydrogel solution until it is completely full.
 3. Stretch a small piece of parafilm over the top of the 5 ml tube and then wrap parafilm around the neck of the tube. Note: Ensure the parafilm contacts the hydrogel solution and there are no bubbles between the hydrogel solution and the parafilm.
 4. Put the 5 ml tube in a 37 °C oven overnight. Observe the hydrogel solution till it becomes a gel.
 5. Take the 5 ml tube out of the oven and remove the gel from the tube with a spanner (such as a shoveled 1 ml pipette tip). Remove the gel from the spinal cord tissue by sticking the coarse tissue to the gel and then take the coarse tissue away (the gel will stick to the tissue and therefore will be removed by the tissue).
 6. After removing the gel from the spinal cord tissue, wash the spinal cord 4 times with 1x PBS (pH 7.4) for 24 hr on a shaker.
 7. Prepare the CUBIC clearing solution by dissolving 3.85 g urea and 3.85 g N,N,N',N'-tetrakis (2-hydroxypropyl) ethylenediamine in 5.38 ml distilled water.
Note: A hot stirrer is used. Add 2.31 g polyethylene glycol mono-p-isooctylphenyl ether/Triton X-100 to the solution once it is clear and cools down to room temperature.
Note: 10 g of these three chemicals are for one mouse.
 8. Cut the mouse spinal cord coronally into 2-3 mm long segments with a razor blade and put them into 5 ml of the above CUBIC clearing solution. Place on a shaker in the oven at 37 °C for 3 days.
 9. Three days later, change the clearing solution to a fresh one. Note: the above solution is prepared prior to use.
 10. Two to three days later after refreshing the CUBIC clearing solution, check the transparency of the tissue against a paper with font 8 letters. If it is transparent, the letters can be seen through the cleared tissue. Remove the clearing solution and add 4 ml of PBST (0.1% Triton-X100) to wash the tissue 4 times a day (every 6 hours).

2. Immunofluorescence Staining

1. Incubate the spinal cord segments in the primary antibody solution (anti-serotonin, raised in rabbit, diluted 1:100 in PBST) for 3 days on a shaker in a 37 °C oven.
2. Remove the antibody solution and add 4 ml of PBST (0.1% Triton-X100) to wash the spinal cord segments 4 times a day (every 6 hr) in a 37 °C oven.
3. Remove the PBST solution and add the secondary antibody solution (594 conjugated goat anti-rabbit IgG, diluted 1:100 in PBST) to incubate the tissue for 3 days on a shaker in a 37 °C oven.
4. Remove the secondary antibody solution and add 4 ml of PBST to wash the spinal cord segments 4 times a day (every 6 hours) on a shaker in a 37 °C oven. The next day the tissue is ready for imaging.

3. Imaging

1. Remove the PBST solution and add 4 ml of 85% glycerol to make the refractive index of the tissue even.

2. Check the clarity of the tissue. Once it is clear in approximately 1 hr, put the tissue onto a large, thin glass slide which fits the holding frame of the multi-photo fluorescent microscope.
3. Put a few drops of 85% glycerol next to the spinal cord tissue and coverslip it with a 22 x 50 mm² coverslip glass.
Note: The orientation of the spinal cord decides how the image looks like.
4. Put the glass slide onto the holding frame of the multi-photon fluorescent microscope and move the tissue into the light pathway.
5. Choose the Helium Neon laser line 594 nm and adjust the intensity of the laser to the optimal level by checking the brightness of the positive signal in the live image.
6. Select the scanning area of the spinal cord tissue using the 20X objective (in water, NA 0.7) and prepare to create a z-stack, and the depth of each step set to 3 μ m.
7. Scan the tissue from the top to the bottom of the z-stack under the 20X objective and then 63X objective (in oil, NA 1.4) (step size was 1 μ m), respectively. Reconstruct 3D videos using a 3D software²⁸.

Representative Results

This section shows results from serotonin antibody staining in the transparent mouse spinal cord using a combination of the CLARITY and CUBIC protocols. We show that serotonergic fibers are present in all laminae of the spinal cord with a predominance in the ventral portion of the ventral horn (**Figure 1**, also see **Video 1**). The control tissue did not have positive fibers (result was not shown). In the ventral horn, densely packed serotonergic fibers are present in the ventromedial portion of the ventral horn and they extend towards the lateral portion of the ventral horn (**Figure 2**, also see **Video 2**). Some immunopositive fibers also extend from the ventral horn towards the dorsal horn or the central canal. Immunopositive fibers are present in all laminae of the dorsal horn but there is a small gap between positive fibers in lamina 2 and 4, especially in the lateral part of the dorsal horn. The majority of serotonergic fibers in the dorsal horn are of small diameter and only a small number of them are of large diameter (**Video 1**). In a horizontal section, densely packed serotonergic fibers in the ventral horn were observed to travel along the longitudinal axis of the spinal cord and form a large fiber bundle. The most interesting finding is that this large fiber bundle issues branches regularly along the longitudinal axis, which are perpendicular to the fiber bundle. These branches further collateralize along their paths to the lateral portion of the ventral horn. Compared with these branches, the ones extending towards the midline are irregular and smaller (**Figure 2**). Under the 63X objective, the serotonergic fibers in the dorsal horn were observed to travel along the axis of the spinal cord and terminate at various points along the path of the tract. The thick fibers are sporadically distributed among the thin fibers (**Figure 3**, also see **Video 3**).

Figure 1: Serotonergic fibers in a coronal section of the lumbar cord at 200X magnification. Left is the ventral horn, right is the dorsal horn, dorsal is the medial part of the spinal cord, and ventral is the lateral part of the spinal cord. The scale bar is 100 μ m. Serotonergic fibers are present in all laminae, especially in the ventromedial part of the ventral horn where the fiber density is high. These fibers extend towards both the lateral part of the ventral horn and the central canal or dorsal horn. The density of fibers in other laminae is low. The majority of serotonergic fibers are thin. Only a small number of fibers are thick and they are seen in both the dorsal horn and the ventral horn ([also see Video 1 \(Right click to download\)](#)).

Figure 2: Serotonergic fibers in the ventral portion of the ventral horn at 200X magnification. Left and right are the lateral sides of the spinal cord, dorsal is the rostral part of the spinal cord, and ventral is the caudal part of the spinal cord. The scale bar is 100 μ m. Serotonergic fibers are shown travelling along the longitudinal axis of the spinal cord and packed in the medial portion of the ventral horn where they form a thick fiber bundle. Out of this bundle, some branches are extended at regular intervals towards the lateral portion of the ventral horn, whereas others that extend towards the midline are irregular and shorter. These fibers terminate at many points along the path of the tract ([also see Video 2 \(Right click to download\)](#)).

Figure 3: Serotonergic fibers in the dorsal horn under at 630X magnification. Left is the lateral part of the spinal cord, right is the medial part of the spinal cord, dorsal and ventral refer to the dorsal and ventral part of the dorsal horn, respectively. The scale bar is 7 μ m. Serotonergic fibers are travelling along the longitudinal axis of the spinal cord and terminate along the path of the tract. These fibers are mainly of small diameter with a small number of thick fibers intermingled. These fibers rarely overlap with each other ([also see Video 3 \(Right click to download\)](#)).

Discussion

The protocol described shows how to image serotonergic fibers in the mouse spinal cord with the combined CLARITY and CUBIC techniques. It introduces a faster clearing process compared to the passive clearing protocol developed by Cheung *et al.*¹⁴ and Tomer *et al.*¹⁵ and allows the spinal cord tissue to be well supported by the hydrogel during clearing.

An important step during fixation of the mouse spinal cord, as reported by Cheung *et al.*¹⁴ and Tomer *et al.*¹⁵, is to keep all the solutions cool on ice, which prevents the polymerization of the chemicals. The other critical step is to perfuse the mouse slowly with the hydrogel solution in order to avoid swelling of the nervous tissue. For the hydrogel solution to become a gel, degassing is required. As an alternative to this, we used parafilm to cover the top of the tube and left no air bubbles between the hydrogel solution and the parafilm. This worked very well and the hydrogel solution became a gel after a few hours. Although some bubbles were found in the gel, they did not interfere with the following experiments, which was demonstrated by the absence of bubbles in the spinal cord tissue. Clearing is the most critical step, and requires a combination of reagents. The clearing solution, containing amino-alcohol, removes the lipid of the spinal cord tissue much faster than the SDS/boric acid based clearing solution used in the original CLARITY protocol¹⁶.

The advantage of CLARITY and CUBIC over traditional immunohistochemical techniques is that the entire tissue block can be imaged without sectioning the tissue, as a result, the continuity of the serotonergic fibers is retained, and it is possible to follow the same fiber for a long distance within the z-stack. With this advantage, collateralization can be confidently identified with the 3D images, and novel conclusions can therefore be made about the nature of serotonergic pathways. The protocol described is a convenient tool for investigation of fiber systems, as well as nuclei by simply labeling the tissue with specific antibodies. This technique is also an ideal choice for interrogating different systems in the same mouse brain by using double or triple labeling. In our laboratory setting, a multi-photon fluorescent microscope was available with a working distance of approximately 300 µm. This limits our image to a maximum of 600 µm if both sides of the tissue are scanned. However, a light sheet fluorescent microscope, can image the entire mouse brain and spinal cord through their full width, length and depth. The other advantage of this technique is that the antibodies can be eluted and then another different antibody or antibodies applied again to the same tissue. This reduces animal usage considerably as well as the costs associated with preparing new brain tissue for each experiment. For the same reason, this technique can be applied to other tissues. There have already been studies conducted on the lungs, kidneys, heart, intestine, and tumor tissue^{26,27}.

There are also limitations to this technique, for example, the raphe nuclei in the mouse hindbrain contains many types of neurons, including GABAergic neurons. Although CLARITY and CUBIC will allow for the study of the distribution of the diverse types of raphespinal fibers in a single mouse brain and spinal cord. Those neurons, which are not identified specifically by their neurotransmitters or protein markers, can not be identified in this setting. An alternative is to label the specific neurons or fiber systems with nucleic acid probes such as in *in situ* hybridization. This is, on the other hand, limited by the labeling system and the available filters for fluorescent imaging. Imaging all the raphespinal fibers therefore remains a challenge. If these techniques were combined with an intracranial injection of an anterograde tracer (such as phaseolus vulgaris leucoagglutinin - PHA-L), it might be possible to see serotonergic fibers, GABAergic fibers, and others.

Disclosures

The authors have nothing to disclose.

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