Journal of Visualized Experiments

Visualizing the Calcitonin Gene-Related Peptide Immunoreactive Innervation of the Rat Cranial Dura Mater with Immunofluorescence and Neural Tracing

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61742R2
Full Title:	Visualizing the Calcitonin Gene-Related Peptide Immunoreactive Innervation of the Rat Cranial Dura Mater with Immunofluorescence and Neural Tracing
Corresponding Author:	Wanzhu Bai China Academy of Chinese Medical Sciences Beijing, Beijing CHINA
Corresponding Author's Institution:	China Academy of Chinese Medical Sciences
Corresponding Author E-Mail:	wanzhubaisy@hotmail.com
Order of Authors:	Jia Wang
	Dongsheng Xu
	Jingjing Cui
	Chen She
	Hui Wang
	Shuang Wu
	Ling Zou
	Jianliang Zhang
	Wanzhu Bai
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, Beijing, China
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please specify the section of the submitted manuscript.	Neuroscience
Please provide any comments to the journal here.	

TITLE:

Visualizing the Calcitonin Gene-Related Peptide Immunoreactive Innervation of the Rat Cranial Dura Mater with Immunofluorescence and Neural Tracing

AUTHORS AND AFFILIATIONS:

Jia Wang[1]*, Dongsheng Xu[1]*, Jingjing Cui[1], Chen She[1], Hui Wang[1], Shuang Wu[1], Ling Zou[1], Jianliang Zhang[1], Wanzhu Bai[1]

[1]Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, Beijing, China

*These authors contributed equally.

Correspondence to:
Wanzhu Bai (wanzhubaisy@hotmail.com)

Email Addresses of Co-Authors:
Jia Wang (wangjia315500@163.com)
Dongsheng Xu (xds402@126.com)
Jingjing Cui (cuijing101@163.com)
Chen She (shechen32@163.com)
Hui Wang (wanghuissy@hotmail.com)
Shuang Wu (18270883568@163.com)
Ling Zou (1184780052@qq.com)
Jianliang Zhang (drzhangjl@yahoo.com)
Wanzhu Bai (wanzhubaisy@hotmail.com)

KEYWORDS:

cranial dura mater, middle meningeal artery, calcitonin gene-related peptide, neural tracing technique, fluorogold

SUMMARY:

Here we present a protocol to visualize spatial correlation of calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibers and blood vessels in the cranial dura mater using immunofluorescence and fluorescent histochemistry with CGRP and phalloidin, respectively. In addition, the origin of these nerve fibers was retrograde traced with a fluorescent neural tracer.

ABSTRACT:

The aim of this study was to examine the distribution and origin of the calcitonin gene-related peptide (CGRP)-immunoreactive sensory nerve fibers of the cranial dura mater using immunofluorescence, three-dimensional (3D) reconstruction and retrograde tracing technique. Here, the nerve fibers and blood vessels were stained using immunofluorescence and histochemistry techniques with CGRP and fluorescent phalloidin, respectively. The spatial correlation of dural CGRP-immuoreactive nerve fibers and blood vessels were demonstrated by

three-dimensional reconstruction. Meanwhile, the origin of the CGRP-immunoreactive nerve fibers were detected by neural tracing technique with fluorogold (FG) from the area around middle meningeal artery (MMA) in the cranial dura mater to the trigeminal ganglion (TG) and cervical (C) dorsal root ganglia (DRGs). In addition, the chemical characteristics of FG-labeled neurons in the TG and DRGs were also examined together with CGRP using double immunofluorescences. Taking advantage of the transparent whole-mount sample and 3D reconstruction, it was shown that CGRP-immunoreactive nerve fibers and phalloidin-labeled arterioles run together or separately forming a dural neurovascular network in a 3D view, while the FG-labeled neurons were found in the ophthalmic, maxillary, and mandibular branches of TG, as well as the C2-3 DRGs ipsilateral to the side of tracer application in which some of FG-labeled neurons presented with CGRP-immunoreactive expression. With these approaches, we demonstrated the distributional characteristics of CGRP-immunoreactive nerve fibers around the blood vessels in the cranial dura mater, as well as the origin of these nerve fibers from TG and DRGs. From the perspective of methodology, it may provide a valuable reference for understanding the complicated neurovascular structure of the cranial dura mater under the physiological or pathological condition.

INTRODUCTION:

The cranial dura mater is the outermost layer of meninges to protect the brain and contains plentiful blood vessels and different kinds of nerve fibers[1,2]. Many studies have shown that sensitized cranial dura mater may be the key factor leading to the occurrence of headaches, involving the abnormal vasodilation and innervation[3–5]. Thus, the knowledge of neurovascular structure in the cranial dura mater is important for understanding the pathogenesis of headaches, especially for migraine.

Although the dura innervation has been previously studied with the conventional immunohistochemistry, the spatial correlation of nerve fibers and blood vessels in the cranial dura mater were less studied[6–9]. In order to reveal the dural neurovascular structure in more detail, calcitonin gene-related peptide (CGRP) and phalloidin were selected as the markers for respectively staining the dural nerve fibers and blood vessels in the whole-mount cranial dura mater with immunofluorescence and fluorescent histochemistry[10]. It may be an optimal choice to obtain a 3D view of neurovascular structure. Additionally, fluorogold (FG) was applied on the area around middle meningeal artery (MMA) in the cranial dura mater to determine the origin of CGRP-immunoreactive nerve fibers, and traced to the trigeminal ganglion (TG) and cervical (C) dorsal root ganglia (DRGs), while the FG-labeled neurons were further examined together with CGRP using immunofluorescence.

The aim of this study was to provide an effective tool for investigating the neurovascular structure in the cranial dura mater for the CGRP-immunoreactive innervation and its origin. By taking the advantage of the transparent whole-mount dura mater and combining the immunofluorescence, retrograde tracing, confocal techniques, and 3D reconstruction, we expected to present a novel 3D view of the neurovascular structure in the cranial dura mater. These methodological approaches may be further served for exploring the pathogenesis of different headaches.

PROTOCOL:

This study was approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (reference number D2018-09-29-1). All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996). Twelve adult Sprague-Dawley male rats (weight 220 ± 20 g) were used in this study. Animals [license number SCXK (JING) 2017-0005] were provided by the National Institutes for Food and Drug Control.

1. Innervation of rat cranial dura mater

1.1. Perfusions

1.1.1. Intraperitoneally inject an overdose of tribromoethanol solution (250 mg/kg) to the rat to induce euthanasia.

1.1.2. Once the breath stops, transcardially perfuse with 100 mL of 0.9% normal saline followed by 250–300 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4).

1.1.3. After perfusion, remove the head skin and open the skull to expose the dura mater and dorsal side of the brain. Then dissect out the cranial dura mater along the brainstem to the olfactory bulb in the whole-mount pattern (**Figure 1**). Perform post-fixation in 4% paraformaldehyde for 2 h, and then cryoprotect in 25% sucrose in 0.1 M PB for more than 24 h at 4 °C.

1.2. Fluorescence immunohistochemistry for CGRP and phalloidin labeling

NOTE: A combination of fluorescent staining of CGRP and phalloidin was applied to reveal the spatial correlation of dural nerve fibers and blood vessels in the rat cranial dura mater in the whole-mount pattern.

1.2.1. Rinse the cranial dura mater in 0.1 M PB for about 1 min.

1.2.2. Incubate the dura mater in a blocking solution containing 3% normal donkey serum and 0.5% Triton X-100 in 0.1 M PB for 30 min.

1.2.3. Transfer the dura mater into mouse anti-CGRP antibody (1:1000) in 0.1 M PB containing 1% normal donkey serum and 0.5% Triton X-100 overnight at 4 °C[11].

1.2.4. Wash the dura mater three times in 0.1 M PB the following day.

1.2.5. Incubate the dura mater in a mixed solution of donkey anti-mouse Alexa Fluor 488 secondary antibody (1:500) and phalloidin 568 (1:1000) in 0.1 M PB containing 1% normal donkey

serum and 0.5% Triton X-100 for 1.5 h at room temperature (26 °C).

1.2.6. Wash the dura mater three times in 0.1 M PB.

1.2.7. Trim the edges and mount it on microscope slides (see **Table of Materials**).

1.2.8. Put on coverslips with 50% glycerin before observation.

1.3. Observation and recording

1.3.1. Observe the fluorescent samples under a fluorescent microscope or a confocal imaging system.

1.3.2. Take the images of the whole-mount dura mater by a fluorescent microscope equipped with a digital camera (4x, NA: 0.13), and use an exposure time of 500 ms. The image mosaics of the dura mater were completed with a software of fluorescent microscope (see **Table of Materials**).

1.3.3. Take images of the CGRP-immunoreactive nerve fibers and phalloidin-labeled blood vessels in the dura mater using a confocal microscope. The excitation and emission wavelengths were 488 nm (green) and 594 nm (red). The confocal pinhole is 110 (20x) and 105 µm (40x). The resolution of image capture is 640 x 640 pixels.

1.3.4. Capture 20 images in 2 µm frames of from each 40 µm thick section and perform single in-focus image integration with a confocal image processing associated software system for 3D analysis as follows: **Set Start Focal Plane | Set End focal plane | Set step size | Choose Depth Pattern | Image Capture | Z series**.

1.3.5. Use a photo editing software to adjust the brightness and contrast of images to optimize the visualization. Pay attention to not remove any data from the images.

2. Retrograde tracing study with FG

2.1. Surgical procedures

2.1.1. Determine the coordinate area of interest in rat cranial dura mater.

2.1.2. Prepare a 10 µL micro-syringe and test it with liquid paraffin.

2.1.3. Anesthetize the rats with tribromoethanol solution (150 mg/kg) via intraperitoneal injection. Check for the depth in anesthesia by the lack of response to toe pinch.

2.1.4. Shave the rat's head with an electric razor.

2.1.5. Put blunt ear bars to the rat and place it on the stereotaxic device. Then put the mouth holder and apply ophthalmic ointment on the eyes.

2.1.6. Clean the surgical site of the head skin using 75% ethanol.

2.1.7. Make an incision along the midline of the scalp.

2.1.8. Bluntly remove the periosteum and muscle tissues away from the skull using sterile cotton-tipped applicators (**Figure 1A**).

2.1.9. Drill a small hole (~5–7 mm) using a burr drill with a round-tip bit (#106) on the left parietal and temporal bones above the middle meningeal artery (MMA)[12], and make sure that the cranial dura mater was kept intact (**Figure 1B**).

2.1.10. Build a bank around the hole with dental silicate cement to limit the spread of the tracer (**Figure 1C**).

2.1.11. Add 2 µL of 2% FG into the hole around MMA with a 10 µL micro-syringe (**Figure 1D**).

2.1.12. Cover the hole with a small piece of hemostatic sponge.

2.1.13. Put a piece of paraffin film on the hole and seal the edges with bone wax to prevent the leakage of tracer and to avoid contaminating to the surrounding tissues.

2.1.14. Suture the wound with sterile thread.

2.1.15. Keep the rats in a warm area until they have fully recovered without any post-operative analgesics.

2.1.16. Return the rats back to their cages.

2.2. Perfusions and sections

2.2.1. After 7 survival days, perfuse these rats as mentioned above in the procedures in section 1.1.

2.2.2. Dissect out the TG and C1-4 DRGs, then post-fix and cryoprotect them as above mentioned in section 1.1.3 (**Figure 1F**).

2.2.3. Cut the TG and DRGs at the thickness of 30 µm on a cryostat microtome system in the sagittal direction and mounted on silane-coated glass slides.

2.3. Double immunofluorescences for FG- and CGRP-labeling in the TG and DRGs

NOTE: Although the FG-labeling can be directly observed with UV illumination under mercury lamp without additional staining[13–16], the labeled neurons with FG were further examined in TG and cervical DRGs using double immunofluorescences with FG and CGRP for revealing the origins of dural CGRP-immunoreactive nerve fibers in the TG and DRGs.

2.3.1. Circle the sections with the histochemical pen.

2.3.2. Incubate the sections for 30 min in a blocking solution containing 3% normal donkey serum and 0.5% Triton X-100 in 0.1 M PB.

2.3.3. Transfer the samples into the solution of rabbit anti-fluorogold (1:1000) and mouse anti-CGRP antibody (1:1000) in 0.1 M PB containing 1% normal donkey serum and 0.5% Triton X-100 overnight at 4 °C.

2.3.4. Wash the sections three times in 0.1 M PB the following day.

2.3.5. Incubate in a mixed solution of donkey anti-rabbit Alexa Fluor 594 (1:500) and donkey anti-mouse Alexa Fluor 488 (1:500) secondary antibody in 0.1 M PB containing 1% normal donkey serum and 0.5% Triton X-100 for 1.5 h at room temperature.

2.3.6. Wash and apply coverslips to the sections as the procedures in steps 1.2.6 and 1.2.8.

2.4. Observation and recording

2.4.1. Take images of the FG-labeled neurons in TG and DRGs under UV illumination by fluorescent microscope equipped with a digital camera.

2.4.2. Capture images of the FG- and CGRP-labeled neurons in TG and DRGs under a fluorescent microscope equipped with a digital camera.

2.4.3. Use the editing software to adjust the brightness and contrast of images and to add labels in the pictures.

REPRESENTATIVE RESULTS:

Neurovascular structure of the cranial dura mater

After immunofluorescent and fluorescent histochemical staining with CGRP and phalloidin, CGRP-immunoreactive nerve fibers and phalloidin-labeled dural arterioles and connective tissues were clearly demonstrated throughout the whole-mount cranial dura mater in a 3D pattern (**Figure 2C,D,E,F**). It was shown that both thick and thin CGRP-immunoreactive nerve fibers run in parallel to the dural arterioles, around the vascular wall, or between the blood vessels (**Figure 2D,E,F**). By taking advantages of the 3D reconstruction, the morphology of dural arterioles and the spatial correlation of the dural CGRP-immunoreactive nerve fibers and arterioles could be clearly demonstrated from different perspectives.

Retrograde-labeled neurons in the TG and DRGs

Seven days after FG application on the region of MMA in the rat cranial dura mater (**Figure 3A**), the FG-labeled neurons were detected in TG and cervical DRGs on the ipsilateral side of the tracer application, which was directly observed under the UV illumination of the fluorescent microscopy (**Figure 3B,C**). FG-labeled neurons were found in all the three branches of TG with higher concentration on the ophthalmic (V1) and maxillary (V2) divisions, and with less on the mandibular division (V3) (**Figure 3B**). Meanwhile, some of the FG-labeled neurons were also observed in the C2-3 DRGs (**Figure 3C**).

In addition, double immunofluorescences were also performed with FG and CGRP on the sections of TG and cervical DRGs. According to the diameter of CGRP-immunoreactive neurons in TG and DRGs, most of them were primarily found in the small- and medium-diameter sensory neurons (<30 μm). Some of these type of CGRP-immunoreactive neurons were also labeled with FG in the TG and C2-3 DRGs, indicating that CGRP-immunoreactive nerve fibers in the cranial dura mater originated from these subpopulation of sensory neurons in the TG and DRGs (**Figure 4**).

FIGURE LEGENDS:

Figure 1: Photographs of main experimental views in the present study. (**A**) An incision along the midline of the scalp. (**B**) A hole above the cranial dura mater showing the middle meningeal artery (MMA). (**C**) A small bank around the hole circled with dental silicate cement for the application of tracer onto the cranial dura mater. (**D**) Application of fluorogold (FG) into the hole with micro-syringe. (**E**) The transparent whole-mount dura mater. (**F**) The outside view of the trigeminal ganglion (TG).

Figure 2: Correlation between calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibers and phalloidin (Pha)-labeled arterioles on the whole-mount cranial dura mater. (**A**) The transparent whole-mount dura mater. (**B**) The whole-mount dura mater was flattened and mounted on the slide. (**C**) Distribution of CGRP-immunoreactive nerve fibers and Pha-labeled blood vessels along the middle meningeal artery (MMA). (**D,E**) The magnified photos from the same areas of D and E in panel C. (**F**) The magnified and adjusted images from panel E with the frame in a 3D pattern.

Figure 3: Distribution of fluorogold (FG)-labeled neurons in the trigeminal ganglion (TG) and cervical (C) dorsal root ganglion (DRG) under UV illumination. (**A**) The region of dura mater with FG application. (**B**) Distribution of FG-labeled neurons in the ophthalmic (V1), maxillary (V2), and mandibular (V3) branches of the TG. (**C**) Distribution of FG-labeled neurons distributed in the C2 DRG.

Figure 4: The representative photographs showing the labeled sensory neurons in trigeminal ganglion (TG) and cervical dorsal root ganglion (DRG) by using double immunofluorescences with fluorogold (FG) and calcitonin gene-related peptide (CGRP). (**A,B**) The labeled neurons with FG, CGRP, and both FG and CGRP were demonstrated in red, green, and yellow, respectively in

TG (A) and DRG (B). A1–B1, (A2–B2): Panels A and B were separately showed with FG-labeling (A1, B1) and CGRP-labeling (A2, B2).

DISCUSSION:

In this study, we have successfully demonstrated the distribution and the origin of CGRP-immunoreactive nerve fibers in the cranial dura mater using immunofluorescence, 3D reconstruction and neural tracing approaches with CGRP antibody and FG neural tracer, providing the histological and chemical evidences to better understand the dural neurovascular network.

As it was known, CGRP plays a critical role in the pathogenesis of migraine[4,17]. It was shown that increased CGRP can lead to vasodilatation and neurogenic inflammation to cause the peripheral and central sensitization along the trigeminal pathway[4,18]. CGRP-immunoreactive nerve fibers belong to the unmyelinated peptidergic sensory axons responsible for the transportation of nociceptive signals[19]. Being consistent with previous studies, here, we clearly demonstrated the distribution of the CGRP-immunoreactive nerve fibers in the cranial dura mater and traced their origin from small- and medium-diameter sensory neurons in the TG and DRGs. These cellular structures could be the sources for synthesizing and releasing CGRP. On the other hand, phalloidin is a specific probe for filamentous actin (F-actin) that is abundant in the smooth muscular and endothelial cells. As a proper candidate, phalloidin was used for labeling the vascular structures and connective tissues[10,20–21]. Our recent study has shown that, in contrast to alpha smooth muscle actin and CD31, phalloidin is more reliable and sensitive for staining dural arterioles, and is the optimal to combine with CGRP for demonstrating the cranial neurovascular network in detail, which has been published eslewhere[10,21].

Neural tract tracing technique is an important tool to investigate the neural origin and termination. In the present study, FG was used for retrograde tracing the origin of CGRP-immunoreactive nerve fibers in the cranial dura mater. Since the cranial dura mater is a thin membrane, the tracer cannot be applied conveniently by the way of injection. Instead, FG was directly added onto the region around MMA in the cranial dura mater according to the method that had been introduced previously[22–23], but care must be taken to keep the dura mater intact. Besides, the effort was made to prevent the leakage of FG to the adjacent tissues. Because the FG-labeling can be directly observed under UV illumination of mercury lamp[24], by this approach, we checked the site of FG application; it was found that besides the neural propagation, FG was limited in the region circled with dental silicate cement without contaminating the surrounding tissues. The other advantage is that FG-labeled neurons can also be stained in the expected color using immunofluorescence with FG antibody, making it more convenient to be used together with other biomarkers[14–16]. Through this study, we proved that FG not only fits for retrograde tracing the dural innervation but is also a proper candidate to combine with CGRP for determining the chemical characteristics of FG-labeled neurons.

It should be noted here that present methods are preferably used for young animals. At the early stage of rat, the cranial dura mater is more transparent in the whole-mount style. This feature makes it more convenient to visualize the neurovascular structure of the cranial dura mater in a

3D pattern without further transparent treating.

In summary, the present study provides a valuable approach to effectively explore the innervation of the cranial dura mater from the sensory neurons in the TG and cervical DRGs, especially the subtype of small- and medium-diameter sensory neurons with CGRP-immunoreactive expression. From the perspective of methodology, it may provide a valuable reference for further investigating the other kinds of nerve fibers in the cranial dura mater, as well as their origins.

ACKNOWLEDGMENTS:

This study was supported by the project of National Key R&D Program of China (Project Code no. 2019YFC1709103; no. 2018YFC1707804) and National Natural Science Foundation of China (Project Code no. 81774211; no. 81774432; no. 81801561).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Kekere, V., Alsayouri, K. *Anatomy, Head and Neck, Dura Mater.* In: StatPearls. Treasure Island (FL): StatPearls Publishing (2020).
2. Shimizu, T. et al. Distribution and origin of TRPV1 receptor-containing nerve fibers in the dura mater of rat. *Brain Research*. **1173**, 84–91 (2007).
3. Jacobs, B., Dussor, G. Neurovascular contributions to migraine: moving beyond vasodilation. *Neuroscience*. **338**, 130–144 (2016).
4. Dodick, D. W. A phase-by-phase review of migraine pathophysiology. *Headache*. **58** (Suppl 1), 4–16 (2018).
5. Amin, F. M. et al. Investigation of the pathophysiological mechanisms of migraine attacks induced by pituitary adenylate cyclase-activating polypeptide-38. *Brain: A Journal of Neurology.* **137** (Pt 3), 779–794 (2014).
6. Keller, J. T., Marfurt, C. F. Peptidergic and serotoninergic innervation of the rat dura mater. *The Journal of Comparative Neurology.* **309** (4), 515–534 (1991).
7. Messlinger, K., Hanesch, U., Baumgärtel, M., Trost, B., Schmidt, R. F. Innervation of the dura mater encephali of cat and rat: ultrastructure and calcitonin gene-related peptide-like and substance P-like immunoreactivity. *Anatomy and Embryology*. 188 (3), 219–237 (1993).
8. Lennerz, J. K. et al. Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. *The Journal of Comparative Neurology*. **507** (3), 1277–1299 (2008).
9. Eftekhari, S., Warfvinge, K., Blixt, F. W., Edvinsson, L. Differentiation of nerve fibers storing CGRP and CGRP receptors in the peripheral trigeminovascular system. *The Journal of Pain: Official Journal of the American Pain Society*. **14** (11), 1289–1303 (2013).
10. Xu, D. S. et al. Characteristics of distribution of blood vessels and nerve fibers in the skin tissues of acupoint “Taichong” (LR3) in the rat. *Zhen Ci Yan Jiu*. **41** (6), 486–491 (2016).
11. Cui, J. J. et al. The expression of calcitonin gene-related peptide on the neurons associated

Zusanli (ST 36) in rats. *Chinese Journal of Integrative Medicine*. **21** (8), 630–634 (2015).
12. Andres, K. H., von Düring, M., Muszynski, K., Schmidt, R. F. Nerve fibres and their terminals of the dura mater encephali of the rat. *Anatomy and Embryology*. **175** (3), 289–301 (1987).
13. Leng, C., Chen, L., Li, C. Alteration of P2X1-6 receptor expression in retrograde Fluorogold-labeled DRG neurons from rat chronic neuropathic pain model. *Biomedical Reports*. **10** (4), 225-230 (2019).
14. Huang, T. L. et al. Factors influencing the retrograde labeling of retinal ganglion cells with fluorogold in an animal optic nerve crush model. *Ophthalmic Research*. **51** (4), 173–178 (2014).
15. Huang, T. L., Chang, C. H., Lin, K. H., Sheu, M. M., Tsai, R. K. Lack of protective effect of local administration of triamcinolone or systemic treatment with methylprednisolone against damages caused by optic nerve crush in rats. *Experimental Eye Research*. **92** (2), 112–119 (2011).
16. Tsai, R. K., Chang, C. H., Wang, H. Z. Neuroprotective effects of recombinant human granulocyte colony-stimulating factor (G-CSF) in neurodegeneration after optic nerve crush in rats. *Experimental Eye Research*. 87(3), 242–250 (2008).
17. Iyengar, S., Ossipov, M. H., Johnson, K. W. The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine. *Pain*. 158(4), 543–559 (2017).
18. Russell, F. A., King, R., Smillie, S. J., Kodji, X., Brain, S. D. Calcitonin gene-related peptide: physiology and pathophysiology. *Physiological Reviews*. **94** (4), 1099–1142 (2014).
19. Kou, Z. Z. et al. Alterations in the neural circuits from peripheral afferents to the spinal cord: possible implications for diabetic polyneuropathy in streptozotocin-induced type 1 diabetic rats. *Frontiers in neural circuits*. **8**, 6 (2014).
20. Alarcon-Martinez, L. et al. Capillary pericytes express α-smooth muscle actin, which requires prevention of filamentous-actin depolymerization for detection. *eLife*. **7**, e34861 (2018).
21. Wang, J. et al. A new approach for examining the neurovascular structure with phalloidin and calcitonin gene-related peptide in the rat cranial dura mater. *Journal of Molecular Histology*. **51** (5), 541–548 (2020).
22. Liu, Y., Broman, J., Edvinsson, L. Central projections of sensory innervation of the rat superior sagittal sinus. *Neuroscience*. **129**, 431–437 (2004).
23. Liu, Y., Broman, J., Edvinsson, L. Central projections of the sensory innervation of the rat middle meningeal artery. *Brain Research.* **1208**, 103–110 (2008).
24. Schmued, L. C., Fallon, J. H. Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. *Brain Research*. **377** (1), 147–154 (1986).

Figure 1

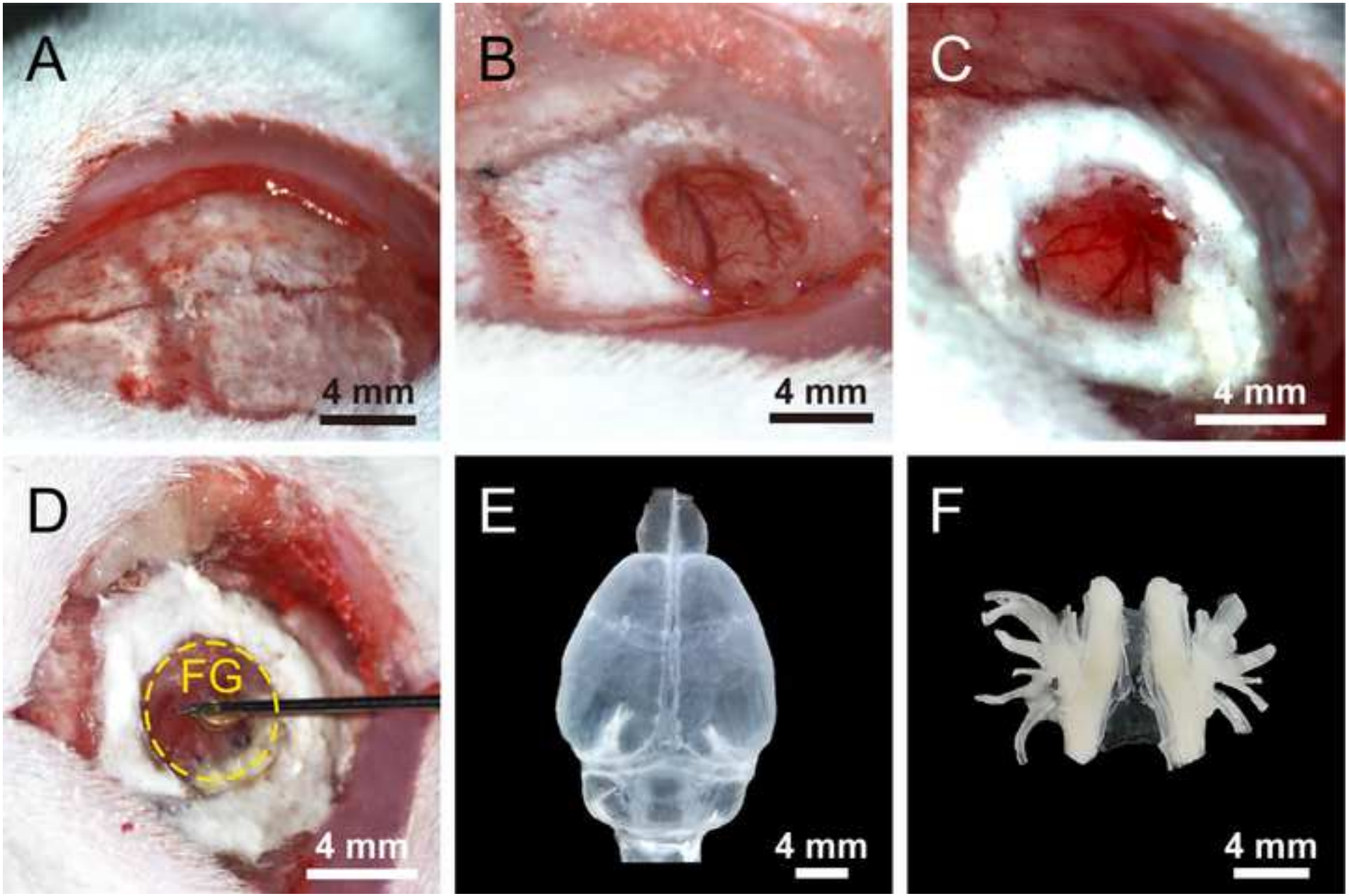

Figure 2

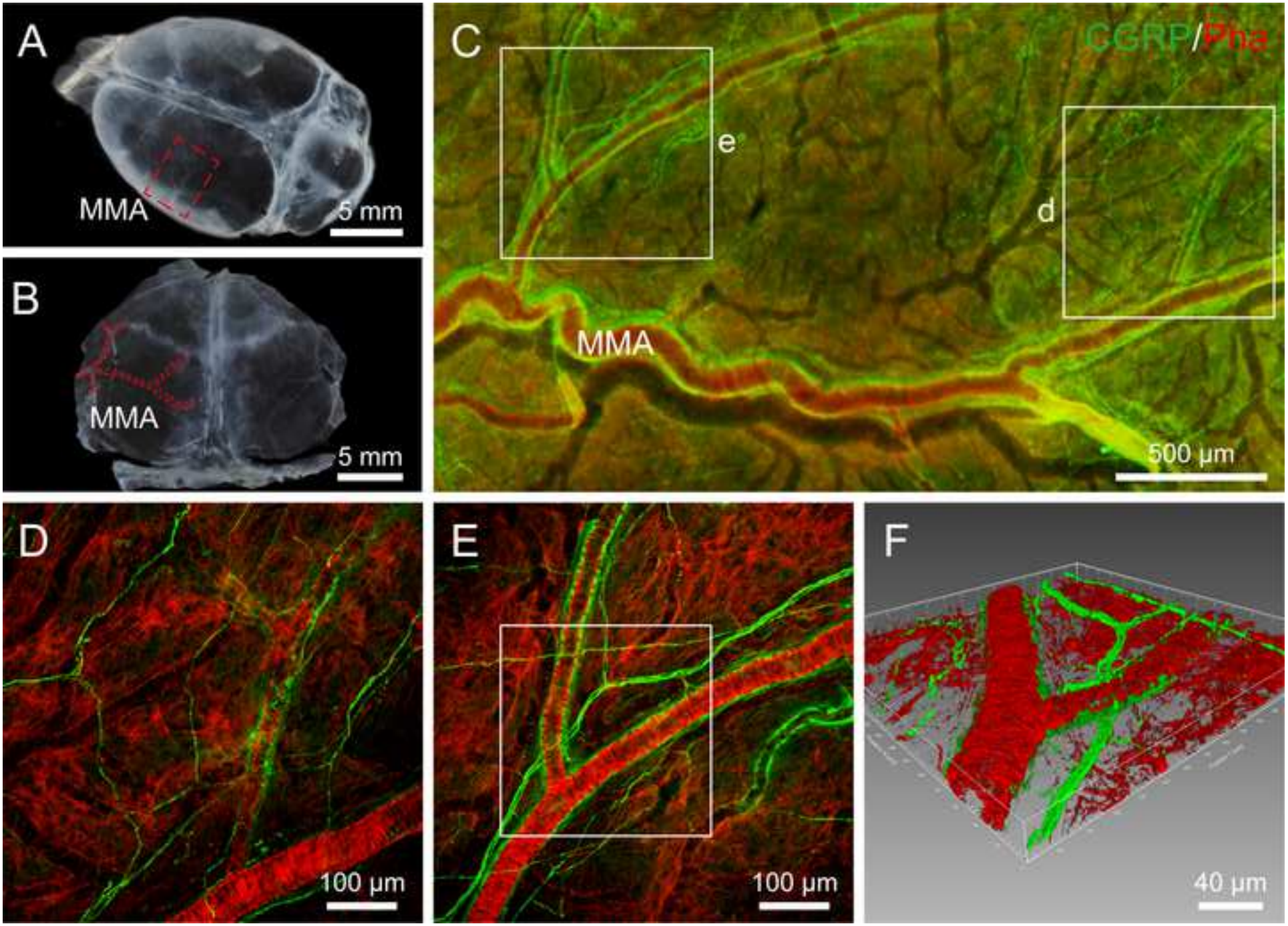

Figure 3

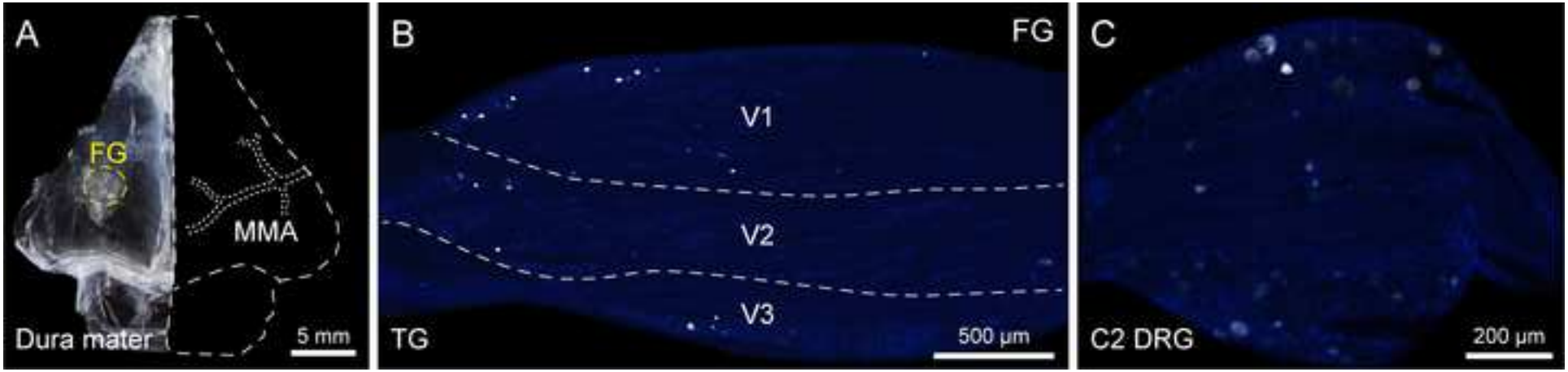

Figure 4

Click here to access/download;Figure;Figure 4.tif

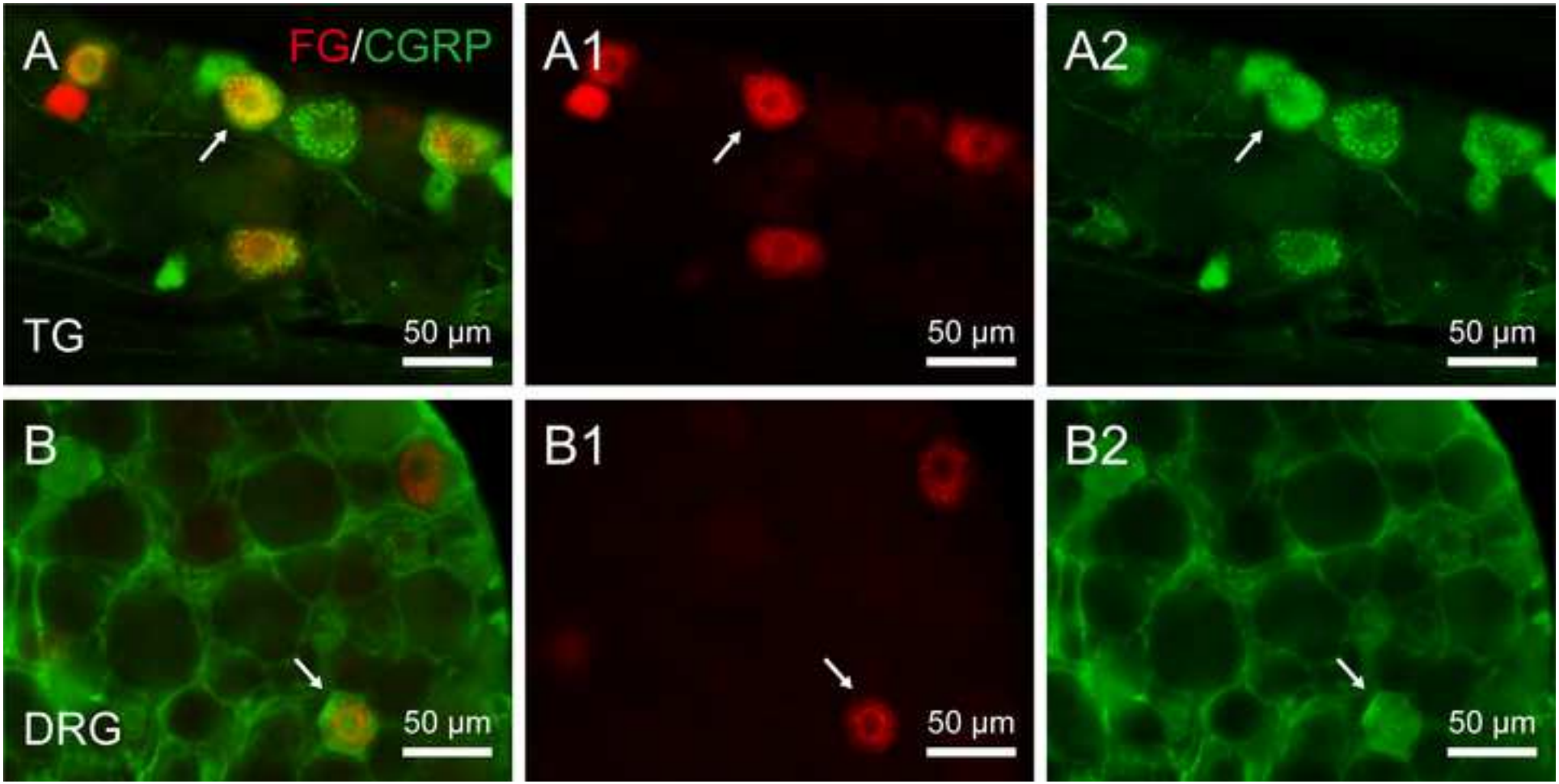

Table of Materials

Click here to access/download;Table of Materials;Table of Specific Reagents and Equipment.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Alexa Fluor 488 donkey anti-mouse IgG (H+L)	Invitrogen by Thermo Fisher Scientific	A21202	Protect from light; RRID: AB_141607
Brain stereotaxis instrument	Narishige	SR-50	
CellSens Dimension	Olympus	Version 1.1	Software of fluorescent
Confocal imaging system	Olympus	FV1200	
Fluorogold (FG)	Fluorochrome	52-9400	Protect from light
Fluorescent imaging system	Olympus	BX53	
Freezing microtome	Thermo	Microm International GmbH	
Olympus FV10-ASW 4.2a	Olympus	Version 4.2	Confocal image processing software system
Micro Drill	Saeyang Microtech	Marathon-N7	
Mouse anti-CGRP	Abcam	ab81887	RRID: AB_1658411
Normal donkey serum	Jackson ImmunoResearch	017-000-121	

Phalloidin 568	Molecular Probes	A12380	Protect from light
Photoshop and Illustration	Adobe	CS6	Photo editing software
Rabbit anti- Fluorogold	Abcam	ab153	RRID: AB_90738
Sprague Dawley	National Institutes for Food and Drug Control	SCXK (JING) 2014-0013	
Superfrost plus microscope slides	Thermo	#4951PLUS-001	25x75x1mm

Dear Editor and Reviewers:

Thank you very much for giving us an opportunity to revise our manuscript. We appreciate you and reviewers for the comments on our revised manuscript JoVE61742R1. We have rewritten, reorganized our text according to all the comments, and expect to meet your requirements. The revised portions are marked in red. The main revision in the paper and the responds to the reviewers' comments are as following.

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

Answer: We have retained and used the attached file for revision.

2. Please address specific questions marked in the manuscript.

2.1 Please include email addresses for all authors here.

Answer: We have added the email address for the co-authors.

2.2 "fluorogold": This is commercial, please use generic term instead. E.g., Non-bleaching bioconjugated diamonds for imaging. The term can be used once in the introduction.

Answer: Fluorogold is a kind of neural tracer, it was not a commercial name.

2.3 "the dura mater and dorsal side of the brain. Then dissected out the cranial dura mater along the brainstem to the olfactory bulb in the whole-mount pattern (Figure 1)": How do you identify these in the brain, citations etc:

Answer: When remove the head skin and open the entire dorsal side of the skull, we can directly find the dura mater, which wraps around the surface of the brain from the brainstem to the olfactory bulb. Then we can dissect out the cranial dura mater along the brainstem to the olfactory

bulb. This is the basic structure in gross anatomy, no citation needed.

2.4 “Rinse the cranial dura mater” : So you use the whole cranial dura matter? How do you differentiate this part of the brain from other parts.

Answer: Yes, we use the whole cranial dura matter for immunofluorescent staining (Figure 2B). After perfusion, we cut off the whole-mount cranial dura mater along the brainstem to the olfactory bulb separately (Figure 1E, Figure 2A).

2.5 “anti-CGRP antibody”: Reasons for using this antibody? citations associated with it?

Answer: This is the primary antibody for staining the CGRP. In the revised version, we have added a citation related to the CGRP staining [11. Cui, J. J., Zhu, X. L., Shi, H., Su, Y. S., Jing, X. H., Bai, W. Z. The expression of calcitonin gene-related peptide on the neurons associated Zusanli (ST 36) in rats. *Chinese journal of integrative medicine*, 21(8), 630-634 (2015).]

2.6 Significance of using Phalloidin?

Answer: The cranial dura mater is the outermost layer of meninges to protect the brain and contains plentiful blood vessels and different kinds of nerve fibers, and the aim of this study is to examine the innervation of dura mater, mainly for the dural blood vessels. Furthermore, according to our published study, phalloidin is a proper maker for staining dural blood vessels. Therefore, in this study, we choose CGRP and fluorescent phalloidin for staining the nerve fibers and blood vessels.

2.7 “Trim the edges of the dura mater and mount it on microscope slides”: The whole dura matter is mounted on the slide? Do you make sections?

Answer: Yes, as shown in the Figure 2B, the whole dura mater is mounted on the slide. And we

don't make sections.

2.8 “on the left parietal and temporal bones above the middle meningeal artery (MMA),” How do you identify these?

Answer: We identified these according to the anatomy and previous published papers. In the revised version, we have added this citation: [12. Andres, K. H., von Düring, M., Muszynski, K., Schmidt, R. F. Nerve fibres and their terminals of the dura mater encephali of the rat. *Anatomy and embryology.* 175(3), 289-301 (1987).]

3. The reviewers' comments were not addressed thoroughly previously. Please ensure that all comments are addressed.

Answer: In the revised version, we have revised all the comments from the reviewers.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

A well-written detailed protocol for studying dura innervation.

Major Concerns:

none

Minor Concerns:

none

Reviewer #2:

Manuscript Summary:

The authors did not address the main concerns:

1) CGRP-immunoreactive innervation of the dura mater is not identical with the sensory

innervation. They have not changed the title of the manuscript accordingly. They have not discussed which subpopulation of sensory neurons are indicated by CGRP-immunostaining.

Answer: In the revised version, we have changed the title of the manuscript as following: "Visualizing the CGRP-immunoreactive Innervation of the Rat Cranial Dura Mater with Immunofluorescence and Neural Tracing Approaches". According to the diameter of CGRP-immunoreactive neurons in TG and DRG, most of them were primarily found in the small- and medium-diameter sensory neurons (<30 μm). CGRP-immunoreactive nerve fibers belong to the unmyelinated peptidergic sensory axons responsible for the transportantion of nociceptive signals.

2) Specificity of the immunostaining is neither controlled nor discussed in the text.

Answer: According to our previous published studies, the immunostaining has been a mature experimental technique in our laboratory, so we don't conduct a control. This have been discussed in the revised version. The related references are as following:

10. Xu, D. S., She, C., Wang, J., Cui, J. J., Cai, H., Bai, W. Z. Characteristics of distribution of blood vessels and nerve fibers in the skin tissues of acupoint "Taichong" (LR3) in the rat. *Zhen Ci Yan Jiu*. 41 (6), 486-491 (2016).

11. Cui, J. J., Zhu, X. L., Shi, H., Su, Y. S., Jing, X. H., Bai, W. Z. The expression of calcitonin gene-related peptide on the neurons associated Zusanli (ST 36) in rats. *Chinese journal of integrative medicine*, 21(8), 630-634 (2015).

21. Wang, J., et al. A new approach for examining the neurovascular structure with phalloidin and calcitonin gene-related peptide in the rat cranial dura mater. *Journal of molecular histology*, (2020).

Reviewer #3:

Major Concerns:

1.My main comment on the original submission was to ask what aspect of the methods was considered noteworthy by the authors, in view of the fact that these basic methods

(ganglionic tracing and immunocytochemical labeling of dural afferents) have been used since 1980 in many papers. The authors' reply is that the key methodological advance is the three-dimensional representation. This does not seem to be a very strong justification for the paper. The method for the three-dimensional representation simply consists of scanning through the tissue with a confocal microscope and using the standard confocal software for making a 3-dimensional representation. This is simply standard use of a confocal microscope. In addition, it is not clear that there is any additional information gained from the 3-dimensional images beyond what is shown in the 2-dimensional images. Although a 3-dimensional image is shown, there is no 3-dimensional organization that is described in the paper.

Regarding my comment that these methods are extremely standard: Figure 1 shows a photo of a rat in a stereotaxic holder. It also shows a photo of the stereotaxic with a manipulator, and a photo of a microscope. These are such standard methodologies that it is hard to imagine that there are investigators for whom these photos would be helpful.

Answer: Thank you for your comments. Although neural tracing and immunofluorescence techniques have been applied since 1980 in many previous studies, but the combination of the neural tracing, immunofluorescence techniques and three-dimensional reconstruction for observing the neurochemical properties of dura mater in the three-dimensional view was few in previous studies. Therefore, the methodological advance of this study is to highlight how conventional techniques can be organically combined for new research. In the revised version, we have added the description for three-dimensional organization.

In addition, we have adjusted the panels in Figure 1in the revised paper.

2. A related point was my question about what type of blood vessels are stained by phalloidin. (In the original manuscript, the authors used the term "arteriors", and I took that to mean "arteries". I assume it does not mean "arterioles" because the phalloidin appears to stain arteries that would be too large to be classified as arterioles.) However, in the revised manuscript, the authors have removed any mention of the types of blood vessels that are stained by phalloidin, and instead simply say that phalloidin stains blood vessels. The

authors must instead specify what classes of blood vessels are stained by phalloidin.

In the revised manuscript, the authors have added a citation of a new paper that they have published (reference 18). This published paper has the same data that is contained in the present submission. The published paper is in fact much more informative than the present submission in its description of the data, and one of the key points of the published paper is to define the type of blood vessels that are stained by the phalloidin.

Answer: Thank you for your comments. The phalloidin stained blood vessels including arteries and arterioles. However, the meninges arteries are mainly presented with the styles of arterioles, which are showed with phalloidin-positive labeling. In the revised version, we have added the type of blood vessels that are stained by phalloidon as arterioles.

Minor Concerns:

The figures should be shown at higher resolution. The images become pixelated when examined closely. The resolution of these images is much higher in the published paper (reference 18).

Answer: The resolution of our figures uploded to the system is 300 dpi. The resolution of these images is the same as these in the reference 18. If the images become pixelated when examined closely at a PDF document, it may be due to the compression of the submission system. In the revised paper, we have changed them to 600 dpi.

Reviewer #4:

The authors have addressed all my concerns.

Reviewer #5:

Manuscript Summary:

The authors provide a method, as I summarized in my original review, of a means to trace afferents of cranial nerve ganglia within the dura mater using Flurogold.

Major Concerns:

I would like to see some comments about sub-regions for the cells they labeled in the ganglia, or at least some comment about their selective uptake relative to the rest of the area. Specifically, in Figs. 3B and 3C, how do they know where they are in the ganglia? Did they use a Nissl section to determine subregional zones in the ganglia or which cervical levels specifically they state they collected from?

Answer: For TG, we dissected out this tissues according to its anatomy and the reference [George, P. *The rat nervous system*. USA: Elsevier academic press. 818 (2004)], in which we can clearly see the ophthalmic (V1), maxillary (V2) and mandibular (V3) branches of TG (Figure 1F). Based on this, we cut the TG on a cryostat microtome system in the sagittal direction and mounted on silane-coated glass slides, in which we differentiate the sub-regions of TG according to the three branches and the distributions of ganglion cells (Figure 3B). For DRGs, we dissected the cervical (C) 2-4 DRGs according to the gross anatomy. After sectioning, some of the FG-labeled neurons were observed in the C2-3 DRGs. The image showed in Figure 3C is the C2 DRG.

Minor Concerns:

None noted.

Best wishes,

Sincerely yours,

Jia Wang, Wan Zhu Bai

Institute of Acupuncture and Moxibustion,

China Academy of Chinese Medical Sciences,

16 Nanxiaojie of Dongzhimennei, Beijing 100700, China.

E-Mail: wanzhubaisy@hotmail.com

Tel: +86-10-64089390 (office)

Mobile: +86-18611170320