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Combined 3DISCO clearing method, retrograde tracer and ultramicroscopy to map corneal neurons in a whole adult mouse trigeminal ganglion

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ABSTRACT

Tissue clearing and subsequent imaging of intact transparent tissues have provided an innovative way to analyze anatomical pathways in the nervous system. In this study, we combined a recent 3-dimensional imaging of solvent cleared organ (3DISCO) procedure, light-sheet microscopy, fluorescent retrograde tracer, and Imaris software to 3D map corneal sensory neurons within a whole adult mouse trigeminal ganglion (TG). We first established the optimized steps to easily and rapidly clear a fixed TG. We found that the 3DISCO procedure gave excellent results and took less than 3 h to clear the TG. In a second set of experiments, a retrograde tracer (cholera toxin B Alexa 594-conjugated) was applied to de-epithelialized cornea to retrograde-labeled corneal sensory neurons. Two days later, TGs were cleared by the 3DISCO method and serial imaging was performed using light-sheet ultramicroscopic technology. High-resolution images of labeled neurons can be easily and rapidly obtained from a 3D reconstructed whole mouse TG. We then provided a 3D reconstruction of corneal afferent neurons and analyzed their precise localization in the TG. Thus, we showed that neurons supplying corneal sensory innervation exhibit a highly specific limited dorsomedial localization within the TG. We report that our combined method offers the possibility to perform manual (on 20 μm sections) and automated (on 3D reconstructed TG) counting of labeled cells in a cleared mouse TG. To conclude, we illustrate that the combination of the 3DISCO clearing method with light-sheet microscopy, retrograde tracer, and automatic counting represents a rapid and reliable method to analyze a subpopulation of neurons within the peripheral and central nervous system.

1. Introduction

The cornea is the most densely innervated tissue of the body (Muller et al., 2003, 1996, 1997). The corneal sensory nerve fibers originate from neuronal cell bodies located in the ipsilateral trigeminal ganglion (TG) (Felipe et al., 1999; Marfurt and Del Toro, 1987; Marfurt et al., 1989). The TG is composed of three nerve branches: (i) the ophthalmic branch, V1, (ii) the maxillary branch, V2, and (iii) the mandibular branch, V3, each branch having its own primary sensory neurons organized inside the ganglion (Marfurt et al., 1989).

Until now, published studies in the literature have reported the distribution of TG neurons supplying the corneal innervation by means of classical histological approaches. All of these studies used serial TG tissue sections from animals whose corneas were treated with various retrograde tracers (such as Cholera toxin subunit B (CTB), Fast blue, Fluorogold, horseradish peroxidase (HRP), wheat germ agglutinin (WGA), etc) (Bron et al., 2014; Ivanusic et al., 2013;

Abbreviations: CTB, Cholera toxin subunit B; TG, trigeminal ganglion; V1, ophthalmic branch; V2, maxillary branch; V3, mandibular branch.

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Marfurt and Del Toro, 1987). However, these procedures required serial sections, microscopic analysis, and manual cell counting, all of which are time consuming (Bron et al., 2014; Ivanusic et al., 2013; Marfurt and Del Toro, 1987). The only way to obtain spatial organization of cells in tissues was to obtain sections and image the cells by epifluorescence, confocal, or two-photon microscopy. Unfortunately, these methods are not suitable to perform spatial reconstruction in large and thick tissues (up to 500 μm of thickness). Nowadays, new technologies have arisen to help scientists overcome this optical limit. For the last three years, several groups have developed new tissue clearing methods for enhanced imaging of entire tissues: ClearT (Kuwajima et al., 2013), SeeDB (Ke et al., 2013; Ke and Imai, 2014), ScaleA (Hama et al., 2011), CLARITY (Chung et al., 2013; Tomer et al., 2014), PACT/PARS (Yang et al., 2014), CUBIC (Susaki et al., 2014; Tainaka et al., 2014), and 3-dimensional imaging of solvent cleared organ (3DISCO) (Erturk and Bradke, 2013; Erturk et al., 2014). When combined with subsequent light-sheet laser scanning microscopy (Dodt et al., 2007), 3DISCO, offers the possibility of imaging entire transparent organs without the need for sectioning (Dodt et al., 2007; Erturk et al., 2012; Erturk and Bradke, 2013).

In this study, we report the 3D mapping of corneal afferent neurons within the adult mouse TG using the 3DISCO clearing method. More interestingly, we combined this procedure with a fluorescent retrograde tracer, light-sheet microscopy, and automated counting using Imaris software to rapidly obtain a 3D somatotopy of corneal neurons in the entire TG. Here, we provide evidence that clearing a whole mouse TG (containing retrograde labeled neurons) with 3DISCO and light-sheet microscopy imaging allows to obtain, in one day, a precise somatotopy of a specific neuronal population in solvent-cleared tissue.

2. Materials and supplies

2.1. Supplies

Surgical blade (Swann-Morton Ref. S0201).

70% ethanol solution (Ethanol 100% Sigma–Aldrich Ref. 32221, v70EtOH100%/v30H20).

Ophthalmic gel (Dechra, Lubrithal Eye Gel, 10 g).

Cholera toxin subunit B (CTB)-Alexa Fluor 594 conjugate (CTB-594, Cell Signaling, Danvers, MA, USA, Ref. C34777).

1× PBS (Gibco, Ref. 10010-023).

Paraformaldehyde (Sigma–Aldrich, Ref. 441244).

Stuart tube rotator (model SB3).

Tetrahydrofuran absolute, over molecular sieve, containing ~ 0.025% 2,6-di-tert-butyl-4-methylphenol as stabilizer ≥ 99,5% THF (Sigma–Aldrich, Ref. 87371).

Dichloromethane (DCM, Sigma–Aldrich, Ref. 270997).

Dibenzyl ether (DBE, Sigma–Aldrich, Ref. 108014).

2.2. Equipment

Binocular microscope (World Precision Instrument, Ref. 502000).

Ultramicroscope (LaVision Biotec) equipped with an Olympus binocular body (MXV10) and an Olympus objective MVPLAPO 2×C with variable magnification from 0.63× to 6.3× and equipped with a PCO Edge SCMOS camera: Scientific low noise CMOS camera Sensors CMOS, CIS 2051 Pixel (2560 × 2160 pixel, pixel size 6.5 × 6.5 μm^2, LaVision Biotec).

ImageJ 1.48 software was downloaded from the website (http://imagej.nih.gov/ij/download.html).

Imaris 7.6.1 software (Bitplane, Zurich, Switzerland, http://www.bitplane.com/imaris/imaris).

Computer Intel® Core™ i7-3930 with powerful graphic card NVIDIA GeForce GTX 560 and 64 gigabytes of RAM. 3D.

2.3. Fluorescent retrograde tracer preparation

CTB-Alexa Fluor 594 conjugate was prepared at a concentration of 1 μg/μL in 1× PBS.

2.4. Experimental animals

Adult male C57BL/6 mice weighing 30 g (Janvier Labs, Le Genest Saint Isle, France) were maintained under controlled conditions (22 ± 1 °C, 60 ± 10% relative humidity, 12/12 h light/dark cycle, food and water ad libitum). All procedures were in strict accordance with the guidelines for the care and use of experimental animals approved by the French National Institute of Medical Research (INSERM) and national and international law and with policies for the use of animals in Research in Vision and Ophthalmology (European Communities Council Directive 86/609/EEC, Authorization No. 75–1235 Granted to A. R-LG).

3. Detailed methods

3.1. Retrograde tracing experiments and tissue preparation

To identify the corneal afferent neurons in the TG, we used CTB-Alexa Fluor 594 conjugate as a tracer. Animals were anesthetized intraperitoneally with a mixture of Ketamine 1000 U (100 mg/kg body weight) and xylazine (10 mg/kg body weight, Virbac, France). Only the left eye was processed in these experiments. The right eye was maintained hydrated during the surgery by placing a drop of ophthalmic gel.

Under a binocular microscope, a filter paper disc (3 mm in diameter) soaked with 70% ethanol solution was positioned in the corneal surface and left in place for 30 s. The disc was then withdrawn and the corneal epithelium of the left eye was removed using a surgical blade. It is important to note that the same experimenter routinely managed this surgical procedure. Using a fluorescein test and under slit lamp examination in another group of animals, the experimenter has previously quantified that nearly 90% of the surface of the corneal epithelium was removed (data not shown).

A total of 1 μl (5 applications of 0.2 μl using a 10 μl tip) of CTB-Alexa 594 solution (1 μg/μl in 1× PBS) or 1 μl of 1× PBS (control mice) was meticulously placed in the damaged left cornea under the binocular microscope to avoid diffusion of the CTB solution outside the de-epithelialized cornea. As control conditions, we also applied CTB solution on healthy cornea. The solution was maintained in contact with the cornea for 10 min. This experimental approach allows the tracer to be taken up by central and peripheral corneal nerve fibers and not surrounding tissues. Animals were left to recover from anesthesia in their cages.

3.2. Tissue preparation

Two days after the CTB application, PBS- or CTB- treated mice were deeply anesthetized with Ketamine 1000 U (100 mg/kg)/Xylazine (10 mg/kg, Virbac, France). Animals were then perfused via the ascending aorta with 10 ml of 0.9% saline followed by 40 ml of 4% paraformaldehyde in PBS (0.1 M, pH 7.4). After fixation, TGs were gently dissected out. During the dissection, it was important to keep the mandibular branch (V3) intact, which constitutes an anatomical landmark during image acquisition under the ultramicroscope. To limit tissue autofluorescence, TGs were not post-fixed in 4% paraformaldehyde, but were placed in 1× PBS solution at 4 °C