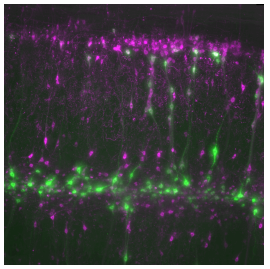
Oct 08, 2025

Immunolabelling and clearing of intact, fixed rat spinal cord for visualization of neurons projecting to major pelvic ganglion



Forked from Immunolabelling and clearing of intact spinal cord for visualization of lower urinary tract afferents

DOI
dx.doi.org/10.17504/protocols.io.n2bvj8bewgk5/v1

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.n2bvj8bewgk5/v1

Protocol Citation: John-Paul Fuller-Jackson, Peregrine B Osborne, Janet R Keast 2025. Immunolabelling and clearing of intact, fixed rat spinal cord for visualization of neurons projecting to major pelvic ganglion. **protocols.io https://dx.doi.org/10.17504/protocols.io.n2bvj8bewgk5/v1**

Protocol status: Working

Created: March 05, 2023

Last Modified: October 08, 2025

Protocol Integer ID: 78163

Keywords: neural tracing, retrograde tracing, clearing, idisco, light sheet microscopy, spinal cord, pelvic ganglion, motor neuron, preganglionic neuron, cholinergic neuron

Funders Acknowledgements:
NIH SPARC
Grant ID: OT2OD023872

Abstract

The whole-mount immunolabeling and clearing method (iDISCO) was used to visualize cholera toxin subunit B (CTB)-labelled lumbar and sacral preganglionic neurons in the spinal cord of the adult rat. Imaging of spinal cord was performed on a light sheet microscope with a 2x and a 12x lens. Choline acetyltransferase immunoreactivity identified preganglionic autonomic neurons and somatic motoneurons within the spinal cord.

Materials

Materials

- Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M3641
- Dichloromethane **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**320269
- Dibenzyl ether **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**108014
- Ethyl cinnamate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**112372
- 1X Dulbecco's Phosphate Buffered Saline (DPBS) **Thermo Fisher Scientific Catalog #**14190094
- Gelatin from porcine skin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G1890
- Hydrogen peroxide 30% **Merck Millipore (EMD Millipore) Catalog #**822287.1000
- Saponin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S4521
- Thimerosal **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T5125
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787-50ML
- Rabbit anti-cholera toxin antibody **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C3062
- Goat anti-choline acetyltransferase antibody **Merck Millipore (EMD Millipore) Catalog #**AB144P
- Cy3 Donkey anti-rabbit IgG **Jackson ImmunoResearch Laboratories, Inc. Catalog #**711-165-152
- AF647 donkey anti-goat IgG **Jackson ImmunoResearch Laboratories, Inc. Catalog #**705-605-147

Equipment

Equipment	
Ultramicroscope II	NAME
Light sheet microscope	TYPE
Miltenyi Biotec	BRAND
NA	SKU
https://www.miltenyibiotec.com/AU-en/products/ultramicroscope-ii.html	LINK

Solutions

PBS: phosphate-buffered saline, 0.1 M, pH 7.2
DPBS: 1x Dulbecco's phosphate-buffered saline
DPBS-T: 1x Dulbecco's phosphate-buffered saline containing 0.5% Triton X100

DPBSG-T: 1x Dulbecco's phosphate-buffered saline containing 0.2% gelatin, 0.5% Triton X-100 and 0.01% thimerosal

Primary antibodies

	A	B	C	D	E
	Abbreviation	**Synonym**	**RRID**	**Host species**	**Dilution**
	ChAT	Choline acetyltransferase	AB_11214092	Goat	1:500
	CTB	Cholera toxin subunit B	AB_258833	Rabbit	1:3000

Secondary antibodies

	A	B	C	D
	Tag-antibody	**Host species**	**RRID**	**Dilution**
	AF647 anti-goat	Donkey	AB_2340437	1:2000
	Cy3 anti-rabbit	Donkey	AB_2307443	1:2000

Spinal cord preparation

30m

1 While immersed in phosphate buffered-saline (PBS), pH 7.2, trim nerve roots of paraformaldehyde-fixed spinal cord to within approximately 2 mm of the spinal cord surface to facilitate the identification of segments later, following imaging. Segments of particular interest are T13-L2 for the lumbar preganglionic neurons, and L6-S1 for the caudal preganglionic neurons.

Bleaching

1d

2 Wash samples in 1x Dulbecco's PBS (DPBS)(6 × 15 mins).

3 Dehydrate samples in a series of methanol in DPBS dilutions while on rotation at 12 rpm:
1. 50% methanol in DPBS (1.5 h)
2. 80% methanol in DPBS (1.5 h)
3. 100% methanol (1.5 h)

4 Bleach samples overnight in 6% hydrogen peroxide in methanol at 4°C, protected from light.

Blocking

2d

5 Rehydrate samples in a series of methanol in DPBS dilutions while on rotation at 12 rpm:
1. 100% methanol (2 × 1.5 h)
2. 80% methanol in DPBS (1.5 h)
3. 50% methanol in DPBS (1.5 h)
4. DPBS (1.5 h)

6 Incubate samples in DPBS containing 0.2% gelatin, 0.5% Triton X-100 and 0.01% thimerosal (DPBSG-T) for 36 h while on rotation at 12 rpm

Primary antibody incubation

1w 3d

7 Incubate samples in primary antibody solution containing DPBSG-T with 0.1% saponin for 10 days at 37°C with agitation. Volume of solution need only be sufficient to cover the sample.

Secondary antibody incubation

5d

8 Wash spinal cords in 1x DPBS with 0.5% Triton X-100 (DPBST) (6 × 15 mins).

9 Incubate samples in secondary antibody solution containing DPBSG-T with 0.1% Saponin for 4 days at 37°C with agitation. Volume of solution need only be sufficient to cover the sample.

Dehydration and delipidation

1d 8h

10 Wash spinal cords in DPBST (6 × 15 mins).

11 Dehydrate spinal cords in a series of methanol in DPBS dilutions while on rotation at 12 rpm:
1. 20% methanol in DPBS (1 h)
2. 40% methanol in DPBS (1 h)
3. 60% methanol in DPBS (1 h)
4. 80% methanol in DPBS (1h)
5. 100% methanol (2 × 1 h)

12 Incubate samples in a solution of 2/3 dichloromethane and 1/3 methanol overnight on rotation. Ensure that samples sink to the bottom of the vial at the end of this step, otherwise continue incubation in freshly made solution.

13 Incubate samples in 100% dichloromethane for 30 mins while on rotation at 12 rpm. Repeat this step until samples sink.

Clearing

2h

14 Incubate samples in dibenzyl ether until the samples have become clear. Ensure each vial is completely filled with dibenzyl ether to minimize sample oxidation as a result of large amounts of air in the vial. This process should not take longer than 2 h.

Storage

15 Store cleared samples in fresh dibenzyl ether. Keep away from light (wrap in foil and store in an opaque container).

Light sheet microscopy

16 Prior to visualization on a light sheet microscope, samples should be transferred into ethyl cinnamate, at least 3 h prior.

17 Remove sample from ethyl cinnamate and gently dry on a tissue. Affix sample to plastic mount using the minimum amount of super glue required. Tips:
- Avoid adhering the sample to the base via any region of the sample that is of interest; both the super glue and proximity to the plastic will reduce imaging quality in that area.
- Mount the sample perfectly in the middle; the light sheet microscope stage has limited mobility, particularly at higher magnifications.
- Orientate the sample such that the thinnest plane of the sample is perpendicular to the light sheet beams. The further light has to travel through a sample, the poorer the image quality.
- Orientate the sample so that the region of interest is facing as close to the lens as possible.

In this protocol, the spinal cord was mounted perpendicular to the light sheet beams, with the dorsal horn facing the lens, and the ventral side of L5 or T13 (depending on sample chosen) being the point of adherence to the mounting platform.

18 In order to visualize the axons and dendrites of preganglionic neurons, use the highest magnification lens available on the light sheet microscope.
For example, a 12x lens is adequate for dendrite visualization.
Look for cholera toxin subunit B labelled projections extending from the intermediolateral nuclei to the dorsal commissural nucleus, as well as projections into the ventral horn of the grey matter.

19 Image acquisition was always performed with 2 µm z-step between each image. Where necessary, mosaic image acquisition was applied to capture the entire region of interest, with 10% overlap between image stacks.

Protocol references

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