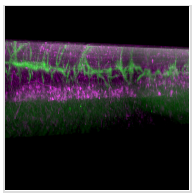

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# Immunolabelling and clearing of intact spinal cord for visualization of lower urinary tract afferents

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The whole-mount immunolabeling and clearing method (iDISCO) was used to visualize cholera toxin subunit B-labelled lower urinary tract afferents in the lumbosacral spinal cord of the rat. Imaging of spinal cord was performed on a light sheet microscope with a 12x lens. Concurrently, choline acetyltransferase identified preganglionic autonomic neurons and motoneurons within the spinal cord, which were used to confirm the rostrocaudal location of afferents.

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**Visualizing lower urinary tract afferent projections in the lumbosacral spinal cord in rats**

neural tracing, retrograde tracing, clearing, idisco, light sheet microscopy

protocol ,

Oct 01, 2021

Dec 15, 2021

53733

Part of collection

Visualizing lower urinary tract afferent projections in the lumbosacral spinal cord in rats

**Materials**

Methanol **Sigma Aldrich Catalog #M3641**

Dichloromethane **Sigma Aldrich Catalog #320269**

Dibenzyl ether **Sigma Aldrich Catalog #108014**

Ethyl cinnamate **Sigma Aldrich Catalog #112372**

1X Dulbecco's Phosphate Buffered Saline (DPBS) **Thermo Fisher Scientific Catalog #14190094**

Gelatin from porcine skin **Sigma Aldrich Catalog #G1890**

Hydrogen peroxide 30% **Merck Millipore Catalog #822287.1000**

Saponin **Sigma Aldrich Catalog #S4521**

Thimerosal **Sigma Aldrich Catalog #T5125**

Triton X-100 **Sigma Aldrich Catalog #T8787-50ML**

Rabbit anti-cholera toxin antibody **Sigma Aldrich Catalog #C3062**

Goat anti-choline acetyltransferase antibody **Merck Millipore Catalog #AB144P**

Cy3 Donkey anti-rabbit IgG **Jackson Immunoresearch Catalog #711-165-152**

AF647 Donkey anti-sheep IgG **Thermo Fisher Scientific Catalog #A21448**

**Equipment**



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Ultramicroscope II
Light sheet microscope
**Miltenyi Biotec** **NA**

**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 |
|---|---|---|
| **Tag-antibody** | **Host species** | **Dilution** |
| AF647 anti-sheep | Donkey | 1:500 |
| Cy3 anti-rabbit | Donkey | 1:2000 |

## Spinal cord preparation 30m

1 While immersed in phosphate buffered-saline (PBS), pH 7.2, trim nerve roots of fixed spinal cord to within approximately 2 mm of the spinal cord surface to facilitate the identification of segments later following imaging.

## Bleaching 1d

2 Wash samples in 1x Dulbecco's PBS (DPBS)(6 x 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)



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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 x 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 x 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 x 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 x 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.



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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 being the point of adherence to the mounting platform.

18 In order to visualize the lower urinary tract afferents in the dorsal horn of the spinal cord, use the highest magnification lens available on the light sheet microscope.
For example, a 12x lens is adequate for afferent visualization.
Look for cholera toxin subunit B labelled projections in the lateral and medial boundaries of the dorsal horn. One should also find dense afferent innervation of the sacral preganglionic nucleus of L6 and S1 spinal cord .



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