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Immunolabelling and clearing of large samples of peripheral nerve tissue

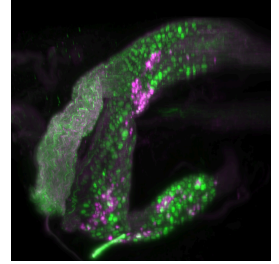
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We use this protocol and it's working

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Abstract

A whole mount immunolabeling and clearing method (iDISCO) combined with the pre-treatment steps in ADIPO-CLEAR is used to visualize neural structures in large samples of peripheral nerve tracts or ganglia. These tissues can be associated with large regions of adipose tissue that are difficult to remove without damaging the adjacent neural tissue. Examples of antibodies used for this type of study are also provided (tyrosine hydroxylase for noradrenergic ganglion neurons and their axons; Beta-tubulin as a pan-neural marker).

Materials

Materials

- ⊗ Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3641**
- ⊗ Glycine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G8790-1KG**
- ⊗ Sodium azide **ChemSupplyAustralia Catalog #SA189-100G**
- ⊗ 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**
- ⊗ Thimerosal **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T5125**
- ⊗ Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8787-50ML**
- ⊗ Anti-class III beta tubulin antibody (rabbit) **BioLegend Catalog #802001**
- ⊗ Anti-tyrosine hydroxylase antibody (sheep) **Merck Millipore (EMD Millipore) Catalog #AB1542**
- ⊗ AF647 donkey anti-sheep antibody **Invitrogen Catalog #10729004**
- ⊗ Cy3 donkey anti-rabbit antibody **Jackson ImmunoResearch Laboratories, Inc. Catalog #711-165-152**

Equipment

Equipment	
Ultramicroscope Blaze	NAME
Light sheet microscope	TYPE
Ultramicroscope	BRAND
140-006-739.04	SKU
https://www.miltenyibiotec.com/AU-en/products/ultramicroscope-blaze.html	LINK

Solutions

PBS: phosphate-buffered saline, 0.1 M, pH 7.2
B1n buffer: 0.3 M glycine, 0.1% Triton X100, 0.01% sodium azide in water

DPBS: 1x Dulbecco's phosphate-buffered saline

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
	TH	Tyrosine hydroxylase	AB_90755	Sheep	1:1000
	Tuj1	Class III beta-tubulin	AB_2564645	Rabbit	1:2000

Secondary antibodies

	A	B	C	D
	Tag-antibody	Host species	RRID	Dilution
	AF647 anti-sheep	Donkey	AB_2535865	1:500
	Cy3 anti-rabbit	Donkey	AB_2307443	1:2000



Large peripheral nerve tissue preparation

1h

- 1 While immersed in phosphate buffered saline (PBS), pH 7.2, use a scalpel blade to sub-dissect large sample of peripheral nerve tissue into smaller samples, generally no larger than 10 mm x 10 mm x 10 mm.

Dehydration and Delipidation

1d

- 2 Wash samples in B1n buffer (1 h) on rotation 12 rpm at 4 °C.
- 3 Dehydrate samples in a series of methanol in B1n buffer dilutions on rotation 12 rpm at 4 °C.
 1. 20% methanol in B1n buffer (1 h)
 2. 40% methanol in B1n buffer (1 h)
 3. 60% methanol in B1n buffer (1 h)
 4. 80% methanol in B1n buffer (1 h)
 5. 100% methanol (1 h)
- 4 Incubate samples in 100% dichloromethane (1 h) on rotation 12 rpm at 4 °C.
- 5 Incubate samples overnight in 100% dichloromethane on rotation 12 rpm at 4 °C.
- 6 Incubate samples in 100% dichloromethane (1 h) on rotation 12 rpm at 4 °C. If sample has not sunk to the bottom of the vessel continue washes with dichloromethane until it has.

Bleaching

1d

- 7 Wash samples twice in 100% methanol (1 h) on rotation 12 rpm at 4 °C.
- 8 Bleach samples overnight in 6% hydrogen peroxide in methanol at 4°C, protected from light.

Blocking

5d

- 9 Rehydrate samples in a series of methanol in B1n buffer dilutions while on rotation at 12 rpm at 4°C:



1. 100% methanol (1 h)
2. 80% methanol in B1n buffer (1 h)
3. 60% methanol in B1n buffer (1 h)
4. 40% methanol in B1n buffer (1 h)
5. 20% methanol in B1n buffer (1 h)
6. B1n buffer (1 h)

- 10 Incubate samples in DPBS containing 0.2% gelatin, 0.5% Triton X-100 and 0.01% thimerosal (DPBSG-T) for 4 days while on rotation at 12 rpm

Primary antibody incubation

2w

- 11 Incubate samples in primary antibody solution containing DPBSG-T for 14 days at 37°C with agitation. Volume of solution need only be sufficient to cover the sample.

Secondary antibody incubation

2d

- 12 Wash samples in DPBSG-T (6 × 1 h).

- 13 Incubate samples in secondary antibody solution containing DPBSG-T for 2 days at 37°C with agitation. Volume of solution need only be sufficient to cover the sample.

Dehydration and delipidation

1d

- 14 Wash samples in DPBSG-T (6 × 1 h).

- 15 Dehydrate samples 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)

- 16 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.

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

Clearing

- 18 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

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

Light sheet microscopy

- 20 Prior to visualization on a light sheet microscope, samples should be transferred into ethyl cinnamate, at least 3 h prior.
- 21 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.
 - Some sample holders are available that can hold the sample in place without glue, try these if they are suitable to the orientation required.
 - 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.
 - Orientate the sample so that the region of interest is flat, the best resolution of the light sheet microscope is in XY.

For viewing sub-dissections of the large peripheral nerve tissue, it is best to begin with lower magnification to view the whole sample at once and then identify regions of interest such as clusters of neurons. These can then be inspected at higher magnification. Sometimes it is necessary to reorient the sample as described above for better illumination and thus imaging.



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