Additional file 1

Laboratory protocols

Spinal cord and dorsal root ganglion harvest and fixation

Based on: *Richner M, Jager SB, Siupka P, Vaegter CB (2017) Hydraulic Extrusion of the Spinal Cord and Isolation of Dorsal Root Ganglia in Rodents. J Vis Exp:55226.*

Materials:

- 1 curved and 1 straight mayo scissor
- 15 mL tube of pre-cooled fresh or frozen 4% paraformaldehyde (PFA) in 1× phosphate buffered saline (PBS) per spinal cord + 1 Eppendorf filled 4% PFA in 1× PBS per dorsal root ganglion (DRG)
- Precooled 1× PBS
- Silicon-coated petri dish, large
- Styrofoam box with ice
- 10 mL syringe
- P200 pipette tip
- Fine anatomical forceps and dissection scissors
- 1-2 mL of pentobarbital (euthanol)
- Aluminum foil
- Dissection microscope

Preparation:

- all steps take place under fume hood to avoid exposure to PFA fumes
- place 15 mL tube with precooled 4% PFA on ice
- cut the proximal 5 mm of the P200 pipette tip and fit it onto the 10ml syringe

Spinal cord harvest:

- Euthanize the rats
- Take the rat to the fume hood (from now on the steps are time sensitive and should be performed as fast as possible to minimize the time to fixation)
- Use curved mayo scissor to decapitate the rat
- Cut the back skin along the spine up to the tail
- Separate the spine from the body by cutting along the spine on both sides up to the tail
- Cut off the spine in the deep sacral part; look for the distal aperture of the spinal canal
- In the wide proximal aperture of the spinal canal you should see the white and mushy spinal cord, if not cut back until you can see it
- Aspirate 10 mL of precooled 1× PBS into the 10ml syringe with the fitted pipette tip
- Insert pipette tip into the distal spinal canal
- Hold proximal spinal aperture downwards above the petri dish

- Straighten the spine with your fingers to facilitate the extrusion
- Inject the PBS in a powerful but steady motion to extrude the spinal cord into the petri dish
- Place the spine on ice to slow autolysis of the DRGs
- Place the spinal cord into the 15 mL precooled tube filled with 4% PFA, mark the tube and wrap it with aluminum foil to prevent photo bleaching
- Place tube for 24 hours at 4°C in fridge (horizontal position to prevent a s-shaped sample)
- Store sample in 1× PBS afterwards at 4°C in the dark

DRG harvest:

- Take the spine on ice to the microscope
- Use straight mayo scissor to carefully cut along the dorsal spinal columns
- Carefully spread the spinal canal
- Identify the insertion of the lowest rib into the vertebra – distal to this vertebra is the 13th thoracic DRG (T13)
- Identify the target DRGs (e.g. L3, L4, L5 for common peroneal nerve) distal to their corresponding vertebra
- Use micro instruments to carefully expose and harvest the DRGs without crushing
- Immerse DRGs in pre-cooled 4% PFA at 4°C overnight (ON) in the dark
- Store sample in 1× PBS afterwards at 4°C in the dark

Immunostaining of intact tissue

Based on: Qi Y, Yu T, Xu J, Wan P, Ma Y, Zhu J, Li Y, Gong H, Luo Q, Zhu D (2019) FDISCO: Advanced solvent-based clearing method for imaging whole organs. Sci Adv 5:eaau8355.

Materials:

- Quadrol (122262, Sigma-Aldrich)
- Primary antibody e.g. rabbit anti-beta 3 tubulin (18207, Abcam, Cambridge, UK)
- Secondary antibody e.g. goat anti-rabbit Alexa Fluor 555 conjugated secondary antibody (A-21428, Invitrogen, California, USA)
- Methanol
- Double distilled water (ddH_2O)
- H_2O_2
- DMSO (Sigma Aldrich, 276855)
- 1× PBS
- Glycine
- Heparin
- Triton X-100 (X100, Sigma Aldrich)
- Tween-20 (P9416, Sigma Aldrich)
- Normal donkey serum

Decolorization (3 days):

- Recommended for heavily colored specimens
- Immerse specimen in 25% quadrol in double distilled water at 37°C for two days with daily changes of the solution
- Wash 4 times in 1× PBS with one ON step at room temperature (RT)

Permeabilization and bleaching (3 days):

- Pretreated sample in ascending methanol series (50%, 80%, 2× 100% methanol in double distilled H_2O) for 30 minutes each
- Incubate ON in 5% H_2O_2/20% DMSO/methanol at 4°C
- 100% methanol for 45 minutes twice at RT
- 20% DMSO/methanol for 45 minutes at RT
- Descending methanol series (80%, 50% methanol in double distilled H_2O for 30 minutes at RT each)
- Wash in 1× PBS for 1 hour twice at RT followed by an overnight step at 4°C
- Incubate in 1× PBS/0.2% Triton X-100/20% DMSO/0.3M glycine ON at 37°C

Alternatively, we recommend the FLASH protocol for fast permeabilization of intact tissue:

Based on: Messal HA, Almagro J, Zaw Thin M, Tedeschi A, Ciccarelli A, Blackie L, Anderson KI, Miguel-Aliaga I, van Rheenen J, Behrens A (2021) Antigen retrieval and clearing for whole-organ immunofluorescence by FLASH. Nat Protoc 16:239-262.

Blocking and staining (11 - 14 days) :

- Immerse in 1× PBS/0.2% Triton X-100/10% DMSO/5% normal donkey serum at RT for 3 days
- Wash in PTwH (PBS/0.2% Tween-20/0.01 mg/mL Heparin) for 2 hours at RT twice
- Immerse in primary antibody dilutions in PTwH/5% DMSO/3% normal donkey serum at 37°C for 2-4 days
- Wash in PTwH on a shaker for 2 days at RT with at least four changes of the washing solution
- Incubate in secondary antibody diluted in PTwH/3% normal donkey serum for 2-3 days at 37°C
- Wash in PTwH on a shaker for 2 days at RT with at least four changes of the washing solution

Solvent based optical tissue clearing of intact tissue

Based on: *Qi Y, Yu T, Xu J, Wan P, Ma Y, Zhu J, Li Y, Gong H, Luo Q, Zhu D (2019) FDISCO: Advanced solvent-based clearing method for imaging whole organs. Sci Adv 5:eaau8355.*

Validated for: Spinal cord, DRGs, full-thickness skin, peripheral nerves, and skeletal muscles (all rats)

Materials:

- 1× PBS
- 4% PFA
- ddH_2O
- Ethanol

- Agarose
- 1.5 mL and 5mL Eppendorf
- Tetrahydrofuran (186562, Sigma-Aldrich)
- Triethylamine (471283, Sigma-Aldrich)
- Dibenzylether (108014, Sigma-Aldrich)
- Dichlormethane (270997, Sigma-Aldrich)
- Glass vials (03-339-22C, Fisher)

! All following steps must be performed with minimal light exposure!

Optional: Agarose embedding for pre-stained small samples e.g. DRGs

- Embedding in agarose may facilitate sample mounting for light-sheet microscopy
- Prepare the embedding solution with 1% agarose / 1× PBS in glass beaker
- Heat in microwave (15 seconds, max watt)→ Cool the gel for 10 minutes at RT (to ~ 40 °C) to avoid exposing the sample to excessive heat.
- While agarose cools down place the samples into a mold and arrange them into the desired position. Avoid any liquid carryover.
- Gently pipette the temperature adjusted agarose gel over the sample. Avoid any air bubbles.
- Let the agarose fully solidify at 4°C for > 2 hours in the dark

Optical tissue clearing (< 24 hours)

- All steps take place under fume hood
- For retrogradely labelled spinal cord we recommend cutting the sample lengthwise in two halves along the anterior spinal artery (use scalpel) to facilitate light penetration during imaging
- Freshly prepare the following solutions in a glass bottle, adjust pH to 9.0 with Triethylamine and precool at 4°C for 1-2 hours
    - 50% tetrahydrofuran (THF) in ddH_2O
    - 75% THF in ddH_2O
    - 100% THF
- For sample immersion use 5ml capped glass vials; fill vial with 50% THF solution and label with tape + lead pencil
- Immerse sample in 50% THF at 4°C under gentle shaking
    - Spinal cord (SC), nerve and skin: 30 minutes incubation
    - Agarose embedded DRGs and muscle: 1 hour incubation
- Pour out THF; leave the sample inside the vial
- Fill with 75% THF solution and immerse at 4°C under gentle shaking
    - SC, nerve and skin: 30 minutes incubation
    - Agarose embedded DRGs and muscle: 1 hour incubation
- Repeat with 100%THF 3× for 1 hour each (for all tissue types)
- Pour out 100%THF and fill with precooled dichlormethane, let sit at RT for

- SC, Agarose embedded DRGs and muscle: 60 to 90 minutes
- Nerve and skin: 30 to 60 minutes or until sample is sunken to the ground

Refractive index matching

Ideal: microscope objectives optimized for high RI around 1.55 (e.g. LaVision)

- Transfer in 5 mL precooled (4°C) dibenzylether (DBE), change DBE at least 3 times with 2 hours in-between and then let equilibrate ON at 4°C
- Image the sample immersed in the equilibrated DBE

Alternative: objectives optimized for glycerol or CLARITY (RI 1.45; e.g. Zeiss Z1 Light sheet)

- Adjust the RI of the sample with ethanol (CAVE lowering the RI may increase light scattering and thus reduce penetration depth)
- Spinal cord: Immerse sample in 0.578 mL DBE + 0.3 mL ethanol (EtOH) (mix thoroughly) in a 1.5 mL Eppendorf at 4°C
- DRG: Immerse sample in 0.578 mL DBE + 0.25 mL EtOH (mix thoroughly) in a 1.5 mL Eppendorf at 4°C
- Change DBE/EtOH solution at least twice after 60 minutes each and let equilibrate at 4°C overnight (longer equilibration times increase transparency but may quench endogenous fluorophores, particularly GFP)
- Image the sample immersed in the equilibrated DBE /EtOH solution

Sample Storability:

- We recommend imaging within 3 days after clearing, however selected fluorophores may be stable in cleared tissue for months if stored in DBE (/EtOH) in the dark at 4°C
    - Fluororuby and Fluoro-Gold > 6 months stable
    - Endogenous GFP days to weeks depending on the animal model
    - Immunostained Cy3 and Cy5 weeks to months depending on the secondary antibody
- If specimen turns opaque following long-term storage dehydrate in 100% THF for 1 hour twice, then immerse in fresh DBE /EtOH solution