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Pericyte Mapping in Cerebral Slices with the Far-red Fluorophore TO-PRO-3

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[Abstract] This protocol describes a method for high-resolution confocal imaging of pericytes with the far-red fluorophore TO-PRO™-3 Iodide 642/661 in cerebral slices of murine. Identification of pericytes with TO-PRO-3 is a short time-consuming, high cost-effective and robust technique to label pericytes with no need for immunostaining or generation of reporter mice. Since the TO-PRO-3 stain resists immunofluorescence, and lacks spectral overlap, the probe is well suited for multiple labelling. Our procedures also combine TO-PRO-3-staining of pericytes with fluorescent markers for astrocytes and vessels in brain slices. These approaches should enable the assessment of pericyte biology in gliovascular unit.

Keywords: Pericyte imaging, TO-PRO-3, Fluorescence confocal microscopy, Brain slices, Astrocytes, vessels

[Background] Fluorescence imaging at the cellular level offers an exceptional tool to track pericytes under confocal or bi-photonic microscopy. Tagging specific pericyte surface antigens, such as chondroitin sulphate proteoglycan neuron-glial 2 (NG2) and platelet-derived growth factor receptor beta (PDGFRβ), proved to be an excellent approach to identify pericytes in the cerebral microvasculature. Antigen labelling is achieved via immunofluorescence with specific antibodies or through fluorescent protein expression under the control of specific promoters for NG2 and PDGFRβ (Ozerdem *et al.*, 2001; Mishra *et al.*, 2014; Hartmann *et al.*, 2015a and 2015b; Jung *et al.*, 2018; Smyth *et al.*, 2018). Notwithstanding, immune techniques involve several steps that take place over hours or days, whereas generation of reporter mice is costly and laborious, mainly in studies employing transgenic mouse models. Herein, we describe a simple, robust and rapid (*e.g.*, min) fluorescent labelling assay to image pericytes in murine brain slices with the far-red fluorophore TO-PRO™-3 Iodide 642/661. This carbocyanine monomer probe has recently been recognized as a pericyte biomarker in both *ex vivo* (Mai-Morente *et al.*, 2021) and *in vivo* (Tong *et al.*, 2021) conditions. TO-PRO-3 stains nucleus in fixed tissue (Van Hooijdonk *et al.*, 1994; de Mazière *et al.*, 1996; Suzuki *et al.*, 1997), but is selectively incorporated by living pericytes *ex vivo* when applied into the physiological saline or *in vivo* after topical

administration (Lacar *et al*., 2012; Mai-Morente *et al*., 2021; Tong *et al*., 2021). Identification of murine brain pericytes by TO-PRO-3 is unambiguous in the tested age range (P06-P90) and, as reported (Mai-Morente *et al*., 2021), TO-PRO-3-stained pericytes express the classical pericyte immunomarkers NG2 and PDGFRβ and incorporate the pericyte dye NeuroTrace 500/525 (Damisah *et al*., 2017). Only a subset of TO-PRO-3 pericytes expresses the contractile protein alpha-smooth muscle actin (α-SMA) (Mai-Morente *et al*., 2021). The far-red emitting TO-PRO-3 dye exhibits negligible autofluorescence and phototoxicity (Suseela *et al*., 2018), which favours its use in live imaging; additionally, TO-PRO-3-loaded slices can be fixed and processed for immunolabelling (Lacar *et al*., 2012; Mai-Morente *et al*., 2021). Since TO-PRO-3 resists immunostaining and fluoresces far from the green and red fluorophores in the light spectrum, it is appropriate for multiple labelling with fluorescent-conjugated probes and antibodies or green fluorescent protein (GFP) reporters. The protocols described here include procedures to identify vessels and astroglia intimately associated with TO-PRO-3-labelled pericytes. Given the ease and reliability of the technique, mapping pericytes with TO-PRO-3 should facilitate future research on pericyte structure and function in cerebral slices.

Materials and Reagents

1. 96-well plate
2. 6-well plate
3. 12/24-well plate
4. Ice plastic tray with silicone bottom for domestic use
5. Nylon mesh of a tea plastic strainer
6. Plastic transfer pipettes (Biologix, catalog number: 30-0138)
7. Sartorius mLINE® mechanical Biohit pipettors 2, 20, 200 and 1,000 µl
8. Six-well, twenty-four-well and ninety-six-well multidishes (DeltaLab, catalog numbers: 657160, 662160 and 655180, respectively)
9. Custom-made strainer
10. Perfusion chamber
11. Transparent (glass or polypropylene) cylindrical test tubes with rounded bottom
12. Conventional 21 gauge (21 G) syringe needles
13. BD Intramedic™ Polyethylene Tubing, 100 ft × 0.034" × 0.050" (Becton Dickinson, catalog number: 427421)
14. Microscope glass slides (Deltalab, catalog number: D 100001)
15. Microscope glass coverslips (Deltalab, catalog number: D 102440) and N1.5 (Knittel Glass, catalog number: VM52440Y1A0.1)
16. Aluminium foil
17. Absorbent tissue
18. Adhesive tape
19. Permanent marker pen

20. Fine-tipped paintbrushes
21. Nail varnish
22. Hippocampal and cortical slices (300-400 µM thick) from P06-P90 male and female mice [*Mus musculus* on a C57BL/6 background (Jackson Laboratory, RRID: IMSR_JAX: 000664)] and *Rattus norvegicus* [Sprague-Dawley (Charles River Laboratories, Strain code 400)]
23. MilliQ-water or double-distilled water (ddH_2O)
24. Quinolinium, 4-[3-(3-methyl-2(3H)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio) propyl]-, diiodide/157199-63-8 or TO-PRO™-3 Iodide 642/661 (Life Thermo Fisher Scientific, catalog number: T3605)
25. Neurotrace™ 500/525 Green Fluorescent Nissl (Life Thermo Fisher Scientific, catalog number: N21480)
26. Poly-L-Lysine (Sigma-Aldrich, catalog number: P4832)
27. Lycopersicon Esculentum (Tomato) Lectin DyLight 488 (LEL-DyLight 488) (Life Thermo Fisher Scientific, catalog number: L32470)
28. Isolectin B4 conjugated to fluorescein isothiocyanate (FITC-ISOB4) (Sigma-Aldrich, catalog number: L2895)
29. Rabbit anti-GFAP-Cy3™ (Sigma-Aldrich, catalog number: C9205)
30. 2-[4-(Aminoiminomethyl) phenyl]-1H-Indole-6-carboximidamide hydrochloride (DAPI) (Sigma-Aldrich, catalog number: D09542)
31. Hoechst 33342 (Sigma Aldrich, catalog number: 23491-45-4)
32. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: 048-46-8)
33. Glycine (Sigma-Aldrich, catalog number: 56-40-6)
34. Glycerol or Fluoromont-G™ Mounting Medium (Life Thermo Fisher Scientific, catalog number: 00-4958-02)
35. NaCl (Sigma-Aldrich, catalog number: 7647-14-5)
36. KCl (Sigma-Aldrich, catalog number: 7447-40-7)
37. $NaHCO_3$ (Sigma-Aldrich, catalog number: S5761)
38. $NaH_2PO_4 \cdot H_2O$ (Sigma-Aldrich, catalog number: 10049-21-5)
39. $Na_2HPO_4 \cdot H_2O$ (Sigma-Aldrich, catalog number: S9763)
40. KH_2PO_4 (Sigma-Aldrich, catalog number: P0662)
41. Glucose (Sigma-Aldrich, catalog number: G5767)
42. $MgSO_4$ (Sigma-Aldrich, catalog number: M7506)
43. $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich, catalog number: C3881)
44. Paraformaldehyde powder (PFA) (Sigma-Aldrich, catalog number: 158127)
45. NaOH and HCl
46. Artificial cerebrospinal fluid solution (ACSF) (see Recipes)
47. Blocking/permeabilizing solution (see Recipes)
48. Diluting solution for antibodies (see Recipes)
49. Fixing solution (see Recipes)

50. Phosphate buffered saline (PBS) (see Recipes)
51. PBST (see Recipes)

Equipment

1. Gas tank 5% CO_2, 95% O_2
2. Digital pHmeter (ORION, model: 410A)
3. Digital Analytical Balance (RadWag, AS82/220.R2)
4. Thermolyne Type 16700 Mixer Maxi-Mix1 vortex mixer
5. TS-2000A VDRL Shaker
6. Fume hood 1300 Series A2 Class II, Type A2 Bio Safety Cabinets
7. Thermo Scientific™ Cimarec™ Basic Stirring Hotplates SP13132033 (ThermoFisher Scientific)
8. Confocal Laser Scanning Microscope (Leica TCS SP5 TANDEM SCANNER) equipped with:
   a. A 40× oil immersion objective Leica N.A 1,3 with UV correction.
   b. 405 nm diode laser, argon gas laser emission at 488 nm and HeNe lasers for 543 nm and 633 emission.
9. Coverslip Clamp Chamber (ALA Scientific Instruments Inc.)
10. HCT-10 Temperature Controller (ALA Scientific Instruments Inc.)
11. Peristaltic Pump (Scientific Industries Inc., Model 203)
12. Refrigerator and freezer
13. Thermostatic water bath

Software

1. Image acquisition and storage system (LAS AF Lite Software)
2. Image analysis software (Fiji, ImageJ version 1.53c)
3. Photo editing software (Adobe Photoshop CS6 13.0 × 64 and Adobe Illustrator CS6 16.0.0)

Procedure

Before getting started, obtain acute cortical and hippocampal slices (300 µm thick) from mice and allow them to stabilize for 45 min in a storage chamber resting on a nylon mesh submerged in ACSF equilibrated with 95% O_2 and 5% CO_2, at room temperature (RT: 22°C-25°C). Detailed protocols for preparing acute cerebral and hippocampal slices from rodents can be found elsewhere (Lein *et al.*, 2011; Pannasch *et al.*, 2012; Mishra *et al.*, 2014; Papouin and Haydon, 2018).

Note: To obtain good quality slices, heed the following recommendations: (1) rapidly remove the brain after confirming the animal's death, (2) use chilled materials (chambers, dishes, instruments) and keep the tissue immersed in ice-cold ACSF carbogenated with 95% O_2 and 5% CO_2 throughout dissection and slicing procedures, and (3) employ one fresh razor blade per brain to prevent tissue deformation.

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A. Vessel and pericyte labelling with lectins in brain slices

For simultaneous identification of lectin-labelled vessels and TO-PRO-3-marked pericytes, follow Procedures A and B. If not interested in visualizing vessels, proceed to Procedure B.

1. In our experiments, we succeeded in visualizing vessel walls and pericyte contours using any of these two probes, (a) Lycopersicon Esculentum (Tomato) Lectin conjugated to DyLight 488 (LEL-DyLight 488) or (b) IsolectinB$_4$ conjugated to FITC (FITC-ISOB$_4$). Prepare the dye solution by dissolving (a) LEL-DyLight 488 in carbogenated ACSF to yield a 10 µg/ml final concentration or (b) FITC-ISOB$_4$ in carbogenated ACSF to yield a 5-10 µg/ml final concentration. Pre-warm (35°C-37°C) the dye solution in a thermostatic water bath protected from light.

   Notes:

   a. *Lectins bind to glycoconjugate residues in basement membranes of endothelium and pericytes (Peters and Goldstein, 1979; Laitinen, 1987; Mishra et al., 2014).*
   b. *Vortex stock solutions prior to use.*
   c. *Use freshly prepared dye solutions.*

2. Transfer acute slices with a plastic pipette (Figure 1A) from the storage chamber to an empty cylindrical transparent tube provided with fine-tipped tubing for gas delivery (95% O_2 and 5% CO_2) (Figure 1B). With a 1,000 µl pipettor, pull the ACSF out of the tube. Keep slices on the bottom and immediately add 1 ml minimum of the pre-warmed dye solution into the tube (Video 1).

   Notes:

   a. *Use the fine-tipped plastic pipette to transport hippocampal slices and a modified transfer pipette (without tip) for cortical slices (see Figure 1A).*
   b. *In one tube accommodate, at least 6-10 hippocampal slices or 3-4 cortical slices.*
   c. *The described procedure decreases the chances of diluting a small volume of dye solution.*

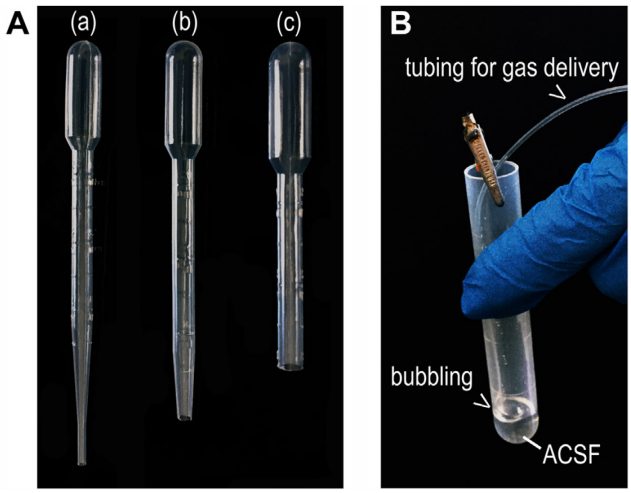
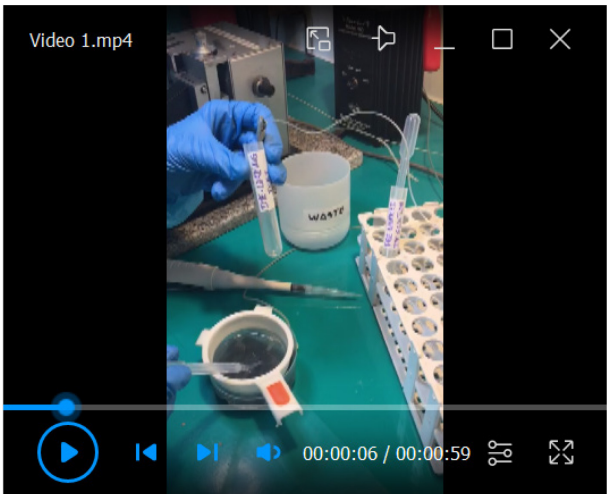


Figure 1. Transfer pipettes and dye-loading tube.

A. Plastic pipettes (3 ml) used to transfer hippocampal (a) or cortical (b and c) acute slices. The length of the pipette in (a) is 16 cms. In (b) and (c), the fine tip of the plastic pipette has been

cut and removed to prevent damage to the cortical slices during their passage through it. B. Rounded bottom dye-loading tube with fine tubing for gas delivery.



Video 1. Slice transfer procedure

3. After transferring the slices, place the dye loading tube with the slices into the thermostatic water bath (35°C-37°C). Keep slices incubated in the dye solution carbogenated with 95% O_2 and 5% CO_2 for 30 min in the dark.
   Note: Maintain the fine-tip of the gas tubing as distant as possible from the bottom of the loading tube to prevent bubbling from disturbing the slices (see Video 1).
4. After 30 min, remove the dye solution from the tube with a 1,000 µl pipettor while keeping slices at the bottom. Rapidly, add a minimum of 1 ml of normal ACSF into the tube to rinse slices for 15 min.
   Notes:
   a. *Rinsing slices will stop labelling and reduce background.*
   b. *During and following the rinsing period, protect slices from light.*

B. Pericyte identification with TO-PRO-3 in brain slices

1. Prepare dye-loading and rinsing chambers. To do so, fill two chambers with 10 ml of ACSF and bubble the solution with 95% O_2 and 5% CO_2 for at least 20 min before submerging the slices. Add 10 µl of the stock solution of TO-PRO-3 into the dye-loading chamber to yield a 1 µM final concentration. Protect solutions from light with aluminium foil. Place a little strainer into a contiguous chamber (Figures 2a-2b).
   Notes:
   a. *Wells of a 6-well plate can be used as adequate dye loading and rinsing chambers, as shown in Figure 2.*
   b. *A little homemade strainer with adequate size to fit into the well allows simultaneous transport of all slices and exchange of both slice surfaces with carbogenated ACSF during*

the whole procedure (Figure 2). To build the strainer, follow the procedure described in Figure 3.

c. *Place recording and rinsing chambers side by side to facilitate the rapid transfer of slices between chambers (Figure 2).*

d. *Wear latex or nitrile gloves to protect yourself when manipulating TO-PRO-3.*

e. *Vortex the stock vial of TO-PRO-3 before use.*

f. *Use freshly and daily prepared dye solution.*

g. *Agitate the dye-loading chamber to dissolve the dye into the ACSF until the dilution becomes homogenous.*

h. *If slices from numerous animals are going to be loaded, it is convenient to prepare an additional loading chamber. In our hands, it is possible to use the same dye solution twice (up to 20 hippocampal slices each).*

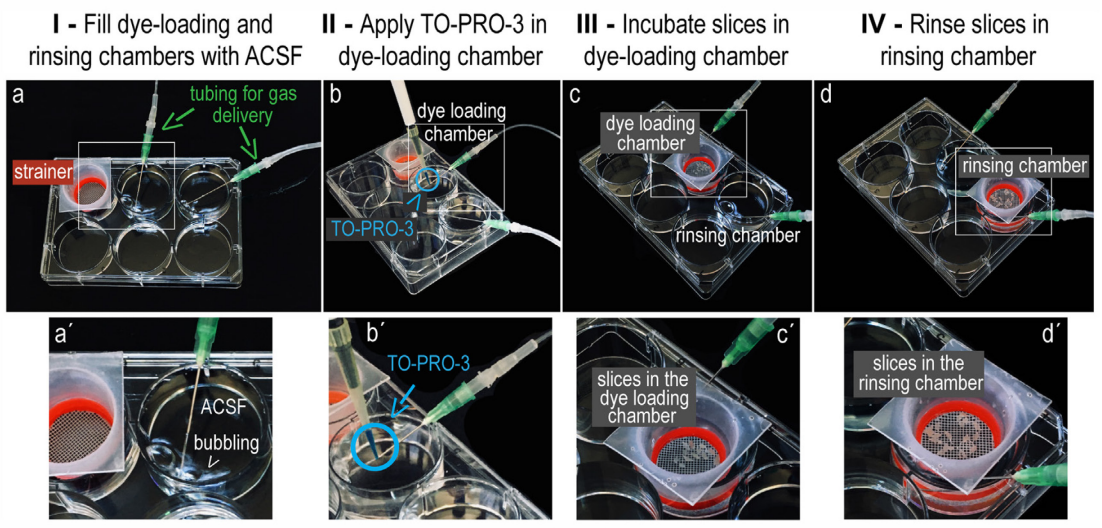


Figure 2. Step-by-step procedure employed to load pericytes with TO-PRO-3 in acute hippocampal slices.

(a, a´) Fill two wells of a six-well plate (12 × 8 cms) with 10 ml of ACSF each and bubble the solution with 95% O_2 and 5% CO_2 through a fine tubing. Place a little strainer into an empty well. (b, b´) Apply a volume (10 µl) of the TO-PRO-3 stock solution into the dye-loading chamber and agitate to facilitate dye dissolution. (c, c´) Pick up brain slices with a transfer pipette and pour them on the top of the strainer (the ACSF contained in the transfer pipette will drop into the empty chamber while the mesh will retain the slices). Rapidly incubate the strainer carrying the slices into the dye-loading chamber. (d, d´) After a dye-loading period of 20 min, transfer the strainer with the TO-PRO-3-loaded slices into the rinsing chamber for 15 min. During the whole procedure, maintain slices and solutions protected from light with aluminium foil. The fields within the white rectangles in pictures (a-d) have been zoomed (2.4×) and are shown below (a´, b´, c´ and d´). Hereinafter, acute slices with TO-PRO-3 loaded pericytes can be either fixed or used in living experiments (*e.g.*, electrophysiological recordings or calcium dynamics analysis).

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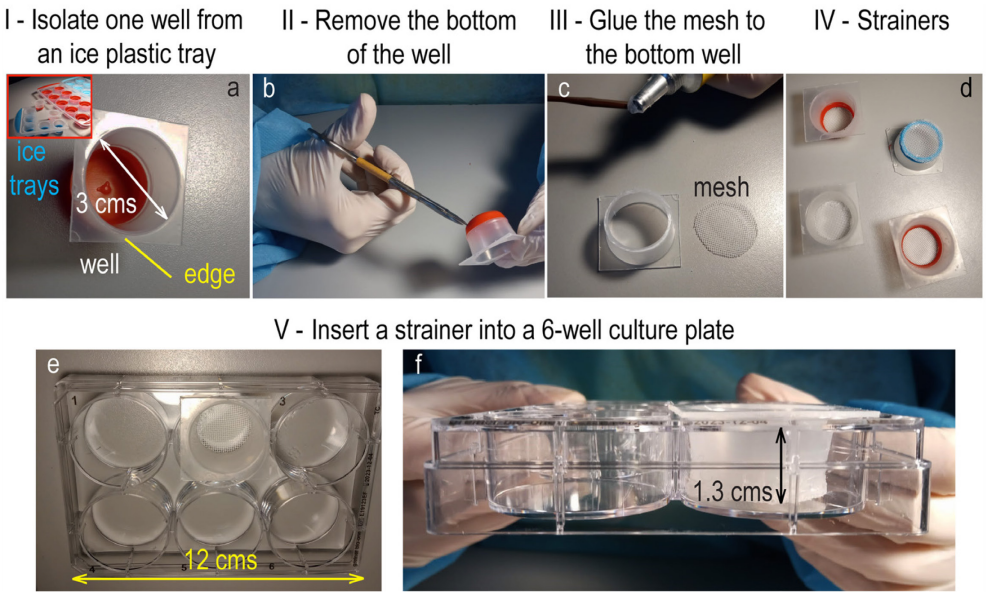

Figure 3. Construction of a custom-built strainer to hold and transfer acute brain slices. (a) Isolate one well from a plastic ice tray and preserve an edge around the well. (b) Remove the bottom well. Be aware that the final height of the strainer measures more than 1,3 cms (shown in f). (c) Cut a round piece of nylon mesh, ensuring that the mesh diameter fits the well diameter. Glue the borders of the mesh to the outside borders of the well hole with a silicone sealer. (d) Allow the strainer to dry for 24 h and rinse with ddH_2O before use. (e and f). Introduce the strainer into the 6-well plate and check that the mesh allows ACSF to pass through it with ease.

2. Pick up the acute slices from the tube or from a storage chamber with a plastic pipette (Figure 1), and place them over the nylon mesh of the little strainer, allowing the ACSF to drop into the empty well. Rapidly introduce the strainer carrying the slices into the loading chamber for 20 min at RT in the dark (Figure 2c).

   Notes:

   a. *Transferring the slices with a strainer instead of using a plastic pipette will prevent diluting the dye solution in the loading chamber.*

   b. *Protect slices and solutions from light with aluminium foil during dye loading and post-loading periods.*

3. Following the incubation period with TO-PRO-3, transfer the strainer carrying the slices from the loading chamber to the rinsing chamber for 15 min (Figure 2d).

   Notes:

   a. *Rinsing slices will stop dye incorporation into cells, reduce background labelling and prevent unspecific uptake.*

b. Since the TO-PRO-3 probe shows a high affinity for DNA (Suzuki et al., 1997), once bound to the nucleic acids, the dye is expected not to leak through the membrane and stay intracellular.

4. Following the rinsing period, counterstain with Hoechst 33342 (0.5 µM in ACSF) for 10 min at RT, if TO-PRO-3-loaded slices are to be used in living experiments (*e.g.*, electrophysiological recordings or calcium dynamics analysis).
5. To use fixed slices, submerge the TO-PRO-3-loaded slices in fixing solution for 40 min at RT under mild shaking. Rinse fixed slices in PBS twice, for 5-10 min each rinse.
   Note: Do not use materials (chambers, tubes, pipettes, tubing, and instruments) in contact with fixed tissue to manipulate living tissue.
6. Counterstain with DAPI (1-5 µM in PBST) or Hoechst 33342 (0.5 µM in PBST) for 10 min at RT under gentle shaking. Rinse fixed slices in PBS, 1-2 times for 5-10 min each.
   Notes:
   a. Use DAPI or Hoechst 33342 to label DNA in fixed slices and Hoechst 33342 to counterstain nucleus in acute slices; Hoechst 33342 is relatively nontoxic and nonmutagenic to living cells (Durand and Olive, 1982).
   b. Counterstaining with DAPI or Hoechst 33342 favours referencing of the slice structure in the hippocampus.
   c. For steps B5 and B6, it is convenient to employ a 12/24-well plate.
   d. Fixed slices can be stored in PBS at 4°C, protected from light for 24-48 h before being mounted.
7. To mount fixed slices, pick up the sections from the well with a fine-tipped paintbrush and place them on a microscope slide. Remove the excess PBS with an absorbent tissue or dry it by incubating slides at 37°C for 5 min. Add a drop of mounting solution (glycerol or Fluoromont-G™ Mounting Medium) to cover the slices. Gently apply a coverslip over the mounting media, avoiding the generation of bubbles, and seal the coverslip by applying nail varnish at its borders. Store slides at 4°C in the dark for at least 24 h before analysis with a confocal laser-scanning microscope.
   Notes:
   a. Waiting overnight before taking photos decreases photobleaching.
   b. In our hands, mounted sections stored in the dark at 4°C preserve the bright TO-PRO-3 stain in pericytes for at least 1-1.5 months (Mai-Morente et al., 2021).
   Figures 4 and 5 illustrate representative examples of TO-PRO-3-labelled pericytes from rodents in fixed hippocampal slices. Figure 5 shows TO-PRO-3-stained pericytes in adjacency to lectin-stained vessels.

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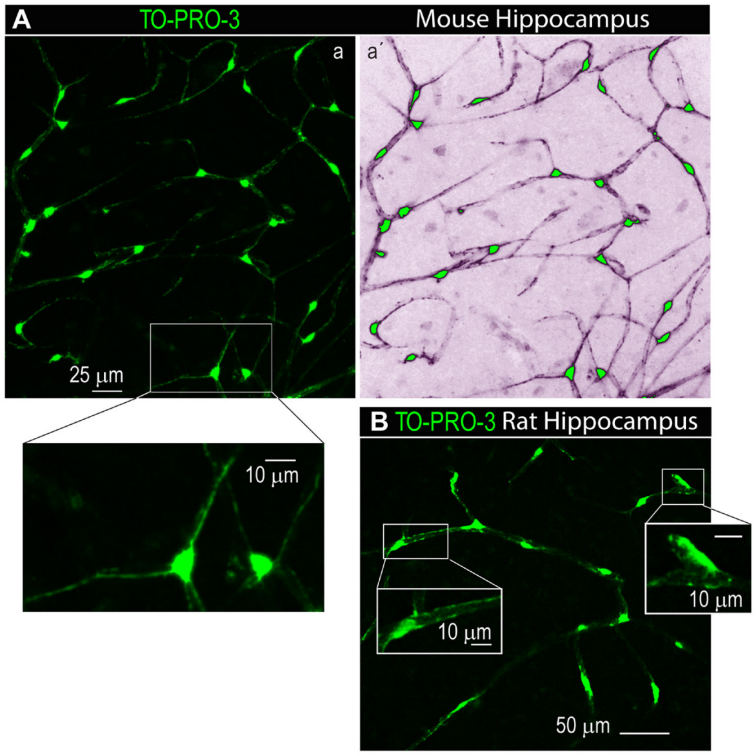

Figure 4. TO-PRO-3-labelled pericytes in fixed slices of the murine hippocampus.

A. Confocal images of mouse hippocampus illustrate a fluorescent field of the stratum radiatum with TO-PRO-3-stained pericytes (a) and the inverted fluorescent version of the same field (a´). The area within the white rectangle in (a) has been zoomed and is shown below. Note the bright spindle-shaped TOPRO-3 somas giving origin to longitudinal processes. The inverted image in (a´) evidences pericyte prolongations marked with the fluorophore. Each image is representative of the hippocampi of 40 mice. B. The fluorescent image illustrates pericytes labelled with TO-PRO-3 in the rat hippocampus. The pericytes within the white rectangles have been zoomed to facilitate the visualization of prolongations stained with TO-PRO-3. Each image is representative of the hippocampi of 10 rats. In all images, the TO-PRO-3 fluorescence is pseudo-coloured in green.

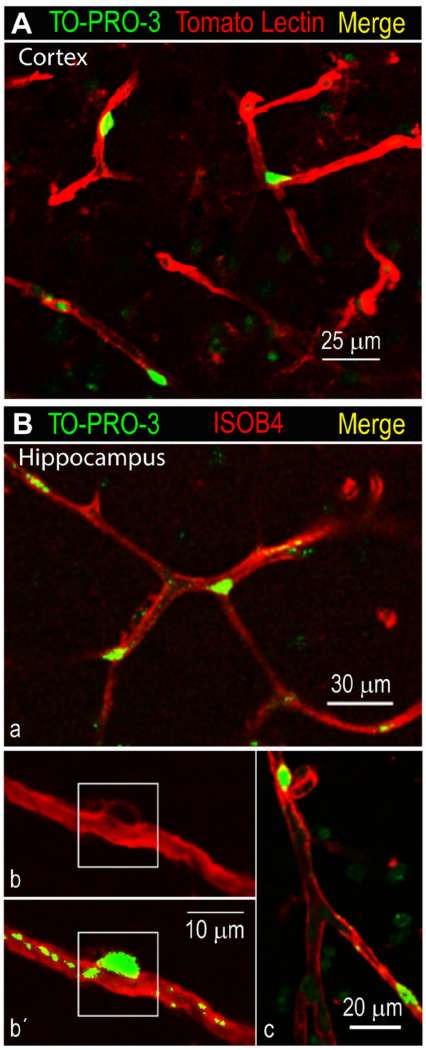

Figure 5. TO-PRO-3-pericyte somas outline the cerebral microvasculature.

A. The fluorescent micrograph illustrates TO-PRO-3-labelled pericytes in the mouse cerebral cortex associated with Tomato Lectin-marked vasculature. The image is representative of five mice. B. Different fluorescent views of mouse hippocampus (a; b, b´; c) illustrate TO-PRO-3-labelled pericytes and Isolectin B_4 (FITC-$ISOB_4$)-marked pericytes and vessels. The picture in (b´) represents the same field as (b) in which the TO-PRO-3 view has been merged. The FITC-$ISOB_4$ probe stains basement membranes of endothelium and pericytes. Note the $ISOB_4$ mark shaping the contour of the TO-PRO-3-labelled soma in (b) and the TO-PRO-3 prolongations delineating the vessel wall in (b´). Each image is representative of 10 mice. In all images, the TO-PRO-3 fluorescence is pseudo-coloured in green, and the fluorescence of Tomato Lectin and $ISOB_4$ is pseudo-coloured in red.

C. Identification of astrocytes and pericytes in brain slices

For simultaneous identification of pericytes and astrocytes, follow Procedures B and C. To include vessel identification, follow Procedures A, B and C.

1. Immerse fixed slices pre-loaded with TO-PRO-3 in a blocking/permeabilizing solution for 2 h in agitation at RT in the dark.

   Notes:

   a. *To do this step, it is possible to use a 12/24-well plate.*

   b. *Blocking/permeabilizing solution and antibody and fixing solutions are freshly prepared. In our experience, these solutions can be stored at 4°C for one week.*

   c. *The blocking/permeabilizing solution blocks unspecific sites and permeabilizes cell membranes prior to antibody application.*

2. Wash slices three times in PBST for 5-10 min each under gentle shaking. Protect the slices from light with aluminium foil.
3. Incubate slices in diluting solution for antibodies with primary antibody anti-GFAP conjugated with Cy3 (1:400) for 2 h at RT under gentle agitation and protected from light.

   Notes:

   a. *Incubate hippocampal slices in antibody solution using a 96-well plate to save antibody. The volume of the antibody solution should be sufficient to cover the slices (200 µl minimum).*

   b. *Use a fine-tip paintbrush to transfer slices into the well or to remove them from it.*

4. Rinse slices three times in PBST for 10 min each under agitation and protected from light.
5. Treat slices for 10 min with DAPI (1-5 µM in PBST) or Hoechst 33342 (0.5 µM in PBST) at RT under mild agitation and rinse them again with PBST.

   Note: In steps C4 and C5, it is possible to use a 12/24-well plate.

6. To mount slices, follow the guidelines detailed in Step B7. Store slides at 4°C in the dark for at least 24 h before analysis with a confocal laser-scanning microscope.

   Figure 6 illustrates different fields of the mouse hippocampus with TO-PRO-3-stained pericytes and GFAP-labelled astrocytes. The intimate rapport between pericyte somata and astrocyte end feet is evidenced. Triple labelling of TO-PRO-3-stained pericytes, GFAP-positive astrocytes and IsoB4-labelled vessels is also shown (Figure 6D).

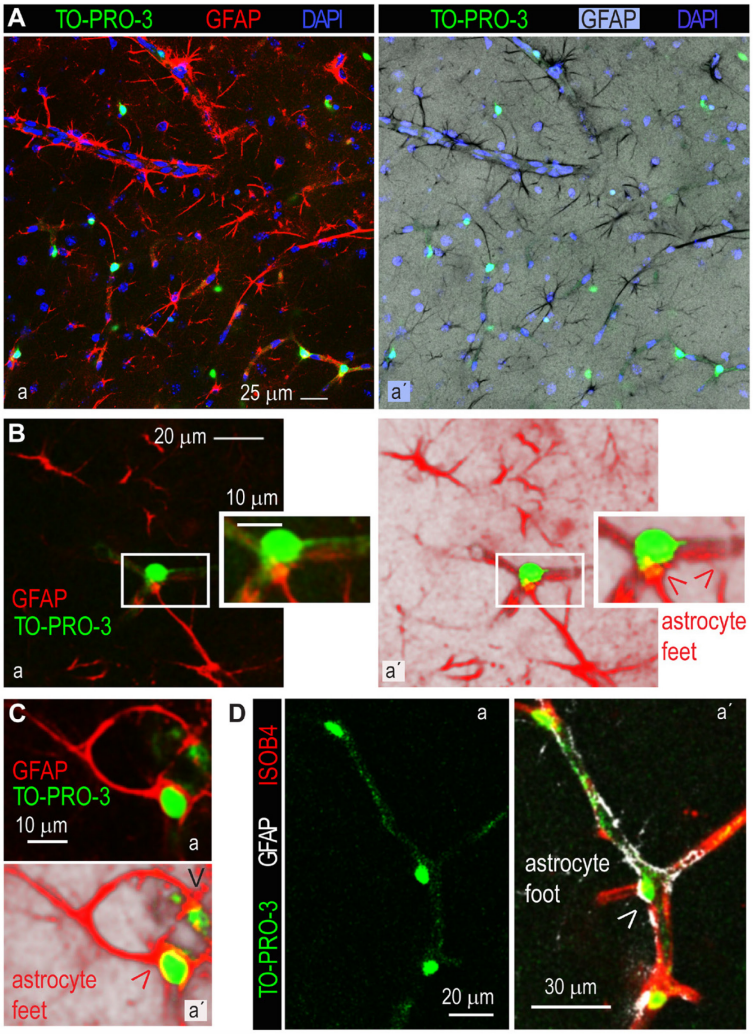

Figure 6. TO-PRO-3-stained pericytes are components of the gliovascular unit.

(A) (a, a´) A fluorescent view of a mouse hippocampus field (stratum radiatum) illustrates TO-PRO-3-labelled pericytes and GFAP-labelled astrocytes (a). The inverted fluorescent version of this field is shown in (a´). Nuclei stained with DAPI have been superimposed. (B) and (C) (a, a´) Photos of mouse hippocampus illustrate the intimate relationship between GFAP-stained astrocyte foot processes and TO-PRO-3-labelled pericyte somas. The inverted fluorescent version of the field shown in (a) is revealed in (a´). The area within the white box in (B) has been zoomed to evidence the rapport between the pericyte and the astrocyte foot process. Images are representative of the hippocampi of six mice. (D) (Same field a, a´) Fluorescent images illustrate TO-PRO-3-labelled pericyte somas lining a vessel in the hippocampus and GFAP-stained astrocyte prolongations reaching the vasculature and the pericyte somas. The basal lamina of the vessel endothelium has been stained with $ISOB_4$. Images are representative of hippocampi of 10 mice. In all images, the TO-PRO-3 fluorescence is pseudo-coloured in green, and the fluorescence of $ISOB_4$ is pseudo-coloured in red.

Notes:

a. *If required, additional immunostaining is possible (e.g., microglial or neuronal markers) in TO-PRO-3 slices (Mai-Morente et al., 2021).*
b. *Waiting overnight before taking photos decreases photobleaching.*
c. *Alternatively, slices derived from transgenic hGFAP-eGFP mice (Nolte et al., 2001) that allow easy identification of astrocytes can be subjected to procedures described in Item B for the simultaneous staining of astrocytes and pericytes in either acute or fixed slices.*

D. Pericyte identification with TO-PRO-3 and NeuroTrace 500/525 in acute slices

1. Repeat procedures described in Steps B1 to B3 but in addition to TO-PRO-3, apply NeuroTrace 500/525 (NeuroTr) (1:50 to 1:500 dilution of the stock solution) into the loading chamber for double labelling of pericytes (NeuroTr/TO-PRO-3). For exclusive staining with NeuroTr just dilute this probe into the loading chamber.

   Notes:

   a. *Adjust the optimal dilution of NeuroTr to your preparation.*
   b. *As for TO-PRO-3, vortex the stock solution of NeuroTr prior to use, prepare fresh dye solutions and protect solutions and slices from light during loading and post-loading periods.*
   c. *For simultaneous identification of pericyte somas with NeuroTr and vessels with lectins, use commercially available lectins conjugated to fluorophores other than FITC, Alexa 488 or DyLight 488 to prevent spectral overlap between these fluorophores and NeuroTr.*
   d. *NeuroTrace 500/525 has been recently identified as a pericyte marker in vivo (Damisah et al., 2017) and ex vivo (Mai-Morente et al., 2021).*

2. If desired, counterstain the nucleus by incubating acute slices in carbogenated (95% O_2 and 5% CO_2) ASCF containing Hoechst 33342 (0.5 µM final concentration) at RT for 15 min. Wash once in carbogenated ASCF for 10 min at RT. After the rinsing period, the dye-loaded living slices are ready to use in living experiments.

Figure 7 illustrates live pericytes co-stained with NeuroTr and TO-PRO-3 adjoining a vessel in a mouse hippocampal acute slice.

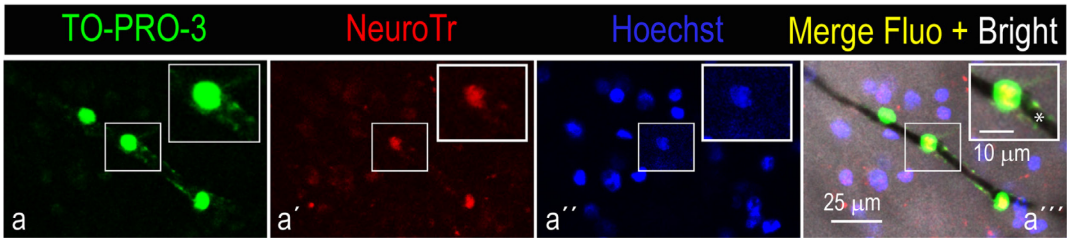

Figure 7. TO-PRO-3 labels live pericytes in acute slices of mouse hippocampus. (a-a´´´) The same field of the mouse hippocampus illustrates live pericytes identified with TO-PRO-3 (a) and NeuroTrace 500/525 (a´); co-localization of both dyes plus Hoechst 33342 is shown in (a´´´) where the brightfield view has been merged to fluorescent images. The pericyte

within the white box has been zoomed. In (a´´´), pericytes lie down adjacent to a microvessel; the asterisk within the zoomed box indicates blood accumulation. Note TO-PRO-3-stained prolongations surrounding the vessel wall. Images are representative of hippocampi of 15 mice. The TO-PRO-3 fluorescence is pseudo-coloured in green, whereas the fluorescence of NeuroTr is pseudo-coloured in red.

Notes:

a. *Counterstaining with Hoechst 33342 enables referencing the slice structure in the hippocampus.*
b. *In addition to NeuroTr-labelled pericytes, living astrocytes might be imaged with the fluorescent dye sulforhodamine 101 (SR101; Exc. 586/Em. 605), which enables staining of glial cells in vivo and ex vivo (Nimmerjahn et al., 2004; Nimmerjahn and Helmchen, 2012; Kafitz et al., 2008). Alternatively, for concurrent labelling of pericytes and astrocytes in living sections, slices derived from transgenic hGFAP-eGFP mice that allow detailed visualization of the astrocyte morphology (Nolte et al., 2001) can be loaded with TO-PRO-3. Notice that the same laser allows visualization of NeuroTr 500/525-loaded pericytes and hGFAP-eGFP astrocytes; therefore, NeuroTr is not appropriate to identify pericytes in acute slices derived from transgenic eGFP-mice.*

E. Image acquisition

1. For fixed preparations, mount microscope slides with TO-PRO-3-loaded hippocampal sections onto the stage of a confocal microscope (Leica SP5 TANDEM SCANNER). Labelled cells are visualized with a 40× oil immersion objective. Capture images and z-stacks with a digital camera connected to the imaging LAS AF Lite Software in data acquisition mode “xyz”, acquisition speed of 400 Hz (*i.e.*, 400 lines/s), image resolution of 1,024 × 1,024 pixels and value of “line average” equal to 2 to reduce noise. Under UV, select the field of interest and focus the area under study. Then, switch to the He-Ne 633 nm filter and tune “gain and offset” parameters in the detection system to optimize the signal intensity/noise ratio of TO-PRO-3, avoiding oversaturation so that bright fluorescent pericytes can easily be discriminated from the background and other cells. Switch to channels 488 nm and 543 nm to visualize lectin-stained vessels and GFAP-labelled astrocytes, respectively, and set up microscope parameter values. Determine the z-stack size and set the “start” and “end” values of the z-stack step. Take images at distances > 15-20 µm from the slice surface, as in surface areas reside damaged cells and reactive astrocytes due to membrane injury during slicing (Takano *et al.*, 2014). These are shown in Figure 8.

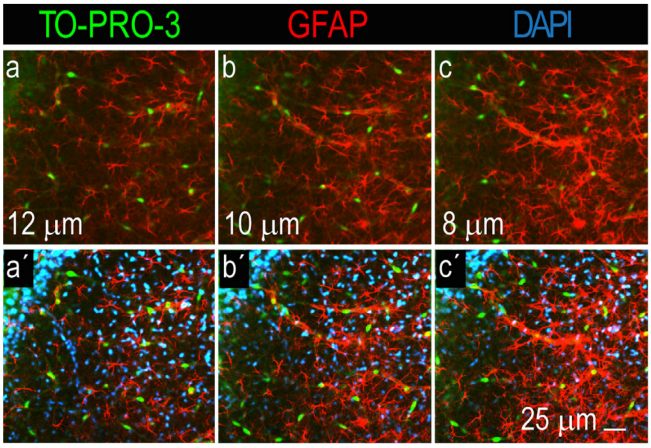

Figure 8. Reactivity gradient of astroglia at different distances from the slice surface. Fluorescent photos of mouse hippocampus acquired at 12 µm (a, a´), 10 µm (b, b´) and 8 µm (c, c´) from the slice surface are shown; note the GFAP up-regulation in the outermost areas of the slice indicative of reactive astrogliosis. Fields (a, b and c) are the same as those (a´, b´ and c´ respectively) in which the DAPI fluorescence has been merged. Images are representative of the hippocampi of seven mice. In all images, the TO-PRO-3 fluorescence is pseudo-coloured in green.

2. For acute slices, after setting the perfusion system, transfer one or two slices to the bottom of the recording chamber. Mount the perfusion chamber onto the stage of a confocal microscope and perfuse with ACSF equilibrated with 95% O_2 and 5% CO_2 at 1 ml/min. Keep the flow rate steady and set the temperature of the ACSF at 34-37°C by using a heating system. Figure 9 displays the setup employed to image brain pericytes from acute slices stained with TO-PRO-3 and NeuroTr. Under UV, select the working area; thereafter switch to 488 nm and 633 nm lasers to image stained pericytes with NeuroTr and TO-PRO-3. To decrease fading, during time-lapse experiments, acquire images at 512 x 512 pixels resolution and 800 Hz. When zooming, laser gains and exposure times should be optimized to decrease fading.

   Notes:

   a. *In the hippocampus, the anatomical segregation of DAPI/Hoechst 33342-stained nucleus facilitates the recognition of different areas (CA1, CA2 and CA3 layers, stratum oriens, stratum radiatum, and dentate gyrus).*
   b. *Minimize the exposure of living slices to UV to preclude phototoxicity (Suseela et al., 2018).*
   c. *As reported by our group, in healthy slices, TO-PRO-3 labelling of pericytes exhibits an excellent signal-to-noise ratio; incorporation of TO-PRO-3 by living pericytes is mediated by an actively operated transport mechanism that concentrates the dye into pericytes, resulting in bright labelling of somas and prolongations (Mai-Morente et al., 2021). Therefore, staining with TO-PRO-3 facilitates the identification of pericyte morphology and location during fluorescence imaging. According to our experience, pericytes from unhealthy slices fail to concentrate the dye.*

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d. *The bottom of the perfusion chamber is made of a poly-L-lysine-coated coverslip (Nr 1.5), which helps to immobilize the slices.*

e. *In our hands, the bright fluorescence of the TO-PRO-3-stain in pericytes is very stable in PFA-fixed slices and lasts up to 1.5 months for sections mounted on slides and stored at 4°C in the dark. Under time-lapse acquisition of living slices, TO-PRO-3 fluorescence is more labile than in fixed slices (Mai-Morente et al., 2021). Indeed, NeuroTr fluorescence is more resistant to photobleaching than TO-PRO-3 fluorescence during image acquisition in acute brain slices.*

f. *Work in the same range distance (e.g., from 20 to 50 µm from the slice surface) for conditions whose data will be compared since responses might depend along a spatial gradient propagation through the slice depth (Tian et al., 2010; Hall et al., 2014; Mishra et al., 2014).*

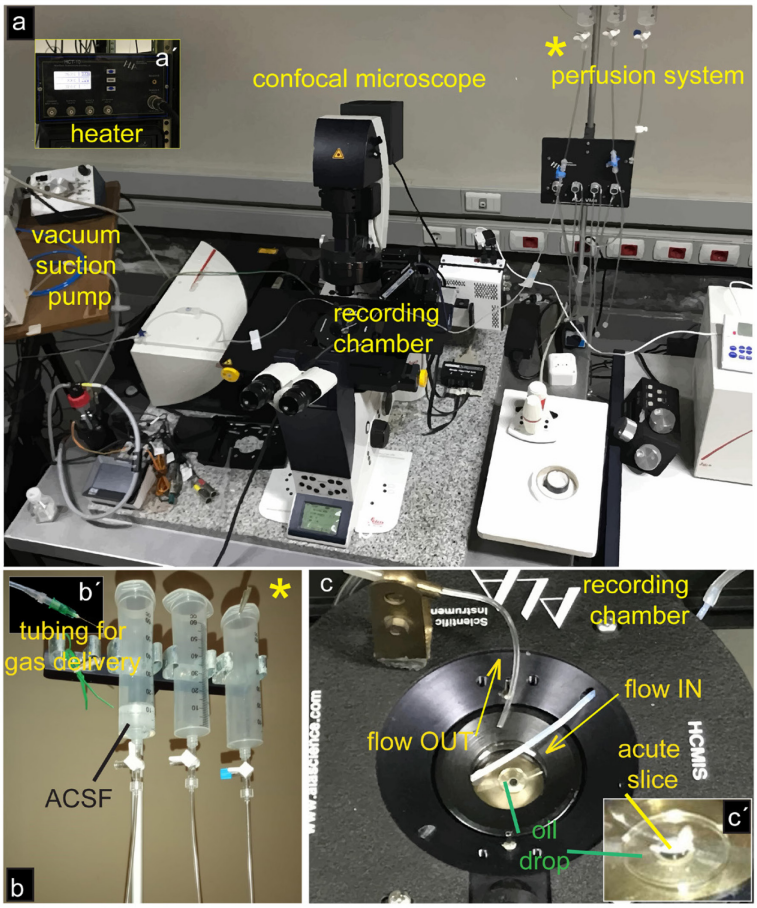

Figure 9. Setup for high-resolution imaging of TO-PRO-3-labelled and NeuroTrace-labelled pericytes in acute brain slices.

(a) General overview of the setup employed for recording TO-PRO-3-labelled pericytes in living hippocampal slices. The inset in (a´) illustrates the system heater employed to regulate the temperature of the recording chamber. (b) (*) Detail of recipients containing ACSF. The solution is equilibrated by a mixture of 95% O_2 and 5% CO_2 delivered by the tubing shown

in (b´) (the tubing should be introduced into the ACSF). (c and c´) Zoomed views of the recording chamber without (c) and with (c´) a hippocampal slice. The oil drop between the 40x oil objective and the bottom of the chamber is visible. The bottom of the chamber is made of a poly-L-lysine-coated coverslip to secure the slice. The tubing for flow perfusion of ACSF (flow IN/flow OUT) has been represented.

Recipes

Note: Use freshly prepared solutions.

1. Artificial cerebrospinal fluid (ACSF)
   In 1 L of MilliQ-water or ddH_2O dissolve:
   7.824 g NaCl
   0.21 g KCl
   2.476 g $NaHCO_3$
   0.155 g NaH_2PO_4
   2.23 g glucose
   0.36 g $MgSO_4$
   Bubble with 95% O_2 and 5% CO_2 for 20 min.
   Add 0.368 g $CaCl_2$ and adjust the pH to 7.4.
   Test osmolarity (300-310 mOsm).
   Store at 4°C.
2. Phosphate-buffered saline (PBS)
   In 1 L of MilliQ-water or ddH_2O, dilute:
   8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
   Adjust pH to 7.4.
   Store at 4°C.
3. Phosphate-buffered saline with 0.5% Triton X-100 (PBST)
   To obtain 100 ml of PBST, add 500 µl of Triton X-100 to 99.5 ml of PBS.
   Store at 4°C.
4. Blocking/permeabilizing solution
   Dilute 2% bovine serum albumin (BSA) and 0.2 M glycine in PBST.
   Store at 4°C.
5. Diluting solution for antibodies
   Dilute 2% bovine serum albumin (BSA) in PBST.
   Store at 4°C.
6. Fixing solution 4% PFA in PBS

Note: Toxicity/Safety. PFA is a toxic, corrosive, irritant, and carcinogenic compound. To avoid skin contact and inhalation, wear protective clothing (e.g., gloves, mask, and robe) and work under a gas extraction cabinet while manipulating PFA.

Dilute 4 g PFA in 100 ml of PBS, stir the solution at 80°C, adjust pH to 7.4 using NaOH, and filter the solution.

Store at 4°C.

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Competing interests

The authors declare no competing interests.

Ethics

Experimental procedures were processed following the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and the local regulation (CDC Exp. 4332/99, Diario Oficial No. 25467, Feb. 21/00, Universidad de la República, Uruguay).

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