Staining protocol for organotypic hippocampal slice cultures

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This protocol details a method to immunostain organotypic slice cultures from mouse hippocampus. The cultures are based on the interface method, which does not require special equipment, is easy to execute and yields slice cultures that can be imaged repeatedly, from the time of isolation at postnatal day 6–9 up to 6 months *in vitro*. The preserved tissue architecture facilitates the analysis of defined hippocampal synapses, cells and entire projections. Time-lapse imaging is based on transgenes expressed in the mice or on constructs introduced through transfection or viral vectors; it can reveal processes that develop over periods ranging from seconds to months. Subsequent to imaging, the slices can be processed for immunocytochemistry to collect further information about the imaged structures. This protocol can be completed in 3 d.

INTRODUCTION

Recent advances in live imaging and transgenic technology have had a substantial impact on the range of experimental tools available to life scientists[1,2]. These include microscopes with greatly improved sensitivity, temporal and spatial resolution and spectral versatility; powerful image acquisition and processing software; and an ever-growing repertoire of fluorescent reagents to monitor second messengers, identify macromolecules and their physiological modifications, and examine subcellular structures *in situ*. In the field of neuroscience, these developments have allowed studies of the structure and function of biologically relevant neuronal circuits to be approached in a noninvasive way and with unprecedented analytical power. To fully exploit these technological developments, adequate biological preparations must be adapted for live-imaging studies of defined neuronal circuits[3–7]. Subsequent immunostaining of the preparations allows retrospective definition of the cellular and molecular identity of the imaged structures and their local surroundings, as well as molecular correlation of the dynamic processes.

Advantages of the method

Key features of the hippocampal organotypic slice cultures[4] from mice include (i) well-defined cellular architecture of the hippocampal circuit, which preserves the organization *in vivo* and allows for the identification and manipulation of defined neurons and synapses[3–7]; (ii) presence of axonal projections (mossy fiber axons extending from dentate gyrus granule cells to the distal end of CA3) that can largely be recovered in the slices in their original state (that is, without lesioning) and that establish stereotype numbers of readily identifiable presynaptic terminals onto excitatory and inhibitory neurons in the hilus and CA3 (refs. 6–8); (iii) a long-term thickness of 100–150 μm, preserving three-dimensional organizations of connectivity[4,5]; (iv) maturation of the slice cultures closely reflecting the corresponding schedule *in vivo*[9]; (v) the option to prepare the slices from mice of any genetic background, including those expressing fluorescent transgenes in selected neurons[6,7,10,11] and those of poor postnatal viability. Imaging coupled to retrospective immunocytochemistry allows the acquisition of information about unlabeled structures in the areas surrounding the imaged (fluorescent) structures, and the investigation of molecular mechanisms at the level of local identified structures within neuronal circuits.

Critical aspects

The main critical issues relate to the extent to which organotypic slice cultures reproduce the properties of hippocampal circuits *in vivo*[5]. This information is important in deciding whether the approach is appropriate to address the particular experimental issues one has in mind. These issues have been investigated in much detail by physiologists, who have demonstrated extensive similarities, but also a few discrepancies, between properties of the corresponding circuits in the neonatal and adult mouse brains[5,9]. Critical limitations of the immunocytochemistry protocol mainly involve issues of antibody penetration and antigen accessibility. Some of these problems can be solved by varying the fixation and permeabilization protocols or by cutting sections of the slices.

Possible results and outlook

Organotypic slice cultures from approximately 1-week-old mouse hippocampus seem to reproduce most anatomical and functional properties of the corresponding hippocampal circuits *in vivo* for at least 6 months *in vitro* as a result of the intrinsic properties of their neurons. Imaging of the slices coupled to *post hoc* immunocytochemistry thus provides an exciting range of possibilities for the exploration of mechanisms controlling the assembly and function of neuronal circuits. Some of these possibilities include (i) time-lapse imaging and molecular analysis over periods ranging from sub-seconds to months, and from individual molecules to entire neuronal projections and circuits; (ii) imaging and analysis of neuronal[6,7,10,11] and glial[12] subtypes; (iii) molecular manipulation using transfection[12,13] or viral approaches[14,15] to knock down or overexpress genes, silence or activate neurons, render neurons responsive to light or selective drugs, and highlight subcircuits; (iv) protocols that combine physiology, imaging and immunocytochemistry; (v) manipulations to investigate lesion-induced plasticity and pathways of neurodegeneration and repair (such as amyloid- or epilepsy-related pathways); (vi) the potential to follow

and characterize the insertion of new neurons, the development of axons and their connections, or the insertion of exogenously added stem cells; and (vii) additional *post hoc* analysis using methods involving tracers, electron microscopy and single-cell genomics.

MATERIALS

REAGENTS

- Organotypic hippocampal slices
- Paraformaldehyde (PFA; Merck, cat. no. 1.04005.1000)
- Methanol (MeOH; Merck, cat. no. 1.06009.1000)
- Triton X-100 (Fluka Chemika, cat. no. 93420)
- BSA (Sigma, cat. no. A3912-100G)
- PBS (with or without magnesium and/or calcium)
- Primary and secondary antibodies suitable for immunohistochemistry
- 4% PFA in PBS (cooled to 4 °C) **! CAUTION** PFA is toxic. Avoid inhalation, ingestion or contact with skin, eyes or mucous membranes.
- 20% MeOH in PBS (cooled to 4 °C)
- Permeabilization solution: 0.5% Triton X-100 in PBS **? TROUBLESHOOTING**
- Blocking solution: 20% BSA in PBS
- Antibody solutions: 5% BSA in PBS + antibodies at specific dilution
- First washing solution: 5% BSA in PBS
- Second washing solution: PBS

EQUIPMENT

- Scalpel or razor blade
- Fine straight forceps
- Microscope slides (e.g., 76 × 26 mm; Menzel-Gläser)
- Thin cover glasses (e.g., 40 × 24 mm, 170 nm thick; Assistant)
- Mounting medium (e.g., ProLong Gold antifade reagent, Invitrogen, cat. no. P36934)
- 12- or 24-well plates (Corning, cat. no. 3513)

PROCEDURE

Fixation of slice cultures (day 1) ● TIMING 15 min

1| Remove the culture medium beneath the membrane by suction.

2| Add 1 ml of cold 4% PFA solution above and 1 ml beneath the membrane insert.
! CAUTION Use gloves to handle PFA, and wear a mask.

3| Wait 5 minutes.
? TROUBLESHOOTING

4| Remove the PFA solution completely.

5| Wash once briefly by adding 1 ml of cold PBS above and 1 ml beneath the insert and then removing by suction.

6| Add 1 ml of cooled 20% MeOH/PBS solution above and 1 ml beneath the insert.

7| Wait 5 minutes.
? TROUBLESHOOTING

8| Wash once briefly with PBS as in Step 5.

Permeabilization of slice tissue (day 1) ● TIMING Minimum 12 h

9| Add 1 ml of permeabilization solution (0.5% Triton X-100 in PBS) above and 1 ml beneath the insert.

10| Incubate overnight, or for at least 12 h, at 4 °C.
■ **PAUSE POINT** Slices can be kept in the permeabilization solution for up to 18 h.
? TROUBLESHOOTING

Blocking (day 2) ● TIMING Minimum 4 h

11| Remove permeabilization solution.

12| Add blocking solution (20% BSA in PBS).
■ **PAUSE POINT** Can be left for 4 h at room temperature (22–24 °C) or overnight at 4 °C. Sections can be kept in the blocking solution at 4 °C for at least 2–3 d.

Cutting slices off membrane of culture plate inserts (day 2) ● TIMING 5 min

13| To reduce the volume of antibody solutions needed, the slices are cut off the membranes of the culture plate inserts. Place the culture plate insert on a plastic cover (preferably a transparent plastic cover lying on a dark background to make the tissue easily visible).

14| Use forceps and scalpel to carefully cut the membrane piece together with the hippocampal slice out of the surrounding membrane. Keep 1–2 mm of distance to the tissue to avoid damage. (Optional: the slices can already be cut off just after the fixation to further limit the amounts of permeabilization and blocking solutions required).
▲ **CRITICAL STEP** Always keep the top side of the membrane facing up, and do not flip it around.

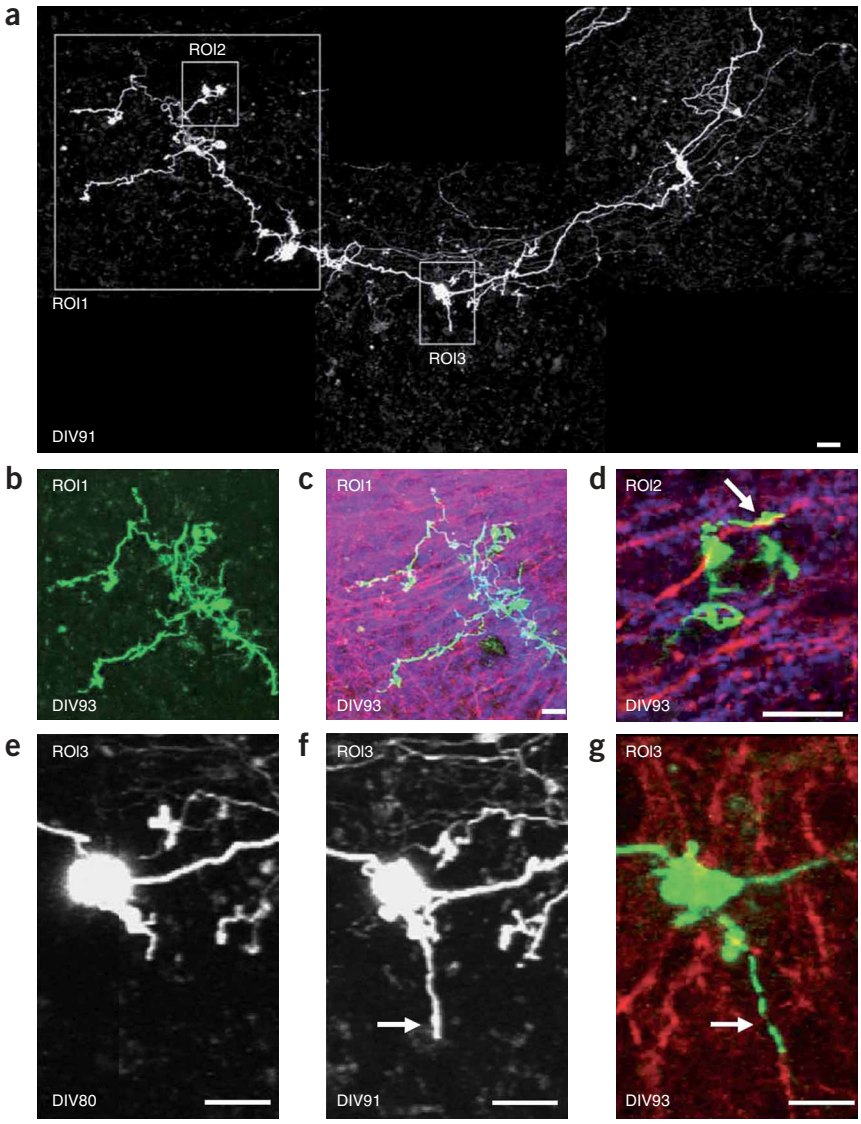

Figure 1 | Example of a time-lapse imaging and immunohistochemistry experiment. A slice from a Thy1-mGFP single-transgenic mouse shows mossy fiber projection expressing membrane-targeted GFP. After imaging, the slice was fixed and stained for Bassoon (active zone marker) and phospho-GluR1 (pyramidal neuron dendrite marker). Live imaging was done on *in vitro* day 80 (DIV80) and DIV91; fixation and staining were done on DIV93. (**a**) Low-magnification view of mossy fiber projection and regions of interest (mossy fiber terminal complexes; ROI1–3). (**b**) Maximum-intensity projection of the GFP signal in ROI1 after fixation. (**c**) Same as **b**, but with superimposed Bassoon (blue) and phospho-GluR1 (red) signals. (**d**) Single confocal plane of ROI2. Note process grown from the mossy fiber terminal between DIV91 and DIV93 (arrow), which exhibits a terminal bouton and contacts the same dendrite (phospho-GluR1) as its mossy fiber terminal of origin. (**e**,**f**) Live imaging of ROI3 (maximum-intensity projection). (**g**) Single confocal plane of ROI3 after fixation and staining (green, GFP; red, phospho-GluR1). Note process that grew from the mossy fiber terminal between DIV80 and DIV91 (arrows in **f** and **g**), extending along a phospho-GluR1–positive dendrite. Scale bars, 25 μm.

15| Place the cut-off membrane pieces (top sides facing up) onto the lid of a culture plate.
▲ CRITICAL STEP To avoid drying, always keep a droplet of 5% BSA/PBS solution on top of each slice.

Incubation with primary antibody (day 2)
● TIMING **Minimum 4 h or overnight**
16| To avoid drying of the slices during the antibody incubations, build a 'wet chamber' by putting wet paper tissues into a box that can be tightly closed and is large enough to hold the culture dish covers of Step 15.

17| Prepare the primary antibody solutions in 5% BSA/PBS (50 μl per slice).

18| Drop 50 μl of the antibody solution onto each slice.

19| Carefully place the lid holding the slices into the wet chamber and close it.
■ PAUSE POINT The primary antibody can be incubated overnight at 4 °C or for 3–4 h at room temperature.

Washing off primary antibody (day 3) ● TIMING **30 min**
20| Fill the wells of a 12- or 24- well plate with 5% BSA/PBS (fill three times as many wells as you have slices to stain).

21| Put each stained slice into one well containing the 5% BSA/PBS washing solution.
▲ CRITICAL STEP Always keep the top side of the slice facing up.

22| To wash off excess antibody, put the plate onto a horizontal shaker for 5–10 min at moderate speed (be careful that the fluid movement does not cause the slice to flip over).

23| Transfer the slices to the next unused wells and repeat this washing twice more.

Incubation with secondary antibody (day 3) ● TIMING **Minimum 3 h**
24| Prepare the secondary antibody solution (50 μl per slice).

▲ CRITICAL STEP If you use fluorescent secondary antibodies, perform the following steps whenever possible in the dark, and keep the antibody-containing solutions away from light.

25| Put the slices back onto a fresh plate lid (as in Step 15).

26| Proceed as for primary antibody (Steps 16–19).
■ PAUSE POINT The secondary antibody can be incubated for 3–4 h at room temperature or overnight at 4 °C.

Washing off secondary antibody ● TIMING 30 min
27| Wash off the secondary antibody as for the primary antibody (Steps 20–23) but using simple PBS solution (no BSA required).

Mounting of stained slice cultures (day 3) ● TIMING 10 min
28| Put the washed slices, top sides facing up, onto a glass microscope slide.

29| Put a droplet of mounting medium directly on the slice.
▲ CRITICAL STEP Avoid drying out the slices.

30| Cover the slice immediately with a thin cover glass.

31| Seal the cover glass with nail polish.
■ PAUSE POINT

32| Store at 4 °C in the dark. The labeled slices can be kept for several months.

● TIMING
Day 1: fixation, 5 min PFA + 5 min MeOH; permeabilization, minimum 12 h (overnight).
Day 2: blocking, minimum 3 h; incubation with primary antibody, minimum 4 h or overnight.
Day 3: washing off primary antibody, 30 min; incubation with secondary antibody, minimum 3 h; washing off secondary antibody, 30 min; mounting sections, 5 min; sealing cover glasses, 5 min.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Transgenic GFP signal is beaded or very weak	Fixation can destroy GFP signal (Steps 3 and 7)	Reduce fixation times (e.g., 3 min each for PFA and MeOH)
		Methanol fixation may not be required for each antibody. Try omitting methanol
		Use anti-GFP antibody to enhance the signal
Antibody does not penetrate deeply into the tissue	Permeabilization too weak (Step 10; especially in young slices, which are more dense)	Increase Triton X-100 concentration during the permeabilization step to 1–2% and/or prolong the incubation

ANTICIPATED RESULTS
Critical factors are the fixation of the slices and penetration of antibodies and reagents. Penetration of reagents can be enhanced by double fixation followed by permeabilization overnight. Unfortunately, fixations are not entirely predictable, even when using the same protocol, so one should plan on processing several copies of crucial data. Suboptimal fixation can lead to a blurred appearance of small structures such as active zones. Optimization of protocols for special needs is recommended.

This protocol should produce good-resolution labeling of cellular and subcellular structures (**Fig. 1**) and unambiguous identification of regions of interest, such as those that had been followed with live imaging before fixation.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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