Long-term live imaging of neuronal circuits in organotypic hippocampal slice cultures

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This protocol details a method for imaging organotypic slice cultures from the 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 after they are isolated on postnatal day 6–9 and for 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 time periods ranging from seconds to months. Imaging can be repeated at least eight times without detectable morphological damage to neurons. Subsequent to imaging, the slices can be processed for immunocytochemistry or electron microscopy, to collect further information about the structures that have been imaged. This protocol can be completed in 35 min.

INTRODUCTION

Recent advances in live-imaging technology have had a dramatic impact on the range of experimental tools available to life scientists[1,2]. These include the following: microscopes with greatly improved sensitivity, temporal/spatial resolution and spectral versatility; powerful image-acquisition and image-processing software; and an ever growing repertoire of fluorescent reagents to monitor second messengers, and to identify macromolecules and their physiological modifications as well as subcellular structures *in situ*. For research in neuroscience, these developments have meant that studying the structure and function of biologically relevant neuronal circuits can now be approached in a noninvasive way, and with unprecedented analytical power. In order to fully exploit these technological developments, adequate biological preparations have to be established in parallel to investigate neuronal circuits. Fortunately, preparations developed by physiologists more than a decade ago[3–5] can be readily adapted for live-imaging studies of defined neuronal circuits[6,7]. Labeling subsets of neurons and their subcellular structures can be achieved using transgenic mice and a mouse Thy1-promoter cassette[8,9]. While cytosolic fluorescent proteins work well[9], expression of membrane-targeted GFP constructs provides optimal visualization of neuronal outlines[6,7]. Further constructs available for Thy1-transgenic mice include, for example, synaptopHluorin[10]. Alternatively, transgenes can be introduced directly into slice cultures using transfection methods[11,12] or viruses[13,14].

Advantages of the method

Key features of the organotypic hippocampal slice cultures[4] include the following: (i) well-defined cellular architecture of the hippocampal circuit, which preserves the organization *in vivo*, and allows the identification and manipulation of defined neurons and synapses[3–7]; (ii) the presence of axonal projections (mossy fiber axons extending from dentate gyrus granule cells to the distal end of CA3), which can largely be recovered in the slices in their original state (i.e., without lesioning), and establish stereotypical numbers of readily identifiable presynaptic terminals onto excitatory and inhibitory neurons in the hilus and CA3[6,7,15]; (iii) a long-term thickness of 100–150 μm, preserving the 3D organizations of connectivity[4,5]; (iv) maturation of the slice cultures closely reflecting the corresponding schedule *in vivo*[16]; and (v) the option to prepare the slices from mice of any genetic background, including those with poor postnatal viability.

Critical aspects

One set of critical issues relates to the extent to which organotypic slice cultures reproduce the properties of hippocampal circuits *in vivo*[5]. This information is important for deciding whether the approach is appropriate to address the particular experimental issues of interest. These issues have been investigated in detail by physiologists, who have demonstrated extensive similarities, but also a few discrepancies, with respect to the properties of the corresponding circuits in the adult brain[5,16]. Further critical issues relate to the manipulations involved in the imaging procedures. The slices can be electrically labile, and gentle handling is important in order to avoid epileptic-like discharges[5]. In addition, it is essential to minimize the times during which the slices are kept outside of the tissue-culture incubator, and to allow sufficient recovery times between single imaging sessions (see PROCEDURE). These factors must be balanced against the requirements of the experimental questions. We recommend always optimizing and standardizing the particular experimental protocols, taking into account reproducibility and negative side-effects. By contrast, contaminations and phototoxicity can largely be avoided through appropriate precautions.

Possible results and outlook

Organotypic hippocampal slice cultures from mice aged ~1 wk appear to reproduce most anatomical and functional properties of the corresponding hippocampal circuits *in vivo* for at least 6 months *in vitro*, due to the intrinsic properties of their neurons. Accordingly, the slices provide an exciting range of possibilities for the exploration of mechanisms controlling the assembly and function of neuronal circuits. These include the following: (i) time-lapse imaging over periods ranging from sub-seconds to

months, and of objects in the slices ranging from individual molecules to entire neuronal projections and circuits; (ii) imaging of neuronal[6,7,9] and glial[12] subtypes; (iii) molecular manipulation using transfection or viral approaches to knock down or overexpress genes, silence or activate neurons, render neurons responsive to light or selective drugs, and highlight sub-circuits; (iv) combined physiology and imaging methods; (v) manipulations to investigate lesion-induced plasticity, and pathways of neurodegeneration and repair (e.g., amyloid-related or epilepsy-related pathways); (vi) following the insertion of new neurons, the development of axons and their connections, or the insertion of exogenously added stem cells; and (vii) *post-hoc* analysis using, for example, tracers, electron microscopy and single-cell genomic methods.

MATERIALS

REAGENTS

- Mouse organotypic hippocampal slice cultures (see REAGENT SETUP)
- Fungizone antimycotic, liquid (Gibco, cat. no. 15290-018)
- Tyrode salt solution (see REAGENT SETUP)

EQUIPMENT

- Single-point scanner upright confocal microscope with spectral detection (e.g., Olympus Bx61 LSM Fluoview or Zeiss LSM 510) equipped with a 40×/0.75W water-immersion objective
- 35-mm Petri dishes (Corning, cat. no. 430165)

REAGENT SETUP

Mouse organotypic hippocampal slice cultures Prepared from mice aged 6–9 d (see PROCEDURE). We have imaged slices at times ranging from 5 d to 6 months *in vitro*. ! CAUTION All procedures must adhere to local laws regulating handling of experimental animals.

Tyrode salt solution 2.7 mM KCl, 0.5 mM $MgCl_2$, 136.9 mM NaCl, 0.36 mM NaH_2PO_4, 1.4 mM Na_2HPO_4, 5.5 mM glucose, 1.8 mM $CaCl_2$ (pH 7.26)

▲ CRITICAL Filter-sterilize through a 0.22-μm membrane

PROCEDURE

Set up of the confocal microscope ● TIMING 10 min

1| Optimal acquisition settings are adapted to the intensity of the labeled cells based on the following criteria: use the smallest laser intensity possible, and enhance the intensity by increasing the gain and photo-multiplier (PMT) strength and/or opening the pinhole; also, use the largest step size possible (adapted to the size of the imaged objects). We obtained the best results for mossy fiber terminals using a step size of 0.62 μm. In order to allow fast acquisition (and, thus, cause minimal damage to the slice cultures), use a low-resolution mode, avoid using averaging functions (e.g., Kalman) and apply the fastest scanning rate available to the microscope. We imaged mossy fiber terminals at 512 × 512 pixels.

? TROUBLESHOOTING

Imaging session ● TIMING 30 min maximum

2| Working in the cell-culture hood, place the cell-culture insert into a 35-mm Petri dish and add 2 ml pre-warmed Tyrode salt solution at 37 °C (1 ml above and 1 ml below the membrane).

3| Move to the confocal microscope. Use the 40×/0.75W water-immersion objective and the mercury lamp to look for labeled cells.

▲ CRITICAL STEP To avoid contaminations originating during the imaging sessions, we clean the objective with 70% (vol/vol) ethanol in water before imaging individual slices. By taking this simple precaution, and using Fungizone and antibiotics in the culture media (see slice-preparation protocol; doi:10.1038/nprot.2006.168) we rarely experience contaminations upon imaging sessions.

4| In order to include all labeled structures in the 3D region of interest (ROI), set the start point of the z-stack slightly below the first labeled structure, and the stop point slightly above the last labeled structure. For example, acquisition of the entire mossy fiber projection required four or five 3D stacks of 40–60 confocal planes in 10–15 min.

▲ CRITICAL STEP The slices should not stay in the Tyrode salt solution and outside the incubator for more than 30 min.

5| After imaging, remove the Tyrode salt solution, return the culture-plate insert into the six-well plate and place it back in the incubator.

▲ CRITICAL STEP From now on, to avoid contaminations, the slices should be kept in culture medium supplemented with Fungizone (0.25 μg ml^{-1}).

6| Repeat Steps 2–5 for the next imaging session, keeping the same settings. In most cases, slices can be imaged repeatedly at least eight times, although some precautions should be taken (see below).

▲ CRITICAL STEP Generally, we have observed that when the experiments require more than two or three imaging sessions, good results depend on allowing long recovery time intervals between individual imaging sessions (e.g., 10–20 d), and keeping slices outside of the incubator for no longer than 20 min during imaging sessions. Our observations suggest that, provided one adheres to the principles outlined above (also see TROUBLESHOOTING), phototoxicity is not the major limiting factor. Instead, most damage to the slices associated with the imaging sessions is due to the changes of medium, and the times when the slices are kept outside of the incubator. It is important to note that our protocol was optimized for imaging granule cells and their mossy fibers. We have noticed that pyramidal neurons in CA3 appear to be more vulnerable to repeated handling, and recommend that repeated

imaging protocols should be initially tested and optimized. Characteristic signs of selective damage include major reductions in the intensity of the GFP signal (Thy1-driven expression of membrane-targeted GFP), thinning of neuronal processes and losses of spines.
? TROUBLESHOOTING

● TIMING
Step 1: 10 min
Steps 2–5: 30 min maximum

? TROUBLESHOOTING
See **Table 1**.

TABLE 1 | Troubleshooting for imaging organotypic slice cultures.

PROBLEM	POSSIBLE REASON	SOLUTION
Phototoxicity Possible signs include the following: abrupt weakening of fluorescence intensity; swellings and breakdowns of axons and dendrites into beaded chains; blurred GFP signal around membranes; formation of large blebs on cell bodies, dendrites or presynaptic terminals; loss of dendritic spines.	Too high and/or long exposure to UV light.	Use appropriate filters to reduce the intensity of the UV light when inspecting the fluorescent signal; reduce the exposure time to UV light to a minimum; search the ROI wherever possible using the live-scanning mode of the microscope avoiding using UV light; use fast and precise shutters.
	Too high laser intensity.	Adapt imaging settings to use the lowest laser intensity possible; optimize the imaging settings outside the ROI; acquire the images using the 16-bit mode, in order to be able to use low laser intensities; select the appropriate emission filter, in order to maximize signal intensity without increasing laser strength.
	Too long exposure to laser energy.	Use the fastest scan mode and the smallest amount of confocal images that still allow proper analysis; avoid time consuming averaging options during acquisition; instead, optimize image quality after acquisition (e.g., by applying deconvolution).
	Too high light energy (UV and/or laser).	Use the smallest magnification objective possible to resolve the structures of interest; choose a high numerical aperture objective; choose an objective lens that is optimized for your emission wavelength.

ANTICIPATED RESULTS

Critical factors for a successful imaging experiment are careful handling of the slices and rapid image acquisition. We strongly recommend always using the same confocal settings for comparable imaging sessions, and practicing the rapid identification of the orientation of labeled slices when first looking at a new type. It is also important to be able to rapidly re-identify the ROI within a given slice. This can be helped by making a schematic drawing, with landmarks of the particular slice, and using it for rapid orientation during the next imaging session (**Fig. 1**).

By standardizing appropriate time-lapse imaging protocols, and adhering to the precautions outlined in the protocol, it should be possible to acquire eight or more images with a resolution of 0.5–1.0 μm in the same slice for further analysis.

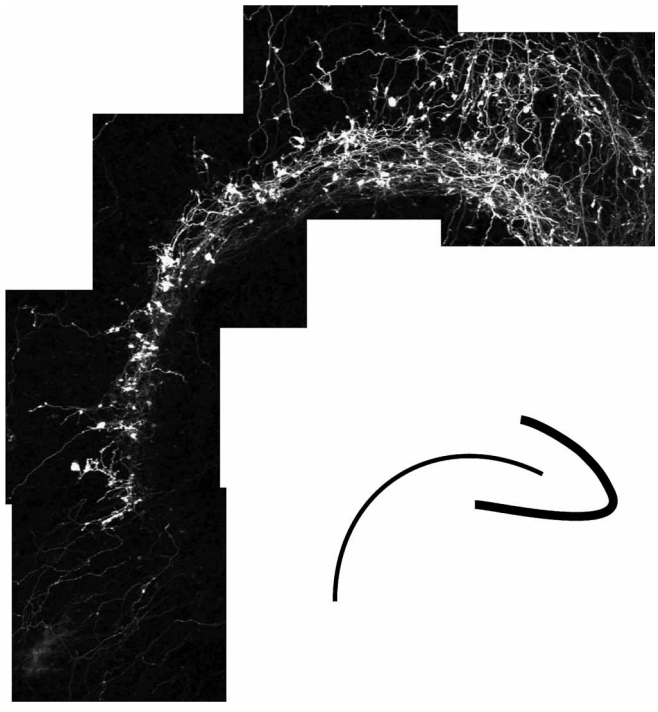

Figure 1 | Example of mossy fiber projection acquisition. The five images were acquired with a 40× objective and then tiled. The schematic on the right indicates the orientation of the hippocampus (dentate gyrus on the right). The axons are labeled by a membrane-targeted GFP construct, as described in the text.

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

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1. Conchello, J.A. & Lichtman, J.W. Optical sectioning microscopy. *Nat. Methods* **2**, 920–931 (2005).
2. Yuste, R. Fluorescence microscopy today. *Nat. Methods* **2**, 902–904 (2005).
3. Gahwiler, B.H. Organotypic monolayer cultures of nervous tissue. *J. Neurosci. Meth.* **4**, 329–342 (1981).
4. Stoppini, L., Buchs, P.A. & Muller, D. A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Meth.* **37**, 173–182 (1991).
5. Gahwiler, B.H. *et al.* Organotypic slice cultures: a technique has come of age. *Trends Neurosci.* **20**, 471–477 (1997).
6. De Paola, V., Arber, S. & Caroni, P. AMPA receptors regulate dynamic equilibrium of presynaptic terminals in mature hippocampal networks. *Nat. Neurosci.* **6**, 491–500 (2003).
7. Galimberti, I. *et al.* Long-term rearrangements of hippocampal mossy fiber terminal connectivity in the adult regulated by experience. *Neuron* **50**, 749–763 (2006).
8. Caroni, P. Overexpression of growth-associated proteins in the neurons of adult transgenic mice. *J. Neurosci. Meth.* **71**, 3–9 (1997).
9. Feng, G. *et al.* Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* **28**, 41–51 (2000).
10. Araki, R. Transgenic mouse lines expressing synaptopHluorin in hippocampus and cerebellar cortex. *Genesis* **42**, 53–60 (2005).
11. Lo, D.C., McAllister, A.K. & Katz, L.C. Neuronal transfection in brain slices using particle-mediated gene transfer. *Neuron* **13**, 1263–1268 (1994).
12. Benediktsson, A.M., Schachtele, S.J., Green, S.H. & Dailey, M.E. Ballistic labeling and dynamic imaging of astrocytes in organotypic hippocampal slice cultures. *J. Neurosci. Meth.* **141**, 41–53 (2005).
13. Ehrengruber, M.U. *et al.* Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures. *Proc. Natl. Acad. Sci. USA* **96**, 7041–7046 (1999).
14. Miyaguchi, K., Maeda, Y., Kojima, T., Setoguchi, Y. & Mori, N. Neuron-targeted gene transfer by adenovirus carrying neural-restrictive silencer element. *Neuroreport* **10**, 2349–2353 (1999).
15. Henze, D.A., Urban, N.N. & Barrionuevo, G. The multifarious hippocampal mossy fiber pathway: a review. *Neuroscience* **98**, 407–427 (2000).
16. De Simoni, A., Griesinger, C.B. & Edwards, F.A. Development of rat CA1 neurones in acute versus organotypic slices: role of experience in synaptic morphology and activity. *J. Physiol.* **550**, 135–147 (2003).