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## Longitudinal two-photon imaging of dorsal hippocampal CA1 in live mice

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**TITLE:**

Longitudinal Two-Photon Imaging of Dorsal Hippocampal CA1 in Live Mice

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**KEYWORDS:**

hippocampus, optical imaging, *in vivo*, longitudinal, mouse, two-photon, surgery, neuronal plasticity.

**SUMMARY:**

This method describes a chronic preparation that allows optical access to the hippocampus of living mice. This preparation can be used to perform longitudinal optical imaging of neuronal structural plasticity and activity-evoked cellular plasticity over a period of several weeks.

**ABSTRACT:**

Two-photon microscopy is a fundamental tool for neuroscience as it permits investigation of the brain of live animals at spatial scales ranging from subcellular to network levels and at temporal scales from milliseconds to weeks. In addition, two-photon imaging can be combined with a variety of behavioral tasks to explore the causal relationships between brain function and behavior. However, in mammals, limited penetration and scattering of light have limited two-photon intravital imaging mostly to superficial brain regions, thus precluding longitudinal investigation of deep-brain areas such as the hippocampus. The hippocampus is involved in spatial navigation and episodic memory and is a long-standing model used to study cellular as well as cognitive processes important for learning and recall, both in health and disease. Here, a preparation that enables chronic optical access to the dorsal hippocampus in living mice is detailed. This preparation can be combined with two-photon optical imaging at cellular and

subcellular resolution in head fixed, anesthetized live mice over several weeks. These techniques enable repeated imaging of neuronal structure or activity-evoked plasticity in tens to hundreds of neurons in the dorsal hippocampal CA1. Furthermore, this chronic preparation can be used in combination with other techniques such as micro-endoscopy, head-mounted wide field microscopy or three-photon microscopy, thus greatly expanding the toolbox to study cellular and network processes involved in learning and memory.

## INTRODUCTION

In mammals, the hippocampus is a key brain region for the encoding and recall of episodic memories as well as for spatial navigation<sup>1-4</sup>. For this reason, the hippocampus has been - and still is - a very important model to study the basic mechanisms that allow the brain to encode and recall memories<sup>5-7</sup> or to navigate in an environment<sup>8,9</sup> collecting rewards and avoiding dangers. In addition, the hippocampal formation is one of the brain regions where new neurons are generated throughout the life of rodents<sup>10,11</sup> and, possibly, of humans<sup>12,13</sup>. Finally, degeneration or impairment of the hippocampal formation are associated with neurological and psychiatric disorders, including Alzheimer's disease<sup>14</sup>.

In mice, the hippocampus is located approximately 1 mm below the brain surface<sup>15</sup>. Its position has prevented optic access in the intact brain and consequently, longitudinal studies of hippocampal dynamics have relied mostly on magnetic resonance (MR) imaging, electrophysiology, and ex vivo imaging analyses. MR imaging methods allow tracking of biological processes (e.g., gene expression changes<sup>16</sup>) in the same animal over multiple days, but lack the spatial resolution to discriminate single neurons. Classic in vivo electrophysiological techniques offer very high temporal resolution and exquisite sensitivity to changes in membrane potential. However, they have a limited spatial resolution and they lack the ability to reliably track the same cells over longer time periods. Optical imaging allows more diverse processes to be studied by virtue of its high temporal and spatial resolutions. However, ex vivo imaging only provides snapshots of ongoing processes, and thus it is not suitable for longitudinal studies during which the animals learn and recall information.

In vivo optical imaging combines some advantages of MR imaging and electrophysiology with those of optical imaging. Therefore, it is very well suited for longitudinal and correlative analyses of mouse brain dynamics and behavior. This is relevant in studies of biological processes with very fast (milliseconds to seconds) or very slow (days to weeks) time scales. Examples for such processes that are relevant for neuroscience are membrane voltage dynamics, Ca<sup>2+</sup> transients, cellular plasticity and structural changes, which are all believed to be very important for memory formation and recall. Different methods have extended in vivo imaging to the dorsal hippocampus<sup>18-22</sup>. Acute preparations have allowed the tracking of pyramidal neuron (PN) activity as well as their dendrites and dendritic spines for several hours<sup>20,22</sup>. This temporal timeframe, however, does not allow long-term structural changes, which might underlie incremental learning, to be studied. Chronic preparations - in combination with micro-endoscopes<sup>23,24</sup> or with long working distance (WD) standard microscope objectives<sup>21</sup> - have enabled repeated imaging of the dorsal hippocampus over several weeks.

Here, we describe a chronic preparation that provides recurrent optic access to the CA1 sub-field of the dorsal hippocampus of living mice using a permanently inserted imaging cannula. This preparation allows repeated access to the CA1 without functional disturbance and is suitable for intravital two-photon (2P) or wide-field epifluorescence imaging. Two examples of 2P deep brain chronic imaging in the dorsal CA1 of live mice are detailed: longitudinal imaging of dendritic structure and dendritic spine dynamics and longitudinal imaging of activity-evoked plasticity. The salient advantages and limitations of the technique are discussed.

#### **PROTOCOL:**

All of the methods described have been approved by the Government of Upper Bavaria (licence 2016\_ROB-55.2Vet-2532.Vet\_02-16-48) and by the Stanford and Max Planck Florida Institute for Neuroscience Administrative Panels on Laboratory Animal Care.

### **1. Preparation of the imaging cannula**

1.1. Hold a precision drill on a drill stand supplied with a movable ruler table.

1.2. Clamp a 3.0 mm diameter stainless steel tube onto the movable ruler table.

1.3. Cut the tube to a 1.6 mm-long metal ring. If the edges of the ring are not blunt after cutting, file out the irregularities.

1.4. Rinse the metal ring and a circular 4 mm-diameter glass coverslip in 100% acetone and leave to dry for ≈5 min.

1.5. Place the metal ring and the glass coverslip onto a smooth, even and clean work place, under a stereoscope.

1.6. Place a drop of UV-curing optical adhesive on a smooth surface, such as a Petri dish. Use a needle or a spatula to spread out the adhesive to form a thin (<0.5 mm) layer.

1.7. Use forceps to dip one side of the metal ring into the adhesive. Pay particular attention not to seal the inside of the ring. If this happens, use a needle to break the optical adhesive in the ring.

1.8. Using the stereoscope, position the metal ring at the center of the glass coverslip with the side of the ring covered by adhesive touching the coverslip. A thin ring of adhesive should form at the interface between the metal ring and the coverslip. Avoid any adhesive spreading to the center of the coverslip.

1.9. Turn on the UV-curing LED driver unit and shine light (365 nm) for 1 min.

**CAUTION:** UV light provokes skin burns and is a mutagenic agent. Wear UV-protecting glasses and cover hands and arms with gloves and a lab-coat to avoid skin exposure.

1.10. To cure the adhesive evenly, make sure all sides of the ring are equally illuminated by changing the direction of the light source.

1.11. Let the adhesive harden for at least 2 h, preferably, overnight.

1.12. Firmly hold the cannula from the open end of the metal ring by means of a hemostat. Using a dental drill fitted with a rotating file and working under the stereoscope, file off the excess glass coverslip until flush with the sides of the ring (**Figure 1A**).

## **2. Implantation of the imaging cannula over the dorsal hippocampus**

### **2.1. Preparation of equipment and setup**

2.1.1. Make sure all surgical instruments are clean and sterile. If using a glass bead sterilizer for sterilization, clean the instruments and place them in the sterilizer (set to 250 °C) for 10 min, prior to use. Alternatively, autoclave the instruments before use.

2.1.2. Prepare all materials required for the surgery, as well as surgical instruments, so that they can be reached readily.

2.1.3. Make sure there is enough freshly prepared medications such as pain killers or anti-inflammatory drugs.

2.1.4. Make sure there is enough isoflurane for the whole duration of the surgery (approximately 1 h per surgery).

2.1.5. Turn on the heating carpet and set it to 37 °C to keep animal temperature stable while under anesthesia.

2.1.6. Make sure the scavenging system for isoflurane is functioning.

2.1.7. Open the valve of oxygen flow.

### **2.2. Anesthesia induction, and animal fixation**

2.2.1. Place the mouse in the anesthesia induction chamber at 3% isoflurane in 1 L/min O<sub>2</sub> and wait until it loses consciousness. Assess anesthesia using the toe pinch reflex test.

2.2.2. Weigh the animal.

2.2.3. Apply anti-inflammatory (Meloxicam, 1mg/Kg) and pain killer (Vetalgin, 200mg/kg) drugs subcutaneously.

2.2.4. Position the mouse on the heating carpet. Make sure that the animal is not in direct contact with the heating carpet to avoid thermal burns.

2.2.5. Secure the head to the stereotactic apparatus and position the nose cone to cover the snout. To maintain anesthesia, decrease isoflurane to 1.5-2%. Throughout the surgery, monitor mouse state by visually monitoring breathing and by testing toe pinch reflex. Regulate isoflurane percentage if necessary.

2.2.6. Apply ophthalmic ointment to the eyes to prevent dehydration and potential blindness.

NOTE: For anesthesia and animal medication, alternative methods and drugs are possible. Please, refer to your animal license and the relative literature.

### **2.3. Optic cannula implantation**

2.3.1. Turn on fiber optic light source.

2.3.2. Remove the hair and disinfect the skin over the mouse head.

2.3.3. Using scissors and forceps, remove the mouse scalp. Start by making a small cut in the scalp in a position close to lambda. Continue by opening the scalp on the two sides. First move laterally in the direction of ears, then rostrally, in the direction of eye orbits, to form a triangle on the sagittal axis, approximately 4 mm rostral to bregma. Pay attention not to cut too close to ears and eyes, but expose bregma and lambda, as well as parietal bones and the posterior half of frontal bones.

2.3.4. Apply one drop ( $\approx 10$  mg) of lidocaine (28.9% v/v in alcohol) to the skull.

2.3.5. Clear the periosteum and dry the skull using a cotton swab.

2.3.6. Position the ear bars, to fix the mouse head.

2.3.7. Make a small craniotomy, using a micro-drill with a 0.5 mm width burr. Position the hole into the frontal bone opposite to the imaged hippocampus, approximately 1.5 mm from the sagittal suture and 2 mm distant from the coronal suture.

2.3.8. Screw a 0.86 mm-width stainless steel bone screw into the skull hole.

2.3.9. If necessary, clean the debris and dry the skull.

#### **2.3.10. Preparation of a mixture of quick adhesive cement**

2.3.10.1. Using a small spoon ( $\approx 4.5$  mm diameter), dispense 1-1.5 level scoops of L-powder into a mixing well.

2.3.10.2. Dispense 3-4 drops of Quick Base into the well.

2.3.10.3. Dispense 1 drop of Universal Catalyst into the well.

2.3.10.4. Stir the mix for 5-10 s using a precision applicator.

NOTE: Alternative cements are available. Please refer to their manufacturer's instructions.

2.3.11. Use precision applicators to apply quick adhesive cement over the skull, screw and surrounding skin. Let it dry for 30 s to 1 min.

2.3.12. Use a 3.0 mm diameter trephine drill to make a craniotomy in the parietal bone. Position the hole approximately 1.5 mm distant from the sagittal suture and 2 mm distant from the lambdoid suture.

2.3.13. Carefully remove the bone flap.

2.3.14. Check the size of the craniotomy using the cannula to make sure that it fits. Use a 0.5 mm or 0.9 mm width micro-drill to enlarge the craniotomy if necessary.

2.3.15. Remove the meninges using Dumont forceps.

2.3.16. Ablate cortical matter, to reach the external capsule. Use a 0.9 mm diameter (19 gauge) blunt needle connected to a vacuum pump. Irrigate with saline to avoid dehydration of the exposed tissue and to wash away residual blood after bleeding is resolved. Suck cortical tissue slowly,  $\approx 50\text{-}100\text{ }\mu\text{m}$  at a time, until cortex detaches from the capsule, exposing the fibers of the *cingulum* or the *corpus callosum*. Change the needle frequently, to prevent clogging.

2.3.17. Fibers extend mainly in three directions (**Figure 1B**). Carefully peel the dorsal fibers until the deepest fibers (*alveus* of the hippocampus) are exposed.

2.3.18. Rinse the tissue with saline. Use thin forceps to dip the bottom cannula into saline and position it over the skull hole. Cannula and tissue have to be sealed by saline to avoid trapping air bubbles between the cannula and the tissue.

2.3.19. Push the cannula into the skull (**Figure 1C**) until the glass coverslip is in contact with the fibers.

2.3.20. Dry the skull well using absorption triangles, cotton swabs and/or the vacuum pump.

2.3.21. Preparation of a mixture of quick adhesive cement (refer to step 2.3.10).

2.3.22. Use precision applicators to apply quick adhesive cement over the skull. Apply adhesive

also on the rim of the cannula. Be careful not to let the adhesive run into the cannula. Let it dry for 30 s to 1 min.

2.3.23. Use a stereotaxic arm to position a head holder plate over the cannula, in contact with the skull.

#### 2.3.24. Preparation of a mixture of dental acrylic

2.3.24.1. Using a spoon ( $\approx 9$  mm diameter), dispense 1 level scoop (1 part) of powder into a mixing well.

2.3.24.2. Dispense 2-3 parts of liquid in the same well. Cover the whole powder with the liquid.

2.3.24.3. Stir for  $\approx 1$  min using a spatula.

NOTE: Alternative acrylics are available. Please refer to their manufacturer's instructions.

2.3.25. Use a spatula or a precision applicator to apply acrylic across the cranium. Cover the entire exposed skull, the screw and the open skin with dental acrylic. This makes the preparation stable. Do not let acrylic run down the neck, ears and eyes.

2.3.26. Let the acrylic dry and harden for about 15 min.

2.3.27. Apply a removable adhesive film onto the head holder plate, to prevent debris from entering the cannula. It is recommended that the film size matches that of the plate.

2.3.28. Turn off isoflurane flow, remove the animal from stereotactic apparatus and position it on a heating plate to maintain physiological body temperature while it wakes up from anesthesia.

2.3.29. Monitor the animal until it has regained sufficient consciousness to maintain sternal recumbency.

2.3.30. Return the animal which has undergone surgery to the company of other animals only when fully recovered.

### 3. Postoperative care

3.1. Check the status of the mouse for 2 days following surgery, by monitoring the weight and general behavior.

3.2. Apply anti-inflammatory (Meloxicam, 1mg/Kg) and pain killer (Vetalgin, 200mg/kg) drugs subcutaneously for 2 days following surgery

NOTE: Alternative monitoring procedures, drugs and dosages are possible for postoperative care.



Please, refer to your animal license and the relative literature.

#### **4. Preparation of the imaging session**

4.1. Turn on the imaging setup in advance and let the laser to warm up and stabilize, if necessary.

4.2. Anesthetize the mouse (refer to step 2.2.1).

4.3. Use forceps to carefully remove the adhesive film from the head holder plate. Hold the mouse in a hand and the head holder plate with the fingers. Remove the film gently, to avoid damaging the preparation.

4.4. Position the mouse under the microscope, over the heating carpet and secure the head plate to the holder (Figure 1D).

4.5. Position the nose cone to cover the snout and decrease isoflurane to 1.5-2%.

4.6. Apply ophthalmic ointment to the mouse eyes.

4.7. Clean the imaging cannula by rinsing with deionized water. Use a syringe and a thin needle to drop water into the cannula and a vacuum pump to remove it.

#### **5. Imaging session**

5.1. Use low magnification, long working distance objectives to visually check the cannula for residual water, dirt, integrity and the presence of fluorescence.

5.2. Align the cannula to the optic axis by adjusting the angles of the head holder arms.

5.3. Switch to the 25X 1.0 NA, 4 mm WD or the 40X 0.8 NA, 3 mm WD, water immersion objectives. Add enough deionized water to fill the cannula and maintain excess water on top of the cannula. Avoid the formation of air bubbles.

5.4. Use 2P excitation and image fluorescent signals.

NOTE: The imaging protocol is highly dependent on the time and spatial scales to be imaged. Please refer to the sections representative results and discussion for details about the imaging settings we have used to produce the images shown in this article.

#### **REPRESENTATIVE RESULTS:**

Since the cannula is placed just dorsal to CA1, the dorsal aspect of the CA1 is more proximal to the microscope if compared to the ventral one. The *alveus* is the most proximal structure followed in order by *stratum oriens* (SO), *stratum pyramidale* (SP), *stratum radiatum* (SR) and

*stratum lacunosum moleculare* (SLM), the most distal layer (**Figures 1B, C**). Longitudinal 2P imaging of neuronal structure with subcellular resolution and of activity-evoked plasticity with cellular resolution are presented as representative results. To excite the fluorophores green fluorescent protein (GFP), d2Venus and TurboFP635, a femtosecond pulsed Ti:Sapphire laser tuned to 920 nm is used and the average laser power is adjusted to 5-25 mW at the sample. The different emission wavelengths are separated using emission filters and different photomultiplier tubes.

### **Longitudinal imaging of dendritic structure and dendritic spines dynamics.**

To sparsely label hippocampal PNs and visualize their structure, a Thy1-GFP transgenic mouse line (M line) is used. Thy1-GFP mice express cytoplasmic enhanced GFP under the control of the *Thy1* promoter in a sparse, random population of PNs<sup>25</sup>. Generally, the major axis of CA1 PNs is roughly perpendicular to the XY-imaging plane (**Figures 1B, Figure 2A and Supplementary Movie 1**). Basal dendrites extend in the SO, from the soma towards the cannula, whereas apical and apical tuft dendrites extend distally to the cannula, in the opposite direction (**Supplementary Movie 1**). Since the preparation leaves the deepest fibers of the *alveus* intact, a few fluorescent fibers traversing the field of view, just beneath the cannula, should be visible (**Figure 2A and Movie 1**). The preparation allows imaging of PN dendrites with spine resolution (**Figures 2 A-C**). To image dendrites and dendritic spines, a 25X 1.0 NA, 4 mm WD, water immersion commercial objective lens is used.

For longitudinal tracking, several brain regions within the field of view of the cannula are defined during the first imaging session. Each region corresponds to an area of approximately 240 x 240  $\mu\text{m}$  and contains between 1 and 7 dendritic segments (**Figure 2B**). These regions are manually mapped to a low magnification three-dimensional stack showing the pattern of GFP expression in the volume below the imaging cannula (**Figure 2A**). Then, 1  $\mu\text{m}$  z-step image stacks of CA1 PN basal dendrites are acquired at different time intervals (from 24 h to 3 days) for up to about 14 days (**Figure 2C**). Longer imaging durations and intervals are possible<sup>26</sup>. Each imaging session lasts approximately 60 to 90 min. Although most images are of dendritic spines in the SO, it is also possible to image dendritic spines in the oblique dendrites of SR (**Figures 2D-F**). In addition to spine density, this method enables the study of spine dynamics by quantifying their survival, gain and loss rates<sup>26-32</sup>. To score and track dendritic spines over time (**Figure 2C**), a custom MATLAB interface is used. This enables the alignment of the image stacks acquired at different time points and supports manual labeling of dendrites and spines while tracking dendritic lengths and spine positions over time<sup>26</sup>. Importantly, this method can be used to distinguish (*per* each time point, excluding the first one) between pre-existing and newborn dendritic spines. This is important as the different classes of dendritic spines are thought to have different roles in memory acquisition and retention<sup>33</sup>.

### **Longitudinal imaging of activity-evoked plasticity.**

To image activity-evoked plasticity in CA1 PNs, the dorsal CA1 hippocampal area is injected with a viral vector expressing green fluorescent destabilized d2Venus via an enhanced form of the synaptic activity-responsive element (E-SARE) within the *Arc* enhancer/promoter and red fluorescent TurboFP635 via the *ePGK* promoter<sup>34</sup>. This allows for imaging levels of activity-evoked

plasticity of hundreds of CA1 PN in each animal<sup>35</sup>. Given the very dense labeling of PN, it is generally not possible to resolve the dendrites of CA1 PN (**Supplementary Movie 2**).

To image the somata of CA1 PN, a 40X 0.8 NA, 3 mm WD, water immersion objective lens is used. For longitudinal tracking, 1 to 9 brain regions are defined *per* mouse during the first imaging session. Each region corresponds to an area of approximately 300 x 300  $\mu$ m and contains between 50 and 150 cells (**Figure 3A**). These regions are manually mapped to local tissue landmarks visible at a lower magnification. Then, 3  $\mu$ m z-step image stacks are acquired, which encompass the SP of CA1 PN (**Figure 3A** and **Supplementary Movie 2**) at different time intervals (from 24 h to 6 days) for up to about 30 days. Each imaging session lasts approximately 60 to 90 min. E-SARE activation peaks 6 to 8 h after an exposure to a new or enriched environment (EE, **Figure 3B**) and decays over the course of a few days. Thus, we generally image 6 to 8 h after experience and allow for 5 days between imaging sessions<sup>35</sup>.

To quantify d2Venus and TurboFP635 fluorescence values, a circular region-of-interest 4.64  $\mu$ m in diameter is drawn, which is smaller than a neural cell body, centered to the cell soma. We then progress to the next time point, score the soma of the same cell in the same way, and iterate this procedure for all time points and all visible cells in the longitudinal dataset. The mean value of each neuron's (activity-dependent) d2Venus emission is normalized by its mean (activity-independent) TurboFP635 emission. This method enables the investigation of long-term dynamics of ensemble plasticity of CA1 PN<sup>35</sup> (**Figure 3C**).

#### FIGURE, MOVIE AND TABLE LEGENDS

**Figure 1: Preparation for in vivo deep brain optical imaging.** (A). Top (left) and side (right) views of example imaging cannulas. Imaging cannulas have a transparent glass bottom to allow optical access to the hippocampus. (B). Schematic description of the preparation highlighting the relative position of the imaging cannula, a CA1 PN and the three layers of fibers from dorsal to ventral. (C, D). Schematic description of the imaging setup (C) and of the animal fixation (D) during an imaging session.

**Figure 2: Longitudinal imaging of dendritic structure and dendritic spines dynamics.** (A). 2P image stack (Maximum Intensity Projection (MIP) of 59 image planes, 2  $\mu$ m z-spacing) of neurons and dendrites labelled by GFP in a live Thy1-GFP mouse. (B). Higher magnification (MIP of 53 image planes, 1  $\mu$ m z-spacing) detailing basal dendrites located in SO. (C). Time-lapse image sequence of a dendritic segment imaged over 14 days. Arrowheads indicate dendritic spines tracked over 14 days. (D, E). 2P image stack of neurons and dendrites labelled by GFP in a live Thy1-GFP mouse; (D) ZY projection (31 image planes, 3  $\mu$ m z-spacing) and (E) XY projections (17 image planes, 3  $\mu$ m z-spacing). (F). Higher magnification (single image plane) detailing apical dendrites and dendritic spines located in SR. Arrowheads indicate dendritic spines. Excitation: 920 nm; emission peak: 510 nm. Scale bars: A, 50  $\mu$ m; B, 10  $\mu$ m; C, 2  $\mu$ m; D and E, 15  $\mu$ m; F, 4  $\mu$ m.

**Figure 3: Longitudinal imaging of activity-evoked plasticity.** (A). 2P images (single image planes) from a live mouse, showing the same cells on Baseline Day 0 and after EE on Day 1. (B). 2P image

stacks (MIPs of 4–6 image planes, 3  $\mu\text{m}$  z-spacing) showing E-SARE activation patterns specific for environment A (Days 1, 19 and 25) and environment B (Days 7 and 13). Green: d2Venus fluorescence. Red: TurboFP635 fluorescence. Excitation: 920 nm; d2Venus emission peak: 530 nm; TurboFP635 emission peak: 635nm. Scale bars: A, 20  $\mu\text{m}$ ; B, 10  $\mu\text{m}$ . This figure has been modified from Attardo et al., 2018<sup>35</sup>.

**Figure 4: Imaging of neuronal structure in hippocampal DG using three-photon (3P) microscopy. (A–H).** 3P images (single image planes) of neurons and dendrites labelled by GFP in a live Thy1-GFP mouse detailing (A-E) PNs in the CA1 and (F-H) granule cells in the DG. Excitation: 1400 nm; emission peak: 510 nm. Scale bar: 40  $\mu\text{m}$ .

**Supplementary Movie 1: Imaging field of view in a live Thy1-GFP mouse.** 2P image stack (83 image planes, 7  $\mu\text{m}$  z-spacing) of neurons and dendrites labelled by GFP (white) in a live Thy1-GFP mouse extending from the bottom of the cannula to SLM. To account for the decay of fluorescence signal with increasing depth, we used a non-linear gradient of photomultiplier tubes' gain.

**Supplementary Movie 2: Imaging of activity-evoked plasticity.** 2P image stack (28 image planes, 3  $\mu\text{m}$  z-spacing) of neurons expressing E-SARE reporter of IEG expression in a live mouse encompassing SP. Green: d2Venus fluorescence. Red: TurboFP635 fluorescence.

## DISCUSSION

Here, a procedure for repeated 2P imaging of the dorsal CA1 in live mice is described. After the surgery, the mouse usually recovers within 2 days. The procedure induces minimal astrogliosis<sup>26,43</sup>. Hemorrhage and edema which might follow the surgery are usually re-absorbed within 10 to 14 days. Generally, from 14 days post-implantation onwards the preparation is sufficiently clear to perform intravital imaging. The success of the surgery does not depend on working in a sterile environment. However, it is crucial to maintain a high level of hygiene, to avoid complications due to surgery-associated infections. This is obtained by meticulously cleaning the surgical instruments before and after the surgery and by heat-sterilizing them immediately prior to each usage (step 2.1.1). The optic cannula is kept into a clean, sterilized container and rinsed with sterile saline just before the implantation. Performing common surgical practices of hands disinfection and cleaning of the surgical station is also very important. The preparation remains stable and allows cellular and subcellular resolution imaging for several weeks<sup>26,35</sup>.

## Critical steps, modifications and troubleshooting.

It is important to peel the external capsule until the deepest fibers are exposed. Failure to expose the *alveus* might result in the inability to focus on the soma of PNs, or in reduced resolution imaging dendritic spines, when using commercial objectives with 3- or 4-mm WD. To this aim, it is useful to ablate the neocortex very slowly using a 0.9 mm diameter needle and then switch to a 0.3-0.5 mm diameter (24-29 gauge) needle for a finer control of suction when removing the most dorsal fibers. Alternatively, fine forceps may be used to remove the remaining cortex after fiber exposure<sup>36</sup>.

Bleeding during surgery can be problematic, as blood obstructs the view. Waiting for the clot to form and then rinsing with saline to wash away residual blood is recommended. Repeat as necessary.

A snug fit between the cannula and the craniotomy helps increase the stability of the preparation by keeping the cannula in place before application of the cement, especially if the outer rim of the cannula is flush with the skull. Since the sizes of the trephine drill and the cannula are matched, a loose fit can arise because of irregularities on the side of the cannula - which require slightly larger craniotomies to fit (see step 2.3.14) - or from an irregular craniotomy. Any cannula irregularities must be filed off (steps 1.3 and 1.12) and the trephine must be held perpendicular to the skull until the craniotomy is completed (step 2.3.12). Removing the trephine from the skull before the craniotomy is completed may result in irregular craniotomies.

#### **Limitations-Invasiveness and stability of the preparation.**

It is difficult to evaluate the effect of cortical ablation as it is arduous to precisely define the areas affected directly and indirectly. In general, the surgery removes part of the parietal cortex and part of the visual and hindlimb sensory cortex<sup>21</sup>. The ablated cortex does not directly project to the hippocampus and hippocampal tissue is neither touched nor injured. Importantly, it has been shown that implantation of an imaging cannula does not grossly alter hippocampal function and specifically hippocampal-dependent learning<sup>21,36-39</sup>. Still, it would be important to quantify to what extent both the cannula and the external part of the implant (head holder plate and dental acrylic cap) are chronic stressors by assessing corticosterone blood levels and adrenal gland weight in comparison to unimplanted mice.

The preparation generally remains stable from weeks to months<sup>26</sup>. In the long term, skin and bone growth tend to displace the acrylic cap and to increase the instability of the imaging preparation.

#### **Optical limitations.**

Conventional 2P microscopy allows imaging up to about 1 mm deep into neocortical tissue<sup>40,41</sup>. Consistent with this, it is possible to image dendrites and dendritic spines located in the SR (**Figures 2D-F**) or SLM<sup>36</sup>. However, imaging through a cannula poses limitations to the effective NA. To achieve the maximum resolution, the diameter and depth of the imaging cannulas should be matched to the imaging NA, as smaller diameters and longer depths will clip light of high NA objectives. For instance, when imaging with a 1.0 NA water immersion objective through a 1.6 mm long cannula, a 3.65 mm inner diameter is needed to keep the full NA. However, using a cannula of this diameter will increase the compression on the hippocampus and might affect the health of the tissue, for this reason, we use a cannula with a smaller diameter. When imaging with a 0.8 NA water immersion objective through a 1.6 mm long cannula, an inner diameter of 2.5 mm would be sufficient to keep the full NA. However, 0.8 NA water immersion objectives have a shorter WD (3 mm in our case), which can prevent from focusing at the SP.

These calculations apply to the center of the field of view at the bottom of the cannula. However,

moving the imaging field of view sidewise - closer to the edges of the cannula - or focusing deeper into the tissue - farther from the glass surface of the cannula - further decreases the effective NA at the focal plane and thus reduces resolution. This will lead to non-homogenous resolution across the different volumes of imaged tissue and can be a concern for quantitative imaging at subcellular resolution, especially when using super-resolution techniques such as 2P-STED microscopy<sup>42</sup>. These issues are less important when imaging at cellular resolution.

#### **Tissue motion.**

Motion within the tissue - originating from breathing and heartbeat in anesthetized animals - tends to become more severe with increased distance from the imaging cannula. This is possibly because the imaging cannula applies mechanical pressure to the brain thus counteracting some of the motion in the vicinity of the cannula (similarly to neocortical preparations). Thus, although imaging of dendritic spines is possible in SR and SLM, in our hands, it is most robust dorsal to the SO up to  $\approx 200\ \mu\text{m}$  from the surface of the cannula. To compensate for motion, we use resonant scanners and offline averaging. Several images (4 to 6 repetitions) are acquired *per* image plane of a z-stack at the maximum available speed (30 frames/s). All the repetitions for each z-plane are then deconvolved (using the commercial software, AutoQuant), registered (using ImageJ) and averaged into a single image<sup>26</sup>. For imaging of somata, motion is often negligible upon anesthesia<sup>35</sup> and two averages are often sufficient to compensate for motion artifacts.

#### **Future applications or directions of the method.**

The preparation can be combined with micro-endoscopes<sup>26,43</sup>. Micro-endoscopes are rigid optic probes which use gradient refractive index (GRIN) microlenses to guide light to and from deep tissue<sup>18</sup>. The use of micro-endoscopes allows cannulas of smaller diameters or even no cannulas at all. However, commercial micro-endoscopes are less well corrected for optical aberrations and have lower NA than commercial objectives. Current probes reach lateral and axial resolutions of  $\approx 0.6\text{--}1\ \mu\text{m}$ ,  $\approx 10\text{--}12\ \mu\text{m}$ , respectively<sup>17,18,44</sup>. The use of micro-endoscopes also enables the combination of this preparation with head-mounted integrated widefield microscopes<sup>45–47</sup>.

The method lends itself also to use in non-anesthetized mice, and it has been used to investigate cellular activity using  $\text{Ca}^{2+}$  sensors in awake head-fixed mice<sup>21,37,48,49</sup>. In these cases, due to the fast time scales of the fluorescence changes, it is advisable to implement line registration<sup>50</sup>. It is also possible to adapt the preparation for imaging of other hippocampal sub-regions such as the dentate gyrus (DG)<sup>39,51,52</sup>. Combining this preparation with 3P excitation<sup>53,54</sup> with 1 MHz frequency pulsed laser tuned to 1400 nm, we were able to image deeper into the hippocampal formation reaching the molecular layer, granule cell layer and the *hilus* of the DG (**Figure 4**) without removing the overlaying CA1.

In conclusion, we present a method that provides optical access to the dorsal hippocampus and allows longitudinal and correlative studies of the dynamics of hippocampal structure and activity. This technique extends the possibilities of analysis of hippocampal function under physiological and pathological conditions.

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## DISCLOSURES

The authors have nothing to disclose.

## REFERENCES

1. O'Keefe, J., Nadel, L. *The hippocampus as a cognitive map*. Clarendon Press; Oxford University Press (1978).
2. Zola-Morgan, S., Squire, L. R. Memory Impairment in Monkeys Following Lesions Limited to the Hippocampus. *Behavioral Neuroscience* **100** (2), 155-60 (1986).
3. Squire, L., Zola-Morgan, S. The medial temporal lobe memory system. *Science* **253** (5026), 1380–1386 (1991).
4. Leutgeb, S., Leutgeb, J.K., Barnes, C.A., Moser E.I., McNaughton, B., L., Moser, M.-B. Independent codes for spatial and episodic memory in hippocampal neuronal ensembles. *Science* **309** (5734), 619–623 (2005).
5. Silva, A. J., Zhou, Yu, Rogerson, Thomas, Shobe, J., Balaji, J. Molecular and cellular approaches to memory allocation in neural circuits. *Science* **326** (5951), 391–395 (2009).
6. Tonegawa, S., Pignatelli, M., Roy, D. S., Ryan, T. J. Memory engram storage and retrieval. *Current Opinion in Neurobiology* **35**, 101–109 (2015).
7. Poo, M. et al. What is memory? The present state of the engram. *BMC Biology* **14**, (2016).
8. O'Keefe, J., Dostrovsky, J. The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Research* **34** (1), 171–175 (1971).
9. Moser, M.-B., Moser, E. I. Functional differentiation in the hippocampus. *Hippocampus* **8** (6), 608–619 (1998).
10. Altman, J. Autoradiographic investigation of cell proliferation in the brains of rats and cats. *Anatomical Record* **145**, 573–591 (1963).
11. Kuhn, H., Dickinson-Anson, H., Gage, F. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *The Journal of Neuroscience*. **16** (6),

2027–2033 (1996).

12. Sorrells, S. F. et al. Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature* **555** (7696), 377–381 (2018).

13. Boldrini, M. et al. Human hippocampal neurogenesis persists throughout aging. *Cell Stem Cell* **22** (4), 589–599.e5 (2018).

14. Polanco, J. C. et al. Amyloid- $\beta$  and tau complexity — towards improved biomarkers and targeted therapies. *Nature Reviews Neurology* **14** (1), 22–39 (2017).

15. Rosene, D. L., Van Hoesen, G. W. The hippocampal formation of the primate brain. in *Cerebral Cortex* (Jones, E. G. et al.) **Chapter 9**, 345–456. Plenum Press. New York (1987).

16. Klohs, J., Rudin, M. Unveiling molecular events in the brain by noninvasive imaging. *The Neuroscientist* **17** (5), 539–559 (2011).

17. Wilt, B. A. et al. Advances in light microscopy for neuroscience. *Annual Review of Neuroscience* **32**, 435–506 (2009).

18. Jung, J. C., Schnitzer, M. J. Multiphoton endoscopy. *Optics Letters* **28** (11), 902 (2003).

19. Levene, M. J., Dombeck, D. A., Kasischke, K. A., Molloy, R. P., Webb, W. W. In vivo multiphoton microscopy of deep brain tissue. *Journal of Neurophysiology*. **91** (4), 1908–1912 (2004).

20. Mizrahi, A., Crowley, J. C., Shtoyerman, E., Katz, L. C. High-Resolution in vivo imaging of hippocampal dendrites and spines. *The Journal of Neuroscience* **24** (13), 3147–3151 (2004).

21. Dombeck, D. A., Harvey, C. D., Tian, L., Looger, L. L., Tank, D. W. Functional imaging of hippocampal place cells at cellular resolution during virtual navigation. *Nature Neuroscience* **13** (11), 1433–1440 (2010).

22. Busche, M. A. et al. Critical role of soluble amyloid- for early hippocampal hyperactivity in a mouse model of Alzheimer’s disease. *Proceedings of the National Academy of Science* **109** (22), 8740–8745 (2012).

23. Jung, J. C., Mehta, A. D., Aksay, E., Stepnoski, R., Schnitzer, M. J. In vivo mammalian brain imaging using one- and two-photon fluorescence microendoscopy. *Journal of Neurophysiology* **92** (5), 3121–3133 (2004).

24. Deisseroth, K. et al. Next-generation optical technologies for illuminating genetically targeted brain circuits. *The Journal of Neuroscience* **26** (41), 10380–10386 (2006).

25. Feng, G. et al. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* **28** (1), 41–51 (2000).

26. Attardo, A., Fitzgerald, J. E., Schnitzer, M. J. Impermanence of dendritic spines in live adult CA1 hippocampus. *Nature* **523** (7562), 592–596 (2015).

27. Trachtenberg, J. T. et al. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* **420** (6917), 788–794 (2002).

28. Zuo, Y., Yang, G., Kwon, E., Gan, W.-B. Long-term sensory deprivation prevents dendritic spine loss in primary somatosensory cortex. *Nature* **436** (7048), 261–265 (2005).

29. Holtmaat, A. J. G. D. et al. Transient and persistent dendritic spines in the neocortex in vivo. *Neuron* **45** (2), 279–291 (2005).

30. Xu, T. et al. Rapid formation and selective stabilization of synapses for enduring motor memories. *Nature* **462** (7275), 915–919 (2009).

31. Yang, G., Pan, F., Gan, W.-B. Stably maintained dendritic spines are associated with lifelong memories. *Nature* **462** (7275), 920–924 (2009).

32. Gu, L. et al. Long-term in vivo imaging of dendritic spines in the hippocampus reveals



structural plasticity. *The Journal of Neuroscience* **34**, 13948–13953 (2014).

33. Fu, M., Zuo, Y. Experience-dependent structural plasticity in the cortex. *Trends in Neurosciences* **34** (42), 177–187 (2011).

34. Kawashima, T. et al. Functional labeling of neurons and their projections using the synthetic activity-dependent promoter E-SARE. *Nature Methods* **10** (9), 889–895 (2013).

35. Attardo, A. et al. Long-term consolidation of ensemble neural plasticity patterns in hippocampal area CA1. *Cell Reports* **25** (3), 640–650.e2 (2018).

36. Schmid, L. C. et al. Dysfunction of somatostatin-positive interneurons associated with memory deficits in an Alzheimer’s disease model. *Neuron* **92** (1), 114–125 (2016).

37. Kaifosh, P., Lovett-Barron, M., Turi, G. F., Reardon, T. R., Losonczy, A. Septo-hippocampal GABAergic signaling across multiple modalities in awake mice. *Nature Neuroscience* **16** (9), 1182–1184 (2013).

38. Lovett-Barron, M. et al. Dendritic inhibition in the hippocampus supports fear learning. *Science* **343** (6173), 857–863 (2014).

39. Hainmueller, T., Bartos, M. Parallel emergence of stable and dynamic memory engrams in the hippocampus. *Nature* **558** (7709), 292–296 (2018).

40. Beaurepaire, E., Oheim, M., Mertz, J. Ultra-deep two-photon fluorescence excitation in turbid media. *Optics Communications* **188**, 25–29 (2001).

41. Theer, P., Hasan, M. T., Denk, W. Two-photon imaging to a depth of 1000 mm in living brains by use of a Ti:Al<sub>2</sub>O<sub>3</sub> regenerative amplifier. *Optics Letters* **28** (12), 1022–1024 (2003).

42. Pfeiffer, T. et al. Chronic 2P-STED imaging reveals high turnover of dendritic spines in the hippocampus in vivo. *eLife* **7**, pii: e34700 (2018).

43. Barretto, R. P. J. et al. Time-lapse imaging of disease progression in deep brain areas using fluorescence microendoscopy. *Nature Medicine* **17** (2), 223–228 (2011).

44. Barretto, R. P. J., Messerschmidt, B., Schnitzer, M. J. In vivo fluorescence imaging with high-resolution microlenses. *Nature Methods* **6** (7), 511–512 (2009).

45. Ghosh, K. K. et al. Miniaturized integration of a fluorescence microscope. *Nature Methods* **8** (10), 871–878 (2011).

46. Ziv, Y. et al. Long-term dynamics of CA1 hippocampal place codes. *Nature Neuroscience* **16** (3), 264–266 (2013).

47. Cai, D. J. et al. A shared neural ensemble links distinct contextual memories encoded close in time. *Nature* **534** (7605), 115–118 (2016).

48. Sheffield, M. E. J., Dombeck, D. A. Calcium transient prevalence across the dendritic arbour predicts place field properties. *Nature* **517** (7533), 200–204 (2015).

49. Basu, J. et al. Gating of hippocampal activity, plasticity, and memory by entorhinal cortex long-range inhibition. *Science* **351** (6269), aaa5694 (2016).

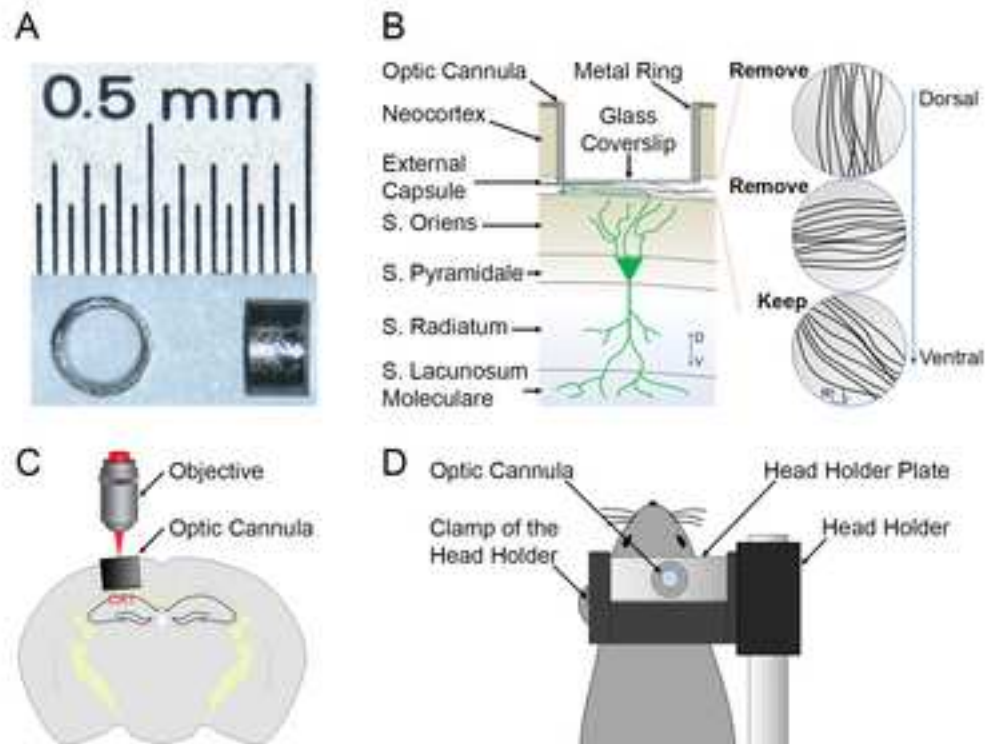
50. Kaifosh, P., Zaremba, J. D., Danielson, N. B., Losonczy, A. SIMA: Python software for analysis of dynamic fluorescence imaging data. *Frontiers in Neuroinformatics* **8**, doi: 10.3389/fninf.2014.00080 (2014).

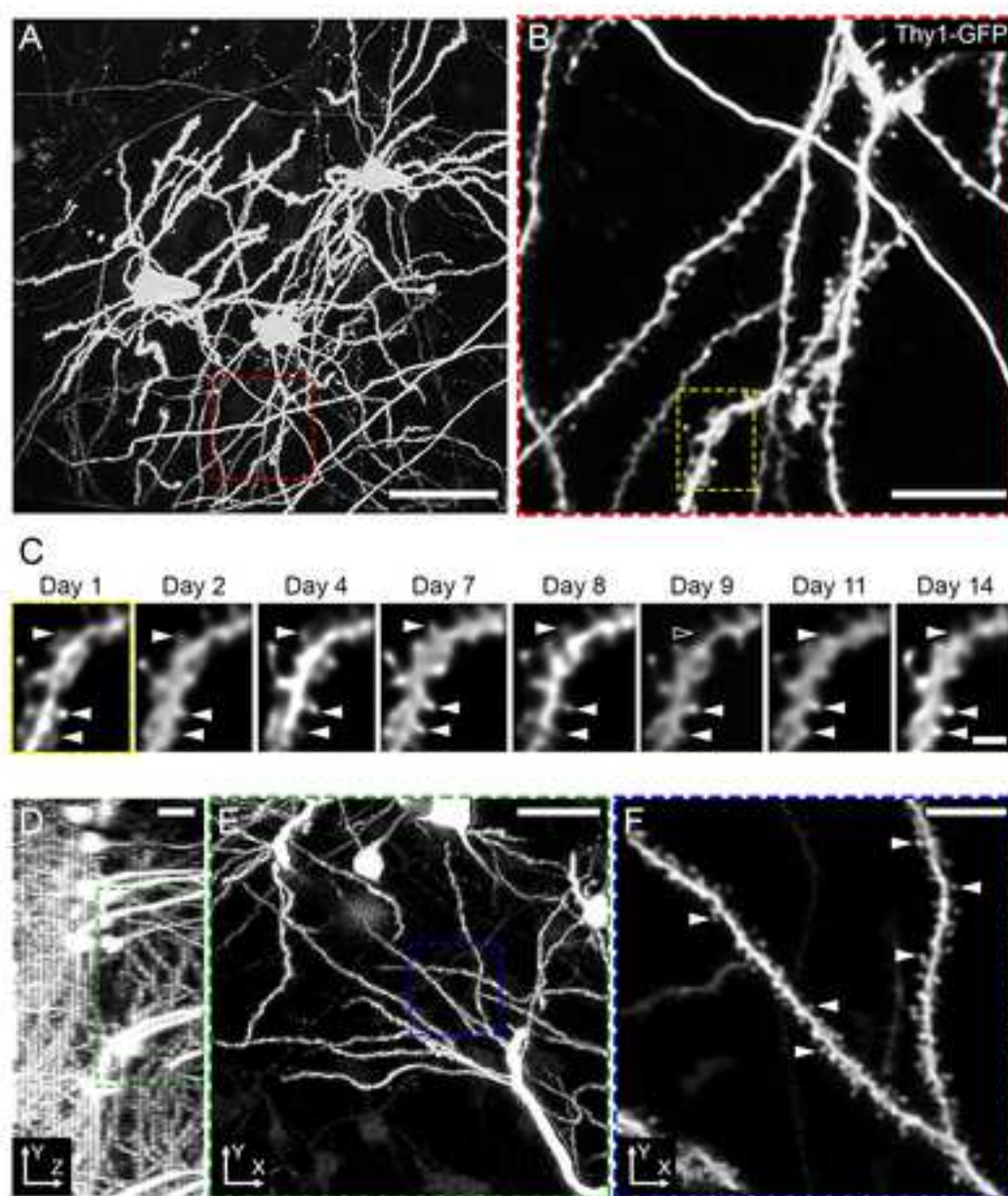
51. Gonçalves, J. T. et al. In vivo imaging of dendritic pruning in dentate granule cells. *Nature Neuroscience* **19** (6), 788–791 (2016).

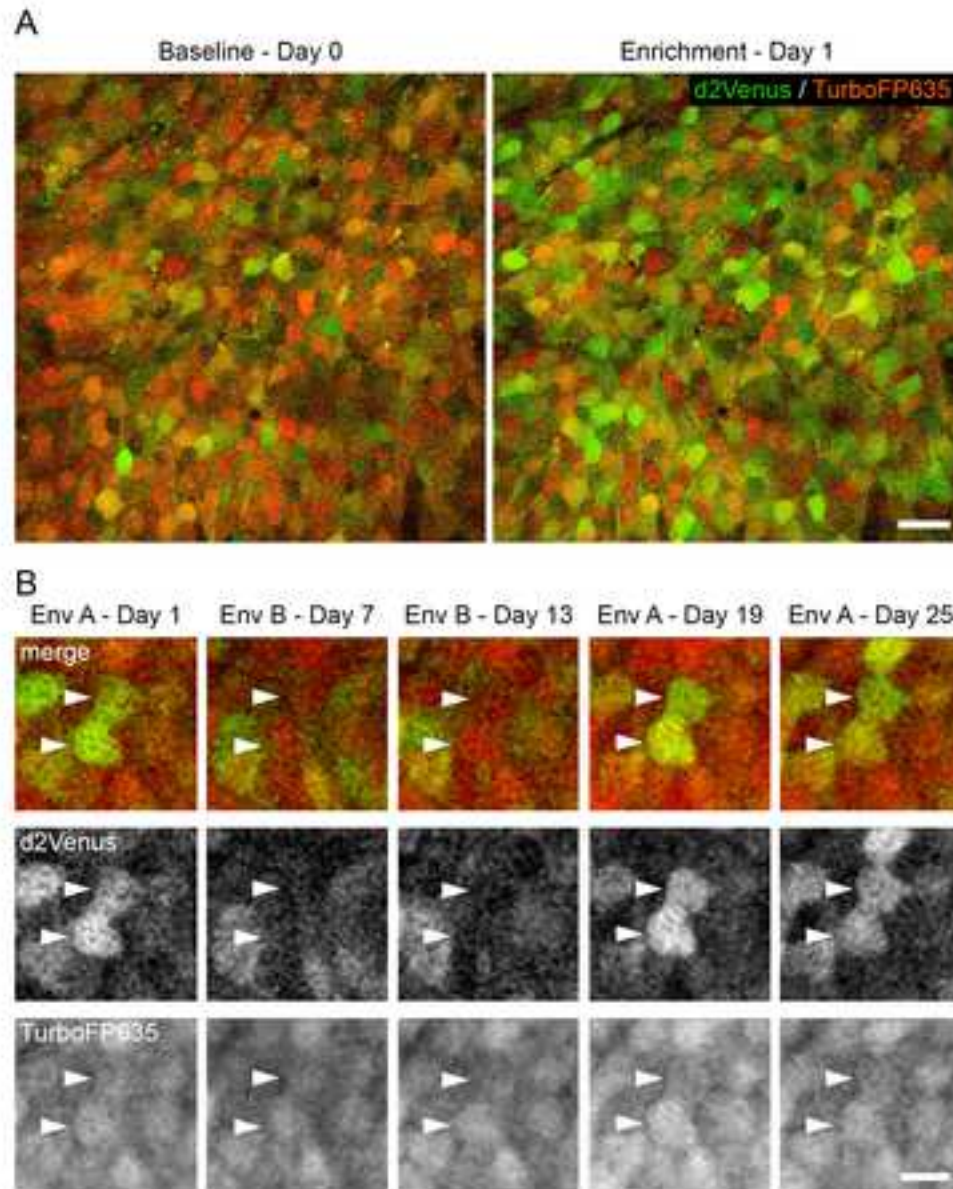
52. Danielson, N. B. et al. Distinct contribution of adult-born hippocampal granule cells to context encoding. *Neuron* **90** (1), 101–112 (2016).

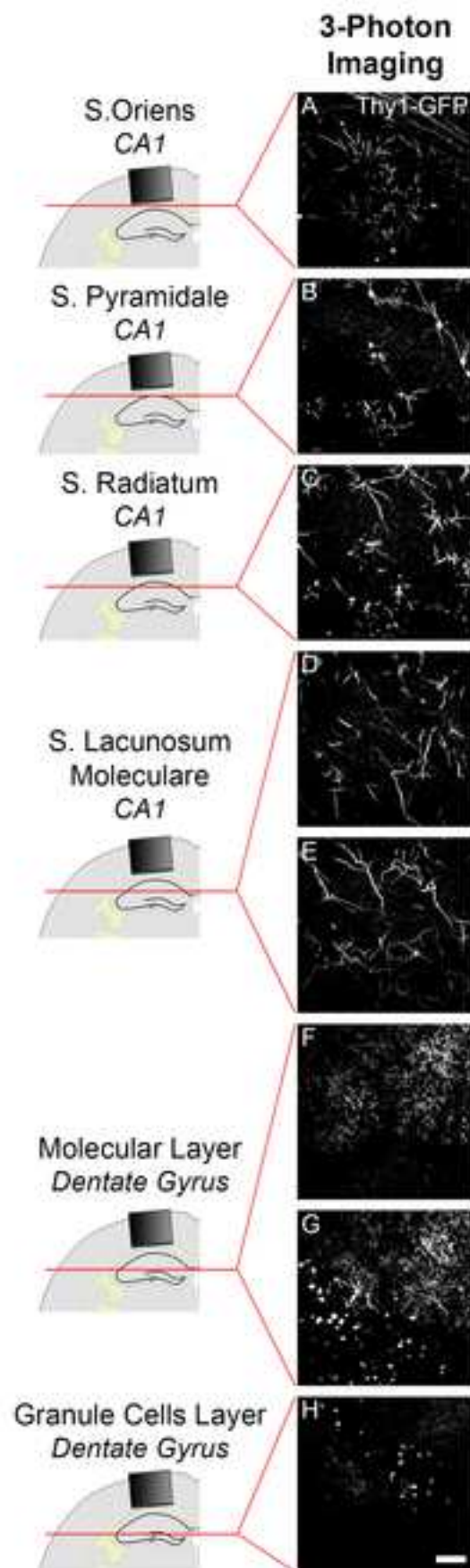
53. Hell, S. W. et al. Three-photon excitation in fluorescence microscopy. *Journal of Biomedical*

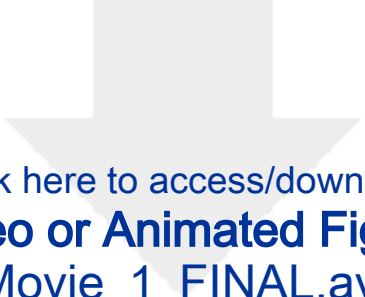
705   *Optics* **1** (1), 71 (1996).  
706   54. Horton, N. G. et al. In vivo three-photon microscopy of subcortical structures within an intact  
707   mouse brain. *Nature Photonics* **7** (3), 205–209 (2013).  
708



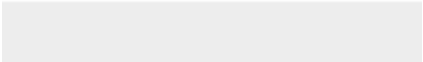









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**Name of Material/ Equipment**

Professional drill/grinder IBS/E  
MICROMOT drill stand MB 200  
MICRO compound table KT 70  
Machine vice MS 4  
Stainless steel tube Ø 3,0 x 0,25 mm (Inner Ø 2,5 mm ) L = 500 mm  
Microscope Cover glass (4 mm round)  
Schlussfeileinsatz 6-tgl. Im Blechset  
Präzisions-Nadelfeile Gesamtlänge 140 mm 4  
UV-Curing Optical Adhesives  
UV Curing LED System, 365 nm  
Stemi 305  
Presto II  
Diamantbohrer FG (5 St.), Zylinder flach, 837-014 fein  
Diamantbohrer FG (5 St.), Zylinder flach, 837-014 grob  
Graefe Forceps - Straight / Serrated  
Burrs for Micro Drill  
Burrs for Micro Drill  
MicroMotor mit Handstück  
Dumont #3 Forceps  
Fine Scissors - ToughCut  
Trepine  
Stainless Steel Self-Tapping Bone Screws  
Stereotaxic apparatus  
3-D-Gelenkarm  
Aufnahme 2SM  
Hot Bead Sterilizers  
Isofluran CP, Flasche 250 ml  
Harvard Apparatus Isoflurane Funnel-Fill Vaporizer  
Lab Active Scavenger  
Metacam 0,5% Injektionslg. (Hund / Katze), Flasche 20 ml  
Vetalgin 500 mg/ml  
CMA 450 Temperature Controller

Bepanthen Augen- und Nasensalbe  
KL 1500 LCD  
Xylocain Pumpspray  
Absorption Triangles - Unmounted  
Parkell C&B Metabond clear powder L  
Parkell C&B Metabond Quick Base B  
Parkell C&B Metabond Universal Catalyst C  
Adjustable Precision Applicator Brushes  
Blunt needles 0.9x23 mm  
Blunt needles 0.5x42 mm  
Blunt needles 0.3x23 mm  
Kallocryl A/C  
Kallocryl  
Hydrofilm transparent roll  
Head plates  
Head plate clamp  
Pedestal post holders  
Stainless steel post  
Stainless steel post  
Stainless steel post  
Post connector clamps  
Aluminum Breadboard, 300 mm x 450 mm x 12.7 mm, M6 Taps  
7" x 4" Lab Jack  
Low profile face mask small mice  
RS4000 Tuned Damped Top Performance Optical Table  
S-2000A Top Performance Pneumatic Vibration Isolators with Automatic Re-Leveling  
Power Meter Model 1918-R  
X-Cite 120Q  
Two-photon microscope  
Two-photon microscope  
Plan N 4x/0.10  $\infty$  /- /FN22  
Plan N 10x/0.25  $\infty$  /- /FN22  
LMPlan FLN 20x/0.40  $\infty$  /- /FN26.5

XLPlan N 25x/1.00 SVM  $\infty$ /0-0.23/FN18

Ultrafast tunable laser for 2P excitation

Ultrafast tunable laser for 2P excitation

Ultrafast tunable laser for 3P excitation

Company	Catalog Number
Proxxon GmbH	28481
Proxxon GmbH	28600
Proxxon GmbH	27100
Proxxon GmbH	28132
Sawade	R00303
Engelbrecht Medizin and Labortechnik	
Hoffmann Group	713750 160
Hoffmann Group	527230 4
Thorlabs	NOA81
Thorlabs	CS2010
Zeiss	
NSK-Nakanishi Germany	Z307015
MF Dental	F837.014.FG
MF Dental	G837.014.FG
Fine Science Tools	11050-10
Fine Science Tools	19008-05
Fine Science Tools	19008-09
DentaTec	MM11
Fine Science Tools	11231-30
Fine Science Tools	14058-09
MW Dental	229-020
Fine Science Tools	19010-10
Kopf	
Hoffmann Group	442114
Hoffmann Group	442100 2SM
Fine Science Tools	18000-45
Henry Schein VET GmbH	798932
Harvard Apparatus GmbH	34-1040
Gropper Medizintechnik	UV17014
Henry Schein VET GmbH	798566
MSD Tiergesundheit	
Hugo Sachs Elektronik - Harvard Apparatus GmbH	8003770

Bayer AG	
Schott	
AstraZeneca GmbH	
Fine Science Tools	18105-03
Hofmeister dental	013622
Hofmeister dental	013621
Hofmeister dental	013620
Parkell	S379
Dentina	0441324
Dentina	0452155
Dentina	0553532
Speiko	1615
Speiko	1609
Hartmann	
Custom made	
Custom made	
Thorlabs	PH20E/M
Thorlabs	TR30/M
Thorlabs	TR75/M
Thorlabs	TR150/M
Custom made	
Thorlabs	MB3045/M
Thorlabs	L490/M
Emka Technologies	VetFlo-0801
Newport	
Newport	
Newport	
Excelitas Technologies	
Bruker	Ultima IV
Thorlabs	Bergamo
Olympus	
Olympus	
Olympus	

Olympus  
Spectraphysics  
Spectraphysics  
Coherent

Mai Tai Deep See  
InSight DS+ Dual beam  
Monaco

## Comments/Description

Precision drill

Movable ruler table

Stainless steel tube for the cannula metal ring

Glass coverslips for the cannula glass

Manual files

UV-curing adhesive

UV-curing LED driver unit

Stereoscope

Dental drill

Files for the dental drill

Forceps for the surgery

0.5 mm width burr for the micro-drill

0.9 mm width burr for the micro-drill

Micro-drill for the craniotomy

Dumont forceps for the surgery

Scissors for the surgery

Trephine drill - 3.0 mm diameter; for the micro-drill

0.86 mm width bone screws

Stereotaxic apparatus

Stereotaxic arm and plate holder

Glass beads sterilizer

Liquid isoflurane for anesthesia

Isoflurane vaporizer

Isoflurane scavenger system

Meloxicam, anti-inflammatory

Vetalgin, pain killer

Heating blanket

Ophtalmic ointment  
Fiber optic light source  
Lidocain, local anesthetic  
Absorption triangles for the surgery

Quick adhesive cement

Precision applicators for the surgery

Blunt needles

Acrylic liquid component  
Acrylic powder  
Adhesive film  
30 mm x 10 mm size; 8 mm diameter hole, titanium

Head plate holder

Microscope stage

Anesthesia facemask holder

Floating table

Power meter  
Fluorescence lamp

Two-photon microscopes

Objectives



Excititon lasers

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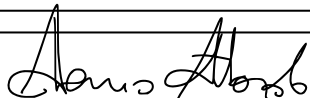
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## **Answers to Reviewer's comment for manuscript: "A preparation for longitudinal two-photon imaging of dorsal hippocampal CA1 in live mice" (59598\_R0)**

### **Reviewer #1:**

*This manuscript by Ulivi et al. reported a protocol of a preparation for longitudinal two-photon imaging of dorsal hippocampal CA1 in live mice. The authors described a chronic preparation that allows optical access to the hippocampus of living mice, and demonstrated in vivo two-photon fluorescence imaging of dendritic spine structure and activated neurons repeatedly over several weeks. Furthermore, authors showed the effect of three-photon microscope for deep fluorescence imaging of hippocampal dentate gyrus neurons. The experimental protocol and the fluorescence imaging results are very clear, should be interesting to the wide readership of Journal of Visualized Experiments.*

We thank Reviewer #1 for her/his positive comment.

*The following are minor comments.*

*1. In the two-photon and three-photon fluorescence imaging, authors should indicate the information of fluorescence emission wavelength for Enhanced GFP from Thy1-GFP mice, d2Venus and TurboFP635 in addition to the excitation information.*

We have added this information in the figure legends (lines 506, 514 and 522 of the revised version of the manuscript).

*2. In Figure 3B fluorescence images, it would be better to show d2Venus (green) and TurboFP635 images separately in addition to the merge images. This will enable to track the each fluorescence changes over the days in addition to the ratio.*

We have updated Figure 3B to include the single channels in addition to the merge images.

*3. It could be helpful for reader to have a schematic drawing how to fix the anesthetized animals under objective lens (perhaps in Figure 1) as authors discussed the tissue motion during the fluorescence imaging due to the animal breathing and heartbeat.*

We have updated Figure 1 to include a new panel describing the head fixation of the mouse.

4. In line 495 "three-photon (3P) excitation with KHz pulsed lasers tuned to 1400 nm", perhaps authors specify the range of pulsed laser frequency as this is unique setup.

Line 658 now reports the repetition frequency of our 3P laser.

5. In line 297 and 455, "1 NA" should be "1.0 NA"

We have corrected these mistakes (lines 392, 435 and 617).

## **Reviewer #2:**

### *Manuscript Summary:*

*The authors describe a protocol to chronically implant optical windows to gain access to the hippocampus for 2-P (or 3-P) imaging. They provide detailed and nice examples of dendritic spine imaging using Thy1-GFP mice, imaging of activity-dependent plasticity, and structural imaging from deep hippocampal areas (DG granule cells). The protocol is clearly described and the data examples convincingly underscore the power of the method. This protocol will therefore be of great use for the scientific community.*

We thank Reviewer #2 for her/his positive comment.

### *Major Concerns:*

*One aspect I feel could further improve the protocol is to explain the actual imaging procedure as part of the protocol (steps of placing the animal in the imaging station, microscope settings etc). This would enable other labs to implement all required steps to achieve live imaging from hippocampal neurons.*

In the first version of the manuscript we focused mostly on the preparation as we thought this would be the scope of our paper. To satisfy the request of Reviewer #2, in the revised version of the manuscript we have added two more subheadings detailing the *in vivo* imaging procedure. However, it is very difficult to describe a "standard" imaging protocol as the details of imaging are highly dependent on the time and spatial scales one wants to image. We then feel that the most appropriate place to report these details is the section *Representative Results* close to the kind of imaging they refer to. So we have decided to add the more procedural details to the *Protocol* section and to keep the more specific details in the *Representative Results* section. We hope that Reviewer #2 will agree with our view and accept the revised manuscript.

*Minor Concerns:*

*1) Line 129: The authors should state the animal license number under which the procedures described in the protocol have been authorized.*

The revised version of the manuscript now contains this relevant information (lines 148-149).

*2) The authors do not apply post-surgical analgesia, which might be required depending on local regulations. The authors could provide specific alternatives for pain relief during the recovery period (e.g. with their statement in lines 189-190).*

We thank Reviewer #2 for this comment. In fact we had overseen this aspect. We do apply both anti-inflammatory drugs as well as pain killers after surgery. This is now detailed from line 358 (*Postoperative Care* section).