Chapter 14

Photomarking Relocalization Technique for Correlated Two-Photon and Electron Microcopy Imaging of Single Stimulated Synapses

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Abstract

Synapses learn and remember by persistent modifications of their internal structures and composition but, due to their small size, it is difficult to observe these changes at the ultrastructural level in real time. Two-photon fluorescence microscopy (2PM) allows time-course live imaging of individual synapses but lacks ultrastructural resolution. Electron microscopy (EM) allows the ultrastructural imaging of subcellular components but cannot detect fluorescence and lacks temporal resolution. Here, we describe a combination of procedures designed to achieve the correlated imaging of the same individual synapse under both 2PM and EM. This technique permits the selective stimulation and live imaging of a single dendritic spine and the subsequent localization of the same spine in EM ultrathin serial sections. Landmarks created through a photomarking method based on the 2-photon-induced precipitation of an electrodense compound are used to unequivocally localize the stimulated synapse. This technique was developed to image, for the first time, the ultrastructure of the postsynaptic density in which long-term potentiation was selectively induced just seconds or minutes before, but it can be applied for the study of any biological process that requires the precise relocalization of micron-wide structures for their correlated imaging with 2PM and EM.

Key words Correlated imaging, Time-lapse live two-photon fluorescence microscopy, Serial-section transmission electron microscopy, Dendritic spine, Synapse, Postsynaptic density, Photomarking, Photoetching, Photobranding, DAB

1 Introduction

Synaptic plasticity has been extensively studied because it is considered the molecular basis for learning and memory. Synapses change over time in an experience-dependent manner, both functionally and structurally [1]. These changes can be studied in vivo or ex vivo by introducing fluorescent markers (such as GFP) and longitudinally imaging synapses and dendritic spines in real time with

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confocal or 2-photon fluorescence microscopy (2PM). In addition to imaging, 2-photon lasers can be used to selectively stimulate individual synapses via photochemical reactions such as glutamate uncaging [2, 3]. However, light microcopy has an inherent limitation that prevents observing structural features below the light diffraction limit. Superresolution techniques can nowadays overcome this limitation and are able to reach nanoscale resolutions [4–7]. Electron microscopy (EM) is more powerful in this sense as it can reach ultrastructural imaging with higher spatial resolution, down to the order of few nanometers. EM is ideal to detect and analyze synapses, including postsynaptic, presynaptic, and extrasynaptic substructures in unaltered tissue [8]. For this reason, it is currently used for brain connectivity mapping projects [9]. However, EM has some critical limitations: it requires sample fixation, thus precluding live time-course imaging of plastic changes, and it cannot detect fluorescence, thus precluding an easy relocalization of the selected neuron or synapses under study.

We developed a powerful methodological approach that bypasses these limitations to allow the correlated imaging of the same specific synapse with both 2PM and transmission EM (TEM). We combined several procedures in a way that permits: (1) the selective stimulation of a single dendritic spine (via glutamate uncaging), (2) the longitudinal observation of the plastic changes occurring in that spine (via 2 PM), (3) the unequivocal localization of the same spine within ultrathin sections (via 2-photon-induced photomarking), and (4) the 3D reconstruction and analysis of the ultrastructural changes that selectively occurred in that spine (via serial section TEM–ssTEM-). The key point is the photomarking step, which is based on the 2-photon-induced precipitation of an electrodense compound, diaminobenzidine (DAB), that generates precise fiducial landmarks visible under both 2PM and EM imaging [10–12]. These landmarks are fine lines, one or a few microns wide, which can be drawn in any region of the tissue following any useful spatial pattern. In this case, they are drawn to flank the selected dendrite and to point to the stimulated spines. After localizing these lines on ssTEM images, it is possible to unambiguously identify the same synapses that were previously stimulated and imaged with 2PM. Two-photon precipitation of DAB was previously used to relocalize single neurons [10] and we adapted it to relocalize single synapses after long-term potentiation (LTP) was selectively induced in that spine and structural changes visualized with 2PM [12–14]. An almost identical method was developed in parallel to us, named near-infrared branding (NIRB) technique, which proved to similarly localize dendritic spines and axons in both optical and EM imaging [11]. Other methods have been used to correlate optical imaging of individually labeled neurons, dendrites, or synapses with EM imaging. However, some of these methods, such as biocytin detection [15], GFP immunostaining [16–18], GFP photo-oxidation [19, 20], or photoconversion of organic fluorescent dyes [21]

suffer from the problem that the spine gets obscured by the precipitate, precluding a clear visualization of the internal subcellular structures such as the PSD. Other approaches can solve this problem but are not suitable for single synapse relocalization in thick tissue. For example, the use of endogenous landmarks, such as blood vessels patterns, is useful to reidentify neurons or groups of neurons [22, 23] but such landmarks are hard to find at microscale resolution. On the other hand, introduction of exogenous fiducial marks, such as fluorescent beads [24] or gold nanorods [25], is difficult to carry out in thick tissues like organotypically cultured slices or in vivo. We had to overcome these problems, since our goal was to image changes in PSD ultrastructure just seconds or minutes after LTP is induced in a single synapse in a hippocampal slice. Plastic changes in PSD morphology had been previously observed with EM after the classic electrical induction of LTP in multiple synapses [26, 27] but not after single-synapse LTP. Using our method we discovered that the structural plasticity of the spine and the PSD are not synchronized during LTP: PSD enlargement is 1 h delayed compared to spine enlargement [12, 28]. The same photomarking technique has recently been employed to correlate in vivo 2PM imaging of cortical neurons with focus ion beam/scanning EM [29, 30]. This method can easily be extended beyond synapse biology and used for the study of any biological process that requires precise relocalization of micron-wide structures for their correlated imaging with live 2PM and EM.

2 Materials

2.1 Hippocampal Slices

Organotypic hippocampal slice culture is a particularly useful experimental approach for studying synaptic plasticity. In this preparation, neurons mature in a relatively similar way as they do in vivo [31–35], and the local hippocampal synaptic circuitry is largely preserved [36, 37]. Acute slices from adult or adolescent transgenic mice expressing fluorescent proteins (e.g., Thy1-YFP mice) are more similar to the intact brain, but the organotypic preparation allows for a more versatile genetic manipulation, since it is easier and faster to transfect any kind of gene or combination of genes using biolistic, viral, or electroporation methods [38, 39]. Hippocampal slice cultures are typically prepared from rat (e.g., SD) or mouse (e.g., C57/BL6) pups of both sexes, at postnatal days 6–8 (P6–8; *see* **Note 1**). Slice cultures can also be prepared from other brain regions, such as cortex, striatum, cerebellum, thalamus, brainstem, etc. [40–43].

2.2 Plasmids Expressing Fluorescent Proteins

In order to longitudinally visualize dendritic spines or synaptic components in a living neuron using 2PM, it is necessary to introduce a source of fluorescence into the cell, for example, a plasmid expressing a fluorescent protein, either alone or as a fusion protein.

For time-course imaging of spine morphological changes, expression of a stable, small, and soluble marker such as EGFP is sufficient. DsRed2 is a good choice if a red-emitting marker is needed (*see* **Note 2**). Fluorescent proteins fused to naturally localized endogenous molecules can be used to target specific cell locations (*see* **Note 2**). To target the synapse or the PSD, for example, we express fluorescently labeled Homer1b, since exogenous overexpression of this protein does not alter normal synaptic physiology and Homer 1b exhibits relatively rapid synaptic turnover, which prevents loss of signal by photobleaching [44, 45]. We routinely use CMV promoter for expression of most genes, or CAG if we need stronger expression.

2.3 Two-Photon Microscope Setup

Time-lapse fluorescence imaging can be carried out with a 1-photon confocal scanning microscope or a 2-photon microscope. Two-photon is recommended for in vivo imaging or in the case of thick tissue such as brain slices, and especially in the case of photochemical reactions such as glutamate uncaging. The best way to perform experiments involving simultaneous imaging and stimulation of single spines by glutamate uncaging is to combine two mode-locked femtosecond-pulse Ti:sapphire lasers, one for imaging and one for uncaging. This expensive equipment can be purchased from several vendors, or can be custom-made provided that the adequate knowledge and equipment are possessed [46–49]. Two-photon imaging in slices is typically performed with the laser tuned between 860 nm and 980 nm, being 910–930 nm the optimum wavelength to excite both green and red fluorophores (such as EGFP and DsRed2). Glutamate uncaging is usually triggered with the same or a second laser tuned at 710–730 nm. Two-photon and 1-photon lasers can be further combined to perform more complex experiments (*see* **Note 3**).

Use an upright microscope equipped with 10× (0.3 NA) and 60× (0.9–1.1 NA) water immersion objective lenses. The microscope chamber must have a set of inlets installed to constantly perfuse artificial cerebrospinal fluid (ASCF) aerated with 95 % O_2/5 % CO_2 (carbogen). As the caged compounds are generally expensive, we recommend recirculating the same solution and using the minimum volume of liquid (e.g., 5–15 ml). This recirculation can be done using a peristaltic pump located close to the stage. It is important to carefully set up the perfusion system to ensure that the level of liquid in the chamber is always constant (*see* **Note 4**).

For such a small volume of recirculating solution, evaporation can become a problem in experiments of long duration. It is convenient to check how severe this problem is in each particular setup. A good solution to prevent evaporation is to cover the chamber with some sort of plastic shield or wet hard paper during the experiment. It is also useful to introduce humidity into the carbogen by bubbling it first through water before bubbling into

the ACSF. For experiments lasting several hours it might be still necessary to add double-distilled (dd) water several times to compensate for the increase in osmolality. Pilot experiments must be carried out to measure the rate of evaporation and to determine how much water to add, and how often (*see* **Note 5**).

2.4 Solutions for Slice Preparation, 2-Photon Imaging, and Photomarking

1. Culture Medium: Modified Eagle's Medium (MEM) with 20% horse serum, 27 mM d-glucose, 6 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 30 mM HEPES, 0.01% ascorbic acid, and 1 μg/ml insulin. Adjust pH to 7.3 and osmolality to 300–320 mOsm/L (*see* **Note 6**). We usually do not add antibiotics. Sterilize by filtering through a 0.22 μm filter and keep at 4 °C no longer than 4 weeks.
2. Dissection Solution: 2.5 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 238 mM sucrose, 1 mM $CaCl_2$, and 5 mM $MgCl_2$ in dd water. Prepare fresh or from partial 10× stock solution (*see* **Note 7**). Bubble with carbogen for 20 min. Use it ice-cold or, optionally, place it at −80 °C for ~10 min or at −20 °C for ~30 min until it acquires a slush-like consistency.
3. Magnesium-free ACSF: 119 mM NaCl, 2.5 mM KCl, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4 11 mM glucose, 3 mM $CaCl_2$, 1 μM tetrodotoxin (TTX), and 50 μM picrotoxin in dd water. Prepare fresh or from a partial 10× stock solution (*see* **Note 8**). Bubble with carbogen for 20 min. Check osmolality is in the range of 320 ± 20 mOsm/L.
4. Uncaging ACSF: 2.5 mM 4-methoxy-7-nitroindolinyl (MNI)-l-glutamate (caged glutamate) in Mg-free ACSF. The concentration of caged glutamate can vary depending on the efficiency of the system and the budget of the researcher (typically 2–6 mM). This compound is expensive, so we recommend testing the intensity and duration of the uncaging laser that triggers the desired stimulation using the minimum concentration of this compound (*see* **Note 27**). Keep this compound under darkness and prepare this solution with the minimum ambient light and maximum protection from UV light (*see* **Note 26**).
5. Fixative Solution: 2% (w/v) paraformaldehyde (PFA), 2% (w/v) glutaraldehyde (GA) in 0.1 M Phosphate Buffer (PB) pH 7.4 (*see* **Note 9**). Make it fresh every day before fixation, using a dedicated glass-ware and plastic-ware. Keep at room temperature (r.t). This solution, as well as PFA and GA, is highly toxic and so the appropriate protection must be used.
6. Photomarking Solution: 7 mM 3,3′-diaminobenzidine (DAB) in ice-cold 0.1 M Tris–HCl buffer. Adjust pH to 7.4 and cool on ice. Filter with a syringe filter (0.2 μm pore size) to remove debris. This solution must be made fresh and pH should be rechecked just before the experiment. DAB is a carcinogen and has to be handled and disposed of appropriately.

2.5 Solutions for Electron Microscopy

1. Osmium Tetroxide Solution: 1% osmium tetroxide (OsO_4) in 0.1 M PB (prepared from a pre-made 2% stock solution). Osmium tetroxide is volatile and extremely toxic in contact with skin and mucosae. Prepare this solution carefully using glassware, under the fume hood to avoid inhalation, and with two layers of latex/nitrile gloves to avoid skin exposure.
2. Osmium Tetroxide/Potassium Ferrocyanide Solution: 1.5% (w/v) potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$) in Osmium Tetroxide Solution.
3. Toluidine Blue Solution: 1% (w/v) toluidine blue in dd water. Filter with a syringe filter.
4. Durcupan Epoxy Resin: 10 parts of component A, 10 parts of component B, 0.3 parts of component C, and 0.2 parts of component D (by volume; ratios may be optimized). Durcupan must be prepared quite in advance before use. It is better to prepare it in a previously dehumidified hood (use Drierite® or a dehumidifier). In order to obtain a hard resin that will not brittle during sectioning, we recommend the proportions above, added in the same order (*see* **Note 10**). Durcupan is toxic and so the appropriate protection measures must be taken.
5. Uranyl Acetate Solution: 1% uranyl acetate in dd water. Filter it through a 0.22 μm disc filter. Uranyl acetate is light-sensitive; keep in darkness at 4 °C. All glass equipment that has been in contact with uranyl acetate should be separated from other labware.
6. Lead Citrate Solution: 3 mM lead citrate, 0.1 N NaOH in degasified dd water. Because CO_2 can precipitate lead in the form of $PbCO_3$, it is necessary to prepare degasified water (CO_2-free) by boiling dd water for 30 min and storing it in an airtight container. All material in contact with lead citrate must be made of plastic.

2.6 Pioloform Slotted Grids

To maximize the viewable area in the serial sections, use grids with oval slots of 1 mm wide by 2 mm long. To support the sections, coat the grids with pioloform (stronger than formvar) or purchase factory-coated grids, which give a highly homogenous and reproducible film thickness.

3 Methods

The complete protocol is a combination of different techniques with multiple steps each: (1) Preparation of hippocampal slice cultures; (2) Transfection of slices with fluorescent markers; (3) Two-photon imaging and stimulation of single dendritic spines; (4) Fixation of the sample; (5) Creation of landmarks by the

photomarking procedure; (6) Processing of the sample for EM; (7) Serial section cutting; (8) EM imaging of the sections; (9) Relocalization and 3D reconstruction of the stimulated spines.

In this section, we will only describe in detail the steps that contain crucial or novel methodologies (steps 3, 4, 5, 9). We will provide some useful basic information but will not describe the other steps in detail (steps 1, 2, 6, 7, 8), as methods are reported in detail in this volume and elsewhere (references below).

3.1 Preparation of Hippocampal Slice Cultures

Slices of 350–400 μm thickness are prepared from P6–8 rat (or other species such as mouse, ferret, etc.) hippocampi using a McIlwain tissue chopper, and cultured at 35–37 °C on interface membranes for 1–4 weeks (*see* **Notes 11** and **12**). Follow the preparation method used in Bosch and colleagues [12, 50], originally developed by Stoppini and colleagues in 1991 [51] based on previous works [34, 52], and technically described in detail elsewhere [53–55]. The experiment is typically carried out at 7–15 days in vitro (DIV), usually 2–4 DIV after gene transfection (to allow for fluorescent protein expression).

3.2 Gene Transfer into Slices

Our preferred method for transfection of plasmids expressing fluorescent proteins is biological ballistics (biolistics, gene gun) [56]. It is easy, rapid, and highly reproducible, and generates well-isolated transfected cells, with minimum background noise. Compared with other methods, it facilitates the expression of more than one protein by combining several plasmids in the same bullet. It also allows the simultaneous downregulation of endogenous proteins (by including a shRNA expression vector), thereby facilitating replacement approaches. Follow the instructions provided by the manufacturer and/or those described in Chapter 19, by Woods and Zito [57] and Soares and colleagues [55]. Assess the quality of slices after ~5 DIV (*see* **Note 13**). Plasmids are typically transfected no earlier than 5 DIV and no later than 15 DIV (*see* **Note 14**). Allow a minimum of 2 days for the expression of exogenous proteins. Some proteins may need >4 days to reach the necessary degree of expression for comfortable imaging. On the other hand, if higher number of transfected cells is needed, or higher spatial accuracy is required (i.e., if a specific region of hippocampus has to be selectively targeted, e.g., only CA3), viral infection is a better choice [38, 58, 59]. This method however may result in too many fluorescent cells, thus making it hard to find an isolated dendrite with dark background. Single-cell electroporation and photostransfection are excellent alternative methods in which one cell can be specifically selected and transfected [60–63], but are much more time-consuming. Additionally, instead of a genetic marker, it is possible to stain the cell by particle-mediated introduction of a lipophillic dye (e.g., DiOlistics) or to fill the cell with a fluorescent organic dye through a pipette, if one is patching the neuron for electrophysiological recording (*see* **Note 15**).

3.3 Two-Photon Imaging and Single-Synapse Stimulation

1. Start the experiment by turning on the lasers to warm the whole system up (*see* **Note 16**). Because stimulation of single synapses requires a high degree of precision, the 910 nm imaging laser and the 720 nm uncaging laser must be aligned daily (with an error of <50 nm). This alignment can be performed using 0.5 μm diameter (Ø) fluorescent beads. Beads are simultaneously or sequentially imaged with both lasers and the light path of each laser must be adjusted so that both images overlap as much as possible (*see* Fig. 1a–f). It is useful to further check the alignment by selecting a group of several beads clustered together and use the uncaging beam to bleach one of them. If lasers are well aligned and reach the sample with the correct precision, only the selected bead should be bleached out but not the neighboring ones (Fig. 1g–i) (*see* **Note 17**).
2. Prepare the Mg-free ACSF (as described in Subheading 2.4; *see* **Note 8**) and the Fixative Solution. Turn on the peristaltic pump and start the Mg-free ACSF perfusion, usually at 2–4 ml/min, while constantly aerating with carbogen. Make sure the level of liquid of the chamber is constant (*see* **Note 4**). It is not necessary to recirculate the same solution at this point, as the caged glutamate is still not present: outflow solution can be discarded and inflow solution can be fresh. Turn on temperature controller and set it at the desired value (we usually set it at 30 °C, although efficient LTP and spine enlargement can be achieved at 23–35 °C).
3. When the microscope setup is ready and all conditions are stable (temperature, perfusion flow, laser power, alignment, etc.), select the appropriate sample to be placed in the chamber (*see* **Note 18**). Pick up one insert from the well and put it on the cover of a petri dish (*see* **Notes 19** and **20**). Quickly, cut a piece of the membrane containing the slices with a surgical scalpel (*see* **Notes 11** and **12**). Cut it in an irregular shape (rectangular or trapezoidal), to be able to later identify the position and the orientation of the selected neuron within the slice and within the piece of membrane. Additionally, cut a small dent in one corner of the membrane to further break symmetry and to be able to identify which side is up and which down (*see* **Note 21**).
4. Put the membrane in the chamber and use a holder to immobilize the membrane (*see* **Note 22**). Use epifluorescence illumination through the 10× objective lens to search for a good transfected pyramidal neuron to image. A good neuron is one with a moderate level of fluorescence expression and typical healthy morphology: long straight dendrites, normal spine density, and normal basal and apical dendritic tree with multiple branches (*see* the example in Fig. 2a). Avoid cells with signs of unhealthy conditions, such as dendritic blebbing, wavy dendrites, small or few spines, or fluorescent aggregates.

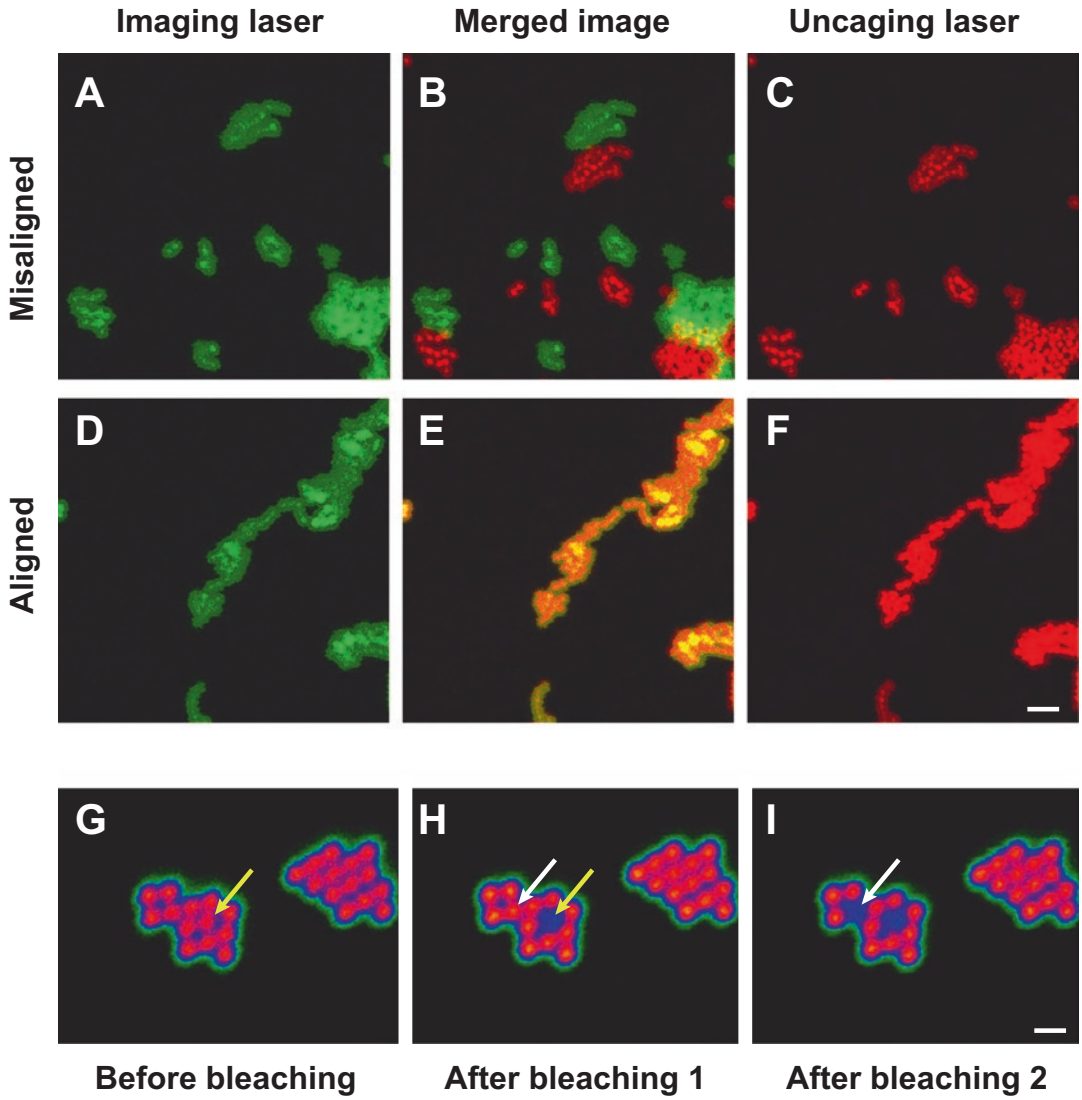

Fig. 1 Laser alignment with fluorescent beads. (**a**–**f**) Fluorescent beads of 0.5 μm Ø are used to align the imaging laser with the uncaging laser. Beads are imaged with the imaging laser (**a**, **d**) and, simultaneously or sequentially, are imaged with the uncaging laser (**c**, **f**). Both images are merged (**b**, **e**) and, if they do not completely overlap (**b**, example of large misalignment), the mirrors that bring the lasers into the microscope must be adjusted until both images match as perfectly as possible (**e**, good alignment). Scale bar in **a**–**f**, 2 μm. (**g**–**i**) Alignment can be further confirmed by imaging a cluster of fluorescent beads (**g**) and using the uncaging laser to bleach one single bead (**h**; *yellow arrow*) and then, for instance, another one (**i**; *white arrow*). Scale bar in **g**–**i**, 1 μm. Images are pseudocolored for presentation purpose

5. Incubate the slices for some time (5–60 min, typically 20 min, but this must be tested empirically) to stabilize some parameters (temperature, metabolism, mechanical drifts due to overall changes of slice shape, etc.). These parameters might change during first minutes of imaging or recording but usually stabilize soon thereafter (*see* **Note 23**). Change the objective lens to the 60× to carry out the subsequent imaging and stimulation.

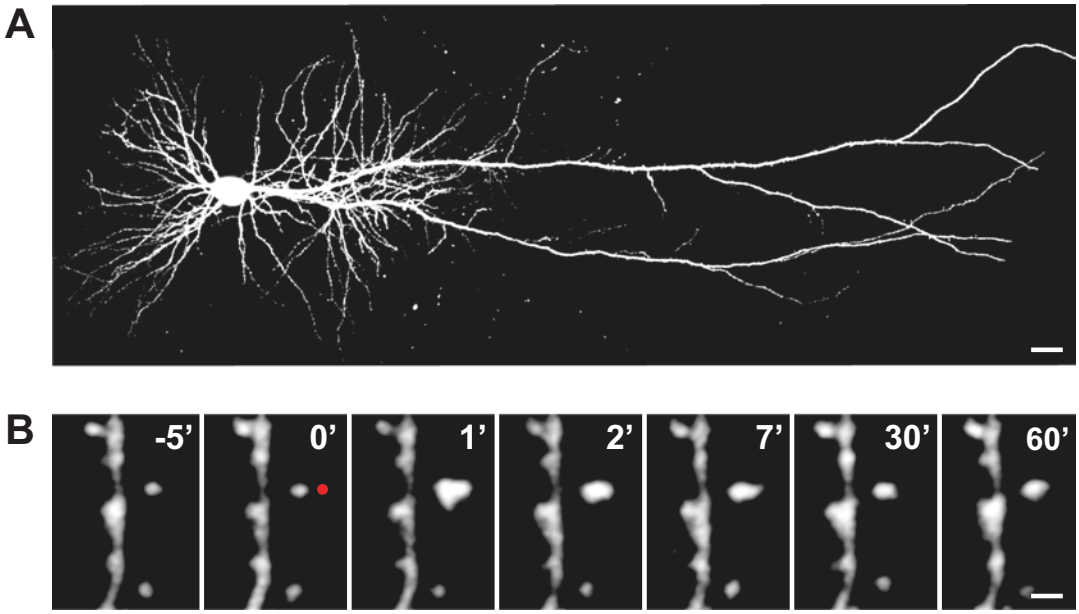

Fig. 2 Neuronal morphology and spine structural plasticity. (**a**) Example of a hippocampal CA1 pyramidal neuron from an organotypic slice culture, transfected with DsRed2. Scale bar, 10 μm. (**b**) Time-lapse 2-photon imaging of the persistent enlargement of a dendritic spine after induction of long-term potentiation by 2-photon glutamate uncaging. *Red dot*: position of the uncaging laser. Time stamp in minutes after stimulation. Scale bar, 1 μm

6. Choose a segment of a dendrite running parallel to the imaging plane, which in turn will be parallel to the bottom surface of the slice, since the slice is sitting flat on the chamber surface. This will increase the chance of the ultrathin sections being cut as parallel as possible to the dendrite and, therefore, will minimize the number of sections later required to reconstruct the whole dendrite with all the selected spines. It is also advisable to select a dendrite close to the upper slice surface to accelerate the cutting process. We choose segments of primary or secondary dendrites from the distal part of the main apical dendrite of CA1 pyramidal neurons, which we have found to have long (20–40 μm) and straight segments that run parallel to the imaging plane (*see* Figs. 2a and 3a).

Fig. 3 (continued) head enlargement of the three stimulated spines at 1 min (**c**), 1–7 min (**d**), and 1–30 min (**e**) after induction of LTP. *Red dot*: position of the uncaging laser. Scale bar, 1 μm. (**f**) Two-photon image of the same dendritic segment after fixation. Scale bar, 5 μm. (**g**) Landmarks created by 2-photon precipitation of DAB in straight fine lines. *Two lines* flanking the dendrite (labeled as "1") are initially drawn and *three semi-perpendicular lines* pointing to the stimulated spines (labeled as "2") are subsequently drawn. (**h**) Additional lines are created several microns above (labeled as "3"). (**i**) Piece of a fixed hippocampal slice visualized under bright field. *Arrowhead* points to the DAB landmarks. *SP* stratum pyramidale, *SR* stratum radiatum. *Asterisk* marks a wound made by the holder. Scale bar, 0.2 mm. (**j**) *Arrowhead* points to the same DAB landmarks (as in **h**) visualized under bright field at higher magnification. Scale bar, 25 μm. (**k–m**) Another example of the photomarking procedure on a different sample. (**k**) Two-photon live image of a dendritic segment. Three spines are also stimulated (*red dots*). (**l**) Two-photon image of the same dendrite after fixation and photomarking. Lines flanking the dendrite (labeled as "1") and semi-perpendicular lines (labeled as "2") pointing to the stimulated spines (*red dots*) are visible. (**m**) Same region under bright field where the flanking ("1") and the semi-perpendicular ("2") DAB landmarks are visible. Scale bar in **k**, 5 μm; in **l**, **m**, 10 μm

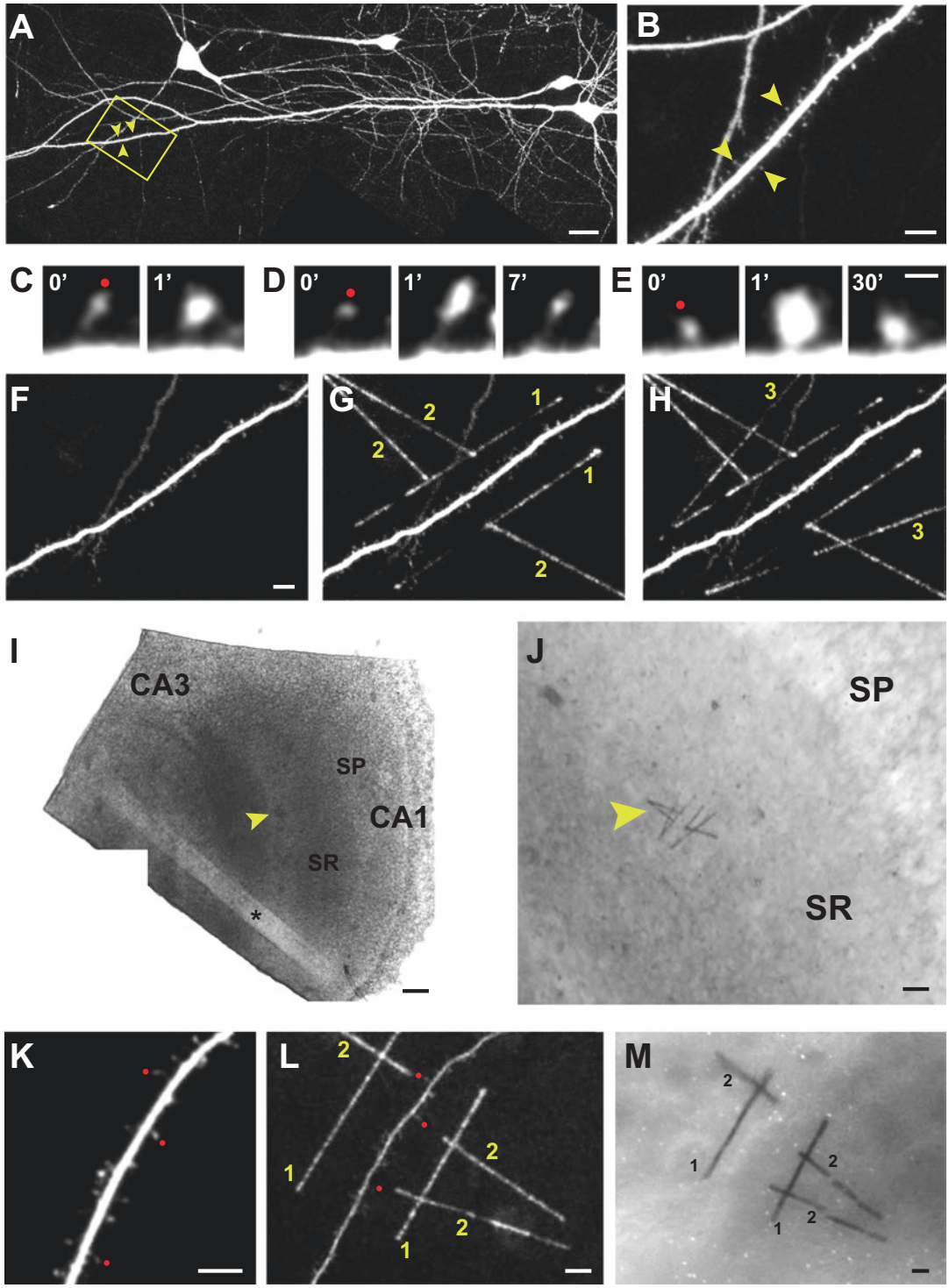

Fig. 3 Generation of landmarks by 2-photon photomarking. (**a**) Two-photon collage image of live hippocampal neurons in an organotypic slice culture. A straight and superficial segment of the dendrite of a pyramidal neuron is selected for the experiment. The *yellow square* highlights the region shown in (**b**). Scale bar, 25 μm. (**b**) Three spines (labeled with *arrowheads* in **a** and **b**) from the selected dendritic segment (*yellow square* in **a**) are chosen to be stimulated at different time points. Scale bar, 5 μm. (**c**–**e**) Two-photon time course live imaging of the spine

Choose one or several dendritic spines of medium-size diameter to stimulate (*see* **Note 24**). We typically choose small mushroom-type spines, with clear head and neck (no stubby, no filopodia, no big spines; *see* Figs. 2b and 3c–e). We target three spines in each experiment, separated at least by >10 μm, and stimulate them sequentially, in intervals not shorter than 10 min between them (*see* Fig. 3b–e; *see* **Note 25**).

7. Imaging parameters must be optimized depending on the type of experiment, type of sample, type of fluorescent gene expressed, etc. We found the following to be good parameters for time-course imaging of EGFP-transfected neurons: Use the 60× objective lens and a digital zoom of around 10× to acquire a *Z*-series of 512 × 512 pixel *XY*-scanned images taken every 0.5–1 μm in depth (*Z*). Usually, three to four images are enough to reconstruct one spine in the *Z*-dimension but it is advisable to take 7–15 images if you also want to acquire neighboring spines. Also, slow drift in the *X*, *Y*, or *Z* dimension is common during long experiments and so it might be necessary to adjust the *XYZ* positions regularly. Time-course imaging requires good signal stability. Photobleaching and phototoxicity are common problems, especially with fusion proteins of slower dynamics than free EGFP. Therefore, it is important to reduce laser intensity, exposure, dwell time, number of images taken, etc., and to enhance sensitivity by increasing PMT voltage as much as possible to minimize photodamage, even though image quality is reduced.
8. Stimulation of single spines has been performed by several methods, of which 2-photon-induced uncaging of caged glutamate is the most selective and efficient [2, 3]. Several laboratories have reported induction of LTP in single spines [12, 64–68] and a few laboratories have reported induction of long-term depression (LTD) in single spines by glutamate uncaging [69–71]. We usually induce LTP using uncaging pulses of 1 ms of duration, repeated at 1 Hz for 1 min, in Mg-free ACSF containing MNI-glutamate, TTX, and picrotoxin (*see* Subheading 2.4). We first recirculate the Mg-free solution (without caged glutamate) while we place the slice into the imaging chamber, search for the neuron, and select the spines to be stimulated.
9. Once this is done, we prepare the Uncaging ACSF and switch the tubes to the reservoir containing this Uncaging ACSF, avoiding any dilution of the solution or calculating and taking into account the expected dilution of the caged glutamate. At this point, recirculate the same solution constantly by returning the outflow liquid to the tube containing the bubbling Uncaging solution. However, you can search for the neuron and spines using epifluorescence with the caged glutamate in

the solution with no evident unwanted uncaging, provided the use of the correct fluorescence filter (typical GFP filters block the UV range of absorption of caged glutamate), avoiding UV light, keeping ambient room light to a minimum, and/or using a red or UV-filtered ambient light (*see* **Note 26**). It is possible, therefore, to replace the samples and perform several sequential experiments using the same Uncaging ACSF.

10. Laser intensity and pulse duration have to be adjusted for each microscope and for any given concentration of caged glutamate (*see* **Note 27**). Aim the laser close to the tip of the spine but not on top. As 2-photon diffraction limit (NA 1.0 lens at 720 nm) is 0.62 μm Ø, it is best to shoot ~0.3 μm away from the spine tip (*see* red dot in Figs. 2b and 3c–e) to avoid direct artefactual excitation of the spine by the light. For 3–5 mM MNI-Glutamate we typically use 3–6 mW of laser power, measured after the objective lens (30–60 mW measured at the back aperture, before the lens). At these intensities, we obtain averaged excitatory postsynaptic currents (EPSC) of ~5 pA with 1 ms pulses (*see* **Note 28**). Take baseline images for the first spine (usually every 1–5 min; *see* **Note 29**), confirm that the size of the spine is stable (*see* **Note 30**), induce LTP as described, and take subsequent time-course images, confirming that the spine has been persistently enlarged.
11. If stimulating several spines, move to the second spine, repeat the process, then to the third spine, etc. Record the position of each spine within the dendrite so that you can come back and image the second or third spine in an interleaved way. It is desirable to draw a schematic sketch of the neuron, the dendrite, and the position of each spine for later identification. If different spines have been stimulated sequentially at different time points, at the moment of fixation you will obtain spines potentiated for different periods (*see* Fig. 3c–e).

3.4 Fixation

1. The Fixative Solution must be prepared fresh each day and kept at r.t. After acquisition of the last image of the last stimulated spine, quickly turn on the red light (*see* **Note 26**), raise the objective lens, remove the plastic cover that prevents dehydration, pick up the membrane with tweezers, and dip it immediately in Fixative Solution (in a beaker or a 6-multiwell plate).
2. Shake the beaker manually or with a mechanical shaker for 5 min at r.t. to accelerate the penetration of the fixative into the tissue. Put it in a shaker at r.t. for 90 min and then at 4 °C for a minimum of 12 h, always shielded from light.

3.5 Two-Photon-Mediated DAB Photo-Marking

1. Rinse slices thoroughly in 0.1 M PB 4–5 times and transfer them back to the 2-photon microscope chamber. Since the photomarking step requires the use of toxic dirty compounds

(PFA/GA-fixed tissue and DAB), we recommend using a different dedicated set of objective lenses, chambers, perfusion tubes, and tweezers exclusively for these steps. Use the asymmetric shape or the dented mark to place the membrane in the appropriate upright position.

2. Prepare the ice-cold DAB solution just before its use (*see* Subheading 2.4). Perfuse continuously with the solution, aerated with pure oxygen (*see* **Note 31**). Use the epifluorescence light to localize the stimulated spines (*see* **Note 32**; *see* Fig. 3f).
3. Photoprecipitate the DAB creating fiducial patterns (*see* below) by line-scanning the tissue with the 720 nm 2-photon laser using line-scan mode. Other wavelengths have been also used (e.g., 910 nm; [11]). We could not achieve photoprecipitation using a 1-photon 488 nm confocal laser (*see* **Note 33**). As the DAB precipitate is also fluorescent, you should be able to see both the dendrite and the lines in the same image at 910 nm excitation (*see* Fig. 3g, h, l). The laser intensity and dwell time have to be adjusted for each sample, as photomarking efficacy can vary between experiments. Typically, laser power can be 15–25 mW as measured after the objective lens (150 and 250 mW measured at the back aperture, before the objective lens), with laser irradiation times around 15–60 s. It is recommended to perform a quick test in the same sample, selecting a similar region at the same depth but several μm away from the selected dendrite, and to draw several lines at increasing laser intensities and/or dwell times to determine which intensity and time creates the best marks, with good autofluorescence signal (not too faint, not too thick). When carefully drawing the lines at the selected region using the chosen laser intensity, it might be necessary to overwrite the same line two to three times, if the first-pass result is too faint. Too much laser intensity or too many rounds of photoprecipitation might result in too much electrodense signal and/or burning and destroying the tissue.
4. Different linear patterns can be drawn for use as landmarks for later identification of the dendrite and spines. We usually draw two lines (e.g., of 40–80 μm long), parallel to the dendrite and as equidistant to the dendrite as possible (e.g., 15 μm away; *see* Fig. 3g, l). Measure (on the computer screen, approximately) the length of the line marks, the width, the distance of each one to the dendrite, etc. Save an image of these lines and the dendrite (*see* **Note 34**).
5. Next, draw several lines, perpendicular or semi-perpendicular to the dendrite, starting ~40–60 μm away from the dendrite, and pointing to the selected spine but without reaching it, i.e., finishing 8–15 μm away from the spine, to avoid photoprecipitating

on the top of the spine. If you have stimulated three spines, draw three lines in an asymmetric pattern (e.g., each one with a different angle with respect to the dendrite; *see* Fig. 3g, l), which will greatly facilitate their unequivocal identification. Save another image and/or measure the length, angle, etc., and write down how precise the line points to the spine by extrapolation (*see* **Note 35**). These pictures and values are important to later compare the pattern of electrodense marks in the EM images to find the dendrite and spines in the absence of fluorescent signal.

6. Finally, move the focal point several microns above the dendrite (e.g., 3–4 μm) and add two more lines, drawn again in any asymmetric pattern that will help its identification (*see* Fig. 3h). These extra upper lines will be very useful in the process of sample sectioning. All these crossing lines are just examples of landmark patterns that we successfully used in our experiments, but any other geometric patterns that the experimenter finds useful can be applied (e.g., squares or rectangles [11, 30]).

3.6 Sample Processing for EM

1. Rinse the membranes in 0.1 M PB several times at r.t.
2. Detach the selected slice from the membrane with a scalpel blade slowly and carefully, avoiding any damage to the sample. Importantly, mark the slice with an asymmetric cut in one or two corners to effectively differentiate top from bottom side of the section at later steps.
3. At this point it is convenient to process several slices at the same time in 4 ml amber vials. Use plastic pipettes to remove liquid and use glass hooks to transfer slices between vials. Transfer slices to Osmium Tetroxide/Potassium Ferrocyanide solution (see Subheading 2.5) and incubate for 30 min at 4 °C in a rotator (*see* **Note 36**).
4. Rinse the slices four times for 5 min in PB at 4 °C and transfer them to the Osmium Tetroxide Solution for an additional 30 min at 4 °C.
5. Transfer slices to new vials and start the dehydration process by rinsing them in alcohol solutions at incremental concentrations: rinse twice for 5 min in 30% ethanol. Repeat with 50% ethanol, then 70% ethanol, then 90% ethanol. Rinse once in 95% ethanol for 10 min and twice in 100% ethanol for 10 min.
6. Rinse twice in propylene oxide for 10 min.
7. Transfer the slices to a 1:1 mix of fresh Durcupan Epoxy Resin and propylene oxide, and incubate them in the rotator overnight at r.t.
8. Transfer the slices into fresh Durcupan and incubate for 4 more hours in the rotator at r.t.

9. Start the process of flat-embedding the samples between two sheets of poly-chloro-tri-fluoro-ethylene (PCTFE) film (e.g., ACLAR®) with new fresh Durcupan resin. Put the slices between the plastic films and use scale calibrating weights (20–50 g) to deliver pressure. Carefully eliminate all bubbles.
10. Heat the samples in an oven at 57 °C and cure them for 2 days.
11. Remove the PCTFE film attached to the bottom side of the slice (the one furthest from the photomarked region) and glue that side with epoxy glue onto cylinders made of Durcupan. These cylinders can be made using standard BEEM cylinder molds. The top side of the section (the one closer to the photomarked region) should be facing up. This orientation is important to minimize the amount of resin we will have to remove during coarse sectioning with the glass knife. More detailed protocols on sample processing for TEM imaging can be found in Bozzola and Rusell [72].

3.7 Cutting Ultrathin Serial Sections

1. Start by manually trimming the block to create a trapezoid with parallel borders using an oil free razor.
2. In order to have perfect parallel top and bottom sides with smooth slopping surfaces in the trapezoid, continue trimming in an ultramicrotome using the angled sides of a 45° Cryotrim diamond knife at 2–3° attack angle [73]. Our block faces are typically ~ 400–500 μm wide and ~ 500–700 μm long to cover as much of the targeted area as possible.
3. Cut semi-thin sections (500 nm) with a glass knife and stain them with Toluidine Blue Solution until the two upper lines of DAB marks appear (*see* Fig. 4a).
4. At this point, initiate the ultrathin serial sectioning of the sample at 60–70 nm thickness. To minimize compression use a 35° diamond knife and try to section using attack angles of ~4°. Sections should come in a straight ribbon but, in our experience, anything longer than 15–20 sections will be hard to handle in the diamond knife boat. The best sectioning can be obtained by programming the optimal cutting speed at 0.8–1 mm/s (*see* **Note 37**). Use an eye-lash to split the ribbon into groups of five to seven sections.
5. Mount sections onto Pioloform-coated slotted grids (*see* Subheading 2.6) using a high-angle insertion of the grid in the boat and with the grid-slot parallel to the ribbon. Gently pull the grid up to prevent excessive shearing force on the ribbon.
6. Dry the grids. We recommend using absorbent paper points instead of letting the grids air dry, as the sections could get dispersed. After the sections are completely dried, proceed with staining.

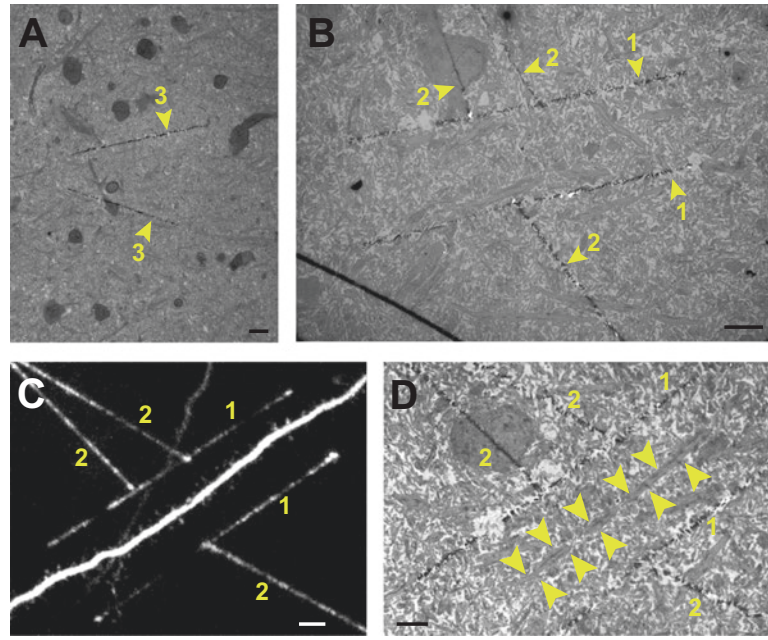

Fig. 4 Relocalization of landmarks in serial section TEM images. (**a**) Electron micrograph of the region shown in Fig. 3a–j where the two upper landmarks are visible (*arrowheads* labeled as "3"; same as those visible in Fig. 3h labeled as "3"). The appearance of these landmarks during cutting semi-thin sections announces the moment to start cutting ultrathin sections. Scale bar, 10 μm. (**b**) Electron micrograph of the same region, 3–4 μm deeper, where the next landmarks are visible, the ones parallel to the selected dendrite (*arrowheads* labeled as "1") and the ones semi-perpendicular to the dendrite pointing to the stimulated spines (*arrowheads* labeled as "2"). Scale bar, 10 μm. (**c**) Two-photon image of the selected region (also shown in Fig. 3g) where the fluorescence of the dendritic DsRed2 and the DAB landmarks is visible, to be compared to **d**. Scale bar, 10 μm. (**d**) Electron micrograph of the selected region, where the electrodense signal of the DAB landmarks is visible. The selected dendrite is delimited with *arrowheads*. Scale bar, 10 μm

7. For staining, put several drops of Uranyl Acetate Solution (*see* Subheading 2.5; *see* **Note 38**), one per grid, on top of a Petri dish covered with parafilm. Place the grid face down on the top of the drop so the sections are in direct contact with the solution. Stain for 1 min and then rinse by letting six to eight drops of dd water run over the tweezers (we found this method less perturbing for the sections than immersing the grids into beakers).
8. After completely drying the grids, repeat the same procedure with the Lead Citrate Solution, always minimizing the contact of this solution with CO_2 (*see* **Note 39**). Rinse the grids with CO_2-free dd water using the same method used with the uranyl

acetate solution. A more detailed protocol on serial sectioning for ssTEM can be found in Harris and colleagues [73] and Hayat [74].

3.8 Relocalization of the Dendrite in Serial Section Transmission EM

1. Load the grids on the sample holder and image them with the transmission electron microscope with a filament tension of 100 kV and at low magnification (3000×) [75].
2. Search for the electrodense DAB landmarks in all serial sections following an established order. Each grid and section must be sequentially numbered. Try to search the landmarks on the grids located in the middle of the sectioning sequence first. If the cutting and sectioning process has been successfully done in a parallel fashion with respect to the dendrite, very few sections should include most of the DAB lines (*see* Fig. 4b). If not totally parallel, the DAB lines will appear as small traces over many sections, which will make the relocalization more difficult, though not impossible. Once the landmarks are localized, start collecting mid-magnification images (5000–8000×) and draw an approximate map of these lines.
3. Localize the two lines that flank the dendrite in parallel and search for the dendrite that best matches the expected position of the selected dendrite, which should be situated at equal distance from these two flanking lines and at the same depth (*see* Fig. 4c, d). At this point, it is not necessary to completely identify the stimulated spines but should have a good guess as to which one is the selected dendrite by thoroughly comparing these mid-magnification images with the previous 2-photon images of the same region (*see* **Note 40**).
4. Once confident of the identification of the dendrite, search for the semi-perpendicular lines pointing to the spines and use them to find the regions where the stimulated spines are expected to be situated. Start collecting images of those regions at high magnification (23,000×) in all the sections. Collect them in a way that builds a virtual cube containing the stimulated spines and the dendrite (i.e., a 3D stack volume comprising several sections above and below the expected position of the spines). This cube will also include several unstimulated neighboring spines. The best method to prevent missing spots is a meander sampling, i.e., taking pictures uniformly spaced in a grid pattern. For that purpose, it is helpful to use the EM stage position display and to set a consecutive numbering scheme (number of section, row, and image within the row) to help during the photo-stitching step.

3.9 Morphometric 3D Reconstruction and Analysis of Spines

1. Use the automated stitching function of an image processing program (e.g., Photoshop) to stitch together all the regions imaged from the same section to create a collage image in 2D.

2. Combine these collage images from different sections to create the 3D stack volume of the whole selected dendrite containing all the stimulated spines and several neighboring spines. If stimulated spines are too far away from each other, build separated 3D stack volumes for each of the selected regions.
3. To perform image alignment, reconstruction, and analysis, we recommend the Reconstruct software package (http://synapses.clm.utexas.edu/synapseweb/software-0) [76]. Perform an initial rough reconstruction to see the overall shape of the dendrite and compare it to the 2-photon fluorescent images. If the dendrite has any feature that permits its identification, such as a turn, a point of branching, a group of spines, a non-spiny region, etc, try to match this feature between the fluorescence and the reconstructed EM images. At this point you must be quite confident that you have correctly identified the dendrite of interest.
4. Proceed to reconstruct all the spines that reside in the regions that have been imaged and stitched, in a blind fashion, i.e., before determining whether they are stimulated or unstimulated (*see* Fig. 5b–d). We focused and reconstructed the same type of stimulated or control spines, i.e., thin or mushroom spines, but not stubby or filopodia (*see* Fig. 5d–g). Reconstructions should include the dendritic shaft, as well as any other features of interest, such as the PSD, endoplasmic reticulum, presynaptic boutons, etc. (*see* Fig. 5a, e–g).
5. Localize the DAB landmarks that point to the stimulated spines and identify those spines from the set of reconstructed spines (*see* Fig. 5c, d). It is convenient to measure the relative distances of these spines to the neighboring spines or other features of the dendritic shaft (turns, branching points, etc.) and compare them to the fluorescence images. It may turn out to be impossible to unequivocally identify the stimulated spines, because they have disappeared, or because it is hard to decide between two spines. In this case, discard this spine from the study because the power of this experimental technique relies on the possibility to unequivocally identify the spines under both 2-photon and electron microscopy, and compare stimulated versus unstimulated synapses with certainty, thus avoiding any reliance on mixed population statistics.
6. Use the Reconstruct software package [76] to measure all desired parameters in each section (length and area of spine head, spine neck, PSD, endoplasmic reticulum, mitochondria, presynaptic bouton, vesicles, etc.) and use them to calculate the desired final 3D volumes and areas (*see* Fig. 5e–g).

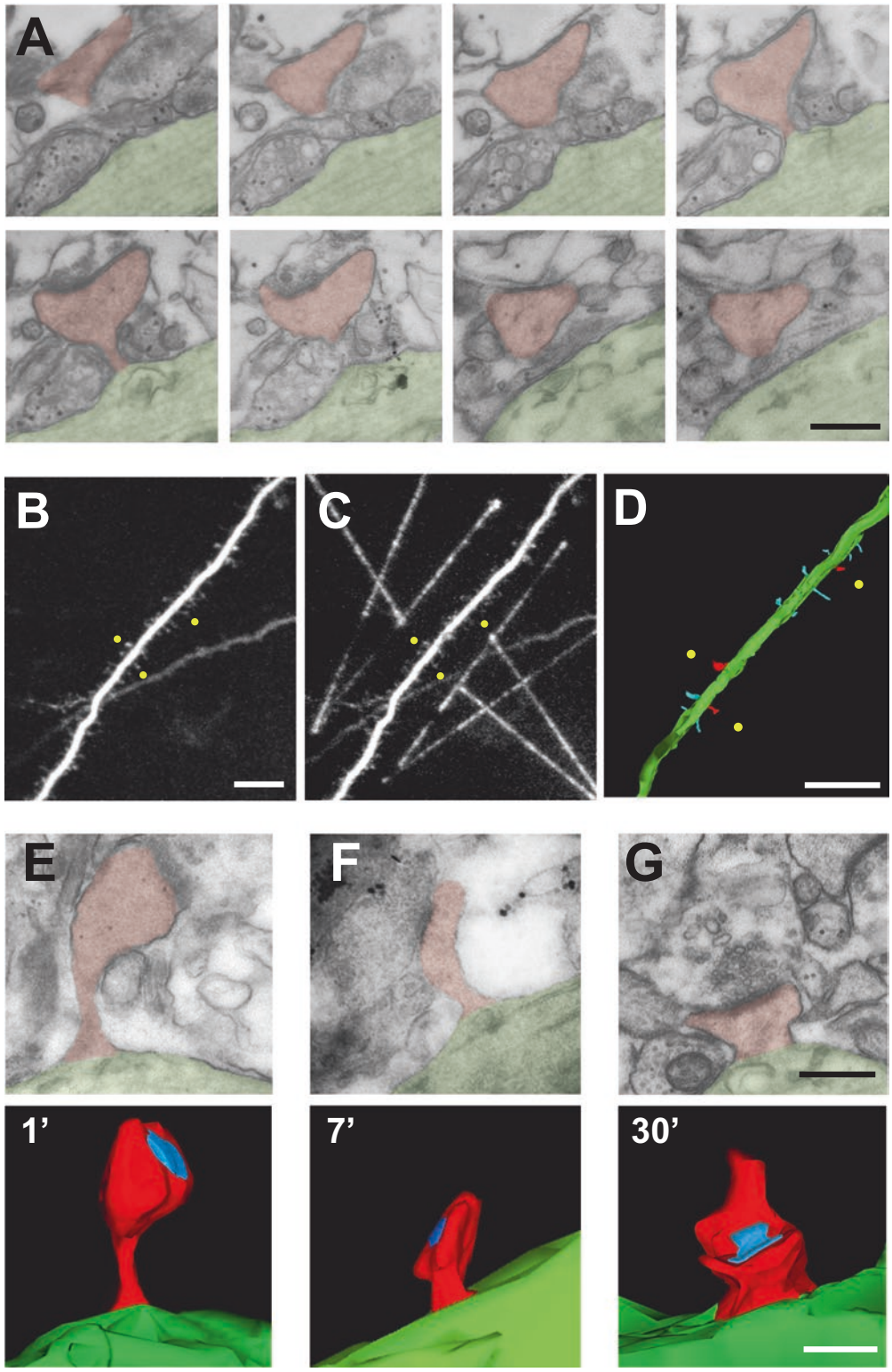

Fig. 5 Reconstruction of the dendrite and stimulated spines in 3D. (**a**) Example of a dendritic spine reconstructed from serial section EM images. *Red*, spine head and neck area; *green*, dendrite area. Scale bar, 0.5 μm. (**b**–**d**) Reconstruction of the whole selected dendrite. (**b**) Two-photon image of the dendrite after fixation (same as Fig. 3f, for comparison with **d**). Stimulated spines are highlighted with *yellow dots*.

4 Notes

1. Viable slices can be prepared from pups as young as P1, but they are less mature in terms of morphological, functional, and circuitry development. Pups older than P8 yield more mature slices but are more fragile in terms of survival and less easy to transfect.
2. Red fluorescent proteins are better excited with longer 2-photon excitation wavelengths (900–1100 nm), which offers lower resolution but deeper tissue penetration. DsRed2 is bright, stable, and well-expressed, but its tetrameric structure makes it inappropriate for fusion proteins, as there is the risk of forming aggregates. GFP can also form weak dimers. For these reasons, mCherry and monomeric-GFP are better options in the case of fusion proteins.
3. It is possible to use a single 2-photon laser for sequential, not simultaneous, imaging and uncaging, by changing the wavelength back and forth between 910 nm (imaging) and 720 nm (uncaging). Another option is to use one 720 nm 2-photon laser for uncaging and a 488 nm 1-photon laser for simultaneous imaging. Many other experimental approaches are possible with two combined 2-photon lasers (or changing wavelengths of one 2-photon laser) and one (or more) 1-photon lasers. It is possible then to perform different compatible photochemical reactions while imaging at the same time. For example, you can use: (1) a 820 nm laser to photoactivate photoswitchable proteins in one spine (e.g., to measure turnover of PAGFP-labeled proteins), (2) a 720 nm laser to photostimulate the same spine by glutamate uncaging (to trigger plastic changes) while (3) using a 488 nm laser to simultaneously image the same spine (e.g., *see* Fig. 4 in Bosch et al. [12]) In the case of 1-photon confocal imaging, always choose a dendrite close to the slice surface.
4. Avoid mechanical vibrations (and electrical noise if combining with electrophysiological recordings). Avoid fluctuations of the level of liquid in the chamber, especially the extreme cases of drying out the chamber or spilling liquid out of the chamber (which can damage the lower optical components of the microscope). A constant level in the chamber can be achieved by

Fig. 5 (continued) Scale bar, 10 μm (**c**) Two-photon image of the dendrite after photomarking (same as Fig. 3h, for comparison with **d**). (**d**) Reconstruction in 3D of the dendrite (*green*), stimulated spines (*red*), and unstimulated spines (*blue*). Scale bar, 5 μm. (**e**–**g**) Reconstruction of the stimulated spines shown in Fig. 3**c**–**e**. (**e**) EM image and 3D reconstruction of a spine stimulated 1 min before fixation. *Green*, dendritic shaft; *Red*, spine; *Blue*, PSD. (**f**) Spine stimulated 7 min before fixation. (**g**) Spine stimulated 30 min before fixation. Scale bar, 0.5 μm. Adapted from [12]

forcing the outflow rate to be faster than the inflow rate. For example, use the pump to remove the liquid from the chamber and elevate it to the reservoir and let gravity to return it to the chamber; or use a higher flow speed for outflow than inflow; or if using the same pump and thus the same speed, use a softer plastic tube for the outflow and a harder tube for the inflow, thus facilitating the pump to more easily displace the liquid through the outflow tube. It is important to replace the tubes often (monthly) with new ones. Check that the pump and tubes are correctly installed and in good condition every day, as this is a common source of problems. Avoid precipitation of salt from ACSF, especially on objective lenses. Recirculate dd water at the end of the day to thoroughly wash all tubes, chambers, and objectives lenses that have been in contact with ACSF or any solution.

5. Measure osmolality every 30 min and determine when it increases significantly. In our setup, we could perform 1.5–2 h long experiments without any major increase in osmolality. For longer experiments, we usually kept a constant osmolality by adding ~100–200 μl of dd water every 1 h, approximately.
6. We recommend testing several batches of horse serum, selecting the one that gives better survival, slice quality, synaptic plasticity, etc., and purchasing large quantities of that batch. Adjust medium pH to 7.3 by adding consecutive small amounts of 10 M NaOH. Typically, up to ~100–200 μl of 10 M NaOH is needed to increase pH from around ~6.9 up to 7.3. Adjust osmolality by adding dd water, even though concentration of all components might be reduced below the suggested values. Osmolality is more important than final concentration of these components. Typically, consecutive small amounts of dd water, up to a final volume of ~90–120 ml, are added to reach 300–320 mOsm/L.
7. Prepare a 10× partial stock solution: 25 mM KCl, 260 mM $NaHCO_3$, 10 mM NaH_2PO_4, and 110 mM glucose. Filter and keep at 4 °C. Before dissection, dilute it 10 times with dd water, and add 238 mM sucrose, 1 mM $CaCl_2$, and 5 mM $MgCl_2$.
8. Prepare a Ca-free Mg-free 10× ACSF stock solution in dd water with 1190 mM NaCl, 25 mM KCl, 260 mM $NaHCO_3$, 10 mM NaH_2PO_4, and 110 mM glucose. Filter and keep at 4 °C. On the day of the experiment, dilute 10 times with dd water, bubble with carbogen for 20 min, and add 3 mM $CaCl_2$. Just before its use, add 1 μM TTX and 50 μM picrotoxin. TTX is added to block the spontaneous neuronal activity that would otherwise increase too much due to the lack of Mg. Picrotoxin is optional, in case you also want to block GABAergic transmission.

9. First prepare 4% EM-grade PFA solution (w/v) in 0.2 M PB. Dissolve it by heating at 55 °C and slightly raising the pH with a few drops of 1 M NaOH. Filter and let cool down to room temperature (r.t.). Mix this solution with a pre-made stock solution of 10% GA and dd water with these proportions (4% PFA:10% GA:dd water; 10:4:6). Adjust pH to 7.4.
10. Because of their viscosity, add components A and B by directly pouring them in a beaker thoroughly cleaned with acetone that will only be used for this purpose. For the less viscous components C and D, use plastic disposable pipettes. Use a scale to measure the quantities and mix thoroughly with a perfectly cleaned glass rod. Transfer to a vacuum bell jar and proceed to degasify the mix. When applying vacuum one should see major bubbles that disappear after a few minutes and a small film of foam in the interphase between Durcupan and air. Use 10 ml syringes to take the resin, avoiding the foamy interphase. Wrap the syringes in parafilm and store in the fridge in a dark compartment to prevent further polymerization. The remaining foamy resin can be used to prepare the 1:1 mix of propylene oxide and resin.
11. Slices are usually cultured on top of semi-permeable (0.4 μm pore) membrane inserts (Millipore) in 6-well plates. Some money can be saved by culturing the slices on top of already-cut pieces of Biopore Millicell hydrophilic polytetrafluoroethylene membrane placed onto these membrane inserts. Membrane inserts can then be resterilized and recycled [55]. Another economical alternative is to use Omnipore (Millipore) membranes [54].
12. It is convenient to culture four slices in each well, placed close to each other in the center of the well (to facilitate the transfection by gene gun), but without touching each other. Culturing more than four slices per well would consume medium nutrients too fast. Less than four slices is possible but then more wells and medium will be required, making the experiment more expensive.
13. Assess the quality of slices by DIV 5 by confirming that: (1) microglia has cleared and the slice becomes transparent, with no dark spots; (2) hippocampal subregions (CA1, CA3, Dentate Gyrus) are clearly visible, and (3) CA3 thickness is comparable to CA1 thickness (CA3 large neurons are more vulnerable to damage).
14. Dead surface neurons will disappear over the first days of culturing and glial response will decrease after that. Transfection of slices too early may result in many unwanted transfected cells: dying neurons or glial cells. On the other hand, efficiency of transfection decreases with time: one will need to prepare and transfect many more slices to have good chances of finding the appropriate fluorescent neuron.

15. If the neuron is whole-cell patched, long-term experiments might be precluded because of the dilution of cellular components into the pipette solution. Some extra experimental conditions might be necessary. For instance, maintenance of spine enlargement after LTP induction during whole-cell patch clamp requires adding 5 μM actin monomers to the pipette [77].
16. Empirically determine how long it takes for the system to reach stable conditions (temperature, etc.) by measuring laser power at final point (chamber) and by checking laser alignment. It can vary from a few minutes to a few hours.
17. This checking point is also useful to confirm the lasers are well aligned in the *Z* dimension. Try to change the position of the objective lens by focusing on several points along the *Z* dimension and confirm that maximum bleaching is achieved when the bead is well focused. If maximum bleaching is shifted and occurs at another *Z* position, you can fix that by a major realignment all optical components, or you can introduce the same *Z* shift when uncaging the dendritic spine.
18. It is possible to prepare the Uncaging ACSF at this point and recirculate it in the system before placing the sample (*see* also **Note 26**). We recommend, however, keeping the working Mg-free ACSF, place the sample, and replace the solution later with Uncaging ACSF.
19. It can sometimes be useful to check the location of fluorescent cells with an epifluorescence microscope before cutting out the membrane from the culture dish, in order to know which well and which slice might contain well-transfected neurons. Alternatively, one can prepare a high number of wells with enough slices. Just cut out a piece of membrane containing the four slices, put it in the chamber and look for the appropriate neuron. By chance, after checking several slices, it is usually possible to find one slice with an appropriate neuron in the appropriate region expressing the appropriate level of fluorescence signals.
20. Minimize the time that the slices are out of the incubator or out of the imaging chamber. Thus, it is worthwhile having a small incubator next to the microscope in which to store the plates on the imaging day, also to avoid disturbing the remaining cultures in the main incubator.
21. Take notes of the shape of the membrane piece, the position of the slice and the position and orientation of the neuron. It will be necessary to know later where to start looking for the landmarks.
22. Slice holders can be purchased or custom-made. Bend a 1 mm Ø platinum wire and make a ~10–14 mm Ø semicircle or U-shape. Cut the vertical strings of a nylon mesh (e.g., stockings) and keep

the horizontal ones. Glue the nylon strings on the two sides of the metal semicircle so that ~5 strings cross it, and cut the ends.

23. Some metabolic parameters, such as neuronal protein synthesis rate, might need >3 h of preincubation at 32 °C [78, 79] to reach a true stable state.
24. If you choose a secondary dendrite or the distal part of the main dendrite, the diameter of the dendrite will be smaller and the spine will be easier to image.
25. This way, you can have three stimulated spines in the same sample and thus reduce the number of samples to process by the factor of 3. However, if spines are too close in space or stimulated too close in time, you may induce metaplastic interactions [65].
26. Keep the caged glutamate under dark conditions. Prepare the Uncaging ACSF under the minimum illumination possible. It is useful to use a red light bulb or LED to illuminate the room or inside the microscope dark box, when needed, and to keep the chamber and the circulating uncaging solution covered all the times (with black curtains or yellow cellophane or UV filters) to protect it from ambient light, computer monitors, etc. If neurons start deteriorating just after the addition of caged glutamate, it could mean the batch is contaminated with free glutamate. This rarely happens and it can be detected by standard amino acid analysis.
27. We recommend performing pilot experiments where simultaneous intracellular recordings are made together with 2-photon imaging. In these pilot experiments one wants to adjust the caged glutamate concentration, laser intensity, and pulse duration to obtain an averaged EPSC response within a range similar to mini EPSCs [12, 36, 45, 80]. Once this is firmly established, subsequent imaging experiments can be performed without simultaneous recordings, which are highly time-consuming and preclude long-term experiments.
28. The efficiency of uncaging depends on the depth in the tissue and on the type of neuron. Therefore, this laser intensity value can be further adjusted for each neuron and for each depth by selecting a test spine, located in the same neuron and at a similar depth but in another dendrite or away from the selected region where the experimental spines will be stimulated, and inducing LTP to obtain a peak increase in spine volume of around 200–300% in the first 1–2 min and a subsequent sustained increase in spine volume of 50–100% for at least 10 min (*see* Figs. 2b and 3c–e) [12].
29. Neurons transfected with EGFP, DsRed2, or any free soluble fluorescent protein are less susceptible to bleaching, and so one can take images more often than when using slow-dynamics

synaptic fusion proteins as markers (e.g., EGFP-PSD-95). Typically, one can take an image every 1–5 min if the total experiment lasts for 30–60 min, or every 10–30 min if the experiment lasts for hours.

30. A variation of 10–20 % of spine head volume between consecutive images is typical in these experiments using Mg-free solutions, possibly due to the endogenous plasticity of spines, and to a lesser extent, also due to technical variations of the microscopic imaging.
31. Do not bubble with carbogen, as the CO_2 will decrease pH and will prevent DAB photoprecipitation. Do not expose fixed slices to hostile conditions that could erase the fluorescence signal, such as drastic changes in pH or strong photobleaching illumination.
32. A decrease in fluorescence signal is expected after the fixation step. A small degree of morphological distortion is also expected. Dendrites might look a little bit wavy and some blebbing may appear. Spine morphology should look as it was in the last live image. This distortion, if it happens, should not preclude a valid comparison between stimulated and control spines, since it should homogeneously affect all of them. However, if the neuron looks unhealthy (too much blebbling, dendrites that are too curvy) and/or the spines look too distorted or disappear, it means the fixation step has not been quick or strong enough. We recommend discarding such samples.
33. This photoprecipitation can be done in any region of the tissue and is probably achieved by the light-dependent generation of free radicals from endogenous intracellular oxidizable molecules. Photoprecipitation seems to occur more efficiently in regions that resemble the cell nuclei (*see* Fig. 3l).
34. Do not take many images and do not use high intensity of imaging laser, as bleaching is now a more significant problem because the sample and the fluorescent proteins are now fixed.
35. It is rather difficult to draw the lines pointing exactly to the spine. And it is actually not totally necessary. So, if the line points not exactly to the spine but *X*μm away, we will just have to search for the spine –*X*μm away from the line on the EM images.
36. Osmium tetroxide solutions are highly susceptible to contaminants and easily reduced by light, so even if using amber vials, cover them with aluminum foil. Any solution that develops black clouding or deposits should be discarded and a fresh solution should be made.
37. It is important to prevent vibrations and air drafts that may interfere with the sectioning process by having the ultramicrotome setup in an enclosed area and minimizing movement around it. It is also important to minimize the levels of static by keeping a humid atmosphere with a humidifier.

38. Use a syringe covered with aluminum foil attached to a 0.22 μm disc filter to drop the Uranyl Acetate Solution. Always discard the first drop.
39. To obtain a CO_2-free environment, wrap the rim of the petri dish with kimwipes soaked in 1 N NaOH solution and add some NaOH pellets. It is also critical to reduce breathing when handling the grids and wear a surgical mask while staining to prevent exposure of the samples to exhaled CO_2.
40. While taking the EM images, it is convenient to simultaneously look at printed copies of the 2-photon fluorescence images of the selected region (or display them on a monitor) and constantly compare the pattern of landmarks to confirm you are in the correct region and correct depth.

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