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Correlative two-photon and light sheet microscopy

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ABSTRACT

Information processing inside the central nervous system takes place on multiple scales in both space and time. A single imaging technique can reveal only a small part of this complex machinery. To obtain a more comprehensive view of brain functionality, complementary approaches should be combined into a correlative framework. Here, we describe a method to integrate data from *in vivo* two-photon fluorescence imaging and *ex vivo* light sheet microscopy, taking advantage of blood vessels as reference chart. We show how the apical dendritic arbor of a single cortical pyramidal neuron imaged in living thy1-GFP-M mice can be found in the large-scale brain reconstruction obtained with light sheet microscopy. Starting from the apical portion, the whole pyramidal neuron can then be segmented. The correlative approach presented here allows contextualizing within a three-dimensional anatomic framework the neurons whose dynamics have been observed with high detail *in vivo*.

1. Introduction

One of the unique features of the brain is that its activity cannot be framed in a single spatio-temporal scale, but rather spans many orders of magnitude both in space and time. Information storage in neural circuits implies a continuous reshaping of the wiring through formation and elimination of μm-sized synaptic contacts, over a time scale of hours and days. Two-photon fluorescence microscopy (TPFM) allows characterizing synaptic turnover through time-lapse imaging of fluorescently labeled varicosities and spines (i.e. pre- and post-synaptic portions) *in vivo* [1–3]. Information processing, on the other hand, is a much faster process (a single action potential can last less than a thousandth of a second) and may involve the communication between neurons several mm apart.

Since both synaptic turnover and signal propagation is highly dependent on the neuronal type [4–6], a reliable neuroanatomical classification of the neuron that has been previously observed *in vivo* could be helpful. This contextualization is often precluded to TPFM, because the penetration depth of TPFM is usually limited to a few hundred microns [1,7,8]. Therefore, neuronal classification is made *a posteriori*, based on the morphological features of axons and dendrites [6,9].

A more reliable reconstruction of the neuron previously observed *in vivo* and of the surrounding network can instead be obtained by combining TPFM with other *ex vivo* imaging techniques that can explore large volumes. Large-scale neuroanatomical data with cellular resolution are provided by a number of techniques including KESM [10], MOST [11], fMOST [12], STP [13], ultramicroscopy [14] and confocal light sheet microscopy (CLSM) [15]. Notably, methods based on light sheet illumination (like ultramicroscopy and CLSM) are currently the only ones in which the sample is preserved without slicing, allowing multiple imaging rounds and therefore providing a greater flexibility. Anyhow, all the above listed approaches are limited to fixed samples, making it impossible to get any information about temporal evolution. These techniques thus produce data which are limited in time but not in space; conversely, TPFM images are limited in space but not in time.

Integration of complementary information from the same sample can be done by correlative strategies [16], like array tomography [17] or correlative light-electron microscopy [18]. For instance, the combination of electron and two-photon microscopy allowed analyzing the ultrastructure of neurons previously observed *in vivo* [19–21]. Generally speaking, the mating between light and electron microscopy allows bridging the gap between the micro- and the nanoscale [22].

To our knowledge, no correlative approach has tried to move from the microscale (accessible with TPFM) towards the mm-scale. In this article, we describe how to combine *in vivo* TPFM with *ex*

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vivo CLSM, in order to contextualize high-resolution time-lapse images of selected neurites in a wider anatomical framework. We describe in detail the procedure to perform both imaging techniques, and to locate the same neuron within the whole brain by using blood vessels as reference (see Fig 1).

2. Materials and methods

2.1. Open skull technique

A craniotomy was performed on ~12 months old thy1-GFP-M mice [23] in correspondence of the somato-sensory cortex. The mice were deeply anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (9 mg/kg). A small dose of dexamethasone (0.04 ml at 2 mg/ml) was administrated to minimize swelling at the site of surgery. A circular portion of the skull (5 mm of diameter) above the somato-sensory cortex was removed and the exposed region was then covered with a coverglass and sealed with dental cement. To minimize the inflammatory phenomena that may take place after the surgery, the mice were treated daily with carprofen (5 mg/kg). The experimental protocols were designed in accordance with the rules of the Italian Minister of Health.

2.2. In vivo two-photon fluorescence microscopy

A mode-locked Ti:Sapphire laser (Chameleon, Coherent; 120 fs width pulses, 90 MHz repetition rate) was coupled into a custom-made scanning system based on a pair of galvanometric mirrors (VM500+, Cambridge Technology). The laser was focused onto the specimen by a physiology objective (XLUM 20, NA 0.95, WD 2 mm, Olympus). A closed-loop piezoelectric stage (P-721, Physik Instrumente) was used for axial displacements of the objective. The fluorescence signal was collected by a photomultiplier module (H7710-13, Hamamatsu Photonics). 3D stacks (2 μm z-axis step) were acquired from the pia mater down to a depth of around 100 μm, setting a field of view of $200 \times 200\ \mu m^2$ at 512×512 pixel^2 with a pixel dwell time of 5 μs. During imaging sessions the mice were lightly anesthetized for a period of 90 min and placed under the microscope (Fig. 1a). The microscope allowed also wide-field reflectance images to be collected with a CCD camera.

2.3. Tissue preparation for light sheet microscopy

Mice were deeply anesthetized with intraperitoneal injection of ketamine (90 mg/kg) and xylazine (9 mg/kg). Then, they were transcardially perfused with 20 ml of 0.01 M phosphate buffered saline (PBS) solution, followed by 100 ml of paraformaldehyde (PFA) 4% in 0.01 M PBS. The pH of both solutions was preliminarily adjusted to 7.6. Brains were extracted from the skull and post-fixed overnight in PFA 4% at 4 °C. Samples were then rinsed three times in 0.01 M PBS, 30 min each. Cerebellum, olfactory bulbs and medulla were removed and the brains were cut in two halves (Fig. 1b). The half containing the region observed with TPFM was dehydrated and cleared following the protocol described by Becker et al. [24]. Briefly, samples were dehydrated in graded tetrahydrofuran/water solution series (50%, 70%, 80%, 90%, 96%, 100% 1 h each, 100% overnight), and cleared with dibenzylether (DBE) 100% (Fig. 1c). DBE was changed three times (2 h each) before imaging. Both tetrahydrofuran and DBE were preliminarily filtered with aluminum oxide to remove peroxides [24].

2.4. Confocal light sheet microscopy

Specimens were imaged using a custom-made confocal light sheet microscope (CLSM) described in [15]. Briefly, in CLSM the light sheet is generated using a laser beam scanned by a galvanometric mirror (VM2500+, Cambridge Technology); a de-scanning system in the detection path creates a fixed image of the excitation scanning line in a secondary image plane, where a linear spatial filter (slit, p/n 35290NR, JML Optical) is positioned. A third scanning system reproduces a wide-field-like image on the chip of an EM-CCD camera (Cascade II: 512, Photometrics). The laser light was provided by a diode laser (Excelsior 488, Spectra Physics), $\lambda = 488$ nm. An acousto-optic tunable filter (AOTFnC-400.650-TN, AA Opto-Electronic) was used to regulate laser power. The sample was mounted on a tipped plate and placed in custom-made chamber which allows brain tissue being immersed in clearing solution during imaging. A motorized stage allowed 3D motion of the sample, as well as rotation around the vertical axis (three M-122.2DD and one M-116.DG, Physik Instrumente). An achromatic doublet (CVI Melles Griot, focal length 50 mm) was used for illumination, while two different objective lenses were alternatively used for

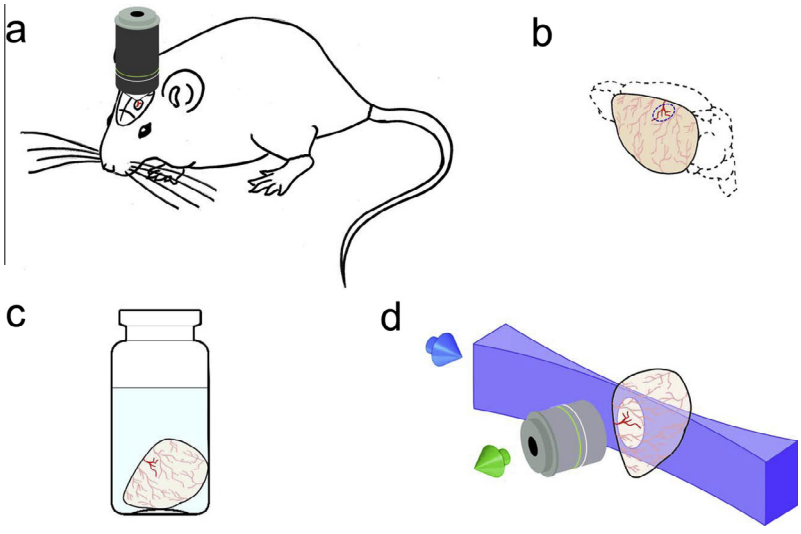

Fig. 1. Experimental pipeline. (a) Mouse brain is observed *in vivo* with TPFM. (b) After tissue fixation, the brain is dissected to keep only the portion of the brain containing the region observed *in vivo*. The blue dashed circle indicates the position of the cranial window, and the blood vessels above the region observed *in vivo* are depicted in bright red. (c and d) The brain portion of interest is subsequently dehydrated and cleared, and finally imaged with CLSM. The brain is oriented in order to reproduce the orientation of TPFM acquisitions. Excitation light in (d) is in blue. Blue and green arrows indicate the propagation directions of excitation and fluorescence light, respectively.