Multiphoton microscopy in brain imaging

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ABSTRACT

Brain imaging is becoming an important field in the frame of the neurophotonics in correlations with other medical ones in neuroscience studying functional and morphological aspects. In this presentation an overview on multi photon imaging of the brain will be presented, together with innovative aspects related to big area imaging and correlative microscopy approaches. Multiphoton imaging applications will be described together with methods to improve the penetration depth and obtain large area detection, or correlating functional aspects in vivo on single neuron with large area, even on whole brain, morphological aspects. Connecting super resolution features at the nanometer level with micro, meso and macroscopic architectures is in fact one of the challenging aspects to understand brain functioning.

Keywords: two-photon microscopy, in vivo imaging, neuroanatomy, neural functionality, correlative microscopy

1. INTRODUCTION

Brain activity involves many levels of organization that spans several orders of magnitude in space and time[1,2]. Neurons communicate between each other at the ms scale through nm-wide synapses. Synaptic contacts appear and disappear on a continuous turnover which takes place on the time scale of hours and days; this process is regulated by the activity of neurons on a circuit scale which extends on volumes greater than a mm^3[3]. On the other hand, neurotransmitters dynamics within a single synapse involves synaptic vesicles and other structures few tens of nanometers wide[4]. This enormously complex system can be investigated exclusively by combining different imaging technique. For example, two-photon fluorescence microscopy (TPFM) can be used to monitor the activity and the plasticity of selected neurons in living mice, with resolution of single spines or varicosities[2, 5].The nonlinear nature of two-photon fluorescence provides an absorption volume spatially confined to the focal region even in strongly scattering tissues, allowing deep high-resolution microscopy of live brain. In combination with fluorescent protein expression techniques, two-photon microscopy has become an indispensable tool to image cortical plasticity in living mice[6]. In parallel to its application in imaging, multiphoton absorption has also been used as a tool for the selective disruption of intracellular structures[7]. A similar approach has also been applied in vivo, where two-photon imaging and laser-induced lesions have been combined[8,9]. In parallel with imaging and manipulation technique, non-linear microscopy offers the opportunity to optically record electrical activity in intact neuronal networks. A central question in neuroscience is how simple processes in neurons can generate cognitive functions and form complex memories like those experienced by humans and animals. In principle, if one were able to record from all the neurons in a network involved in a given behavior, it would be possible to reconstruct the related computations. Unfortunately, this is not possible with current techniques for several reasons. Generally, the more precise the method of neuronal recording is (e.g. patch-clamp), the more limited the number of simultaneously recorded neurons becomes. Conversely, global recordings (e.g. field recordings) collect activity from many neurons but lose information about the computation of single neurons. Current optical techniques for recording membrane potential (Vm) can potentially overcome these problems[10,11]. In order to record deep Vm activity in intact systems maintaining a high spatial resolution, nonlinear optical methods are needed[12,13]. By combining fast scanning at high speed by acousto-optic deflectors (AODs) and two-photon microscopy it is possible to optically record fast Vm events[14]. This system is capable of resolving APs occurring simultaneously in several neurons in a wide-field configuration, and with deep tissue penetration in living brain slices[15].

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However, the resolution of TPFM is not enough to achieve subcellular details at the nanometer scale. Correlative microscopy strategies conveys multiple levels of complementary information from the same sample in an integrated dimension. A fruitful example of correlative approach is the combination of scanning electron and two-photon microscopy, which allowed analyzing the ultrastructure of neurons after observing neuronal dynamics in vivo[16,17]. The mating between light and electron microscopy, by bridging the gap between the micro- and the nanoscale, attracted the interest of many neuroscientist. An interesting example of this fruitful combination has been provided by imaging with EM the ultrastructural details of a regenerated axonal branch previously laser dissected and imaged with TPFM[9].
TPFM though able to investigate the long-term dynamics of events that last over months, is limited in the space it has access to. Indeed, the penetration depth of TPFM is usually limited to the cortex[18], preventing the observation of deeper structures. It might be hard therefore to perform a neuroanatomical classification of the neuron imaged with TPFM from superficial anatomical details. On the other hand, large-scale neuroanatomical data can be obtained, with cellular resolution, by a number of techniques like ultramicroscopy[19] and confocal light sheet microscopy (CLSM)[20]. Notably, these methods based on light sheet illumination are currently the only in which the sample is preserved without slicing, allowing multiple imaging rounds and therefore a greater flexibility. Anyhow, these approaches can only be applied to fixed samples, making it impossible to get any information about the temporal evolution of brain structure.
Here we show that by combining *in vivo* TPF microscopy with ex vivo confocal light-sheet and electron microscopy we can place high-resolution time-lapse images of selected neurites in a wider or more detailed anatomical framework, respectively.

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2. RESULTS

2.1 TPFM to investigate brain activity in vivo with voltage sensitive dyes.

Random access multi-photon (RAMP) microscopy is a very versatile and promising method complementary to existing electrophysiological techniques to record action potentials (APs) in clusters of cortical neurons[15]. This novel optical method allows recording of multi-unit electrical activity in intact neuronal networks in brain slices. The bulk loading of di-4-AN(F)EPPTEA, combined with RAMP microscopy, provides a fast and noninvasive approach to measure APs in neuronal assemblies. Acute cerebellar slices were labeled by bulk loaded with the dye di-4-AN(F)EPPTEA. We were able to record spontaneous AP spikes from multiple Purkinje cells using two-photon excitation with a 1,064-nm mode-locked fiber laser (Fig. 1). This recording is achieved by rapidly positioning the laser excitation with an acousto-optic modulator to sample a patch of membrane from each cell in <100 μs; for recording from five cells as in Fig. 1B, multiplexing permits a temporal resolution of ~400 μs—sufficient to capture every spike. Fig. 1A shows spontaneous activity recorded in this manner over 800 ms from the five neighboring cells, showing that spiking is not temporally correlated. Simultaneous optical and electrical recording from PC_1 shows the high fidelity of the optical measurement. The expanded trace for PC_5 in Fig. 1A reveals the characteristic after hyperpolarization and shows the high temporal resolution of the random access microscope. The dye and the optical recording protocol do not produce any significant photodamage to the preparation.

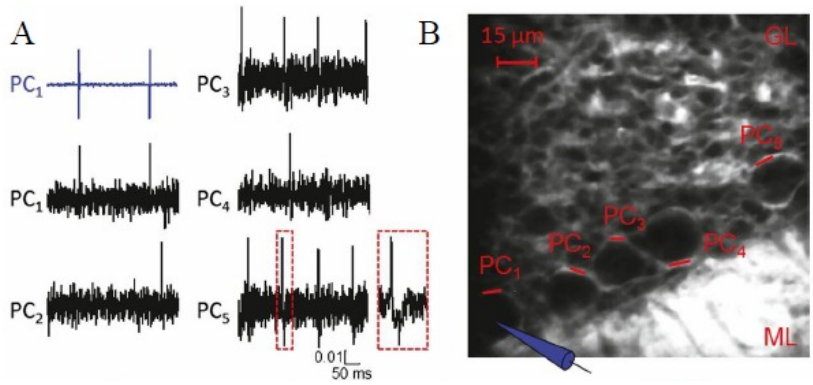

Figure 1. Real-time multicellular AP recording by random access multiphoton microscopy. (*A*) Multiplexed optical recording of spontaneous activities from the five PCs (black traces) with a temporal resolution ~400 µs. PC1 electrical activity measured by the electrode (blue trace) shows the reliability of the optical recording in spike detection. A simple spike in PC5 trace (red box) is temporally magnified, revealing the undershoot phase. (*B*) TPF image of a parasagittal acute cerebellar slice stained with di-4-AN(F)EPPTEA. The molecular (ML) and granular (GL) layers are clearly distinguishable. The multiunit optical recording was carried out from the lines drawn (red) on the five Purkinje cells (PCs). The electrical activity (cell-attached recording) of PC1 was also monitored.

2.2 Correlative TPFM and electron microscopy

In order to study how regenerated axon interacts with possible postsynaptic targets, we combined two-photon *in vivo* imaging with focused ion beam milling and scanning electron microscopy (FIB-SEM)[17]. Immediately after the last *in vivo* imaging session, the animals were deeply anaesthetized and intracardiacally perfused with aldehyde fixative. The cerebellum was then vibratomed, and the section containing the imaged region positioned in the two-photon microscope. Fiducial marks were then burned around the area of interest in this section which was then further stained and embedded for electron microscopy[21]. The laser marks were then used to position the resin block in the electron microscope and serial images taken at 5 nm pixel size through this volume, with 10 nm spacing between each image. Axonal branches regenerated few days after being dissected by laser axotomy could be retrieved back with EM and 3D reconstructed[9]. These reconstructions shows the close proximity of this newly formed branch with a spiny branch of a Purkinje cell, the postsynaptic neuronal target (Fig. 2). The EM serial images allows visualizing both the distribution of mitochondria and synaptic vesicles inside the axon, and the structural interplay between the axon and the dendrite. The combination of two-photon imaging with electron microscopy allowed tracking back a single fluorescent structure (like an axon or a dendrite) previously imaged *in vivo*, and seeing its ultrastructure within significant volumes of brain tissue[9].

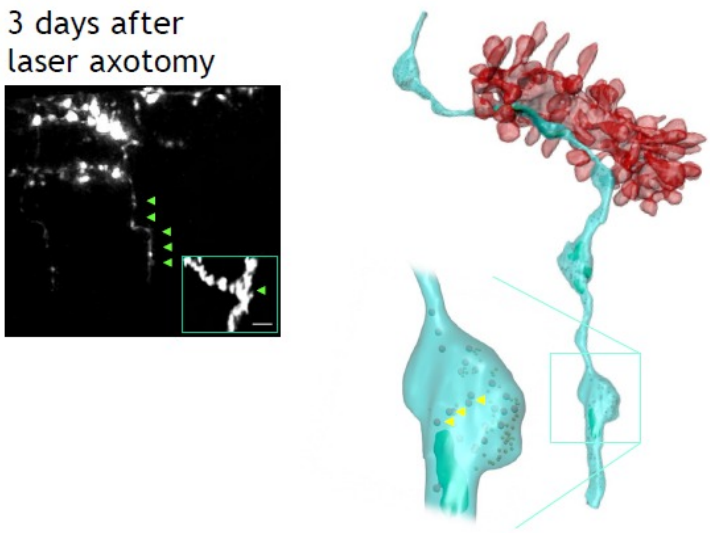

Figure 2. Correlative light and electron microscopy on a sprouted axonal branch. The panel on the left is a maximum intensity projection from an in vivo stack acquired with TPFM three days after laser axotomy. The green arrowheads point at the newly formed branch. On the right, the 3D rendering of the images obtained with FIBSEM on the same axonal branch is displayed. The adjacent Purkinje cell is shown in red; the yellow arrowheads point at the synaptic vesicles.

2.3 Correlative TPFM and light sheet microscopy

Two-photon fluorescence microscopy allow following the real-time dynamics of single neuronal processes in the cerebral cortex of living mice. The structural rearrangements can be imaged in vivo first, and then the same neuron could be retrieved on the fixed tissue and imaged with light sheet microscopy. We could image the apical portion of a neuron in living mice using TPF microscopy, and afterwards we could find the same neuronal process after tissue fixation, dehydration and clearing. For correlative two-photon and confocal light-sheet microscopy the TPF stacks were acquired on a regular 2-dimensional lattice, allowing for subsequent reconstruction of a larger field of view. Before removing the animal from the microscope, a wide-field image was acquired in reflection mode, to obtain a map of major blood vessels on the brain surface. Afterwards, maximum intensity projections (MIPs) were extracted from all the acquired image stacks using the Fiji software (Fiji.sc). By acquiring many adjacent image stacks with two-photon microscopy, it was possible to observe a relatively large field of view with high resolution, high enough to detect single dendritic spines. By using major blood vessels as landmark points, the same neuronal process observed in vivo was found again in the cleared tissue. Starting from this portion, the dendritic arbor can be reconstructed down to the cellular soma (see Fig. 3)[22]. With the correlative approach presented here, researchers can now place in a three-dimensional anatomic context the neurons whose dynamics have been observed with high detail in vivo.

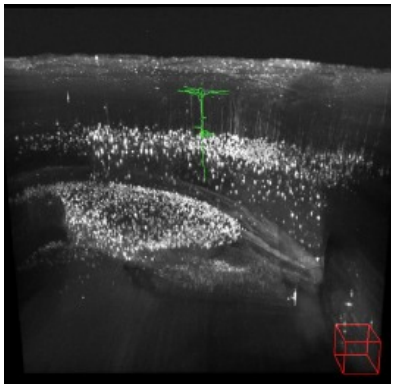

Figure 3. 3D rendering showing the reconstruction of a neuron (green) whose temporal reshaping has been previously imaged in vivo with TPFM. Image modified with permission from Silvestri et al. [22]

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