

Correlation of Two-Photon in Vivo Imaging and FIB-SEM Microscopy



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Author: Dr. Eric Hummel, Hans Zimmermann,
Carl Zeiss Microscopy

Prof. Dr. Jochen Herms, Lidia Blazquez-Llorca,
Institut für Neuropathologie, LMU München

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Introduction

Neuroscience and the understanding of brain functions is closely linked to the technical advances in microscopy. Cajal founded the field of modern neuroanatomy with routine staining of the Golgi-Apparatus in brain cells. Structural interaction between different neurons via synapses could be illustrated for the first time using microscopy. The contact zones between the neurones became the mayor focus point of modern neuroscience until now. Ongoing advances in light and electron microcopy have enabled new experimental approaches to inspect the nervous system across multiple spatial scales, confirming a critical aspect of the neuron theory: The presynaptic and the postsynaptic elements in both invertebrate and vertebrate nervous tissues are physically separated. Especially the dynamics of neurodegenerative processes are of mayor interest for medical research i.e. plaque disposal during Alzheimers disease (Schön et al. 2012). Development of two-photon microscopy in combination with electrophysiology allows the visualization of functional interactions within the brain of a living organism. Thanks to its superior imaging capability of deeper tissue penetration and efficient light detection, this method becomes an increasingly inspiring tool for neurobiologists. Two-photon imaging is therefore emerging as new tool to study the dynamics of neurodegenerative diseases, such as Parkinson's or Alzheimer's disease, i.e. plaque disposal (Burgold et al. 2011) or alterations of the dendritic spines (Bittner et al., 2010) during Alzheimers disease.

However, it is important to bear in mind that the light microscope level is in general rather coarse (e.g., connections between brain regions, synaptic nature of the dendritic spines), and has important limitations regarding resolution of important structures of synapses. Focus ion beam techniques have given already new insights into ultrastructure of brain tissue

(Merchán-Pérez et al., 2009; Blazquez-Llorca et al., 2013) The quantification and measurement of synapses is a major goal in the study of brain organization. However, a major limitation is that obtaining long series of ultrathin sections is extremely time-consuming and difficult. The most common method employed to estimate synaptic density in the human brain is indirect, by counting at the light microscopic level immunoreactive puncta using synaptic markers (DeFelipe, 2010). Correlative microscopy offers the possibility to combine dynamic deep-in-tissue NLO (two-photon) imaging and ultra-structural resolution. Using focused ion beam milling and crossbeam imaging we managed to observe the same dendrites at 7 nm isotropic resolution, that were formerly imaged over weeks with a NLO-system. ZEISS offers the whole portfolio of solutions to make this new way of correlative imaging possible, which will open up new horizons and opportunities to unravel the complexity of the nervous system.

Experimental procedure and results

Two-photon microscopy

Female mice expressing green fluorescent protein (GFP) under control of Thy-1 promoter were used in all experiments (GFP-M line). All procedures were in accordance with an animal protocol approved by the University of Munich and the government of upper Bavaria.

For in vivo imaging, a chronic cranial window was placed above the somatosensory cortex. Imaging began after a 3–4-week recovery period post surgery utilizing a ZEISS LSM 710 system being equipped with a MaiTai laser (Spectra Physics). For in vivo imaging, mice were anesthetized by an intraperi-

toneal injection of ketamine/xylazine. Imaging sessions lasted for no longer than 60 min, and laser power was kept below 50 mW to avoid phototoxic effects. Two-photon excitation of GFP was performed at 880 nm, a Plan-APOCHROMAT 20x 1,0 water-immersion objective was used. The selected neurons were imaged over several weeks. To find each time the same position, the motorized stage of the microscope was put in the corner limit left/front and this position was set as (0, 0). The coordinates of the brain regions that were imaged were established in reference to the (0, 0) point and noted down. Each imaging day, the same (0, 0) point was set and afterwards a brain position was easily found by just entering its coordinates in the ZEN Software. We observed that the error finding the same position was $\pm 100 \mu\text{m}$. For the fine relocation of the area of interest the blood vessels were additionally used to fine calibrate the area of interest (Figure 1 A). Between 7 – 10 dendrites located in layer I were selected for live cell imaging in each animal. The in vivo imaging was performed at three time points (t), the time between t1 and t2 was one week and one day between t2 and t3. In this way we are able to distinguish between stable spines (present at least for one week) and transient spines (present only during one day) (Figure 1 C-E). Figure 1 B shows the seven selected dendrites of neurons in one of the animals. Immediately after the last 2P in vivo imaging point (t3), the animals were intracardially perfused. Four hours after the perfusion, the cranial window was removed but strictly around the window region not in the whole head. In this way the metal bar holder can be maintained in the head, this helps to keep the same orientation of the 2P in vivo imaging which is crucial to find the same structures ex vivo. The area of interest was cut into 50 μm thick vibratome sections. After perfusion, the dendrites of interest were imaged ex vivo (Figure 1F) and then, laser marks were applied using the 2P-laser system according to the NIRB technique (Bishop et al., 2011) (Figure 1G-I). Marks were placed around the dendrite of interest in the central plane of the dendrite (around 5 – 10 μm far from the dendrite, Figure 1 H). Ten microns above this central plane other marks were placed resembling the profile of the dendrite (Figure 1G). These laser marks could be easily identified around the dendrite of interest (Figure 1 I). After the preparation of the tissue for electron microscopy the fluorescence is lost and the marks are the references to find the dendrites of interest with the FIB-SEM.

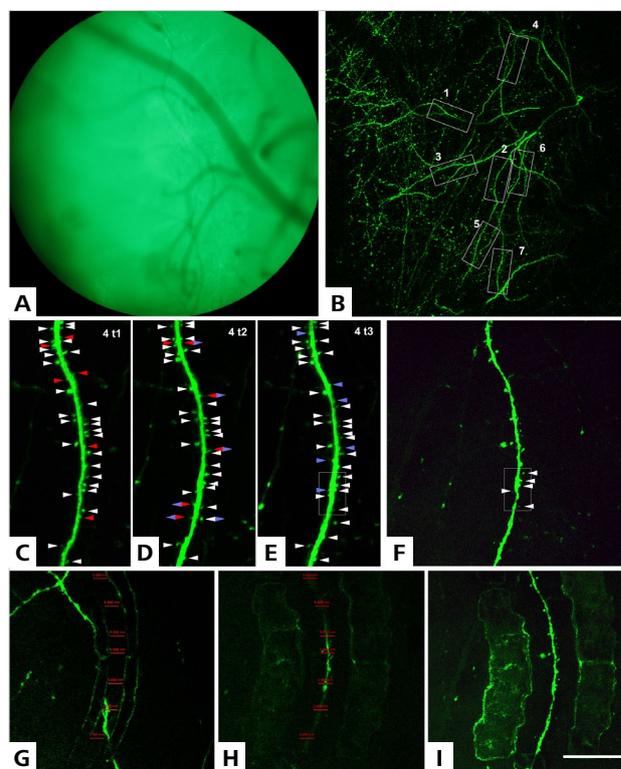


Figure 1: Two-photon imaging of mouse brain neurons in living mice. (Bars: A = 135 μm , B = 90 μm , C-F = 20 μm , G-I = 25 μm)

- A: Blood vessel distribution was used in addition to coordinate setting to accurately relocate the same position for two-photon imaging.
- B: Selected dendrites for the correlative approach.
- C-E: Dendrite 4 imaged at different time points (1 week between t1 and t2; 1 day between t2 and t3). White arrow heads show spines present during the whole imaging period. Red arrow heads: Spines disappearing at the next timepoint. Blue arrow heads point out spines that appear in this time point.
- F: Dendrite 4 imaged ex vivo after the perfusion of the animal. Note that the white rectangle present in E and F surround the dendritic segment that was further reconstruct with the FIB-SEM (see Figure 3).
- G-H: Two focus planes of dendrite 4 relocated after perfusion and with laser marks around it, 10 μm over the dendrite (G) and in the same focal plane (H). Marks are necessary to know the region that has to be scanned with the FIB-SEM.
- I: Maximum intensity projection of dendrite 4. Laser marks are clearly visible.

The sections were further trimmed around the laser marks. We have chosen two laser marks within one area of interest (Figure 2 A) for further preparation for electron microscopy. Selected sections were osmicated for 1 h at room temperature in PB containing 1% OsO₄ and 7% glucose. After washing in PB and one wash in 50% ethanol, the sections were stained for 30 min with 1% uranyl acetate in 50% ethanol at 37°C, dehydrated afterwards and flat embedded in Araldite.

After the osmication the fluorescence is lost but the laser marks around the dendrites are still visible (Figure 2 B). Shuttle and Find was used to relocate the area with the laser marks for FIB imaging. Laser marks of the selected neurons are only partly visible on the surface due to the difference in z or are covered with platinum after sputter coating. Shuttle & Find enables to recover the exact surface position for FIB imaging (Figure 2 C-D).

The ultrastructural 3-D study of these samples was carried out using a CrossBeam AURIGA electron microscope. This instrument combines a high-resolution field-emission SEM column (Gemini column, ZEISS Germany) with a focused gallium ion beam (FIB), which permits to remove a few nanometer thin layers of material from the sample (i.e. FIB milling). As soon as one layer of material has been removed, the exposed surface of the sample is imaged by the SEM using the backscattered electron detector. The sequential automated use of alternating FIB milling and SEM imaging allowed us to obtain long series of photomicrographs that represent three-dimensional sample volumes of selected regions. Images of 2048 x 1536 pixels, at a resolution of 5.99 to 9.97 nm per pixel were taken; each individual photomicrograph therefore covered a field of view ranging from 12.27 x 9.20 to 20.42 x 15.31 μm . The layer of material milled by the FIB in each cycle (equivalent to section thickness) was 10 nm in all samples. The number of serial sections obtained for each sample varied between 135 and 1300. The milling current of the FIB ranged from 500 pA to 1 nA and the SEM was set to 1.1 to 2.0 kV acceleration potential.

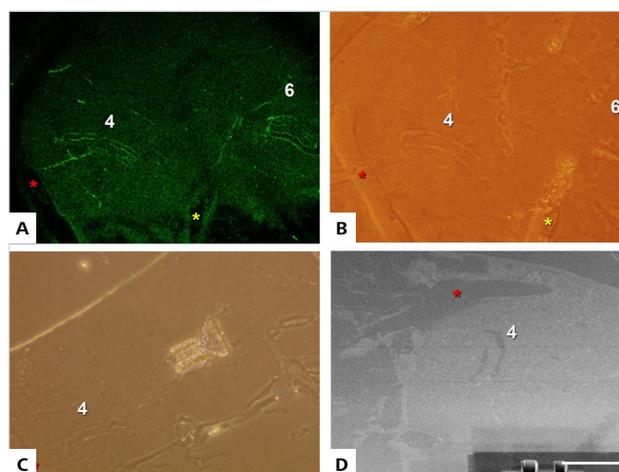


Figure 2: Areas of interest with laser marks before and after embedding (Bars: A,B, D = 70 μm , C = 75 μm)

- A: Two laser marks are visible within the thick vibratome section (50 μm). Blood vessels appear as dark areas (asterisk). B: The same area imaged post embedding. Laser marks could be easily identified and were recovered with Shuttle & Find in the SEM. Even blood vessels could be identified (asterisk)
- C: A semithin section showing the laser mark around dendrite 4 that was selected for further FIB analysis. D: Position recovery on the CrossBeam. Laser marks are only partly visible in the SEM image.

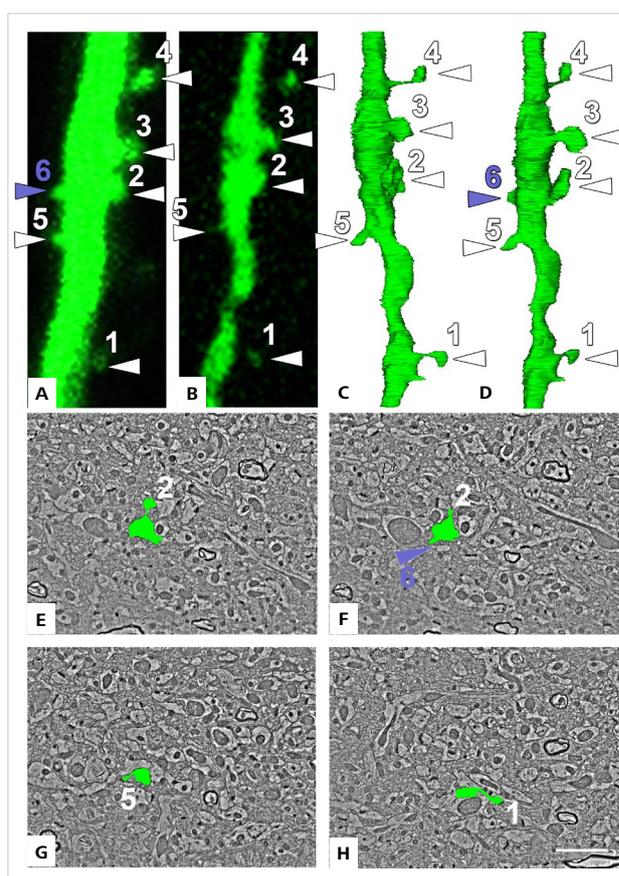


Figure 3: Correlative imaging of mouse neuron using two-photon and FIB CrossBeam imaging. (Bars: A-D = 2 μm , E-I = 2.7 μm)

- A,B: *In vivo* (last time point) and *ex vivo* imaging.
- C,D: Reconstructions of the area. Dendritic spines can be clearly colocalized. Note that the reconstruction in C resembles better the segment of the dendrite that was imaged *ex vivo* (B). D Rotation show dendritic spine number 6.
- E-H: CrossBeam images out of the focus ion beam stack. Dendrite of interest segmented within the stack. Note that the spines in white (1-5) were present during the whole imaging period and thus, they correspond to "stable spines". All the stable spines established synapses with excitatory axons. The transient spine number 6 (purple) that was only present in the last imaging time point did not establish synapses with axons, thus it is a non-synaptic dendritic spine (H).

Conclusion

Two-photon imaging enables scientists to study dynamic processes within living animals. The analysis of dendritic spines and their dynamics is of strong interest for many open questions in brain research. The dynamics of dendritic spines could be analysed in detail. We were able to distinguish between stable dendritic spines (visible over the whole imaging period) and transient dendritic spines (visible only at some time-points). Combining NLO imaging with focused ion beam milling and imaging allows to analyse large volume dynamics and correlate these at the ultra structural level semi-automatically. Correlative microscopy offers the possibility to exactly relocalize the dendrites of interest and provide ultrastructural insights which so far was only possible using

tedious and time consuming serial sectioning. This approach opens the door for many investigations in neuroscience which are so far unanswered. The combination of NLO and FIB-SEM methods avoid the typical problems which are normally encountered using serial section and serial tomography methods. Missing wedges (due to the Crowther criterion) are no issue. Missing sections or differences of section thickness are not present. CrossBeam systems provide large volumes that can be analysed with precision and high spatial resolution. Time consuming alignment procedures generally used in tomography are not needed, simple cross correlation methods are sufficient to achieve precise alignment of the obtained data.

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Carl Zeiss Microscopy GmbH
07745 Jena, Germany
BioSciences
microscopy@zeiss.com
www.zeiss.com/microscopy



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