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Towards a comprehensive understanding of brain machinery by correlative microscopy

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Abstract. Unraveling the complexity of brain structure and function is the biggest challenge of contemporary science. Due to their flexibility, optical techniques are the key to exploring this intricate network. However, a single imaging technique can reveal only a small part of this machinery due to its inherent multilevel organization. To obtain a more comprehensive view of brain functionality, complementary approaches have been combined. For instance, brain activity was monitored simultaneously on different spatiotemporal scales with functional magnetic resonance imaging and calcium imaging. On the other hand, dynamic information on the structural plasticity of neuronal networks has been contextualized in a wider framework combining two-photon and light-sheet microscopy. Finally, synaptic features have been revealed on previously *in vivo* imaged samples by correlative light-electron microscopy. Although these approaches have revealed important features of brain machinery, they provided small bridges between specific spatiotemporal scales, lacking an omni-comprehensive view. In this perspective, we briefly review the state of the art of correlative techniques and propose a wider methodological framework fusing multiple levels of brain investigation. [DOI: 10.1117/1.JBO.20.6.061105]

Keywords: neurons; two-photon microscopy; light-sheet microscopy; *in vivo* imaging; MRI; electron microscopy.

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1 Understanding Brain Machinery Requires Multilevel Investigation

The brain encompasses vast numbers of interconnected neurons that constitute anatomical and functional networks. In order to understand how the specific wiring of neurons accounts for brain functions, neuroscientists need various types of data. First, they need to build three-dimensional (3-D) maps of neurons within their circuits on multiple scales, ranging from interhemispheric axonal connections to single synaptic contacts. Second, the flow of electrical signals through brain circuits needs to be computed. Third, since the circuitry undergoes continuous modifications, long-term monitoring of dynamic remodeling like structural reshaping and functional adaptation is required. A vast repertoire of experimental tools is currently available to map neuronal connectivity at multiple levels (Fig. 1). Functional and structural maps of (animal models and) human brain in their entirety can be obtained with low-resolution imaging techniques like functional magnetic resonance imaging (fMRI), electroencephalography (EEG), diffusion magnetic resonance imaging, and polarized light imaging.[1–7] These methods achieve maximum spatial resolutions of hundreds of micrometers (fMRI) and temporal precision of the order of tens of milliseconds (EEG). These minimally invasive techniques play a key role in the exploration of the human functional and structural connectome.[8,9] Moving toward finer details on smaller samples (human brain samples or entire mouse brain), light microscopy-based techniques like light sheet microscopy (LSM) are well suited to obtain mesoscopic maps of connectivity with micrometric resolution.[10–12] Other techniques such as the knife-edge scanning microscopy,[13] micro-optical sectioning tomography (MOST),[14] fluorescence MOST (fMOST),[15] and serial two-photon tomography[16] (lateral resolution $\leq 1\ \mu$m) can achieve similar goals with slightly better contrast, though they do not preserve the sample and usually require a longer acquisition time. The subcellular resolution over the whole mouse brain is entangled by the need for sparse fluorescent labeling; brainbow techniques might overcome this limitation.[17] Wide-field electron microscopy (EM) can achieve better spatial resolution (10 to 100 nm) than light microscopy, but cell types cannot be specifically labeled and the imaging process is orders of magnitude slower, making the reconstruction of the whole mouse brain really demanding. All of these techniques for whole brain reconstruction work *ex vivo* on mammalian samples and cannot reveal functional and/or dynamic features. The activity of microcircuits of neurons can be characterized with cellular and subcellular resolution over spatial scales of a few hundreds of micrometers by confocal microscopy and two-photon fluorescence microscopy (TPFM). With its unique ability to image with high resolution and high sensitivity inside scattering tissue,[18] two-photon optical recordings combined with fluorescent reporters of cellular activity (e.g., calcium indicators or voltage sensitive probes) revealed crucial features of neural computation *in vivo*,[19,20] in head-fixed awake animals[21] and virtually[22] or truly[23] freely moving. Longitudinal studies (temporal scale of the order of weeks/months) of neuronal reshaping with *in vivo* TPFM revealed the structural correlation of neuronal

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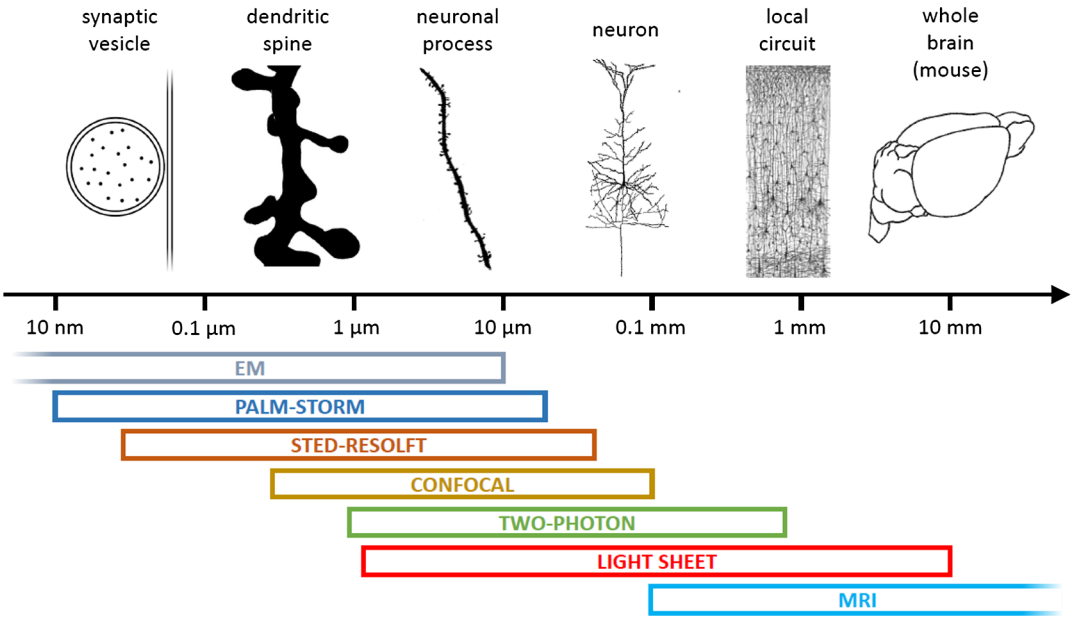

Fig. 1 Multiple spatial scales in the brain. On the top, different structures are depicted in proximity to their typical size, showing how relevant spatial scales in the brain span several orders of magnitude. On the bottom, typical working ranges of state-of-the-art imaging technologies.

functional adaptivity within its dynamics perspective. The structural features of medium-sized local circuits (e.g., a cortical column or the hippocampus) can be reconstructed in their entirety by EM-based techniques like serial block-face scanning electron microscopy (SBSEM)[24] or Automatic Tape-Collecting Lathe UltraMicrotome (ATLUM)[25] (voxel size $16 \times 16 \times 25$ nm^3), which mechanically slice the sample. For smaller neural circuits (up to 100 μm side) at higher resolution (near-isotropic voxels of ~4 nm), focussed-ion beam scanning electron microscopy[26] (FIBSEM) may be the most suitable. The identification of synaptic connections at the subcellular level and the molecular properties of individual synapses can be reached quite exclusively by electron microscopy and optical super-resolution techniques like photo-activated localization microscopy[27] and stimulated emission depletion[28] that break the diffraction barrier (nanometer resolution).

However, despite many decades of studies, we are still far from achieving comprehensive descriptions of brain machinery across all those levels. Recently, multiple attempts have addressed the multilevel complexity of this machinery by combining multiple imaging techniques providing different information. This integrated approach might overcome the intrinsic limit of spatial and temporal resolution and can provide multiple level information on the same sample. Here, we describe the state of the art of correlative approaches for investigating brain structure and functionality.

2 Correlative Imaging Overcomes the Limitation of Single Techniques

Novel approaches may provide new ways to bridge the gap between "postmortem" microscopic and *in vivo* macroscopic worlds through functional measures reflecting neural connectivity. Several orders of magnitudes can be crossed by combining the spatiotemporal resolution of complementary techniques. Here, we provide four examples of correlative approaches that linked different temporal/spatial scales, going from noninvasive whole brain functional MRI to subsynaptic structure imaging with FIBSEM.

2.1 *Simultaneous fMRI and Optical Functional Imaging*

The functional connectivity at macro- and mesoscopic scales can be inferred with different neuroimaging techniques. fMRI enables noninvasive monitoring of activity in healthy and diseased brains with submillimeter spatial resolution in humans and animals.[2] Alterations in the blood oxygenation levels (BOLD contrast) arising from changes in cerebral blood flow, blood volume, and oxygenation are used to estimate brain activity.[29,30] In order to understand the link between BOLD signals (macroscale) and the underlying neural activity (mesoscale), Helmchen et al. used a combination of BOLD fMRI and simultaneous recording of calcium activity[31] [Fig. 2(a)]. The authors provided a proof of principle of the integration of fluorescence measures of brain functionality through an optical fiber with fMRI scanners. Further, they demonstrated the close relationship between the two signals by predicting BOLD signals from the fluorescence responses measured with the optical fiber. This study highlights the complexity of fMRI BOLD signals, involving both neuronal and glial activity. The hybrid method for simultaneous recording of BOLD fMRI and calcium transients imaged with TPFM could facilitate further understanding of cellular mechanisms of neurovascular coupling.

2.2 *Correlative Light Sheet and In Vivo Two-Photon Microscopy*

TPFM is a powerful tool for longitudinal studies of brain reshaping, both in terms of functional and structural plasticity. The structural plasticity of selected neuronal populations *in vivo* can be visualized by time-lapse imaging of fluorescently labeled neuronal structures like varicosities, spines (i.e., pre- and postsynaptic portions), axons and dendrites.[36–40] The wide-ranging

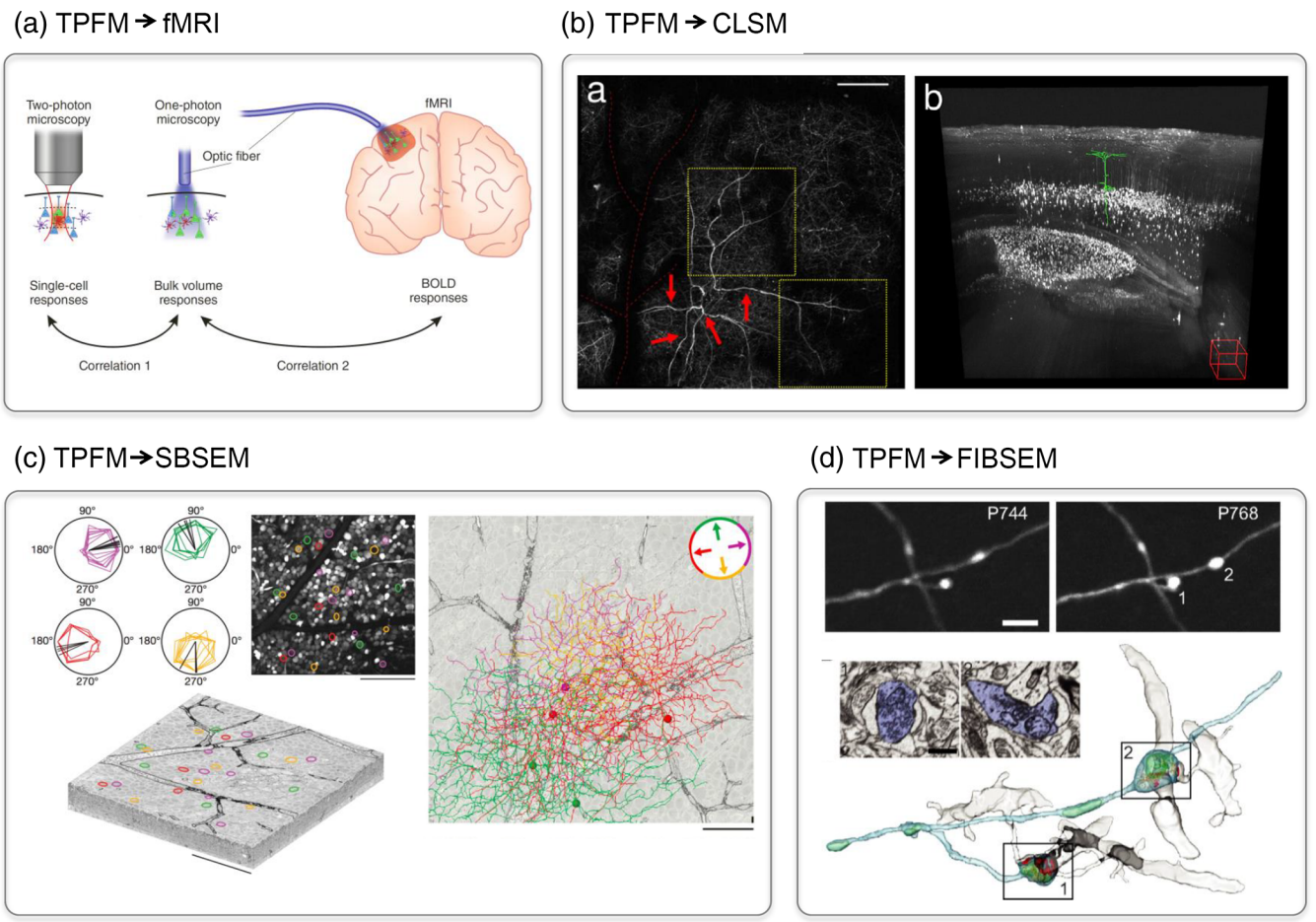

Fig. 2 Correlative microscopy in neuroscience. (a) Experimental approach for examining the relationship between single-cell activity and fMRI signals. Single-cell Ca^{2+} measured by two-photon microscopy were correlated with bulk responses in the same region measured by one-photon fiber-optic microscopy. Then the bulk one-photon responses were correlated with the BOLD fMRI signals. Figure modified with permission from Ref. 32. (b) On the left, maximum intensity projections of several stacks TPF stitched together in a single image. The red dashed line highlights blood vessel shadows. Red arrows highlight characteristic features of a dendritic arbor, to help finding it back in the CLSM images. On the right, CLSM imaging of the same neuron observed with TPF. Starting from the apical portion of the dendritic tree, the neuron has been segmented and is shown inside a maximum-projection 3-D rendering. The scale of the figure can be inferred from the red cube down on the right, which has 100 μm side. Figure modified with permission from Ref. 33. (c) Functional characterization of direction-selective retinal ganglion cells (DSGCs) and their localization within SBSEM volume. On the left, polar tuning curves for 25 DSGCs sorted and color-coded by preferred direction. The corresponding soma locations superimposed onto a two-photon image from the recorded region of the ganglion cell layer and the acquired SBSEM volume (scale bars: 100 nm). On the right, skeleton reconstructions of DSGCs. DSGCs, color-coded by preferred direction (inset), normal to the plane of the retina (scale bars: 50 μm). Figure modified with permission from Ref. 34. (d) Correlative in vivo TPF and focused ion beam scanning electron microscopy of cortical neurons. In vivo TPF imaging of an axon showing two stable boutons (scale bar: 5 μm). Both boutons make multiple synaptic contacts, as visible in a single plane of the correspondent EM images, with multiple dendritic spines (scale bar: 500 nm). 3-D rendering of the same axon imaged in TFP microscopy. The cytoplasm of the axon is represented in light blue, mitochondria in green, synaptic vesicles in yellow and synapses in red. The postsynaptic spiny neurons are shown in gray. Figure modified with permission from Ref. 35.

neuroanatomical tracing of the neuron previously observed *in vivo* allows associating univocally the dynamic fingerprint of a neuron to a specific neuronal type and to its partners of connectivity. A reliable correlative reconstruction of the neuron previously observed *in vivo* and of the surrounding network can be obtained by combining TPFM with other *ex vivo* imaging techniques that can explore large volumes. Unlike other large-scale neuroanatomical techniques, ultramicroscopy,[11] confocal light sheet microscopy (CLSM),[10] and CLARITY optimized light sheet microscopy (COLM)[12] are the only ones in which the sample is preserved without slicing, allowing multiple imaging rounds and, therefore, providing a greater flexibility. By using the blood vessel pattern as an internal reference, Silvestri et al. were able to image the apical portion of a dendritic arbor in a living mouse using TPFM, to find the same neuronal process after tissue fixation, dehydration and clearing, and to trace the entire

neuron from CLSM images[33] [Fig. 2(b)]. The same correlative two-photon and LSM approach could allow reconstructing the distribution and the anatomy of neurons whose activity has been monitored through *in vivo* functional imaging.

2.3 *Correlative In Vivo Two-Photon Calcium Imaging and Serial Block-Face Scanning Electron Microscopy*

Linking functional TPFM with the structural connectivity obtained with large-scale electron microscopy allows answering otherwise intractable neurobiological questions. For example, though discovered 50 years ago,[41] the computation of motion direction by direction-selective retinal ganglion cells (DSGCs) lacks a complete explanation. Though both light microscopy and EM studies independently attempted to study direction-selectivity circuit anatomy, the results were either contradictory[42,43] or incomplete.[44,45] Until the recent advancement in serial processing, EM was not capable of reconstructing large fractions of cells in the same piece of tissue, covering the spatial distances over which individual neurons project (hundreds of micrometers) with the resolution of single axonal projections (tens of nanometers). In addition, since DSGC's preferred direction cannot be inferred from its dendritic morphology,[46,47] functional optical imaging techniques are needed to complement structural reconstructions to determine these neural circuit diagrams.

In a very accurate and in depth study, Briggman et al. combined *in vivo* calcium imaging in the intact retina and SBSEM-based reconstruction of the circuitry in the same piece of tissue to explain the behavior of retinal ganglion cells (DSGCs)[34] [Fig. 2(c)]. They show that dendrites of mouse starburst amacrine cells make highly specific synapses with direction-selective ganglion cells depending on the ganglion cell's preferred direction. This pattern provides the structural substrate for the functional asymmetry in the inhibitory input currents observed in DSGCs. This study directly correlated a structural (wiring) asymmetry with the functional properties of the cell, i.e., to the computation of direction selectivity. Accurate 3-D maps of connectivity between neurons will be essential for the implementation of the algorithms used in neural computations, such as the detection of directed motion by the retina.

2.4 *Correlative In Vivo Two-Photon Imaging of Structural Plasticity and Focused-Ion Beam Electron Microscopy*

The synaptic connections between axons and dendrites can quite exclusively be visualized with EM. When combining high-resolution EM with *in vivo* light microscopy, the time lapse structural remodeling can be linked with the underlying ultrastructural morphology.[48,49] *In vivo*-imaged dendrites and axons in adult mouse brains can subsequently be prepared and imaged with EM. The growth of dendritic spines in the adult mammalian brain, seen with two-photon *in vivo* microscopy, has been verified as new synaptic connections by using *post hoc* serial section transmission electron microscopy (TEM).[50,51] The advent of FIBSEM greatly increased the level of automation, reliability, and speed of EM imaging.[26] One of the most commonly used tricks to retrieve the position of the *in vivo* imaged structure is to burn fiducial marks with the pulsed laser next to it in the fixed tissue.[52,53] Since this combination of techniques avoids the use of specific labels to identify the structures of interest in the electron microscope, optimal structural preservation for 3-D analysis is guaranteed [Fig. 2(d)]. In the last years, several studies provided proof of evidence of the added value of correlating *in vivo* TPFM and FIBSEM for targeting micro- and ultrastructural features of synaptic plasticity.[35,54,55] Recently, two parallel works by Allegra Mascaro et al.[56] and Canty et al.[57] combined two-photon *in vivo* imaging with FIBSEM to study how laser axotomized and regenerated axonal branches interact with the surrounding neuropil and possible postsynaptic targets. These EM reconstructions allowed visualizing both the distribution of mitochondria and synaptic vesicles inside the axon and inferring the structural interplay between the axon and the dendrite.

3 Wider Methodological Frameworks Fusing Multiple Levels of Investigation might Boost Our Understanding of the Brain

The correlative methods presented in the previous section showed fundamental insight into different spatiotemporal scales of brain functioning. The small though solid bridge they provided between different types of data can be promisingly expanded toward a unified approach covering most perspectives. In detail, *in vivo* imaging by noninvasive human-targeted techniques like fMRI could be the starting point of a long pipeline that interrogates the long-term plasticity of small populations of neurons through *in vivo* TPFM functional and structural imaging. Actually, this translational step is the most critical and less explored, probably because fMRI remains a clinically oriented technique while TPFM is a basic science research tool. Nonetheless, understanding the cellular activation patterns underlying fMRI signals would be beneficial for diagnostic purposes, and it would boost the translational potential of light microscopy. Several studies on neurovascular coupling[58–61] are now helping to fill this gap, and hopefully new correlative investigations combining simultaneous one- or two-photon fluorescence microendoscopy (e.g., Jung et al.)[62] and MRI will come. Once functional and structural data are obtained *in vivo* at the small-circuitry level, the same sample shall be processed with LSM or analogous techniques for *ex vivo* long-range anatomical analysis. This contextualization into a wider framework is refined up to the synaptic scale when imaged through electron microscopy and/or super-resolution techniques. The need for sample processing procedures (e.g., clearing methods) compatible for all the techniques along the pipeline is one of the main limiting factor. In this view, the CLARITY technique, developed in Deisseroth's lab to drastically reduce tissue scattering and perform optical imaging and immunolabeling through entire intact brains, preserves ultrastructural features and is thus EM compatible.[63] Anyway, different degrees of tissue deformation throughout the pipeline are unavoidable and have to be considered when building multilevel maps of the brain. Informatics tools to align data at different scales within a common framework (i.e., stereotaxic atlases of murine and human brain) and big data storage facilities need to be further implemented and routinely used. Big transnational research partnerships like the Human Brain Project are working in this direction by "developing the integration and algorithmic reconstruction processes required for high fidelity reconstruction of the mouse brain across all levels of biological organization, from genes to cognition."[64] Since a completely integrated correlative approach implies that the same animal should be studied by multilevel analysis on several devices, the possibility of

having this wide set of tools near to each other, e.g., in the same campus, is not a negligible issue. Multidisciplinary facilities provided with the above-described imaging devices are essential requirements for setting up this working strategy. In addition, considering the intrinsic difference between individuals (even between mice of the same strain), a multilevel investigation of the same sample would be extremely beneficial to reduce statistic variability and to cut the number of animals used in the experiments.

We will try now to speculate on the information that can be gained on a neurological disease like stroke by following this pipeline. Stroke alters and triggers changes in intra- and inter-hemispheric connectivity; this rewiring aims at compensating for the loss of function.[65] fMRI on (human and) mouse brain affected by stroke can tell the progression of the pathology over time, showing the plastic remapping of distant regions over the whole brain.[66] MRI does not have enough resolution to infer what the cellular trigger of this remodeling is; simultaneously performed TPF imaging of labeled neuronal cells could reveal the structural and functional rewiring underlying fMRI signals in the newly activated cortical area of the same mouse with subcellular detail.[67] Optical imaging on stroke animal models is capable of providing fundamental insight into dendritic remodeling, axonal rewiring, and spine plasticity[67–69] while accurately depicting the functional remapping of the damaged cortex,[70,71] and revealing angiogenesis and hemodynamic adaptation over time.[72–74] Alterations in long-range projections underlying inter-hemispheric plasticity can be studied *ex vivo* on the same brain once cleared. Moreover, stroke-induced expression of several molecules and proteins, like growth-associated factors and inflammatory chemokines, can be addressed with multiround immunohistochemistry over the entire clarified brain by LSM or similar techniques. Once having the big picture in terms of long-term dynamics and wide-range remodeling, fine details like the presence of synaptic contacts on regenerated axons are available by electron microscopy on targeted regions of the same sample.

In our opinion, this multidimensional hybrid strategy could be extremely useful in the investigation of complex brain diseases and would speed up the translation of neurobiology studies to clinical settings. Moreover, the setup of pharmacological treatments would crucially benefit from this multilevel investigation given the multitude of information that can be gained at once. We believe this kind of cross-disciplines multiscale studies is the missing tile to boost our knowledge of the brain.

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