Functional optoacoustic neuro-tomography (FONT) for whole-brain monitoring of calcium indicators

Gali Sela1, Antonella Lauri1,2, X. Luis Deán-Ben1, Moritz Kneipp1,3, Vasili Ntziachristos1,3, Shy Shoham4,*, Gil G. Westmeyer1,2,3,*, and Daniel Razansky1,3,*

1Institute for Biological and Medical Imaging (IBMI), Helmholtz Center Munich, Neuherberg, Germany
2Institute of Developmental Genetics, Helmholtz Center Munich, Neuherberg, Germany
3Department of Medicine, Technische Universität München, Munich, Germany
4Department of Biomedical Engineering, Technion – Israel Institute of Technology, Haifa, Israel

Non-invasive observation of spatiotemporal neural activity of large neural populations distributed over entire brains is a longstanding goal of neuroscience. We developed a real-time volumetric and multispectral optoacoustic tomography platform for imaging of neural activation deep in scattering brains. The system can record 100 volumetric frames per second across a 200mm³ field of view and spatial resolutions below 70µm. Experiments performed in immobilized and freely swimming larvae and in adult zebrafish brains demonstrate, for the first time, the fundamental ability to optoacoustically track neural calcium dynamics in animals labeled with genetically encoded calcium indicator GCaMP5G, while overcoming the longstanding penetration barrier of optical imaging in scattering brains. The newly developed platform offers unprecedented capabilities for functional whole-brain observations of fast calcium dynamics; in combination with optoacoustics’ well-established capacity in resolving vascular hemodynamics, it could open new vistas in the study of neural activity and neurovascular coupling in health and disease.

Neuronal activation occurs concurrently or in a highly coordinated fashion in different areas across the nervous system, reflecting functional interconnection between specialized neuronal sub-circuits. Imaging neuronal activation with high temporal and spatial resolution over an entire intact brain, including deep and normally inaccessible areas, could thus play a critical role in the attempt to decipher the fundamental operating principles underlying neural circuit activity. Major efforts are underway to advance the ability to optically image the activity of large, distributed neural populations1,2. Recently, these efforts have led to whole-brain activity imaging in transparent organisms using single3 and multi-photon4 light-sheet and light-field5 microscopy. However, these approaches are unable to resolve deep neural activity in intact scattering brains where current state-of-the-art optical imaging strategies based on rapidly scanning multiphoton microscopy are limited to volumes below 1mm³ due to limited penetration depth and field of view (FOV).

Conversely, optoacoustic imaging, an extremely powerful approach with demonstrated capacity for centimeter scale penetration into highly scattering tissues6, can potentially provide functional neural imaging beyond the limit of current optical imaging technologies. To date, functional optoacoustic brain imaging mainly focused on probing hemodynamics and blood oxygenation variations3,4, slow and delayed processes that only indirectly reflect neural activation. In contrast, modern approaches towards imaging neural activation are largely based on measuring calcium dynamics, which provides a much more direct correlate of neural activity. Calcium dynamics are conventionally tracked using fluorescent sensors, which change their fluorescence output because of a change in their extinction coefficient, fluorescence quantum yield or both as a function of intracellular calcium concentrations6,7,8. Recent advances in the field of genetically encoded calcium indicators (GECIs) have provided a variety of genetically modified reporter animal models with calcium indicators in specific neuronal structures9,10, which in some cases can give rise to signals down to the level of single action potentials11,12.

We hypothesized that some members of the GCaMP5G family of fluorescent GECIs could provide a suitable calcium dependent optoacoustic contrast due to their strong calcium-dependent absorption changes in the presence of calcium13. To test this hypothesis, we selected the zebrafish reporter line HuC:GCAMP5G that exhibits expression of the calcium sensor in a large fraction of neurons to obtain high-resolution optoacoustic measurements from larvae and isolated adult brains.

To enable tomographic imaging of the whole scattering brain in this model system with high spatiotemporal resolution, we developed a new generation functional optoacoustic neuro-tomography (FONT) system that can simultaneously monitor calcium activity in all five dimensions, i.e. space, time and wavelength (spectrum) (Fig. 1A). The experimental setup and accompanying image reconstruction algorithms enable real-time rendering of 200mm³ volumes at a frame rate of 100Hz; this temporal resolution is high enough to allow tracking of freely moving organisms (e.g., a freely swimming 6 day old wild type zebrafish larva, supplementary video 1). This major improvement over previous state-of-the-art implementations14 further enables the acquisition of over 10⁶ informative voxels over a 6 · 6 · 6 mm FOV with spatial resolution down to 52µm and 71µm in the axial and lateral directions, respectively (Fig. 1B). The laser also has fast wavelength tuning capability, enabling the fast acquisition of spectroscopic information regarding chromophores of...
To explore the fundamental spectral signature of our experimental system, 6 days old HuC:GCaMP5G zebrafish larvae were imaged using two distinct wavelengths: 488nm where GCaMP5G has high absorption and 530nm where GCaMP5G absorption is close to zero (Fig 1C). The larva’s eyes, with their characteristically highly absorbing melanin pigmentation are readily visible in both optoacoustic images, whereas tissues expressing GCaMP5G do not yield a strong signal at 530 nm but provide clear contrast at 488nm, where the protein is highly absorbing (Fig. 1D). Optoacoustic measurement of calcium-related dynamic changes in GCaMP5G absorption were compared with planar fluorescence by coupling the system to a fast sCMOS camera.

To elicit and image strong neural activation we exposed immobilized HuC:GCaMP5G zebrafish larvae (n=5) to a neuroactivating agent (Pentylenetetrazole, PTZ) that induces fast ictal-like spikes in the larva’s nervous system and changes in their swimming behavior, most likely by interfering with GABAergic signaling. PTZ exposure caused robust calcium waves propagating from the larva’s posterior (site of drug injection) to the anterior part of its spinal cord (Fig. 2). Strong correlations between the simultaneously acquired optoacoustic and fluorescent signals were observed during evoked calcium transients both at the tip ($R^2$=0.98) and midtail ($R^2$=0.97) regions, providing confirmation that GCaMP5G calcium sensing can be read out via optoacoustics (Fig. 2 and supplementary video 2).

Next, we examined FONT’s imaging performance in isolated brains of adult HuC:GCaMP5G zebrafish (n=4), measuring 2-3 mm on their short axis. The system was found to provide high-quality time-resolved 3D reconstructions across these highly scattering brains (Fig. 3); note that only the forebrain and part of the optic tectum were effectively illuminated and thus visualized. Five volumes of interest (VOIs) were then selected for analysis of the dynamics of GCaMP5G using time series of both the optoacoustic (absorption) and the planar fluorescence signals during neural activation (Fig. 3B). According to the optoacoustic signal traces, activation patterns associated with high contrast calcium-related changes occurred mainly in deep brain areas with maximal contrast $\Delta OA/OA_0$=8.5, while voxels close to...
the surface actually showed a slight decrease in activity (blue VOI). However, as compared to true 3D information provided by the tomographic optoacoustic reconstructions, whose spatial resolution is not affected by the intense light scattering, the planar fluorescence lacks optical sectioning thus may result in wrong conclusions based on smeared sub-surface information averaged over large volumes. The signal traces from the gray VOI placed over a superficial part of the optic nerve show a similar overall trend in both the fluorescence and optoacoustic modality, although the optoacoustic signal shows some faster signal fluctuations. However, signal changes can be detected in the pink VOI by optoacoustics that are absent in the planar fluorescence images. Similarly, the time course averaged over the yellow VOI placed deeper in the brain shows signal changes that are not detected by the fluorescence read out from an approximately corresponding planar region. This simultaneous dual-mode imaging demonstrates that epi-fluorescence fails to faithfully identify the calcium fluxes that optoacoustics localized deep inside scattering brain.

Finally, we examined whether the system's exceptionally large FOV opens up the capability to non-invasively measure neural activity while simultaneously monitoring ongoing natural behavior. Indeed, one of neuroscience's major challenges is to study neural processing during unrestrained motion, motivating the successful recent introduction of a number of experimental paradigms for studying behaving zebrafish larvae using e.g. bioluminescence imaging as well as light sheet and light field microscopies (note however that these strategies cannot provide volumetric information in scattering brains). We therefore tested whether our optoacoustic system enables high-speed volumetric imaging in larvae that were allowed to swim freely in ~0.5 cm² chamber and then exposed to the neurostimulant PTZ - leading to rapid movements followed by long resting periods. The optoacoustically-recorded responses indeed revealed an increase up to \( \Delta OA/OA_0 = 1.8 \) in calcium signal (see arrows in Fig. 4), just before the fish moves.

Figure 3. Activity in isolated scattering brains. (A) Typical 3D optoacoustic image acquired from a highly scattering brain of an adult fish in its resting state (two side views and one isometric view of the 3D reconstruction are shown). The telencephalon (T) and most of the optic tectum (OT) are clearly visible. Five 300µm x 300µm x 300µm VOIs were chosen at different locations and depths within the brain. (B) Traces of the fluorescence (top) and optoacoustic (bottom) signal changes are shown for the five regions in corresponding colors (all signal changes are normalized to the resting signal levels). Note that the optoacoustic traces are calculated over volumes whereas the fluorescent signals are calculated over the roughly corresponding planar areas. Snapshots acquired at 5 different time points before and after introduction of the neurostimulant are shown (scale bar - 500 µm). The injection phase caused image artifacts and is therefore excluded from the graphs.

Figure 4. Activation in freely swimming larvae. Two separate activation events, as captured by volumetric optoacoustic tomography, are shown. Following injection of the neurostimulating agent at approximately \( t=0 \), the larva occasionally stops swimming while experiencing a surge of activation through its tail (the arrows point to the location of optoacoustic signal increase) before it starts moving promptly to a new position (notice movement of the tail in the two rightmost frames). Scale bar - 500µm.
In summary, we demonstrated a novel, state-of-the-art optoacoustic imaging platform for direct imaging of spatio-temporal neural activity across an entire light-scattering brain while maintaining similar values of spatial resolution at highly scalable depths. Our study is also, to the best of our knowledge, the first to examine the optoacoustic signature of modern GECIs, showing that the strong changes in GCaMP5G fluorescence are directly related to their optoacoustic signature. Since FONT uses a widely-tunable nanosecond OPO laser technology, it can be conveniently tuned to work with a large array of other functional probes, including for instance the newer generation of GCaMP6 probes and red shifted probes like RGEQC, or future sensors optimized for in vivo deep tissue optoacoustic detection providing high extinction coefficient changes in the near-infrared window as well as lower quantum yield. Furthermore, the ability to simultaneously track the movement and neural activation of living unrestrained organisms could form a basis for behavioral studies not currently possible with other neuroimaging techniques. While the current spatial resolution does not allow to distinguish individual cells, future generations of the described tomographic approach could utilize higher-frequency transducer technology and/or super-resolution strategies for fast functional observations at the cellular-scale. In addition, the high sensitivity of optoacoustics to a variety of intrinsic absorption tissue contrasts, most prominently the oxy- and de-oxy hemoglobin, is well established. This may provide highly complementary information to the functional calcium imaging and thus enhance the amount of spectrally- and time-resolved volumetric information available for the five dimensional optoacoustic studies looking at coupling between the vascular changes and nervous system in more complex animal models. While we recently demonstrated high-resolution imaging of near-infrared fluorescent proteins (iRFP) in mouse brain in vivo using multispectral optical tomography, development of similar calcium sensitive indicators in the near-infrared is expected to greatly expedite translation of our technology into mammalian brains.

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