Fast three-dimensional two-photon scanning methods for studying neuronal physiology on cellular and network level

Doctoral thesis

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INTRODUCTION, AIMS

How does the brain work? This ancient question was in the focus of many clever minds in the past, from Aristotle through Szentágothai all the way to recent high profile laboratories. However, with the development of technology new approaches could emerge. Aristotle could only rely on thinking and deduction, while with the invention of Golgi, the novel era of neuroscience could emerge focusing on how the brain looks like, how it is organized. This detailed anatomical knowledge is accompanied in the recent decades with the emergence of new investigation technologies fuelling a new boom in the functional knowledge about our initial question.

The systematic understanding of brain function requires methods that allow neuronal activity to be recorded at different spatial scales in three dimensions at a high temporal resolution. At single neuron level activity is differentially distributed in space and time across the dendritic and axonal segments. Therefore, in order to understand neuronal signal integration, activity should be simultaneously recorded at many spatial locations within the dendritic and axonal tree of a single neuron. At neuronal circuit level, closely spaced neurons can have vastly different activity patterns; on the other hand, widely separated cells may belong to the same functional circuit. Therefore, recording techniques are required that collect information near-simultaneously with sub-millisecond temporal resolution from many cells of a neuronal population situated in an extensive volume of tissue.

Light microscopy inside living tissues is hampered by the degradation of resolution and contrast, caused by absorption and light scattering in every tissue. Deeper within the tissue, images become more degraded and eventually even discriminating cells becomes impossible. The combination of

low phototoxicity enabled by the single-point two-photon microscopy and the efficient use of fluorescence – even scattered – enables more than one millimeter depth penetration and subcellular level resolution. These make single-point two-photon microscopy a unique tool for observing function in relatively intact tissues. Its main drawback, however, is speed as compared to camera based approaches. As only one point might be illuminated at a time, special scanning methods need to be developed to sample the important biological features with enough speed to resolve their functionality.

Our aim was to develop novel laser scanning algorithms that enable functional investigations. My work is dedicated on finding out and implement such scanning methods, devices, electronics and software and to perform cellular level biological measurements in acute brain slices and in the brain of living animals.

METHODS

The work detailed in the thesis is closely related to the multidisciplinary development of the microscope hardware. The microscopes were designed in specialized software: the mechanics was designed in SolidWorks, the optics in Zemax, and the electronics in OrCAD. In the *in vitro* experiments using the newly developed scanning methods we used rat or mouse acute brain slices and the fluorescent dyes were introduced by the use of the patch-clamp technique. In the *in vivo* experiments we exposed the brain surface of anesthetized mice through surgery and introduced the fluorescent marker into the cells using the bolus loading technique. To implement the new scanning methods we developed new printed circuit boards; the measurement algorithms have been implemented

in Matlab and C++ programming languages. Matlab software and scripts were used for data analysis and visualization too.

NEW SCIENTIFIC RESULTS

We made three major steps forward in the development of new scanning methods for fast neurophysiology measurements.

Thesis 1*: I developed a new method to image dendrites; by limiting the measurements only to the functionally investigated neuronal locations increased speed and signal-to-noise ratio of the measurements significantly.*

Publication related to the thesis: Lorincz et al., 2007, Rozsa et al., 2008; Chiovini et al., 2010.

In two-photon microscopy the scanning is traditionally used to form images. Images are formed by measuring the fluorescence intensities in all pixels, moving the focal spot line-by-line. To record neuronal signaling and action potentials we should give up recording entire images and instead of sampling all pixels we need to scan repetitively only the regions containing interesting information for the experimenter (ROIs). This can be achieved by using galvanometer based scanning mirrors controlled by analog voltage signals, boosting repetition speed to the 100 Hz - 1 kHz rate. Scanning only the interesting parts not just increases repetition speed of the measurement at a given ROI, but also increases signal to ratio (SNR) of the measured fluorescence signals. The higher the ratio of the area of the field of view (FOV) to the area of the ROIs is the larger the possible SNR gain is.

We developed a complex line-scanning method called Multiple Lines Scanning (Lorincz et al., 2007) and built a microscope utilizing it. With this technique we could increase

the SNR theoretically by a factor of 29, in practice, typically by a factor from 3 to 4. We were able to use this feature to show Na^+/Ca^{2+} exchanger (NCX1) activity in the case when multiple synaptic inputs arrive in close succession to the same dendritic branch (Lorincz et al., 2007). The major benefit of using the Multiple Line Scanning method was that by simultaneously scanning multiple neighboring spines we were able to isolate the cases where only single spines were activated. We also took advantage of this method to record multiple locations with good SNR in other studies, where we studied the attenuation of backpropagating action potentials (bAPs) in interneuron dendrites (Rozsa et al., 2008; Chiovini et al., 2010).

Thesis 2*: I developed a new method to measure activity across neurons by using a new implementation of piezoelectric objective positioning, and a new driving principle. The method is capable of depth-scanning ten times faster than previous realizations. I experimentally proved the usability and parameters of the method.*

Publication related to the thesis: Katona et al., 2011.

Using scanning mirrors to deflect the laser beam enables rapid positioning of the focal point only in the focal plane of the objective. Biological structures are, however, rarely planar. Our aim was to sample activity along long tortuous dendritic segments so we needed to develop a method capable of scanning 3D trajectories with high speed.

We extended our two-dimensional Multiple Line Scanning Method with the use of a high-speed, piezoelectric objective positioner to image points along a 3D trajectory with high spatial as well as temporal resolution. Line-scanning using galvanometric mirrors was precisely synchronized to the phase of the *z*-axis movement of the nonlinearly resonating objective. We named this method Roller Coaster Scanning (Katona et al., 2011). The method allows *in vitro* imaging of up

to 250 μm long dendrites situated in a wide field of view (up to 650 μm x 650 μm). It has a suitable *z*-scanning range (up to 25 μm) with a resolution characteristic of two-photon microscopy (< 450 nm) and enables high repetition rates (150-690 Hz) without limiting pixel dwell time. Using Roller Coaster Scanning we had approximately 27 times larger chance to image 40 μm dendritic segments than we would have had with 2D scanning approaches.

These parameters allowed us to detect spontaneous events on spatially extensive dendritic arbors of hippocampal CA1 stratum radiatum interneurons (Katona et al., 2011). Here we searched for active synaptic inputs on long dendritic segments of these interneurons during spontaneous network activities *in vitro* and found spatially extensive dendritic spikes and small compartmentalized unitary events. These events were reproduced using focal electrical stimulation and two-photon glutamate uncaging to be able to investigate their pharmacological properties and their dependence on the number and distribution of coincident synaptic inputs driving them. We found that NMDA dependent dendritic spikes appear when ~10 spatially clustered inputs arrive synchronously and trigger supralinear integration in relatively small (~14 μm) dynamic interaction zones.

Thesis 3*: I developed a new technique by using new electronic and software driving algorithms furthermore, specialized measurement and data analysis principles on a 3D scanning AO microscope. The method can collect measurement data in a 700*700*2000 μm³ volume in transparent samples in random-access mode. I modified the method to be able to measure activity across neuronal networks at high temporal resolution.*

Publication related to the thesis: Rozsa et al., 2007; Katona et al., 2012.; Chiovini et al., 2014.

In the third step of developments our goal was to create a 3D random-access laser scanning two-photon microscope which overcomes mechanical limits posed by earlier solutions. An ideal 3D microscope for neuroscience applications needs to simultaneously satisfy two different needs in the largest possible scanning volume. The first need is to record activity across the dendritic tree of a single neuron at high spatial and temporal resolution in 3D in a way that dendritic spines remain resolvable. The second need is to record in a more extensive volume at high speed in order to capture activities of a large number of cell bodies in a neuronal population.

Several novel technologies have been developed to generate 3D readouts of fast population and dendritic activities; however, in 2012 there were limitations in the use of these methods both *in vitro* and *in vivo*. Acousto-optical (AO) scanning combined with the single-point two-photon ROI scanning approach can penetrate deep in the living tissue and simultaneously increase the measurement speed and the signal-to-noise ratio as compared to classical raster scanning.

In an earlier design we proposed to use optical fibers to achieve 3D random access point scanning and used two AO deflectors to couple light alternating into the fibers (Rozsa et al., 2007). Recent efforts used rather four AO deflectors with which in sequence it is possible to position the focal point in all three dimensions when using synchronized and chirped driving signals.

We created a detailed optical model of the 4 AO deflector sequence. Following the arrangement suggested by the model, a large aperture (15-17 mm) optical assembly was constructed and coupled into a two-photon microscope (Katona et al., 2012). The major difference between the system described here and previous designs is that here the AO deflectors form two functionally and physically different

groups. The first AO deflector pair is used for *z*-focusing, whereas random-access positioning in the *x*-*y* plane is restricted only to the second group of deflectors. This arrangement increased the diameter of the lateral scanning range by a factor of about 2.7. Furthermore, not only electronic driver function, but also deflector geometry, TeO_2 crystal orientation and bandwidth are different between deflectors of the two groups. Altogether these factors increased the diameter of the lateral scanning range up to 720 μm using the Olympus 20× objective and over 1100 μm with the Nikon 16× objective. Although spatial resolution decreased with radial and axial distances from the center of objective focus, PSF size remained small (xy <0.8 μm, z <3 μm) in the central core of the volume (approximately 290 × 290 × 200 μm^3), allowing the resolution of fine neuronal processes and remained below 1.9 μm diameter and 7.9 μm axial length in the whole FOV (now over 1100 × 1100 × 3000 μm^3 in transparent samples) allowing the resolution of cell somata.

We created specialized electronics and software to generate driving signals for the AO scanning. The position and the movement of the focal point is determined by eight values. Four of them control the starting acoustic frequency on the four AO deflector drivers, while the other four define the frequency ramp speeds (chirping). By perturbing calculation of these parameters we could dynamically compensate for various optical errors. Finally, all of the eight parameters need to be continuously updated to the syntheser electronics in every sweep cycle (typically 33.6 μs is used) while the photomultipliers of the system are sampled synchronously.

We extended the microscope control software used in the previous chapters and added new measurement modes for *xy* scanning, 3D Multiple Line Scanning and *z*-stack creation using the AO scanner. We also added new cell localization, point handling and trajectory selecting tools supporting the 3D

requirements. We also added a 3D virtual reality environment to the system.

To examine the temporal resolution of our system we chose imaging propagating activity of single hippocampal neurons in acute brain slices. We patch-clamped CA1 pyramidal cells in whole-cell mode and filled the cells with the green fluorescent Ca^{2+} sensor Fluo-5F and the red fluorescent marker Alexa 594. The imaged subvolume containing the cell was 700 × 700 × 140 μm^3. Action potentials were evoked by somatic current injection while we were able to measure dendritic Ca^{2+} signals near-simultaneously at even eighty-seven 3D locations selected by the experimenter. In other experiments we could measure in fine processes the propagation speed of action potentials or dendritic spikes in an all optical way.

In a recent study we used AO scanning to investigate dendritic spikes on parvalbumin containing interneurons during SPW activity (Chiovini et al., 2014) proving that the AO deflector based 3D scanning methods are indeed usable to address questions unanswerable with other techniques.

To test the performance of our imaging system *in vivo*, we recorded Ca^{2+} responses from a population of individual neurons in the visual cortex of adult anesthetized mice. We injected a mixture of OGB-1-AM to monitor changes in intracellular Ca^{2+} concentrations, and sulforhodamine-101 to selectively label glial cells. The total imaging volume was 400×400×500 μm. First, we recorded a reference *z*-stack and, using an automated algorithm, identified the neuron and glial cell bodies. The algorithm listed 3D coordinates of the centers of each neuronal cell body and these coordinates were used for high speed random-access activity imaging. Next, we presented the mouse with visual stimuli consisting of movies of a moving white bar oriented at eight different angles. We then compared the simultaneously measured responses of the 375 individual

cells to the bar's moving direction (a total 28,125 Ca^{2+} transients were recorded) and found orientation-selective, direction-selective, and orientation-non-selective cells within the neuronal population. In such a population measurement SNR is increased compared to classical raster scanning typically with a factor of 50. These experiments showed that AO scanning can perform simultaneous measurement of activity in large 3D neuronal networks *in vivo*, even during complex measurement scenarios.

APPLICATIONS

In summary, the AO scanning technology has been proved outstanding in biological experiments when one would like to perform functional imaging from sparse regions of interest in 3D. For example, when one would like to follow the activity of multiple dendritic segments of a single neuron (Chiovini et al., 2014) or to map activity of large neuronal networks (Katona et al., 2012).

Optically, 3D AO microscopes are now close to the theoretical maximum which can be realized using the currently available objective lenses, but many other aspects of AO scanning could still be improved. With the developments we would like to extend the system to be able to scan at multiple brain areas simultaneously in order to study communication between sensory, motor and higher order areas and to incorporate photostimulation features during 3D scanning to be able to functionally map connectivity within neuronal networks. We plan to develop novel scanning strategies enabling previously unseen flexibility and precision in fast physiology measurements, and I hope with these enhancements this technology will gain a significant share among the tools used to study brain function.

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LIST OF PUBLICATIONS

Journal articles related to the thesis

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