Real time 3D nonlinear microscopy

J. Fekete, Á. Bányász, R. Szipöcs*
Research Institute for Solid State Physics and Optics, P.O. Box 49, H-1525 Budapest, Hungary
szipoecs@sunserv.kfki.hu

G. Katona, A. Lukács, B. Császár
R & D Ultrafast Lasers Kft. P.O. Box 622, H-1539 Budapest, Hungary

Z. Várallyay, A. Sághy, P. Maák
Budapest University of Technology and Economics, Budafoki út 8, H-1111 Budapest, Hungary

B. Rózsa, E. S. Vizi
Institute for Experimental Medicine, Department for Pharmacology, P.O. Box 67, H-1450 Budapest, Hungary
**also with Institute for Experimental Medicine, Department for Pharmacology*

Abstract: We propose a nonlinear microscope scheme being capable of simultaneous, 3D investigation of the electric activity pattern of neural networks or signal summation rules of individual neurons in a 0.6 x 0.6 x 0.2 mm^3 volume with sub-micrometer spatial resolution.

OCIS codes : 170.5810 Scanning microscopy; 320.2250 Femtosecond phenomena

1. Introduction

It has been a decade since the importance of using nonlinear effects such as two photon absorption for high spatial resolution microscopy was realized [1]. Inspite of the fact that pioneers in the field initially adopted this technique for investigation of electric signaling of neurons, it is still a challenge to describe and understand the arithmetics that neurons use to integrate ongoing inputs from connected neighboring neurons. The major problem of current nonlinear microscope systems is that the image is typically obtained by scanning the (femtosecond pulse) laser beam in a plane and a 3D image is obtained only after subsequent recording of hundreds of 2D images along the optical axis (z-axis). This procedure typically takes a few minutes being several orders of magnitude longer than the signal transition time (< 1 ms) of a neuron. In order to characterize the arithmetic function performed by a neuron, snapshots on its electric activity in the 3D volume should be taken in those points (such as dendritic spines) that were identified as inputs and outputs of a neural "circuit". Unfortunately, these inputs and outputs lay randomly distributed in the 3D volume that can not be investigated simultaneously by laser scanning microscope schemes. This fact is demonstrated in Fig.1, in which a reconstructed 3 D image of an interneuron (Fig. 1a) and a few of the dendritic spines to be characterized (Fig. 1b) are displayed.

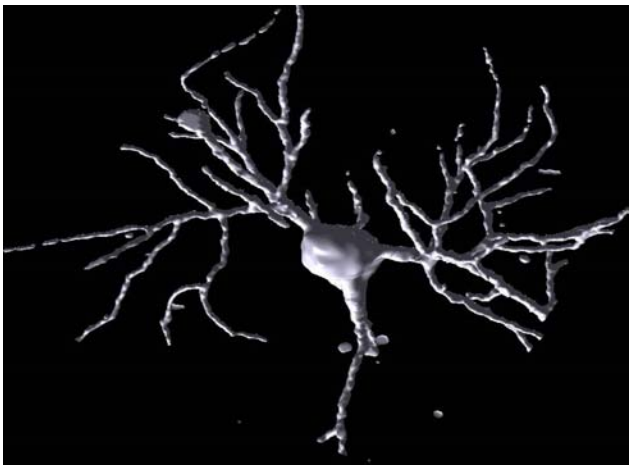

Fig. 1a Reconstructed 3D two-photon image of an interneuron

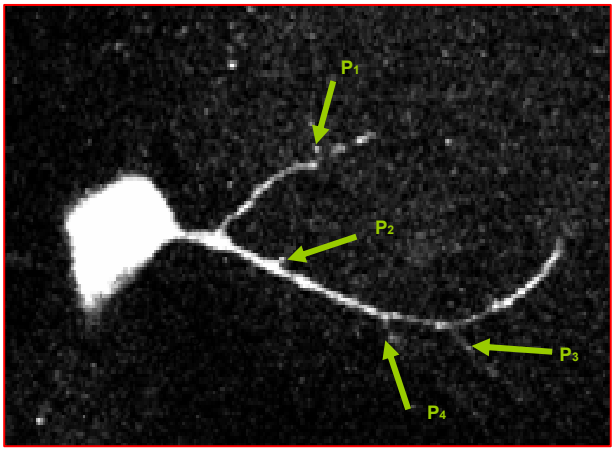

Fig. 1b Two-photon image of dendritic spines to be measured (multiple images are projected in z direction)

In this paper we present a novel two-photon microscope scheme being capable of high speed measurement of neural networks or tiny neuronal structures such as spines in a 3D volume of appr. 0.6 x 0.6 x 0.2 mm^3, while keeping the advantages of a convention two-photon microscope (e.g. high spatial resolution and high penetration depth). The basic idea is as follows: after taking a conventional 3D two-photon image of a biological sample, we determine the coordinates of those points (P_1, P_2 …P_n) in the 3D volume that are to be investigated. During measurement of the neural activity, only these points are sequentially addressed by a high speed acousto-optic (AO) switch combined with a fiber bond (comprising n pieces of single mode optical fibers) and a properly designed optical imaging system.

2. Setup

Our experimental setup is shown in Fig. 2. In our measurements, we used a modelocked Ti-sapphire laser oscillator (FemtoRose 20 MCD [2]) with a central wavelength of 795 nm and FWHM bandwidth of 18 nm. In order to achieve effective two-photon excitation, high energy density is needed, which requirement can be met by the minimization of pulse duration and its spatial extension in the biological sample.

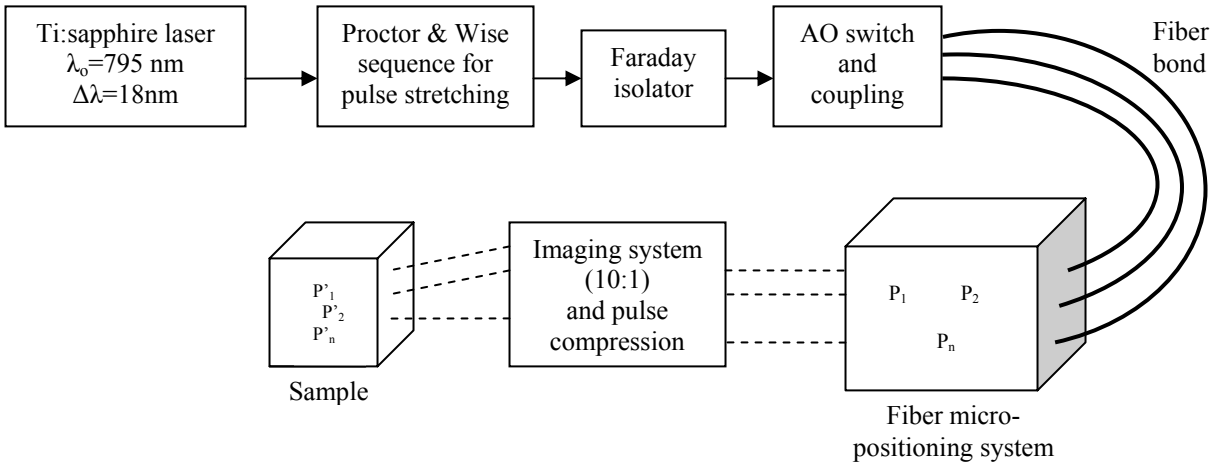

Fig. 2. Experimental setup for real time 3D two-photon imaging

Fluorescent dyes used for detecting Ca^{2+} etc. ions typically have broad absorption bands with FWHM bandwidths ($\Delta\lambda$) of tens of nm-s, which support excitation pulses with time durations around 20 fs and above. In our work, we applied a chirped pulse fiber delivery system for transmission of fs laser pulses through standard single mode optical fiber, in which the temporal shape of the laser pulses and the Gaussian shape of the laser beam profile are restored by minimizing nonlinear effects during pulse propagation in the optical fiber. It is achieved by (1) applying relatively broadband (i.e., temporally short) infrared excitation pulses and (2) applying the chirped pulse concept widely used in chirped pulse amplification (CPA) systems. We found that the transmitted pulse energy can be scaled up to nanojoule energy levels by increasing the spectral width of the laser oscillator being limited by the two-photon absorption bandwidth of the dye applied. An alternative approach is application of large mode area (LMA) photonic crystal fibers [3], which allows distortion free, single mode delivery of femtosecond pulses without applying the CPA concept. Currently, we are adopting this latter approach for our proposed setup. We must note, however, that in case of using LMA fibers, the pulse duration of the mode-locked laser is limited at around 100 fs (at similar energy levels) due to higher nonlinearity and higher sensitivity for dispersive effects in case of shorter laser pulses.

In order to construct a distortion free single mode fiber delivery systems for fs pulses, we applied the CPA concept discussed above: in order to deliver our relatively short ($\tau < 50$ fs) pulses through single mode optical fibers with no temporal distortion, we stretched the pulses by introducing a high negative chirp (GDD ≈ -14000 fs^2) by using a Proctor & Wise four-prism sequence [4]. In front of the acousto-optic switch [5], a Faraday isolator was placed in order to avoid any disturbance of the laser operation by back reflection from the optical fiber bond, which reduced the negative dispersion by GDD $\approx$ 2,700 fs^2. Switching between the optical fibers is carried out by an acousto-optic switch [5] controlled by a computer. Switching time between the fibers could be reduced down to 1-3 μs depending on the number of fibers (up to 10 in one dimension) to be addressed [5]. The acousto-optic switch exhibit an additional positive GDD of $\approx$ 1,500 fs^2. Angular dispersion of the AO switch is compensated by properly

designed, AR coated prisms made of SF11 glass. The acousto-optics switch is then imaged onto a fiber coupling lens by a large diameter doublet lens free of spherical aberration. Optimization of coupling into the single mode fibers is performed by fine frequency adjustment of the AO switch.

3. Measurement of the temporal resolution

In our experiment, we used single mode optical fiber pieces with a length of ≈ 130 mm. After collimating the light exiting the single mode fiber, we measured spatial, spectral and temporal properties of the femtosecond pulses transmitted through the fiber. The dispersion compensating unit built into the imaging system (with physical dimension of 12x12x12 mm^3, that provides GDD ≈ 10,000 fs^2) was placed into the beam path as well before the autocorrelation measurements were performed.

The fiber delivery system was tested by measuring the temporal and spectral properties of the femtosecond pulses at different energy levels. Interestingly, we found that the transmitted spectrum becomes narrower at higher pulse energy levels as a result of interplay between the negatively chirped propagating pulse and the positive frequency chirp induced by self-phase modulation in the optical fiber. The measured spectra and one of the corresponding autocorrelation traces are shown in Fig. 3. According to these measurements, we can say that our chirped pulse delivery system supports sub-100 fs pulse transmission up to ≈ 0.5 nJ energy levels in our proposed experimental setup, which pulse energies are suitable for nonlinear microscopy.

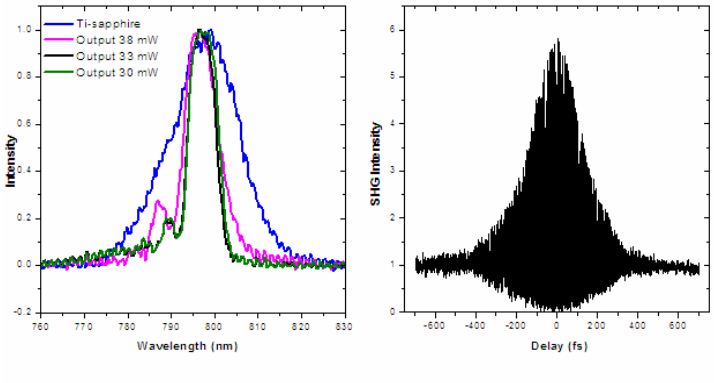

Fig. 3 Input and output spectra of femtosecond laser pulses transmitted through our chirped pulse fiber delivery system (left) and the measured autocorrelation trace at the fiber output (dispersion compensating unit included) at power level of 38 mW (right)

4. Measurement of the spatial resolution

The imaging system located between the fiber micropositioning system and the sample exhibit a 1:10 magnification. In order to measure the focusability of the beam exiting the single mode fiber and the spatial resolution of imaging system, we placed fluorescent beads as samples. The beads had a diameter of 10 μm and were condensed on the surface of a slide glass. We took three dimensional two-photon images of the beads and determined the spatial resolution of the system uing the method described by Kuba et al. [6]. We found that the lateral resolution of our optical system is better than 1 μm in the examined volume of 0.6 x 0.6 x 0.2 mm^3 and 0.6 μm in the middle region.

In conclusion, we can say that our proposed system is suitable for high spatial resolution, 3D nonlinear microscopic investigation of neuronal microcircuits that aren't located in a 2D focal plane and investigation of cortical firing patterns, activity of large neuronal populations that require 3D fast scanning at kHz repetition rates with number of datapoints (n) as high as 100.

4. References

1. Denk, W., Strickler, J. H. and Webb, W. W. 2-photon laser scanning fluorescence microscopy. *Science* 248, 73-76. (1990)
2. For details on laser performance, visit www.fslasers.com
3. Ouzounov DG, Moll KD, Foster MA, Zipfel WR, Webb WW, Gaeta AL, "Delivery of nanojoule femtosecond pulses through large-core microstructured fibers", Optics Letters 27 (17) 1513-1515 (2003)
4. Proctor B, Wise F, "Quartz prism sequence for reduction of cubic phase in a mode-locked $TiAl2O_3$ laser", Optics Letters, 17 (18): 1295-1297 (1992)
5. Maák P, Jakab L, Barócsi A, Richter P, "Improved design method for acousto-optic light deflectors", Optics Communications 172, 297-324 (1999).
6. Kuba K, Nakayama S., "Two-photon laser scanning microscopy: test of objective lenses and Ca+ probes", Neuroscience Research 32, 281-294 (1998).