Real time 3D nonlinear microscopy

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Abstract. We propose a nonlinear microscope scheme capable of real time, 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 mm³ volume with sub-micrometer spatial resolution.

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Introduction

Recent investigations in neurology pointed out the importance of nonlinear effects such as two-photon absorption and second-harmonic generation in high spatial resolution microscopy [1]. Dendritic integration at the cell level and arithmetics of neuronal signaling at the network level however show a need for real time measurements meanwhile keeping the advantages of nonlinear microscopy.

The problem of measuring within the signal transition time across neurons or neural networks has not been solved yet. The reason for this is the following: current nonlinear microscope systems obtain images by scanning a laser beam in 2D and the 3D volume can be reconstructed only after subsequent recording of hundreds of 2D images along the optical axis (z-axis). This procedure typically takes a few minutes which is several orders of magnitude longer than the signal transition time (< 1 ms) of a neuron. Looking at signal propagation in neurons the points to be investigated (such as dendritic spines) lie randomly distributed in 3D space. This requires the ability to carry out measurements on arbitrary points within a 3D volume well within the signal transition time.

In this paper we present a novel two-photon microscope scheme being capable of high speed measurement of signal transitions in neural networks or through single neurons in a 3D volume of approx. 0.6 x 0.6 x 0.2 mm³.

Concept

As an initial step, a conventional 3D image is obtained by taking 2D scanning two-photon images of the biological sample. From this image the coordinates of those points (P₁, P₂ ... Pₙ) are determined in the 3D volume that are to be investigated (see Fig. 1(a)). During the measurement of neural activity, only these points are sequentially addressed by a high speed acousto-optic (AO) switch combined with a fiber bundle (of n optical fibers) and a properly designed optical imaging system (see Fig. 1(b)).
Experimental setup

In order to achieve effective two-photon excitation, high energy density is needed. This requirement can be met by minimization of pulse duration and focused beam diameter. The minimization of pulse duration is limited by the finite absorption bandwidth of fluorescent dyes. Fluorescent dyes used for detecting Ca\textsuperscript{2+} etc. ions typically have a full width at half maximum (FWHM) absorption bandwidth (\(\Delta\lambda\)) of tens of nm-s. This bandwidth supports excitation pulses with time durations of around 20 fs and above.

Initially we used standard single mode optical fibers (SMF) (Thorlabs 780HP) in our work. The temporal shape of the fs laser pulses and the Gaussian shape of the laser beam profile are maintained by minimizing nonlinear effects during propagation. Distortion-free fiber delivery is achieved by (1) applying relatively broadband (i.e. temporally short, \(\Delta\tau \approx 25\) fs transform limited) infrared laser pulses and (2) applying the chirped pulse concept widely used in chirped pulse amplification (CPA) systems. Briefly, the CPA consists of the following steps: (a) stretching of the initially short pulses by high second order dispersion (GDD) in order to decrease the maximum intensity hence the nonlinearity in the fiber/amplifying media; (b) propagation in the fiber/nonlinear media, (c) recompression of the laser pulses by introducing nearly the same dispersion of the opposite sign. Since the free space between the two objectives (OBJ1, OBJ2, see Fig. 2) is limited we use the high space demanding negative GDD setup before the fiber. High negative chirp (GDD \(\approx -14000\) fs\(^2\)) – i.e. pulse stretching – was introduced by a Proctor & Wise four-prism sequence which is adequate for the control of third-order dispersion (TOD) as well \([2]\). High positive GDD after the fiber is realized by a highly dispersive polarizing beam splitter cube (PBS) built into the imaging system.

Our experimental setup is shown in Fig. 2. In our measurements, we used a mode-locked Ti:sapphire laser oscillator (FemtoRose 20 MCD \([3]\)) with a central wavelength of 795 nm and FWHM bandwidth of \(\sim 20\) nm.

A Faraday isolator (FI) was placed after the Proctor & Wise four prism sequence in order to avoid any disturbance of the laser operation by back reflections from the fibers. This FI reduced the negative dispersion by GDD \(\approx 2,700\) fs\(^2\). Switching between the optical fibers is carried out by computer controlled acousto-optic switches \([4]\). Switching time between the fibers could be reduced down to 1-3 \(\mu\)s depending on the number of fibers (up to 10x10) to be addressed. The acousto-optic switches exhibit an additional positive GDD of \(\sim 1,500\) fs\(^2\). Angular dispersion of the AO switches is compensated by properly designed, anti-reflection coated prisms made of SF11 glass. The AO switches are then imaged onto a fiber coupling lens by a large diameter doublet lens free of spherical distortion.

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Fig. 1 (a) Dendritic spines to be measured in a 3D volume (projection of several two-photon images in the z-direction) and (b) the scheme of the acousto-optic switch, optical fibers and imaging system which label the chosen points of the sample for 2-photon measurements.
and chromatic aberration. Optimization of coupling into the single mode fibers is performed by fine frequency adjustment of the AO switches.

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

The output ends of the optical fibers are imaged onto the sample by a 10:1 telescope imaging system. This consists of a collimating objective, the high dispersion PBS cube and a focusing objective. The focusing objective has high numerical aperture (NA = 0.8) for strong focusing and for collecting most of the fluorescent photons exiting the sample after excitation. The fluorescent signal has a blue-shifted spectrum compared to the two-photon excitation (IR) spectrum, consequently the spectral filtering can be performed by dichroic mirrors (D). Fluorescent photons are detected by photo-multiplier tubes (PMT).

The lower part of Fig. 2 is a conventional scanning two-photon microscope (TPM) that is illuminated by the Ti:sapphire laser oscillator when flipping a beam-steering mirror. This unit consists of two scanning galvano-mirrors (that rotate the polarization by 90°) and a lens imaging the mirrors onto the input aperture of the focusing objective (OBJ2). According to its polarization state, the laser beam is reflected on the PBS onto the sample through OBJ2. Fluorescence light has a random polarization so it can be collected on any of the PMTs shown in Fig. 2.

**Temporal characteristics**

The spectral and temporal characteristics of our laser are shown in Fig. 3. Our fiber delivery system was designed according to the following steps. We measured the spatial resolution of the telescope imaging system in order to find the possible maximum dimensions of the PBS that can be built into the imaging system while maintaining acceptable resolution for two-photon microscopy. We also measured the dispersion curve of the PBS and calculated the dispersion parameters (GDD, TOD) that have to be compensated for before the fiber. We made computer simulations for pulse propagation
through the optical fiber in order to control the pre-chirp and optimize pulse parameters for our system. In order to find the optimum arrangement of the Proctor & Wise four-prism sequence [5], we measured dispersion all of the optical components in the system and calculated the corresponding distance values for pulse stretching.

In our computer simulations modeling pulse propagation through the optical fiber, we took into consideration nonlinearities and dispersion of the fiber. This was done by solving the nonlinear Schrödinger equation with the Split-Step Fourier Method [6]. Using the simulation we determined the chirp parameters before and after the fiber and checked the stability of pulse duration at different pulse energies. Fig. 4(a) shows the effect of the recompression of the initially stretched pulse by the high positive dispersion imaging system. Pulses stretched to ~ 1 ps time durations can be recompressed to ~ 40 fs. Fig. 4(b) shows that the pulse duration remains about 40 fs if the coupled energy is 40 mW or below (20, 10 mW).

However, the pulses with the same duration at 80 mW average power (E ~ 1 nJ) can be recompressed only to ~ 100 fs due to nonlinear effects in the fiber. Let us note that if we use a laser with ~ 40 nm FWHM bandwidth it is possible to generate ~ 25 fs pulses at the sample even at 80 mW. This is due to much higher stretching of shorter pulses (more sensitivity to dispersion of shorter pulses) at the same parameters which causes smaller nonlinearities in the fiber.

The fiber delivery system was tested by measuring the temporal and spectral properties of the femtosecond pulses at different energy levels. In the experiment we used single mode optical fibers with a length of 130 mm. After collimating the light exiting the single mode fiber, the dispersion compensating unit built into the imaging system (with a length of 12 mm provided GDD ~
10,000 fs$^2$) was placed into the beam path as well. The measured spectra and one of the corresponding autocorrelation traces are shown in Fig. 5. We found that the transmitted spectrum becomes narrower than the laser spectrum 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.

![Fig. 5](image)

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. Such pulse energies are suitable for nonlinear microscopy.

5. Photonic crystal fibers

As an alternative approach, large mode area (LMA) photonic crystal fibers (PCFs) [7] allow distortion free, single mode delivery of femtosecond pulses instead of applying the CPA concept discussed above. This is demonstrated in Fig. 6a and Fig. 6b, in which the far field intensity distribution of the laser beam exiting the PCF and the laser spectra recorded before and after a PCF sample are shown for 0.5 nJ, ~150 fs pulses, respectively. In our recent experiments we adopted this technique for our proposed setup. We must note that when using LMA fibers, the pulse duration of the mode-locked laser is limited to around 150 fs (at similar energy levels) due to higher nonlinearity and higher sensitivity for dispersive effects for shorter pulses.

![Fig. 6](image)
Measurement of the spatial resolution

The imaging system located between the fiber micro-positioning system and the sample exhibit a 10:1 magnification. In order to measure the focusability of the beam exiting the single mode fiber and the spatial resolution of the telescope imaging system, we placed fluorescent beads as samples. The beads had a diameter of 10 μm. We took three dimensional two-photon images of the beads and determined the spatial resolution of the system using the method described by Kuba et al. [8]. 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 mm³ and even better, 0.6 μm in the central region of the field of view.

![Fig. 7 (a) Two-photon microscope image of 10 μm diameter fluorescent beads measured in different depths of the sample and (b) intensity distribution along the diameter of a bead for evaluation of the resolution](image)

Conclusion

In conclusion, we can say that our proposed system is suitable for high spatial resolution, real-time 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 as high as 100 datapoints.

References

3. For details on laser performance, visit www.fslasers.com