

Real time 3D two-photon microscopy for neurology

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Abstract: We propose a two-photon microscope scheme capable of real time, 3D investigation of the neural activity in a $0.6 \times 0.6 \times 0.2 \text{ mm}^3$ volume with sub-micrometer spatial resolution.

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OCIS codes: 170.5810 Scanning microscopy; 170.2520 Fluorescence microscopy

1. 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 neural activity within the signal transition time 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 after subsequent recording of hundreds of 2D images along the optical axis. This procedure typically takes a few minutes which is several orders of magnitude longer than the signal transition time ($< 1 \text{ ms}$) of a neuron. The points to be investigated 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. $0.6 \times 0.6 \times 0.2 \text{ mm}^3$.

2. 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 that are to be investigated are determined in the 3D volume. 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 imaging system.

3. 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 ($\Delta\lambda$) of fluorescent dyes, used for detecting Ca^{2+} etc. ions. This bandwidth is typically few tens of nm which supports excitation pulses with time durations of around 20 fs and longer.

Initially we used standard single mode optical fibers (Thorlabs 780HP) in our work. The temporal shape of the fs laser pulses is maintained by minimizing nonlinear effects during propagation. Distortion-free fiber delivery is achieved by (1) applying relatively broadband (i.e. temporally short, $\Delta\tau \sim 25 \text{ fs}$ 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; (b) propagation in the fiber, (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. 1) is limited we use the high space demanding negative GDD setup before the fiber. High negative chirp ($\text{GDD} \sim -14000 \text{ fs}^2$) – 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.

The experimental setup is shown in Fig. 1. 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 ~ 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 $\text{GDD} \sim 2,700 \text{ fs}^2$. Switching between the optical fibers is carried out by computer controlled acousto-optic switches [4]. Switching time between the fibers could be reduced to 1-3 μs . The acousto-optic switches exhibit an additional positive GDD of $\sim 1,500 \text{ fs}^2$. 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 and chromatic aberration. Optimization of coupling into the single mode fibers is performed by fine frequency adjustment of the AO switches.

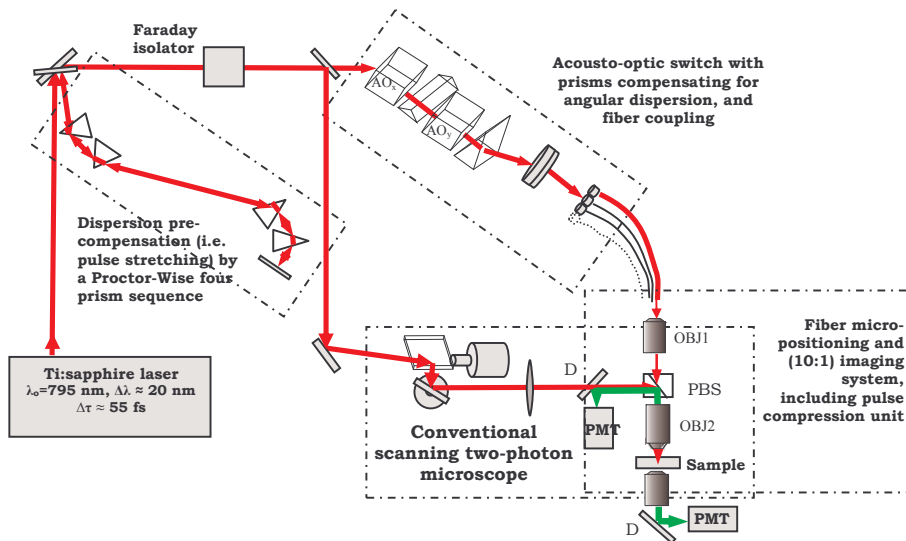


Fig. 1. Experimental setup for real time 3D two-photon imaging

The output ends of the optical fibers are imaged onto the sample by a 10:1 imaging system. This consists of a collimating objective (OBJ1), the PBS cube and a focusing objective (OBJ2). The focusing objective has high numerical aperture ($\text{NA} = 0.8$) for strong focusing and for collecting most of the fluorescent photons. The fluorescent signal is blue-shifted compared to the two-photon excitation spectrum, consequently spectral filtering can be performed by dichroic mirrors (D). Fluorescent photons are detected by photo-multiplier tubes (PMT).

The lower part of Fig. 1 is a conventional scanning two-photon microscope that is illuminated by the Ti:sapphire laser oscillator by 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. 1.

4. Temporal characteristics

We measured and/or estimated the dispersion parameters (GDD, TOD) of all of the optical components that have to be compensated for before the fiber. We made computer simulations for pulse propagation through the optical fiber and optimized pulse parameters and the pre-chirp for our system. We set up the Proctor & Wise four-prism sequence in accordance with these results [5].

In our computer simulations modeling pulse propagation through the optical fiber, we took into consideration the nonlinearities and the 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. Results show that, pulses stretched to ~ 1 ps time durations before the fiber can be recompressed to ~ 40 fs. Stability is verified by showing that the pulse

duration remains about 40 fs when coupled energy is varied (40, 20, 10 mW). However, pulses with 80 mW average power ($E \sim 1$ nJ) can be recompressed only to ~ 100 fs due to nonlinear effects in the fiber.

The fiber delivery system was tested by measuring the temporal and spectral properties of the pulses. We used a single mode optical fiber with a length of 130 mm. The measured spectra and a corresponding autocorrelation trace are shown in Fig. 2(a) and (b). 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.

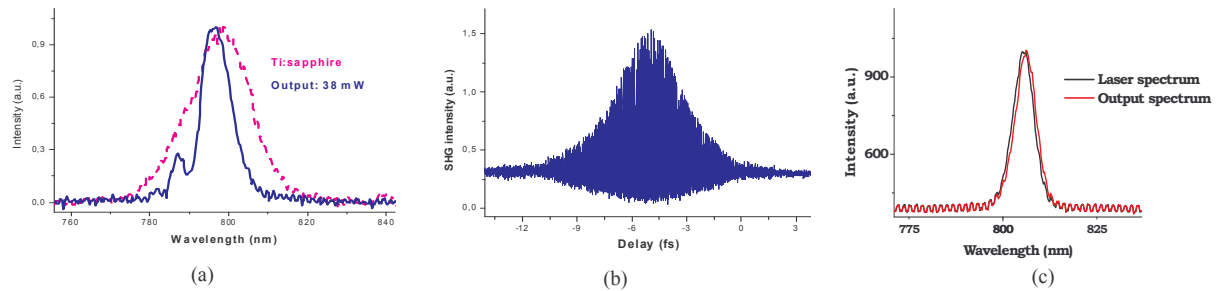


Fig. 2. (a) Input and output spectra of femtosecond laser pulses transmitted through the chirped pulse fiber delivery system and (b) the measured autocorrelation trace at the fiber output (dispersion compensating unit included) at 38 mW (c) Laser spectrum recorded before and after the PCF sample for optical pulses of $\Delta\lambda = 5.5$ nm and pulse energy of ~ 0.5 nJ.

According to the measurements, we can say that our chirped pulse delivery system supports sub-100 fs pulse transmission up to 0.5 nJ energy levels. Such pulse energies are suitable for two-photon microscopy.

5. Photonic crystal fibers

As an alternative approach, large mode area (LMA; core diameter = 15 μm) 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. 2(c), in which 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.

6. Spatial resolution

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 3D 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 $0.6 \times 0.6 \times 0.2$ mm³ and even better, 0.6 μm in the central region of the field of view.

7. Conclusion

Our proposed system is suitable for high spatial resolution, real-time 3D two-photon microscopic investigation of neuronal microcircuits that aren't located in a 2D 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.

8. References

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