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**Visit Patrycjusz Stremplewski to Department of Pharmacology, Case Western Reserve University, Cleveland USA report
[WP2]**

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TABLE OF CONTENT

<i>Introduction</i>	3
<i>Aim of the visit</i>	3
<i>Conclusion</i>	5

Noninvasive two-photon imaging of murine retina *in vivo*

1. Introduction

Two-photon excitation fluorescence imaging of the back of the eye allows visualization of subcellular structures in the living animal eye[1]. This method is helpful for investigating mechanisms of retinal diseases and development of ophthalmic therapies. Endogenous fluorophores, necessary for replenishing visual chromophore, and thus sustaining vision have absorption maxima in the range from 320 – 400 nm. However, anterior optics of the animal eye poorly transmit light at those wavelengths[2, 3]. Two-photon excitation fluorescence (TPEF) imaging employing 75 fs laser pulses overcomes this barrier and visualizes subcellular organelles in the living animal eye.

Reported earlier systems for two-photon imaging, in living small animal eye required application of eye flattening custom contact lens. This technology extended to large animals would require measurement of the eye and fitting the custom contact lens for each animal, both cumbersome and costly[4]. Later we demonstrated optical path for coupling light energy into the mouse eye and capturing emitted fluorescence without using contact lenses[5].

Here we demonstrate further improvement of the TPEF imaging system by introducing new excitation sourcedelivering reduced duration pulses, centered at optimized wavelength and new dispersion compensation unit, capable of compensating high order dispersion components.

2. Aim of the visit

Our goal was to improve the quality of TPEF and reducing laser power needed for imaging by reducing duration of excitation pulse. It is known that the TPEF signal is inversely proportional to the excitation pulse duration[6], thus we can expect, that by using pulse of duration of 30 fs instead of 75 fs, centered at the same wavelength, we should obtain 2.5 fold higher signal. However reduced pulse duration is associated with increased bandwidth of the laser, thus dealing with the dispersion by the optical elements becomes more difficult. For such a short pulses as 30 fs the higher order dispersion (higher than group delay dispersion GDD) become significant, thus traditional dispersion compensation techniques, like chirped mirrors, pairs of gratings or prisms often fail to give satisfactory results, especially in the case of complicated, commercial microscope setup. Multiphoton intrapulse interference phase scan (MIIPS)[7] setup seems well suited for this purpose, since it is capable of compensating higher order dispersion components, it might be easily adjusted when dispersion of the setup changes, and is capable of compensating huge amount of dispersion.

In order to compare results obtained with the lasers generating pulses of 75 fs and 30 fs, we introduced new excitation source and new dispersion compensation unit to our previously reported mutiphoton microscope. Switching between light sources was realized by changing the position of the mirror (Fig. 1). Switching can be realized in approximately 30 seconds, so the conditions of the experiments, especially important when imaging live animals, can be preserved when comparingthe intensities of the images collected with both lasers. Before imaging the power of both lasers was adjusted to the same level (at the output of the objective, and after the experiment (colleting images with both lasers) it was rechecked.

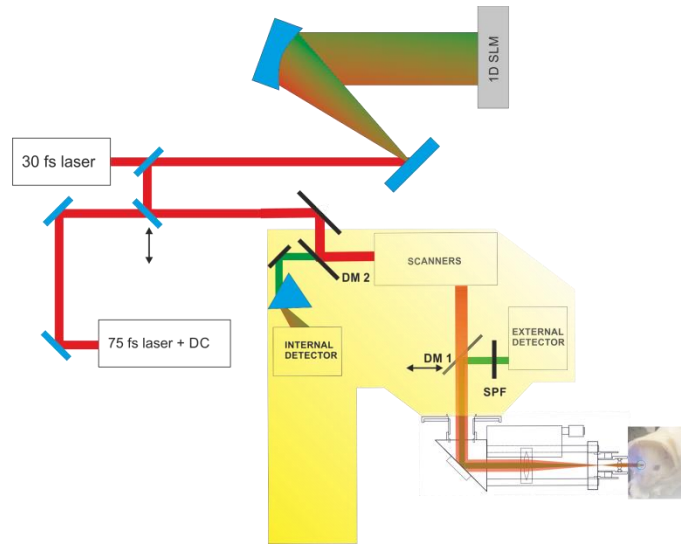


Fig. 1. Experimental setup, 75 fs Coherent Chameleon laser is equipped with dispersion compensation unit (DC), precompensation of pulsed beam of 30 fs Femtolaser Element is realized with commercial MIIPS setup (diffraction grating and SLM), TPEF signals are filtered with dichroic mirror and short pass filter (SPF) or dichroic mirror and monochromator inside the microscope body. For experiments presented in this article we used external detector.

Before the comparison of the intensities of images obtained with both excitation sources we checked the pulse duration of the new laser at the output of the microscope by measuring autocorrelation function with nonlinear crystal placed at the microscope output (Fig. 2a). The first imaging test was performed with a piece of paper. We verified that paper absorbs 745 nm through two photon absorption and emits visible light. In order to compare functions of the signal intensity versus excitation power we collected sets of images of the same area of the object with both lasers using average excitation power ranging from 0.5 mW to 4.5 mW (Fig. 2b). We used mean gray pixel as a measure of the signal intensity. This simple method was correct, since noise level is extremely low for all collected data and we avoided saturation.

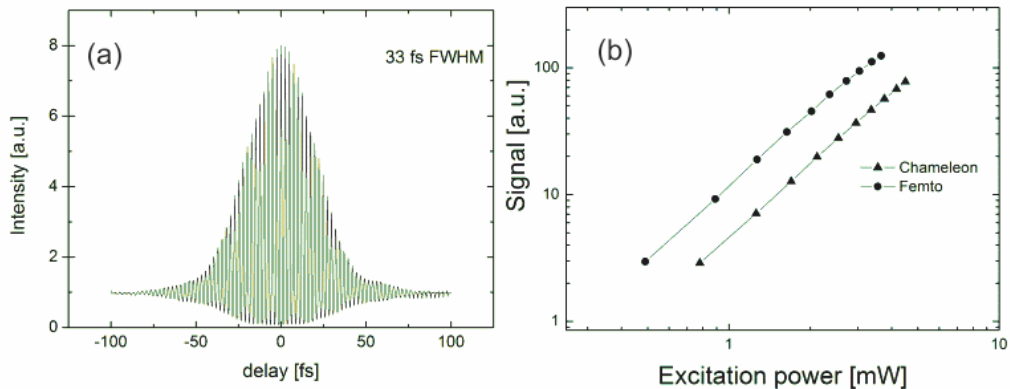


Fig. 2(a) - result of ACF measurement of new laser pulses, (b) - comparison of intensities of the images of TPEF in paper collected with both excitation sources (log-log scale).

For shorter pulses and the same average excitation power we obtain signals 2.85 times higher than with the longer pulse excitation and as expected, for both sources we obtain TPEF signal proportional to excitation power squared.

Another test was performed with the living mouse eye. Mean excitation power at the cornea was 7.5 mW for both sources (Fig. 3). Object remained at the same position during switching the lasers, thus exactly the same area was imaged.

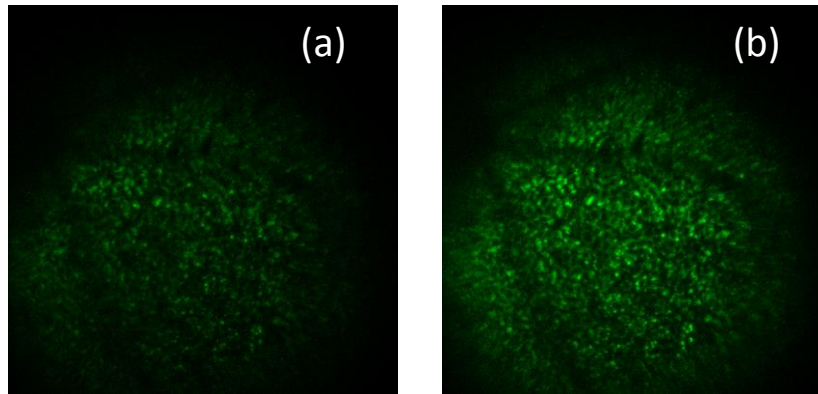


Fig. 3(a) - TPEF image obtained with 75 fs laser, (b) TPEF image obtained with 30 fs laser

Mean intensity of the TPEF is 2 times higher for the image obtained with shorter pulse laser, ratio of the signals is similar to the one obtained with paper as an object.

3. Conclusion

Here we demonstrate progress in TPEF imaging by incorporating new excitation source to complicated microscope setup (of large amount of dispersion). Combination of short pulse laser and previously reported periscope which provides an interface with a commercial microscope allows for obtaining high quality TPEF images of the RPE in vivo.

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