Interferometric Spectral Modulation of sub-100-fs Pump Pulses for High Chemical Contrast, Background Free, Real Time CARS Imaging

Gábor Molnár[1], Ádám Krolopp[2], Norbert Kiss[3,4], Gábor Tamás[1], Róbert Szipőcs[2,4,*]
[1]*MTA-SZTE Research Group for Cortical Microcircuits, University of Szeged, Közép fasor 52, Szeged, H-6726, Hungary*
[2] *R&D Ultrafast Lasers Ltd, P.O. Box 622, H-1539 Budapest, Hungary*
[3]*Department of Dermatology, Venereology and Dermatooncology, Semmelweis University, Budapest, Hungary*
[4] *Wigner RCP, Institute for Solid State Physics and Optics, P.O. Box 49, H-1525 Budapest, Hungary*
** r.szipocs@szipocs.com*

Abstract: A simple, fast interferometric spectral modulation technique is proposed for nonresonant background suppression during CARS imaging. We demonstrate that the proposed setup is also suitable for real time stain-free histopathology of the brain.
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1. Introduction

Coherent anti-stokes Raman scattering (CARS) [1] microscopy is widely used in label-free biomedical imaging applications. For *in vivo* diagnostic applications of CARS microscopy, wide field detection is preferred to descanned configurations [2]. Chemical selectivity poses a major difficulty when femtosecond pulse lasers are applied, since their spectral bandwidth is typically significantly higher (~5-10 nm) than the optimum value (~ 1 nm) matching the bandwidth of molecular vibrations. This fact leads to the appearance of an enhanced non-specific background and the decrease of spectral sensitivity in CARS imaging. In a recent publication, Li et al. [3] proposed a „multi-wavelength time-lens source" for nonresonant background suppression in a CARS imaging system utilizing a mode-locked Ti-sapphire laser (~100 fs, ~80 MHz) for the generation of the pump pulses. In their experiment, they used two picosecond pulse, Yb-fiber amplifiers that were synchronized with the Ti-sapphire laser. One of the Yb-amplifier was tuned "on resonance", the another "off resonance" relative to the CH_2 stretching frequency of 2845 cm^{-1}. Using the electronic pixel clock signal of their microscope, they were continuously switching between the resonant and anti-resonant Stokes lasers, so the recorded CARS image contained both the resonant and the anti-resonant CARS signals detected practically at the same time in subsequent pixels. In this way, after some post-processing of the image, the nonresonant background was successfully removed.

In this paper, we propose an alternative, fast (and cost efficient) spectral modulation technique for sub-100 fs pulse Ti-sapphire lasers, which allows us to modulate the laser spectrum on a ms time scale with the use of a piezo-driven Michelson interferometer. Switching between the properly shaped "on-resonance" and "off-resonance", laser spectra can be synchronized either to the electronic "line" or to the "frame" signals of our laser scanning microscope, which allows us to perform real time nonresonant background suppression during CARS imaging. In an alternative setting, we can modulate the relatively broad laser spectrum in such a way, that CARS imaging for "lipids" and "proteins" does not require any tuning of the pump (Ti-sapphire) laser or readjustment of the delay between the pump and Stokes (Yb-amplifier) pulses, which paves the way for real time stain-free histopathology [4].

2. Experimental setup

We used a CARS imaging setup similar to that was reported in Ref. 4. For our present studies, we constructed a small size Michelson interferometer (see Fig.1). In the beam path of the Ti:sapphire laser (inside our sealed *FemtoCARS Unit*), we replaced one of the 45 degree folding mirrors by our small size interferometer. One of its mirrors (M2) was placed on a piezo-electric linear actuator with a maximum travelling distance of 2.8 μm. The optical path difference (2*ΔL) had an offset value of ~0.1 mm, which resulted in different modulated laser spectra at the interferometer output depending on the phase difference of the two arms. We tested our setup for nonresonant background suppression at CH_2 stretching frequency of 2845 cm^{-1}, for which the laser central wavelength was set to 796 nm. Depending on the phase difference at the central wavelength, we were capable to generate "on resonance" and "off resonance" laser pulses with a spectral maximum or minimum at central wavelength (phase difference 0 or π in the interferometer). In this case, we faced the problem of different average power of the pump laser after the interferometer, that is why nonresonant background suppression required some post-processing of the images. For our stain-free histopathology imaging experiments [4] on in vitro brain slices, we used different interferometer

settings: we switched the phase difference between $\pi/2$ and - $\pi/2$ at the laser central wavelength of 975 nm. As a result we obtained two different laser spectra with maxima at 793 and 796 nm, respectively. In this latter case the power measured at the output of the interferometer was practically the same for the two different settings. In the following, we show representative images recorded from *in vitro* brain slices using the latter settings.

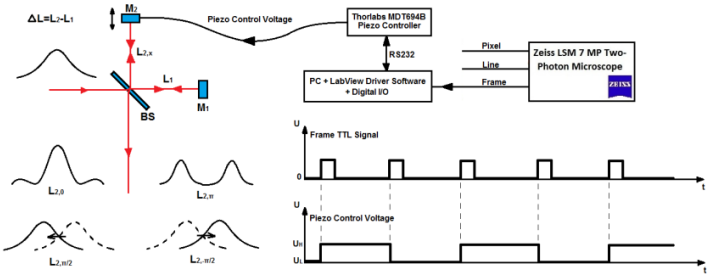

Fig. 1 A Michelson-interferometer is used for spectral modulation of the pump pulses. Depending on the electronically controlled phase difference of the two arms, different spectra can be generated for CARS imaging. ΔL has a properly set offset value depending on the spectral bandwidth of the laser applied. The phase difference is electronically controlled and synchronized to the frame (or line) signal of our microscope.

3. Results

Myelin sheaths - wrapping around the axons of neurons - are rich in lipid therefore we can record high quality 3D CARS images [5]. Degradation of the myelin sheath is the cause of neurodegenerative diseases, such as multiple sclerosis (MS), but a clear mechanistic understanding of myelin loss is missing. Previously we studied myelin breakdown in murine models with multiple sclerosis (MS) using the toxin cuprizone [5]. We found that myelin debris form lipid droplets alongside myelinated axon fibers. For quantification of lipid debris we used a custom-made software for segmentation and three dimensional reconstruction. For automatic lipid reconstruction, however, strong and specific lipid signal is needed. Therefore, to exclude CH_3 signal originating primarily from proteins and nonresonant background we introduced spectral modulation with our interferometer. In coronal brain slices of Wistar rat (P21, somatosensory cortex) we aimed to map and characterize lipid distribution in areas of layer 6 and in the lipid rich white matter tracks (Fig.2). We performed CH_2 (lipid) and CH_3 (protein) signal separation by spectral modulation tuning its peak from 793 nm to 796 nm using an interferometer (Fig.2, A,B). After subtracting images (CH_2-CH_3) we observed strong CH_2 contrast but less CH_3 contrast from white matter lipid structures. Using inverse image subtraction (CH_3-CH_2) we observed rich CH_3 contrast of the background neuropil and neuron somata, and less lipid structures from CH_2 vibration (Fig.2, C,D). Composite image shows minimal overlap of the images, which indicates more specific chemical selectivity (Fig.2, E).

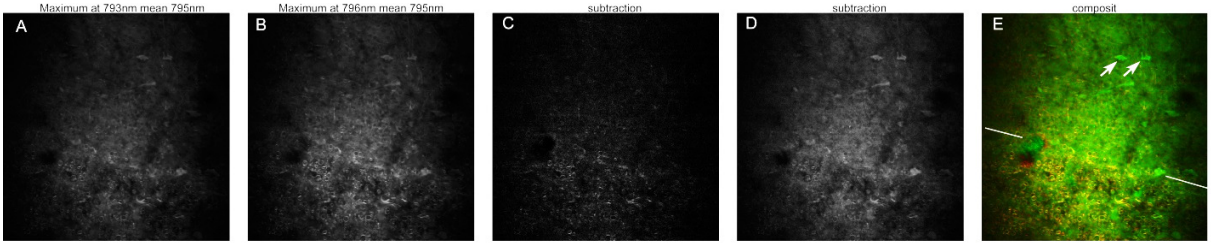

Fig. 2 IF-CARS imaging of *in vitro* brain slices of Wistar rat with a Mai Tai pump laser tuned to 795 nm. A-B) Images of somatosensory cortex coronal slices of layer 6 and white matter were recorded for two different, spectrally modulated pump pulses with spectral maxima at 793 nm ("CH_3") and 796 nm ("CH_2"). C) Subtraction of images (CH_2- CH_3) shown in Fig. 3 highlight more lipid structures. D) Inverse image subtraction (CH_3- CH_2) reveals protein rich background and somata of neurons. E) Composit image of lipid (red, CH_2- CH_3) and protein (green, CH_3-CH_2). Arrows show somata of neurons in layer 6, sidelong lines show the border of layer 6 and the white matter. Scalebar 50 µm.

4. References

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