OPTICAL IMAGING USING COHERENT ANTI-STOKES RAMAN SCATTERING AND OTHER NONLINEAR MECHANISMS

Application notes

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In this work multimodal operation of commercially available CARS microspectrometer (EKSPLA CARSCOPE) equipped with a picosecond dual wavelength laser source (EKSPLA PT259) was demonstrated for the imaging of various biological objects using coherent anti-Stokes Raman scattering (CARS), two photon excited fluorescence (TPEF) and second harmonic generation (SHG) contrast mechanisms. Obtained CARS-images of yeast cell and flower pollen clearly demonstrate label-free and 3D imaging capabilities of CARS technique. Multi-contrast images of green algae and plants containing starch demonstrate a variety of research facilities, where the method can be applied.

1. INTRODUCTION

In recent decade the coherent anti-Stokes Raman scattering (CARS) phenomenon has found multiple applications in microscopy as an imaging technique [1-6]. This tool enables chemically selective visualization of various objects using vibrational contrast. CARS microscopy is primarily marketed as label-free technique, where the contrast mechanism is based on intrinsic molecular vibrations. The CARS process is nonlinear light-matter interaction. Thus, similarly as two photon excited fluorescence (TPEF) it allows 3D sectioning, circumventing the need for confocal detection. Moreover, using near infrared excitation wavelengths CARS microscopy enables a probe of thick tissue up to 100 μm in depth. Detected anti-Stokes signal is blue shifted with respect to the excitation wavelengths and therefore CARS signal is free from linear fluorescence. In comparison to spontaneous Raman microscopy, CARS signal is coherent and directional, with several orders of magnitude higher intensity. All mentioned advantages make this technique very attractive to imaging purposes.

Fundamentals of the coherent anti-Stokes Raman scattering imaging may be found in detailed reviews [7, 8]. Briefly, the CARS signal originates from the four-wave mixing process involving three laser pulses – pump, probe and Stokes with frequencies $\omega_p \geq \omega_p' > \omega_s$, respectively. In most of implementations pump and probe frequencies coincide ($\omega_p = \omega_p'$). Thus two-color light sources are used in this technique and the CARS signal is obtained at the frequency $\omega_{as} = 2\omega_p - \omega_s$. Optical parametric oscillator (OPO) pumped by a high repetition rate ultrashort pulsed laser currently is considered as a most suitable source for CARS microscopy. It features widely tunable two-color output in near-infrared region. Using near infrared excitation wavelengths for microscopy eliminates or significantly reduces possibility of photochemical damage.

The pulse duration of several picoseconds is a good compromise between high intensity and narrow spectral bandwidth necessary for the CARS microscopy [3]. Its intensity is sufficient also for observation of other nonlinear responses of the matter, e.g.: TPEF or second harmonic generation (SHG). Considering CARS microspectrometer as a multimodal platform for nonlinear optical imaging extends capabilities of equipment and helps to reveal different properties of investigated samples.

In this work multimodal operation of commercially available CARS microspectrometer CARSCOPE from EKSPLA was demonstrated for the imaging of various biological objects using CARS, TPEF and SHG contrast mechanisms.

2. EXPERIMENTAL SETUP

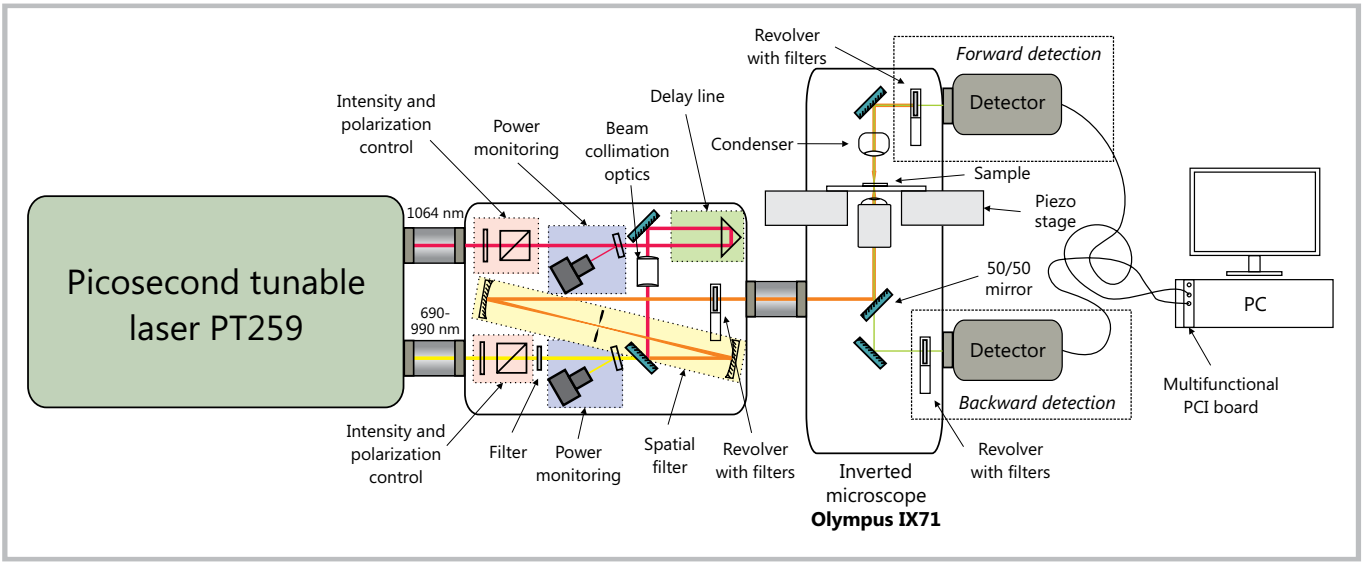

Fig. 1. Block diagram of microscpectrometer CARSCOPE (EKSPLA).

The block diagram of experimental setup is shown on *Fig. 1*. Picosecond tunable laser PT259 (EKSPLA) is used as an excitation source. Both excitation beams (Stokes and pump) are collinearly combined in main optical unit of microspectometer and directed to an inverted microscope Olympus IX71. A spatial filter was used to improve the beam profile. The excitation light was focused on the sample with an oil-immersion objective (Olympus, Plan Apochrom., 60X, NA 1.42). In the forward detection scheme the CARS signal was collected by a long working distance condenser. Long-pass and short-pass filters were used as a blocking tool for spectral separation of the CARS signal. CARS radiation was detected with the avalanche photodiode (Perkin Elmer), which was connected to a multifunctional PCI board (National Instruments Ltd.) A piezo scanning system (Physik Instrumente GmbH & Co) was used for scanning the sample.

EKSPLA tunable laser PT259 consists of picosecond Nd:YVO_4 pump laser with the pulse repetition rate of 1 MHz equipped with a frequency doubling unit and traveling wave optical parametric generator (OPG) integrated into single compact housing. It features 700-2300 nm tuning range, 5 ps pulse duration and nearly Fourier transform-limited linewidth. It also contains additional port for fundamental pump laser radiation (1064 nm) access, which is specially designed for CARS application.

Using this laser source, three different combinations of excitation beams coupling can be realized: OPG signal wave (700–990 nm) with fundamental wave (1064 nm), OPG idler wave (1150–2300 nm) with fundamental wave or OPG signal wave with OPG idler wave. In all cases the range of accessible vibrations is approx. 700–4000 cm^{-1}, which covers all most important vibrational modes. In our scheme for CARS implementation OPG signal wave was coupled with fundamental wave (1064 nm) and used as pump and Stokes excitation beams, respectively. This configuration was chosen, because it allows using simpler, commercially available microscopy optics and achieving best possible spatial resolution. Furthermore, in longer than approx. 1300 nm wavelengths water absorption increases significantly. This effect drastically reduces damage threshold of investigated biological objects.

Measurements of the CARS spectra were performed by tuning OPG wavelength and changing filter sets within five different spectral ranges, enabling investigations with the typical detection rate of 5 cm^{-1}/s. The single frame scans of 200×200 pixels images were obtained with 2 ms pixel dwell time. Excitation pulse energies from 0.1 to 1 nJ at the sample for both pump and Stokes beams were used. Scanning control, data processing and the laser wavelength tuning were controlled by a computer.

TPEF and SHG signal detection was realized using the epi- and forward detection, respectively, and utilizing appropriate band-pass and interference filters.

3. RESULTS AND DISCUSSION

3.1 CARS CONTRAST

For CARS imaging demonstration we chose a cell biology model organism – the yeast cell in water. *Fig. 2* shows a 3D picture of yeast cell distinguishing the distribution of lipids inside cell.

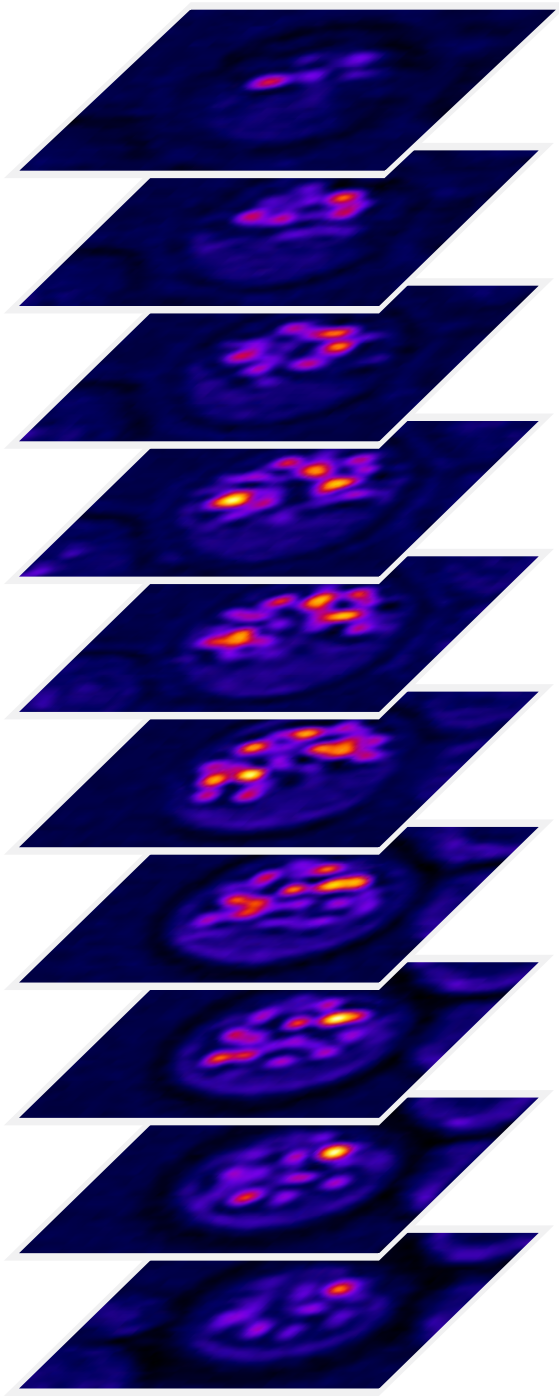

Fig. 2. CARS image of yeast cell recorded at 2850 cm^{-1} vibrational frequency. Intensity scale bar indicates the number of photons for a single pixel from 0 to the maximal value.

Image was recorded at the 2850 cm^{-1} vibrational frequency – the region where C-H vibrations are active. The spectrum of the one of lipid droplet inside the yeast cell was measured as well (*Fig. 3*). The spectrum contains several peaks at 2850 cm^{-1} and 2900, 2920 cm^{-1} which were assigned to the -CH_2, -CH_3 symmetrical, and -CH_2, -CH_3 asymmetrical stretches correspondingly.

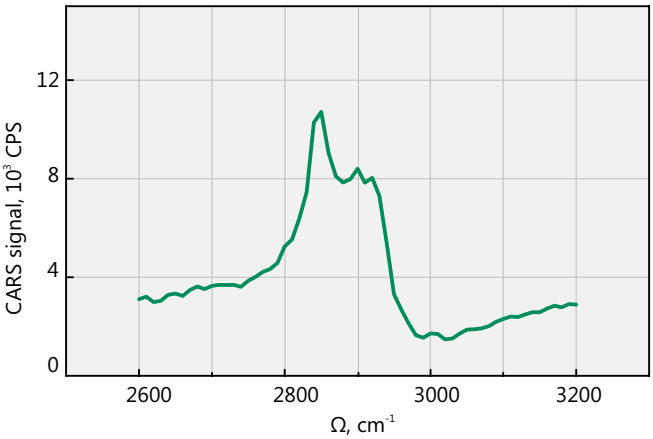

Fig. 3. The spectrum of the one of lipid droplet inside the yeast cell.

The resolution of image is limited by the excitation wavelengths and numerical aperture (NA) of focusing objective. In our case, when 813 nm and 1064 nm excitation light was focused on the sample using objective with 1.42 NA the resolution was of the order of 0.5 μm and 1.5 μm in lateral and axial directions respectively.

Fig. 4 shows 3D projection of flower pollen grain located on the surface of objective cover glass.

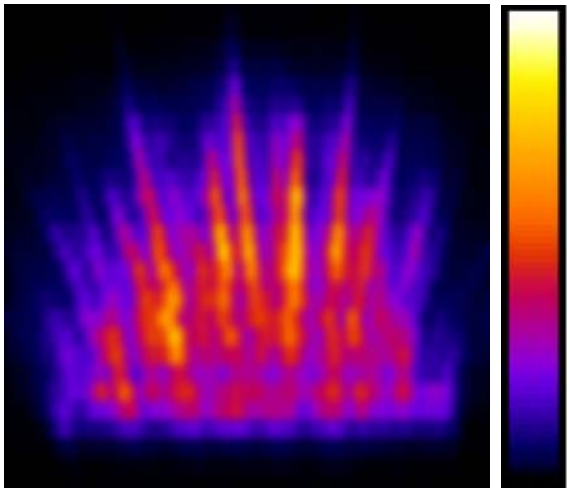

Fig. 4. Three-dimensional projection of flower pollen grain located on the surface of objective cover glass. The scanned volume is cube with size of 15 μm.

Image recorded at the 2880 cm^{-1} vibrational frequency. The pointed growths on the grain surface are clearly visible in picture.

3.2 TPEF CONTRAST

Operation of CARS microspectrometer in TPEF imaging mode can be realized in single beam excitation scheme utilizing either OPO or fundamental beam output for sample excitation. The contrast of TPEF image can be enhanced by entering of special fluorophores (labels) into the sample. Often the object under investigation has a strong autofluorescence property enabling label-free TPEF imaging. For instance, the plants contained a lot of chlorophyll give a strong autofluorescence signal. In such type of objects the label-free studying using both TPEF and CARS contrasts can be realized.

The imaging of green algae Nostoc commune with CARS and TPEF contrast mechanisms are shown in *Fig. 5*. This algae has two types of cells: normal cells which have chlorophyll, and heterocyst cells which do not have chlorophyll. *Fig. 5(a)* shows CARS image tuned to CH_2 vibration contrast where both types of cells are visualized. *Fig. 5(b)* shows TPEF image of chlorophyll fluorescence. In this figure heterocyst cell is not visible. Combined image (*Fig. 5(c)*) of two contrasts revealed all type of *Nostoc commune* cells.

In general, TPEF is a competitive process in the CARS technique. It should be noted that correct measurement of CARS signal is possible only in the case when at least one of the excitation beams does not induce the TPEF. Such a beam (not exciting the TPEF) should be used at maximum acceptable intensity, while the beam generating TPEF must be attenuated. Recording CARS image of algae Nostoc commune contribution from TPEF in our experiment was less than 3%.

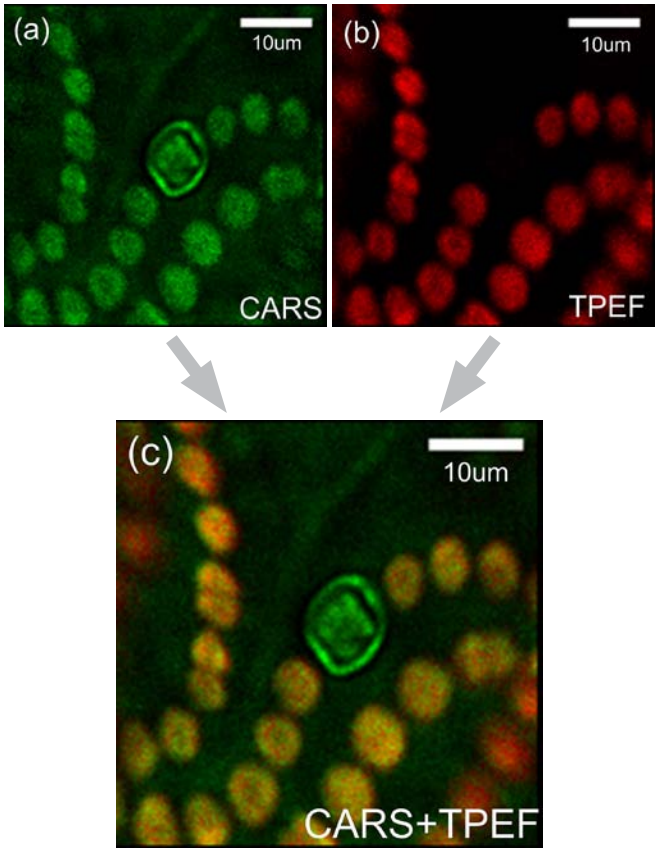

Fig. 5. Pseudo color images of green algae Nostoc commune using different contrast mechanisms: coherent anti-Stokes Raman scattering (a), two-photon excitation fluorescence (b) and overlay of CARS and TPEF images (c).

3.2 SHG CONTRAST

SHG occurs only in the presence of the compound having a non-centrosymmetric structure. In biological objects the second harmonic is obtained in compounds such as collagen and starch. The imaging based on SHG contrast mechanism reveals the distribution of collagen in life tissue or starch distribution in plant cells.

Most distinguishing SHG can be observed in starch grains pulled from potatoes. *Fig. 6* demonstrates combined image of some starch grains located on the surface of objective cover glass. In linearly polarized light the SHG picture (green) has a two-lobed pattern. Such a pattern is due to radial orientation of glucose polymers inside the granule [9].

SHG images of some crop plants contained a lot of starch are depicted in *Figs. 7-9*. For better perception the combined SHG and CARS image is depicted. CARS signal painted red color.

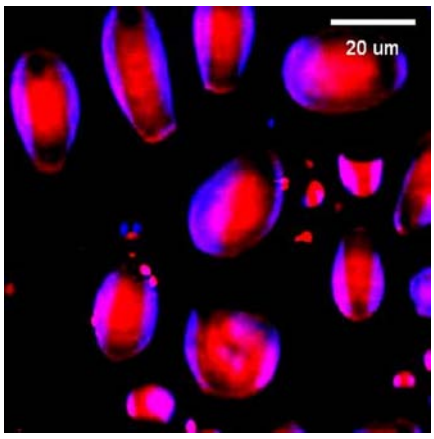

Fig. 6. Pseudo color (overlay) image of starch grain of potatoes recorded using two contrast mechanisms: CARS and SHG.

Fig. 7 shows the image of starch grains of buckwheat seed (*Fagopyrum*) recorded in two contrasts: SHG (green) and CARS (red). The SHG signal comes from the starch granules for which the orientation of glucose polymers (nonlinear dipoles) coincide with linearly polarized incident light oriented horizontally [9].

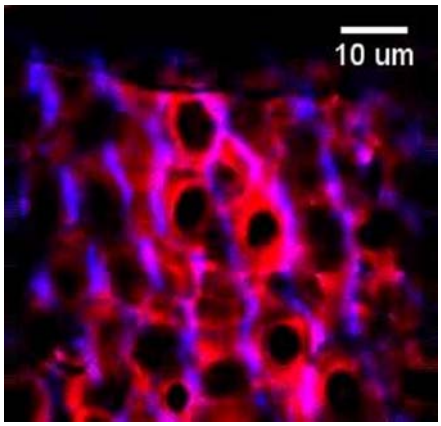

Fig. 7. Pseudo color (overlay) image of *Fagopyrum* recorded using two contrast mechanisms: CARS and SHG. The image recorded at the 2880 cm^{-1} frequency.

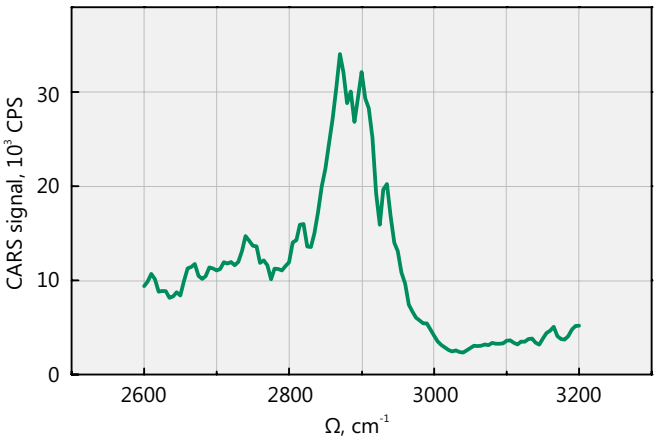

Fig. 8. CARS spectra of *Fagopyrum*.

Fig. 9 shows SHG/CARS image of starch grain of barley (*Hordeum vulgare*) seeds. The SHG-image (green color) of the starch grain has a typical pattern when the linearly polarized incident light is used.

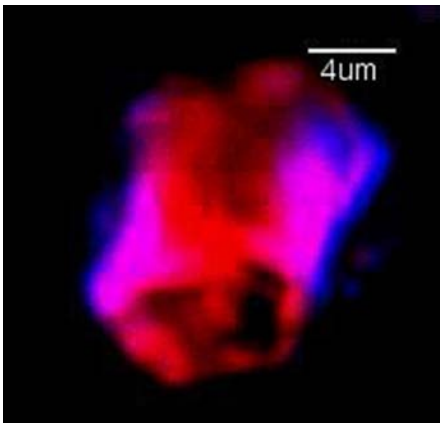

Fig. 9. Pseudo color (overlay) image of starch grain of barley (*Hordeum vulgare*) recorded using two contrast mechanisms: CARS and SHG.

CONCLUSIONS

We have demonstrated that the CARS microspectrometer equipped with a picosecond dual wavelength laser source provides the unified multimodal platform suitable for microscopic investigations using second-order and third-order nonlinear optical contrast mechanisms such as TPEF, SHG and CARS. Obtained CARS-images of yeast cell and flower pollen clearly demonstrate label-free and 3D imaging capabilities of CARS technique. Multi-contrast images of green algae and plants containing starch demonstrate a variety of research facilities where the method can be applied.

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