

# CARS microscopy for biomedical imaging

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## Introduction

Recognition of diseases on a molecular level is of utmost importance for diagnosing and the developing novel therapeutic approaches. Up to now staining of the samples and a highly experienced pathologist is needed. Therefore, the development of objective microspectroscopic tools for inspection of chemical and morphological composition of tissue is of large interest. Especially noninvasive, computer aided routines are desirable. As a promising imaging tool we use coherent anti-Stokes Raman scattering (CARS) providing a label-free and fast method with 3D sectioning capabilities<sup>[1]</sup>.

It has been shown that certain cancer types can be distinguished by Raman measurements<sup>[2]</sup>. Furthermore comparative studies between Raman and CARS have been performed<sup>[3]</sup>. We present CARS as a method which allows us to visualize morphological as well as chemical contrast in tissue samples without staining. In addition image processing can be used to enhance contrast and highlight several features.

## Methodology

For these investigations we concentrated on the CH stretching vibration ( $\sim 2900\text{ cm}^{-1}$ ) due to its high Raman cross section. The CARS images were collected with the setup shown in figure 1. The Ti:sapphire laser produces 3 ps pulses with a spectral width of 1 nm and a repetition rate of 76 MHz. The average power of  $\sim 3.5\text{ W}$  can be achieved within a wavelength range from 700 to 1000 nm. The Ti:sapphire output is split into two parts by a 60:40 beam splitter. One of the beams is used to pump an optical parametric oscillator (OPO) (APE, Germany). The wavelengths for Stokes and pump-laser employed are 831 nm and 669 nm, respectively. To adjust the wavelength difference between pump and Stokes-laser, i.e. to scan over the CH stretch region, the pump wavelength was tuned from 665 nm to 675 nm. The radiation was focused on the sample with an 20x, NA 0.4 objective (Olympus) and the CARS signal was detected by photomultipliers (Hamamatsu R6357) in the forward direction.

Colon tissue samples are dried on quartz slides having a thickness of 10  $\mu\text{m}$ . Polystyrene beads with a diameter of 5  $\mu\text{m}$  are embedded in 1% agarose (Invitrogen) gel. Thin slides have been cut and fixed on slides.

The CARS images were exported from the Zeiss LSM software as bitmaps and converted (without loss of information) into portable graymaps (pgm). All consecutive computational steps were carried out with the Gnu R software (<http://www.r-project.org>).

## Results and discussion

Images of pathological and physiological tissue have been recorded for different Raman shifts. The whole CH stretching band has been covered ( $2800\text{ cm}^{-1}$  to  $3000\text{ cm}^{-1}$ ) including nonresonant regions. Figure 2 shows raw CARS images of tissue under resonance and non-resonance conditions. For both regions morphological

contrast is obtained. This occurs due to ubiquitous nonresonant, frequency-independent contributions. Tuning to the resonance one can see higher contrast in the images due to the CARS process which has been investigated in detail<sup>[4], [5]</sup>. The images obtained show the distribution of the excited vibrations in the cells highlighting several areas where either the density of the tissue differs from the environment or more molecules of interest are located.

Hence, with CARS we can provide a method of fast imaging (one image of 512 x 512 pixels can be obtained in 10 seconds) showing the structure of the tissue without any staining. Furthermore the chemical contrast in the resonant regions provides additional information on the distribution of certain functional groups. By tuning to other Raman shifts one can obtain information not only on the CH stretching vibration, e.g. on the ring breathing vibration or vibrations of the protein backbone.

The nonresonant background mentioned before significantly reduces the molecular contrast. Therefore several methods have been introduced to this background. One can either physically change the image recording process to suppress<sup>[6], [7]</sup> or use image processing tools for reducing the background (noise). Investigations concerning this procedure concentrated on recovering the shape and size of the examined objects<sup>[8]</sup>.

Several effects occurring in the CARS process have been described, e.g. interference effects<sup>[9]</sup>. To investigate those effects in order to improve contrast or to highlight certain for tissue samples we chose polystyrene beads as a model system. The CARS images of the polystyrene beads under resonance conditions show mainly CARS signal (cf. Figure 3c). Under non-resonance conditions fringe effects can be observed as black circles surrounding the beads (cf. Figure 3a). An algorithm has been developed to distinguish between the fringe effects, the nonresonant background and the signal using the intensity information of the images. The computed information allows for not only reducing but mainly removing the background and separately plotting the extracted information what can be seen in Figure 3 for resonant and nonresonant CARS images of the polystyrene beads.

We are now able to correct the CARS images in a way that several information can be extracted for simple systems. This procedure has then to be tested for the much more complex biological samples containing a broad variety of molecules.

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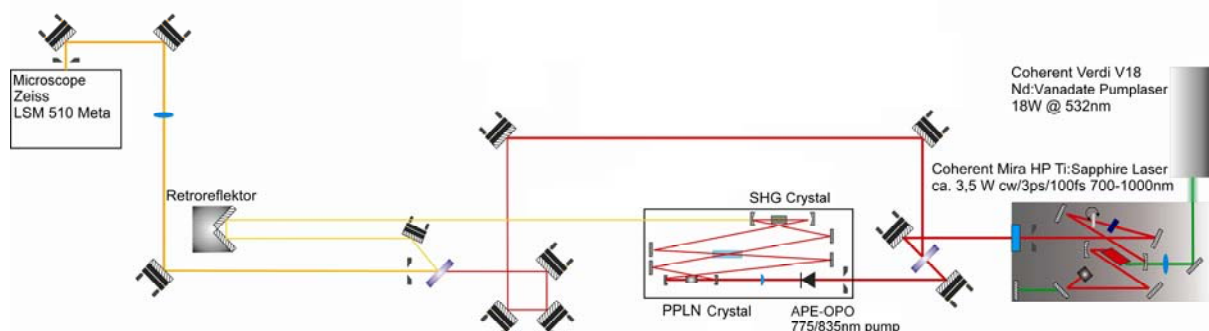


Figure 1: Diagram of the CARS setup.

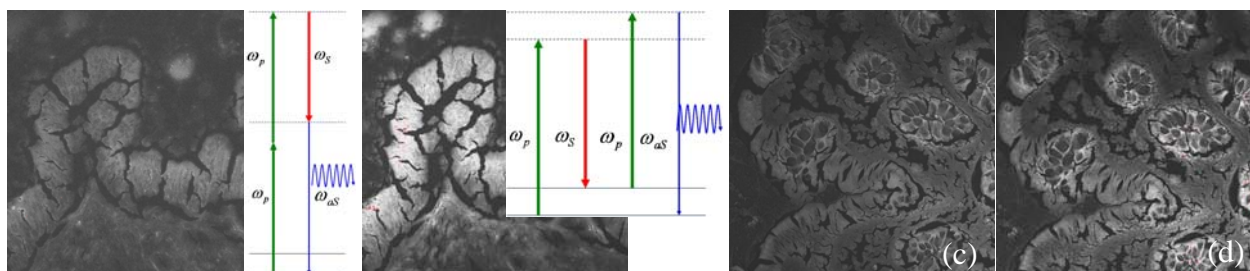


Figure 2: Raw CARS images of colon tissue with insets showing the energy scheme for the four-wave mixing (a) and CARS process (b). (a), (b) Cancerous tissue recorded at  $2817\text{ cm}^{-1}$  and  $2850\text{ cm}^{-1}$ , (c), (d) Healthy tissue recorded at  $2849\text{ cm}^{-1}$  and  $2881\text{ cm}^{-1}$

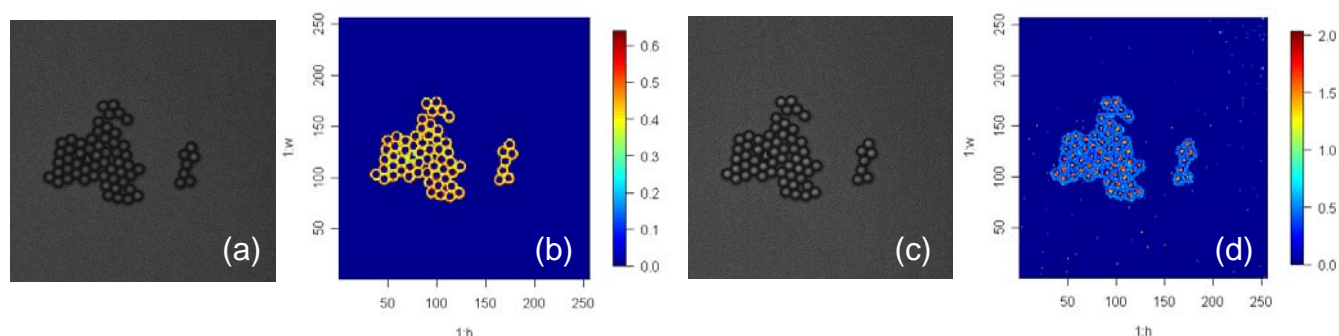


Figure 3: Raw CARS images of polystyrene beads taken at  $2746\text{ cm}^{-1}$  (a) and  $2861\text{ cm}^{-1}$  (c) and corresponding processed images. (b) Fringe effects visible when being off any resonance and (d) Signal and fringe effects in resonance. Diameters of the beads are reproducible.

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