Looking at life through molecular vibrations: biomedical applications of CARS spectro-microscopy
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3 Experimental methods

In this chapter we outline the experimental methods used in the thesis. For CARS experiments two different setups were employed, a single frequency and a broadband CARS microscope. The single frequency CARS setup was used for the research projects outlined in Chapter 4, Chapter 5, and Chapter 6. This setup allows for simultaneous measurements with other non-linear optical techniques which were also used in this thesis, such as Multi-photon Induced Luminescence (Chapter 5) and 2-Photon Fluorescence (Chapter 6). Broadband CARS was used for the projects in Chapter 6 and Chapter 7.

Transmission Electron Microscopy was used for the project in Chapter 5 to validate the results on the uptake of gold nanoparticles in cells. Finally, standard fluorescence was used as a complementary technique for the study in Chapter 7, using a commercial Olympus IX70 inverted microscope.

3.1 Single frequency CARS

The single frequency CARS microscopy setup consists of a picosecond pulsed laser system generating two synchronized beams collinearly aligned into an inverted microscope (Eclipse TE-2000, Nikon, Tokyo, Japan) via a beam scanning unit (C1, Nikon). A fraction of the fundamental output of a Nd:Van laser (Pico-train, HighQ Lasers GmbH, Hohenems, Austria) at 1064 nm is directly coupled into the microscope serving as the Stokes beam in the CARS process, while its frequency-doubled output (532 nm) is used to synchronously pump an Optical Parametric Oscillator (Emerald OPO, APE GmbH, Berlin, Germany). The tuneable output is provided by the OPO, set to 817 nm in this work to form a beating excitation field with the Stokes beam at the frequency 2845 cm$^{-1}$ corresponding to the resonant vibration of the CH$_2$ group in the acyl chain of lipids, so that CARS images predominantly organelles with high lipid content. The laser beams were focused on the sample by an oil immersion objective (Plan Fluor 40x, NA 1.30, Nikon), resulting in a power of 10 mW for each of the beams at the sample position. The CARS signal was collected by an aspherical lens (NA 0.68) in the forward direction and detected by a single-photon counting photomultiplier tube (PMC-100, Hamamatsu) connected to a time-correlated single-photon counting unit (SPCM-830, Becker and Hickl). Bandpass filters (650/50 nm, Chroma Technologies, USA) in front of the detector suppressed the radiation at the laser wavelengths and preferentially transmitted the CARS signal generated at 663 nm. A detailed outline of the setup has been given by Enejder et al. [64]. The setup is schematically shown in Fig. 3.1.
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#### 3.2 Broadband CARS setup

The broadband CARS setup is based on a dual-output laser source (Leukos-CARS, Leukos) providing the pump and the Stokes beams. The source is a passively Q-switched 1064 nm microchip laser, delivering $< 1 - ns$ pulses at 32 kHz repetition rate and 300 $mW$ average power. The laser beam is equally divided into two parts with a beam splitter. One part is sent through a bandpass filter ($FL_{1064 - 10}$, Thorlabs) and used directly as the pump beam. The other part is introduced into a photonic crystal fiber that creates supercontinuum emission from 420 to 2400 nm at the fiber output, with $> 100 \mu W \text{ nm}^{-1}$ spectral power density from 1.05 $\mu m$ to 1.6 $\mu m$. The supercontinuum is coupled out of the fiber with a reflective collimator ($RC_{04APC - P01}$, Thorlabs) and passed through 700 nm ($FEL_{0700}$, Thorlabs) and 880 nm ($LP_{02 - 830RS - 25}$, Semrock) longpass filters. The Stokes and pump beams are overlapped at a dichroic mirror ($LP_{02 - 1064RU - 25}$, Semrock) and introduced into a modified inverted microscope (Eclipse Ti-U, Nikon). The pump and Stokes pulses are tightly focused onto the sample with a near IR objective (PE IR Plan Apo 100X, NA 0.75, Seiwa). The sample is mounted on nested stepper-motor driven (Microstage, Mad City Labs) and piezo driven stages (Nano-PDQ 375 HS, Mad City Labs) that together provide 25 mm travel range with $< 1 \mu m$ resolution. The CARS signal generated by the sample is collected in the forward direc-
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Figure 3.2. Schematic representation of the broadband CARS setup. (1) reflective collimator; (2) 880 and 700 nm long pass filters; (3) mounts for N.D. filters; (4) 250 mm lens used to collimate the pump/probe output; (5) Rail for ns timing adjustment; (6) dichroic mirror and 1064 nm notch filter; (7) 1064 nm notch and 1000 nm short pass filters

Figure 3.2. Schematic representation of the broadband CARS setup. (1) reflective collimator; (2) 880 and 700 nm long pass filters; (3) mounts for N.D. filters; (4) 250 mm lens used to collimate the pump/probe output; (5) Rail for ns timing adjustment; (6) dichroic mirror and 1064 nm notch filter; (7) 1064 nm notch and 1000 nm short pass filters

...tion by another objective \((M - 20X, NA 0.4, Newport)\) and sent through notch \((NF03 - 532/1064E - 25, Semrock)\) and short-pass filters \((FES1000, Thorlabs)\) to remove the pump and Stokes beams. The filtered CARS beam is dispersed by a spectrometer (Shamrock 303i, 300 lines \(mm^{-1}\), 1000 nm blaze, Andor) and detected on a deep-depletion CCD (Newton DU920P – BR – DD, Andor). The schematics of the broadband setup are outlined in Fig. 3.2.

This setup allows to acquire CARS spectra with spectral resolution of \(8 - 15 cm^{-1}\) depending on the grating used. The acquisition time can be as short as 2 ms, although typical experiments on biological samples were performed with acquisition times between 100 ms and 1 s. The optimized spatial resolution of the system is less than 1 \(\mu m\) in plane and about 2 \(\mu m\) axially.

3.3 Transmission Electron Microscope

In order to validate the results on the uptake of gold nanoparticles in cells, transmission electron microscopy was used. The sections of the cells were collected on copper grids and contrasted with uranyl acetate and lead citrate before exami-
nation in a Zeiss 912AB electron microscope (Carl Zeiss SMT AG, Oberkochen, Germany) equipped with a LaB6 electron source operated at 120 kV. Images were collected using a MegaView III CCD camera (Olympus SIS, Münster, Germany).