Looking at life through molecular vibrations: biomedical applications of CARS spectro-microscopy
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SUMMARY

In this thesis we present a series of studies employing non-linear vibrational microscopy, in the form of Coherent anti-Stokes Raman Scattering (CARS) spectro-microscopy. The underlying motivation of this thesis was to explore the limits and demonstrate the technical feasibility of CARS imaging in a variety of biological systems. We begin with examples using CARS in conventional morphological microscopy and end with multivariate spectral analysis, thereby showing the additional information afforded in vibrational imaging that is unattainable with any other technique.

Microscopy is arguably the most invaluable tool in life science research, as it can provide insights into the local organization of biological systems, as well as the underlying biochemical interactions. Also, microscopy can be used to investigate the interaction of biological matter with inorganic particles, widely used in medical therapies both as labeling agents and as target-specific shuttles for drugs. The most prevalent imaging modality offering molecular specificity is fluorescence microscopy, which unlike CARS relies on electronic excitation rather than vibrational excitation to provide the contrast in the recorded images. Because of the necessity of electronic states, fluorescence often requires exogenous molecules attached to macromolecular species of interest. In contrast to fluorescence, CARS uses the inherent vibrational modes of molecules as contrast agent to access the local chemical composition of a biological sample. As vibrations are intrinsic to all molecules, exogenous labels are not required for CARS measurements. This inherent contrast is the crucial difference with fluorescence microscopy, and the main advantage when compared to it.

In CARS experiments, three photons interact with the sample, resulting in the emission of a fourth photon - the anti-Stokes photon. The CARS signal is resonantly enhanced when the frequency of a molecular vibration is matched by the frequency difference of two of the incoming photons. Due to the nonlinear nature of the process, the response of the sample is collected from the tiny (∼ 1 µm³) region in the focal volume only, where the probability of a four-photon event is higher. This provides the technique with intrinsic sub-diffractive resolution and axial-sectioning capabilities. Higher resolution and larger response due to the resonant interaction are the main advantages of CARS spectro-microscopy compared to traditional vibrational techniques such as infrared or spontaneous Raman microscopy.

CARS microscopy is often used in the single line implementation (single frequency CARS), to provide a picture of the spatial distribution of an individual compound, or more accurately of an individual chemical group, in the sample. The other implementation is multiplex CARS, where a vibrational spectrum at
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Each location in the sample is obtained (hyperspectral CARS imaging). Hyperspectral imaging provides richer information as it contains the full vibrational fingerprint of the sample, though it comes at the price of longer acquisition times. Both experimental approaches have been used in this thesis, and are described in chapter 3.

The work presented in this thesis covers a number of selected biomedical topics, demonstrating the successful applicability of CARS spectro-microscopy. Chapters 4 and 5 detail the study of inorganic particle uptake in living cells, which is of interest in medical therapeutics and diagnostics. In chapter 4 we report on the visualization of Magnetic Resonance Imaging (MRI) contrast agents in living cells. Micrometer-sized iron oxide particles (MPIOs) are used as labels for cellular tracking by clinical MRI after cell transplantation. Our single frequency CARS experiments, employing a combination of resonant and non-resonant CARS responses, allowed us to determine the localization of MPIOs in living hepatoma cells. Specifically, we were able to visualize the morphology of individual cells by collecting the resonant response from the stretching vibrations of the CH bonds. Subsequently, we could localize MPIOs taken up by the cells with a second CARS measurements probing an off-resonant region of the spectrum, i.e. where biological molecules have no vibrations. MPIOs, which present a larger density of electrons compared to biological components, were clearly visible in both measurements, whereas the cells were visible in the resonant measurement only. Hence, an overlay of the two measurements provided the exact localization of individual MPIOs in the cells, which complements the MRI meso-scale localization. Using the intrinsic axial resolution of the technique, the position of the particles inside the cells could be determined in three dimensions. This is a particularly useful result as brightfield microscopy, commonly used to visually inspect the internalization process, is not able to distinguish along the axial direction the particles that have been internalized from those sitting outside the cell membrane. Our results suggest the possibility that MPIOs also penetrate cell nuclei.

In chapter 5, results on the uptake of gold nanoparticles (AuNPs) in healthy and tumor cells are reported. AuNPs are used for cell-targeted drug delivery e.g. for cancer treatment, although the mechanism of internalization of these particles in living cells is still under debate. We used the resonant CARS response of lipids to obtain cellular images, and multi-photon induced luminescence (MIL) to localize the AuNPs in three dimensions. The two approaches could be implemented on the same experimental setup as they share the excitation source, yet derive contrast from fundamentally different physical processes. Our results show that particles were internalized into the cells over a 10 hours incubation window. We found that healthy and tumor cells differentially respond to exposure to PEG-coated AuNPs. Limited internalization of the particles was found in tumor cells, with little increase over time. Isolated AuNPs were found localized primarily at the cell membrane or in endosomes in the perinucleic region of the cells. In contrast, healthy keratinocytes presented a large uptake of the particles with a dramatic increase of the number of internalized particles with time. These results, which were independently confirmed with Transmission Electron
Microscopy, indicate that the uptake mechanism of AuNPs is highly dependent on the cell line used, and possibly on the composition of the incubation medium.

The final two studies presented in this thesis focus on the identification of functional components in tissues based on the differences in their vibrational signature. In chapter 6 we report on the visualization of cellular compartments in the developing wing disc of Drosophila melanogaster. The compartments that play a role in tissues development had previously been identified by fluorescent staining, but little information was available on the chemical differences between the compartments. In our experiments we were able to visualize the cell compartments by determining the differences in the local chemical composition in a label-free manner. We employed hyperspectral CARS imaging to measure a vibrational spectrum at each position in the disc. This dataset was first used to provide morphology maps of the entire disc in three dimensions based on the distribution of CH bonds. In order to deconvolute the differences in the vibrational spectra, Principal Component Analysis (PCA) of the hyperspectral CARS was employed. This analysis uses co-variance matrices to determine the spectral features with largest spatial variance and reduce the dimensionality of the dataset. We found that the maps obtained for the first and second principal components could distinguish the anterior/posterior and the hinge/notum compartmentalizations in the wing disc, respectively. The analytic form of the second component also showed that the hinge and the notum compartments differ in their chemical composition due to the predominant presence of lipids and proteins respectively, as indicated by the dispersive lineshape of the component in the CH stretching region. These results show that hyperspectral CARS imaging provides valuable chemical information to complement fluorescence in the determination of cell compartments, and serves as a basis for investigating the origin of these changes during development.

In chapter 7 we present a study where we use hyperspectral CARS imaging to visualize lipid droplets and other biological components in thin sections of muscle tissues, and simultaneously investigate their chemical composition. The protein PLIN5, known to increase the production of intra-myocellular lipids, was selectively overexpressed in the investigated tissues. First, CARS maps were obtained, showing the local concentration of CH rich molecules in the tissue. Hierarchical Cluster Analysis (HCA), an alternative multivariate analysis to PCA, allowed identification of the spectral features bearing larger correlation at different locations in the tissue. The maps obtained by HCA provide clear identification of the lipid droplets present in the cells overexpressing PLIN5 as confirmed by fluorescence measurements of anti-PLIN5 staining. These lipid droplets were matched with lipid rich regions in a map of the spectral region from -2845 to -2905 cm$^{-1}$. We could also distinguish the protein-rich regions of the samples, corresponding to the vibrations between -2913 and -2970 cm$^{-1}$. Finally, features were found in the HCA maps compatible with the morphology of nuclei and mitochondria in muscle cells. These results demonstrate the utility of CARS for the research of complex tissue.