Looking at life through molecular vibrations: biomedical applications of CARS spectro-microscopy
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4 Visualization of MRI contrast agents in living cells

Micrometer-sized iron oxide particles (MPIOs) attract increasing interest as contrast agents for cellular tracking by clinical Magnetic Resonance Imaging (MRI). Successful cellular uptake of the MPIOs is the crucial prerequisite for determining the outcome of cell transplantation by MRI. Despite the great potential of MPIOs for *in vivo* imaging of MPIO-labeled cells, little is known about the intracellular localization of these particles following uptake due to the lack of techniques with the ability to monitor the particle uptake *in vivo* at single-cell level. Here, we show that single frequency CARS microscopy enables non-invasive, label-free imaging of MPIOs in living cells with sub-micron resolution in three dimensions. CARS allows simultaneous visualization of the cell framework and the MPIOs, where the particles can be readily distinguished from other cellular components of comparable dimensions, allowing accurate determination of their location inside the cell.

4.1 Introduction

Cell transplantation using *e.g.* stem cells, progenitor cells and adult cell lines constitutes a promising approach for treatment of several human diseases [115, 138], and has already been tested for clinical treatment of cardiovascular, neurological, and metabolic disorders [53, 70, 90, 115, 137]. The advantages compared to whole organ transplantation are many: the treatment is less invasive, cryopreserved cells can be used, and it provides the possibility that an organ from a single donor can be used for treatment of multiple patients [52, 71, 110]. To understand- and ultimately improve - cell transplantation schemes, knowledge has to be gained regarding the fate of cells following transplantation: monitoring cell transplantation is crucial for the success of the therapy. As a result, substantial effort has been devoted to the development of techniques to image individual cells in live organisms. Such imaging allows the detection of the early stages of cell homing, tracking cell migration, and the visualization of complications such as microembolization of transplanted cells [23, 141, 170, 172].

The outcome of cell transplantation in clinical trials has been investigated
via biopsies from the target organ, using visualization by radioisotope imaging [11]. This approach has serious limitations, however: it is associated with a risk for the patient due to the invasive procedure and constrained by the fact that only a limited part of the organ can be investigated, and only at limited numbers of moments in time. Alternatively, optical labels have been tested in the visualization with Near Infrared Fluorescence (NIRF) Optical Imaging of the development of transplanted cells in vivo in a preclinical rat-model [114]. A major drawback of this method is that NIRF-dyes are only detectable up to a tissue thickness of about 2 to 3 cm in vivo [121] and that the NIRF-dye must present antigen specificity. The discovery of immunospecific NMR contrast agents [176] has enabled magnetic resonance imaging (MRI) as a suitable tool for non-invasive tracking of transplanted labeled cells. The technique was first employed for imaging single cells in vitro [56,187] and has since been extended to cells in vivo [5,185]. MRI is currently the most common imaging technique for tracking in vivo labeled cells owing to its high resolution, and enhanced tissue contrast [89]. This technology further benefits from its widespread availability in clinical environments. The progress of the technique in vivo relies primarily on the choice of cell labels that are easily internalized by cells and can be readily visualized by MRI. However, to date it has remained challenging to fully elucidate the cellular uptake mechanism of these particles. In this study, we present a combination of resonant and non-resonant single frequency CARS microscopy as a useful tool for the visualization of MRI labels in living cells.

Paramagnetic Gadolinium (Gd) chelates are commonly used as T₁ contrast agents, although their low permeability through the cell membrane requires high concentrations combined with long incubation times for efficient internalization, and the detectability of labeled cells was found to be insufficient for clinical applications [40]. Gd-based metalloporphyrins, e.g. gadophrin-2 [46], and amphiphilic chelates, e.g. Gadofluorine M [99], are readily internalized by cells, but high concentrations are still required for MRI detection. Perfluorocarbons have also been investigated as cell labels [191] with the advantage that ¹⁹F MRI provides background-free imaging of the cells. Regrettably, they exhibited inadequate signal intensity levels. Superparamagnetic iron oxide (SPIO) nanoparticles have successfully been used as T₂ and T₂* contrast agents and exhibit higher contrast compared to normal paramagnetic particles [100,160]. SPIOs possess very high molar relaxivity, giving rise to contrast that well exceeds the physical dimensions of the particles in vitro. In addition, their magnetic properties can be tailored by modifying the particle size and aspect ratio [129]. These particles are negative contrast agents, appearing as pronounced hypointense regions in MR images. The major drawback of nanometer-sized SPIOs as labels is that a significant number of particles is required within a voxel for efficient detection. Thus, the dilution of the label as a result of cell division inevitably reduces the local concentration below the detection limit after a few cell division cycles [187]. SPIOs have primarily been used for detecting single cells in vitro [143] (for a general review on primary human hepatocytes see [127]), but also in vivo by clinical MR equipment [49]. Clinical MRI was found to be limited by the relatively low field strength and resolution compared to what
was shown in experimental studies [135]. The conclusion from these efforts is that larger particles creating a greater magnetic moment within the cells would be desirable for efficient detection of labeled cells under clinical conditions. Labeling with micrometer-sized iron oxide particles (MPIO) with higher magnetic relaxation compared to SPIOs constitutes a promising approach towards optimized imaging capabilities. It has been shown that MPIOs exhibit increased relaxation compared to nanometer-sized particles with the same total iron content per unit sample volume [101]. MPIOs can be easily internalized by several types of cells and allow for labeling capacity up to hundreds of picograms of iron per cell without affecting cellular viability [186]. For instance, primary human hepatocytes can be labeled with MPIOs without negative effects on cellular integrity or metabolic activity [175]. MPIOs have been used for MR imaging of cells both in vitro [101, 187] and in vivo [185], and it has been shown that even a single MPIO can be detected by MRI at a resolution of 100 \( \mu m \) [186]. Single particle sensitivity implies that MRI detection is not compromised by cell division due to dilution of the label.

Efficient uptake of the label is a crucial step for cell tracking, and an understanding of the underlying mechanisms is crucial for optimizing the labeling process. Also, unambiguous determination of the average number of MPIOs taken up per cell is crucial to evaluate MRI results and adapt the labeling protocol. Much research is expected to be dedicated to structural and functional modifications of the particles in order to achieve both better MRI contrast and specific cellular uptake. Any imaging technique that would support these studies must fulfill quite stringent requirements. The technique must firstly be non-invasive in order not to affect the viability of the cells, and label-free in order to study the interaction between the cell and the MPIOs under realistic and biologically relevant conditions: all invasive forms of sample preparation such as staining and sectioning are undesirable; finally, it must offer sub-cellular resolution, three-dimensional imaging capabilities and contrast from both MPIOs and cells. The ability of present technology to monitor intracellular localization of MPIO is still rudimentary due to the difficulties of co-localizing the particles with cellular components without the use of labels. Conventional brightfield microscopy based on light transmission is the fastest and least invasive approach for visualizing the particles in vitro [175,186], but unfortunately provides neither chemical selectivity nor sufficient axial resolution. In this technique, MPIOs are identified from their shape, with the risk of false positive identifications from large lipid droplets or other circular organelles in the cell. Due to the limited axial resolution, it is challenging to retrieve information on the localization of the particles relative to intracellular features from this approach. Knowledge of the location of particles is especially important relative to the cytoplasmic membrane; brightfield microscopy is not able to differentiate between a microparticle sitting outside the cell from one that has been internalized but still located in proximity to the membrane. Multi-channel confocal and two-photon fluorescence microscopies provide a viable alternative, allowing for visualization of fluorescently labeled components of the cells and labeled particles [185]. For instance, one among many possible options is double staining for cytoplasmic
proteins and nucleus, combined with additional staining of MPIOs \[175\]. While multi-channel fluorescence microscopy overcomes the spatial resolution issues of light microscopy \[145\], the approach is limited to the visualization of the particles relative to one or a few intracellular components, which in addition are studied under artificial conditions in the presence of multiple fluorescent marker molecules. In systems so critically dependent on local chemical and physical properties as the nano-bio interface, the presence of additional labels is undesirable as it can perturb the nature and dynamics of biological and physical interactions. Transmission electron microscopy (TEM) has also been used to visualize the uptake of MPIOs \[181\]. TEM has the ability to resolve both particles and cellular components on nanometer length scales. However, significant limitations of this approach may be noted, including the time-consuming and highly invasive sample preparation associated with potential artefacts \[180\]; cell fixation, resin embedding and slicing of the cells. This technique also precludes studies of the temporal characteristics of the particle internalization process.

As we will show here, the combination of resonant and non-resonant CARS microscopy represents a useful label-free approach to the visualization of MPIOs in living cells. The particles are readily distinguished from micrometer sized cellular features such as cytoplasmatic lipid bodies based on their high density of electrons. The approach is shown to provide accurate localization of the particles with respect with the cell body. The visualization of both particles and cells is achieved with CARS, as outlined in Chapter 1, high spatial resolution is ensured both laterally (300 nm) and axially (1 \(\mu m\)) with three-dimensional imaging capabilities \[28\]. We refer here to CARS as a special case of Four Wave Mixing (FWM) where three incident fields, two with degenerate frequency \(\omega_{pu}\) (pump/probe beam), and a third with frequency \(\omega_S\) (Stokes beam), interact through the third-order susceptibility of the probed material, generating a blue shifted fourth field at the anti-Stokes frequency \(\omega_{AS} = 2\omega_{pu} - \omega_S\).
Keeping in mind the expression of the total molecular susceptibility of Eq. (2.2) as the sum of the resonant and non-resonant term, performing an on-resonance CARS microscopy measurement means tuning the energy difference of the exciting beams to match a vibrational resonance of a specific chemical present in the sample, e.g. the methylene stretch abundant in lipids. In the off-resonance measurement on the other hand, the beams are tuned away from all vibrational resonances, and the intensity of the response only depends on the magnitude of $\chi^{(3)}_{NR}$ and in turn on the electron density of the compound being illuminated. The bioorganic molecules of the cell and the inorganic oxide of the MPIOs can be visualized by tuning the frequency difference of the fields to be on- ($\omega_{pu} - \omega_S = 2845 \text{ cm}^{-1}$) and off-resonance ($\omega_{pu} - \omega_S = 3000 \text{ cm}^{-1}$) with the symmetric stretch vibration of the $CH_2$ groups. Fig. 4.1 shows the normalized CARS spectrum of tripalmitin as an example of the response of biological matter at the frequencies used for the on- and off-resonance measurement indicated in figure by arrows. On-resonant excitation yields an enhanced CARS signal from lipid-rich components of the cells [64], accompanied by a strong non-resonant electronic signal from the MPIOs due to the high electron density of the iron oxide. Off-resonant excitation provides a weak non-resonant signal from the cells, but the strong non-resonant electronic signal from the MPIOs remains. The difference in the magnitude of the non-resonant signals arises from the large difference of the electronic susceptibility of iron oxide ($4 \times 10^{-10}$ esu [95]) compared to that of biological matter (typically $10^{-13}$ esu [147]). It is important to underline that due to the large dimensions of MPIOs, the particles have the same physical properties of bulk iron oxide. Owing to the very small bandgap of this material ($E_{gap} = 0.14$ eV, corresponding to a band edge absorption of 8.8 $\mu$m), the optical response in the visible and near-IR wavelength range is largely frequency independent. The results obtained here for our CARS setup with its specific combination of wavelengths should therefore be generally applicable to other CARS setups as well, as this wavelength range includes the excitation wavelengths of the beams normally employed in CARS experiments. As the contrast is derived from molecular properties, no additional staining of the sample is required for imaging [48, 98]. Additionally, the use of excitation beams in the near-infrared is particularly suitable for imaging biological samples as the absorption cross-section of water is low in this region [152].

CARS has previously been employed to visualize gold nanoparticles in cells [173], as well as wide bandgap semiconductor oxide nanoparticles in biological systems [142, 214]. In the latter works, the energy of bandgap absorption of a material is matched by the second harmonic of one of the excitation beams, resulting in a third term that contributes to the molecular susceptibility arising from two-photon electronic resonance in Eq. (2.2). The response of these particles is enhanced and gives rise to very high signals that allow to readily distinguish the particles from their environment [207]. The enhancement is nevertheless limited to the coupling of the two photons to the electronic states in the vicinity of the energy gap, where the effects of excitons are expected to be larger [25, 198]. This effect is negligible in magnetite as, contrary to other oxides, this material has a small bandgap of 0.14 eV. Two consecutive CARS
measurements, on-and-off-resonance, are hence necessary to distinguish MPIOs from the biological components. This apparent drawback turns out to be an advantage as the overlay of on- and off-resonance CARS measurements of the same region provides unambiguous determination of the location of each MPIO within the cell, and intrinsically discriminates between them and micrometer-size lipid structures that may easily be confused in brightfield images of the cells.

4.2 Materials and methods

4.2.1 Micron-sized iron oxide particles
MPIOs were obtained from Microparticles GmbH (Berlin, Germany). The average diameter of each microparticle is 1.18 +/- 0.08 µm. Microspheres consist of a silica based matrix with homogeneously incorporated iron oxide nanoparticles (40 vol-%). A hydrophilic polymer layer surrounds the core to prevent leaching of iron species. Additionally streptavidin functionality was introduced at the surface of the particles via 1-ethyl-3-(3-dimethylaminopropryl) carbodiimide (EDC)-coupling. The particles have a narrow size distribution, are superparamagnetic and show a very good colloidal stability in phosphate buffered saline (PBS) solution. The concentration of the particles in 1% w/v aqueous stock suspension was 7.088 x 10^9 particles per mL, resulting in an iron content of approximately 2.88 mg/mL. Subsamples from the stock solution were dissolved in PBS to a suspension concentration of 10^7 particles/mL and stored at 4°C under sterile conditions until cell incubation.

4.2.2 Cell cultures and incubation with MPIOs
Cryopreserved HuH7 cells, from a well-differentiated human hepatoma cell line, were purchased from JCRB Cell Bank (Osaka, Japan). Cells were thawed in a water bath and cultured in 25 mm² culture flasks (Sarstedt, Nrnberg, Germany) using Dulbeccos minimal essential medium (Biochrom AG, Berlin, Germany), supplemented with 10% fetal bovine serum (FBS), 1% L-Alanyl-L-Glutamine (200mM), 1% sodium pyruvat (100mM) and 1% penicillin-streptomycin at 37°C with 5% CO₂ atmosphere and 100% humidity. The cultures were passaged until a confluent layer was formed. Cells were washed with PBS and then released from the flask by incubating with 0,05% trypsin/0,02% EDTA for 4 min at 37°C. Growth medium at 4°C with 10% FBS was added to the cell suspension in order to stop the enzyme activity. A representative number of HuH7 cells were stained with Tryphan blue and counted in a hemocytometer. 100,000 living cells were seeded on sterile WillCo petri dishes (series GWSt-5030) with a 0.17 mm thick glass bottom and allowed to attach for 1 h at 37°C in 5% CO₂. Cells were washed and fresh medium was supplied.

Particles were gently resuspended prior to incubation, and 1mL particle solution was added to 1 mL growth medium in the dish. The MPIO amounted to 100 beads per cell. The cells were incubated for 4h at 37°C in 5% CO₂. As
controls, native cells were treated identically, but without particles. To remove free particles after incubation, the cells were extensively washed with PBS. The slides with the living cells were immediately transferred to the microscope for imaging. After the procedure it was visually confirmed that the cells were still intact. The time span between seeding the cells, particle incubation and CARS measurements lasted no longer than 48h.

4.2.3 On- and off-resonance CARS

The combination of on- and off-resonance CARS measurements was implemented on the experimental setup described in Paragraph 3.1 of this thesis. The output of the OPO was set either to 817 nm or 807 nm in order to form a beating excitation field with the Stokes beam at the frequencies of 2845 cm\(^{-1}\) and 3000 cm\(^{-1}\) respectively. The Raman shift of 2845 cm\(^{-1}\) corresponds to the symmetric stretch vibration of the \(\text{CH}_2\) group in the acyl chain of lipids (on-resonance measurement), whereas the Raman shift of 3000 cm\(^{-1}\) corresponds to a spectral region where lipid structures present a low response (off-resonance measurement). This can be seen in the CARS spectrum in Fig. 4.1 collected from a tripalmitin crystal as an example of a typical saturated fatty acid compound, where the intensity of the response at the above frequencies is marked with arrows. Prior to the imaging measurements, we tested the optimal experimental conditions and found that this laser power combined with an imaging time of 20 s per image (256x256 pixels) provided optimal results in that both the cells and the particles are clearly visible in the images without compromising the viability of the cells.

Three-dimensional imaging was achieved by scanning a sequence of horizontal planes at different vertical positions by translating the objective with a motorized stage. Samples were first imaged in brightfield mode and the regions of interest, typically covering an area of 30×30 \(\mu\text{m}^2\) (256×256 pixels), were then measured by CARS, and eventually imaged at different vertical positions with 1 micron spacing. On-resonance and off-resonance images of the same region of the sample were collected consecutively by changing the wavelength of the OPO and allowing for \(\sim\) 1 minute stabilization of the laser system. The output of the OPO was optimized for the on-resonance measurement, and the same settings were kept in the off-resonance measurement resulting in a small loss of power. The total acquisition time for each layer was 20 s at each wavelength. The tripalmitin CARS spectrum of Fig. 4.1 was measured under similar experimental conditions as the cell studies, images were collected on a tripalmitin crystal with the OPO tuned to wavelengths in the range 802-823 nm, thus probing frequencies between 2750 and 3050 cm\(^{-1}\). For each tripalmitin image a corresponding reference image of the sample cover glass was measured for CARS signal normalization, and the spectrum shows the average normalized crystal signal versus probed frequency.
4.3 Results and discussion

4.3.1 Imaging of dried MPIO solution

To ascertain selective imaging of MPIOs in cells, on-resonance and off-resonance images were first collected on a reference sample of pure MPIOs. A droplet of solution containing $10^8$ particles/mL of MPIOs was left to dry for one hour on a conventional microscope cover slip before imaging with both brightfield and CARS microscopy. Fig. 4.2 (A) shows a brightfield microscopy image of a region of the sample where three MPIOs can be identified. The same region was consecutively imaged with CARS, first with the pump and Stokes beams tuned to match the excitation energy of CH$_2$ bonds (on-resonance measurement), presented in Fig. 4.2 (B), and then with the beams tuned away from the resonance (off-resonance measurement), presented in Fig. 4.2 (C). Each of the three images covers an area of $10 \times 30 \, \mu m^2$.

By overlaying the on- and off-resonance CARS images in Fig. 4.2 (B) and (C), perfect colocalization of the features is obtained. Due to the lack of resonant signal together with a strong non-resonant contribution, the three particles are clearly visible in both CARS images, indicating that the optical contrast is not of vibrational origin but is related to a purely electronic response. Hence, for the measurements reported in Fig. 4.2 (B) and (C), one would expect the same signal strength. The decrease in signal intensity in off-resonance mode (Fig. 4.2 (C)) is not of physical origin but can be ascribed to the experimental procedure; the set-up is initially optimized for the on-resonance measurements.
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To minimize the time between the two consecutive measurements no additional optimization was performed after tuning the instrument to off-resonance excitation wavelength, resulting in a power loss in the output of the OPO. This can be confirmed by observing that also the reference signal measured from the glass cover slip is reduced accordingly between the two measurements. Within the signal fluctuation and variations resulting from frequency tuning, the signal intensities measured on- and off-resonance are identical after normalization by the reference response measured in glass.

We conclude from the CARS images presented in Fig. 4.2 that the contrast, or signal to background ratio, is in both cases (on- and off-resonance) large enough to enable precise identification of the particles. Since the dimensions of the particles are larger than the excitation wavelengths, detection of the CARS signal occurs here in forward direction. Extending this imaging approach to nanometer sized magnetite particles would instead be likely to benefit from epi-detection of the back-scattered signal, as backward collection geometry is preferable for the visualization of objects with lateral dimensions comparable to or smaller than the excitation wavelength [29]. The intensity profile taken along the diameter of any of the three round shapes fits well with the response expected from a 1.2 $\mu m$ sized particle (Fig. 4.3).

4.3.2 Imaging of HuH7 cells in absence of MPIOs

Before interpreting the images of HuH7 cells incubated with the microparticles, the typical CARS response of these cells was characterized and compared with conventional brightfield microscopy. Fig. 4.4 (A) shows a brightfield microscopy image of a single cell. The cell is flat and spread over a large area, with peripheral terminals visible at its edge, giving rise to additional contact between the cell and the glass support. The nucleus is visible in the center of the image. Its irregular shape is typical for tumor cell lines. Cellular features have low

![Figure 4.3](image)
contrast, except for the many anonymous circular features with diameters of \( \sim 1 \mu m \) that surround the nucleus. From this image it is clear that MPIOs will be difficult to distinguish from other intracellular features by conventional bright-field microscopy. The on-resonance CARS response of the same area \((30 \times 30 \mu m^2)\), imaged at an axial position \( \sim 2 \mu m \) above the glass surface is shown in Fig. 4.4 (B). This image appears similar to the brightfield microscopy, but it is important to underline that the CARS image is not merely a map of density or light transmission variations, but contains local chemical information as the intensity of the response is correlated to the concentration of \( CH_2 \) bonds within the focal volume. The peripheral terminals are not as clearly visible in the CARS image, indicating that these are located at an axial position closer to the supporting glass. Several features with particularly high intensity appear, leading us to conclude that these are lipid bodies, known to exhibit large CARS signals in this frequency range. The number of droplets is significantly smaller than the number of micron-sized features in the brightfield image, which illustrates an important limitation of conventional microscopy; the brightfield image is a projection of the entire cell volume showing all cellular features irrespective of their chemical composition or axial position. The CARS image instead gives a chemically specific picture of the distribution of lipids within a 1-\( \mu m \) thick focal plane. These lipids are present both in dense lipid droplets and in the cytoplasm (see Fig. 4.4 (B)). The on-resonance CARS signal is particularly helpful to identify the outline of the cell and the nucleus. The nucleus can be identified in an indirect way as the round region in the center of the cell where the high intensity features indicating the presence of lipid-rich aggregates are not present. The nucleolus is barely visible, indicating that it is located at a different axial position (compare Fig. 4.5 (A)). These imaging capabilities of CARS microscopy are of particular importance in order to determine whether the MPIOs actually have entered the cells, and if so, to distinguish the particles from natural intracellular components of similar size. The off-resonance CARS image of the area is presented in Fig. 4.4 (C). The image appears as a negative of the on-resonance image, with intensities lower or similar to that of the medium surrounding the cell. The appearance of the lipid bodies as dark regions in Fig. 4.4 (C), \textit{i.e.} giving a lower CARS signal off-resonance than both the water surrounding the cell and the aqueous solution inside the cell, can be traced to the relatively large signal from the water at the off-resonance frequency \((3000 \text{ cm}^{-1})\) that arises from the flank of the broad OH stretch, and hence presents low but non-zero CARS response. In the lipid bodies, where no (or very little) water is present, the CARS response is truly off-resonance; in both water environments the tail of the water response gives rise to small, but finite signal. It is particularly remarkable how this effect makes the edges of cell and nucleus well defined and readily visible in comparison with the background. This negative-like response is useful as it still gives an indication on the localization of cellular components in the off-resonance measurements where primarily MPIOs are detected. It is important to note that the lipid bodies in the cytoplasm are likely to slightly change their position over time, but in most cases the time between different CARS images is short enough so that the lipid
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Figure 4.4. Brightfield microscopy (A), on-resonance CARS (B), and off-resonance CARS (C) images of a HuH7 cell in absence of MPIOs labels. Greyscale values vary from 3 to 60 in (B) and from 6 to 20 in (C).

bodies can be tracked in time.

4.3.3 Imaging of HuH7 cells incubated with MPIOs

The localization of MPIOs in cells was investigated after incubating the HuH7 cells with a 1 mL solution of iron oxide particles at a concentration of $10^7$ particles/mL and 1 mL growth medium. The low concentration ensured that a limited number of particles were taken up by the cells. We note that the use of MPIO solutions with higher concentration imposes no additional complications to the imaging process. Fig. 4.5 (A) shows the on-resonance CARS image of a $30 \times 30 \, \mu m^2$ region depicting an isolated cell of elongated shape. The outside membrane of the cell is visible, as well as the contour of the nucleus. The image was collected from a focal plane near the center of the cell. The very large, circularly shaped nucleus can be identified by the lack of C-H signal, indicating lipids-rich aggregates are not present. The nucleus region presents uniform signal with intensity comparable to the surrounding aqueous growth medium. As shown previously in Ref. [148], the bright region inside the nucleus can be identified as the nucleolus, containing high densities of proteins and nucleic acids generating intense CARS signals. Several round features with a diameter of $\sim 1 \, \mu m$ can be identified inside the cytoplasm. The challenge of our imaging approach is exemplified by this image; are we able to resolve which of the features in Fig. 4.5 (A) are MPIOs and distinguish them from lipid-rich cell components? The CARS response in Fig. 4.5 (B) shows that only one such feature also presents a high intensity also in the off-resonance image of the cell, indicating the presence of an iron oxide particle. All other cellular features appear in the negative-like fashion similar to that in Fig. 4.4 (C); the cell is still visible with negative contrast relative to the water background. In this case also the nucleolus is visible as a dark region in the center of the nucleus.

Visual inspection already reveals that the bright feature in the off-resonance image finds immediate correspondence in the on-resonance image. This observation is confirmed by the colour-coded overlay of the two images presented in
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Figure 4.5. (A) on-resonance CARS and (B) off-resonance CARS images of a HuH7 cell incubated with MPIOs solution. (C) is the overlay of the on- and off-resonance images where the former appears in red, and the background corrected off-resonance response appears in green. Greyscale values vary from 3 to 40 in (A) and from 3 to 25 in (B). The overlay image obtained from on- and off-resonance measurements allows for the identification of a single iron oxide particle in the lower part of the cell (green spot).

Fig. 4.5 (C), where the on- and off-resonance images were added after subtraction of the background in Fig. 4.5 (B), where the background is defined as the average value of the intensity of the signal collected from the medium surrounding the cell. The on-resonance image of the cell appears in red, superimposed with the MPIO signature obtained from the off-resonance image in green. The identification of the particle is here unambiguous despite the many morphologically similar lipid droplets present in the cell, and we have obtained a label-free and non-invasive fast image of the intracellular distribution of MPIOs. The particle has been internalized by the cell, and is located within the cytoplasm in the proximity of the cellular membrane in an axial plane that contains as well the nucleus and the nucleolus located $\sim 2 \mu m$ above the surface of the glass support.

We can infer, a posteriori, that one feature of the MPIO allows a first-hand identification already in the on-resonance image, as the high intensity spot where the particle is located is surrounded by a dark halo. This effect is due to the large refractive index mismatch between the particle and the surrounding medium, resulting in a distortion of the beams foci that alters the CARS response at the interface [78]. This effect is instead not present in the brightfield images of cells incubated with MPIOs.

4.3.4 Three-dimensional intracellular localization of MPIOs

The optical sectioning capabilities of CARS are best exploited in three-dimensional images of the cell. Such 3D-images allow direct inspection of the spatial distribution of MPIOs within the cells. The brightfield microscopy
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image shown in Fig. 4.6 (A) shows a projection of a cell with the nucleus located to the left and a collection of circular features are visible to the right in the cell, some of them presenting a diameter in the order of 1 µm. Again, it is not straightforward to distinguish MPIOs from lipid bodies in the image and from the optical image no information is available on the vertical position of the different structures relative to the upper and lower boundaries of the cell. In fact, inspection of Fig. 4.6 (A) does not allow one to conclude that particles have been internalized by the cell, rather than being located at the outside of the membrane. Fig. 4.6 (B-F) is a sequence of overlays of on-resonance (red) and off-resonance (green) CARS images of the cell taken at descending vertical positions separated by 1 µm. The full outline of the cell can be observed. In Fig. 4.6 (F) the interface between the cell and the underlying glass support is imaged. From Fig. 4.6 (C-D) we conclude that the nucleus as well as the collection of lipid droplets on the right side of the cell are both located in the mid-sections of the cell. The colocalization with the non-resonant image also allows the identification of the volume distribution of MPIOs, which is particularly important in order to determine whether the internalization process has been successful. Two particles can be observed, the leftmost of which located in the upper region of the cell (see Fig. 4.6 (B-D) ) and the rightmost in the lower region of the cell closer to the glass substrate (see Fig. 4.6 (C-E) ). Hence, from this three-dimensional CARS image we can conclude with certainty that two particles have been successfully internalized, appearing with a maximum signal at locations 2 µm (lower right particle, Fig. 4.6 (D) ) and 3 µm (upper left particle, Fig. 4.6 (C) ) above the glass support. The signature of the particles clearly extends beyond their physical dimensions, as the axial resolution is larger (∼ 1 µm) than the lateral resolution (∼ 300 nm). The slight discrepancy between the location of the MPIOs in the on- and off-resonance images is most likely due to a slight relocation of the particles between the two measurement series. We can rule out the possibility that the change in position is due to optical tweezing by noting that this effect is not present in the combination of single layer images where the time interval between consecutive measurements is shorter. The set of on/off resonance CARS images provides unambiguous insight into the presence and three dimensional distribution of single MPIOs, granting access to information that is otherwise impossible to obtain from the brightfield image in Fig. 4.6 (A).

These results demonstrate that CARS microscopy enables objective verification of the particle uptake in living cells. This is of particular interest, since the particles and lipid droplets present similar morphology under brightfield microscopy, making the evaluation of their uptake dependent on the experience of the investigator. Moreover, the access to the exact intracellular localization of incorporated particles enables investigations of the interactions between particles and cell organelles, paving the way for long-term toxicity studies of importance from a clinical perspective.
Figure 4.6. (A) Brightfield microscopy image of a HuH7 cell incubated with MPIOs. (B-F) Overlay of on-resonance (red) and off-resonance (green) CARS images of the same cell with descending axial position (separated by 1 µm). Two internalized iron oxide particles (green) can be identified from the overlay images.
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Figure 4.7. (A) Brightfield microscopy image of a HuH7 cell incubated with MPIOs. (B) Overlay of on-resonance (red) and off-resonance (green) CARS images of the same cell. One MPIO (green) appears to be located within the nucleus of the cell.

4.3.5 Outlook: nuclei penetration

One interesting feature of MPIOs labels and of functionalized particles used for cell penetration is their capability to penetrate nuclei. Engineering particles capable of penetrating the nuclei is challenging due to the lack in our knowledge of the different factors that regulate uptake in cell nuclei and the concomitant difficulty in formulating effective functional agents. Although the streptavidin functionalization used here was not designed specifically for transport into the nucleus, evidence of nuclear penetration was found in about 10% of the cells imaged with the on-/off-resonance CARS approach.

Fig. 4.7 (A) shows the brightfield microscopy image of a HuH7 cell incubated with MPIOs. The nucleus of the cell is visible, although the identification of the edges is not straightforward. A large number of round intracellular features are also visible, several of them accumulating in the perinucleic region. Inside and close to the nucleus four large features are visible, potentially related to the presence of MPIOs. Fig. 4.7 (B) shows the overlay of the on-resonance (red) and off-resonance (green) CARS images of the same cell. Here the shape of the nucleus appears more clear and seems to occupy the majority of the volume of the cell. One single MPIO is visible in a location that appears to be inside the nucleus. The quality of this image is not sufficient to unambiguously claim that the MPIO has penetrated the nucleus, although it provides strong evidence that this could be the case. Additional measurements are required to confirm this result, especially volume views of other cells. This unexpected result is an encouraging example of the possibility of visualizing MPIOs with nuclei penetration capabilities with CARS, and certainly warrants follow up experiments.
4.4 Conclusions

We have shown that CARS microscopy is a suitable tool for intracellular visualization of micrometer-sized iron oxide particles and has the potential to become an important instrument for the development of tracers for cell tracking in clinical MRI. Compared to other techniques available for this purpose, CARS has the advantage of being a label-free non-invasive technique that gives sufficient contrast both for the visualization of the particles and the cellular environment without need of additional labels. As magnetite is a small bandgap semiconductor, the four-wave mixing response of the particle does not benefit from additional enhancement of two-photon electronic excitation when the excitation beams are tuned to match the lipid CH stretches used for achieving contrast from the cellular body, hence the particles are not unambiguously identifiable from the on-resonance image of the cell alone. However, as non-resonant CARS is almost frequency-invariant, MPIOs are easily distinguished in the off-resonance image of the system. Overlays of the on- and off-resonant measurements enable the visualization of the position of the particles in the cells with sub-micron accuracy. The immediate advantages of the use of CARS for this task are the chemical specificity that allows us to distinguish unambiguously the MPIOs from intracellular lipid-bodies of comparable dimensions, as well as the intrinsic three-dimensional imaging capabilities that allows us to identify the axial position of the particles in the cell with high precision. The issues that CARS microscopy is capable of addressing are fundamental questions that naturally arise in the development stages of engineered particles, where physical properties of the particles are modified and additional surface functionalizations are added, modifying the interaction between the particles and the cell at the molecular level. Besides quantitative assessment of the efficacy of the internalization, additional information on the exact intracellular position of the MPIOs in comparison with other cell components are readily available in three-dimensions. It is easy to imagine scenarios where this information is particularly relevant if, e.g. nucleus penetration is desirable. Preliminary results suggest that nuclear penetration is already taking place for the MPIOs used in this work, although the streptavidin functionalization was not specifically designed for this aim. Further measurements are required to confirm this claim. As an important outlook, we note that the CARS approach allows for following the kinetics of the particle uptake in real-time. Moreover, other subcellular components can be selectively visualized in CARS by probing molecular vibrations characteristic for e.g. mitochondria or nucleic acids [152], allowing the co-localization of the particles with other cell components with high specificity and without labeling. Also, CARS measurements can readily be combined with two-photon fluorescence (2PF) on most CARS setups. Hence, using markers for specific internalization processes and combining CARS and 2PF, information on the process responsible for the uptake of the particles can be obtained.
4.5 Appendix: THG visualization of MPIOs

Parallel to the combination of on- and off-resonance CARS outlined in section 4.3.1, another approach for the visualization of MPIOs was tested using Third Harmonic Generation (THG). THG is a non linear effect taking place when three photons from the excitation beam interact with the material, causing the emission of a fourth photon presenting three times the frequency of the incoming photons. Due to the symmetry of the interaction, when the medium at the focal point is homogeneous, the generated signal before and after the focal point interfere destructively, and the net THG response is zero. When instead inhomogeneities are present at the focal point, such as an interface between two different media, the third harmonic response becomes very strong [188]. THG microscopy can hence be used to obtain high resolution images of systems presenting sharp interfaces, as the case of visualizing MPIOs in cells [207]. THG can be easily implemented on a CARS setup as the two techniques share one of the excitation beams. We employed the 1064 nm pump beam to excite the third harmonic, while the other beam was kept blocked. The signal was detected around 350 nm after spectral filtering to avoid contaminations from direct illumination and other high order effects.

A droplet of solution containing $10^8$ particles/mL of MPIOs was left to dry for one hour on a conventional microscope cover slip before imaging with THG. Fig. 4.8 (A) shows the brightfield microscopy image of a $20 \times 20 \mu m$ region of the sample. Here, despite the poor resolution of the technique at this magnification, several MPIOs are visible as the round objects with a slight red dominance. Fig. 4.8 (B-L) shows the THG images obtained from eleven consecutive vertical positions of the same area, separated of $0.3 \mu m$. Three round features corresponding to MPIOs are clearly visible in most of the layers, with maximum contrast in images F-H.

THG provides very clear images with high signal to background ratio of the particles. The axial resolution can be determined indirectly from Fig. 4.8, considering that each of the particles is visible with clarity in eight vertical layers, hence over a distance of $2.1 \mu m$, compared to the physical size of the particles of $\sim 1.2 \mu m$. The resolution is clearly better in plane, as shown in Fig. 4.9 (A) where the profile intensity of the signal across the diameter of one of the particles (see panel) is displayed. Compared to the intensity profile of the CARS signal (Fig. 4.3), the THG signal presents steeper edges extending over $\sim 0.5 \mu m$ and a central plateau of $\sim 1 \mu m$ in length. Fig. 4.9 (B) presents a three dimensional rendering of the particles, reconstructed from the images in Fig. 4.8. The particles present a slightly elongated shape as originated by the different resolution in the planar and axial directions.

The visualization of the MPIOs with THG hence results very clear and provides unambiguous identification of the particles. Regretfully, this technique turns out to be inefficient for the visualization of MPIOs in living cells. When measuring the THG signal from the cells incubated with MPIOs, the signal collected in the background from the biomaterial becomes non negligible preventing the discrimination of the particles from their environment. This strong
Figure 4.8. (A) 20 × 20 µm brightfield microscopy image of dried solution of MPIOs. (B-L) THG microscopy images of the corresponding area. The images refer to vertical planes separated by 0.3 µm. Scale bar 2 µm. Three MPIOs clearly appear in the THG images, with maximum contrast in layers F-H.

Figure 4.9. (A) Intensity profile of the THG signal along the red line drawn in the top right panel across the diameter of one of the MPIOs. The profile is compatible with the response expected from a particle with 1.2 µm lateral dimension. (B) three dimensional rendering of the THG response of the MPIOs. The image is reconstructed from the layers in figure Fig. 4.8.
and inhomogeneous background signal arises from the sharp interfaces between the different cell components, which are characterized by media with different composition [212].

In conclusion, THG microscopy is a suitable technique for the visualization of MPIOs, although it becomes inefficient for their localization in cells in vivo, due to the high background response produced by the cells.