Nonlinear vibrational microscopy applied to lipid biology

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Optical microscopy is an indispensable tool that is driving progress in cell biology. It still is the only practical means of obtaining spatial and temporal resolution within living cells and tissues. Most prominently, fluorescence microscopy based on dye labeling or protein fusions with fluorescent tags is a highly sensitive and specific method of visualizing biomolecules within sub cellular structures. It is however severely limited by labeling artifacts, photo bleaching and cytotoxicity of the labels. Coherent Raman Scattering (CRS) has emerged in the last decade as a new multiphoton microscopy technique suited for imaging unlabeled living cells in real time with high three dimensional spatial resolution and chemical specificity. This technique has proven to be particularly successful in imaging unstained lipids from artificial membrane model systems, to living cells and tissues to whole organisms. In this article, we will review the experimental implementations of CRS microscopy and their application to imaging lipids. We will cover the theoretical background of linear and non linear vibrational micro spectroscopy necessary for the understanding of CRS microscopy. The different experimental implementations of CRS will be compared in terms of sensitivity limits and excitation and detection methods. Finally, we will provide an overview of the applications of CRS microscopy to lipid biology.

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1. Introduction

For centuries, developments in optical microscopy have led to new insights in cell biology which in turn stimulated the evolution of these optical tools. In particular, the advent of the laser providing a bright light source with a well defined spatial and temporal mode has had a profound impact on the contrast techniques available in optical microscopy. Today, most prominent among the laser based microscopy techniques are confocal and two photon fluorescence (TPF) microscopy [1], which together with the development of highly specific fluorescent labeling techniques have shaped modern cell biology. In confocal microscopy, continuous wave lasers are employed to excite the fluorophore of interest via one photon absorption. An image is generated by raster scanning the focused beam across the sample and the emitted fluorescence is recorded sequentially with a single channel detector. Three dimensional (3D) spatial resolution is achieved by filtering the detected light by a confocal pinhole, suppressing out of focus light. An alternative way to obtain 3D resolution is realized in TPF microscopy where two near infrared photons, for which one photon absorption is negligible, are absorbed simultaneously by the fluorophore. As in confocal fluorescence, an image is obtained by raster scanning the focused beam over the sample while collecting the emitted fluorescence into a single channel detector. Since two photon absorption is non linear and occurs dominantly in the focal volume where high photon densities are reached, TPF has an intrinsic 3D resolution without the need of a detection pinhole. In turn the collection efficiency is improved, since light generated in the focal volume and subsequently scattered within the sample is not rejected. An important advantage of TPF is the use of near infrared light which enables deeper penetration into thick specimens due to the reduced Rayleigh scattering at longer wavelengths and the reduced one photon absorption from endogenous cellular material. Importantly, TPF requires pulsed lasers to generate sufficient photon densities at the pulse peak while maintaining moderate average powers. Typical laser sources for TPF microscopy, which are now found in many biomedical laboratories, provide pulses with durations of approximately 100 fs and repetition rates on the order of 100 MHz.

The success of TPF microscopy paved the way for research into the use of other non linear optical phenomena for the generation of contrast in high resolution optical microscopy. It was quickly realized that non linear processes are especially well suited for the investigation of unlabeled samples. While fluorescence labeling is a mature technique to specifically tag and hence visualize biomolecules in living cells, it also has inherent drawbacks. Firstly, fluorescence labeling requires a sample preparation procedure, conditioning the types of samples which can be studied, and adding time delays and cost. Secondly, the labeling changes the system under investigation. Although in many cases these changes are acceptable, there are numerous examples of labeling artifacts. In the field of lipid biology, this concerns for example the observation of fusion of lipid droplets which can be induced by standard labeling protocols [2–4]. A third problem of fluorescence microscopy is the photobleaching of organic fluorophores. Electronic excitation of organic fluorophores leads with a certain probability to chemical reactions towards products which do not absorb at the excitation wavelength and/or do not fluoresce. This often severely limits the observation times in fluorescence microscopy and is accompanied by cytotoxic effects.

One should note that many linear label free optical microscopy techniques are widely used already for decades. Examples are differential interference contrast (DIC) microscopy and phase contrast microscopy. The contrast in these techniques relies on refractive index differences in the sample which gives little information on

the chemical composition. On the other hand, the ability to distinguish chemically specific structures in cells and tissues (e.g. lipid vesicles from other cellular vesicles) is crucial to the understanding of their functional behavior within the cellular machinery. One way to generate chemical contrast without the need for external labeling is to exploit the vibrational spectra of biomolecules. Vibrational resonances depend on the masses of the constituting atoms and their respective bond strengths. A typical vibrational spectrum therefore contains a large number of resonances relating to the set of vibrational modes of the molecule reflecting the chemical composition of the sample. Conventional techniques for vibrational microscopy are the absorption of infrared (IR) light and Raman scattering (see Section 2.1). IR absorption microscopy is a well established technique. Yet, it is much more widely used in material science than in biological applications. The main two reasons for this are the poor spatial resolution achievable with IR wavelength and the strong IR absorption of water, which makes IR investigations of biological samples difficult. Raman microscopy, by contrast, utilizes light sources in the visible wavelength range. For this reason, it delivers spatial resolutions comparable to those achievable in confocal fluorescence microscopy. The application of Raman microscopy is not hampered by the presence of water in the sample. However, Raman scattering is weak, such that it can be overwhelmed even by weak autofluorescence in the sample. In addition, the measurement times for the generation of a well resolved Raman image often are prohibitively long.

By exploiting nonlinear optical effects, novel microscopy techniques have been developed over the last ten years to rapidly image living cells in a chemically specific label free way. As a matter of fact, most non linear optical processes do not rely on the presence of a chromophore since a nonlinear signal is generated, with varying efficiencies of the process, in any polarizable medium, i.e. in any sample. This said, it is important to note that different symmetry restrictions and resonance conditions apply to different non linear effects. For example, second harmonic generation (SHG), that is the generation of light at exactly half the wavelength of the exciting light, has been used extensively for imaging collagen fibers [5,6] since the formation of SHG signal reflects the local symmetry and order of the sample. Specifically, SHG is forbidden in isotropic media, which causes the SHG signal to be suppressed in most of the liquid cell interior due to the random orientation of the molecules. Structures like collagen fibers, however, are ordered, allowing the coherent addition of the SHG from the individual non inversion symmetric molecules. Another non linear process used for label free optical microscopy is third harmonic generation (THG), producing light with exactly a third of the wavelength of the exciting light. THG is created also in inversion symmetric structures, but due to the significantly different refractive index at the THG wavelength, the coherent superposition oscillates in space and is suppressed away from interfaces or structures having sizes comparable to the excitation wavelength [7,8]. Since lipids have a much higher nonlinear susceptibility [9,10] for THG than water, this microscopy modality is useful for imaging lipid rich structures [11,12]. On the other hand, SHG and THG do not provide vibrational contrast, since the involved light frequencies are well above the vibrational frequency range. This situation changes when laser fields containing frequency differences in the vibrational frequency range are used for excitation. By tuning the frequency difference in the exciting light field to match the frequency of a molecular vibrational resonance, this resonance is driven coherently, such that the resulting Raman scattering of each molecule is mutually coherent. This leads to coherent Raman scattering (CRS), in which the Raman scattering fields of all molecules constructively interfere, which results in a quadratic scaling of the scattered intensity with the number of molecules, as opposed to

the linear scaling in spontaneous Raman scattering. High concentrations of molecules with similar vibrational resonances, as can be found in lipids, and especially in lipid droplets, therefore give a strong CRS signal. The most common implementation of CRS is to use two excitation frequencies, and to detect the frequency up shifted Raman scattering of the higher excitation frequency, called coherent anti Stokes Raman scattering (CARS). CARS microscopy has first been described in the early 80's [13], however for practical reasons it has found widespread application only in the last decade [14]. More recently also stimulated Raman scattering (SRS), which detects the CRS components spectrally overlapping with the excitation fields, has been implemented [15–17]. Similar to THG, CRS is a four wave mixing (FWM) process in which three exciting waves result in one signal wave. Differently from THG, however, CRS enables spectroscopic investigations, i.e. one can tune the frequency difference of the exciting lasers, which allows the identification of different chemical species according to their vibrational spectra. The advantages of CRS microscopy have soon become apparent such that over the last decade many different applications in biology have been published and dedicated microscopes are now commercially available.

Most CRS applications so far have been aimed at label free imaging of lipids in a variety of samples from artificial model systems, to living cells and tissues to whole organisms. On the one hand this prevalence is due to the important role of lipids in biology. On the other hand, lipids exhibit favorable signal strengths in CRS experiments. CRS microscopy can therefore help to answer many open questions on lipid related processes inside cells and tissues, which due to the limitations and artifacts associated with fluorescence lipid staining have been left open. The ability of CRS microscopy to image lipids label free and with high spatial and temporal resolution in living cells offers a unique tool for the study of lipid biology. In this article, we will review the experimental implementations of CRS microscopy reported to date

and their applications to image lipids in various systems from artificial membranes to living cells, tissues and model organisms. The paper is organized as follows. Section 2 covers the theoretical background of vibrational micro spectroscopy from linear to nonlinear effects, necessary for the understanding of CRS microscopy. Section 3 discusses and compares different experimental implementations of CRS in terms of sensitivity limits and excitation and detection methods. An overview of the applications of CRS microscopy ranging from membrane model systems to live model organisms is given in Section 4.

2. Principles of vibrational micro-spectroscopy

2.1. Linear micro spectroscopy

Let us recall some basic principles of vibrational spectroscopy, starting with the case of linear optical phenomena, namely infrared absorption and Raman scattering.

IR absorption originates from the motion of partially charged atoms in molecules. If the corresponding electric dipole moment changes during the vibration, then the transition is IR active. Homonuclear diatomic molecules, such as N_2 , have no partial charges and their vibrations are consequently not infrared active. Examples of simple IR active molecules are shown in Fig. 1. Carbon dioxide is a linear molecule with inversion symmetry and partial charges are indicated. The symmetric stretch does not result in a change in electric dipole moment and hence is not IR active, while the other two modes, the asymmetric stretch and the bend, are IR active. For water, a non linear molecule with reflection symmetry, all modes are IR active. In larger molecules, typically all modes are to some extent IR active due to missing symmetry.

In general, an N atomic non linear molecule has $3N - 6$ vibrational normal modes. Therefore, in contrast to electronic

Fig. 1. Sketch of vibrational modes of CO₂ (left) and H₂O (right). The vibrational frequencies are given, together with their relevance in infrared absorption (IR) or Raman scattering. Partial charges of the atoms are indicated.

Fig. 2. Extinction coefficient of liquid water. The mechanisms relevant for the different wavelength ranges are labeled. The dotted line indicates an extinction coefficient equal to the inverse wavelength λ^{-1} , and the dashed line indicates the scaling λ^{-4} of the extinction by Rayleigh scattering.

absorption spectra, vibrational spectra show a large number of different bands. An example of an IR transmission spectrum is given in Fig. 3 for cholesterol ($C_{27}H_{46}O$), the principal sterol synthesized in cells and a building block of cell membranes. In organic molecules, vibrations in two main spectral ranges can be distinguished. The high frequency range $2700-3600\text{ cm}^{-1}$ (frequencies are given as wavenumbers, which are the inverse of the corresponding light wavelength in vacuum) is related to hydrogen stretching modes, with C-H bonds being at lower frequencies ($\leq 3100\text{ cm}^{-1}$) than O-H bonds. Lower frequencies ($< 1800\text{ cm}^{-1}$) relate to stretching modes of heavier atoms such as C, N, O but also deformation modes involving hydrogen. Strongly delocalised modes (e.g. breathing modes in the sugar ring of nucleic acids) give rise to vibrational resonances $< 1000\text{ cm}^{-1}$. The lower frequency range is commonly known as the fingerprint region, since it exhibits a characteristic band pattern which can be used to identify chemical compounds.

Although IR absorption is a powerful spectroscopic technique for chemical characterization, its application to cell microscopy has two main drawbacks. The first one is the spatial resolution which scales with the light wavelength λ . A resolution of in the order of 10 micrometers over the typical vibrational spectral range (1000 cm^{-1} vibrational frequency equates to $\lambda = 10\text{ }\mu\text{m}$) is not suited for subcellular imaging. The second drawback is the limited penetration depth by the absorption process itself in particular when taking into account that water, a dominant component of most living materials, has a strong IR absorption for $\lambda > 3\text{ }\mu\text{m}$ corresponding to an extinction length $< 10\text{ }\mu\text{m}$ (see Fig. 2).

Differently from the process of light absorption, in which light is converted into an internal excitation, light can also be scattered into a different optical mode. Vibrational Raman scattering may be regarded as an inelastic collision of light with a vibrating molecule, where following the collision the light has a different frequency. The energy difference between the incident and scattered photon equates the vibrational energy gained or lost by the molecule. Scattered light with higher (lower) frequency than the incident one is called anti Stokes (Stokes) component.

Compared to IR absorption, Raman scattering uses visible light. Hence, in Raman microscopy the spatial resolution is greatly improved and equals that of confocal fluorescence microscopy. Selection rules for Raman scattering are different from those of IR absorption (see Fig. 1) since Raman scattering does not rely on the electric dipole moment from charge displacements. Instead, Raman activity of a transition requires a change in the molecular polarizability during the vibration (for a detailed account of Raman scattering theory and selection rules see Ref. [18]). Consequently the vibrational spectra of organic molecules detected by IR or Raman techniques show the same resonances but with different

weights. An example of a Raman spectrum of cholesterol is shown in Fig. 3. In comparison to the IR spectrum, the same resonances are visible, but with different relative strength.

A limitation of Raman spectroscopy is that tiny amounts of fluorescent material can create a significant background, as the cross section for absorption is many orders of magnitude larger than for Raman scattering. For example, glass often used for mounting samples in optical microscopy is a disordered material with a tail of electronic states which creates a spectrally broad luminescence background. Fused silica (quartz) has a higher band gap and thus a lower density of localized states at a given wavelength, reducing this background. Using a pure crystalline material such as CaF_2 avoids this tail of electronic states and results in a low luminescence background. Typical values of Raman scattering cross sections are 10^{-29} cm^2 per vibrational mode, which is equivalent to a disc of 0.03 fm diameter, about 50 times smaller than the nucleus of carbon. For comparison, absorption cross sections for electronic transitions are typically 10^{-15} cm^2 , 14 orders of magnitude larger and equivalent to 0.3 nm diameter, about the diameter of the carbon atom. Due to the small cross section, Raman microscopy with a laser power of 10 mW at 532 nm, limited by photodamage, requires integration times of at least 0.1 s per point, and accordingly minutes to hours for images of 10×10 to 100×100 pixels, which is generally incompatible with live cell imaging.

2.2. Nonlinear micro spectroscopy: coherent Raman scattering

In linear Raman scattering, individual vibrational modes of molecules within the excitation volume are scattering the incident light incoherently with each other. Since thermally excited vibrations have random phases, also the spontaneous Raman emission has a random phase. The scattered fields are thus interfering randomly such that the time averaged intensity of the scattering scales linearly with the number of modes N_m in the focal volume. If the scattering was coherent, the fields would constructively interfere and the scattered field would scale linearly with N_m , with the intensity scaling as N_m^2 , a factor of N_m enhancement compared to the incoherent case. The typical focal volumes in high resolution optical microscopy are of the order of a femtoliter ($1\text{ }\mu\text{m}^3$), and a typical molecule volume is 1 nm^3 . Thus up to about 10^9 molecules can fit into the focal volume, providing an intensity enhancement of up to 10^9 . Even without reaching such densely packed molecular arrangement, an enhancement in the 10^6 range would allow for an increase in the imaging speed enabling video rate imaging with pixel dwell times of 100 ns.

Coherent Raman scattering microscopy exploits such a coherent enhancement effect by driving the oscillation of modes using the

Fig. 3. Vibrational spectra of cholesterol having a chemical structure as shown. Raman scattering intensity (black) and infrared transmission (red) are shown [19].

interference between two light sources (called pump and Stokes). Light wavelengths are typically chosen in the so called biological window (λ 600–1300 nm) i.e. the spectral range of minimum absorption from water (extinction length is larger than 1 cm) and away from electronic absorption of most organic molecules. The frequency *difference* between pump and Stokes light fields (and hence the low frequency beating term of their interference) is then adjusted to be resonant to the molecular vibration of interest.

Without discussing the details of a mathematical description (which can be found in Ref. [20]) the coherent Raman process is described by light induced transitions between molecular energy levels, as shown in Fig. 4a. A semiclassical picture is used in which light is a classical field and molecular vibrations are quantized levels. Pump and Stokes fields ($\mathcal{E}_p$ and $\mathcal{E}_s$ respectively) create, via a virtual intermediate state, a coherence between the first two vibrational levels $\Psi_{0,1}$ of the electronic ground state S_0 . For a given driving frequency, vibrational resonances at significantly higher frequency are providing a constant real background in the coherent Raman polarization. Furthermore, other third order nonlinearities (so called electronic contributions) are contributing to the CRS, as summarized in Fig. 4b. They have only virtual intermediate states and contribute to a non resonant real susceptibility χ_e , which is also known as Kerr effect. If the upper intermediate state of this process is in resonance with e.g. an excited electronic state, two photon absorption occurs, which increases the photo damage significantly. The total CRS polarization can thus be written as a sum over all the vibrational resonant parts and an electronic contribution which we will assume to be non resonant in the following. This defines a susceptibility in frequency domain as

$$\tilde{\chi}_s(\omega) = \chi_e + \sum_j \frac{f_j}{\Omega_j^2 - \omega^2 + 2i\gamma_j\omega} \quad (1)$$

The sum runs over all vibrational modes numbered by j and we have introduced the Raman oscillator strength f_j , the resonance frequency Ω_j and the linewidth γ_j of the j th vibrational mode. In time domain, the susceptibility χ_s relates to the induced dipole moment p of a molecule via

$$p(t) = E(t) \int_{-\infty}^t \chi_s(t-t') E^2(t') dt' \quad (2)$$

where E is the real electric field amplitude. Substituting $\mathcal{E}_p$ and $\mathcal{E}_s$, oscillating at optical frequencies ω_p and ω_s respectively, into the field E in Eq. (2) produces the different frequency components of CRS (see Table 1). In practical applications, $\mathcal{E}_p$ and $\mathcal{E}_s$ are trains of pulses to provide sizeable peak fields, needed to generate nonlinear effects, whilst maintaining moderate average powers to limit sample heating. In this case ω_p and ω_s indicated in Table 1 are their

Table 1

Components of the coherent Raman polarization induced by the pump and Stokes field $\mathcal{E}_p, \mathcal{E}_s$ with frequencies ω_p and ω_s .

Frequency	Acronym	Amplitude
$2\omega_p - \omega_s$	CARS	$\mathcal{E}_p \mathcal{E}_p \mathcal{E}_s^*$
ω_p	SRL	$\mathcal{E}_p \mathcal{E}_s \mathcal{E}_s^*$
ω_s	SRG	$\mathcal{E}_p \mathcal{E}_p^* \mathcal{E}_s$
$2\omega_s - \omega_p$	CSRS	$\mathcal{E}_p^* \mathcal{E}_s \mathcal{E}_s$

center optical frequency. Note that due to the resonant nature of the vibrational parts in $\chi_s(\omega)$, information about individual modes is localized in frequency domain, while the non resonant electronic background is localized in time domain. Given that for an excitation consisting of pump and Stokes there are four CRS fields of similar amplitude, one can ask the question which one to detect, which we will discuss in the following section.

3. Coherent Raman scattering: sensitivity limits, excitation and detection methods

3.1. CARS and CSRS

CARS and Coherent Stokes Raman Scattering (CSRS, see Table 1) are free from excitation background since they can be spectrally well separated from the frequency of the exciting pump and Stokes pulses. They are typically 10 orders of magnitude weaker in intensity than the excitation pulses, yielding for 10 mW average power in the excitation a CARS power of 1 pW, corresponding to 10^6 – 10^7 photons per second. These can be detected with sensitive detectors such as photomultipliers (PMTs) or avalanche photodiodes (APDs). If the CARS signal is spectrally resolved, also CCD Cameras can be used, which are however much slower to read out, typically milliseconds compared to nanoseconds for APDs and PMTs. Of the two signals, CARS is advantageous due to its shorter wavelength, at which these detectors typically have a higher quantum efficiency. Furthermore, incoherent background due to fluorescence and Raman scattering is mostly present at longer wavelengths, spectrally overlapping only with CSRS.

When detecting the scattered intensity proportional to the modulo square of the detected field, the non resonant response χ_e interferes with the resonant response, giving rise to a complex spectral shape (see Fig. 5). Different methods to mitigate this issue have been demonstrated in the literature. One approach is to measure the CARS spectrum over a large vibrational range and using appropriate algorithms such as the maximum entropy method [21,22] or the time domain Kramers–Kronig method [23,24] to retrieve from $|\chi_s(\omega)|^2$ the complex quantity $\chi_s(\omega)$, containing the

Fig. 4. Sketch of optical transitions in coherent Raman scattering. (a) Vibrationally resonant scattering. (b) Non-resonant scattering. S_0 : electronic singlet ground state, S_1 : first electronic singlet excited state of the molecule.

Fig. 5. Intensity of CRS signals as function of the detuning of $\omega - \omega_p - \omega_s$ for different Raman oscillator strengths f and linewidths γ as labelled. The CARS intensity $|\chi_s(\omega)|^2$ and the SRG intensity $2\Im(\chi_s/\chi_e)$ are scaled relative to the non-resonant background field. Note that typically the Stokes field $\mathcal{E}_s$ is 10^5 times larger than the non-resonant background, increasing the SRG intensity by the same factor.

resonances in the imaginary part. To measure the spectrum $|\chi_s(\omega)|^2$, one can either simultaneously excite a range of frequencies using broad band pulses covering $200 - 4000 \text{ cm}^{-1}$ vibrational range (multiplex CARS), and use a spectrally resolved detection, or use a sequential approach tuning the center optical frequency of narrow band ($\sim 10 \text{ cm}^{-1}$) excitation by picosecond pulses (single frequency CARS). Note that typical vibrational resonances of biomolecules in liquid have linewidths in the 10 cm^{-1} range, i.e. are matched to picosecond pulses. A limitation of the spectral retrieval approach is the acquisition speed, since a broad band spectral readout is typically in the millisecond time range. This is due to the limited light throughput of spectrometers and the finite acquisition speed of CCD cameras used in multiplex CARS, or the scanning time needed to sequentially cover large ranges in single frequency CARS. To increase the acquisition speed for real time imaging applications, one can resort to measuring only a small number of spectral points simultaneously. This has been done by a fast switch (in the MHz range) of the pump Stokes frequency difference hence exciting two (or more) vibrational frequencies, and demodulating the CARS intensity detected by a fast and efficient single PMT. This method implemented in slightly different variants has been called in the literature frequency modulated [25,26] (FM) CARS, Dual/Differential [27–29] CARS (D CARS, see Fig. 6), and Quadruple [30] CARS.

An alternative approach to reduce the influence of the non resonant response in the detected CARS intensity uses the vectorial polarization of light. The resonant CARS signal typically has a non isotropic Raman susceptibility characteristic for the specific resonance, such that by choosing specific excitation polarizations of pump and Stokes fields, the resonant and non resonant parts in CARS have different polarization directions [31–33]. Detecting the field polarized orthogonal to the non resonant signal eliminates the latter. However, this technique suppresses also the resonant intensity by typically an order of magnitude, and the depolarization depends on the molecular vibration, complicating the analysis of the signal. Furthermore, birefringence in the sample or in the optics can give artifacts by altering the polarization of the non resonant contribution.

Propagation effects of the CARS signal [32] can be used to suppress background from homogeneous media, similar to dark field contrast microscopy. CARS created by a bulk medium has a propagation direction given by the wavevector conservation of the four wave mixing process, namely $\mathbf{k}_{\text{CARS}} = 2\mathbf{k}_p - \mathbf{k}_s$ with $\mathbf{k}_{\text{CARS}}, \mathbf{k}_p$, and $\mathbf{k}_s$ being the wavevectors of CARS, pump and Stokes fields respectively. Due to the dispersion of the refractive index, for parallel beams there is a wave vector mismatch limiting the interaction length. Typically, for microscopy applications one focuses the pump and Stokes beams onto the sample using a high numerical aperture (NA) microscope objective in order to have a small focal

volume and hence a good spatial resolution. In this case, the directions of $\mathbf{k}_p$ and $\mathbf{k}_s$ are distributed over a range in the forward direction, such that the combination of wavevector directions and the limited interaction lengths given by the axial focus size allow to satisfy the wavevector conservation. The resulting directional range of $\mathbf{k}_{\text{CARS}}$ is centered in forward direction and distributed over a somewhat smaller solid angle [32] than $\mathbf{k}_p$ and $\mathbf{k}_s$. On the other hand, if the medium is spatially structured, χ_s is distributed over a range of wavevectors which are convoluted with the excitation directions and extend the wavevector distribution of the signal. Detecting the CARS signal at wavevectors created outside the range for a bulk sample can thus be used to eliminate the contribution of the bulk like material. Therefore, if the sample investigated consists of a small volume fraction of sub wavelength sized structures with strong resonant signal surrounded by a bulk like non resonant medium, this method is beneficial. Most noted implementations are epi collection [32] and wide field CARS [34–36] which is a non scanning technique allowing for single shot image acquisition. Furthermore, by modifying the angular phase distribution of pump and Stokes, the bulk CARS signal can be suppressed in forward direction [37]. In principle the most straightforward method to distinguish the resonant contribution in χ_s is to actually measure the CARS signal in amplitude and phase, which however requires an interferometric detection which adds to the complexity. This approach was shown in a modulated heterodyne detection scheme [38,39], allowing for a fast single channel detection, or in a multiplex CARS scheme by spectral interferometry [40,41].

3.2. SRL and SRG

Stimulated Raman loss (SRL) and stimulated Raman gain (SRG) processes, generally called stimulated Raman scattering (SRS), give rise to signals spectrally superimposed to the excitation fields (see Table 1), which have typically $10^4 - 10^6$ times higher amplitude [16,17]. The excitation fields have a fixed phase relation to the signals, and thus represent phase stable homodyne fields for the SRG and SRL signals. The imaginary part of χ_s ($\Im(\chi_s)$) creates an in phase signal and the intensity change of the transmitted excitation field is therefore proportional to $\Im(\chi_s)$ (see Fig. 5), which is directly the resonant part (the non resonant term χ_e has no imaginary part) and proportional to the spontaneous Raman intensity spectrum (in case of exponential dephasing). The combined effect of SRL and SRG transfers photons from the pump to the Stokes beam under the creation of a vibrational excitation receiving the photon energy difference $\hbar(\omega_p - \omega_s)$, hence it requires a vibrational resonance. Moreover, incoherent backgrounds in the detection of these signals are negligible due to large homodyne gain. However, the small change in intensity of the transmitted excitation by the SRL/SRG

Fig. 6. (a) Sketch of differential/dual CARS (D-CARS) with spectral focussing. A pump-Stokes pulse pair Π_1 is split into two orthogonally polarized pairs using a half-wave plate ($\lambda/2$) and a polarizing beam-splitter (PBS). The second pair Π_2 travels through a thin SF57 glass block which introduces a delay time between Stokes and pump pulses due to their different group velocity and in turn results in the excitation of a vibrational resonance at a different frequency (via spectral focussing) compared to Π_1 (adapted from Ref. [27]). (b) Example of D-CARS spectra and images showing the ability to distinguish lipid droplets made from different lipid types (GTC: glyceryl tricaprilate, GTO: glyceryl trioleate, GTL: glyceryl trilinolenate. Adapted from Ref. [29]). (c, d) In plane (x, y) and in depth (x, z) images of a polystyrene bead in agarose gel (on a logarithmic gray scale over 2 orders of magnitudes) using single-frequency CARS at the resonance frequency of the CH aromatic band at 3050 cm^{-1} and D-CARS at the difference between the in-resonance and out-of-resonance (3110 cm^{-1}) frequencies, showing the ability of D-CARS to suppress the non-resonant background (adapted from Ref. [27]). (e-g) CARS, Sum-CARS (SCARS) and D-CARS spectra and corresponding images in stem-cell derived human adipocytes (adapted from Ref. [42]).

process, typically in the order of 10^{-5} , is superimposed to classical laser noise of the excitation and modulation by the spatially varying sample transmission during scanning. To allow discrimination of the signal in this condition, one can modulate the amplitude of the non detected excitation field which modulates the signal accordingly (see Table 1). Using an excitation modulation in the 1–10 MHz range [16,43,44] allows one to suppress classical laser noise and microphonics. The modulation of the sample transmission has a frequency cutoff given by the scan speed and the resolution. Allowing for several excitation modulation periods per pixel, and a pixel size half the spatial resolution for Nyquist sampling, this frequency cutoff is significantly below the excitation modulation frequency and can thus be suppressed. However, in a modulated technique the signal is created only during half of the total measurement time, reducing the efficiency. On the other hand, photodiodes with a quantum efficiency (QE) above 80% can be used for detection, roughly a factor of two larger than the QE of PMTs used for CARS.

Similar to FM CARS or D CARS, it is possible also in SRS to measure a few vibrational frequencies simultaneously by modulating

and detecting at different modulation frequencies [45]. The Stokes pulse can also be spectrally shaped to measure the projection of the SRL response onto non trivial vibration frequency distributions [46]. The implementation of multiplex SRS using broadband laser sources and spectrally resolved detection is possible in principle, but is presently limited [47] by the multiplex detectors available, due to the high power and high frequency modulation to be detected. Typical line scan CCD cameras which could be used for spectrally resolved detection have a pixel read rate of 100 MHz and a full well capacity of 10^5 electrons, and thus can detect about $2 \mu\text{W}$, which is 3–4 orders of magnitude less than the excitation powers. Furthermore, spectra can be read with a rate of about 100 kHz, allowing for a modulation frequency 1–2 orders below the one used for fast scanning. These limits are due to the design of these cameras and can be overcome in the future within the same basic silicon technology by increasing full well capacity and allowing for on chip modulation. Multiplex SRS images are presently taken sequentially by tuning the frequency of the modulated excitation beam with step times down to the millisecond range [48,49].

Another notable difference between SRS and CARS is that wave vector conservation is respected by definition in the process ($\mathbf{k}_{\text{SRS}} = \mathbf{k}_p - \mathbf{k}_s$). Owing to the third order nonlinearity of the process the point spread function of the SRS is smaller than the one of the exciting beam such that the emitted signal has a wider directional range.

Without entering into detailed derivations (which can be found in Ref. [20]) it is important to point out that CARS and SRS have similar signal to noise ratio limits, basically because the related fields are of similar amplitude (see Table 1).

3.3. Coherent control in CRS

Coherent quantum control techniques such as Rabi oscillations or adiabatic passage have been proposed in the literature [50] to improve certain aspects of the CRS contrast. However, to avoid sample damage by avalanche breakdown or multiphoton ionization [51], CRS is typically in the small perturbation regime, i.e. pump and Stokes pulses drive the vibrational coherence well below the level of a full coherent population of the excited vibrational state (see Fig. 4a). Such coherent quantum control techniques are therefore not applicable for typical biological samples.

3.4. Excitation schemes of CRS

As already partly introduced in Section 3.1, various excitation schemes for CRS have been proposed in the literature, using broad band femtosecond pulses or narrow band picosecond pulses. Here we will discuss the advantages and limitations of the different excitation schemes in more detail.

3.4.1. Single frequency excitation

Single channel detection is easy to implement in commercially available microscopes and can be fast with pixel times down to the sub microsecond range, enabling video rate imaging [52,44]. Since the information about individual vibrations is localized in frequency domain χ_s , it is advantageous to measure CRS at a specific drive frequency. The best compromise between signal strength and background suppression [53,54,28] is achieved using a pulse duration in the excitation which matches the dephasing time of the vibration, typically in the picosecond time range. An excitation scheme with pump and Stokes pulses of picosecond duration was the first one used [14] and is still popular [55] due to its conceptual and practical simplicity. The related spectrogram is shown in Fig. 7 on the right. However, a practical issue with this approach is that for other multi photon contrasts, such as two photon fluorescence or second harmonic generation which are valuable to access in the same instrument, 100 fs pulses are better suited since for a given average power their intensity typically scales inversely with the pulse length down to below 100 fs.

An alternative approach, named spectral focussing [56], uses femtosecond pump and Stokes pulses, linearly chirped with an equal slope. In this way the instantaneous frequency difference (IFD) between them (which determines the driving frequency via the interference of pump and Stokes in CRS) is constant, and the spectral bandwidth of the excitation is given by the inverse of the chirped pulse duration which can easily reach picosecond values (see also Fig. 6). The related spectrogram is shown in Fig. 7 in the middle. Furthermore, since the IFD can be tuned within the pulse spectral width by changing the delay time between pump and Stokes pulse, it allows spectroscopy by simply moving a delay line without the need for laser wavelength tuning. Using broad band Stokes pulses created by a fibre continuum [57] or a sub 10 fs laser [58,42], a large vibrational frequency range of 1000–4000/cm can be accessed. Moreover, simultaneous CARS/TPF and SHG excitation and detection is possible with the same laser source. Noticeably, in Ref. [42] the optimum chirp requirement for CARS together with the shortest (hence unchirped) pulse duration requirement for TPF/SHG were met simultaneously owing to the large spectral range of the 5 fs laser source available and the selection of fully separated portions of the laser spectrum to generate pump, Stokes and two photon excitation pulses.

3.4.2. Single time excitation

The counterpart of single frequency excitation is single time excitation, which is impulsively exciting the system, and probing $\chi_s(t)$ at $t = 0$. The related spectrogram is shown in Fig. 7. This is efficient in the simultaneous drive of many vibration, but inefficient in the readout. Specifically, the response is largely affected by the non resonant χ_e , which has no information concerning the vibrational resonances and is instantaneous in time domain compared to the resonant part which decays with the time scale of the vibrational dephasing time hence in the picosecond range. To gain more resonant information, two [59] or multiple [60] pulses of adjustable time delay τ have been used, where the CRS signal of the second pulse has a component $\chi_s(\tau)$, and the delay is scanned to measure χ_s which is then converted to χ_s by Fourier transformation. Generally this is a slow technique, not well suited for imaging. However, due to the possibility of reaching long delay times, the vibrational spectrum can be measured down to small frequencies and with a high spectral resolution.

3.4.3. Multiplex excitation

In multiplex excitation a spectrally broad pump pulse and a spectrally narrow probe pulse are used to excite a range of vibrational resonance frequencies simultaneously and read them out with high spectral resolution. Historically the first implementation used a combination of a picosecond and a femtosecond Ti:Sapphire lasers [61] for pump and Stokes pulse, respectively, yielding a frequency time spectrogram as shown in Fig. 8a. The Stokes pulse is

Fig. 7. Sketch of spectrograms in single frequency and single time CRS. The contributions of pump and Stokes excitation and CARS are labeled, and the excitation spectral intensity I are shown. Left: chirp free excitation. Middle: Chirped excitation for spectral focussing. The dotted circles indicate the spectrograms for un-chirped pulses of the same spectrum. Right: Single time CRS. The pump and probe excitation separated in time by τ , and the CARS and CSRS signal are indicated.

Fig. 8. Sketch of spectrograms in multiplex CRS. The contributions of pump and Stokes excitation and CARS are labeled. (a) coherent femtosecond Stokes and picosecond pump pulses (b) incoherent picosecond Stokes and picosecond pump pulses. (c) Coherent sub 10 fs pump pulse, picosecond probe pulse created by spectral filtering of pump. (d) 8 fs pump pulse at 1–1.5 μm wavelength, and frequency doubled picosecond probe pulse at 775 nm [66].

typically chirped by the dispersion of the microscope optics. Its interference with the pump pulse drives the vibration, which is subsequently probed by the pump pulse. This method has been widely used in the literature [62,22], but a femtosecond picosecond dual laser system is complex and costly. It can be simplified by using a single ~ 100 fs laser, which is spectrally filtered to provide the picosecond pulse while the remaining spectral components are used for continuum generation in an optical fibre [63] to provide a broad band Stokes pulse. Alternatively, one can also use picosecond pulses from simpler laser systems such as Nd:YVO₄, and continuum generation in a fibre for the Stokes pulse [64,65]. Noticeably, this continuum is incoherent since its generation involves stimulated Raman gain and soliton instabilities, and the resulting spectrogram is shown in Fig. 8b. The compromised time ordering leads to a somewhat weaker CRS, and the intensity fluctuations of the continuum are adding noise to the signal.

Another approach is to use a single femtosecond source of 5–20 fs duration, having sufficient bandwidth to excite the relevant vibrations, and spectrally shape the pulse to create a longer probe pulse on the short wavelength edge. The corresponding spectrogram is shown in Fig. 8c. The CRS is then detected by a multiplex spectral detector. The spectral shaping can be done by a liquid crystal based grating shaper demonstrated for CARS [67] and SRG/SRL [68], or with a notch filter demonstrated in CARS [69].

An alternative recent approach uses a sophisticated fibre laser system [66]. A 200 fs Erbium fibre oscillator at a wavelength of 1.5 μm is split and amplified. One part is frequency doubled to produce picosecond probe pulses at 775 nm, the other part is spectrally broadened in a fibre and compressed to deliver 8 fs pulses at 1000–1400 nm, providing the pump pulses. The related spectrogram is shown in Fig. 8d. Due to the rectangular shape of the probe pulse, its rising edge can be placed a delay time τ after the pump, suppressing the instantaneous response χ_e by measuring $\chi_s(t)$ for $t > \tau$. The rather long wavelength of the pump pulse is also advantageous due to the small glass dispersion in this wavelength range, reducing the complexity of chirp compensation of the pulse in the microscope focus, and the reduced photo damage being below the threshold for three photon absorption in typical biological material such as proteins, lipids and sugars.

4. Applications to lipid systems

Coherent Raman micro spectroscopy has already found a variety of applications ranging from material science to *in vivo* investigations of live mice. The CRS signal from lipids is particularly strong due to the large number of identical bonds in the focal volume providing a large coherent enhancement as discussed in Section 2.2. Investigations of lipid rich materials have consequently been an early focus of CRS microscopy applications.

4.1. Membrane model systems

Soon after the development of the modern CARS microscope [14], experiments on membrane model systems have been published. These systems, which include multilamellar vesicles, small, large and giant unilamellar vesicles (MLV, SLV, LUV, and GUV, respectively) and deposited bilayers are attractive samples for CARS microscopy, since their chemical composition is well defined. This allows subtle changes in the spectra, which can occur for example by temperature induced phase transitions, to be monitored directly. While a huge number of fluorescence microscopy based work has been published on these systems, label free CARS microscopy is attractive here, since it precludes the occurrence of labeling artifacts.

The first CARS microscopy work on multilamellar vesicles introduced multiplex CARS microscopy as a new variant of this approach [70]. As explained in 3.4.3, multiplex CARS microscopy offers the possibility to record a CARS spectrum over a certain vibrational frequency range at every image pixel. In this respect, the approach is similar to spontaneous Raman micro spectroscopy, where also a whole vibrational spectrum is recorded at every sample position. However, multiplex CARS spectra and Raman spectra cannot be compared directly since CARS spectra are affected by the non resonant background (see Section 2.2). In Ref. [70] the authors show that monitoring skeletal C–C stretch vibrations of the lipid acyl chains at approximately 1100 cm^{-1} can be used to differentiate between regions on vesicles, or whole vesicles, in gel phase or liquid crystalline phase. Specifically they show that it is possible to distinguish DSPC and DOPC vesicles which have high and low phase transition temperatures, respectively. The multilamellar vesicles investigated in Ref. [70] were approximately 2 μm in diameter, resulting in an excitation volume which is completely filled with lipids. The situation changes drastically, when unilamellar vesicles are investigated. For these, the number of molecules in the focus is three to four orders of magnitude smaller. Given that the CARS intensity depends quadratically on the number of scattering molecules, this corresponds to a CARS signal decrease of six to eight orders of magnitude. Detection of a single lipid bilayer therefore pushes CARS microscopy to its sensitivity limits. The first successful detection of lipid bilayers by CARS microscopy was reported by Potma and coworkers on DOPC DOPG GUVs and on erythrocyte ghosts (Fig. 9) [71]. In these experiments, the authors used single frequency CARS measurements both in a transmission type geometry and a backscattering (epi) detection configuration. They show that in the epi detection the signals can be amplified by non resonant signal from the glass/water interface, which facilitates the detection, but also leads to interference effects in the images. In imaging lipid bilayers with CARS microscopy, polarization effects occur due to the orientational order of the lipid chains, resulting in a higher CARS intensity [71] for a linear excitation polarization in the bilayer plane, i.e. orthogonal to the lipid chains.

Fig. 9. Forward-CARS image of an erythrocyte ghost. The image was taken in the equatorial xy plane of the vesicle at a Raman shift of 2845 cm^{-1} . The arrows point toward the orientation of the bilayer, giving rise to a higher CARS intensity (top and bottom) when averaged C-H bond orientation is parallel with the incident beams (double arrow), and a lower CARS intensity (left and right) when C-H bond orientation is perpendicular to the incident polarization. Scale bar is $2\text{ }\mu\text{m}$. Image size is 256×256 pixels and pixel dwell time is 1 ms . Adapted from [71].

Following these initial proof of concept investigations, CARS microscopy has been used to investigate structures and their dynamics in various membrane models. It has been shown earlier for spontaneous Raman microscopy, that chain order and packing as well as the presence of *gauche* configurations can be analyzed by rationing intensities for symmetric and antisymmetric CH_2 stretch vibrations and of CH_2 and CH_3 vibrations [72,73]. Similar work was possible using CARS microscopy, with the advantage that meaningful spectra with sufficient signal to noise ratios could be obtained within 20 ms as compared to 120 s for the spontaneous Raman measurements [74]. In order to improve the CARS microscopy sensitivity, the authors again used a multiplexing scheme, with the advantage that the non resonant background from the environment is amplifying the resonant signal [62].

While CARS spectra can be acquired by either scanning the excitation frequency or by using the multiplex approach (see Section 3.4), the interpretation of the spectra is non trivial. As discussed in Section 2.2 the CARS spectrum is composed of a resonant and a non resonant part. The CARS intensity spectrum therefore reflects the interference term between the resonant and non resonant parts. Depending on the relative strengths of both components with respect to each other, this can result in CARS lineshapes, which are different from those of spontaneous Raman spectra. Quantitative interpretation of CARS spectra requires the knowledge of the non resonant signal alone and the application of appropriate algorithms in order to derive the resonant spectrum (see Section 3). While this makes a spectral analysis cumbersome, the interference term can also serve as an inherent signal amplification. The usefulness of these approaches has been demonstrated in studies of the main lipid phase transition in DMPC LUVs and SUVs [22]. In this phase transition from the solid phase to

the liquid crystalline phase, the rotational and translational mobility of the lipids changes significantly. In addition, an increase in *trans gauche* isomerizations is observed. The intensity rationing of different CH stretch vibrations (after retrieval of Raman like spectra from CARS intensity spectra) was used to quantify the changes and follow the phase transition as the temperature is changed [22].

Besides spectral information, insights into the sample structure can be gained by exploiting the polarization properties of the excitation light and the sample (see also Section 3). Raman scattering for a given molecular vibration is described by a scattering tensor. This means that the observed Raman and CARS signals have a well defined polarization which depends on the excitation polarization, the tensor and the orientation of the molecules. Since symmetry considerations simplify the tensor, experiments in which the excitation and the detection polarization are varied can be used to draw conclusions about the orientation of the chains. This approach allows the quantification of changes in the acyl chain ordering in MLVs composed of different phosphatidylcholines upon addition of small amounts of cholesterol. Significant increases in ordering have been observed for amounts of approximately $10\text{ mol\%}$. [75].

CARS microscopy has also been used in a number of investigations on phase segregation and domain formation in mixed compound systems. Most often, deuterated compounds are used in these experiments for the preparation of the vesicles and planar bilayers containing lipid mixtures. The advantage of this approach is that CD stretch vibrations have frequencies around 2100 cm^{-1} , hence are well distinguished from the CH stretch around 2900 cm^{-1} and fall into a spectral range void of common resonances, specific for rarely occurring groups such as C=N or $\text{C}\equiv\text{C}$.

Fig. 10. (A) Fluorescence image of 1:1 DOPC/DPPC GUVs labeled with 0.4% BODIPY PC. (B) Fluorescence image of a bilayer patch of a 1:1 mixture of DOPC and DPPC. (C) and (D) CARS images of the bilayer patch shown in (B) detected in forward- and in epi-direction, respectively. In this case, the contrast in the epi-image is better as in the forward-image, since the bilayer is much thinner than the excitation wavelength. The intensity profiles along the red lines are shown below the images. The CARS images were averaged over 40 frames with an acquisition time of 0.44 s for each frame of 256×256 pixels. The pump and Stokes powers at the sample were 3.0 and 1.4 mW, respectively. Bar 10 μm . Adapted from [77].

It is therefore straightforward to isolate a specific lipid from a mixture if that lipid is deuterated. CARS microscopy experiments of phase segregation were first done on mixed DOPC/[D70]DSPC GUVs in which only the [D70]DSPC phase was monitored using CARS microscopy [76]. For the visualization of the DOPC phase, 1% of fluorescently labeled DPPE was added and detected using simultaneous two photon. Combined two photon fluorescence and CARS experiments of this type are easy to implement, since they require only the addition of a second detection channel for a different spectral window. Monitoring of domain formation is also possible solely based on CARS microscopy by using deuterated and non deuterated lipids and measuring CARS at the CD and at the CH vibrations. The dependence of the CARS signal on the number of scattering molecules also enables the monitoring of different domains in lipid bilayers. This is possible if the domains differ in density of CH_2 groups either because of their chemical composition or because of their packing density (See Fig. 10.) [77]. More quantitative data analysis is possible if deuteration is used in addition. In this way, the content of [D62] DPPC in coexisting domains of DOPC/d62 DPPC bilayers was quantitatively measured as a function of cholesterol content in Ref. [78].

4.2. Cellular systems

Membrane model systems of the types described above have the advantage that they consist of only a few different molecular species often only one or two with known vibrational spectra and well defined concentrations. Compared to cellular systems, which contain a large number of species with different spectra in unknown concentrations, this makes the spectral interpretation much simpler. However, potential applications in live cell and live animal imaging have been the most important motivation for the development of non linear vibrational microscopy techniques. Apart from difficulties due to the complex nature of real world biological samples, live cell and animal investigations are faced with the additional problem that the excitation light intensities should be kept at a level which does not induce any photodamage. For

all the techniques discussed in this review, the situation is comparable to that encountered in two photon excited fluorescence microscopy. Since all approaches employ pulsed lasers excitation in order to induce non linear optical effects in the samples, there are two damage regimes which deserve attention. In a linear regime, i.e. when considering effects caused by linear absorption of laser light by the sample, mainly local heating is the cause of the damage. In order to reduce this problem, the excitation wavelengths used are usually chosen to lie in the near infrared spectral region, where the absorption of most cells and tissues is rather weak. Use of intense pulsed lasers, however, can also lead to undesired non linear effects which might e.g. result in multi photon excitation or avalanche breakdown and the ionization of molecules in the microscope focus. Several studies have addressed the problem of phototoxicity of pulsed laser excitation [79–81]. While these give some insight into the parameters relevant for the cause of photodamage, the complexity of the damage process complicates any prediction of the amount of photodamage. This is due to the broad range of important variables such as the exact nature of the biological sample, the treatment of the cells (temperature, culture medium, atmosphere), the laser pulse duration, the pulse energy, the excitation wavelength, and the laser repetition rate, to name only the most important ones. A general issue which has to be mentioned in this respect is that the term photodamage is ill defined. Often biological samples can cope with seemingly dramatic photodamage such as membrane blebbing, commonly regarded as a clear sign of photodamage [81]. Earlier blebbing will, for example, not necessarily inhibit cell division. On the other hand, careful inspection of the samples often shows laser induced modifications such as enhanced autofluorescence even if no other apparent sign of damage is present. Thus, care has to be taken to avoid laser excitation related data artifacts.

For the proper interpretation of cellular images obtained with non linear vibrational microscopy, it is necessary to recall the signal generation mechanisms and imaging schemes described in 3. In nearly all experiments, the signal will be generated in a focal spot of the size of $1\mu\text{m}^3$. This means that a concentration of $1\mu\text{mol}$

Fig. 11. Multiplex CARS spectral imaging of lipid droplets in fixed 3T3-L1 cells. (a) Bright-field image of an adipocyte incubated with a 1:3 mixture (mol/mol) of linolenic and palmitic acid. Marked region was imaged with multiplex CARS microscopy. Images are derived with contrast based on (d) lipid concentration (integrated intensity), (e) C = C concentration derived from CC-stretch region (I_{1650}/I_{1450}), and (f) acyl-chain order (I_{2880}/I_{2845}). (b and c) Representative $\text{Im}(\chi^{(3)})$ spectra were recorded at locations indicated (in d) for CC-stretch and CH-stretch spectral regions, respectively. A least-squares fit of a sum of three Lorentzians to the data is also shown (b). Acquisition time was 20 ms for a multiplex CARS spectrum at every pixel position (~ 1 min/image). Adapted from Ref. [86].

corresponds to roughly 10^3 molecules per excitation volume. Typical sensitivity limits of CARS as well as of SRS, however, are in the range of 10^5 – 10^6 oscillators. As a result, CRS imaging techniques can detect small sample structures down to sizes of a few hundred nm in diameter, as long as the local concentrations of molecules is high. These conditions are met e.g. in cellular vesicles. CRS instead is less suited for monitoring small variations in evenly distributed diluted compounds. For this reason and due to the mentioned strong CRS signal of lipids, non linear vibrational microscopy has extensively been used to study lipid droplets (LD). During the last two decades, it has become apparent that LDs are cellular organelles with a well defined internal structure. A monolayer of phospholipids forms their outer membrane, into which a broad variety of proteins are inserted [82]. The interior of the LD contains neutral lipids (mainly triglycerides and sterol esters). LDs participate in a broad variety of physiological processes and are found in many different cell types. Their sizes vary from some tens of nm to tens of μm in diameter in differentiated adipocytes. Since they have an active role in fat metabolism, LDs are dynamic organelles both concerning their intracellular location as well as their size. CARS microscopy has been used to study the movement of LDs in live cells. These tracking experiments, which can be performed at video rate image acquisition times, reveal that LDs are actively transported in cells. This transport is mediated by microtubule related motor proteins [83,84] and is commonly bidirectional, i.e. it can be directed towards the plus or the minus end of the microtubules, which can be explained by the simultaneous presence of different motor proteins on the LDs. The balance between the two transport directions can be changed e.g. after infection with the hepatitis C virus [85]. Multiplex CARS microscopy has been used to monitor the fat composition of LDs in HeLa cells and

adipocytes under different dietary conditions [86,87]. In these works, ratios between intensities observed for different vibrational bands were used to infer information about the acyl chain order and the ratio between saturated and unsaturated fatty acids. While some of these markers provided the expected results, it was also seen that the prominent resonance of unsaturated fatty acids at 3020 cm^{-1} failed to provide quantitative information about the local concentration. Nevertheless, use of this approach allowed the identification of variations in the composition of LDs in individual cells. (See Fig. 11.)

A major application of CRS microscopy in quantitative studies of LDs has been the monitoring of LD numbers and sizes. As mentioned before, CRS microscopy is suited for LD sizes down to diameters of approximately 100 nm. Small LDs are mainly observed in cells not specialized for fat storage, but are also present in adipocytes held under lipolytic conditions. CARS microscopy has successfully been used to quantitatively monitor the size of LDs in yeast cells [88]. The morphological dynamics of LDs are most striking in adipocytes which can be derived from mesenchymal stem cells. CARS microscopic inspection of these stem cells shows only few LDs of very small size. Differentiation into adipocytes leads to a drastic change in LD number and size over approximately two weeks. Shortly after the induction of differentiation, numerous small LDs are formed which fuse to finally form one unilocular lipid droplet per cell with diameters exceeding $10\text{ }\mu\text{m}$. This process has been followed using CARS microscopy (Fig. 12). The specific virtue of CARS microscopy here is the possibility to perform long term experiments over the course of more than a week on the same sample without the occurrence of marker bleaching. Analysis of the data gives detailed insight into the fusion behavior of LDs [89]. However, large LDs are not only found in adipocytes.

Fig. 12. CARS imaging of LD growth in human adipose-derived stem cells. Lipid acceptors can turn into lipid donors: A lipid donor (solid arrow, first panel) is almost completely absorbed over time by a closely associated lipid acceptor below. Later on, this lipid acceptor in turn serves as a lipid donor (open arrow, second panel) for a LD, which simultaneously absorbs a second LD (solid arrow, second panel). Finally, this LD starts to absorb a third LD (solid arrow, last panel). The completion of one LD fusion takes more than 2 h. During the fusion process, one contact point is maintained. CARS images were acquired at 2845 cm^{-1} . Maximum-intensity projections of 3D stacks acquired at the indicated time points. Bar = $10\text{ }\mu\text{m}$. Adapted from [89].

Fig. 13. CARS images showing changes in lipid distributions in Huh-7.5 cells after treatment with the small molecules BA and lovastatin. (a) Structure of the PPAR α (peroxisome proliferator activated receptor α) antagonist 2-chloro-5-nitro-N-(pyridyl) benzamide (BA) and (b) structure of the HMG (3-hydroxy-3-methylglutaryl)-CoA reductase inhibitor lovastatin. (c) Images depicting the effects of $75\text{ }\mu\text{M}$ BA on Huh-7.5 cells incubated in culture for 6 h. (d) Images showing the effects of $50\text{ }\mu\text{M}$ lovastatin treated Huh-7.5 cells for either 24 or 48 h. The values on the bottom left corner of the CARS images represent the results of a voxel analysis indicating the lipid droplet volume per cell (average of 5 cells per sample). Scale bar: $10\text{ }\mu\text{m}$. Adapted from [95].

CARS microscopy has revealed that also enterocytes, absorptive cells of the small intestine, contain pools of LDs which can grow to sizes of nearly $10\ \mu\text{m}$ in diameter [90]. The pool of these LDs is dynamic and the enzymatic regulation of these dynamics has been investigated using CARS microscopy in Ref. [91,92].

CRS microscopy has also been used to monitor pathological changes in LD structures and dynamics. Changes in the lipid metabolism are induced by various different diseases. One example are infections by the hepatitis C virus (HCV) which are known to affect the lipid metabolism of the host [93] and which result in changes in the cellular accumulation of LDs. CARS microscopy has been used to show that HCV replication can be suppressed by the action of a small molecule inhibitor. Application of the inhibitor to infected HUH 7 cell cultures thereby leads to a clearly detectable increase of cellular lipid accumulation [94] and a disruption of HCV replication. A combination of two photon excited fluorescence experiments and CARS microscopy shows that the latter effect is caused by dispersion of the HCV RNA (Fig. 13) [95].

CRS microscopy has also proven to be a valuable tool for studying lipid related mechanisms of cancer metastasis. While it has been noticed that lipid rich tumors exhibit a stronger tendency to metastasis, the details of this process have not been understood. Application of CARS microscopy *in vitro* and *in vivo* has shown that excess free fatty acids lead to a phase separation in the cell membranes disrupting cell cell contacts resulting in a higher cell motility [96]. In these experiments, CARS based intravital flow cytometry was used. For this purpose, a superficial vein in a mouse ear was monitored using CARS microscopy. Simultaneous two photon excited fluorescence imaging then served for the identification of tumor cells. This example shows that the fast imaging capabilities of CARS microscopy lend themselves also to the application in analytical techniques such as cytometry, possibly complementing existing fluorescence based cytometry approaches [97,98].

4.3. Tissues and model organisms

As a label free multiphoton microscopy technique with intrinsic optical sectioning (3D resolution) and substantial penetration depth, CRS offers unique opportunities to image tissues and model

organisms. The first application of CRS to live imaging was shown in Ref. [52] where the skin tissue of a mouse ear was imaged at video rate using CARS in a backscattering geometry, namely for ward CARS which was scattered in the tissue and collected in epi direction. A similar demonstration with SRS was subsequently reported in Ref. [44,99]. These works showed the ability to distinguish sebaceous glands, corneocytes and adipocytes label free and to follow dynamic processes such as the diffusion of mineral oils and drugs into the skin. The potential of CARS combined with two photon autofluorescence for 'optical skin biopsy' *in vivo* and in turn diagnosis of skin cancer in human patients was shown in Ref. [100].

Live animal imaging with CRS has also been shown on the model organism *C. elegans* specifically addressing lipid metabolism. Owing to its small size, optical transparency, its ability to support lipid droplet expansion to $>10\ \mu\text{m}$ size and its extensively studied genome, *C. elegans* has become an attractive model for studying fat storage *in vivo* combining sub cellular resolution with whole animal behavior. CARS microscopy was used to quantify changes in lipid storages in genetic mutants [101–103] and the effect of cholesterol lowering drugs [104]. Noticeable findings included the visualization of hypodermal lipid droplets that were not visible or quantifiable by fluorescence using Nile red, thus highlighting the advantage of label free microscopy [101] (see also Fig. 14A). Non linear optical imaging has also been used to study embryo genesis in model organisms. Here, by following the lipid droplet distribution the different stages of development can be visualized, as has first been shown in zebrafish embryos using THG microscopy. Recently, early stage development based on lipid droplet tracking has also been studied in *Drosophila* embryos using femto second SRL microscopy [105] (see also Fig. 14B).

Nerve tissues have also been widely investigated with CRS, since the myelin sheath that coats nerve axons is lipid rich and thus provides strong CRS contrast. Demyelination is a hallmark for many neurodegenerative diseases. Real time imaging of intact nerve tissues by CRS therefore offers a unique opportunity to elucidate the cellular mechanism of demyelination disorders. Wang et al. [109] reported the first application of CARS to visualize myelin sheets in explanted spinal cord tissues of guinea pigs. Following studies included the characterization in real time of

Fig. 14. (A) CARS microscopy of lipid droplets in *C. elegans* (adapted from Ref. [101]). (B) SRL image of lipid droplets in a *Drosophila* embryo (adapted from Ref. [105]). (C–E) CARS of a crushed rat sciatic nerve ranging from the healthy (C), proximal (D) and distal (E) region of the lesion (adapted from Ref. [106]). (F, G) CARS and multimodal SHG (blue)/TPF (green)/CARS (red) images of the entire descending aorta of an ApoE-deficient mouse fed a Western diet. (adapted from Ref. [107]). (H) SRS multicolor image of an equine articular cartilage (Blue: carbonate resonance at $1070\ \text{cm}^{-1}$, Green: Phosphate resonance at $959\ \text{cm}^{-1}$, Red: Lipid resonance at $2840\ \text{cm}^{-1}$) (adapted from Ref. [108]).

lysophosphatidylcholine induced myelin degradation *ex vivo* and *in vivo* in mice [110], the study of the microenvironment of the sciatic nerve in a living mouse by CARS and SHG [111], the ability of block copolymer micelles to repair spinal cord injuries [112], the response of myelin to high frequency electrical stimulation for therapeutic purposes [113], and the real time *in vivo* monitoring of demyelination and subsequent remyelination of a rat sciatic nerve after controlled injury [106] (see also Fig. 14C E). In experiments of this type, the spectroscopic information of CARS microscopy can be complemented with other non linear optical responses of the sample, which can be read out simultaneously. Thus, different aspects related to the healing of spinal cord injuries has been followed by simultaneous monitoring of two photon excited autofluorescence from macrophages (inflammatory response), of SHG indicative for the formation of collagen fibers (scar formation), and of CARS depicting nervous tissue [114].

The application of CRS to brain imaging has also been investigated. Evans et al. [115] demonstrated the use of CARS microscopy in identifying normal brain structures and primary glioma in fresh unfixed and unstained *ex vivo* mouse brain tissue. More recently, chemoselective imaging of fresh mouse brain tissue via multiplex CARS microscopy [116], and multimodal CARS, SHG, and TPF on unstained tissue sections from pig brain have been demonstrated [117]. Furthermore, a novel combination of SRS and two photon photothermal lensing was recently shown to provide simultaneous visualisation of cortical microvascular morphology and surrounding cellular structures in mice brain slices [118].

Another major clinically relevant application of CRS is the study of lipids in atherosclerotic plaques. Existing diagnostic tools for atherosclerosis such as magnetic resonance imaging and ultrasound tend to detect only advanced calcified plaques and are inadequate for early diagnosis. In addition the diagnosis of vulnerable plaques require a chemical evaluation of their composition which is lacking in these techniques. CRS imaging overcomes these limitations as shown in Ref. [119] where multiplex CARS has been used to identify the chemical profile of four types of atherosclerotic lipids in mice aortas *ex vivo*. Multimodal CARS/SHG/TPF was used in Ref. [120,121] to visualize the composition of arterial walls and atherosclerotic plaques. The impact of a high fat high cholesterol diet on the composition of atherosclerotic plaques in apolipoprotein E deficient mouse model was reported in Ref. [107] using multimodal CARS/SHG/TPF (see also Fig. 14F and G), and the identification of cholesterol crystals in plaques of atherosclerotic mice using multiplex CARS imaging was shown in Ref. [122]. Furthermore, multimodal CARS/SHG/TPF was used to characterize atherosclerotic plaque development in myocardial infarction prone rabbits [123] and stent induced coronary artery stenosis in pigs [124].

Other applications of CRS to tissues included imaging of liver and intestine. Specific imaging of fat droplets in intact liver tissues was performed by CARS microscopy in Ref. [125] and used to quantify the hepatic fat content through image analysis while eliminating laborious procedures required by traditional histopathological examination. Multimodal CARS/SHG/TPF was shown to be a valuable tool to assess liver steatosis and fibrosis in rats [126]. *Ex vivo* and *in vivo* CARS imaging of small intestine of mice during dietary fat absorption was shown in Ref. [90,92].

Also to be noted are CRS imaging of breast tissue and lung tissue, for the purpose of cancer diagnosis. Specifically, a knowledge based CARS microscopy system was developed in Ref. [127] for label free imaging, pattern recognition, and classification of cells and tissue structures from human patients in order to differentiate lung cancer from non neoplastic lung tissues and identify lung cancer subtypes. The differential diagnosis of breast cancer using CARS in human breast tissues was reported in Ref. [128]. The use of combined confocal Raman and high speed CARS

imaging for label free determination of apical polarity of human live mammary acini in 3D culture was shown in Ref. [129] to be an effective method for the rapid identification of risk factors that initiate the very early stage of epithelial neoplasia. Moreover, in Ref. [130] CARS was used to validate an adipose tumor epithelial cell co culture system designed to reproduce the *in vivo* mammary environment.

Within the remit of tissue engineering, multimodal CARS/SHG has been used recently to visualize the 3D cell arrangement in microporous bacterial cellulose scaffolds seeded with osteoprogenitor cells [131], to study the interaction between rat marrow stromal cells and engineered extracellular matrices [132], and to characterize bovine smooth muscle cells and their proliferation, migration, and differentiation in hydrogel cellulose scaffolds [133]. SRS has also been applied to unstained samples of articular cartilage enabling the investigation of living cells within fresh tissue and the identification of variations in protein and lipid content within the cells, pericellular matrix and interterritorial matrix [108] (see also Fig. 14H).

Finally, an upcoming area of biomedical interest is the study of nanoparticle uptake in cells and tissues. The motivation for these studies is either an interest in the toxicity of nanoparticles or in their pharmaceutical application. While CARS microscopy has been applied in studies of nanoparticle toxicity [134], much more work is currently undertaken to study drug delivery using non linear vibrational microscopy. Examples include Ref. [135] where CARS microscopy was used to image peptide drug nanofibers delivered across the blood brain barrier in mice brain tissues *ex vivo*. A combination of CARS, SHG and TPF microscopy was used in Ref. [136] as a multimodal label free method for pinpointing orally administered polymeric nanoparticles within the stomach, intestine, gall bladder and liver in mice tissue sections.

5. Conclusions and outlook

In conclusion, we have shown that CRS microscopy is a versatile new tool for studying a variety of different samples. With its specific strength of allowing video rate imaging of unlabeled samples while providing chemically specific contrast, it complements existing linear and non linear optical microscopy techniques. After more than a decade of technological development of different CRS approaches, the techniques are now mature and can be applied also by non specialists. This is witnessed by the fact that the majority of publications in the field are now focused on biological applications of CRS microscopy, and commercial CRS microscopes are now available. Specifically, we pointed out many applications relevant to lipid biology due to the strong CRS signals of lipids. This will most likely continue to be the case, with biomedical applications receiving even more attention in the future.

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