Vibrational imaging based on stimulated Raman scattering microscopy

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Abstract. A stimulated Raman scattering microscope with near-infrared picosecond laser pulses at high repetition rates (76 MHz) and radio-frequency lock-in detection is accomplished. Based on stimulated Raman loss detection, we demonstrate noninvasive point-by-point vibrational mapping of chemical and biological samples with high sensitivity and without the requirement for labeling of the sample with natural or artificial fluorophores. We experimentally demonstrate a major benefit of this technique, which is the capability to respond exclusively to the linear Raman-resonance properties of the sample, thus allowing a direct quantitative interpretation of image contrast in terms of the number density of Raman-active modes.

Recent advances in nonlinear coherent microscopy have attracted much interest because it offers three-dimensional visualization of microscopic objects within a complex and heterogeneous biological sample, i.e. a living cell or tissue, without the requirement for labeling the object of interest with external fluorophores or auto-fluorescent proteins. Among the coherent microscopies, those based on coherent Raman scattering (CRS) provide vibrational contrast intrinsic to and characteristic of chemical species, which is similar to confocal spontaneous Raman scattering microscopy. The applicability of the latter, however, is often limited by the inherently weak Raman scattering cross sections. In contrast, because of the coherent nature of the CRS signal, in which the molecular bonds oscillate in phase and the vibrational states are actively pumped, the Raman signal is significantly enhanced. Here, pump and Stokes incident electromagnetic fields with frequencies \( \omega_p \) and \( \omega_S \) (\( \omega_p > \omega_S \)), respectively, interact

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with the sample that is most generally described by a nonlinear susceptibility of the form $\chi^{(3)} = \chi^{(3)\text{nr}} + \chi^{(3)r}$. Third-order nonlinear polarizations at distinct frequencies are induced, which are sources of coherent radiation fields in the corresponding phase-matched directions [1]–[3]. The complex quantity $\chi^{(3)r}$ represents the nuclear response of the molecules and provides the intrinsic vibrational contrast mechanism in CRS-based microscopies. The contrast is enhanced when the Raman shift, $\omega_p - \omega_S$, matches the resonance frequency $\Omega_r$ of a Raman-active mode (figure 1(a)). $\chi^{(3)\text{nr}}$ represents the nonresonant electronic contributions to the total third-order nonlinear susceptibility, which is a real quantity, and thus is independent of Raman shifts [4].

While this underlying physics is shared by all CRS processes, the most predominant realization of CRS microscopy to date is CARS microscopy where the generated anti-Stokes field at $\omega_{\text{AS}} = 2\omega_p - \omega_S$ is detected [5, 6]. CARS microscopy has emerged as a highly sensitive tool for label-free vibrational imaging and microspectroscopy in the life and material sciences [7, 8]. With the output field being in a mode different from the input fields, CARS microscopy benefits from the fact that the CARS signal can be selectively detected through spectral filtering against the pump and Stokes beams. For the same fundamental reason, however, the following main disadvantages in CARS detection concurrently exist [3]. Firstly, the detected signal is proportional to the modulus square of the total third-order susceptibility, $I_{\text{CARS}} \propto |\chi^{(3)}|^2$, causing distorted Raman line shapes and limiting the detection sensitivity due to unavoidable nonresonant background. Secondly, the CARS signal scales as the square of the spontaneous Raman scattering response and as a cube of laser power. Consequently, weak Raman resonances or low densities of scatterers are not easily detected. Finally, the need to fulfill the phase-matching condition in CARS results in a signal that is dependent on both the dimension of the nonlinear sample and the microscope’s geometry for the propagation directions of the input and output fields [9, 10]. During recent years, methods have been developed that can significantly improve the detection sensitivity in CARS microscopy either by suppressing the nonresonant background signals [9]–[13] or by implementing optical heterodyne detected CARS (OHD-CARS) [14]–[16].

In this work, we explore a particularly attractive approach to avoid the difficulties inherent to CARS microscopy, which is based on the detection of stimulated Raman scattering (SRS). SRS is quantum mechanically described as a two-photon stimulated process where one pump photon at $\omega_p$ is annihilated (stimulated Raman loss: SRL) and one Stokes photon at $\omega_S$ is created (stimulated Raman gain: SRG), while the Raman medium makes a transition from the initial electronic ground state to the final vibrationally excited state [2] (figure 1(a)). SRS then appears in the form of a gain or a loss of the Stokes and pump laser beams, as first observed by Woodbury and Ng in 1962 [17] and by Jones and Stoicheff in 1964 [18], respectively (figure 1(b)). SRS spectroscopy has long been recognized as a highly sensitive spectroscopic tool for chemical analyses in the condensed and gas phases [19]–[22]. In 1980, Heritage and Allara demonstrated the shot-noise limited SRS detection of a single molecular monolayer [23]. Here, we demonstrate SRS as a contrast mechanism for label-free optical microscopy, which provides readily interpretable and background-free chemical image contrast. We show that SRS is linearly proportional to the Raman response and is inherently insensitive to any nonresonant background contributions [1, 3]. This is especially important for biomedical imaging applications where water represents the predominant source of nonresonant background signal in the sample.

The combination of femtosecond SRS spectroscopy and optical microscopy has been recently reported using an amplified femtosecond laser system operating at 1 kHz with pulse
energies at the sample exceeding 270 nJ and multiplex detection of difference spectra with a photodiode array [24]. Although spatially resolved SRS spectroscopy and subsequent image reconstruction of 30 µm polystyrene beads has been demonstrated, this approach uses high pulse peak powers that may cause photo-damage in biological samples. It has a limited SRS detection sensitivity reported to be 0.01 and limited image pixel dwell times in the order of a second due to the low laser pulse repetition rate. Consequently, this scheme is inappropriate for fast vibrational bioimaging with high sensitivity. In contrast, here we present an approach [25] that relies on the use of high-repetition rate (76 MHz) picosecond pulse trains with pulse energies at the sample <2 nJ and radio-frequency lock-in detection. Compared with the previous report [24], our approach thus allows SRS imaging with more than three orders of magnitude lower peak powers, with more than five orders of magnitude higher sensitivity, and with image pixel dwell

Figure 1. (a) Energy-level diagram for the SRS four-wave mixing process, in which a pair of spatially and temporally overlapped pump and Stokes laser pulses at frequencies $\omega_p$ and $\omega_S$, respectively, interact with a nonlinear Raman-active medium. $g$ and $v$ denote the electronic ground state and the vibrational state of a Raman mode with resonance frequency $\Omega_r$, respectively. (b) Illustration of the SRS process in a nonlinear Raman medium ($\chi^{(3)r}$) of thickness $L$, in which under Raman resonance conditions the focused Stokes and pump laser beam experiences a gain and a concomitant loss, respectively, while propagating along the optical axis $z$. (c) Schematic of the SRS microscope configured for SRL detection. (P, polarizer; HWP/QWP, half/quarter-wave plate; BC, dichroic beam combiner; Obj, objective lens; F, filter; A, analyzer; L, lens; S, sample; AOM, acoustooptical modulator; PD, photodiode detector; Preamp, pre-amplifier; RF-LIA, radio-frequency lock-in amplifier.)

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times in the order of milliseconds rather than of seconds. These beneficial properties combined facilitate direct online vibrational bioimaging with high SRS detection sensitivity and fast image acquisition, as will be presented in this work.

We first describe SRS classically as a parametric four-wave mixing process, which is the picture commonly used to theoretically describe the properties of CARS microscopy [9, 10]. We consider the pump and Stokes beams as monochromatic plane waves collinearly propagating along the z-axis through an isotropic Raman-active slab medium of thickness L (figure 1(b)). For the detection of SRL, the signal field $E_{sig}(\omega_p, L)$ generated in the slab is in the same mode as the input pump field $E_p(\omega_p)$ with the total intensity at the detector being $I_p(\omega_p, L) \propto |E_p(\omega_p) + E_{sig}(\omega_p, L)|^2$ [3]. This is reminiscent of an optically heterodyned detection scheme, where the pump field acts as an intrinsic local oscillator wave automatically injected along with the generated signal wave. Compared to OHD-CARS microscopy, no additional external local oscillator field is required. Furthermore, because $E_{sig}(\omega_p, L)$ is in phase with $E_p(\omega_p)$ the phase-matching condition is automatically fulfilled. Consequently, the quantity physically detected in the SRL experiment is then a change of the optical pump intensity, which in the weak signal limit, $E_{sig}(\omega_p, L) \ll E_p(\omega_p)$, is given by [1]

$$\Delta I_p(\omega_p) = I_p(\omega_p, L) - I_p(\omega_p, 0) \approx \frac{3\omega_p\mu_0}{n_p n_s \varepsilon_0} \text{Im} \left[ \chi^{(3),\text{SRL}}_{1111}(\omega_p; \omega_S, \omega_p, -\omega_S) \right] I_p(\omega_p, 0) I_S(\omega_S, 0) L. \quad (1)$$

Here, the input intensities are assumed to be independent of the interaction pump length (undepleted pump approximation). $n_p$ and $n_s$ are the indices of refraction of the medium at $\omega_p$ and $\omega_S$, respectively. $\varepsilon_0$ and $\mu_0$ are the permittivity and permeability of vacuum, and $I_S(\omega_S, 0) \propto |E_S(\omega_S)|^2$ and $I_p(\omega_p, 0) \propto |E_p(\omega_p)|^2$ are the intensities of the Stokes and pump input beams, respectively. Because in SRL $\text{Im}[\chi^{(3),\text{SRL}}_{1111}(\omega_p; \omega_S, \omega_p, -\omega_S)] \geq 0$, equation (1) describes a pump intensity attenuation. The analogous equation describing the detection of SRG is obtained by exchanging the subscripts p and S and by replacing the imaginary part of the third-order susceptibility with $\text{Im}[\chi^{(3),\text{SRG}}_{1111}(\omega_p; \omega_S, -\omega_p, \omega_S)] = -\text{Im}[\chi^{(3),\text{SRL}}_{1111}(\omega_p; \omega_S, \omega_p, -\omega_S)]$ [2]. In SRG, equation (1) thus describes an increase in Stokes intensity, the Raman gain. In transparent and optically inactive media, where the input frequencies are away from any electronic transition frequencies, and only the molecular ground state is populated, the selection rules of both SRS and spontaneous Raman scattering are identical [4, 26]. Accordingly, for a given Raman-active resonance $r$, the amplitudes of $\chi^{(3)r}$ can be expressed in terms of the isotropy and symmetric anisotropy invariants of the corresponding spontaneous Raman scattering tensor, $\alpha^2$ and $\gamma^2_s$, respectively. In the case of parallel linearly polarized pump and Stokes fields, which is the arrangement used in our experiments and implied in equation (1), the only relevant tensor component assumes the following form:

$$\chi^{(3)r}_{1111} = A_{r,1111} \delta_r - i\Gamma_r = C N \Gamma_r \frac{\alpha^2 + 4/45\gamma^2_s}{\delta_r - i\Gamma_r}. \quad (2)$$

$\delta_r = \Omega_r - (\omega_p - \omega_S)$ is the detuning from the $r$th Raman resonance at frequency $\Omega_r$ with a half-width at half-maximum (HWHM) of $\Gamma_r$. $N$ is the number density of Raman-active scatterers and $C$ is a proportionality constant. The parallel polarized spontaneous Raman scattering line shape is then given by $I_{\text{SRL}}(\omega_p - \omega_S) \propto \text{Im}(\chi^{(3)r}_{1111})$ [26].

The quantity detected in SRS microscopy (equation (1)) thus provides a particularly attractive approach to vibrational imaging of biological samples for the following reasons.
(i) the signal is linearly proportional to the imaginary part of $\chi^{(3)}_{1111}$, thus being directly proportional to the corresponding spontaneous Raman cross section, to the Raman line shape, and via equation (2) to the number density of Raman-active modes within the focal volume. (ii) The signal scales linearly with the pump and the Stokes input laser intensities. (iii) The signal is inherently insensitive to real nonresonant background contributions. Being a nonlinear optical microscopy with signal generation confined to the focal volume, SRS microscopy exhibits the additional advantage of a three-dimensional sectioning capability.

The validity of these beneficial SRS imaging properties is constrained to the weak coherent coupling regime in SRS. In biological and biomedical applications of SRS microscopy, we are primarily concerned with both molecular vibrations of organic molecules in aqueous solvents at room temperatures and weak input field strengths kept below the onset of sample photo-damage. Under these conditions, which are satisfied in the experiments presented here, typical Raman line widths are spectrally broad (within the range of 10–20 cm$^{-1}$, unlike narrow and intense Raman line features present in atomic vapors or crystalline solids) with corresponding ultrafast decays of vibrational coherences (in the subpicosecond time range) [12], and the applied pump and Stokes field strengths are far from saturating the Raman transitions. Consequently, the occurrence of nonlinear effects that are characteristic for the strong coherent interaction regime and that might deteriorate the SRS image contrast are considered negligible (vide infra).

To demonstrate the feasibility of SRS microscopy, we have set up an SRS microscope based on the detection of SRL. The experimental layout of our SRS microscope is schematically depicted in figure 1(c). The pump and Stokes pulse trains are provided by two electronically synchronized mode-locked Ti:sapphire oscillators (Coherent Inc., Mira 900D/900P/Synchro-Lock AP) operating at a repetition rate of 76 MHz with a mutual pulse timing jitter of less than 50 fs [27]. The wavelengths of both lasers are independently tunable within the Ti:sapphire gain curve from 700 to 950 nm, which allows the coverage of Raman shifts up to $\sim$3750 cm$^{-1}$. The temporal widths of the near-transform-limited pump and Stokes laser pulses are 5 ps, which correspond to a spectral bandwidth of $\sim$3 cm$^{-1}$ being narrower than a typical molecular Raman line width at room temperature. In the SRL experiment, the Stokes beam is modulated acoustooptically at 1 MHz. Both the pump and Stokes beams are expanded to fill the back aperture of the microscope objective before being combined on a dichroic beam combiner and directed into an inverted microscope. A water immersion objective lens (Olympus, PlanApo 60 $\times$ IR, NA = 1.2) is used to focus the collimated and collinear pump and Stokes beams into the sample. The transmitted pump beam is collected in the forward direction by a second objective lens (Olympus, LumPlanFl 60 $\times$ IR, NA = 0.9), and spectrally isolated from the Stokes beam by a stack of short-pass filters. The modulation of the pump intensity due to SRL is detected by a Si-PIN-diode, a current pre-amplifier (Stanford Research SR570), and a radio-frequency lock-in amplifier (Stanford Research SR844), thus directly providing the quantity $\Delta I_p(\omega_p)$ described by equation (1). Images were collected by raster scanning the sample respective to the fixed laser beams, using a closed-loop $xyz$-piezo-driven scanner, which limits pixel dwell times reported in this work to a few milliseconds. Pixel dwell times can be significantly reduced down to a few microseconds when laser-scanning schemes are used instead.

To experimentally demonstrate the characteristic properties of SRS microscopy, we performed SRL imaging of a self-assembled layer of 2.4 $\mu$m polystyrene beads on a coverslip surrounded by water. Figure 2(a) shows an image acquired within $\sim$90 s at a Raman shift of 2904 cm$^{-1}$, which resides within the line width of the aliphatic anti-symmetric
Figure 2. SRS microscopy of a polystyrene bead. (a) SRL image of self-assembled 2.4 μm polystyrene beads in water recorded with pump and Stokes beams at 716 nm and 904 nm, corresponding to a Raman shift of 2904 cm$^{-1}$. The average power of each beam was 5 mW. The image size measures 23.1 μm × 25.6 μm (140 × 155 pixels) with a pixel dwell time of 4 ms. Shown in (b) and (c) are the measured dependences of the SRL signal on the average power of the pump and Stokes beams, respectively, when focused on a single bead. In (b) the average Stokes power was kept constant at 10 mW, whereas in (c) the average pump power was 5 mW. Fitting the measured SRL signal (filled circles) to a power law (solid lines) reveals linear proportionality to both the pump and Stokes power. (d) Measured SRL spectrum (filled circles) of a single 2.4 μm polystyrene bead recorded with the Stokes wavelength at 901 nm and the pump wavelength tuned from 711 to 719 nm. The average power of each beam was 10 mW. Both the aliphatic symmetric $\nu_s$(CH$_2$) and anti-symmetric $\nu_{as}$(CH$_2$) Raman modes of polystyrene at 2853 and 2912 cm$^{-1}$, respectively, are clearly resolved. Shown for comparison as a solid line is the parallel-polarized spontaneous Raman spectrum $I_{s}^{\parallel}$ of bulk polystyrene.

CH$_2$-stretch Raman resonance, $\nu_{as}$(CH$_2$), of polystyrene (vide infra). The image contrast completely vanishes when the pump and Stokes pulses were not spatially or temporally overlapped, proving that the signal depends on both input pulses. No signal from water is detected, proving that SRS detection is free of nonresonant background. To test the linear properties of SRS detection as expressed by equation (1), we focused on a single bead. First, we recorded the dependence of the SRS signal on the average pump and Stokes powers, shown in figures 2(b) and (c), respectively. The fit to a power law reveals linear proportionality to both average laser
Figure 3. (a) SRL image of a polystyrene bead of ~500 nm diameter in water recorded with pump and Stokes beams at 752 and 854 nm, corresponding to a Raman shift of 1588 cm$^{-1}$ on resonance with the C=C stretching vibrations. The average power of each beam was 20 mW. The image size measures 3.4 $\mu$m $\times$ 3.8 $\mu$m (40 $\times$ 45 pixels) with a pixel dwell time of 5 ms. Shown in (b) is the lateral intensity profile along the line indicated by the arrows in (a). The fitted FWHM amounts to 900 $\pm$ 40 nm.

Next, we measured the spectral dependence of the bead signal on the Raman shift, $\omega_p - \omega_S$, by tuning the pump pulse wavelength with respect to a fixed Stokes pulse wavelength. Figure 2(d) displays the recorded spectrum covering a Raman shift range from 2800 to 2970 cm$^{-1}$ where CH-stretching vibrations reside. Both the aliphatic symmetric $\nu_s$(CH$_2$) and anti-symmetric $\nu_{as}$(CH$_2$) Raman modes of polystyrene at resonance frequencies of 2853 and 2912 cm$^{-1}$, respectively, are clearly resolved. The observed SRS spectrum qualitatively reproduces the characteristic features of the corresponding parallel-polarized spontaneous Raman spectrum, $I_{\text{Raman}}^{\parallel}(\omega_p - \omega_S)$, of bulk polystyrene, which is also shown for comparison. This observation verifies that the information content obtained by SRS and by spontaneous Raman scattering are identical. Thus, the image contrast is directly proportional to $\text{Im}(\chi^{(3)})$. All observations combine to prove that the detected signal is SRL and obeys equation (1). Neither an indication for a nonlinear dependence of the SRL signals towards higher irradiance levels nor a deviation from the expected linear spectral Raman response was observed. We conclude that at the power levels used in this study strong coherent interaction effects are not present. Consequently, the recorded image pixel intensities in figure 2(a) are interpreted as being linearly proportional to the number densities of $\nu_{as}$(CH$_2$) Raman modes of polystyrene inside the focal volume.

Figure 3(a) shows the SRL image of a 500 nm polystyrene bead spin-coated on a coverslip and embedded in water. The pump and Stokes beams are tuned to a Raman shift of 1588 cm$^{-1}$, which is on resonance with the C=C stretching vibration of the benzene rings in polystyrene. The bead contains $\sim 2.5 \times 10^8$ benzene rings. The lateral intensity profile shown in figure 3(b) is fitted with a Gaussian function with an FWHM of 0.90 $\pm$ 0.04 $\mu$m. The signal-to-background ratio at the maximum signal amounts to about 10. The background is limited by the noise floor of our current detection system. It can be further suppressed by the implementation of frequency-modulation techniques that have been previously demonstrated to provide shot-noise limited detection in SRS spectroscopy [20, 28].

SRL imaging of an unstained differentiated human HL-60 cell (Human promyelocytic leukemia cell) in an aqueous environment is demonstrated in figure 4. The cells have been stimulated to express a high concentration of lipid-rich, micron-sized droplets throughout the cytoplasm, which was first confirmed by the brightfield image shown in the insert for a representative cell. Next, we recorded the SRL image of such cells at a Raman shift of
Figure 4. SRL image of unstained human HL60 cells in an aqueous environment. The size of the image is $38.3 \mu m \times 33.6 \mu m$ (200 $\times$ 170 pixels). The pump and Stokes beams were at 746.5 and 852 nm with powers of 7.5 and 30 mW, respectively. The corresponding Raman shift was $1659 \text{ cm}^{-1}$ on resonance with the C=C stretching vibrations. The image pixel dwell time was 10 ms. The lateral intensity profile along the line marked by the arrows identifies the smallest features with FWHM of $\sim 970 \text{ nm}$ at $x = 8.1$ and 22.4 $\mu m$. The insert shows a bright-field image of a representative cell.

$1659 \text{ cm}^{-1}$, on resonance with the C=C stretching vibrations. The subcellular distribution of small features with high densities of C=C bonds is clearly visible, as one would expect to observe for lipid droplets rich in unsaturated lipids [29]. Shown in the bottom of figure 4 is the intensity profile across the line marked by the arrows in the cell image. The FWHM of the smallest features is $\sim 0.97 \mu m$, the actual size may be even smaller. The image contrast vanishes when the pump and Stokes pulses were not temporally or spatially overlapped, and clearly changed when recorded at a different $z$ position, indicating the three-dimensional sectioning capability.

In conclusion, SRS microscopy using near-infrared picosecond pulse trains at MHz-repetition rates allows label-free and quantitative vibrational imaging of submicron-sized objects in the whole spectral region of molecular vibrations. Being demonstrably (i) insensitive to electronic nonresonant background nonlinearities, (ii) linearly proportional to the spontaneous Raman response of the sample, and (iii) linearly proportional to each of the input laser intensities, the SRS image contrast can be linearly related to the number density of Raman-active modes inside the focal volume. Thus, the difficulties commonly encountered in CARS microscopy due to interferences of resonant and nonresonant CARS signals leading to dispersive CARS line shapes and coherent image artifacts are inherently avoided in SRS microscopy. Combined with a high sensitivity at a biologically tolerable laser power level, which is particularly attractive for the detection of weak Raman resonances or low densities of scatterers, SRS microscopy provides exciting possibilities for acquiring a point-by-point chemical map inside living cells or tissues based on Raman spectroscopy.
Note added. After submission of this work, a paper by Freudinger et al was published [30], which independently of our work also reports the successful demonstration of SRS bioimaging based on a concept that is similar to ours.

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