FULL ARTICLE

Fingerprint-to-CH stretch continuously tunable high spectral resolution Stimulated Raman Scattering microscope

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Stimulated Raman scattering (SRS) microscopy is a label-free method generating images based on chemical contrast within samples, and has already shown its great potential for high-sensitivity and fast imaging of biological specimens. The capability of SRS to collect molecular vibrational signatures in bio-samples, coupled with the availability of powerful statistical analysis methods, allows quantitative chemical imaging of live cells with sub-cellular resolution. This application has substantially driven the development of new SRS microscopy platforms. Indeed, in recent years, there has been a constant effort on devising configurations able to rapidly collect Raman spectra from samples over a wide vibrational spectral range, as needed for quantitative analysis by using chemometric methods. In this paper an SRS microscope which exploits spectral shaping by a narrowband and rapidly tunable Acousto Optical Tunable Filter (AOTF) is presented. This microscope enables spectral scanning from the Raman fingerprint region to the CH-stretch region without any modification of the optical setup. Moreover, it features also a high enough spectral resolution to allow resolving Raman peaks in the crowded fingerprint region. Finally, application of the developed SRS microscope to broadband hyperspectral imaging of biological samples over a large spectral range from 800 cm^{-1} till 3600 cm^{-1} , is demonstrated.

KEYWORDS:

Stimulated Raman scattering, hyperspectral imaging, coherent Raman imaging, lipid droplets

1 | INTRODUCTION

Coherent Raman scattering (CRS) microscopy has recently established itself as a rapid label-free technique for chemical specific biomedical imaging, allowing significant improvement in the acquisition speed and the advantage of an intrinsic 3D sectioning capability when compared to Spontaneous Raman scattering microscopy in important applications^[1,2]. In particular, Stimulated Raman scattering (SRS) microscopy is a CRS modality which features a signal linearly dependent on the concentration of

molecules and not affected by the presence of spectral artifacts, which would considerably increase the complexity in obtaining precise chemical information on the sample^[1,3]. In SRS microscopy, two pulsed laser beams at different wavelengths, a Pump beam and a Stokes beam, are temporally synchronized and spatially overlapped on the sample, giving rise to a coherent vibration of all molecular groups within the focal volume that are vibrationally resonant with the difference in optical frequencies between the two beams. Such a coherent vibration is then measured by using a lock-in detection scheme, either as a decrease of intensity on the Pump beam or as an increase of intensity on the Stokes beam, with

a sensitivity level which enables even video-rate imaging on biological samples^[4]. In order to select a specific Raman wavenumber for measurement, and eventually scan that wavenumber to obtain a hyperspectral image, most SRS setups have a single detector and use scanning elements according to different strategies^[5–7], which might be generally summarized as being of two types: wavelength tuning of one of the two beams^[3,8], or temporal scanning with an optical delay line (in the so-called Spectral-Focusing method^[9–11] and in the Fourier-transform based method^[12]). Other approaches have been presented which use parallel detection of different wavenumbers by using an array of detectors coupled with high sensitivity electronics^[13,14] or modulation-frequency multiplexing with a complex setup^[15].

Recently, SRS microscopy has seen a few technical advancements, moving from an imaging mainly performed at a single Raman wavenumber towards hyperspectral imaging^[7]. This has been motivated by the need to collect a large enough spectral content to allow quantitative analysis by using chemometric methods. In particular, most of the recently proposed and sensitive SRS setups use femtosecond lasers instead of picosecond lasers, notwithstanding the spectral width of the latter is often better fitting the requirements for Raman spectral resolution in measurements of biological samples. The rationale for this choice is the fact that a broadband laser source, as is a femtosecond laser, generates simultaneously a broad range of wavenumbers instead of just one, and the ability to perform a fast spectral scan is then up to a scanning mechanism (spectral or temporal) external to the laser, which is usually intrinsically faster than the tuning speed of the laser wavelength (of note, a fast laser tuning scheme exists^[16], which uses wavelength-swept Fourier Domain Mode Locking-FDML lasers, but some development is still needed to improve sensitivity, as the optical power delivered by this laser is still relatively low). Additionally, the use of femtosecond lasers has an important strength on allowing to opt for two different SRS spectral imaging schemes, namely either a "sequential" scheme, where different Raman wavenumbers are probed one at a time, or a "multiplexed" scheme, where multiple Raman wavenumbers are probed simultaneously.

One of the limiting factors in most femtosecond laser-based broadband SRS setups presented in the literature is that they require the replacement of optical components (e.g. diffraction gratings, glass rods, etc.) when switching between different spectral regions (e.g. fingerprint and CH stretch), especially if one doesn't want to compromise in spectral resolution of measurements. Therefore, acquisition of very broad SRS vibrational spectra might become cumbersome.

Here we present an SRS setup where spectral shaping of a femtosecond laser is performed through a fast and narrow-band Acousto-Optical Tunable Filter (AOTF), which allows

scanning all the way from the Raman fingerprint region to the CH-stretch region without any modifications of the setup. An AOTF with high enough spectral resolution (8 cm^{-1}) has been selected, which is paramount to properly resolve Raman peaks in the spectrally crowded fingerprint region.

2 | MATERIALS AND METHODS

2.1 | SRS microscope

The laser source used to drive our SRS setup (see Figure 1 (a)) is a commercial Optical Parametric Oscillator pumped by a femtosecond fiber-laser (Chameleon Discovery, Coherent Inc., Santa Clara, CA) featuring two 80 MHz temporally synchronized outputs, one at fixed wavelength (1040 nm, 2.5 Watts average power), which acts as a Stokes beam, the other tunable between 680-1300nm (with > 1.8 Watts of average power between 780-950 nm), which acts as a Pump beam. The pulsewidth is around 140 fs for the Stokes beam, while it's around 100 fs for the Pump tunable beam. In order to have high enough spectral resolution in Raman measurements, the spectral width for both the Stokes and the Pump beams is reduced to a bandwidth of about 8 cm^{-1} (corresponding to a ~ 3 ps transform limited pulse duration), which is an ideal resolution to well resolve Raman bands in biological samples. The spectral shaping for the two beams is done according to two different strategies: for the Stokes beam, a transmission grating (LightSmith, 1000 lines/mm, LSFSG-1000-3225-94) and a slit (Thorlabs, VA100/M) in a folded 4f configuration are used to obtain a fixed 8 cm^{-1} bandwidth at around 1040 nm; the Pump beam is instead passed through an AOTF (Gooch&Housego, TF950-500-1-5-NT2) with a spectral bandpass of 8 cm^{-1} (see Figure1 (b)) and whose central wavelength (i.e., ultimately, the chosen Raman wavenumber) can be switched very quickly over the 700-1200 nm spectral region. Of note, an SRS setup using an AOTF has been already proposed^[17], however its spectral resolution of 33 cm^{-1} is not narrow enough to properly resolve Raman peaks, even in the less demanding CH-stretch region.

The wide band spectral scanning strategy in our SRS system relies on the combination of a slower scan of the laser central wavelength and of a faster scan with the AOTF. In particular, the generated laser pulse spectral bandwidth, which is of about 150 cm^{-1} for every selected laser central wavelength, is fast scanned with the 8 cm^{-1} AOTF window and the signal is recorded for each of the selected corresponding wavenumbers. Then, the laser central wavelength is changed and an AOTF scan is performed again. In this way, and with the here described set of optical elements, the $800 - 3600\text{ cm}^{-1}$ spectral region, i.e. from fingerprint to CH stretch, can be covered with high spectral resolution and without the need to realign

FIGURE 1 (a) Schematic of our SRS microscope. PBS: polarizing beamsplitter; PD: signal photodiode; PDR: reference photodiode; PMT: Photomultiplier; DC: low frequency electrical output; RF: high frequency electrical output; LIA: Lock-in amplifier. (b) Spectrum of the Pump laser beam (black line) filtered the by one channel of the AOTF (red line).

the beam path, nor changing any of the optical elements of the setup. Of note, extension of the spectral scan range towards smaller wavenumbers is possible by a different choice of the dichroic elements/filters in the setup. The time needed by the AOTF to move to any other filter's central wavelength is about $40 \mu\text{s}$, while the time needed by the tunable laser to change its central wavelength is about 4 seconds. **As an example, a full scan between $800\text{--}3600 \text{ cm}^{-1}$, with a uniform step of 3 cm^{-1} and with a 1 ms integration time per spectral point, would require around 90 seconds as it is dominated by the laser wavelength tuning times (88 s due to 22 laser wavelength changes needed in present configuration). A measurement over a spectral range which can be scanned by relying on the AOTF only, es. $2800\text{--}2950 \text{ cm}^{-1}$ would instead take only 50 ms.** While the slow tuning of the laser central wavelength is a bottleneck for the speed of the system, this current limitation can potentially be overcome by using a Pump laser with a larger bandwidth or by spectral broadening of its output spectrum (e.g. by nonlinear effects in a suitable optical fiber). With this scheme it is easy to perform spectral scans in a continuous mode, eventually skipping unwanted regions to speedup acquisition, but also in a "sparse" mode, where only a small set of desired wavenumbers, even belonging to very different spectral regions, are measured. Additionally, our scheme, while being sequential in this described configuration (therefore only one wavenumber measured at every time instant) lends itself to be

upgraded to a multiplexed scheme by exploiting the possibility to amplitude-modulate the 8 independent channels of the AOTF, in a similar way as done by Fu et al^[17]. To allow high-sensitivity lock-in detection, the amplitude of the Stokes beam is modulated at 5 MHz by an acousto-optic modulator (Gooch&Housego, AOMO 3080-197). **An even faster modulation speed could be potentially obtained by replacing the AOM with an Electro-Optical Modulator, without any limitation by the other elements of the setup.**

The difference in optical pathlength between the modulated narrowband Stokes beam and the narrowband Pump beam is compensated by a software-controlled mechanical delay line. After being recombined on a dichroic mirror (Semrock, NFD01-1040-25x36), both beams travel collinearly via a pair of galvanometric mirrors (Cambridge Technology, 6215H) and a tube lens-scan lens sequence to an inverted microscope (Nikon Eclipse Ti-E), where they are focused into the sample by a water-immersion high numerical aperture objective (Nikon CFI Plan Apo IR SR 60XWI, NA = 1.27). The Pump beam focused on the sample is then collected in forward direction by a water dipping microscope objective lens (Olympus LUMPLFLN 60XW, NA = 1) and directed to a silicon photodiode (Hamamatsu, S3994-01). A pair of short pass filters (Thorlabs FESH1000) are set in front of the photodiode to avoid detection of the Stokes beam.

With the described configuration, the maximum available powers before the microscope objective for Stokes and

Pump beams, after spectral filtering, are 70 mW and 80 mW, respectively.

In order to compensate for the relatively high intensity noise of our Pump laser (its Relative Intensity Noise is around $-125\text{dBc}/\sqrt{\text{Hz}}$ at 1 MHz), which ultimately relates to the intrinsic noise of the fiber-based laser oscillator at the core of the laser system, a balanced detection scheme has been implemented starting from the configuration used by Freidiger et al^[18] (see Figure S1). In short, part of the Pump beam is directed, by using an achromatic quarter-wave plate (Thorlabs AHP05M-980) and a polarizing beam splitter (Thorlabs PBS102), to a reference silicon photodiode (Thorlabs SM05PD1A) placed before the entrance to the microscope. The outputs from the two photodiodes (reference and main) are then sent through two Bias-Tee filters (Mini-Circuits ZFBT-4R2GW+) with a 100kHz cut-off frequency to separate the low-frequency (LF) from the high-frequency (HF) components of the photo-generated signals. The separated signals are then fed on the four inputs of a 4-channel variable gain amplifier (AD8264). The difference of the LF signals from both photodiodes is used in an auto balancing loop, implemented by using a software-based proportional-integral-derivative controller (written in LabVIEW). The balanced HF output of the two photodiodes is then connected to the differential input of a Lock-in amplifier (HF2LI, Zurich Instruments) for final noise subtraction and subsequent SRS signal demodulation.

2.2 | Cell culture

Human hepatocellular carcinoma cells (American Type Culture Collection, Hep G2 [HEPG2] ATCC HB-8065) were cultured in flask (430639, Corning) in a 37 °C humidified incubator with 5% CO₂ supply. The Gibco (Thermo Fisher Scientific) media and reagents were used for Hep G2 cell culture maintenance: Dulbecco's Modified Eagle Medium (DMEM), high glucose supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin Streptomycin (P/S) and 1% L-Glutamine. For SRS imaging with deuterium labeled glucose, Hep G2 cells were directly seeded at about 900 cells/cm² on a glass coverslip (No. 1.5, Menzel-Glazer) in a cell culture dish (430165, Corning) for adherent culture with 25 mM of glucose-d₇ (DLM-2062, Cambridge Isotope Laboratories, Inc.) in glucose-free media (A14430, Gibco, Thermo Fisher Scientific) supplemented with 10% FBS, 1% P/S and 1% L-Glutamine for 4 days. Cells were fixed with 4 % paraformaldehyde before imaging.

FIGURE 2 (a)-(b): SRS imaging at two characteristic wavenumbers of a cluster of three beads from a mixture of 5 μm PMMA and PS beads (images are normalized to the maximum of SRS signal for each wavenumber). **Integration time is 50 μs per pixel, giving a 2 sec total acquisition time for each 200x200 pixel image.** (c) overlay of two SRS images acquired at resonance with PMMA (2955 cm^{-1} , in red), and with PS (3060 cm^{-1} , in green). (d) SRS spectra collected from the beads **with a in integration time of 3 ms; the time to collect each spectrum is 83 s (dominated by the laser wavelength tuning time).** The Stokes and Pump beam powers used for these measurements are 25 mW and 15 mW, respectively, on the sample plane .

3 | RESULTS AND DISCUSSION

For an initial assessment of the broadband imaging capability of our SRS microscope, a sample made by a mixture of poly-methyl methacrylate (PMMA) and polystyrene (PS) beads, both with 5 μm diameter (Sigma-Aldrich), was used. Figure 2 (a),(b) shows SRS images (20x20 μm Field of View) collected at 2955 cm^{-1} and 3060 cm^{-1} , which correspond to resonances of PMMA and PS Raman vibrational response respectively, and Figure 2 (c) shows an overlay of two SRS images acquired at resonance with PMMA (2955 cm^{-1} , in red), and with PS (3060 cm^{-1} , in green). The average Pump and Stokes powers on the sample were 15 and 25 mW respectively. Pixel dwell time was set to 50 μs , with a spatial step for galvo scan set to 100 nm per pixel. The broadband SRS spectra collected from the two types of beads are shown in Figure 2 (d).

A remarkable application of broadband SRS microscopy is the imaging of Lipid Droplets (LD) in cancer cells, to study the dysregulation of their lipid metabolism^[19]. Lipid Droplets are organelles present in living cells and are composed of neutral lipids, primarily triglycerides and sterol esters. Recent literature reveals a clear relationship between alteration of LD

FIGURE 3 Broadband SRS Raman spectra of three different edible oils with different fatty acid saturation levels: coconut oil, sunflower oil and olive oil. Inset: selected range within the fingerprint region. **Stokes power on sample plane is 35 mW, Pump power depends on the wavenumber (around 15 mW when filtered at the maximum of the laser spectral intensity). Integration time is 3 ms per wavenumber, giving a total scan acquisition time of 83 s (dominated by the laser wavelength tuning time).**

expression and cancer cell stemness, high tumor grade and aggressiveness^[19–21]. To show the potential of our SRS microscope towards quantitative imaging of lipids, and in particular its ability to characterize their saturation level through a high resolution spectral scan of the whole fingerprint-to-CH-stretch bandwidth, we have measured three vegetable edible oils with different lipid saturation levels as test samples. In particular we have used coconut oil -which contains mostly saturated fatty acids- sunflower oil and olive oil, which are instead composed by a mixture of mostly unsaturated fatty acids. The measured SRS broadband spectra are shown in Figure 3 and show that all the main Raman peaks that can be used to determine the saturation level of lipids are well resolved. In particular, in the fingerprint spectral region, the bending CH_2 vibration at 1445 cm^{-1} can be used for normalization of spectra, while the 1660 cm^{-1} , 1300 cm^{-1} and 1265 cm^{-1} peaks, which correspond to the $\text{C}=\text{C}$ stretch, CH_2 twisting and $=\text{CH}$ bending vibrations respectively, can be used to quantify the saturation level of the lipids. The smaller peak at 1750 cm^{-1} is assigned to the ester $\text{C}=\text{O}$ vibration^[22,23]. Our measurements show a decrease of the lipid saturation level starting from the mostly saturated coconut oil, then moving to olive oil and finally to the most unsaturated sunflower oil, as expected^[23]. In the CH-stretch region, the main peaks related to lipid saturation level are 2855 cm^{-1} and 3010 cm^{-1} , that can be assigned to CH_2 and $=\text{CH}$ stretching vibration respectively. **By calculating the amplitude ratio of the above listed Raman peaks related to saturation/unsaturation of the lipids, we can estimate a 1.8-fold reduction of lipid unsaturation in the olive oil compared to sunflower oil, consistently with the typical fatty acid composition of these oils^[23,24].** It's clearly evident from the measured SRS spectra how the information on the CH-stretch region well correlates with what observed in the

fingerprint region. Overall, the amount of information in the fingerprint region is more easily interpretable and more complete, therefore the ability to well resolve this spectral region would greatly improve quantitative analysis.^[6,25]

In order to demonstrate the imaging capability of our SRS microscope for measurements on biological samples, we have collected hyperspectral SRS images on human liver cancer cells (Hep G2). Figure 4 shows SRS images acquired at a selected set of wavenumbers, specific to lipids and proteins^[26] (1440 cm^{-1} , 1660 cm^{-1} , 2850 cm^{-1} , 2930 cm^{-1} and 3015 cm^{-1}), of a Hep G2 cell over a $40\text{ }\mu\text{m}$ field of view, with 30 mW and 15 mW sample-level average power for Stokes and Pump beam, respectively, and with a $200\text{ }\mu\text{s}$ pixel dwell time.

Broadband SRS spectra on LDs of Hep G2 cells are shown in Figure 5. In particular, spectra from a single point measurement on a LD (black line), acquired with 3 ms integration time per wavenumber, and as retrieved from the stack of an hyperspectral image of the same cell (red line), acquired with $200\text{ }\mu\text{s}$ integration time per wavenumber, are shown and demonstrate consistent results and high spectral resolution.

The broadband operation of our SRS microscope allows also to perform experiments with isotope-labelled molecules, which have recently shown great potential to study the dynamics of metabolic processes in live cells^[27–29]. For example, culturing cells with deuterated glucose (glucose- d_7) allows direct observation of *de novo* lipogenesis^[29], e.g. the process by which glucose is used as a substrate to synthesize new lipid molecules, which can be subsequently stored in cellular LDs as esterified fatty acids. In this regard, the possibility to simultaneously collect the signal in the silent region, typical of the isotope-labelled molecules, and in the fingerprint and/or CH-stretch region

FIGURE 4 (a) Bright-field and (b)-(f) hyperspectral SRS Raman images of a Hep G2 cell, shown at different wavenumbers specific to lipids and proteins (images are normalized to the maximum of SRS signal for each wavenumber; for images normalized to the maximum SRS signal across the whole spectrum, see Figure S5; for non-resonant wavenumbers see Figure S6). Stokes power on sample plane is 35 mW, Pump power depends on the wavenumber (around 20 mW when filtered at the maximum of the laser spectral intensity). An integration time of 200 μ s per pixel has been used, leading to an 8 s acquisition time for each 200x200 pixel image.

FIGURE 5 Broadband SRS spectra measured on the Lipid Droplet identified by a white square in Figure 4 . Black line: SRS spectrum from a scan on a single spatial point in the LD (3 ms integration time per wavenumber for a total measurement time of 79 s; laser powers same as for Figure 4). Red line: SRS spectrum on the same LD, as retrieved from a SRS hyperspectral image stack of the cell, rather than a spectrum collected on a single spatial point. Inset: selected range within the fingerprint region.

can add significant information on the biomolecular processes under investigation in the cells. To demonstrate application of our SRS microscope to this kind of experiments, we have performed hyperspectral SRS imaging on Hep G2 cells cultured in glucose- d_7 medium. In Figure 6 images of a Hep G2 cell collected at 2140 cm^{-1} , 2850 cm^{-1} and 2930 cm^{-1} are reported.

The spectrum in Figure 6 (e) represents the broadband SRS spectrum collected from a single point within a LD in the cell. One can clearly see appearance of the C-D stretch Raman peak at 2140 cm^{-1} in the silent region of the spectrum, due to the presence of newly synthesized lipid molecules

FIGURE 6 (a) Bright-field and (b)-(d) 200x200 pixel hyperspectral SRS Raman images of a Hep G2 cell incubated with glucose- d_7 medium (images are normalized to the maximum of SRS signal for each wavenumber). Images at 2850 cm^{-1} and 2930 cm^{-1} are measured with a 300 μs integration time per pixel (12 s per frame), while image at 2140 cm^{-1} is measured with a 500 μs integration time per pixel (20 s per frame); (e) SRS spectrum from a single point measurement on a LD (indicated by a white square in (a)-(d)) in the cell. Inset: selected range within the fingerprint and silent region. Time needed to measure the SRS spectrum is about 96 s (dominated by the laser wavelength tuning time).

through *de novo* lipogenesis from the supplied deuterated glucose.

4 | CONCLUSIONS

We have implemented a broadly tunable SRS microscope based on a narrowband and fast scanning AOTF filter. The main advantage of our scheme is that it allows to scan from the Raman fingerprint region to the CH-stretch region without any modification of the optical setup, and with consistently high spectral resolution. By using this setup we have demonstrated measurement of several samples over very broad vibrational spectral ranges, noticeably showing excellent capability to spectrally resolve Raman peaks in the crowded fingerprint region, enabling quantitative chemical imaging on bio specimens.

The relatively long settling time for tuning of the laser central wavelength is currently a bottleneck for the spectral scan speed of the system, but this limitation can be potentially overcome by using a Pump laser with a larger bandwidth or by a spectral broadening (e.g. by propagation in a suitable optical fiber) of its output spectrum. Indeed, by using a tunable source with a 30 fs pulselength, the whole CH stretch region (2700-3100 cm^{-1}) would be measured with just the AOTF scan mechanism, and no need of laser wavelength tuning. Additionally, our

scheme, while being "sequential" in this described configuration (therefore only one wavenumber measured at every time instant), lends itself to be upgraded to a, faster, multiplexed scheme by exploiting the possibility to amplitude-modulate the 8 independent channels of the AOTF.

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SUPPORTING INFORMATION

The following supporting information is available as part of the online article:

Figure S1. Schematic the balanced detection electronics.

Figure S2. Effect of the balanced detection scheme on the laser noise level.

Figure S3. Comparison of SRS images of a PMMA bead at 2955 cm⁻¹, collected without (left) and with (right) noise cancellation with the balanced detection scheme and with 5 μs

integration time.

Figure S4. Examples of AOTF filtered spectra.

Figure S5. Bright-field and hyperspectral SRS Raman images of a Hep G2 cell, shown at different wavenumbers specific to lipids and proteins (SRS images are all on the same intensity scale, normalized to the maximum of SRS signal at 2850 cm^{-1}).

Figure S6. SRS images of the cell of Figure S3, shown at non-resonant wavenumbers.

Detailed calculation of the acquisition times for presented measurements.

GRAPHICAL ABSTRACT FIGURE