Stimulated Raman scattering microscopy with long wavelengths for improved imaging depth

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Abstract
Stimulated Raman scattering (SRS) imaging is a fast, label-free, and sensitive technique to map the distribution of a vibrational species in a microscopy setting. It has great potential for applications in many fields, such as lipid imaging in biomedicine. However, depth penetration of the light into the sample is an issue with any light-based technique, especially with multiphoton techniques such as SRS. Using longer wavelengths allows deeper penetration into densely scattering materials, but applying wavelengths above 1,500 nm is challenging technically. We have built a flexible SRS microscope system capable of imaging with a combination of 1,064 nm and wavelengths over 1,500 nm, using the idler output of an optical parametric oscillator (OPO). For comparison, the same system was also operated in the more common configuration, using 1,064 nm in combination with the OPO signal output around 800 nm. With the long-wavelength settings, we show improved depth penetration in polyethylene plastic material and in a silicone phantom with embedded polymer microbeads, and we report images of lipid structure in biological tissue. These results demonstrate the technical feasibility of using these long wavelengths for SRS imaging. Disadvantages such as poorer spatial resolution and lower signal strength are also discussed. The application of this new approach to SRS microscopy can allow greater insight into deep-lying structures in a non-invasive way.

KEYWORDS
lips, NIR wavelengths, penetration depth, spatial resolution, SRS

1 | INTRODUCTION

Many imaging methods have been developed for visualising biological systems such as cells or tissues. Methods based on Raman scattering are of particular value because the contrast is based on intrinsic molecular vibrations and no labelling is required. However, the applicability of spontaneous Raman spectroscopy for imaging is limited by low signal strength and fluorescence backgrounds. A major breakthrough was the practical implementation of the stimulated Raman scattering (SRS) technique. It enables much higher mapping speeds and lacks the interference from fluorescence. SRS imaging presents a promising new field for visualising biological systems.

Raman scattering can be described as an inelastic process where a quantum of energy equal to a vibrational...
energy level of the molecule is transferred from a photon to the molecule, resulting in a scattered photon of lower energy and longer wavelength. For SRS, a second laser beam is introduced, and stimulated energy transfer occurs such that the Raman photons are scattered coherently (Figure 1a). The power increase in this second beam (called the Stokes beam) or the power decrease in the first (called the pump beam) is a measure for the number of Raman scatterers in the focal volume of the two laser beams. This change in power is very small (on the order of $10^{-5}$ to $10^{-7}$) and can therefore be lost in the laser relative intensity noise (RIN). To overcome this issue, one of the beams is modulated and a lock-in detection scheme is used, which measures the amplitude changes on the pump or Stokes beam at the chosen modulation frequency for which the laser RIN is low. Whether an SRS signal is generated depends on the energy difference between the two incident beams, not the exact wavelengths used. This means that the same Raman transition can be probed with different combinations of pump and Stokes wavelengths. It is possible to image with a beam pair at a longer wavelength and still obtain the same imaging contrast and information.

The Raman spectrum is often divided in the fingerprint region from about 1,000 to 1,600 cm$^{-1}$ and the CH stretch region at 2,800 to 3,100 cm$^{-1}$. For biological applications, the CH stretch region is particularly interesting, because it allows anatomical imaging of lipids and proteins that have strong peaks due to the large number of similar C–H bonds.[3] Most SRS imaging systems described in the literature are based on a combination of circa 1,064- and 800- to 900-nm laser beams, as produced by an optical parametric oscillator (OPO) system, or similar wavelength ranges. Alternatively, when 1,064-nm light is combined with a long-wavelength tunable laser, the fingerprint vibrations are accessed with up to 1,300-nm Stokes beams, and the CH stretch region requires wavelengths above 1,500 nm.

Working at longer wavelengths has many benefits, most importantly the increased penetration depth into turbid samples due to reduced scattering, allowing imaging of deep-lying structures. For instance, in medical applications, current imaging methods are limited in penetration depth and any increase would be a welcome improvement.[4] The depth limit is caused by the loss of excitation power by scattering before the light reaches the focus. Theoretical and experimental investigations in two-photon excitation fluorescence microscopy have shown that a fundamental depth limit is reached at approximately 5 to 6 attenuation lengths of the excitation light.[5–7] Longer wavelengths have longer attenuation lengths, allowing for deeper penetration of the light and signal generation at deeper locations. Another advantage is that longer wavelengths cause less photodamage because the energy is too low to cause electronic excitation by absorption; however, heating effects due to overtone absorption at these wavelengths can be an issue.[8]

Technically, imaging at these long wavelengths is challenging, because of the limited availability of components such as appropriately coated or corrected optics and detectors suitable for longer wavelengths. Lack of optimised optics leads to lower transmission and issues with chromatic aberrations. The available long-wavelength detectors also have higher electrical capacitances.

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**FIGURE 1**  Principle and experimental set-up of stimulated Raman scattering (SRS) microscopy. (a) Principle of SRS: When the photon energy difference between the beams matches a vibrational transition in a molecule, the result is a loss of the pump beam power (higher photon energy) and a gain in the stokes beam power (lower photon energy), independently from the exact wavelengths. (b) Schematic representation of the experimental set-up, showing the light path of 1,064 nm, signal, and idler wavelengths. AOM: acousto-optic modulator; PBS: polarising beam splitter; HWP: half-wave plate; QWP: quarter-wave plate. The AOM amplitude modulates the 1,064-nm beam, and the resulting intensity changes in the signal beam (loss) or the idler beam (gain) are detected with the lock-in amplifier.
compared to the short-wavelength equivalents, generally resulting in detection systems with poorer sensitivity. This sensitivity is generally lower because the maximum possible transimpedance amplification gain is limited at a given resonance frequency. The longer wavelength also intrinsically means a lower lateral resolution for diffraction limited imaging.[9]

Whether due to these technical difficulties, or a lack of interest or necessity, few research papers have so far been published employing these long wavelengths in SRS imaging applications. The time-correlated SRS applications of Karpf et al.[10] showed SRS signals up to 1,600 nm, but no imaging was performed. The applications of stimulated Raman gain and opposite loss detection (SRGOLD) employed the short and long wavelengths of the OPO but only showed imaging up to the fingerprint region at 1,555 cm\(^{-1}\).[11] Work in two-photon excited fluorescence imaging has shown the benefits of employing longer wavelengths to gain penetration depth,[12] but the collection of fluorescence signals is at about half the wavelength of the excitation. This is different from the situation in SRS, where the detected photons are also of near-IR wavelengths, the wavelength region where the transmission of optics is low and collection of the generated signal is a challenge. Also, in SRS, the signal on the detector is an intense background with a small amplitude modulation, which is very different from the situation in fluorescence detection where very sensitive photo-multiplier tubes are employed to detect low numbers of photons.

Imaging of biological tissues is an area of great interest, and coherent Raman methods have started to take their place in this research field.[3] In particular, for the non-invasive mapping of cellular structure on the basis of the signals in the CH stretch region, SRS microscopy has great potential. Adipose tissues are composed of adipose cells, a type of cell specialised in the storage of neutral lipids for use in times of nutrient scarcity. These lipids are composed almost entirely of CH\(_2\) groups and therefore give strong SRS signals at the CH\(_2\) stretch vibrational frequency of 2,845 cm\(^{-1}\).[13] In this paper, we first determine the signal attenuation with depth in a block of polyethylene and in silicone rubber phantoms with embedded polymer beads, comparing long-wavelength and short-wavelength laser combinations. We then show the potential of imaging with wavelengths over 1,500 nm for visualising lipids in biological tissues and discuss the advantages and limitations of long-wavelength SRS imaging.

2 | EXPERIMENTAL

The experimental set-up is shown in Figure 1b. An OPO (Levante Emerald, APE Germany) is pumped with 532-nm light at 80 MHz, pulse width 8 ps (Lumera Plecter); from this laser, also, the fundamental 1,064-nm output is available. The OPO splits these 532-nm photons into two near-infrared (NIR) wavelengths called the signal and idler, spectral resolution circa 3 cm\(^{-1}\). From energy conservation, it follows that these signal and idler wavelengths are at an equal energy distance from 1,064 nm. Because in SRS, the signal is dependent on the energy difference between the pump and Stokes light, both sets (signal with 1,064 nm and idler with 1,064 nm) can be used to probe the same Raman vibration. For imaging CH\(_2\) stretch vibrations, the Raman vibration is found at 2,845 cm\(^{-1}\), which corresponds to a signal wavelength of 816.7 nm or an idler wavelength of 1,528 nm. We choose to perform stimulated Raman loss (SRL) with the signal wavelengths and stimulated Raman gain (SRG) with the idler wavelengths. The amount of idler light available is limited, and amplitude modulation of this beam would further reduce the applied power. Instead, we use only one acousto-optic modulator (Crystal Technology 3080-194) in the 1,064-nm beam path, modulating at 3.63 MHz for short wavelength and at 1.11 MHz for long-wavelength SRS.

Different detectors are required for the two wavelength ranges. The detection for the signal wavelengths has been described before.[14] For the idler wavelengths, a custom detector with amplifier was developed in house based on an InGaAs PIN photodiode (Hamamatsu G12180-030A) and a home built transimpedance amplifier (based on a Texas Instruments OPA656). The amplifier provides an 81-dB gain at 1.11 MHz, which is a lower frequency than used for signal wavelength SRS at 3.636 MHz. Modulating at this lower frequency leads to slightly higher laser RIN. The balance between photodiode capacitance, amplification factor, and resonance frequency was carefully optimised to deliver maximum amplification at a frequency that still provided low laser RIN. A combination of electronic low pass and high pass filters (Mini-circuits SLP-30+ and ZFHP-1R2+) acted as a <3-dB loss bandpass filter between 0.85 and 35 MHz to block the laser repetition rate and decrease low-frequency RIN, which could otherwise saturate the input of the lock-in amplifier (LIA). Figure 2 shows the noise characteristics of the detection system at 1.11 MHz, with a shot noise limited range of laser powers from 0.2 to 1.1 mW of 1,528-nm light on the detector. At the lower end, the thermal (electronic) noise of the amplifier dominates, and above 1.1 mW, the laser RIN becomes the main noise source.

It is required for SRS generation that the focal spots of both laser colours overlap in the sample. The microscope system used (Zeiss LSM7MP with 32× C-Apochroplan objective, NA = 0.85) has been optimised by the manufacturer for long-wavelength transmission and minimal chromatic aberrations at the wavelengths used. Deuterated water (D\(_2\)O, Sigma Aldrich) was used as the
immersion medium between the objective and the sample, because it shows negligible absorption of light at the wavelengths used.\textsuperscript{[15]} The working distance of the objective was 1.1 mm. In addition, the number of optical components in the beam path of the idler wavelengths was reduced to minimise losses. Nevertheless, the total transmission to the sample was still low, with only 2.7% of light at 1,528 nm reaching the sample, compared with 23% at 816.7 nm. All experiments were performed in epi-detection with a polarising beam splitter plate (Edmund Optics 48–545) and a quarter wave plate system to separate the light going towards the sample and returning. The polarising beam splitter plate was a broadband wire grid polariser specified for visible light applications from 420 to 670 nm. However, it performed very well at the wavelengths used in this study, with reflectivity values of 85%, 94%, and 96% for 816.7, 1,064, and 1,528 nm, respectively.

It has previously been reported that the returning light is completely scrambled in polarisation,\textsuperscript{[16]} but for the samples shown here, we found that it is beneficial to use the quarter wave plate, as most polarisation is maintained in reflection. Optical filters were used to block the 1,064-nm light before the detector surface. The detector signal was demodulated with a LIA (Zurich Instruments HF2LI). The phase of the LIA was matched to the phase of the SRS signal, noting that SRL and SRG are out of phase by 180°. The time constant of the LIA was set to 1 ms, and the demodulated signal was sent to the microscope software (ZEN2011) for data processing. Image processing was done in ImageJ with a Gaussian blur with radius 0.8 applied in the y-direction to match the smearing in the x-direction caused by the mismatch between the time constant and the pixel dwell time. This mismatch is a consequence of the limitation in the slowest scan speed of the laser scanning microscope, which is faster than the optimal time constant for demodulating the SRS signal.

3 | RESULTS AND DISCUSSION

3.1 | Effective SRS focal volume

It is known from Gaussian optics that the spot size and focal depth of an imaging system scale linearly with the wavelength of the light used and will be larger with the longer wavelengths used in this study. Therefore, the maximum resolution obtainable in both the axial and lateral directions is lower. The resolution for SRS imaging is defined as the smallest separation where two point sources can be resolved and is directly related to the overlap of the spots of the two wavelengths of light used.\textsuperscript{[9]} For our set-up, we measured the beam size of each wavelength separately by laterally scanning the edge of a bar on a USAF resolution target and measuring the intensity of the reflected light, and repeated this process over a depth range of several microns. For each depth, the discrete derivative of the raw data was fitted to a Gaussian beam function in MATLAB. The FWHM Gaussian width was taken as the beam diameter, where the minimum width was taken as the lateral beam diameter at the focus. Next, we determined the focal depth (defined as twice the Rayleigh length) by finding the depth locations where the beam diameter is $\sqrt{2}$ times the beam diameter at the focus. The FWHM diameters were $0.68 \pm 0.01 \mu m$ for 816.7 nm, $0.85 \pm 0.01 \mu m$ for 1,064 nm, and $1.26 \pm 0.03 \mu m$ for 1,528 nm. As expected, the beam diameter increases roughly linearly with the wavelength. The focal depths were $3.47 \pm 0.07 \mu m$ for 816.7 nm, $5.05 \pm 0.11 \mu m$ for 1,064 nm, and $6.90 \pm 0.34 \mu m$ for 1,528 nm.

To determine the dimensions of the SRS focal volume, we calculated the power density product of two Gaussian beams. At the focus, the effective SRS focal width is described here as the product of the two individual Gaussian functions:

$$w_{\text{SRS}} = \frac{w_p w_S}{\sqrt{w_p^2 + w_S^2}}$$

where $w_p$ and $w_S$ are the widths of the pump and Stokes beams and $w_{\text{SRS}}$ is the effective SRS focal width. This parameter (FWHM) was $0.53 \pm 0.01 \mu m$ for SRS with

\[ FIGURE 2 \] Noise characteristics of the detection system as a function of 1,528-nm light intensity on the detector. Measured data points (black squares) match the sum of theoretical shot noise (blue line), measured electronic or thermal noise (Johnson–Nyquist noise level, grey line) and linear laser noise (dotted line). The grey area indicates the shot noise limited detection window from 0.2 to 1.1 mW on the detector.
816.7 and 1,064 nm, and $0.71 \pm 0.01 \mu m$ for SRS with 1,064 and 1,528 nm. Similarly, we calculated the effective SRS focal depth and found $2.63 \pm 0.04 \mu m$ for short-wavelength SRS and $3.73 \pm 0.09 \mu m$ for SRS at long wavelengths. The lateral resolution of SRS with longer wavelengths was lower by 33%, and the axial resolution was lower by 42%.

A larger focal volume causes a lower power density and was expected to result in a lower SRS signal at the long-wavelength combination. On the other hand, SRS

![FIGURE 3](image-url)
is generated over a larger volume. We estimated the overall SRS signal by integrating over the focal volume using the three-dimensional power density product of two Gaussian beams with the diameters determined previously. We assumed equal total beam intensities and sample homogeneity across the focal volume. The signal of the longer wavelength SRS was calculated to be 25% lower compared with the shorter wavelengths SRS from this effect alone.

### 3.2 Comparison between short and long wavelengths

For a direct comparison between imaging with shorter and longer wavelengths, a uniform block of polyethylene was measured. The sample was translated in the z-direction to measure a depth profile of the SRS signal at 2,845 cm\(^{-1}\), corresponding to the CH\(_2\) stretch Raman vibration. The maximal available power for the idler wavelengths was used: 11.2 mW at 1,528 nm and 28.8 mW at 1,064 nm on the sample. For a fair comparison, this total power of 40 mW on the sample was also used for the signal wavelengths, but here, the optimal ratio of 1:2 of unmodulated and modulated beams was used.\(^{[14]}\)

The results are shown in Figure 3a with the short-wavelength data in grey and the long-wavelength data in black. The background intensity slowly reduces with depth as the amount of light collected, and therefore, the shot noise level decreases. This background was measured off-resonance at 2,780 cm\(^{-1}\) and was subtracted from the data points. In the time between the measurements of the signal and background data points, there can be a minor drift in the offset applied to the input voltage by the microscope analogue-to-digital converter. This was resolved by fitting an exponential decay to the raw data, resulting in an offset at maximum depth, where no light reaches the detector. This offset was used to properly calculate the SRS amplitudes from the raw data. A single exponential decay with a single exponent was fitted to the data (red lines in Figure 3a).

Decay constants (defined as the depth over which the intensity decreases with a factor of e) were found of 29.2 ± 0.1 μm for SRL at 816.7 nm and 60.3 ± 0.3 μm for SRG at 1,528 nm. At greater depth, the data deviated from a single exponential slightly, possibly as a consequence of lower lock-in sensitivity. Because the collection efficiency and detector sensitivity are higher for the shorter wavelengths, the signal at the surface was about seven times stronger, but decays to the background level much faster. For the shorter wavelengths, a greater dynamic range is required to acquire SRS signal from both the surface and greatest depth in a sample. In contrast, with SRG at 1,528 nm, the signal intensities are much more similar across the depth scan and images can be recorded and visualised with a single sensitivity setting indicating another advantage of using longer wavelengths. Combined, all this means that using these long wavelengths in SRS can extend the available imaging depth. The improved imaging depth can be appreciated from the side projections in Figure 3b, which show that the SRS signal drops to the background level at a greater depth for the long wavelength compared with the short wavelength.

In addition to imaging a uniform block of polyethylene, we show the imaging of polyethylene microbeads with a size of 10–22 μm (CPMS-0.96, Cospheric LLC, Santa Barbara, USA) embedded in polydimethylsiloxane (PDMS) elastomer with short and long-wavelength SRS at 2,845 cm\(^{-1}\) down to 450-μm depth. The PDMS phantom contained 0.5% ZnO particles for uniform light scattering throughout. The imaging parameters were the same as those described for Figure 3a,b. Figure 3c shows the SRS signal amplitudes from individual beads at both short- and long-wavelength combinations, plotted against the depth in the phantom. Similar to the situation in Figure 3a, the SRS signal in Figure 3c shows a clear negative trend with both short and long wavelengths, with a faster decay of the short-wavelength SRS signal when compared with the long-wavelength SRS signal. The ratio between the short and long-wavelength SRS signal, plotted in red data points in Figure 3c, clearly shows the negative trend, indicating that long-wavelength SRS becomes more useful at larger depths in a scattering medium. The representative images at different depths in Figure 3d also illustrate this point. Although the SRS signal is clearly stronger for the short-wavelength combination at more shallow depths, the SRS signal of the long-wavelength combination does not decay as fast, and the bead is clearly more visible at a depth of 256 μm. Again, the long-wavelength SRS approach requires a smaller dynamic range to capture signals from the entire depth range.

For a fair comparison, we imaged exactly the same location in the phantom to compare individual beads. Any bead visible in both measurements was included in Figure 3c. When multiple beads were present at one depth, the average intensity was used. However, some beads were not visible in both sample stacks. Sample degradation during short-wavelength SRS measurements was an issue with this phantom sample. We hypothesised that this degradation is caused by absorption of the 816.7-nm light causing local heating and degradation. Regions of this damage obscured features in both the short and long-wavelength SRS measurements at the same location. For long-wavelength SRS, the defects produced transient...
absorption signals, which are out of phase with SRG, and therefore are seen as areas of black pixels. Examples of this effect can be seen in the right-hand panels in Figure 3d.

3.3 | Imaging lipid-rich fat tissue

To illustrate the potential of this technique, SRS images are acquired of the lipid distribution (CH₂ vibration at 2,845 cm⁻¹) in a piece of fat tissue, at the long-wavelength combination of 1,064 and 1,528 nm. A sample of fresh subcutaneous fat was taken from the top side of a pig, and a section a few millimetres thick was cut in the transverse direction and placed on a microscope slide. No additional sample handling procedures were necessary. A natural saline solution (0.9% NaCl in D₂O) was used as the immersion medium to avoid osmotic damage to the tissue during measurements. Image size was 100 × 100 μm and the spacing in the z-direction 4 μm; single images took half a minute to record. The results are shown in Figure 4; the separate adipocytes, or fat storage cells, filled with stored lipids can be clearly identified. The maximum intensity orthogonal projection in YZ (Figure 4b) shows lipid signals collected over 100 μm below the surface.

3.4 | Benefits and limitations of long-wavelength SRS imaging

We have shown that imaging at long wavelengths is possible, but the technique has some specific benefits and limitations. The limitations are mainly the restriction to samples with low water content, the lower spatial resolution, and the technically more challenging experimental set-up.

For applications in biological imaging, long-wavelength SRS is mostly suitable for tissues with low water content, such as fat tissue, because water absorption within the sample is an issue when working with these long wavelengths. The (decadal) extinction length of H₂O at 1,528 nm is 1.2 mm,[15,17] corresponding to a transmission of only 15% per millimetre in the case of pure water. For a depth of 250 μm (assuming a total path length of 500 μm) and 70% water content, the transmittance would be 51%. This limitation is also why D₂O was used as the immersion medium in this study, which is one method to deal with this issue. Other options include the use of fixed tissue samples or a minor adjustment of the wavelengths used to take advantage of a minimum in the water absorption spectrum.

The longer wavelengths used lead to a larger focal volume, which in turn leads to lower spatial resolution and lower total signals. However, the difference in signal level was calculated to be only 25%, which can be acceptable if the application prioritises deep penetration into the sample.

The main benefit of using very long wavelengths (1,064 nm combined with a Stokes beam above 1,500 nm) for SRS imaging is the potential for increased penetration depth, as demonstrated in the direct comparison in Figure 3. Depending on the properties of the sample, at some depth, the SNR of long-wavelength SRS surpasses that of short-wavelength SRS. Additionally, although customised detection systems are required, a smaller dynamic range is necessary in the detection systems to visualise the signals from the surface and maximum depth at the same sensitivity level. Finally, using long wavelengths can reduce photodamage effects.

4 | CONCLUSION AND OUTLOOK

We show for the first time SRS imaging with wavelengths above 1,500 nm in a commercial microscope system with
minimal adaptations and a custom detector. In a direct comparison, the longer wavelength allows for deeper light penetration into model samples and information collection over a larger depth range. The feasibility of this approach for biomedical applications is demonstrated with measurements on subcutaneous fat tissue, where an imaging depth of over 100 μm is achieved.

In the future, the improved availability of appropriately coated and corrected optics and sensitive detector materials could make this technique even more universally applicable. A set-up with more laser power available on the sample, combined with a more efficient collection path, would make imaging at long wavelengths a viable option for improving depth penetration of SRS imaging techniques in practical applications. The latter would include nonbiological samples and biological samples with a low water content, such as agrochemical seed coatings.[18]

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AUTHOR CONTRIBUTIONS

M. J. B. M. and J. F. d. B. conceived the research and designed the experiments, M. J. B. M. and L. Z. built the experimental set-up, M. J. B. M. performed and analysed the PE and biological experiments, L. Z. performed and analysed the phantom experiments, B. F. and L. Z. designed, performed, and analysed the resolution measurements, F. A. and J. F. d. B. provided conceptual advice and supervision, and M. J. B. M. wrote the paper with critical revision by L. Z. and F. A.

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