Background-Free Stimulated Raman Spectroscopy and Microscopy

Pascal Berto, Esben Ravn Andresen, and Hervé Rigneault
Aix-Marseille Université, CNRS, Centrale Marseille, Institut Fresnel, UMR 7249, Domaine Universitaire de Saint Jérôme, F-13397 Marseille Cedex 20, France
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We propose a three-color, double-modulation scheme for the background-free detection of stimulated Raman scattering (SRS). We call the scheme stimulated Raman gain and opposite loss detection (SRGOLD). It exploits the symmetric nature of potential parasitic signals (cross phase modulation, two-photon absorption, and thermal effects) to the end of suppressing them. Conversely, the antisymmetric nature of SRS provides for a twofold increase in the magnitude of the SRS signal. We experimentally demonstrate SRGOLD spectroscopy and microscopy on test samples as well as on mice skin samples.

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Although known for more than fifty years, stimulated Raman scattering (SRS) [1,2] was only recently implemented as a contrast mechanism in a microscope yielding label-free chemical imaging with great potential in the biomedical sciences [3–5]. The appeal of SRS is its fast imaging modalities [6], sensitivity orders of magnitude greater than spontaneous Raman scattering, and access to vibrational spectroscopic information that is readily interpretable as Raman spectra [2]. The initial reports suggested that SRS microscopy—unlike its predecessor coherent anti-Stokes Raman scattering (CARS) microscopy [7]—was free of spurious background signal. This is true only in a relative sense; the SRS signal is in general overlaid with parasitic signals stemming from other quasi-instantaneous nonlinear optical processes whose sources are ubiquitous. The ratio of SRS signal to spurious background thus decreases with the concentration and cross section of the Raman scatterers which one seeks to detect. This renders SRS microscopy on biological samples exceedingly difficult in the fingerprint spectral region where molecules present their unique vibrational signature. Indeed, few examples of SRS images in the fingerprint region have been published [8]. The spurious background signals will be spatially nonuniform in heterogeneous or scattering samples, which translates into increased risk that parasites will be mistaken for the SRS signal.

The purpose of this Letter is to develop a detection scheme insensitive to parasitic signal that could open the door to reliable SRS imaging in the fingerprint spectral region, in particular, and of weak Raman scatterers, in general. We will first detail the nature of potential sources of parasitic signal, and then we will introduce the principle of the stimulated Raman gain and opposite loss detection (SRGOLD) scheme. We will finally validate the SRGOLD scheme first on model samples, then on a mouse skin sample.

SRS is an energy transfer between a pump pulse and a probe pulse; the pulse at the higher frequency experiences a gain while the one at lower frequency experiences a loss. SRS is particularly appealing because this energy transfer is mediated by optical phonons associated to molecular Raman vibrations whose frequencies equal the frequency difference between the pump and probe beam. SRS stands as a label-free, chemically specific, contrast mechanism as Raman spectra are the fingerprints of molecules. Nonlinear transient absorption and nonlinear transient scattering processes are the two major classes of phenomena that give rise to parasitic signals overlaid on an SRS signal. (i) Nonlinear absorption, also known as two-photon absorption (TPA), arises from the concomitant absorption of one pump and one probe photon. The resulting attenuation of the probe beam is misinterpreted as an SRS signal [9]. (ii) Nonlinear scattering arises from the refractive index change induced by the pump beam and experienced by the probe beam. It is known as cross phase modulation (XPM) when it has a pure nonresonant electronic origin [10] and thermal lensing or thermal scattering [11,12] when it results from temperature-induced refractive index variation. In both cases, nonlinear scattering affects the angular distribution of the probe beam that is further converted into an intensity variation by aperture clipping along the detection beam path. Here again this probe beam intensity variation is misinterpreted as an SRS signal but has no chemical specificity. We report below how an SRS detection scheme can be made free of TPA, XPM, and thermal lensing or scattering artifacts by exploiting the fact that the artifacts are symmetric under sign change of the pump probe frequency difference whereas SRS is antisymmetric. The proposed SRGOLD scheme, furthermore, enhances the chemically specific signal by a factor of 2 and proves to be efficient in heterogeneous samples where linear scattering is at work.

Consider first the standard two-color stimulated Raman gain (SRG) detection scheme often employed in SRS microscopy. The involved pump and probe frequencies are \(\omega_0 + \Omega\) and \(\omega_0\), with \(\Omega\) the targeted vibrational molecular frequency. Figure 1(a) details the energy transfer...
to $\omega_0$ from the intensity-modulated $\omega_0 + \Omega$ due to SRS as well as the three effects responsible for artifacts: XPM, TPA, and thermal effects. While SRS results in a gain $\Delta I_{\text{SRG}}$ at $\omega_0$, the Kerr lens resulting from XPM changes the divergence of $\omega_0$ that due to aperture clipping is interpreted as a gain or a loss. Likewise, the effect of the thermal lens is interpreted as a gain or a loss. In Fig. 1, we arbitrarily assume a gain for XPM and a loss for thermal effects. TPA always results in attenuation at $\omega_0$ and is always interpreted as a loss.

Consider now the standard two-color stimulated Raman loss (SRL) scheme also employed in SRS microscopy where the involved pump and probe frequencies are $\omega_0 - \Omega$ and $\omega_0$. Note that the difference compared to the SRG scheme is a sign change on the frequency difference $\Omega$. Figure 1(b) details the energy transfer to $\omega_0$ from the intensity-modulated $\omega_0 - \Omega$. The antisymmetry of SRS is readily appreciated by comparison with what is seen in Fig. 1(a); SRS now results in a loss $\Delta I_{\text{SRL}}$ rather than a gain. Conversely, the XPM, TPA, and thermal artifacts are seen to be symmetric; they are unchanged compared to that in Fig. 1(a).

Figure 1(c) again shows a standard two-color SRL process but for $\omega_0 - \Omega$ modulation phase shifted by $180^\circ$ (denoted SRL-180$^\circ$) as compared to that of Fig. 1(b). Consequently, SRL signal and the XPM, TPA, and thermal artifacts are similarly phase shifted. The SRGOLD scheme in Fig. 1(d) comes about as the addition of SRL seen in Fig. 1(a) and SRL-180$^\circ$ seen in Fig. 1(c). Here, the three frequencies $\omega_0 - \Omega$, $\omega_0$, and $\omega_0 + \Omega$ are incident on the sample whereas $\omega_0 - \Omega$ and $\omega_0 + \Omega$ are modulated in opposite phase. The net effect is (1) to multiply by 2 the SRS signal at $\omega_0$ as it experiences successively a loss and a gain ($\Delta I_{\text{SRL}} + \Delta I_{\text{SRL}}$) and (2) to cancel all the parasitic effects, namely, XPM, TPA, and thermal effects. The physical picture of the cancellation is that the XPM, TPA, and thermal effects are made perpetual in the SRGOLD scheme to which signal detection by modulation transfer is insensitive.

In practice, the implementation of SRGOLD proceeds as presented in Fig. 1(e). The three IR beams are provided by a commercially available synchronously pumped OPO system (APE Levante OPO pumped by a frequency doubled Nd: YVO, picotrain HighQ laser) $\omega_0 - \Omega = 1150$–2450 nm (tunable idler beam), $\omega_0 = 1064$ nm, and $\omega_0 + \Omega = 690$–990 nm (tunable signal beam). These three beams (pulse duration ~5 ps, repetition rate 76 MHz) are overlapped in time and space and sent into a custom-made scanning microscope [13]. The signal at $\omega_0$ is detected in the forward direction by means of a high-speed photodiode (Thorlabs PDA10CS) and a fast lock-in amplifier (Signal Recovery 7280). Idler ($\omega_0 - \Omega$) and signal ($\omega_0 + \Omega$) beams are modulated at 1.5 MHz in opposite phase by two acousto-optic modulators (AAOptoelectronic MT200 A0,2-800). The collection objective lens (NA = 0.6, LUCPlanFL N 20×, Olympus) has a larger numerical aperture than the excitation one (NA = 0.45, LCLPlanFL N 20× IR, Olympus) to minimize the parasitic nonlinear scattering (XPM, thermal lensing or scattering) effects. For imaging, the sample is raster scanned.

As a first experimental demonstration, we performed SRGOLD spectroscopy. Figure 2(a) presents the stimulated Raman spectra of pure chlorobenzene as recorded in SRG, SRL, SRL-180$^\circ$, and SRGOLD schemes. These spectra were recorded with an iris diaphragm located before the photodiode [see Fig. 1(e)] in order to exemplify the contribution of XPM. Indeed, TPA and thermal lensing have negligible contribution in chlorobenzene at the considered IR wavelengths due to its transparency. In Fig. 2(a), SRG and SRL-180$^\circ$ spectra have different negative and positive offsets. These result from the XPM Kerr lens experienced by $\omega_0$ while interacting with $\omega_0 + \Omega$ and $\omega_0 - \Omega$ (180$^\circ$ phase shifted). In this case, the refractive index variations in the SRG and SRL cases experienced by the $\omega_0$ are given by

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\delta n_{\omega_0+\Omega}^{\omega_0+\Omega} = \frac{2}{3} \chi^{(3)}_{\text{XNR}} (\omega_0 + \Omega) I_{\omega_0+\Omega} n(\omega_0)n(\omega_0 + \Omega) c
\delta n_{\omega_0-\Omega}^{\omega_0-\Omega} = \frac{2}{3} \chi^{(3)}_{\text{XNR}} (\omega_0 - \Omega) I_{\omega_0-\Omega} n(\omega_0)n(\omega_0 - \Omega) c
$$

(1)

FIG. 1 (color online). SRGOLD principle and practical implementation. [(a)–(d)] Sketch of incident pumps ($\omega_0 + \Omega$, red, or $\omega_0 - \Omega$, blue) and probe ($\omega_0$, orange) and modulation transfer to the probe in (a) SRG scheme, (b) SRL scheme, (c) stimulated Raman opposite loss (SRL-180$^\circ$) scheme, and (d) SRGOLD scheme. Comparison of (a) to (c): The modulations induced on the probe due to SRS are in phase while the artifacts induced by XPM, TPA, and the thermal effect are in opposite phase. Conclusion on (d): In the SRGOLD scheme, the SRG and SRL signals add up while the artifacts cancel out. (e) Practical SRGOLD implementation.
where \( I_{\omega_0+\Omega} \) and \( I_{\omega_0-\Omega} \) are the signal and idler intensity, respectively, \( \varepsilon_0 \) is the vacuum permittivity, and \( c \) is the speed of light. In Eq. (1), \( \chi_{\text{XNR}}^{(3)} \) are the cross phase nonlinear susceptibilities given by \( \chi_{\text{XNR}}^{(3)}(\omega_0 + \Omega) = \chi_{\text{XNR}}^{(3)}(-\omega_0, \omega_0 + \Omega, \omega_0, -\omega_0 + \Omega) \) and \( \chi_{\text{XNR}}^{(3)}(\omega_0 - \Omega) = \chi_{\text{XNR}}^{(3)}(-\omega_0, \omega_0 - \Omega, \omega_0, -\omega_0 - \Omega) \) that are assumed to be independent of frequency. It is possible to set \( \delta I_{\omega_0+\Omega} = \delta I_{\omega_0-\Omega} \) by adjusting the ratio \( (I_{\omega_0+\Omega})/(I_{\omega_0-\Omega}) \) while being off molecular resonance. In this case, the XPM Kerr lens effects experienced by \( \omega_0 \) while interacting sequentially with \( \omega_0 + \Omega \) and \( \omega_0 - \Omega \) are set equal and the resulting net effect is the cancellation of the XPM background. In Fig. 2(a) this XPM cancellation was performed at \( \Omega = 960 \text{ cm}^{-1} \). This cancellation is also accompanied by a significant signal improvement.

Figure 2(b) shows an example of SRGOLD microscopy. A 20 \( \mu \text{m} \) polystyrene bead, embedded in a nonresonant liquid (Cargille immersion liquid OHZB), is successively imaged in the SRG, SRL-180°, and SRGOLD modalities both on the C–C skeletal vibrational resonance (\( \Omega = 1003 \text{ cm}^{-1} \)) and off resonance (\( \Omega = 930 \text{ cm}^{-1} \)). Here again, an iris diaphragm is placed before the photodiode to exemplify the effect of XPM. Whereas SRG and SRL images present a significant XPM background both at and off resonance, the SRGOLD scheme is completely background free and shows a signal at resonance that is the summation of the SRG and SRL-180° signals. The “\( \omega_0 \)” image is the signal with the sample present and with \( \omega_0 + \Omega \) and \( \omega_0 - \Omega \) beams blocked. This image represents the instrumental background in the detection scheme; i.e., SRGOLD is deemed successful if it can decrease the image background to this level.

We wish now to evaluate the SRGOLD modality in a more realistic case when the sample presents scattering, a situation that is found when imaging biological tissues. We concentrate first on a model sample consisting of 20 \( \mu \text{m} \) polystyrene beads sandwiched between two cover slips (spacing 150 \( \mu \text{m} \)) on the top of which a 60 \( \mu \text{m} \) scattering layer (Scotch tape 3 M) is placed [Fig. 3(a)]. The iris diaphragm [Fig. 1(e)] is now removed. Figure 3(b) shows
images of the \( \omega_0 \) laser light at the collection objective lens back aperture when the scattering layer is present and not present. In the absence of scattering layer, the collection objective lens (NA = 0.6) collects the totality of the focused (NA = 0.45) \( \omega_0 \) beam, whereas this is not the case when the scattering layer is present since this results in overfilling the collection lens back aperture. In this situation, the collection objective is clipping the \( \omega_0 \) beam and XPM background is expected. Figure 3(c) shows the SRG, SRL-180°, and SRGOLD images of a polystyrene bead at resonance (\( \Omega = 1003 \text{ cm}^{-1} \)) in the presence of the scattering layer. In this example, SRGOLD is efficient at removing the XPM background that is clearly present on the SRG and SRL images while it also proves to be efficient in enhancing image contrast in the presence of scattering for weak Raman bands. Figure 3(d) shows the SRG, SRL-180°, and SRGOLD images of polystyrene beads at \( \Omega = 1034 \) and 1041 cm\(^{-1} \) in the presence of the scattering layer. These two vibrational bands are 10 and 50 times weaker than the previously considered \( \Omega = 1003 \text{ cm}^{-1} \) C–C band, respectively. In both cases, the SRGOLD image is of superior quality and makes the beads stand out more clearly for the \( \Omega = 1041 \text{ cm}^{-1} \) case.

In order to evaluate the ability of SRGOLD to reveal vibrational contrast in biological samples, we concentrate now on its implementation in mice skin samples. Samples of 20 \( \mu \text{m} \) thick cryosectioned mice skin were placed on cover slips for SRS investigation without any further preparation. Figure 4(a) shows a white light image of a selected skin sample together with the SRG, SRL-180°, and SRGOLD images when targeting the amide II band at \( \Omega = 1450 \text{ cm}^{-1} \). Note that this amide band is about 10 times weaker than the \( \Omega = 2950 \text{ cm}^{-1} \) CH\(_3\) stretch commonly imaged with CARS and SRS. The \( \omega_0 \) image presents, as before, the SRGOLD image with \( \omega_0 + \Omega \) and \( \omega_0 - \Omega \) beams blocked. In the SRGOLD scheme, the artifact balance [realized by adjusting \( (I_{\omega_0+\Omega})/(I_{\omega_0-\Omega}) \)] was performed arbitrarily at point 0 for off-resonance frequency \( \Omega = 1550 \text{ cm}^{-1} \). Whereas SRG and SRL images show a poor contrast, the SRGOLD image correlates much better with the white light image showing details invisible with conventional SRG and SRL contrast mechanisms. To further appreciate the spectroscopic ability of the SRGOLD scheme, Fig. 4(b) presents the SRG (blue circles), SRL-180° (red triangles), and SRGOLD (black squares) spectra in the amide band (1350 cm\(^{-1} \) < \( \Omega < 1700 \text{ cm}^{-1} \)) obtained by scanning \( \omega_0 + \Omega \) and \( \omega_0 - \Omega \) [13]. On the same graph is also shown the signal with \( \omega_0 + \Omega \) and \( \omega_0 - \Omega \) blocked (gray circles). Two sets of spectra are presented here at points 1 and 2 as labeled in the white light image in Fig. 4(a). The opposite signs of the backgrounds in the SRG and SRL spectra prove that artifacts (XPM, TPA, thermal) are at work. Those are significantly suppressed in the SRGOLD spectra at both points 1 and 2. We can appreciate that the SRGOLD artifact balance performed at point 0 holds over most of the field of view and can be practically used over an area as large as 200 x 200 \( \mu \text{m} \) depending on the sample. Although SRGOLD artifact can be nicely balanced spatially over a quite large field of view, the balance as a function of frequency deserves some more attention. Indeed, contrary to XPM, TPA can exhibit chromatism between \( \omega_0 \) and \( \omega_0 + \Omega \) which is not present for \( \omega_0 - \Omega \). Figure 4(c) shows the opposite signs of the backgrounds in the SRG and SRL scheme than it does in the SRL scheme. We have found that, in the 1000 cm\(^{-1} \) < \( \Omega < 1700 \text{ cm}^{-1} \) range, the artifact cancellation in SRGOLD holds over 400 cm\(^{-1} \) without further adjusting \( (I_{\omega_0+\Omega})/(I_{\omega_0-\Omega}) \) (see the Supplemental Material Fig. S1 [14]).

The proposed SRGOLD scheme uses the fundamental antisymmetric nature of SRS and the symmetric nature of potential artifacts (XPM, TPA, and thermal effects) to cancel all spurious signals not pertaining to Raman processes. Not only does it provide true background-free SRS imaging and spectroscopy, it also enhances by a factor of 2 the SRS signal. This is because in SRGOLD, one is not modulating between on and off resonance. Rather, one is modulating between SRG detection and SRL detection. No post processing nor a priori knowledge of the targeted Raman band is required. The experimental implementation of SRGOLD is straightforward and takes full advantage of the commercial OPO source technology that generates at the same time \( \omega_0 - \Omega \), \( \omega_0 \), and \( \omega_0 + \Omega \) over the full molecular vibrational spectrum. All these features are unique to the SRGOLD scheme and differ fundamentally from previously reported efforts aimed at improving the
sensitivity in SRS [15–17]. Whereas the artifact cancellation has been demonstrated here in the forward detection scheme, it would also work in the backward detection modality as the XPM, TPA, and thermal effects are made perpetual and, hence, invisible to the lock-in detection. We believe SRGOLD can bring valuable sensitivity and signal to noise ratio enhancement in label-free molecular imaging and spectroscopy.

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*Corresponding author.
herve.rigneault@fresnel.fr