Abstract: Acquiring images of biological tissues and cells without the assistance of exogenous labels with a fast repetition rate and chemical specificity is what coherent anti-Stokes Raman Scattering (CARS) imaging offers. Nonresonant background (NRB) is one of the main drawbacks of the CARS microscopy technique because it limits the detection of weak Raman lines and the detection of low-concentration molecules. We show that a six-wave mixing process with two beams, which is a cascade effect of CARS, show better signal/NRB ratio and can be utilized for biological tissues imaging. The cascade CARS (CCARS) depends on chi-3 to the fourth power, instead of chi-3 squared as in the usual CARS signal; therefore, the contrast ratio with NRB is higher for CCARS than for CARS. We present analytic calculations showing that CCARS have better contrast over CARS in any situation. Comparison of the signals of both techniques generated on water-ethanol solutions confirm these results. Finally, we acquired CCARS images of fresh biological tissues, attesting that it is a useful tool for biological studies.

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1. Introduction

Coherent Anti-Stokes Raman Scattering (CARS) is a four-wave mixing process showing resonance with Raman active vibrations and signal with orders of magnitude stronger than spontaneous Raman [1]. CARS process requires at least two laser beams at different frequencies, usually called pump, $\omega_p$, and Stokes, $\omega_s$, which are set at an energy difference matching a Raman active vibration, $\omega_p - \omega_s = \Omega_R$, in order to resonantly enhance CARS signal at $2\omega_s - \omega_p$. The response CARS signal emerges at a different frequency from the two interrogation beams, pump and Stokes, making it easy to isolate the response beam from the excitation ones by using dichroic filters. CARS became a powerful tool for bioimaging after it had been successfully demonstrated that, on a tight focusing and with small interaction length of the incident beams, the phase matching conditions were eased [2]. Due to its chemical sensitivity, it has been widely used for imaging biological samples without the need of dye labeling [3–5] specially for lipids [6], an important characteristic for in vivo imaging.
One of the main challenges of CARS microscopy is to reduce the influence of the nonresonant background (NRB), which decreases the contrast ratio of chemically selective images. For example, for samples with low concentration of resonant species, the contribution of the nonresonant background due to the presence of water, particularly with non-fixed or in vivo biological tissue, and other biological structures, may overwhelm the resonant CARS signal when acquiring images [7–9].

Variations of the CARS technique have been developed to reduce the nonresonant background [10,11]. For example, employing NIR lasers pulses diminishes nonresonant signal generated by electronic transitions [2] but it is still not sufficient for acquiring CARS images with negligible NRB. Another NRB free Raman microscopy technique is the stimulated Raman scattering [12–14] in which energy is transferred via Raman from pump to Stokes beam. However, in this technique the response beam has the same wavelength of the interrogation beams, and can only be isolated by the use of laser modulation and lock-in amplifiers, adding complexity to the detection. Recently, Min et al. [13] have shown the influence of the NRB when comparing epi-CARS with SRS imagens, even when acquiring images from CH$_2$ resonance.

While significant effort has been devoted to reduce the NRB by improvements on the experimental technique, computational models for the subtraction of off-resonance image from an on-resonance image have been widely used [15–17]. In fact, according to Meyer et.al [18], for situations in which the signal from resonant CARS is at least an order of magnitude larger than the NBR, this can be subtracted without affecting the Lorenzian lineshape of the signal. One spectral approach to reduce the nonresonant background is to use time-domain Kramers–Kronig transform to isolate the resonant only component from the spectrum [19–21], but that still demands the acquisition of a NRB spectrum, and later, the mathematical treatment of the measured spectrum. In our understanding, the current scenario still demands different techniques that could improve the biological imaging acquisition of on-resonance CARS signal.

The ideal technique for Raman microscopy would require the signal enhancement of non-linear optical processes, with response wavelength different from the interrogation ones, and with negligible NRB or, at least, lower than that for CARS, but carrying the same chemical information of CARS. Therefore, it is worth searching for other resonant wave mixing optical processes that could improve the signal to NRB ratio. Among those, the six-wave mixing, or cascade CARS (CCARS), is a process in which the signal of a first CARS process is used to generate the second CARS process. CCARS intensity depends on the cube of the pump beam and on the square of the Stokes beam intensities, therefore, on the fourth power of $\chi^{(3)}$, instead on the second power of the conventional CARS, and the signal emerges at the frequency $\omega_4 = 3\omega_p - 2\omega_s$. The fact that CCARS signal depends on the fourth power of $\chi^{(3)}$, instead of the typical CARS second power of $\chi^{(3)}$ dependence, means that the signal/NRB ratio is higher in any limit, regardless if CARS<NRB, or CARS>NRB. For the limit where CARS<<NRB, the signal/NRB for CCARS shows a two-fold enhancement when compared to CARS signal/NRB, as we will show in details in the next section.

There are several six-wave mixing nonlinear optical processes generating a response signal at $\omega_4 = 3\omega_p - 2\omega_s$. Compaan et al. published a theoretical discussion [22] of these processes where they show one process related to $\chi^{(5)}$ and two cascade four-wave mixing processes, proportional to $\chi^{(3)} \otimes \chi^{(3)}$. They also demonstrated, both theoretically and experimentally, that cascade CARS is five orders of magnitude higher than direct second order CARS in benzene [22,23]. The two possible cascade processes are called parallel and sequential, Fig. 1. It has been shown previously [24,25] that the sequential cascade process is overwhelmed by the parallel process. On two-dimensional Raman spectra of CS$_2$ the direct fifth order is at most 2% of the cascade process [24]. This is the expected result considering that the sequential process is an overtone contribution, while the parallel process is a fundamental contribution.
In this report, we demonstrate the generation of CCARS signal and its use to acquire images of biological tissues. The image was acquired by collinearly scanning a pump and a Stokes beam in the sample and detecting the generated CCARS light. We verified that cascade four-wave mixing was the source of optical signal by measuring its intensity on solution of various proportions of water-ethanol. The generated six-wave mixing signal, even being a cascade four-wave mixing process, was strong enough to be easily distinguished from the auto fluorescence. To demonstrate the difference between CARS and CCARS we acquire images with both techniques in the CH$_2$ stretch region commonly used for CARS imaging. These lines are almost isolated, with several hundreds of wavenumbers from the spectrally dense fingerprint region. This was also important to avoid lines superposition due to our broadband laser system. Our results show the properties of the CCARS signal and its use as an imaging technique for living tissues. The fact that the coherent non-linear optical process of CCARS generates another beam at different wavelength makes the technique easy to implement, avoiding the detection of any incident beams with optical filters, with no need of lock-in amplifier techniques. User-friendly turnkey high energy laser sources can be used to observe higher order phenomena, going beyond the commonly used nowadays, (CARS, Second/Third Harmonic Generation and Two Photon Excitation Fluorescence) and, more important, allow to observe all these signals in parallel, encoding much more biological relevant information and allowing digital manipulation of signals to obtain other properties.

2. Theory

In CARS the pump and Stokes beams drive the molecules in the focal volume to vibrate in the beating frequency between the $\omega_p$ and $\omega_s$, and a fourth wave in the Stokes frequency is generated where the two beams overlap. If this frequency matches an active Raman level, then the resonant signal will be enhanced [26]. The beating frequency, even when far from resonance, can drive the molecules to vibrate, which gives rise to the nonresonant contribution to the CARS signal. The $\chi^{(3)}$ has a nonresonant and a resonant term given by $\chi^{(3)} = \chi^{(3)}_{nr} + \chi^{(3)}_r$ [27,28], shown by Eq. (1):

$$\chi^{(3)}_r = \frac{A}{\Omega - (\omega_p - \omega_s) - i\Gamma}.$$
where $\Omega_R$ is the Raman active frequency with $\Gamma$ Raman line bandwidth.

Six-wave mixing CARS can be generated by both a direct six-wave mixing and by a cascade four-wave mixing [25]. On the direct six-wave mixing process, the nonlinear polarization is proportional to the nonlinear susceptibility of fifth order, $P_{\text{CARS}} \propto \chi^{(5)}$. On the cascade four-wave mixing, the first order CARS signal generated will be used in a second CARS process with the same two lasers beams to generate a signal in a fourth wavelength with twice the Raman shift from the pump laser, $\omega_4 = 3\omega_p - 2\omega_s$. The nonlinear polarization generated by this cascade process is proportional to $P_{\text{CARS}} \propto \chi^{(3)}(\omega_p - \omega_s)$ (Fig. 1) [22]. Here we consider $\chi^{(3)}(\omega_p - \omega_s) = \chi^{(3)}(\omega_p, \omega_s, \omega_s, \omega_0)$, therefore the intensity is proportional to $\chi^{(3)}$.

CCARS intensity can be written in the expanded form:

\[ I_{\text{CCARS}} \propto \left| \chi^{(3)} \right|^2 + 4 \text{Re}\left\{ \chi^{(3)}(\omega_p - 2\omega_s) \right\} + 2 \text{Re}\left\{ \chi^{(3)}(\omega_p - \omega_s) \right\} \text{Re}\left\{ \chi^{(3)}(\omega_p - \omega_s) \right\} + 4 \text{Re}\left\{ \chi^{(3)}(\omega_p - \omega_s) \right\} \text{Re}\left\{ \chi^{(3)}(\omega_p - 2\omega_s) \right\}. \]  

The first term of Eq. (2) is purely nonresonant, independent of the Raman shift. The second term is purely resonant, independent of NRB, bringing the desired Raman shift information. The other terms all have resonant and nonresonant mixtures. This equation shows that CCARS signal has terms which depends on $\chi^{(3)}$ from the 4th power to 0th (NRB), while CARS dependence goes from 2nd to 0th power of $\chi^{(3)}$ [29]. The intensity for CARS is by Eq. (3) as follows:

\[ I_{\text{CARS}} \propto \left| \chi^{(3)} \right|^2 + \left| \chi^{(3)}(\omega_p - \omega_s) \right|^2 + 2 \text{Re}\left\{ \chi^{(3)}(\omega_p - \omega_s) \right\} \text{Re}\left\{ \chi^{(3)}(\omega_p - \omega_s) \right\}. \]  

When the NRB is much larger than the CARS signal, CARS<<NRB, we only consider the term of Eq. (2) and Eq. (3) with the lower resonance power, which gives a signal/NRB of order of $4\left[ \text{Re}\left\{ \chi^{(3)}(\omega_p - \omega_s) \right\} \right]$ for CCARS, compared to $2\left[ \text{Re}\left\{ \chi^{(3)}(\omega_p - \omega_s) \right\} \right]$ of usual CARS, with a gain factor of two. On the other hand for CARS>NRB the enhancement in contrast can be higher.

As a nonlinear process, CCARS intrinsically allows for the capability of three-dimensional sectioning, and as CARS, CCARS provides contrast based on the intrinsic molecular vibrations of a specimen, without the assistance of exogenous labels. For comparison between each signal and the NRB, Fig. 2 shows two calculated theoretical spectral plots for CARS and CCARS, where we normalized the NRB for both techniques by setting $\chi^{(3)} = 1$. In Fig. 2-A the curves were calculated for $\chi^{(3)} = 2\chi^{(3)}(\omega_p - \omega_s = \Omega)$ with CCARS presenting a higher peak than CARS. For the case when the NRB dominates both CARS and CCARS signals, Fig. 2-B, CCARS has twice the maximum CARS signal in comparison with the NRB. Our theoretical calculations shows that CCARS will present better contrast in any situation.
Fig. 2. Comparison of calculated CCARS (dashed), CARS (continuous) and NRB (doted), for \( \Gamma = 1 \text{ cm}^{-1} \), with \( \Delta = (\omega_p - \omega_s - \Omega_\omega - \Omega) \). Plotted lines are normalized to \( \chi^{(3)}_{NR} = 1 \). (A) \( \chi^{(3)}_{NR} = 2\chi^{(3)}_{SR} (\Delta = 0) \). (B) \( \chi^{(3)}_{NR} = 10^3\chi^{(3)}_{SR} (\Delta = 0) \), for comparison when \( \chi^{(3)}_{SR} \ll \chi^{(3)}_{NR} \).

3. Experimental setup

The beams were generated using a commercial femtosecond laser system. To generate the 800 nm beam (pump) we sampled the beam of a Ti:Sapphire amplified laser [Legend, Coherent] capable of generating pulses width shorter than 130 fs at 1 kHz repetition rate. The remaining light was fed into an optical parametric amplifier [OPerA, Coherent] where the Stokes beam was generated. Figure 3 shows the setup in details. The beams were combined using an 805SP dichroic mirror [Thorlabs DMSP805], the backward signals were reflected to the spectrometer [Princeton Instruments - Acton 2300i] by a 776LP dichroic mirror [Semrock - FF776-Di01]. The beams were aligned directly to the galvo mirrors of the laser scanning system [Olympus - FV300] through a lateral port, and from there, to the inverted microscope [Olympus - IX81]. We used a 10X NA0.3 Olympus, and a 20X NA 0.4 Leitz Wetzlar objectives, and a 0.55NA condenser to collect the forward light.

Fig. 3. Experimental setup showing in: 1 - Dichroic 805SP mirror; 2 - Dichroic 776LP mirror.

The laser beams blocking filter was a 680 short pass [Semrock - FF01-680/SP] that was utilized for the imaging and the spectra data acquisition, while a 710 short pass was used for the acquisition of the detuned images. The CCARS images were acquired with a Zeiss filter cube including a 525-560 band pass filter and a 610SP filter to make sure no CARS signal
would go through, while for the CARS imaging the filter cube included only a 555LP filter. When acquiring on/off resonance CCARS imaging we changed the 525-560 band pass filter for a 500LP filter. Both CCARS and CARS filters were placed over the condenser, and followed by hollow metal tube, 100mm long and 25mm wide, to avoid detecting any light coming from outside the filter cubes.

To measure the light intensity when working with water-ethanol solution, we placed a photodiode [PD] [Thorlabs - DET100A], but we used a photomultiplier tube [PMT] [Hamamatsu - R3896] to acquire the images in the forward position supplied by the microscope controller. Because the microscope software did not allow a pixel dwell time long enough to work with a 1 kHz repetition rate pulse, we controlled the scanning mirrors with a multifunction data acquisition [DAQ] [National Instruments - NI USB-6356]. The same DAQ was used to acquire the signal from the PMT. The pixel dwell time was set to 1 ms with one pulse per pixel. The approximated calculation for the spot size for 1000 nm and 0.4 NA objective is 3 μm, and for a 0.3 NA is 4 μm.

4. Results and discussion

The first characterization for the nonlinearity of the CCARS signal at 3ωp - 2ωs was to check if the dependence of the signal intensity on the pump and Stokes intensities follow the expected three pump and two Stokes incident photons dependence proportion, i.e., if

\[ I_{\text{CCARS}} \propto I_{\text{pump}}^3 I_{\text{Stokes}}^2. \]

We first characterized the nonlinearity measuring the CCARS peak intensity in water varying pump and Stokes power. For that, we placed a 5X objective before the galvo mirrors, focused the beams in a water sample, and measured the peak counts on the spectrometer for the CCARS generated signal as a function of pump and Stokes power. The nonlinear signals were strong enough to be observed at naked eye at different angles as shown in Fig. 4(A). Its spectrum is shown in Fig. 4(B). Figure 4(C) shows the CCARS intensity dependence on the pump and Stokes power, measured with the spectrometer maximum count for each emission wavelength, showing the expected cubic and quadratic dependence.

We then measured the CCARS signal intensity concentration dependency in a water-ethanol solution for several ethanol concentrations, from 99.3% of the solution to 9.93%, with the lasers wavelength adjusted for 2847 cm⁻¹ to observe the CH₂ symmetric stretching vibration, which is Raman active in ethanol but not in water [30]. The results are shown in figure Fig. 4(D), CCARS signal shows a stiffer curve than CARS, which is expected accordingly to Eq. (2), because the CCARS intensity dependence with N⁴. However, that behavior will not be visible on the concentration plot from Fig. 4(D) due to mixture terms with the nonresonant signal.

We also measured CCARS intensity on and off resonance in an ethanol volume by changing the Stokes beam wavelength, figure Fig. 4(E).
Fig. 4. (A) Photography of the scattered nonlinear signals - red is the CARS and Green the CCARS. One can even see a less intense blue signal of higher nonlinear orders. (B) Backscattered spectrum of (A). (C) Dependence of the CCARS intensity in water at 3240 cm\(^{-1}\), with pump at 800 nm and Stokes at 1080 nm Log-Log plot shows slope of fitted line fixed on 3 for pump beam and 2 for Stokes beam. (D) Normalized signal intensity vs. (ethanol volume)/(Solution volume), on a solution of water ethanol. Pump beam at 800 nm and Stokes beam at 1036 nm, for 2847 cm\(^{-1}\). (E) CARS and CCARS in and out resonance normalized signal intensity by changing Stokes beam wavelength - ethanol concentration 99.3%.

To demonstrate the capability of the CCARS to generate chemical selective images we chose to work with fresh mouse ear sample because it has an easily identifiable layer of lipid cells, and it is thin enough to detect forward signals. No animal have been sacrificed only for its ear, we utilized young male isogenic mouse from C57BL/6Junib lineage that have already been sacrificed, (approved by the local Committee for Ethics in Animal Research, CEUA - Unicamp). The lasers wavelength were adjusted for 2847 cm\(^{-1}\) to image CH\(_2\) symmetric stretching vibration, the pump beam sample from the regenerative amplifier was set to 800 nm, and the Stokes beam, from the OPA, was tuned to 1036 nm.
The results are shown in Fig. 5. After acquiring each image, we placed the same filter in front of the spectrometer and performed a scanning on the same area to acquire its spectrum. Figure 5(A) shows the spectrum for the CARS image (Fig. 5(C)) plotted in red, and the spectrum for the CCARS image (Fig. 5(D)) plotted in green, the merge of both images is shown in figure Fig. 5(E). The fact that one can see red spots on the merged images shows that CCARS image is not the same of CARS, although the yellow spots shows that both coincides in the majority of pixels. As observed in both, CCARS and CARS spectra, the signals are strong enough to suppress any possible two-photon excited auto fluorescence generated in the sample. The auto fluorescence generated in the tissue was not strong enough to be detected on the imaging and was not visualized in the spectra.

Figure 5(B) shows the bright field image acquired by a camera placed on the microscope ocular lens position. It is possible to see a small damaged area in the center of the image generated during the alignment procedure, when we kept both lasers at the sample and change the focus for signal optimization. That was the only considerable damaging caused on the tissue. After that, we could perform a series of images without any damage. The sample was able to support a peak power of over 3.7 GW for a pulse with approximately 150 fs, with an average power of 560 μW.

We then performed a series of images changing the OPA beam wavelength. Figure 6 shows the CARS and CCARS images for 2744 cm$^{-1}$ (Stokes beam at 1025 nm), 103 cm$^{-1}$ off peak resonance, and 2657 cm$^{-1}$ (Stokes beam at 1016 nm), 190 cm$^{-1}$ off peak resonance. We shall keep in mind that the resonances with our 90 cm$^{-1}$ broad laser pulse are not so sharp compared to 10 cm$^{-1}$ picosecond usual CARS laser sources.
Although CCARS and CARS images were acquired in the same region, sequentially, just by changing the filters, each CCARS/CARS image pairs were acquired from different regions because the timing to change the wavelength was too long and the sample moved. We kept the same PMT voltage for all CARS images in order to compare the detected light, the same was true for all CCARS images. By the images shown in figure Fig. 6 it is clear that on resonance CARS and CCARS images are similar. For a 103 cm\(^{-1}\) detuning off resonance we can still detect a reasonable amount of CARS arising from the tail of the broad incident beams together with the NRB. Both the intensity and the bright area of the CARS image are larger than the correspondingly pixels of CCARS image, demonstrating higher contrast of CCARS. For a 190 cm\(^{-1}\) detuning, both CARS and CCARS images are much weaker than the resonant ones, but the CARS still shows more intensity and features than CCARS.

Using the Measure analyses from the ImageJ software, we calculated the mean value of the pixels inside each region of interest delimited by a yellow rectangle showed in each image in Fig. 6. We chose regions with not a great number of pixels with the maximum value of 255, because this could mean a saturation of the detector, which would influence the results, showing less signal than was actually generated. Starting with a comparison between on resonance and 103 cm\(^{-1}\) off resonance, the results are 38.534 for A and 14.109 for B, which has a 2.73 CARS signal/NRB. For the CCARS images E and F, the results are respectively 40.096 and 5.41 with a signal/NRB of 7.41, that is 2.71 times greater the measurement from CARS. For the images acquired with 190 cm\(^{-1}\) off resonance, Fig. 6(C),(D),(G) and (H), the results are 49.341 for C and 8.524 for D, which has a 5.78 CARS signal/NRB. For the CCARS images G and H, the results are respectively 37.889 and 3.702 with a signal/NRB of 10.23, that is again 1.76 times greater the measurement from CARS. The results of Figs. 5 and 6 show, therefore, that CCARS images possesses the same intrinsic chemical information as CARS with higher signal/NRB ratio.

5. Conclusion

In this work, we showed, for the first time, to the best of our knowledge, acquisition of cascade CARS images in biological tissue samples. We acquire both CARS and CCARS images on and off resonance in the same focal volume to compare the two techniques. We have shown that CCARS can be strong enough to acquire images with the same chemical selectivity of CARS but with better signal/NRB ratio. We have performed tests of intensity and concentration dependence by measuring both CARS and CCARS signal from a solution
of water-ethanol with different proportions and verified the $I_{CCARS}$ and $I_{CARS}$ dependence. We have also shown that CCARS has better contrast compared to CARS, by taking the spectra of non-linear signals in different water-ethanol solutions. These results agree with our theoretical modeling.

This work also shows that there are higher order nonlinear optical signals that can be accessed by the modern high energy and tunable femtosecond synchronized multiple sources and can be used to acquire images with relevant biological information. Among the nonlinear optical signals the ones that are resonant with molecular vibrations, such as CCARS, are especially interesting because they allow performing spectroscopy and chemically selective image acquisition simply by tuning the wavelength of one beam with respect to the other, that is, by tuning the beating between the two sources. Moreover, the detection of these signals is simple, because the response wavelength is different from the two interrogation wavelengths, which also means that the detection of CCARS does not interfere with the detection of CARS as they are at different wavelengths, a simultaneous detection that gathered information from both is possible.

All the complexity of the experimental apparatus, therefore, is embedded in the laser system, not in the detection. The light source with the regenerative amplifier laser, with 1 kHz repetition rate, was the major downside for the image acquisition. Because of the low repetition rate, we found an increased difficulty in focusing the sample to the correct position, which caused the tissue damage shown in Fig. 5(B). We believe that amplified laser systems tend to become smaller, more powerful, with MHz repetition rate, turnkey, easy to use and affordable in the future, which could then be used to acquire all non-linear optical (NLO) signals, including CCARS and CARS, harmonic generation (SHG/THG/SFG), Two-Photons Exited Fluorescence and others simultaneously in a multimodal platform. In this scenario, CCARS and CARS resonance with molecular vibrations will be very valuable, particularly when performed in parallel with the other NLO techniques.

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**Disclosure**

The authors declare that there are no conflicts of interest related to this article.