Refractive effects in coherent anti-Stokes Raman scattering microscopy

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Coherent anti-Stokes Raman scattering (CARS) microscopy with high sensitivity and high three-dimensional resolution has been developed for the vibrational imaging of chemical species. Due to the coherent nature of the CARS emission, it has been reported that the detection of epi-CARS and forward-CARS (F-CARS) signals depends on the size and shape of the sample. We investigate theoretically and experimentally the effects on the CARS signal of refractive index mismatches between the sample and its surroundings. Backward-CARS and F-CARS signals are measured for different polystyrene bead diameters embedded in different refractive index solvents. We show that index mismatches result in a backward-reflected F-CARS signal that generally dominates the experimentally backward-detected signal. Simulations based on geometrical and wave optics comparing forward- and backward-detected signals for polystyrene beads embedded in different index solvents confirm our findings. Furthermore, we demonstrate that the maxima of forward- and backward-detected signals are generated at different positions along the optical axis in the sample if refractive index mismatches are present between the sample and its surroundings. ©2006 Optical Society of America

OCIS codes: 180.6900, 190.4380, 170.5650, 290.5910, 300.2570, 300.6230.

1. Introduction

Coherent anti-Stokes Raman scattering (CARS) is a four-wave mixing process in which a pump and Stokes laser beams, with center frequencies of $\nu_p$ and $\nu_S$, respectively, are spatially overlapping, and interact with a sample. When the frequency difference between the pump and the Stokes lasers coincides with the frequency of a molecular vibration present in the sample, the CARS signal at the anti-Stokes frequency $\nu_{AS} = (2\nu_p - \nu_S)$ is strongly enhanced leading to a light contrast mechanism with chemical selectivity.

As a third-order nonlinear process, the CARS-induced polarization can be expressed as

$$P^\text{NL}(\omega_{AS}) = \chi^{(3)}E_p(\omega_p)^2E_s(\omega_s)^*,$$

where $\chi^{(3)}$ is the third-order nonlinear tensor, $E_p(\omega_p)$ and $E_s(\omega_s)$ are the respective electromagnetic fields of the pump and Stokes beams, and $\omega_p$, $\omega_S$, and $\omega_{AS}$, respectively, refer to pulsations of the pump, Stokes, and anti-Stokes fields.

The $\chi^{(3)}$ tensor splits between a resonant vibrational contribution and a nonresonant electronic contribution, the latter leading to a nonspecific signal that usually limits the desired CARS specific molecular sensitivity. First implemented in microscopy by Zumbusch et al. under tight focusing conditions and collinearly propagating pump and Stokes beams, CARS microscopy appears today as a powerful contrast mechanism to study living matter with chemical selectivity, reduced photodamage, and 3D sectioning capability.

As a coherent process, the CARS signal generation relies on a phase-matching condition so that its spatial distribution proves to depend on the shape and size of the sample. In the collinear beam geometry (see Fig. 1) and under tight focusing the usual plane wave phase-matching condition $\Delta k \cdot L \ll \pi$ [where $\Delta k = k_{AS} - (2k_p - k_S)$] is strongly relaxed due to the large cone angle of wave vectors and the small interaction length $L$ that is located at the very vicinity of the objective focal point. Consequently, as reported in Refs. 6 and 7, a strong CARS signal is generated in the forward direction (denoted F-CARS) if the sample
extension along the $z$ axis, $d$, is larger than the excitation fields wavelengths ($d \approx \lambda_p$). On the other hand, if $d \approx \lambda_p$ the sample is too small to take advantage of the constructive interference in the forward direction and the radiation pattern is dipolelike leading generally to a similar CARS signal in the forward and backward directions, (the latter denoted E-CARS).

Although these general considerations are valid, the accurate CARS far-field emission depends on the shape of the object. To match our experiments, we have modeled the CARS far-field emission of beads. Taking into account the 3D vector structure of the excitation field, the total CARS signal is computed at different spatial positions by summing the CARS radiation of an ensemble of coherently induced Hertzian dipoles located inside the scatterer (i.e., inside the bead). For simplicity we assume that $\chi^{(3)}$ is a scalar and that the polarizations states of the incoming fields are linear along the $x$ axis at the back aperture of the microscope lens (see Fig. 1). Furthermore, we neglect the near-field terms in the dipolar emission since we are interested only in far-field radiation. This technique of calculation will be detailed in a future paper and is equivalent to the methods presented in Refs. 6 and 7.

The computation reported in Fig. 2 was performed assuming no refractive index mismatch between the bead and the surrounding medium. This hypothesis is rarely fulfilled in most experiments, and the present paper addresses the issue of how the E-CARS and F-CARS signal are affected by refractive index mismatches between the beads and surrounding media. For clarity we will denote backward-CARS (B-CARS), as the CARS signal that is detected in the backward direction and includes refractive effects. As refractive effects are difficult to introduce in a full CARS electromagnetic modeling for wavelength scale scatterers, we pursue here an alternative by using experimental results together with simple geometric optics and wave-optics modeling.

The influence of refractive index mismatch in high-resolution 3D confocal microscopy was studied by several authors. Visser et al.\textsuperscript{8,9} compared measured thickness values of fluorescent micrometer spheres immersed in liquids of varying refractive indices. In other experiments, using an oil-immersion objective, cross-sectional images through a fluorescent layer were compared, with and without $n$ mismatch.\textsuperscript{10,11} Moreover, refractive index mismatch effects on the image formation in a two-photon microscope were

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**Fig. 1.** Experimental setup used for collinear CARS microscopy ($F$, filter; BS, beam splitter; BC, beam combiner; $L_x$ and $L_y$, lenses; $C$, condenser).

**Fig. 2.** Radiation pattern of a spherical shaped sample with different diameter $d$, ($d = 100$ nm and $d = 2$ $\mu$m). Ratio E-CARS–F-CARS versus the diameter of the sample ($\lambda_p = 740$ nm, $\lambda_S = 840$ nm). Polarizations of the pump and Stokes beams are set along the $x$ axis.
studied, by using fluorescent nanometer beads at different focusing depths.\cite{12,13} Finally, a procedure to determine the correct thickness of an object with confocal microscopy in case of refractive index mismatch was tested by using water–polymer layers.\cite{14} We clearly demonstrate that in the case of index mismatch, distortions are introduced in the CARS image formation process while detected B-CARS signal results in most cases of back-reflected F-CARS emission.

The paper is organized as follows: in Section 2 we briefly present our CARS experimental setup. Section 3 focuses on the experimental results for the B-CARS and F-CARS signals of polystyrene beads embedded in different refractive index media, and Section 4 presents the simulations and a discussion.

2. Coherent Anti-Stokes Raman Scattering (CARS) Setup

Similar to other nonlinear optical processes, high peak powers are necessary for the efficient generation of the CARS signal. These are readily available using picosecond or femtosecond light pulses, the choice of which is determined by the spectral resolution required. We use near-IR excitation pulses that do not give rise to direct electronic excitations in the sample and therefore avoid photochemical damage due to photobleaching.

Our CARS microscope, as depicted in Fig. 1, uses two synchronized picosecond pulse trains.\cite{16} The pump and Stokes beams (with respective frequencies $\nu_p$ and $\nu_s$) are generated by two picosecond Ti-sapphire lasers operating at 80 MHz (Mira-900, Coherent) tunable from 700 to 1000 nm to cover the entire spectrum region of molecular vibrations in biological systems (up to 3000 cm$^{-1}$). The two pulses trains (pulse duration 3 ps horizontally polarized) are electronically synchronized (SynchroLock System, Coherent), with a typically measured jitter of $\sim 250$ fs. The pump and Stokes beams are synchronously pulse picked through two Bragg cells (Pulse Picker, APE, Germany) to reduce the repetition rate of the pulse trains to several hundred kilohertz, thus avoiding photodamage of the sample while still maintaining high peak power for CARS generation. The colinearly combined and expanded beams are sent into an inverted microscope (Carl Zeiss, Axiovert 200M, Germany), and focused onto the sample by a water-immersion objective lens (C-Apochromat, Carl Zeiss, Germany) with an NA of 1.2. The B-CARS signal is collected by the same objective lens while the F-CARS signal is collected by a condenser lens with an NA of 0.5. The B-CARS and F-CARS signals are filtered and detected by two avalanche photodiodes with a 200 $\mu$m $\times$ 200 $\mu$m active area (Perkin Elmer, Canada). As the B-CARS signal represents the total back-collected CARS signal in the 1.2 NA, the F-CARS signal represents only one fifth of the actually emitted F-CARS light. This limitation in F-CARS signal detection is due to the poor imaging properties of the 0.5 NA condenser lens combined with lens $L_1$, which produces a spot five times bigger than the avalanche photodiode active surface. The CARS images are collected by raster scanning the sample, using an XY piezo-translation stage (Physike Instrument, Germany).

3. Experimental Coherent Anti-Stokes Raman Scattering Imaging With Refractive Effects

To characterize the imaging properties of the CARS microscope, we use polystyrene beads (Sigma Aldrich) of well-defined sizes. Figure 3 shows an F-CARS image of a 2 $\mu$m polystyrene bead (Raman shift centered at 1600 cm$^{-1}$ corresponding to C=C vibration stretch) sitting on the microscope slide in air. In the same figure are shown the raw F- and B-CARS signals simultaneously recorded while scanning along the z axis. Similar results are obtained for beads of different diameters. Several intriguing features are present in these traces. First, the B/F ratio is approximately 0.20, whereas from Fig. 2, no more than a few 10$^{-4}$ for a 2 $\mu$m polystyrene bead, is expected. Second, we note that the z positions of the F-CARS and B-CARS signal maxima do not coincide, as already observed in a melanin bead embedded in an agarose gel.\cite{18} Finally the FWHM of both the F-CARS and B-CARS curves is approximately 4 $\mu$m, a value much larger than the actual bead diameter (2 $\mu$m).

To investigate the origin of these discrepancies, the refractive index $n$ of the medium surrounding the beads was changed. Figure 4 shows the F-CARS and B-CARS signals while scanning along the z axis for 2 $\mu$m diameter polystyrene beads embedded in air.
When the bead refractive index differs significantly from that of the surrounding media (air or agarose gel), a B-CARS signal is detected, which disappears when the refractive index mismatch is lowered (the oil case).

Figure 4 proves the refractive origin of the B-CARS detected signal. In air and agarose gel, the B-detected signal is interpreted as a backward-reflected F-CARS emission. To understand further the experimental results and study the refractive effects on the CARS signal detection, we have performed several numerical simulations.

4. Numerical Simulations and Discussion

Although a full electromagnetic theory of the CARS emission process in beads including the refractive effects is, in principle, possible, its numerical implementation is extremely computation consuming. As a comprehensive approach, we report, here, two simulation methods to study the refractive effects in CARS microscopy. The first one, based on geometrical optics, permits to model the CARS emission (anti-Stokes) and collection while the second one based on wave optics gives access to the excitation fields.

A. Geometrical Optics: Coherent Anti-Stokes Raman Scattering Emission and Collection Functions in Beads

We use a ray tracing software (Oslo, Lambda Research) to investigate how F-CARS and E-CARS emitted beams are refracted by a bead (see Fig. 5). We simply model the CARS emission by a forward-propagated set of beams whose NA matches that of the experimental pump and Stokes beams.

Working with a 2 μm bead, we consider the B-CARS signal as a pure reflection of the forward-emitted CARS signal on the upper bead interface. To do so, we set a reflecting mirror on the upper bead interface and calculate, for each position $z$ of the focal point in the bead along the $z$ axis, the NA of the reflected beams cone angle $NA_B = \sin \theta_B$. To simulate the F-CARS signal we use upper media with different refractive indices, and we calculate, for each position $z$ of the focal point in the bead along the $z$ axis, the NA of the transmitted beams cone angle $NA_F = \sin \theta_F$. 

Fig. 5. Refractive model system used in geometrical optics simulations. The bead stands on a glass cover slip and is embedded in a solvent of refractive index $n$. (Left) H-CARS signal is considered as a pure reflection of the forward-emitted CARS signal on the upper bead interface. (Right) F-CARS signal is refracted by the bead–solvent interface.
The CARS emission is considered only along the z axis with the origin set at the bead–cover glass interface (see Fig. 5).

Figure 6 shows the calculated NAs \( N_A(z) \) and \( N_B(z) \) as a function of the emitted CARS position \( z \) in a 2 \( \mu \)m polystyrene bead. From Fig. 6(a) it can be seen that the forward-refracted beams are poorly collected by the \( N_A = 0.5 \) condenser lens used in the experiment. At least only the positions satisfying \( z < 0.3 \) \( \mu \)m for a bead in air are fully collected by the condenser lens.

For the backward reflected beams [Fig. 6(b)], \( N_B(z) \) is always shorter than 1, a value smaller than the NA (\( NA = 1.2 \)) of the water-immersion objective lens used to detect the B signal. Although this B signal is well collected, it is interesting to see that it mostly comes from the bead center whatever the surrounding media are.

From \( N_A(z) \) and \( N_B(z) \), we can define the collection functions \( C_F(z) \) and \( C_B(z) \) that are the ratio of the collected power to the total emitted power in the forward and the backward direction, respectively. Making the simple assumption that the emitted CARS power per unit solid angle is constant we get the expression

\[
C_F(z) = B(z) \frac{1 - \left[ 1 - ON_{col}^2 \right]^{1/2}}{1 - \left[ 1 - ON_{coli}^2 \right]^{1/2}},
\]

where \( B(z) = 1 \) in the bead and \( B(z) = 0 \) outside the bead, \( ON_{coli} = 0.5 \) is the condenser NA used for the F-signal detection. A similar expression is obtained for \( C_B(z) \) replacing index \( F \) by \( B \). As an example, Fig. 7 shows \( C_F(z) \) and \( C_B(z) \) for a 2 \( \mu \)m bead diameter (in air) centered at \( z = 10 \) \( \mu \)m. As expected from Fig. 6, \( C_F(z) \) concentrates in the lower z positions and decreases quickly for larger \( z \), whereas \( C_B(z) \) is stronger in the bead center. We believe that this asymmetry between \( C_F(z) \) and \( C_B(z) \) explains why the F-signal maximum appears before the backward-signal maximum in Figs. 4(a) and 4(b).

B. Wave Optics: Excitation Fields in Beads

The second numerical simulation we have implemented is based on wave optics using a commercial finite-difference time-domain (FDTD) software (Fullwave, Rsoft). In this Subsection, 2D calculations are reported for focused excitation beams (pump and Stokes) in beads embedded in various media. For simplicity we concentrate on an excitation field whose wavelength (\( \lambda = 800 \) nm) is close to those of the pump (\( \lambda_p = 740 \) nm) and Stokes (\( \lambda_S = 840 \) nm) fields.

Let us first concentrate on the excitation Poynting intensity \( I_{ex}(x,z) \) and its maximum (beam waist) when a scan is performed along the bead z axis. The calculations are performed in a space composed of two semi-infinite media (glass and upper medium) separated by an interface (located at \( z = 3 \) \( \mu \)m) on which a polystyrene bead can be settled. First, considering no bead at the interface, a Gaussian beam (waist 0.5 \( \mu \)m) is launched from the glass side. This beam focuses at \( z_{laser} \) in the upper medium. Then, a polystyrene bead is settled at the interface. We observe how the intensity pattern \( I_{ex}(x,z) \) is affected, and we concentrate on the new location \( z_{max} \) of the intensity maximum. Figure 8(a) gives an example of results obtained with a 2 \( \mu \)m diameter bead (centered at \( z = 4 \) \( \mu \)m), settled at the glass surface (the solid white line at \( z = 3 \) \( \mu \)m) and embedded in air. While the bead is scanned along the z axis, the high refractive index mismatch between air (\( n = 1 \)) and polystyrene (\( n = 1.6 \)) clearly confines \( z_{max} \) into the bead as compared to \( z_{laser} \) (dashed line). Figure 8(b) plots \( z_{max} \) versus \( z_{laser} \) for a 2 \( \mu \)m bead diameter embedded in various media (oil, agarose, air).

From Fig. 8(b), the excitation beam focal point \( z_{max} \) clearly depends upon the refractive index mismatch between the polystyrene bead (\( n = 1.6 \)) and its surrounding medium. As the refractive index contrast increases, \( z_{max} \) remains confined in the bead although \( z_{laser} \) is set well above the bead. The strongest effect is obtained in air [Fig. 8(a)]. In this case, for \( z_{laser} \) varying between 3.5 and 7 \( \mu \)m, the location of the excitation beam focal point \( z_{max} \) remains in the bead and a CARS signal is still generated. This phenomenon explains qualitatively why the CARS bead extension

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Fig. 6. Calculated numerical apertures as a function of the emitted CARS position \( z \) in a 2 \( \mu \)m polystyrene bead. (a) Resulted \( N_A \) of forward-refracted beams. (b) Resulted \( N_B \) of backward-reflected beams.

Fig. 7. Calculated collection functions \( C_F(z) \) (solid triangles) and \( C_B(z) \) (open squares) in a 2 \( \mu \)m diameter polystyrene bead (dotted curve).
along the $z$ axis is larger than the actual bead diameter in our experiments (see Fig. 4).

Let us concentrate now on the ratio of the F-CARS to the B-CARS signals shown in Fig. 3. In air, we have found experimentally a ratio of 5:1 in favor of the F-CARS detected signal. FDTD simulations report that 4.5% of the F-CARS emission is backreflected by the upper bead interface and penetrates into the glass substrate while 90% is transmitted to the F detection. Since our F detector collects roughly 20% of the F-CARS signal, we reach an expected ratio of 4:1 in favor of the F-CARS detected signal, which is in acceptable agreement with the experiment. In agarose, the experimental ratio of 10:1 is also in good agreement with the simulation. These results demonstrate again the back-reflected F-CARS origin of the backward detected signal.

C. Coherent Anti-Stokes Raman Scattering Profile Modeling along the $z$ Axis

To take the refractive effects fully into consideration we now combine our results from the geometrical and wave optics simulations. We simply model the CARS signal obtained along the $z$ axis as a convolution between the collection function and the cube of the excitation intensity. This is clearly a crude approximation that does not take into account the coherent nature of the CARS emission. Nevertheless, this simple analysis is motivated by the inclusion of refractive effects in both the collection function and the excitation intensity. With the above mentioned notations, the F-CARS signal $I_{F-CARS}$ is given by

$$I_{F-CARS}(z) = K \int_{z'} C_p(z') I_{ex}^3(z - z') dz',$$

where $K$ is a constant. A similar expression is obtained for the B-CARS signal $I_{B-CARS}$. $I_{F-CARS}$ and $I_{B-CARS}$ can now be compared to their respective fitted experimental values (recorded in Fig. 4). Figure 9 compares, in the same graph, theoretical and experimental results for the agarose and air cases. When agarose is taken as the surrounding medium [Fig. 9(a)], a good agreement is found between theory and experiment. The B-CARS signal maximum is shifted 1.3 $\mu$m after the F-CARS signal maximum. Similarly, the experimental FWHM of both F- and B-CARS signals are larger than the bead diameter, still in good agreement with theory. When agarose is replaced with air [Fig. 9(b)], the main features still operate. The B-CARS maximum is shifted 1.8 $\mu$m after the F-CARS signal maximum. Similarly, the experimental FWHM of both F- and B-CARS signals are still larger than the bead diameter. Nonetheless, the discrepancy between experiment and theory is stronger, pointing out the limitations of our model. Indeed, it does not fully take into consideration the coherent nature of the CARS emission process and considers the B-CARS signal as a sole reflection of the F-CARS

![Fig. 8. FDTD calculation in a 2 $\mu$m polystyrene bead centered at $z = 4$ $\mu$m. (a) Excitation Poynting intensity mapping $I_{ex}(x, z)$ ($\lambda = 800$ nm, waist 0.5 $\mu$m) in the bead settled on a glass-air interface ($z = 3$ $\mu$m; white line). In the absence of bead the beam focuses at $z_{laser} = 4$; 5 and 6 $\mu$m (dashed line). (b) $z_{max}$ versus $z_{laser}$ for the bead embedded in various media (oil, agarose, air).](image)

![Fig. 9. Comparison between experimental and theoretical CARS signals obtained when scanning along the $z$ axis for a 2 $\mu$m bead diameter ($z$ scan performed along the symmetry axis of the bead). Experimental F-CARS signal (solid triangle), B-CARS (open squares), $I_{F-CARS}$ and $I_{B-CARS}$ (continuous black curve). (a) Bead embedded in agarose, (b) bead embedded in air.](image)
signal. In the above results we have shown the important contribution of refractive effects in CARS imaging and more generally in any nonlinear microscopy process.

Two important results have been pointed out. First, we have demonstrated the prominent contribution of the strong forward-emitted CARS signal to the epidetected signal via backreflection due to the refractive index mismatch between the sample and its surrounding medium. This does not preclude that in the backward-detected signal, a true E-CARS emission exists and, in this case, mixed with the backreflected F-CARS emission. As a consequence, the radiation pattern shown in Fig. 2 has to be reconsidered, taking into account the refractive effects to match the experimentally detected B-CARS–F-CARS ratio.

Second, we have highlighted the distortion of the excitations and CARS emitted fields due to the refractive index mismatch, and induced distortion of CARS images. On the one hand, in the scope of our experiment, the F-CARS signal was well collected only close to the glass interface (see Fig. 7). On the other hand, the excitation fields remained trapped in the high refractive index bead while the beam was scanned upward (see Fig. 8). Combining both effects in the final CARS imaging process [as depicted in Eq. (2)] induced image distortions such as altered FWHM, or maximal F-CARS and B-CARS signals detected at distinct positions.

5. Conclusion

In this paper we have investigated the effects of refractive index mismatch between the sample (polystyrene bead) and its surrounding medium in CARS microscopy. We have pointed out the influence of a backreflected F-CARS signal on the B-CARS detected signal, and in some cases this could be a major contribution to the epi-detected signal. Moreover, we have emphasized the leading effect of the refractive index mismatch on CARS images distortion. Perturbations induced to excitations and CARS fields were bound to the shape and the refractive index of the sample. Although refractive effects are believed to play a minor role in nonlinear microscopy, the diversity of compounds found in a living cell—lipid \((n = 1.48)\), protein \((n = 1.50)\), melanin \((n = 1.7)\), and mitochondria \((n = 1.40)\)—justifies a special care for fine imaging investigation.

We acknowledge financial support from the CNRS, the French National Education Ministry, the P.A.C.A. region, and the E.U. (FEDER). We thank Kien Phan Huy for his help with the FDTD computation.

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