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Detecting lateral interfaces with focus-engineered coherent anti-Stokes Raman scattering microscopy

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Focus-engineered coherent anti-Stokes Raman scattering (FE-CARS) microscopy is used to highlight the lateral interfaces between chemically distinct media. Interface highlighting is achieved by using a HG10 mode for the Stokes laser beam and a HG00 mode for the pump laser beam in the forward detection scheme. The spectral and the orientation dependence of FE-CARS are found to be in agreement with theoretical predictions. A brief discussion on the relevance of this technique for imaging third-order nonlinear susceptibility interfaces in thin samples of biological or chemical importance is presented.

KEYWORDS: coherent Raman spectroscopy; CARS microscopy; focus engineering; chemical interfaces

INTRODUCTION

Coherent anti-Stokes Raman scattering (CARS) microscopy is rapidly gaining ground as an imaging tool for biological samples.[1] CARS provides vibrational contrast and circumvents the need for labeling for visualizing select compounds such as water, lipids, and proteins in cells and tissues. While the number of imaging applications continues to grow, the need for improved CARS techniques becomes apparent as well. In particular, efficient suppression of the nonresonant background, while retaining strong resonant signals, is crucial for many anticipated biological applications of CARS microscopy. Several contrast-improving methods have been developed, including polarization sensitive detection,[2] time-delayed detection,[3] heterodyne mixing,[4] and frequency-modulation techniques.[5]

One way to achieve contrast improvement is to make efficient use of the coherent nature of the CARS emission. CARS intensity detected in the far field is a result of coherent superposition of waves emanating from the focal volume.[6] Hence the CARS output intensity, unlike the incoherent emission in fluorescence, depends closely on the spatial phase distribution of the Raman oscillators in the excitation volume. Modifying the phase profile of the focal volume can thus lead to altered CARS signals. We recently proposed spatial phase shaping of the focus as an effective approach to improve image contrast in CARS microscopy.[7,8] In particular, on the basis of numerical calculations, we pointed out the enhanced sensitivity of focus-engineered CARS (FE-CARS) imaging to interfaces of chemically distinct media. In this article, we present experimental results to demonstrate that the FE-CARS imaging technique can be employed to highlight lateral $\chi^{(3)}$-interfaces in the forward detection mode of the microscope.

In CARS, a pump beam of angular frequency ω_p and a Stokes beam of angular freqeuncy ω_S are utilized to generate a vibrationally sensitive anti-Stokes signal at angular frequency $2\omega_p - \omega_S$. Changing the spatial phase profiles of either of the beams results in modifications to the anti-Stokes signal. Here, we restrict our discussion to the case of HG10 excitation, where a Gaussian beam is used as the pump and the first order Hermite–Gaussian (HG10) beam is used as the Stokes beam. A HG10 Stokes beam carries a transversal π step across the beam profile. This π-phase jump leads to a corresponding π-phase discontinuity in the CARS excitation volume through the phase relationship $\phi_{CARS} = 2\phi_p - \phi_S$, where ϕ_p and ϕ_S are the relative phases of the pump and the Stokes beams respectively. Consequently, the CARS waves generated in the focal volume interfere destructively at the far-field. This leads to negligibly small CARS signal even for a highly resonant medium. However, according to theoretical predictions, a stronger CARS signals is expected at $\chi^{(3)}$-interfaces oriented parallel to the applied phase step.[7,8] The phenomenon of interface highlighting is a result of incomplete destructive interference of the waves at the $\chi^{(3)}$-edge. In this article, we experimentally ascertain these predicted effects using simple model systems. We compare the results with conventional

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CARS excitation (or HG00 excitation) where both the beams are Gaussian.

EXPERIMENTAL

Figure 1 shows a sketch of the experimental setup. The laser beam of wavelength 1064 nm from a mode-locked Nd : Vanadate laser (Picotrain, HighQ GmbH: pulse width = 7 ps, and repetition rate = 76 MHz) is split into two beams at the beam-splitter BS. The reflected portion of the beam is used to synchronously pump an intracavity doubled optical parametric oscillator (OPO, Levante, APE: pulse width = 7 ps, and repetition rate = 76 MHz). The output of the OPO is wavelength tunable in the range 775–960 nm and acts as the pump light for the CARS measurements. The transmitted part of the beam through the BS serves as the Stokes light in the CARS experiment.

The experimental realization of HG10 excitation is achieved by including a computer-controlled liquid crystal phase modulator (SLM2256D, Meadowlark) in the Stokes beam. The phase modulator is positioned in the beam path such that one half of the beam is phase shifted by π radians with respect to the other half, thus converting the HG00 mode from the laser to a HG10 mode.[9] Note that by turning the phase-modulator off, the standard beam geometry for CARS microscopy is retrieved. Our setup thus allows for quickly switching between FE-CARS and conventional CARS by a simple button click on our computer interface.

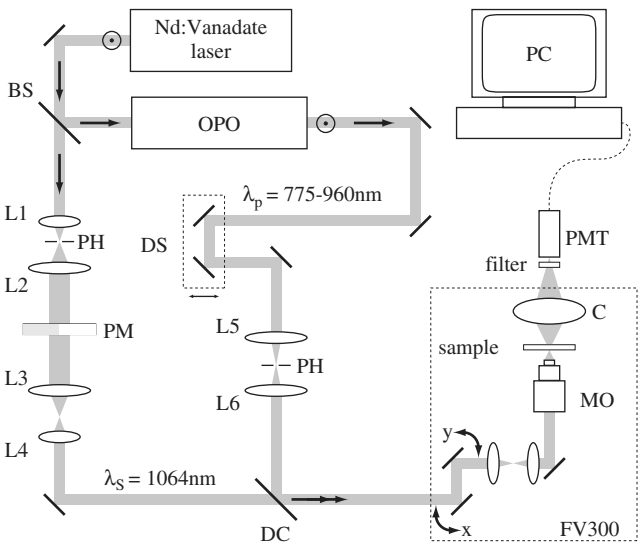

Figure 1. Sketch of the experimental setup: OPO - optical parametric oscillator; BS - beam splitter; L1-L6 - lenses; PH - pinhole; PM - liquid crystal phase modulator; DC - dichroic; DS - delay stage; FV300 - Fluoview microscope system; MO - microscope objective; C - condenser; PMT - photo-multiplier tube; PC - computer. Both the pump and the Stokes beams are *s*-polarized.

After passing through the modulator, the Stokes beam is combined with the pump beam from the OPO on a dichroic (DC) mirror and directed into an inverted laser-scanning microscope (Fluoview FV300, Olympus). An infinity-corrected water-immersion microscope objective (UApo, Olympus: 40x, and 1.15 NA) is used to focus the two beams at the sample. The generated CARS signal is collected with a 0.55 NA condenser in the forward direction. The signal is subsequently isolated using a set of band-pass interference filters (central wavelength = 650 nm; bandwidth = 38 nm, Chroma Technology) and detected with a red-enhanced photomultiplier tube (R3896, Hamamatsu Corporation).

Spontaneous Raman spectra are measured using the 532-nm radiation from a Verdi V5 pump laser (Coherent Inc.), which is guided into the CARS microscope and focused by the same objective onto the sample. Raman emission is detected in the epi-direction, passed through the backport of the microscope and separated from the excitation light by a holographic notch filter (Kaiser). The Raman signal is resolved by a grating spectrometer (SpectraPro-150, Acton) equipped with a cooled CCD camera (Princeton Instruments).

PHASE SHAPING OF CARS EMISSION

Figure 2(a) shows a typical CARS image of a thick homogeneous sample. The sample here is a mixture of 6 : 100 dodecane : paraffin visualized at wavenumber 2936 cm^{-1}. The CARS intensity distribution across the image is not uniform – it is brighter at the center and becomes gradually weaker toward the edges. Such images are typical of beam-scanning microscopes – where the scanning mechanism using the galvanometer scanner in combination with the microscope optics leads not only to a continuous translation of the focal spot across the sample, but also to a continuous change in the angle of incidence of the excitation beams.[10] Consequently, the spatial overlap between the Stokes and the pump beams and the quality of the focal spot deteriorate at the outer edges of the image (which correspond to larger angles of excitation) due to the off-axis aberrations and the chromatic dispersion of the microscope optics. This generally leads to a lower CARS signal toward the margins of the image. Typically, for reliable CARS imaging, the area near the central portion of the image is considered. Note that a wider detection area is obtained if the chromatic shift between the pump and the Stokes beams is reduced.

Figure 2(b) shows the CARS image of the same homogeneous sample under HG10 excitation. The FE-CARS image looks completely different from the conventional CARS image – two intensity lobes appear with a dark region in the central portion of the image. This can be explained by vignetting at the back aperture of the objective lens. Vignetting arises because the scanning mirrors and the back aperture plane are not placed in exact conjugated planes. This leads to walking of the beam and partial clipping of the Stokes

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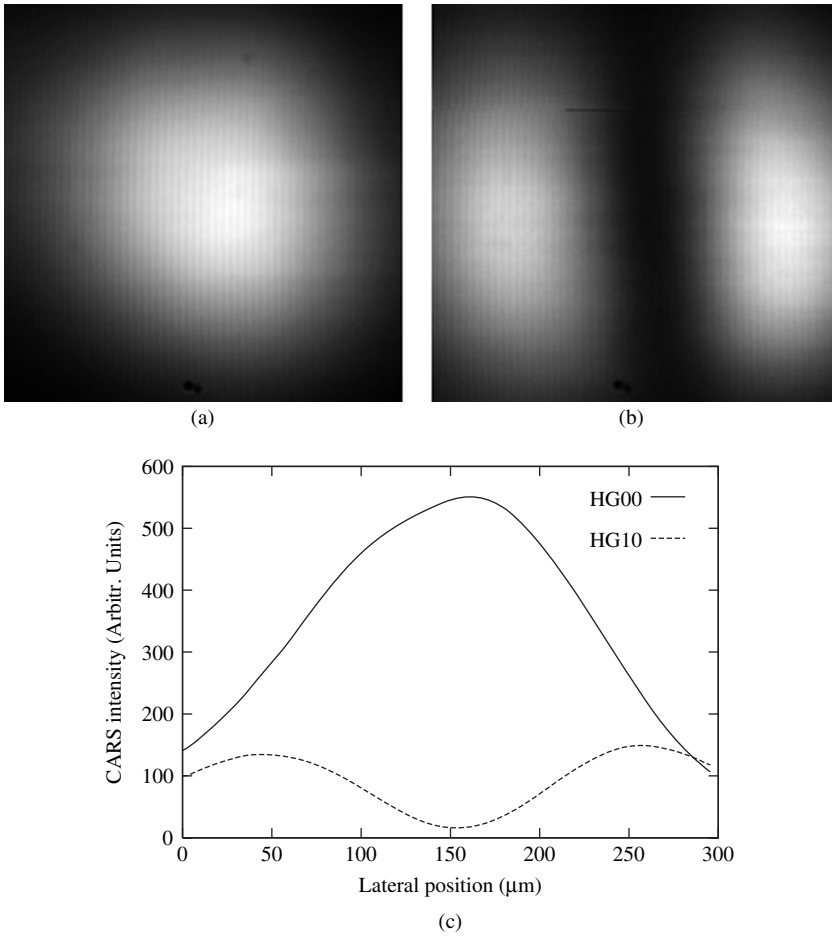

Figure 2. CARS image of the mixture of paraffin and dodecane under (a) conventional excitation and (b) HG10 excitation. (c) Comparison of the intensity profiles of the two images. The Raman shift was 2936 cm^{-1}. The size of the images correspond to 300 μm × 300 μm.

HG10 profile at the back aperture for larger angles. Partial clipping in the direction of the phase step introduces incomplete destructive interference, and hence stronger signals with increasing angle. In addition, a small tilt in the lobed CARS emission at larger scanning angles may also cause slight vignetting at the condenser. Closer to the margins of the images, again the afore-mentioned effects of aberrations and chromatic dispersion are expected to adversely affect the CARS intensity. Figure 2(c) compares the two cross-sectional profiles from the center of the two images. Analogous to the case of conventional CARS excitation, in FE-CARS only the points close to the center of the field of view are used for imaging and analysis.

The limited field of view in CARS imaging applies only to microscope systems that use galvanometer-based beam-scanning technique with nondegenerate wavelengths. In contrast, if the imaging is performed by scanning the sample stage, the image quality is independent of position. However, for practical applications, the stage-scanning microscopes are much slower than their beam-scanning counterparts. The beam-scanning approach, for instance, has been successfully used for video rate CARS imaging of live animals,[11] acquisition speeds that are out of reach for sample scanners. To maintain rapid scanning capability, the imaging studies in this work were carried out with a beam scanner.

LATERAL INTERFACE DETECTION

The chemical interface that is being considered in this work is the one formed between a 6 : 100 dodecane : paraffin mixture and deuterated dimethyl sulphoxide (d-DMSO). Both these chemicals have similar refractive indices of about 1.478 at room temperature and hence display negligible refractive effects at their interface. However, they have different third-order nonlinear susceptibilities in the spectral region of interest which spans from 2800 to 3050 cm^{-1}. d-DMSO is vibrationally nonresonant, whereas the dodecane : paraffin mixture is highly resonant. The CARS spectrum of the dodecane : paraffin mixture is plotted in Fig. 3, along with its spontaneous Raman spectrum.

Figure 4 shows the CARS images and the intensity profiles of the interface formed between d-DMSO on the right

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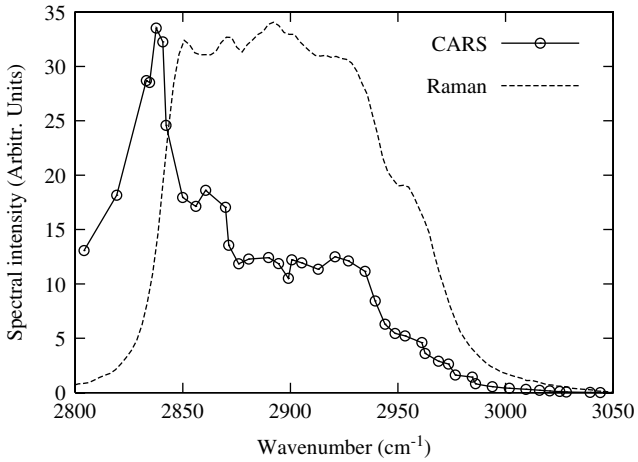

Figure 3. CARS and the Raman spectra of the mixture of paraffin and dodecane. The solid line joining the points in the CARS spectrum is meant to serve as a guide to the eye.

side and dodecane : paraffin mixture on the left side. Images labeled from a(*i*)–d(*i*) correspond to CARS images obtained using HG00 excitation; and those labeled from a(*ii*)–d(*ii*) correspond to images obtained under HG10 excitation. The plots in Figs 4a(*iii*)–4d(*iii*) compare the cross-sectional intensity profiles under both HG00 and HG10 excitations at different wavenumbers.

The conventional CARS images show that the CARS intensity of the dodecane : paraffin mixture decreases gradually from 2900 to 3025 cm^{-1}. Two points that are of particular interest are (1) the appearance of an intensity dip at the interface and (2) the strength of modulation of this intensity dip. The appearance of intensity modulation in the CARS profiles for excitations from 2941 to 3027 cm^{-1} is a well known spatial effect brought about by the interplay of the $\chi^{(3)}$-spectral phases of the two media. In this spectral range, the CH_2 vibration of the dodecane : paraffin mixture is driven at the blue-side of its resonance, and exhibits a phase shift relative to the nonresonant background. Hence, the CARS emission from the mixture is out of phase with respect to the nonresonant signal from d-DMSO. At the interface of the media, the generated CARS waves interfere destructively, resulting in a dip in the detected CARS signal. This intensity dip is more pronounced for 2977 cm^{-1} compared to the excitation at 2941 or 3027 cm^{-1}. This is

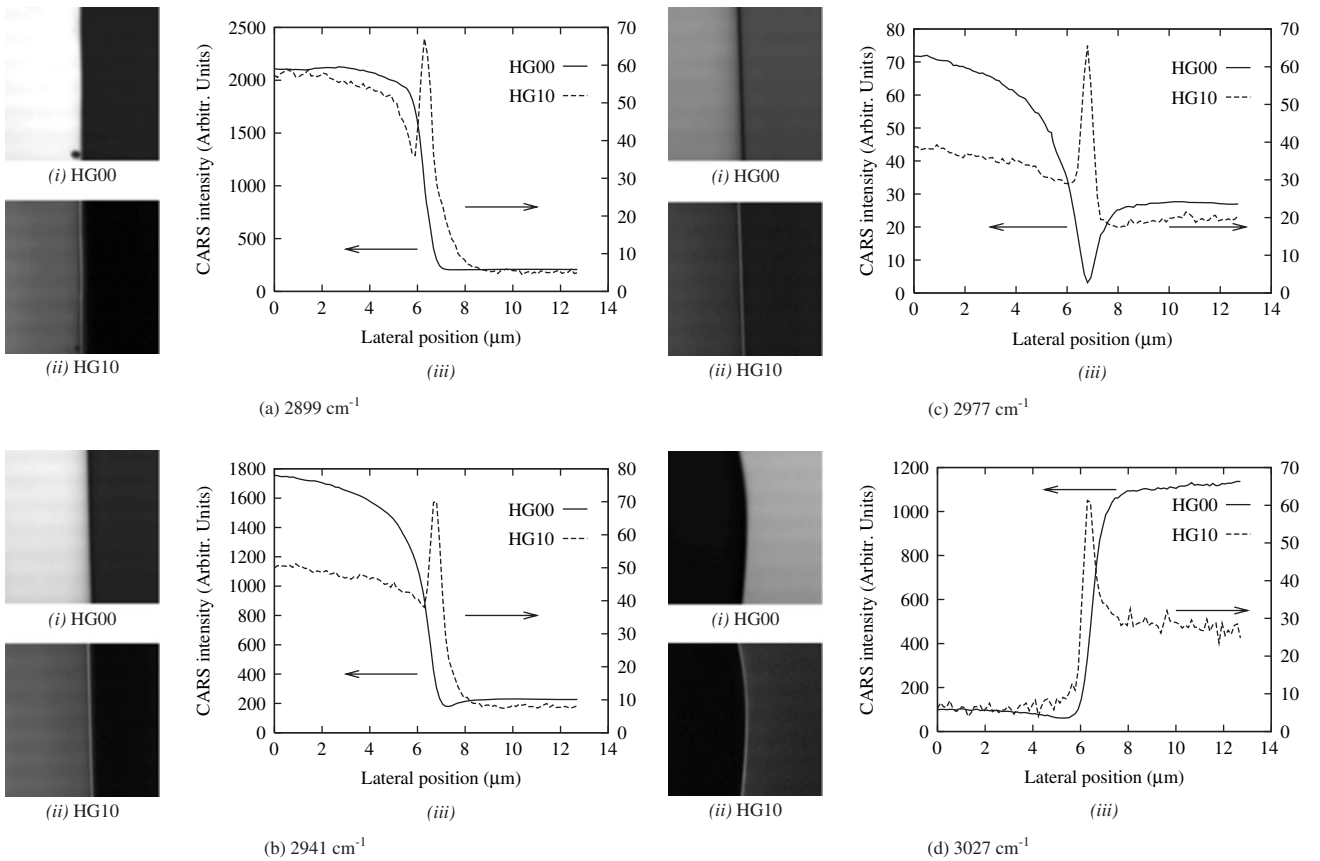

Figure 4. Experimental demonstration of lateral interface detection with focus-engineered CARS and its spectral dependence. Images labeled as *(i)* correspond to CARS images under HG00 excitation and those labeled as *(ii)* correspond to HG10 excitation. All images in the above panels are of the size 30 μm × 30 μm. The plots, labeled as *(iii)*, compare the intensity profiles of the images in *(i)* and *(ii)*.

because the magnitudes of $\chi^{(3)}$ for the two chemicals are nearly equal at 2977 cm^{-1} (as seen in Fig. 4c(*iii*)) and thus the destructive interference of the generated CARS waves is stronger, which results in a stronger dip.

The most prominent feature of the FE-CARS images (Figs 4a(*ii*)–4d(*ii*))and the intensity profiles (Figs 4a (*iii*)–4d(*iii*)) obtained with HG10 excitation is the presence of an intensity spike at the chemical interface. The sub-micron width of the spike corresponds to the lateral resolution of the microscope. Under HG10 excitation, the CARS waves generated from the either side of the π phase discontinuity in the focal volume interfere destructively. Consequently, the total intensity from the bulk in FE-CARS is less by almost 2 orders of magnitude. However, at the interface, since the third-order susceptibility on either side of the engineered π phase jump are different, the destructive interference of the generated CARS waves is incomplete. This results in an intensity spike that highlights the presence of a chemical interface.

The degree of interface highlighting is dependent on the differences in both the amplitude and phase of the $\chi^{(3)}$ of the media. Qualitatively, the CARS intensity at the interface can be written as

$$I_{\mathrm{CARS}} \propto |\chi^{(3)}_{\mathrm{dmso}} \pm |\chi^{(3)}_{\mathrm{paraffin}}| e^{i\phi(\omega)}|^2 \qquad (1)$$

where $\chi^{(3)}_{\mathrm{dmso}}$ and $\chi^{(3)}_{\mathrm{paraffin}}$ are the third-order susceptibilities of d-DMSO and dodecane : paraffin mixture, respectively, and $\phi(\omega)$ is the spectral phase of the paraffin mixture. In the above expression, the plus sign corresponds to HG00 excitation, and the minus sign corresponds to HG10 excitation. When the spectral phase of paraffin swings toward π radians (*i.e.*, when the excitation is on the blue side of the resonance), the sign in front of $\chi^{(3)}_{\mathrm{paraffin}}$ flips. Equation (1) shows that under these conditions, the interfacial CARS signal for HG00 excitation dips while the signal with HG10 excitation is maximized. The strong influence of the spectral phase, a distinct property of the vibrationally resonant part to the signal, is confirmed in Fig. 4. Note that Figs 4(a), (b), and (d) correspond to large differences in the magnitudes of the susceptibilities, while Fig. 4(c) corresponds to a large difference in the relative phases of the susceptibilities. In each of these cases, the interface between the two chemicals is clearly highlighted.

If the chemical interface is perpendicular to the π phase jump in the focal volume of HG10 excitation, no highlighting is expected.[7] This is because, in the focal plane one can always find two equally spaced points on either side of the π phase jump, such that the CARS signals generated from them interfere destructively at the far field. Because of this, direction-specific interface highlighting is expected under HG10 excitation. Figure 5 depicts this directional dependence of interface highlighting. Figure 5a and 5b correspond to the interface parallel and perpendicular to the phase jump in HG10 excitation, respectively. Clearly, the intensity spike seen in Fig. 5a does not appear in Fig. 5b. This direction specificity and the spectral dependence

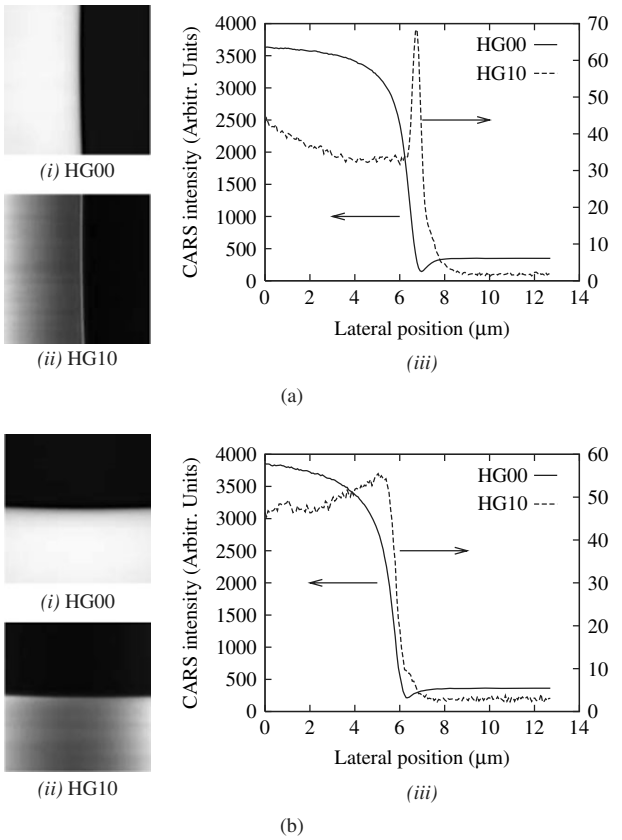

Figure 5. Directional dependence of interface highlighting with FE-CARS of the sample (a) with the interface being parallel to the phase jump in HG10 excitation and (b) with the interface being perpendicular to the phase jump in HG10 excitation. For comparison, the CARS images and intensity profiles under conventional excitation are also shown. All images in the above panels are of the size 30 μm × 30 μm. The Raman shift was 2936 cm^{-1}.

discussed above are conclusive evidence to the fact that the interface highlighting is a consequence of engineering the focal excitation volume rather than an outcome of spurious linear refractive effects.

In the present detection configuration, the contrast of interface highlighting increases with smaller NA of the collection lens.[7] This, however, leads to low detected signal levels. Hence, a balance has to be found between efficient signal collection and the highest possible contrast. In the case of high NA excitation objective (NA = 1.15; water immersion), we have found experimentally that the 0.55 NA of standard condensers provides excellent contrast and signal collection characteristics.

DISCUSSION

This article demonstrates experimentally that FE-CARS is sensitive to chemical interfaces. The observation of interface highlighting under HG10 excitation in FE-CARS corroborates

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previous theoretical predictions. We also observe that the spectral trend of this interface highlighting feature is strongly dependent on the spectral phase of the CARS spectrum, which emphasizes that the effect is highly sensitive to the vibrationally resonant part of the nonlinear susceptibility. Therefore, the FE-CARS technique is a promising method for extracting vibrationally resonant information from the sample.

Although the contrast of interface highlighting as seen in Fig 4a(*ii*)–d(*ii*) is high, the overall intensity of FE-CARS images can be up to 2 orders of magnitude lower. One of the reasons for the lower FE-CARS signals is the chosen experimental arrangement, in which the pump beam is a Gaussian beam and the Stokes beam is a higher-order Hermite–Gaussian beam. The intensity maxima of these two modes do not overlap. Because CARS is a nonlinear process, the effective signal intensity is accordingly lower. To address this problem, Liu and Kim[12] have recently suggested using Laguerre–Gaussian beams for both the pump and the Stokes beams and showed numerically that the resulting interface images show higher contrast with better signal-to-background ratio.

A successful application of this technique critically depends on the amplitude and phase quality of the focal volume. In biological applications, such as tissue imaging, where one encounters large refractive index variations, the very propagation of the beams through the sample may distort the optimal spatial phase distribution in the focal region. Therefore, this technique is of limited relevance to imaging studies of thick and turbid samples unless adaptive techniques are used to retain the focal amplitude distribution.[13] On the other hand, this imaging methodology could be of tremendous interest for investigating chemical interfaces in thin samples. One such example is the imaging of polymer blends composed of a mixture of polymers.[14,15] In these samples, the refractive index variations are minimum and the samples are usually thin. Thus the acquisition of FE-CARS images along with conventional CARS images would provide a consummate picture, and may lead to a better understanding of the chemical composition of thin film samples.

The attractive feature of FE-CARS is that it is available alongside regular CARS contrast. FE-CARS offers additional contrast mechanisms without compromising the image quality of the standard CARS microscope. During CARS imaging sessions, in case additional contrast in the image is required, a quick FE-CARS inspection of the sample is easily obtained by switching on the phase modulator. The interface highlighting feature of FE-CARS may reveal details otherwise hidden in the image.

The lateral interface highlighting is a result of a single phase step in the Stokes beam. By synthesizing more advanced phase masks, other contrast mechanisms can be obtained. For instance, it has been shown that a circular phase step leads to enhanced interfaces along the axial direction in forward detected CARS.[8] We expect that additional shaping of the focal volume may result in further improved contrast mechanisms, including higher spatial resolution.

CONCLUSION

We demonstrated the feasibility of FE-CARS in a conventional beam-scanning microscope. FE-CARS was conducted by combining a Stokes beam with HG10 beam mode and a pump beam with HG00 mode. We verified the spectral dependence and orientation dependence of $\chi^{(3)}$-interface highlighting in a chemical sample composed of two chemicals – nonresonant d-DMSO and a resonant mixture of dodecane and paraffin – with equal linear refractive properties. Our results are in concert with theory. The FE-CARS technique provides an additional contrast mechanism sensitive to vibrational resonances in the sample. In combination with conventional CARS imaging, the new interface contrast provided by FE-CARS offers a more thorough analysis of the sample under study.

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