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Wide-field coherent anti-Stokes Raman scattering microscopy based on picosecond supercontinuum source

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We present a wide-field coherent anti-Stokes Raman scattering microscopy setup based on picosecond-laser-pumped supercontinuum and use it to demonstrate video-rate imaging with chemical specificity. The broadband excitation allows simultaneous imaging of a wide range of Raman modes, and chemically selective imaging is achieved by applying filters corresponding to the anti-Stokes Raman bands. © 2018 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/). https://doi.org/10.1063/1.5045575

I. INTRODUCTION

Optical microscopy is a widely adopted technology for imaging targets down to sub-micrometer scale. Modern microscopic technologies such as bright-field, dark-field, phase contrast, confocal, and fluorescence microscopy have served as vital tools in material science and biology.1 Raman scattering, a noninvasive technique that probes the vibrational and rotational molecular levels, has also played a significant role in microscopy due to its ability to provide chemical specificity. Raman microscopes are now widely available and have been used in a broad range of fields such as petrography, pharmacy, and polymer science.2 However, the use of Raman microscopy in practical imaging systems, especially those requiring fast imaging speed and a high frame rate, is limited due to the low efficiency of Raman scattering. Another difficulty in this technology is the autofluorescence background from the sample, which is often strong near resonance and can veil the weak Raman signal.

Coherent anti-Stokes Raman scattering (CARS), as a variation of the commonly adopted spontaneous Raman scattering technique, can provide much higher signal level while avoiding the fluorescence background problem altogether.3 CARS is a four-wave mixing (FWM) process, in which a pump field at frequency \( \omega_p \) and a Stokes field at \( \omega_s \) are tuned such that their frequency difference matches the frequency of a vibrational mode of the target molecule, generating coherence between the vibrational levels. This coherence is subsequently probed by a probe field at frequency \( \omega_{pr} \), creating an anti-Stokes signal at \( \omega_{sig} = \omega_p - \omega_s + \omega_{pr} \). Due to the nature of the nonlinear process, efficient CARS generation requires a phase-matching condition,4 which can be satisfied over a short interaction length in a tight focus.5 Therefore, a majority of the CARS microscopy schemes demonstrated so far are based on scanning of tightly focused excitation beams over the sample.6–9 While taking advantage of the high spatial resolution offered by the small focal spot, such an imaging scheme is typically time-consuming, and high-frame-rate microscopy over a significant area has been demonstrated only for strong scattering materials.10

Another illumination scheme known as wide-field CARS has been proposed,10 in which a CARS image is directly obtained by using wide-field illumination (Fig. 1). Early wide-field CARS schemes

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used an ultra-dark-field condenser to produce a cone-shaped pump light, and the generated CARS signal satisfied the phase-matching condition and propagated backward through the Stokes path.\textsuperscript{11,12} This, however, requires complicated optics to achieve. Later on, simpler illumination geometries were adapted, such as samples being excited by loosely focused pump and Stokes beams with a finite offset angle.\textsuperscript{13,14} In these cases, phase-matching conditions were not met, and it has been shown that CARS signals from such non-phase-matched illumination stemmed mostly from refraction and scattering inside the sample.\textsuperscript{13,15} By using wide-field excitation, video rate imaging and even single-shot imaging of standard and biological samples have been demonstrated.\textsuperscript{12,14–16} All these schemes were based on narrowband CARS excitation and required sophisticated wavelength extension units like an optical parametric oscillator (OPO) or an optical parametric amplifier (OPA); a simpler scheme is to use a Raman shifter to produce the Stokes beam, yet in that case the detection band was limited by the shifter element.\textsuperscript{14}

Here we present a wide-field CARS microscopy setup based on the multiplex CARS excitation scheme. By using a high energy supercontinuum (SC) source generated through large-mode-area photonic crystal fiber (LMA PCF), a broad range of Raman bands can be excited over a wide spatial region, and CARS imaging of specific Raman vibrations can be achieved by applying corresponding bandpass filters. We demonstrate its chemical selectivity by imaging a mixture of polystyrene (PS) and poly(methyl-methacrylate) (PMMA) microspheres, as well as nanodiamond flakes. We also show its capability of video-rate imaging by using the standard sample and discuss its applicability as well as limitations.

FIG. 1. (a) Schematic drawing of the wide-field coherent anti-Stokes Raman scattering (CARS) imaging. Excitation beam (red) illuminates a wide area at the sample plane (black rectangle), and the CARS signal (green) from a small object (black cross) is collected by the objective and imaged onto the camera after filter and tube lens. (b) Experimental setup of the wide-field CARS microscopy. D: dichroic beam splitter; OAP: off-axis parabolic mirror; S: sample; Obj: microscope objective; F: filter; L: tube lens; Cam: imaging camera. The inset sketches the PCF input coupling. (c) The supercontinuum spectrum obtained from the PCF, with a red vertical line indicating the excitation wavelength (1064 nm).
II. EXPERIMENTAL SETUP

The schematic setup of the SC generation has been discussed earlier and is shown in Fig. 1(b). In brief, the output of a picosecond Nd:YVO₄ laser (APLX-10, Attodyne), with 1 MHz repetition rate and 10 µJ pulse energy at 1064 nm, was divided into two parts: one was used to pump a 2-m-long LMA PCF (NKT Photonics) to generate SC light and was used as the Stokes beam and the other part was sent through a delay stage and served as the pump beam. The spectrum of the SC pulse is shown in Fig. 1(c). The Stokes beam then passed through a long-pass filter (FELH1150, Thorlabs), which transmitted the wavelength component above 1150 nm. The two beams were recombined on a dichroic beam splitter (LPD02-1064RU-25, Semrock) and were focused collinearly onto the sample using an off-axis parabolic mirror with a focal length of 100 mm. The diameter of the focal spot was measured to be 32 µm.

Figure 1(a) illustrates the illumination and collection scheme. After the sample, the forward-scattered beam was collected by using a microscope objective (100× LE Plan, Nikon). The excitation light was then blocked by spectral filters, while the CARS signal passed through. The sample image was projected onto the camera through a tube lens with a focal length of 200 mm. A common short-pass filter (FESH1000, Thorlabs) was used to block the excitation light, while different bandpass filters were used to address different Raman bands: a bandpass filter centered at 800 nm (FBH0800, Thorlabs) was used to image the aromatic C—H stretch around 3050 cm⁻¹; another bandpass filter centered at 810 nm (FBH0810, Thorlabs) was used for the aliphatic C—H stretch around 2950 cm⁻¹; a tunable bandpass filter consisting of a tunable long-pass (TL01-995-25 × 36, Semrock) and a tunable short-pass (TSP01-995-25 × 36, Semrock) filter was used to select Raman bands in the fingerprint region. The filters chosen resulted in a spectral resolution of 140 cm⁻¹ and 35 cm⁻¹ when detecting in the C—H stretching region and fingerprint region, respectively. For the imaging camera, we tested the system with both a CCD (iKon-M 934, Andor) and a scientific CMOS (sCMOS) camera (DHY ANA 95, Tucsen).

PS and PMMA microspheres were obtained in the form of aqueous solutions (Sigma Aldrich), with bead sizes of 3 µm and 4 µm, respectively. The mixed sample was prepared by mixing the two solutions at 1:1 ratio and depositing the bead mixture onto a low-density polyethylene (LDPE) film (Glad Corporation). Nanodiamonds were purchased in the form of aqueous solution (Columbus NanoWorks) and were deposited on the LDPE film to form clustered flakes. L-cystine powder (Sigma Aldrich) was deposited on a thin polyethylene terephthalate (PET) film (GoodFellow).

III. RESULTS AND DISCUSSION

To demonstrate chemical selective imaging, we first show the results obtained from the PS and PMMA bead mixture. The bright-field image of the sample is shown in Fig. 2(a), and the PS and PMMA beads can clearly be distinguished by their sizes. Figures 2(b) and 2(c) show the CARS images obtained using the 800 nm and 810 nm filters, respectively. By using the 800 nm filter, the aliphatic C—H stretch signal from PMMA was rejected, and therefore only PS beads were visible in Fig. 2(b). On the other hand, when applying the 810 nm filter, the PMMA bead in the center became bright, while the PS beads were dimmed but still visible due to the residual PS signal in this spectral region. In Fig. 2(d), we plot the CARS spectra acquired when focusing on either a PS or PMMA bead using a home-built CARS microscope. Notice that there were also contributions from the LDPE substrate in these spectra, but such a background signal was not significant in the CARS image as the surroundings appeared to be dark, and the bead signals were dominant, with a signal-to-noise (SNR) ratio exceeding 100. However, we note that the bead samples in the wide-field CARS image in Figs. 2(b) and 2(c) appeared to be much smaller as compared to their bright-field image [Fig. 2(a)]. This can be attributed to the index mismatch between the bead and the surrounding medium (air), and the beads acting effectively as micro-lenses and focusing the incident light, causing a bright center spot with a dark periphery. Here images were acquired using the CCD camera, and the integration time was set to 100 ms. The power of the pump and Stokes beams on the sample was measured to be 30 mW and 15 mW, respectively.
FIG. 2. CARS images of the polystyrene (PS) and poly(methyl methacrylate) (PMMA) mixture captured by the CCD camera. (a) Bright-field image of the bead mixture, with a red circle indicating the CARS imaging area. (b) CARS image of the mixture using the 800 nm bandpass filter, corresponding to the 3050 cm\(^{-1}\) band of PS. (c) CARS image of the mixture using the 810 nm bandpass filter, corresponding to the 2950 cm\(^{-1}\) band of PMMA. (d) CARS spectra of a PS bead (black straight line) and a PMMA bead (red dashed line). The bandpass region of the 800 nm and 810 nm filters is marked in blue and green, respectively. The scale bars in the figures correspond to 5 µm.

We also demonstrate CARS imaging in the fingerprint region using nanodiamond flakes as the sample. Figure 3(a) shows the bright-field image of the clustered diamond flakes. We used the tunable bandpass filter to select the imaging wavelength. The off-resonance and on-resonance CARS

FIG. 3. CARS images of the diamond flake captured by the CCD camera. (a) Bright-field image of the flake, with a red circle indicating the CARS imaging area. (b) CARS image of the flake when tuned off-resonant around 1400 cm\(^{-1}\). (c) CARS image of the flake when tuned on-resonant at 1320 cm\(^{-1}\). (d) CARS spectra when focused on diamond, and the bandpass region of off-resonance and on-resonance states is marked in blue and green, respectively. The scale bars in the figures represent 5 µm.
images of the flakes are shown in Figs. 3(b) and 3(c), respectively. In Fig. 3(d), we also plot the CARS spectrum when focused on a single spot within the diamond cluster. By tuning to the diamond resonant peak around 1320 cm$^{-1}$, we can see bright spots showing up on the flake in Fig. 3(c), while in the off-resonant case, no detailed feature is visible. The constant background signal as shown in Figs. 3(b) and 3(c) can be seen when tuning the bandpass filter throughout the fingerprint region, and it could be attributed to the non-resonant signal originating from the interface of diamond and the polymer substrate. Again images were acquired by the CCD camera with the same integration time and power.

Taking advantage of the high signal-to-noise ratio (SNR) in the bead sample images, we can perform video-rate imaging with the sCMOS camera. Figures 4(a) and 4(b) show the images obtained from the PS and PMMA mixture using the sCMOS camera, with the 800 nm and 810 nm bandpass filters, respectively. Again we can see that PS and PMMA beads glowed separately when the corresponding CARS band was selected. The integration time used here is 38 ms, with the power of the pump and Stokes beams increased to 50 mW and 40 mW, respectively. Under such settings, we can achieve a frame rate of 25 fps, with an SNR larger than 20. A video recording of the CARS signal from the PS beads at different positions is shown in Fig. 5 (Multimedia view).

As a demonstration of a possible practical application, we performed microscopy on L-cystine microcrystalline. L-cystine is an oxidized dimeric form of cysteine and is highly concentrated in the immune system, skeletal and connective tissues, skin, digestive enzymes, and hair. Figure 6(a) shows the bright-field image of the microcrystalline, and Fig. 6(b) is the CARS image obtained by applying the 810 nm bandpass filter, corresponding to its C–H stretch signal. Note that because non-phase-matched scattering was dominant in this case, the signal occurred mostly in regions where internal refraction and scattering were significant. Therefore only certain spots appeared to be bright. The CARS nature of the signal was verified by switching to the 800 nm filter and noticing that the image

FIG. 4. Video-rate CARS imaging of the PS and PMMA bead mixture. (a) CARS image in the 3050 cm$^{-1}$ region (800 nm bandpass filter). (b) CARS image of the 2950 cm$^{-1}$ region (810 nm bandpass filter). The scale bars in the figures represent 5 μm.

FIG. 5. The video-rate CARS imaging of the PS beads in the 3050 cm$^{-1}$ region (800 nm bandpass filter). (a)–(c) correspond to different positions on the film. The scale bars in the figures represent 5 μm. Multimedia view: https://doi.org/10.1063/1.5045575.1
FIG. 6. CARS imaging of L-cystine microcrystalline. (a) Bright-field image of the microcrystalline and (b) the corresponding CARS image in the 2950 cm\(^{-1}\) region (810 nm bandpass filter). The scale bars in the figures represent 5 \(\mu\)m.

turned completely dark. The pump power and Stokes power used here were 35 mW and 30 mW, respectively, with an image integration time of 200 ms.

The estimated average laser fluence in the current setup is 10 mJ/cm\(^2\), which is lower compared to the system using an amplified ultrafast laser\(^\text{14}\) but higher than that using oscillator-only pulses.\(^\text{15}\) This is reasonable due to the relatively low intensity contained in each spectral component in the SC pulse, which in turn calls for higher pulse energy to reach a significant level of the CARS signal. Yet the advantage of the SC excitation as compared to previous wide-field CARS microscopy schemes is that multiple bands can be stimulated simultaneously such that more information from the target could be obtained in a single collection. The idea of broadband wide-field excitation has been demonstrated earlier using amplified femtosecond pulses for relatively large scale gas imaging,\(^\text{21}\) whereas we believe our setup is more suitable for microscopy due to the longer wavelength, less pulse energy, and longer pulse duration used, which induce less sample damage.\(^\text{22}\) Still, depending on the sample used, additional studies are needed to determine and to achieve optimized parameters and pulse settings that provide optimal balance between the signal level and sample damage.

In terms of spectral performance, we note that the resolution of the system can be further enhanced simply by choosing filters with narrower passband, but this has to be compensated by the increase in integration time so as to achieve desired SNR. Also, with more Raman bands involved in imaging, significant complication to the detection system is expected. Therefore when it comes to hyperspectral imaging where many Raman bands need to be imaged simultaneously, the current scheme will be less favorable compared to laser-scanning-based CARS.\(^\text{8,9}\) The fundamental limit is due to the low spectral intensity in the excitation and can be resolved with an improved SC source. On the other hand, if the sample components are known and only a few well-separated Raman bands are of interest, our setup can provide faster imaging speed as opposed to the raster-scanning method.

The observed bead image distortion due to refractive index mismatch has also been seen in previous wide-field CARS using non-phase-matched illumination.\(^\text{15,16}\) On the other hand, phase-matched wide-field CARS displayed no significant distortion on the oil droplet image,\(^\text{12}\) possibly due to less distortion caused by a planar object and a correspondingly better sectioning capability. Refractive index mismatch distortion has been reported in laser-scanning CARS imaging as well\(^\text{23,24}\) but with less serious effects in terms of planar imaging of bead samples as compared to our wide-field CARS scheme. We expect our system to perform better on planar structures where refractive index mismatch distortion is less severe, but image correction of more complicated structures requires further research.

Other limitations include non-uniformity in the excitation field—the Gaussian beam profile causes the center of the image to be brighter while the edge to be completely dark. Although numerical correction is possible by normalizing the signal to the excitation intensity profile, this will also enhance the noise level on the edge. A preferable way to flatten the field is to enhance the illumination area, which will in turn call for more power of the excitation beams, particularly the supercontinuum beam. The current setup allows a useful power of 140 mW in the Stokes beam, but higher power can be obtained by increasing the length of the fiber or by using a polarization-maintaining PCF.\(^\text{17}\) Another issue is the instability in the signal, which largely arises from the nonlinear nature of the
CARS process and the SC generation and further deteriorated by other fluctuations such as the laser-pointing instability and air turbulence. It is of interest in the future to develop a wide-field scheme that does not require nonlinear broadening or is based on an all-fiber setup.

We note that the substrates are chosen to be thin films here to minimize the background FWM signal. When depositing samples onto coverslip up to hundreds of microns thickness, we noticed a strong non-resonant background appearing around the microspheres despite the phase-mismatch. This background could be generated from the vicinity of the sample, and due to the depth of focus of the objective, these signals finally end up in the imaging camera. A more elegant solution to this problem could be using the tunable filter and performing a frequency-modulation measurement such that when taking the difference between two images with close-wavelength separation, the non-resonant background is eliminated and the resonant signal is enhanced.

Lastly, given that a majority of the spectral components are not used when imaging with a specific Raman band, it can be advantageous to put a tunable filter in the Stokes path of our setup to allow programmable excitation that can be rapidly switched (such as an acousto-optic tunable one), for high speed biological imaging and cytometry. Another promising application is to combine the ideas of compressive spectral imaging to achieve simultaneous spectral and spatial resolution with high efficiency. Also, with the advancement in SC generation, a more powerful SC pulse with high spectral coherence could be implemented to achieve better background suppression through temporal control and better sensitivity through coherent intra-pulse excitation.

IV. CONCLUSION

In conclusion, we have demonstrated a multiplex wide-field CARS imaging scheme. By using the high energy picosecond supercontinuum pulse for excitation, different Raman band imaging was achieved by applying corresponding filters. We were able to perform video-rate CARS imaging of the standard sample. Such a scheme can be applied in cytometry, gas sensing, and other biomedical disciplines where high-frame-rate imaging of various species is required. It is also promising to combine with compressed sensing to achieve high-speed hyperspectral imaging.

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