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Coherent anti-Stokes Raman scattering imaging under ambient light

Yinxin Zhang^{1,2}, Chien-Sheng Liao², Weili Hong², Kai-Chih Huang², Huaidong Yang³, Guofan Jin³, and Ji-Xin Cheng^{2,4,5,6,*}

¹Key Laboratory of Opto-electronics Information Technology of the Ministry of Education, College of Precision Instrument and Opto-Electronics Engineering, Tianjin University, Tianjin 300072, China

²Weldon School of Biomedical Engineering, Purdue University, West Lafayette, Indiana 47907, USA

³State Key Laboratory of Precision Measurement Technology and Instruments, Tsinghua University, Beijing 100084, China

⁴Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, USA

⁵Birck Nanotechnology Center, Purdue University, West Lafayette, Indiana 47907, USA

⁶Purdue Institute of Inflammation, Immunology, Infectious Disease, West Lafayette, Indiana 47907, USA

Abstract

We demonstrate ambient light coherent anti-Stokes Raman scattering (AL-CARS) microscopy that allows CARS imaging to be operated under environment light for field use. CARS signal is modulated at megahertz frequency and detected by a photodiode equipped with a lab-built resonant amplifier, then extracted through a lock-in amplifier. Filters in both spectral domain and frequency domain effectively blocked room light contamination of the CARS image. *In situ* hyperspectral CARS imaging of tumor tissue under ambient light is demonstrated.

Coherent anti-Stokes Raman scattering (CARS) microscopy is a recently developed vibrational spectroscopic imaging technology with broad applications in biology and medicine [1–4]. CARS is a third-order nonlinear optical process in which a pump-probe beam (ω_p) and a Stokes beam (ω_s) interact with molecules in the specimen. When the beat frequency ($\omega_p - \omega_s$) is tuned to be resonant with a given vibrational mode, a strong anti-Stokes signal is generated at the frequency of $\omega_{as} = 2\omega_p - \omega_s$ [1,4]. CARS microscopy holds the promise for noninvasively imaging complex systems with high spatial resolution, high sensitivity, and label-free chemical specificity. Its clinical applications have been promoted by advanced developments [5], including epi-detection [6], multiplex acquisition [7–9], single-beam excitation [10,11], spectral focusing [12,13], nonlinear fiber wavelength

*Corresponding author: jcheng@purdue.edu.

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conversion [14], time-resolved probing [15] and interferometry [16, 17]. Despite these advances, current CARS microscope can only be operated in a dark environment because the CARS signal currently relies on highly sensitive photomultiplier tubes (PMT) which can be easily saturated by room light. This remaining challenge has blocked the *in situ* applications of CARS microscopy in an operating room and in the field.

Here, we address this challenge through the development of ambient light CARS (AL-CARS) microscopy. In our scheme, the laser is modulated at megahertz frequency. Under ambient light, a photodiode equipped with a lab-built resonant amplifier selectively picks up the CARS signal at the same modulation frequency. Bandpass filters are further used to spectrally block the laser and the room light. Together, by filtering in both frequency domain and spectral domain, we have succeeded in blocking the environment light and sensing CARS signal with sufficient signal-to-noise ratio (SNR). By our AL-CARS setup, spectroscopic images of human breast cancerous tissue *in situ* were obtained. Through multivariate curve resolution (MCR) analysis [18], the fibrosis and cytoplasm were distinguished.

In AL-CARS, both band pass filters and the modulation serve as the obstacle to ambient light. The band pass filters block the excitation laser as well as most of the ambient light, releasing only the CARS signal and room light around the CARS wavelength to the detector. By modulation of the laser and demodulation of the signal at megahertz, we further reject the environment light in the frequency domain. The ambient light entering the detector has an optical frequency of about several hundred THz and is accompanied with the alternating current frequency of 60 Hz. Since the modulation frequency is quite different from either the optical frequency of the ambient light or the alternating current frequency, we are able to extract the CARS signal selectively by a resonant circuit and a lock-in amplifier.

The system is depicted in Fig. 1a. An ultrafast laser (InSight, Spectra Physics) with dual outputs provides two synchronized pulse trains. The tunable beam with 120 fs pulse duration served as the pump beam and was tuned to 798 nm for excitation of C-H bonds. The 1040 nm beam with 200 fs pulse duration serves as the Stokes beam and is modulated by an acousto-optic modulator (AOM, 1205-C, Isomet) at the frequency of 2.31 MHz. The two beams are combined and chirped by two 15 cm long SF57 glass rods. In this way, the Raman shift can be controlled by the time delay between the pump and Stokes beams. The pump and Stokes beams are sent into a lab-built microscope [19]. A 60 $\times$ water immersion objective lens (NA=1.2, UPlanApo/IR, Olympus) is used to focus the light onto the sample, and an oil condenser (NA=1.4, U-AAC, Olympus) is employed to collect the scattering signal. Two band pass filters (D680/100, HQ650/60, Chroma) are housed in a lens tube and attached to the photodiode case. The signal is collected by a large-area photodiode (S3994-01, Hamamatsu) and amplified by a lab-built resonant circuit with a 2.25 MHz central frequency and 300 kHz bandwidth (Fig. 1b). The lock-in amplifier (HF2LI, Zurich Instrument) extracts the modulated CARS signal. Hyperspectral CARS images are acquired by scanning the temporal delay between the chirped pump and Stokes beams [12, 20]. By this spectral focusing scheme, the Raman shift of each frame in hyperspectral images varies as the time delay changes.

The calibration of the Raman shift with respect to the temporal delay was completed by using Raman peaks of known chemicals. A modified Kramers-Kronig method [21–23] was employed to extract the equivalent Raman spectra from measured CARS spectra which contain a vibrationally resonant signal and a nonresonant background. By comparing the phase retrieved CARS spectral profile of known chemicals with their spontaneous Raman spectra, the relation between motor step position and Raman shift is obtained and shown in Fig. 1c. The relation can be described by a linear fitting with $R^2=0.99$.

In order to compare our AL-CARS setup with “dark” CARS detected by PMT, we recorded the AL-CARS image of Dimethyl sulfoxide (DMSO) (Fig.2a) and the “dark” CARS image of the same sample (Fig.2b), respectively, under the 60 Hz room light. The AL-CARS setup worked well in the lighted room, where the photodiode was not saturated and the modulated CARS signal was successfully extracted from the ambient light. In contrast, Fig.2b revealed that the PMT was saturated by the lamp light when the voltage for PMT was 250 V. Because the CARS signal from a biomedical sample is much weaker than that of DMSO, for CARS imaging of tissues, the voltage for PMT must be much higher than 250 V. Under such condition, the environment light would definitely saturate the PMT or even damage the detector. Together, this comparison shows the advantage of our scheme for ambient light applications of CARS microscopy.

To characterize the signal and the source of noise in AL-CARS, we measured the CARS signal and noise level as a function of the excitation laser power. The intensity of CARS signal can be expressed as [4]:

$$I_{CARS} \propto |\chi^{(3)}|^2 I_p^2 I_s \quad (1)$$

$\chi^{(3)}$ is the third-order nonlinear susceptibility containing a vibrationally resonant and a nonresonant component. Fig. 3a shows that the CARS intensity is proportion to $I_p^2 I_s$, which is in agreement with equation (1). There are three major noise resources in a CARS microscopic system, namely the shot noise, the laser intensity noise and the detector electronic noise [4]. The relationship between these noise resources can be written as:

$$total\ noise = \sqrt{shot\ noise^2 + laser\ intensity\ noise^2 + elec.\ noise^2} \quad (2)$$

The electronic noise is a constant, and the shot noise is known to be proportional to the square root of the power, here, the intensity of the CARS signal. In our AL-CARS microscope, the total noise in the signal raises with the increase of the excitation intensity (Fig. 3b). When $I_p^2 I_s$ is below $5 \times 10^5 \text{ mW}^3$, the electronic noise is dominant and the total noise increases slowly with the laser intensity. As $I_p^2 I_s$ exceeds $5 \times 10^5 \text{ mW}^3$, the total noise level increases linearly with the laser intensity. As the CARS signal level has a linear relationship with $I_p^2 I_s$, the shot noise is proportional to the square root of $I_p^2 I_s$ accordingly. Thus, the linear relationship between noise and $I_p^2 I_s$ in Fig. 3b indicates that the AL-CARS setup is laser noise dominant when $I_p^2 I_s$ exceeds $5 \times 10^5 \text{ mW}^3$. Consistently, the SNR

increases quickly at the very beginning, and becomes a constant as the excitation intensity is raised (Fig. 3c).

To evaluate the detection sensitivity of our AL-CARS imaging setup, we used DMSO at different volume concentrations as a test bed. Fig. 4a shows a representative phase-retrieved image of DMSO solution diluted by Deuterium Oxide (D_2O). The CARS spectra of different concentrated DMSO samples can be found in Fig. 4b. When the DMSO volume concentration is as low as 1%, the peak of 2913cm^{-1} Raman shift is still clear, which indicates that the detection limit of the setup can reach 1% DMSO.

In equation (1), $\chi^{(3)}$ is the term that generates CARS signal. From microscopic view of molecules, $\chi^{(3)}$ is proportional to the bulk number density [24,25]. Connecting the macroscopic tensors and molecular tensors, there should be a quadratic relationship between CARS signal intensity and DMSO density. The results in Fig. 4c are consistent with the theory. Meanwhile, the SNR of each image is related to the DMSO concentration by linear fitting, resulting in a linear correlation with $R^2=0.99$ (Fig. 4d).

Finally, we demonstrated the capability of AL-CARS scheme for *in situ* spectroscopic imaging of breast tissue. Forward-detected hyperspectral CARS imaging mapped the human breast cancer cells and the stroma based on their distinct chemical composition. The CARS image at 2920cm^{-1} shows the morphology of the cancerous tissue (Fig. 5a). By phase retrieval and MCR analysis, we were able to decompose the hyperspectral CARS data set into a chemical map containing two major components (Fig. 5b). The spectral profile (Fig. 5c) with a strong peak around 2930cm^{-1} is assigned to the fibrosis in the stroma, and the weaker and broader peak is assigned to the protein-rich cytoplasm. The nuclei showed a dark contrast.

In summary, the current work demonstrated a new scheme that allows CARS imaging under ambient light, in which a photodiode with a lab-built resonant amplifier is used as the detector. The band pass filters in the spectral domain as well as modulation in the frequency domain blocks the environment light. We note that stimulated Raman scattering (SRS) [26, 27] works well under ambient light. Nevertheless, SRS microscopy suffers from laser noise and requires a high-quality solid state laser as the excitation source. Because the CARS signal appears at a new wavelength, the laser noise is not a big issue. Our AL-CARS scheme is applicable to a compact fiber laser as the excitation source. Moreover, epi-detected CARS imaging is doable. These features collectively render AL-CARS a promising tool for *in situ* and *in vivo* clinical applications and field uses.

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Full references

1. Evans, Conor L., Sunney Xie, X. Coherent Anti-Stokes Raman Scattering Microscopy: Chemical Imaging for Biology and Medicine. *Annu Rev Anal Chem.* 2008; 1:883–909.
2. Krafft, Christoph, Dietzek, Benjamin, Schmitt, Michael, Poppa, Jürgen. Raman and coherent anti-Stokes Raman scattering microspectroscopy for biomedical applications. *Journal of Biomedical Optics.* 2012; 17(4):040801. [PubMed: 22559673]
3. Camp, Charles H., Jr, Cicerone, Marcus T. Chemically sensitive bioimaging with coherent Raman scattering. *Nature Photonics.* 2015; 9:295–305.
4. Zhang, Chi, Zhang, Delong, Cheng, Ji-Xin. Coherent Raman Scattering Microscopy in Biology and Medicine. *Annu Rev Biomed Eng.* 2015; 17:16.1–16.31.
5. Tu, Haohua, Boppart, Stephen A. Coherent anti-Stokes Raman scattering microscopy: overcoming technical barriers for clinical translation. *J Biophotonics.* 2014; 7:9–22. [PubMed: 23674234]
6. Evans, Conor L., Potma, Eric O., Puoris'haag, Mehron, Côté, Daniel, Lin, Charles P., Sunney Xie, X. Chemical imaging of tissue in vivo with video-rate coherent anti-Stokes Raman scattering microscopy. *PNAS.* 2005; 102(46):16807–16811. [PubMed: 16263923]
7. Camp, Charles H., Jr, Lee, Young Jong, Heddleston, John M., Hartshorn, Christopher M., Hight Walker, Angela R., Rich, Jeremy N., Lathia, Justin D., Cicerone, Marcus T. High-speed coherent Raman fingerprint imaging of biological tissues. *Nature Photonics.* 2014; 8:627–634. [PubMed: 25621002]
8. Cheng, Ji-xin, Volkmer, Andreas, Book, Lewis D., Sunney Xie, X. Multiplex Coherent Anti-Stokes Raman Scattering Microspectroscopy and Study of Lipid Vesicles. *J Phys Chem B.* 2002; 106:8493–8498.
9. Müller, Michiel, Schins, Juleon M. Imaging the Thermodynamic State of Lipid Membranes with Multiplex CARS Microscopy. *J Phys Chem B.* 2002; 106:3715–3723.
10. Katz, Ori, Levitt, Jonathan M., Grinvald, Eran, Silberberg, Yaron. Single-beam coherent Raman spectroscopy and microscopy via spectral notch shaping. *Optics Express.* 2010; 18:22693–22701. [PubMed: 21164608]
11. Dudovich, Nirit, Oron, Dan, Silberberg, Yaron. Single-pulse coherently controlled nonlinear Raman spectroscopy and microscopy. *Nature.* 2002; 418:512–514. [PubMed: 12152073]
12. Hellerer1, Thomas, Enejder1, Annika MK., Zumbusch, Andreas. Spectral focusing: High spectral resolution spectroscopy with broad-bandwidth laser pulses. *Appl Phys Lett.* 2004; 85:25–27.
13. Jones, Gareth W., Marks, Daniel L., Vinegoni, Claudio, Boppart, Stephen A. High-spectral-resolution coherent anti-Stokes Raman scattering with interferometrically detected broadband chirped pulses. *Optics Letters.* 2006; 31:1543–1545. [PubMed: 16642166]
14. Baumgartl, Martin, Chemnitz, Mario, Jauregui, Cesar, Meyer, Tobias, Dietzek, Benjamin, Popp, Jürgen, Limpert, Jens, Tünnermann, Andreas. All-fiber laser source for CARS microscopy based on fiber optical parametric frequency conversion. *Optics Express.* 2012; 20:4484–4493. [PubMed: 22418207]
15. Volkmer, Andreas, Book, Lewis D., Sunney Xie, X. Time-resolved coherent anti-Stokes Raman scattering microscopy: Imaging based on Raman free induction decay. *Applied Physics Letters.* 2002; 80:1504–1597.
16. Potma, Eric O., Evans, Conor L., Sunney Xie, X. Heterodyne coherent anti-Stokes Raman scattering (CARS) imaging. *Optics Letters.* 2006; 31:241–243. [PubMed: 16441043]
17. Marks, Daniel L., Boppart, Stephen A. Nonlinear Interferometric Vibrational Imaging. *Phys Rev Lett.* 2004; 92:123905. [PubMed: 15089675]
18. Wang, Ping, Liu, Bin, Zhang, Delong, Belew, Micah Y., Tissenbaum, Heidi A., Cheng, Ji-Xin. Imaging Lipid Metabolism in Live *Caenorhabditis elegans* Using Fingerprint Vibrations. *Angew Chem Int Ed.* 2014; 53:11787–11792.
19. Wang P, Li J, Wang P, Hu CR, Zhang D, Sturek M, Cheng JX. Label-Free Quantitative Imaging of Cholesterol in Intact Tissues by Hyperspectral Stimulated Raman Scattering Microscopy. *Angew Chem, Int Ed.* 2013; 52:13042.

20. Pegoraro, Adrian F., Ridsdale, Andrew, Moffatt, Douglas J., Jia, Yiwei, Pezacki, John Paul, Stollow, Albert. Optimally chirped multimodal CARS microscopy based on a single Ti:sapphire oscillator. *Optics Express*. 2009; 17:2984–2996. [PubMed: 19219203]
21. Liu, Yuexin, Lee, Young Jong, Cicerone, Marcus T. Fast extraction of resonant vibrational response from CARS spectra with arbitrary nonresonant background. *Journal of Raman Spectroscopy*. 2009; 40:726–731.
22. Liu, Yuexin, Lee, Young Jong, Cicerone, Marcus T. Broadband CARS spectral phase retrieval using a time-domain Kramers–Kronig transform. *Optics letters*. 2009; 34:1363–1365. [PubMed: 19412273]
23. Camp, Charles H., Jr, Lee, Young Jong, Cicerone, Marcus T. Quantitative, comparable coherent anti-Stokes Raman scattering (CARS) spectroscopy: correcting errors in phase retrieval. *Journal of Raman Spectroscopy*. 2015
24. Zhang, Chi, Wang, Jie, Ding, Bei, Jasensky, Joshua. Quantitative Spectral Analysis of Coherent Anti-Stokes Raman Scattering Signals: C–H Stretching Modes of the Methyl Group. *J Phys Chem B*. 2014; 118:7647–7656.
25. Davis, Ryan P., Moad, Andrew J., Scott Goeken, G., Wampler, Ronald D., Simpson, Garth J. Selection Rules and Symmetry Relations for Four-Wave Mixing Measurements of Uniaxial Assemblies. *J Phys Chem B*. 2008; 112:5834–5848. [PubMed: 18419167]
26. Freudiger, Christian W., Min, Wei, Saar, Brian G., Lu, Sijia, Holtom, Gary R., He, Chengwei, Tsai, Jason C., Kang, Jing X., Sunney Xie, X. Label-Free Biomedical Imaging with High Sensitivity by Stimulated Raman Scattering Microscopy. *Science*. 2008; 322:1857–1861. [PubMed: 19095943]
27. Saar1, Brian G., Freudiger, Christian W., Reichman, Jay, Michael Stanley, C., Holtom1, Gary R., Sunney Xie, X. Video-Rate Molecular Imaging in Vivo with Stimulated Raman Scattering. *Science*. 2010; 330:1368–1370. [PubMed: 21127249]

Fig. 1. Schematic and performance of a AL-CARS imaging setup. (a) Schematic of a lab-built hyperspectral AL-CARS imaging setup. HWP, half wave plate; PBS, polarizing beam splitter; L, lens; M, mirror; AOM, acousto-optic modulator; DM, dichroic mirror; SF57, SF57 glass; GM, galvo mirror; MS, motored stage; PD&RA, photodiode and resonant amplifier. (b) Pictures of the lab-built photodiode and resonant amplifier model. (c) Correlation of Raman shift with respect to the motor step numbers. The relation was fitted by a linear curve ($R^2=0.99$).

Fig. 2.

Comparison between CARS images of DMSO solution detected by photodiode and by PMT under 40W lamp light of 60Hz frequency. (a) Image detected by the photodiode in AL-CARS setup. The power of the pump and Stokes beam were 200mW and 100mW respectively. (b) Image detected by PMT. The voltage for PMT was 250 V. The PMT was saturated by the lamp light.

Fig. 3.

Signal and noise characteristics of our AL-CARS setup. (a) Measured (dot) and linear fitting (line) relationship between excitation intensity and CARS emission. (b) The noise level of the setup, which was laser noise dominant when $I_p^2 I_s$ exceeds $5 \times 10^5 \text{ mW}^3$. (c) The signal to noise ratio (SNR) as a function of $I_p^2 I_s$.

Fig. 4.

Detection sensitivity of AL-CARS determined by measuring different concentrations of DMSO in D₂O. Pump: 400 mW, Stokes: 200 mW before the microscope. (a) Phase-retrieved CARS image (5% DMSO diluted by D₂O). (b) CARS spectra of different DMSO concentrations. (c) Measured (dot) and polynomial fitting (line) relationship between DMSO density and CARS signal intensity at 2913 cm⁻¹ Raman peak. (d) Measured (dot) and linear fitting (line) relationship between DMSO density and SNR of CARS images at 2913 cm⁻¹ Raman peak.

Fig. 5.
In situ mapping of human patient breast cancer and stroma by ambient light hyperspectral CARS. (a) A single-frame CARS image of breast cancer tissue at 2920cm⁻¹ Raman shift. (b) MCR concentration maps of fibrosis (red) and cytoplasm (green). (c) MCR output spectra of two components.