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CARS Microscopy
Lights Up Lipids in Living Cells

by Xiaolin Nan, Dr. Wei Yuan Yang and Dr. X. Sunney Xie, Harvard University

Coherent anti-Stokes Raman scattering microscopy has matured into a powerful imaging technique with promising applications for studying lipids in living cells.

Biologists desire imaging techniques that provide contrast with high sensitivity and selectivity but without altering the sample. Fluorescence microscopy has high sensitivity, but dyes can alter the sample. Phase-contrast and differential-interference-contrast microscopes avoid this problem at the cost of chemical specificity. Coherent anti-Stokes Raman scattering (CARS) microscopy fits between these techniques, imaging with chemical selectivity in unstained samples.

CARS microscopy is a nonlinear imaging technique that produces images of chemical species based on their vibrational signatures. The past five years have seen advances in both the understanding of the method's contrast mechanism and the available instrumentation.[1] Conventional laser scanning microscopes can routinely carry out these imaging experiments with benign excitation powers of 1 to 2 mW and image acquisition rates up to that of video.

The technique is shedding light on many emerging and exciting biological applications. Among the chemical species that it has revealed in living cells,[2] lipids provide the best contrast, offering great potential to augment biomedical research in lipid-related diseases such as obesity and atherosclerosis.

Imaging without staining

CARS microscopy relies on the Raman effect. In the spontaneous Raman process, molecules scatter photons, modifying the photon energy with energy quanta that corresponds to the molecules' vibrational modes. Hence, spontaneous Raman

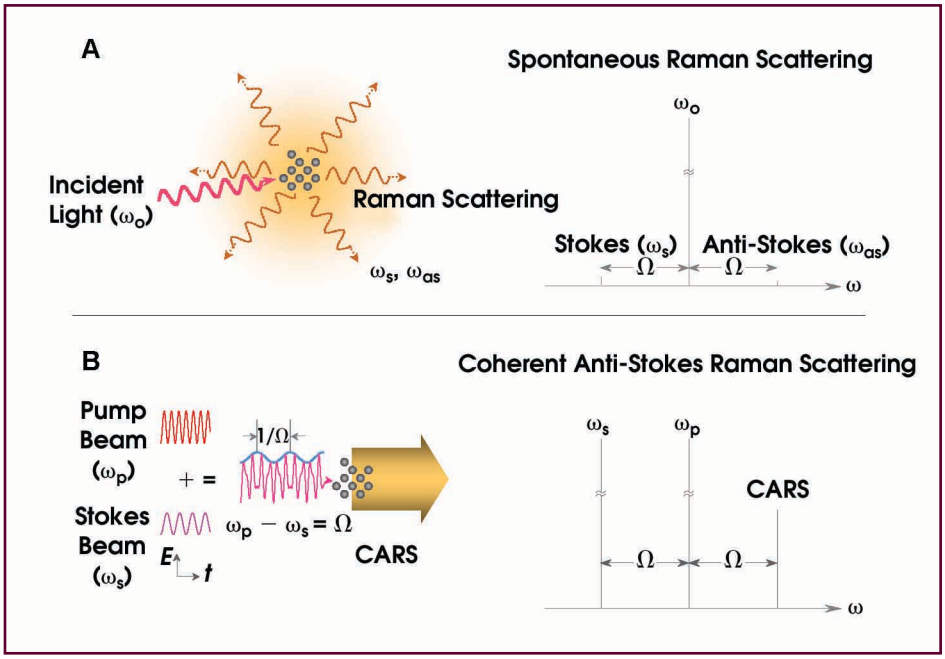

Figure. 1. In Raman scattering processes, incoming light changes frequency according to a vibrational frequency (Ω) of the molecules. Red- and blue-shifted components are termed Stokes and anti-Stokes lines, respectively. In spontaneous Raman (A), thermally driven and random-phased molecular vibrations cause inefficient scattering in all directions. In coherent anti-Stokes Raman scattering (B), two excitation beams at frequencies ω_p and ω_s form a beating field with frequency $\omega_p - \omega_s$. When $\omega_p - \omega_s$ matches Ω, the molecular vibrations occur in-phase and efficiently, resulting in a strong directional signal.

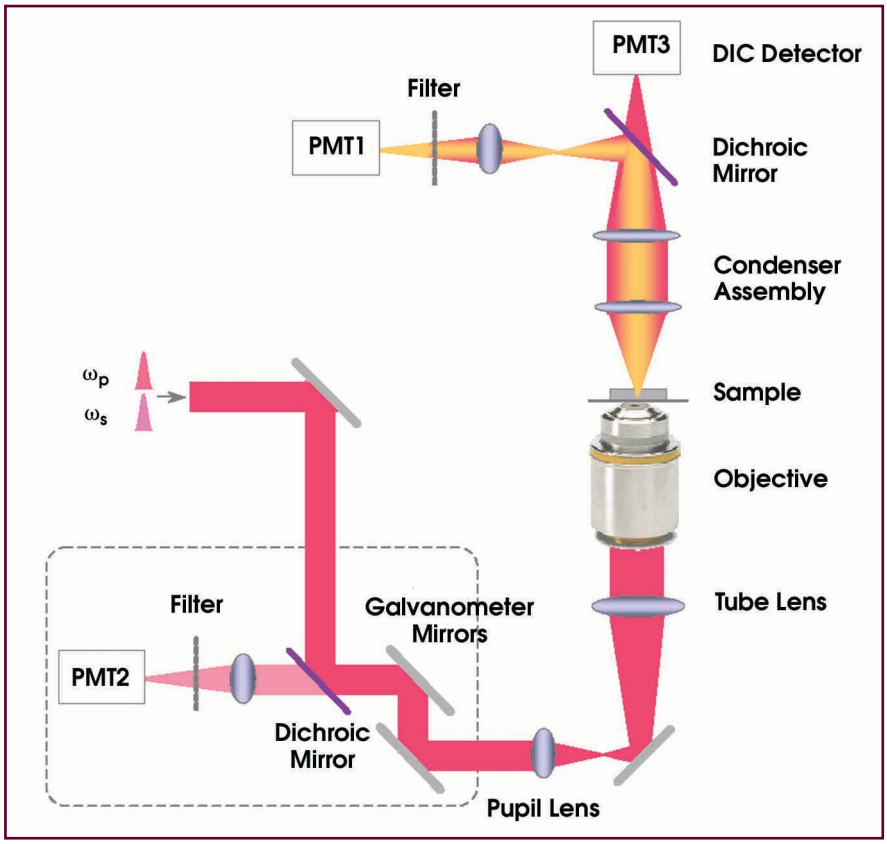

Figure 2. In a laser-scanning CARS microscope, two near-IR beams combine collinearly, reflect off a pair of scanning mirrors and reach into the microscope. High-sensitivity photomultiplier tubes detect the CARS signals, one for forward and one for backward. A third photomultiplier tube also can record differential interference contrast (DIC) or transmission images.

scattering probes molecular vibrations. However, spontaneous Raman signals are so weak (~$1/10^{16}$ of fluorescence) that using the technique to produce images requires high excitation powers (~100 mW) and long acquisition times (hours for an image). To image live cells with moderate average powers (<10 mW) in real time (>1 fps), the technique would have to produce signals four to five orders of magnitude higher.

CARS achieves this by using two laser beams instead of one. A pump beam at frequency ω_p and a Stokes beam at frequency ω_s ($\omega_s<\omega_p$) focus tightly onto the sample together. This creates a composite electric field beating at the frequency $\omega_p - \omega_s$. When this beating frequency matches a vibrational frequency of the molecules, it synchronizes the molecular oscillators, producing strong excitation across the whole focal volume. The resulting signal (at the anti-Stokes frequency $2\omega_p - \omega_s$) is orders of magnitude stronger than the incoherent spontaneous Raman signal.

The nonlinear process uses pulsed laser sources. The signal intensity has quadratic and linear dependence on pump and Stokes powers, respectively. As a result, it generates signal only within the focus, where the laser intensity is the highest, enabling three-dimensional resolution.

Pushing sensitivity

Strong signals do not necessarily deliver high sensitivity. One problem with

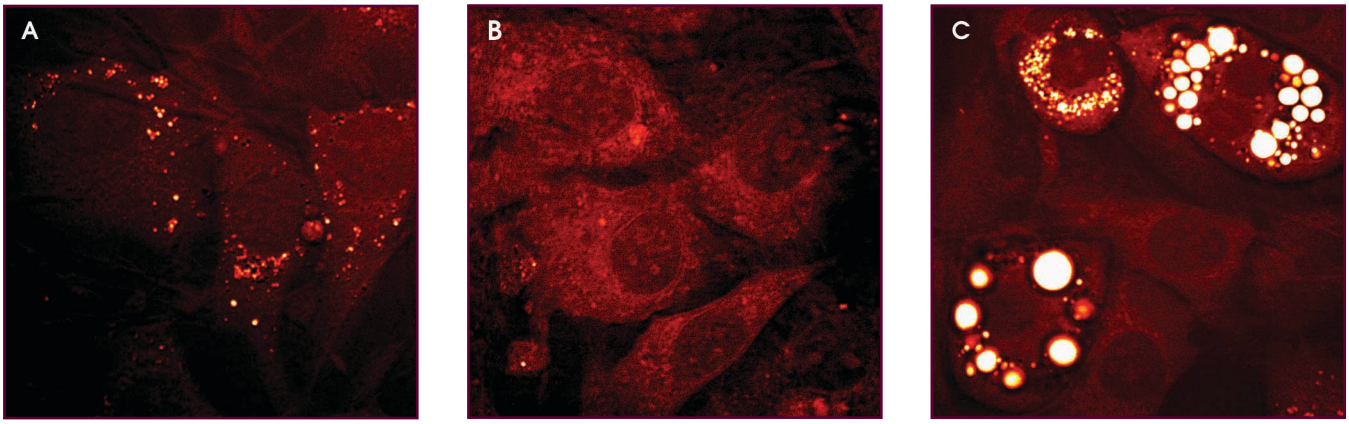

Figure 3. CARS images (2845 cm^{-1}) show live mouse fibroblast (3T3-L1) cells during their conversion to fat cells at 12 (A), 48 (B) and 108 (C) hours after conversion. At 48 hours after the conversion was triggered, lipid droplets were cleared from the cytoplasm. Images are 75 µm wide.

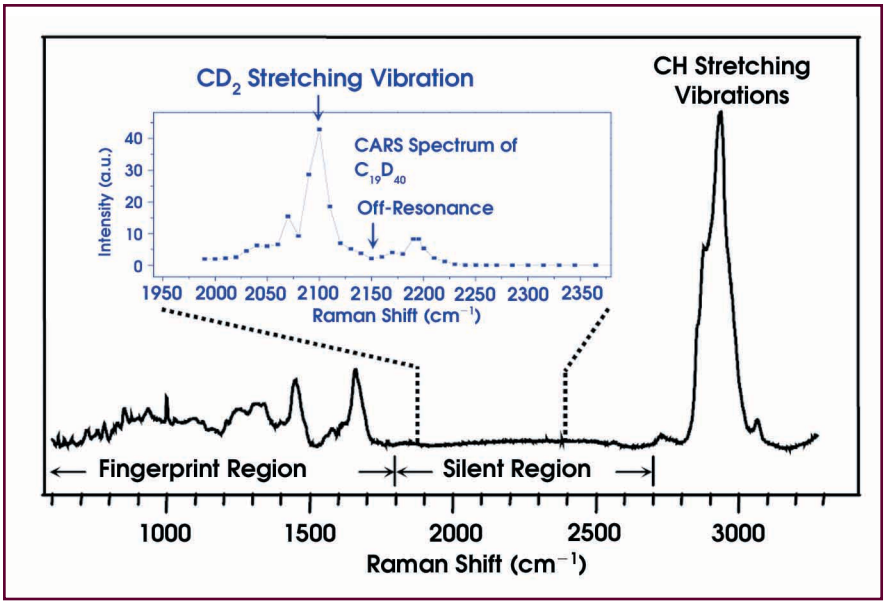

Figure 4. The Raman spectrum of dried HeLa cells shows a silent region between 1800 and 2700 cm^{-1}. In the inset, the CARS spectrum of deuterated nonadecane ($C_{19}D_{40}$) shows the main peak of CD_2 stretching vibrations at 2100 cm^{-1}.

the technique is that a nonresonant background always accompanies the signal. This background does not have vibrational selectivity, and two-photon absorption enhances the effect. Under certain conditions, the background intensity can be as high as or higher than the vibrational resonant signal, significantly limiting the signal-to-background ratio, which is equivalent to the sensitivity of CARS microscopy.

Several strategies can alleviate this problem.

First, using two near-IR excitation beams instead of visible ones reduces the background generation.[3] One added attraction of this strategy is that living samples tolerate the IR beams better. Moreover, near-IR beams penetrate deeper into turbid media, which is advantageous for tissue imaging.

Second, early CARS experiments employed femtosecond lasers, but picosecond pulses (2 to 5 ps) provide better signal-to-background ratio because their linewidths (10 to 20 cm^{-1}) match the typical Raman bandwidth (~20 cm^{-1}) far better than femtosecond pulses (~440 cm^{-1} for 100-fs pulses). This means that all the spectral components in the pulse can contribute to generating the resonant signal. In addition, the picosecond pulses provide better spectral selectivity.

These and other enhancements enable high-contrast CARS images with excitation power at 1 to 2 mW and acquisition speeds of a few frames per second and potentially up to video rate.

Visualizing lipids

Lipids are vital to cells, making up the membranes of cellular compartments and serving as fuels, biosynthetic precursors and signal transducers. They are smaller molecules than proteins or DNA, so their structure and functions are more susceptible to changes that might occur when adding fluorophores for imaging. CARS microscopy offers a rapid and noninvasive technique for visualizing lipid structures in live cells.

Lipids are abundant in CH_2 groups. In the Raman spectrum, these groups have a strong band at 2845 cm^{-1}, attributed to the symmetric stretching of CH_2 groups. Tuning $\omega_p - \omega_s$ to 2845 cm^{-1} illuminates lipids because of the strong CARS signals from the CH_2 bonds. The technique is so sensitive that it can image a single lipid bilayer with decent contrast.[4]

In CARS images of cells taken at this frequency, the highest contrast comes from lipid droplets,[5] which are solid neutral lipid structures typically found in fat and liver cells and in some types of adrenal cortical cells. Traditionally, researchers imaged the droplets with fluorescence microscopy by labeling fixed cells with neutral-lipid specific dyes. For long-term imaging, fluorescence-labeling methods are undesirable because of photobleaching and phototoxicity of the dyes, which damage the samples before providing information about the droplets' dynamic behaviors.

With CARS microscopy, we can monitor the changes in lipid droplets continuously during cellular processes. For example, in the conversion of mouse fibroblast (3T3-L1) cells to fat cells, cells accumulate triglycerides in the form of lipid droplets. The conventional belief was that the droplets continue to grow during differentiation, but continuous observation on the same cell culture with this microscopy method identified a period when they are cleared from the cytoplasm (Figure 3).

Although the CH_2 vibrational signature can distinguish lipids from other chemical species, it cannot differentiate specific lipids because their CH_2 vibrational frequencies are so similar. To selectively image a subset of lipids, we replace their CH_2 groups with CD_2. This dramatically changes the vibrational frequencies of the CH_2 groups (Figure 4) without affecting the lipid structure. In addition, the new vibrational frequencies fall in a silent region in the Raman spectrum of the cell, so other chemical species do not interfere

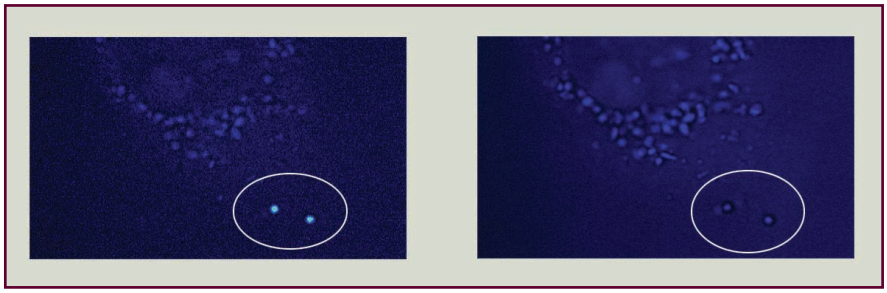

Figure 5. A mouse macrophage takes up deuterium-labeled cholesterol ester emulsions (in circle), imaged on the left with CARS microscopy at 2100 cm^{-1} and on the right at 2150 cm^{-1} (off-resonance). Images are 50 μm wide.

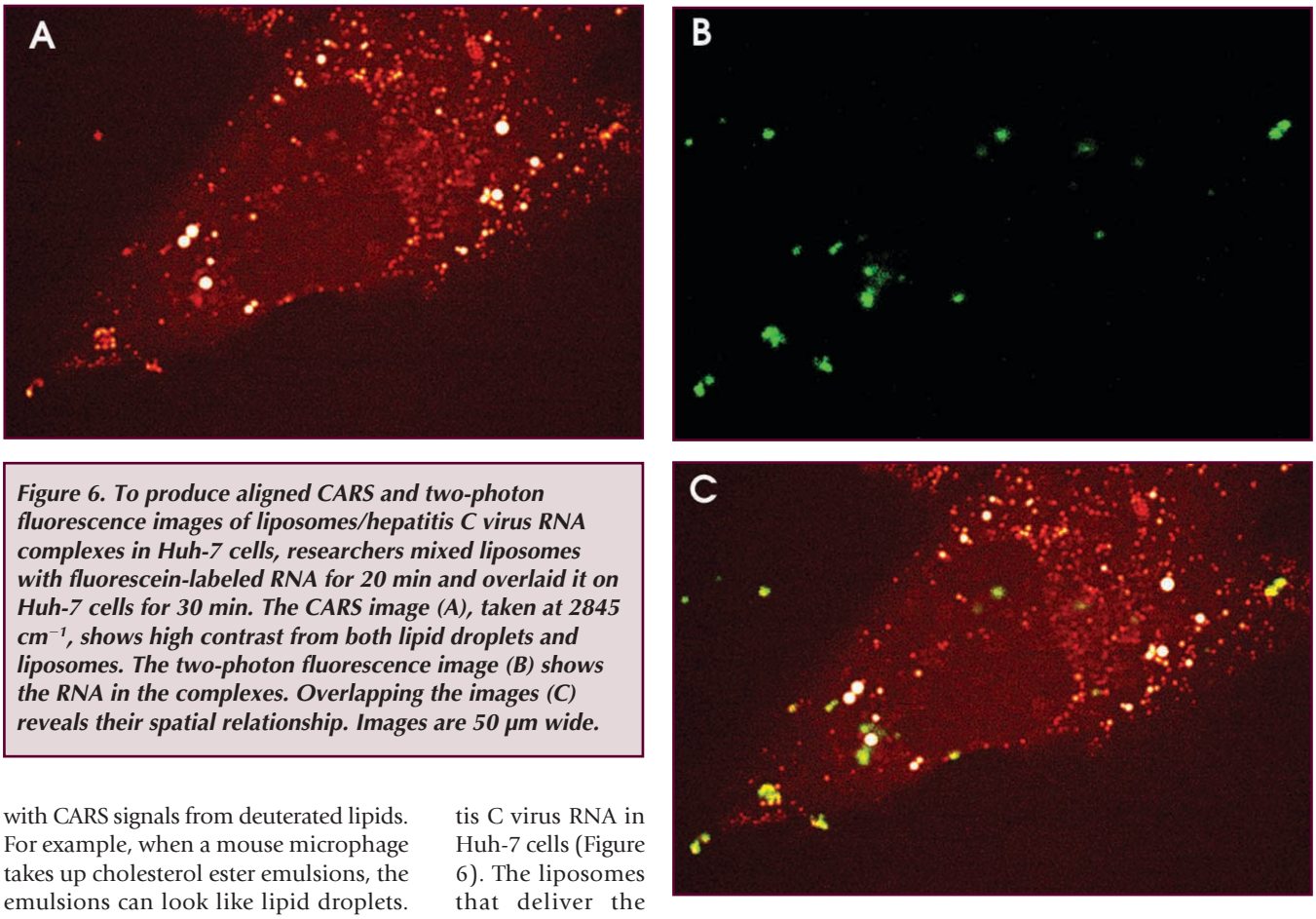

Figure 6. To produce aligned CARS and two-photon fluorescence images of liposomes/hepatitis C virus RNA complexes in Huh-7 cells, researchers mixed liposomes with fluorescein-labeled RNA for 20 min and overlaid it on Huh-7 cells for 30 min. The CARS image (A), taken at 2845 cm^{-1}, shows high contrast from both lipid droplets and liposomes. The two-photon fluorescence image (B) shows the RNA in the complexes. Overlapping the images (C) reveals their spatial relationship. Images are 50 µm wide.

with CARS signals from deuterated lipids. For example, when a mouse microphage takes up cholesterol ester emulsions, the emulsions can look like lipid droplets. But CARS images can clearly distinguish deuterated lipids (Figure 5).

Combining techniques

To show lipids with other species, we can combine CARS microscopy with fluorescence microscopy. We can generate all of the signals with the same laser beams, thereby naturally aligning the images.

For example, in collaboration with Drs. John Pezacki and Angela Tonary at the National Research Council of Canada, we are using CARS and two-photon fluorescence to study the propagation of hepatitis C virus RNA in Huh-7 cells (Figure 6). The liposomes that deliver the RNA into the cells condense into crystalline structures that give strong CARS signals at 2845 cm^{-1}. We can simultaneously image fluorescein-labeled RNA with two-photon fluorescence excited by the pump beam (usually at 710 nm). Aligning the signals from liposomes and RNA will allow us to study the release of RNA and then possibly target it onto lipid droplets.

The past few years have witnessed a rapid evolution of CARS microscopy toward higher sensitivity, turnkey instrumentation and faster scanning speeds. The lipid imaging studies described here indicate that biological applications are beginning to emerge. Looking into the future, video rate tissue imaging is the prime target for technological advancement. Biomedical research involving lipid structures, such as obesity and atherosclerosis, will be one of the foci of exciting applications of CARS microscopy. □

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References

1. J.-X. Cheng and X.S. Xie (January 2004). Coherent anti-Stokes Raman scattering microscopy: instrumentation, theory and applications. *JOURNAL OF PHYSICAL CHEMISTRY*, 108, pp. 827-840.
2. J.-X. Cheng et al (July 2002). Laser-scanning coherent anti-Stokes Raman microscopy and applications to cell biology. *BIOPHYSICAL JOURNAL*, pp. 502-509.
3. A. Zumbush et al (May 1999). Three-dimensional vibrational imaging by coherent anti-Stokes Raman scattering. *PHYSICAL REVIEW LETTERS*, pp. 4142-4145.
4. E.O. Potma and X.S. Xie (September 2003). Detection of single lipid bilayers with coherent anti-Stokes Raman scattering microscopy. *JOURNAL OF RAMAN SPECTROSCOPY*, pp. 642-650.
5. X. Nan et al (November 2003). Vibrational imaging of lipid droplets in live fibroblast cells with coherent anti-Stokes Raman scattering microscopy. *JOURNAL OF LIPID RESEARCH*, pp. 2202-2208.

Meet the authors

Xiaolin Nan, Wei Yuan Yang and X. Sunney Xie are with the department of chemistry and chemical biology at Harvard University in Cambridge, Mass; e-mail: xie@chemistry.harvard.edu.