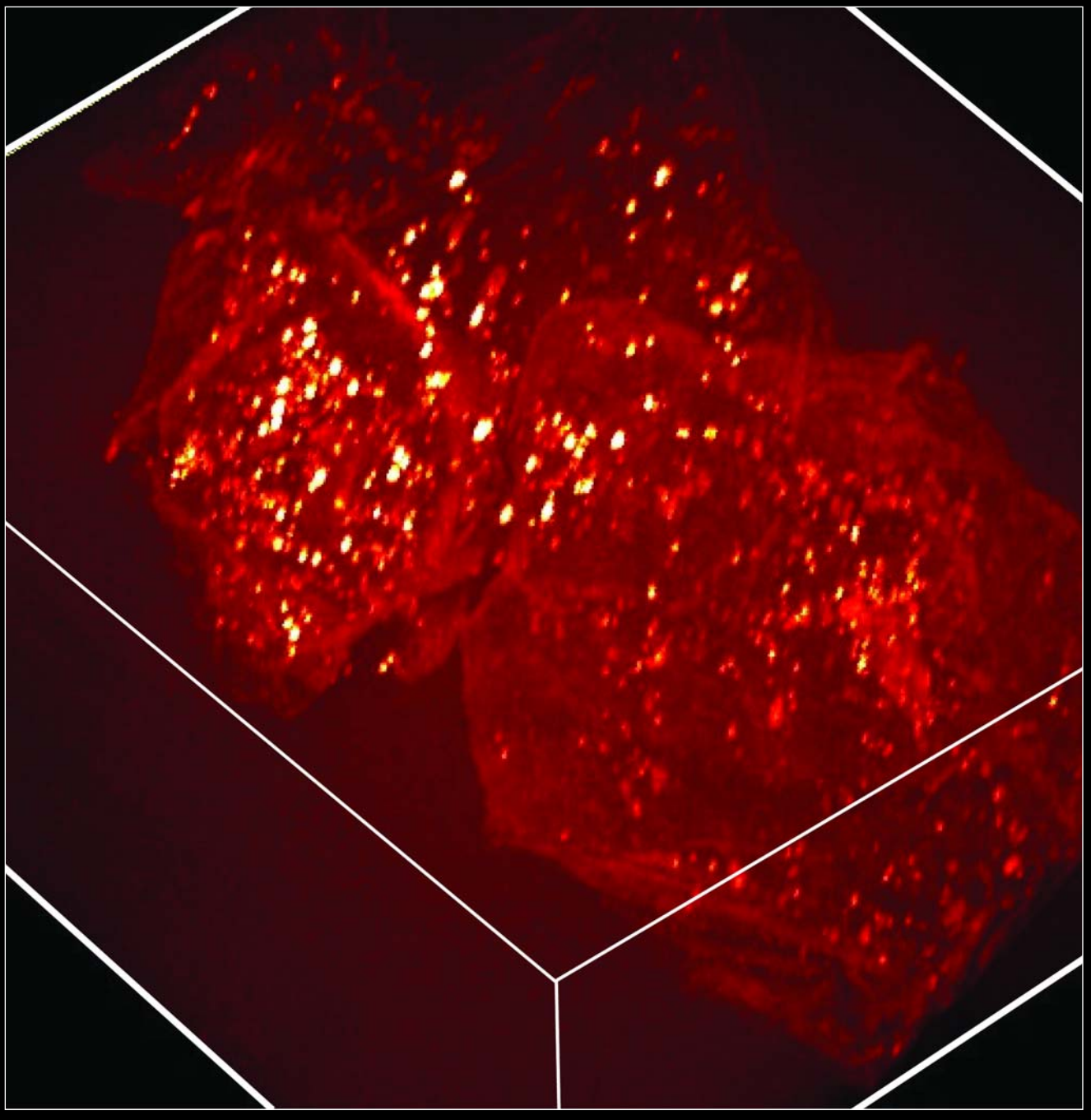

CARS Microscopy
For Biology and Medicine

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Conor Evans and Eric Potma of the Harvard team with their CARS microscope.

The key requirements imposed on optical imaging techniques for the visualization of living biological specimens are noninvasiveness, chemical selectivity and high sensitivity. Conventional Raman microscopy can satisfy the first two conditions but not the final one. Coherent anti-Stokes Raman scattering (CARS) microscopy, which meets all three requirements, casts a new light on vibrational microscopy and paves the way for exciting new applications in cell biology and medicine.

In his Nobel lecture of 1930, Sir Chandrasekahra V. Raman predicted that the effect of inelastic scattering of light by molecules was likely to give rise to a vast new field of spectroscopy. His prediction was not off the mark. The phenomenon we know today as spontaneous Raman scattering [Fig. 1(a)] is an extremely powerful tool for the characterization of molecules based on properties of chemical bonds. By the end of the 20th century, the technique had become a routine optical tool, with applications in fields as diverse as forensic research, pharmaceuticals, the mineralogy of planetary materials and the analysis of art objects, to name just a few.

The molecular sensitivity associated with the Raman effect originates from the specific frequencies of molecular vibrations. Compared to infrared absorption spectroscopy, Raman spectroscopy has the advantage of allowing for background-free detection: spectrally dispersed Raman signals can be collected against the strong background of the scattered excitation light. Another characteristic of Raman scattering is that it is based on the use of visible light, which has frequencies much higher than the molecular vibrational frequencies.

For microscopists, this is good news. Visible excitation confers on Raman microscopy a level of spatial resolution much higher than that associated with infrared absorption vibrational microscopy. Raman microspectroscopy has found many applications in the microscopic characterization of solid-state materials. Raman microscopy has also attracted the attention of biologists and biomedical researchers. Their interest in the technique is motivated by its chemical selectivity, which makes it unnecessary to stain samples with artificial agents [Fig. 1(c)].

A significant limitation

Yet despite its unique capabilities, spontaneous Raman imaging has found limited application in the study of live cells. This can be explained by a drawback associated with Raman scattering: it is a very weak optical effect. The weakness of the inelastic scattering phenomenon was recognized by Raman himself. Raman and Krishnan, in their 1928 letter to *Nature*, wrote that the fact that "the effect is a true scattering and not a fluorescence is indicated in the first place by its feebleness in comparison with the ordinary scattering."[1]

To express the concept in numbers, in the condensed phase the "excessive feebleness" that Raman described translates into one Raman photon scattered out of 10^{10} incident photons propagating through 1 μm of the Raman active sample. To visualize a typical biological specimen with Raman contrast requires long image acquisition times, even when intense laser beams are used. It is obvious

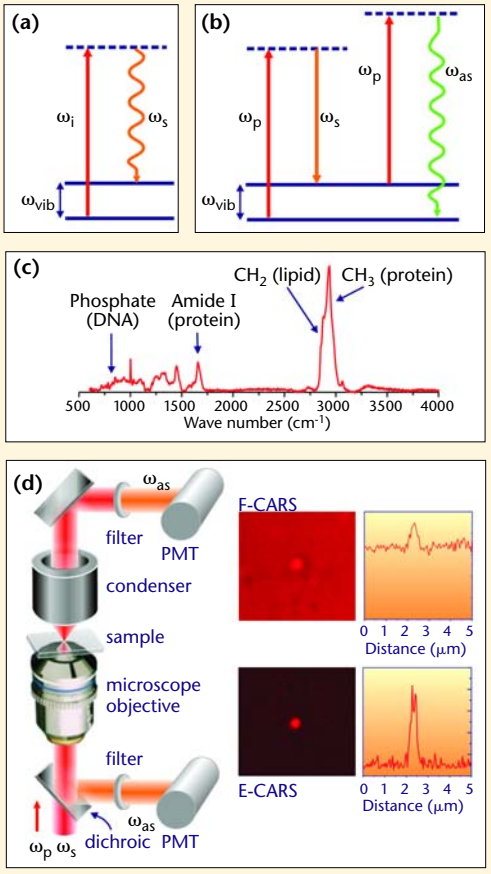


Figure 1. Raman and CARS microscopy. (a) Spontaneous Raman energy diagram based on inelastic scattering of incident radiation resulting in a red-shifted (ω_s, Stokes) emission. (b) CARS energy diagram in which the vibrational oscillators are actively driven at $\omega_p - \omega_s$. Upon further interaction, a blue-shifted (ω_{as}, anti-Stokes) photon is emitted. (c) Raman spectrum of dried HeLa cells showing the wealth of molecular information contained in the vibrational spectrum. (d) Basic layout of CARS microscope with a collinear excitation geometry. Either the sample or the beams are scanned. Signal is detected simultaneously in the forward (F-CARS) and the backward (E-CARS) direction, yielding different contrast mechanisms. F-CARS signals are strong and are accompanied by a nonresonant background from the medium while E-CARS signals are weak and the background is suppressed. This is illustrated by the images of a 0.2 μm polystyrene bead in agarose taken at the 3,050 cm^{-1} aromatic CH vibration of polystyrene.

that long image acquisition times severely complicate the visualization of living specimens.

Fortunately, there are several techniques available to boost Raman signals. One uses the strong enhancement of the Raman signal generated at nanometric metallic objects. Surface enhanced Raman scattering (SERS), an example of this approach, has made possible increases of more than 15 orders of magnitude in signal levels. A drawback of the method is that it requires close contact of the sample with a metal surface and are thus not easily applied to three-dimensional (3D) biological samples.

Another approach is based on coherent Raman effects, in which molecular vibrations are set in motion through stimulated excitation by laser beams. Unlike the situation with spontaneous and incoherent scattering, the Raman oscillators are driven coherently by the strong optical fields. In this manner it is possible to generate strong vibrational signals that have a nonlinear dependence on the excitation intensity; in this way, we have entered the realm of coherent nonlinear Raman spectroscopy. Nobel laureate Nicolaas Bloembergen summarized the prospects of the field in 1978, saying: "As spontaneous Raman spectroscopy has blossomed and grown during one half-century, it may be predicted with some confidence that coherent nonlinear Raman spectroscopy will yield many new results in the next half-century."[2]

Coherent anti-Stokes Raman scattering, a member of the family of coherent nonlinear Raman techniques, was first demonstrated in 1965 by Maker and Terhune at Ford Motor Company.[3] In the decade that followed, the technique went by different names until Begley et al., with reference to its place of inception, named it coherent anti-Stokes Raman spectroscopy, or CARS for short.[4] In CARS, a pump (ω_p) and a Stokes (ω_s) beam drive the molecular oscillators at the difference frequency $\omega_p - \omega_s$. Under their combined action, a vibrational coherence—a coherent superposition of the ground state and the first excited vibrational state—is created. Through further interaction with the pump beam, the vibrational coherence can be converted into a detectable signal at frequency $2\omega_p - \omega_s$ [Fig. 1(b)].

The phase-matching legacy

As a direct result of the active driving of the molecular oscillators and the coherent build-up of the signal, CARS typically offers conversion efficiencies 10^5 higher than those associated with conventional Raman scattering. CARS emission is also highly directional, a factor that facilitates efficient collection of the signal. Overlap with the red-shifted fluorescence background can be avoided because the CARS signal is blue-shifted with respect to the incident beams. Researchers quickly recognized that such advantages over spontaneous Raman scattering dramatically improved vibrational spectroscopic measurements. CARS has been extensively used for the analysis of molecular gases, solids and liquids.

To ensure a high signal in CARS, the signal waves must propagate in step with the incident driving fields so that efficient coherent buildup of the signal occurs. In dispersive media, however, waves of different colors have different propagation velocities, which can introduce a mismatch between their respective phases. As a result, waves can destructively interfere and in this case much lower signals are obtained. For this reason, to compensate for the phase-mismatch, a special noncollinear overlap of the incident beams is usually chosen. The concept of phase-matching has consequently become synonymous with nonlinear coherent spectroscopy.

Duncan et al. anticipated that CARS microscopy might overcome the shortcomings of the vibrational approach to biological imaging. In 1982, they constructed the first CARS microscope by using picosecond visible dye lasers as the light source.[5] They adopted a noncollinear excitation geometry similar to the phase-matching condition in conventional CARS spectroscopy. And indeed, they obtained images with strong CARS signals from samples such as onion skin

cells. But along with the advantages, some problems were encountered. Spatial resolution—particularly along the axial dimension—was low. In addition, the nonresonant background, an inherent electronic contribution to the CARS signal which is independent of the Raman shift, overwhelmed the vibrational contrast.

After the initial demonstration, the avenue CARS had opened for microscopy remained unexplored for almost 18 years. It was only in 1999 that Zumbusch et al. resuscitated the field by circumventing several of the limitations that had been encountered earlier on.[6] First, they abandoned the legacy of non-collinear phase-matching because they realized that along the propagation direction of the beams, there is virtually no phase-mismatch within the tight laser focus of up to several micrometers. They combined the beams in a simple collinear fashion and focused them using a high numerical aperture objective lens. It is important to note that nonlinear signal generation only occurs at the focal point where the excitation density is the highest, which results in the 3D sectioning capability associated with CARS microscopy (Fig. 2). Second, near-infrared light sources were employed by Zumbusch et al. At these wavelengths, far from ultraviolet (UV) electronic transitions, the nonresonant background is much weaker than in the case of visible excitation. These improvements enabled the first recording of high quality CARS images of live cells.

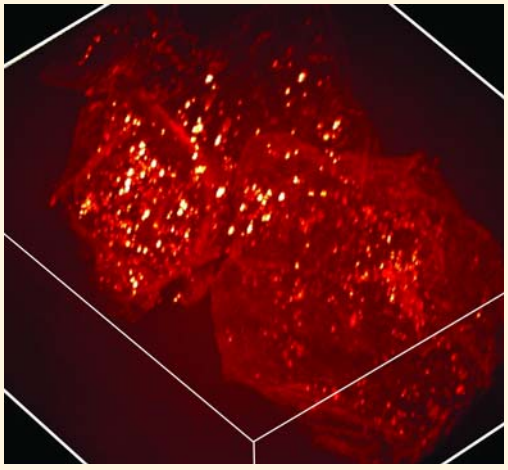
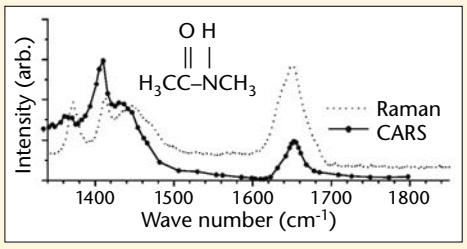
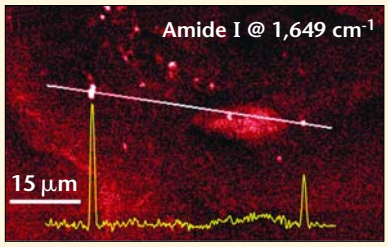


Figure 2. CARS microscopy has 3D sectioning capability. Shown is a 3D reconstruction from a stack of sectioned xy images of three epithelial cells. Contrast arises from the CH_2 stretching vibration at 2,845 cm^{-1}, showing lipid droplets and the cells' membranes. The dimensions of the box are 150 x 150 x 40 μm.





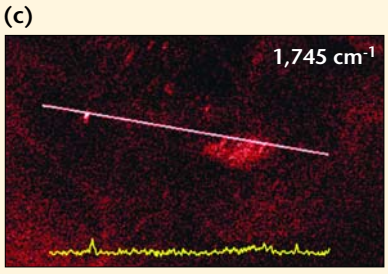


Figure 3. CARS imaging at different Raman shifts. (a) Raman and polarization CARS spectrum of N-methylacetamide, an amide that serves as a model system for polypeptides. (b) Protein distribution in an epithelial cell visualized through the amide I vibration at 1,649 cm^{-1} using polarization CARS. (c) Off resonance, at 1,745 cm^{-1}, the protein contrast disappears. [Adapted from Cheng et al., Opt. Lett. **26**, 1341 (2001).]

A continuous evolution

The advances made in 1999 opened the door to further developments in CARS microscopy. An important improvement was the use of high repetition rate, near infrared picosecond light sources. Conceptually, many nonlinear processes are optimized by use of short pulses of high peak power while the average power is kept low to limit heating of the sample. This is why femtosecond pulse trains are commonly used in multiphoton fluorescence microscopy. In CARS microscopy, however, the spectra of femtosecond pulses (~ hundreds of cm^{-1}) are much wider than the Raman bands (~10 cm^{-1}). Poor spectral matching implies that many spectral components will contribute to the nonresonant background instead of generating resonant signal. Cheng et al. showed that a much higher signal-to-background ratio is obtained if 2-3 ps pulses (~10 cm^{1}) are used instead.[7]

CARS microscopy as a field has been propelled by the incessant development of appropriate ultrafast light sources more than by anything else. The use of two picosecond Ti:sapphire lasers gave a boost to CARS microscopy by providing a cheaper light source with ease of operation. Recently, the technology of actively synchronizing two independent mode-locked lasers has been marketed by Coherent Lasers (Santa Clara, Calif.) and Spectra-Physics (Mountain View, Calif.). Even tighter synchronization was achieved through a collaboration between Jun Ye's group at JILA and by our group at Harvard; this development yielded crisp CARS images free of temporal pulse jitter. The use of synchronously pumped optical parametric oscillators (OPO) completely eliminates the problem of temporal pulse jitter. Commercial near-infrared picosecond OPO solutions based on periodically poled nonlinear crystals pumped by a mode-locked laser with semiconductor saturable absorber mirrors have recently been introduced by APE (Berlin) and by High Q Lasers (Hohenems, Austria). These new technologies will probably elevate

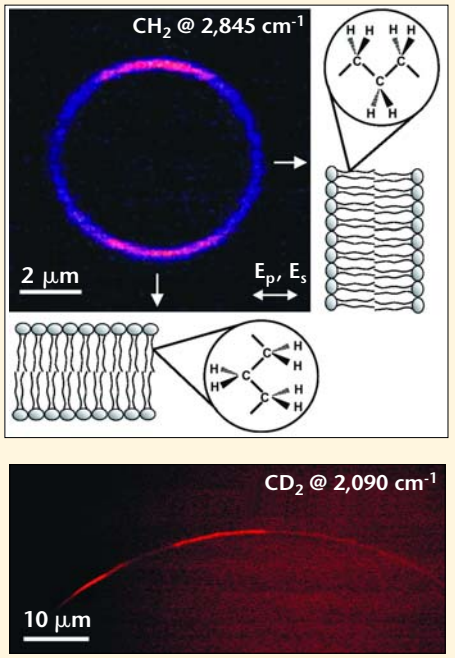


Figure 4. (a) Visualization of the membrane of a lysed erythrocyte cell through the CH_2 stretching vibration at 2,845 cm^{-1}. The brighter and darker parts of the ring result from the polarization of the beams being parallel or perpendicular to the CH_2 polarizable bonds, respectively. (b) Unilamellar vesicle composed of a binary mixture of phospholipids. Phase segregated lipid domains are observed by making use of deuterated and non-deuterated lipids and tuning to the CD_2 vibrational band at 2,090 cm^{-1}. [Adapted from Potma et al., J. Raman Spectosc., **34**, 642 (2003).]

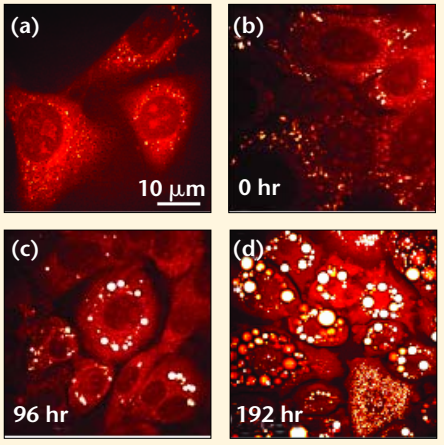


Figure 5. Growth of lipid droplets in live cells. Lipid contrast at 2,845 cm^{-1} was used to visualize lipids without staining. (a) Cells before addition of drugs. (b) Additon of drugs when the cell distribution is dense. (c) Differentiation of cells after 96 hours in insulin-rich medium. The lipid droplets have grown in size. (d) Terminal differentiation after 192 hours. Very large lipid droplets are observed. [Adapted from Nan et al., J. Lipid Res. **44**, 2202 (2003).]

CARS imaging of biological materials to the next level.

Thanks to advances in laser technology, CARS images of living cells have been acquired with high sensitivity and rapid scanning speeds. Images with Raman contrast can now be obtained noninvasively at video rate instead of hours, an improvement of more than four orders of magnitude compared to the work carried out in 1999.

For some applications however, the nonresonant background that remains may overshadow weak vibrational signals. Several techniques have been developed to reduce these unwanted signal contributions. For example, polarization sensitive detection, which exploits polarization differences between resonant and nonresonant signal contributions, can be employed to fully suppress the nonresonant background.[7]

Another leap forward was the introduction of the Epi-CARS microscope, which detects signals in the backward direction. Conventional wisdom states that because the interacting waves are phase matched only in the forward direction, no signal can be observed in the reverse direction. Although this is indeed true for homogeneous samples such as those used in regular spectroscopy, it no longer holds for samples that display variation of optical properties on the micrometer scale. Volkmer et al. realized that waves emitted in the backward direction by subwavelength structures still constructively interfere.[8] This mechanism is explained in the box on page 45. Epi-CARS makes microscopy easy: the setup is simple and permits imaging of thick samples with limited transmission. The Epi-CARS microscope also suppresses the nonresonant background from the aqueous medium, which enables detection of small structures against a large nonresonant background. Today's CARS microscope, equipped with an Epi-detection module or polarization sensitive detection, has matured into a powerful tool for unveiling cellular dynamics with chemical selectivity.

Seeing the invisible

Most cellular structures do not absorb or emit visible light, a fact which makes them literally invisible to researchers. Specially engineered fluorescent tags are commonly used to enable observation of specific molecular compounds. But alongside the advantages offered by fluorescence techniques, toxicity and photobleaching of the dyes are often a concern.

Thanks to the high sensitivity of CARS, molecules in live cells can now be made visible without labeling, simply by use of their intrinsic vibrational signatures. For instance, cellular protein distributions can be mapped out by tuning the Raman shift to the amide I vibration at 1,649 cm^{-1} which is characteristic of polypeptides. In Fig. 3, polarization sensitive detection was used to detect CARS signals from dense protein clusters in cultured fibroblast cells. When the Raman shift is tuned off-resonance, the sharp contrast from the protein disappears, confirming the chemical contrast obtained.

The strongest cellular CARS signals are observed from lipids. Fatty acids are very rich in CH_2 modes, which give them a prominent feature at 2,845 cm^{-1}. The signal is strong enough to resolve natural single lipid bilayers. In Fig. 4(a), the membrane of a lysed red blood cell is shown. The single bilayer sensitivity of CARS can be used to study the segregation of lipids into domains with different thermodynamic phases. Figure 4(b)

displays part of a unilamellar vesicle composed of two types of lipid residing in different phases. By making use of one deuterated component, a sharp contrast is observed when tuning the Raman shift to the symmetric CD_2 vibration of the lipid.

Bright signals from lipids are observed in cells; this is especially true for lipid droplets that contain a high concentration of triglycerides. This signature can be used to track the growth and motion of the droplets in live cells (Fig. 5). The rapid scanning capability of CARS microscopy allows for study of the dynamics of motor-protein assisted motion of lipid droplets along microtubules. Cells can be repeatedly imaged in CARS without the fading problems caused by photobleaching. This is in contrast with what occurs in the case of fluorescence microscopy.

In addition to cell structures and solutes, the surrounding water host can also be made to light up under the CARS microscope. By use of the OH-stretch vibration of water, cellular hydrodynamics have been studied with subsecond time resolution.[9] The resonant CARS signal from water has also been used to determine the orientation of the water molecules in the hydration layer at the surface of lipid membranes.[10]

Future light

CARS microscopy is useful for more than imaging unstained cells in culture dishes. The ability to noninvasively probe biomaterials with chemical selectivity offers attractive solutions for medical tissue imaging. In collaboration with Charles Lin's group at Massachusetts General Hospital, we recently began video-rate Epi-CARS imaging of skin tissue in vivo. The excellent contrast obtained has opened doors for the entry of CARS into hospitals.

Along with rapid imaging of cells and tissues, the marriage between CARS and microscopy has spurred other research activities as well. For instance, CARS excitation has recently been combined with near field optical methods, pushing the spatial resolution down to less than 50 nm.[11] Yet another focus is the development of wideband CARS analogues to Raman microspectrometry to measure CARS spectra at selected sites in the sample.[12, 13]

From the years of dormancy that followed its inception in 1982, CARS microscopy has made an impressive comeback. It is now recogized as a powerful tool for the investigation of biological specimens. The current state-of-the-art CARS microscope is capable of imaging unstained living cells with a high vibrational sensitivity at video rates. The demands of emerging applications in cellular biology and biomedical science are likely to push the technology even further. With potential clinical applications in sight, the reach of the Raman effect has extended quite a bit farther than C. V. Raman himself could ever have predicted.

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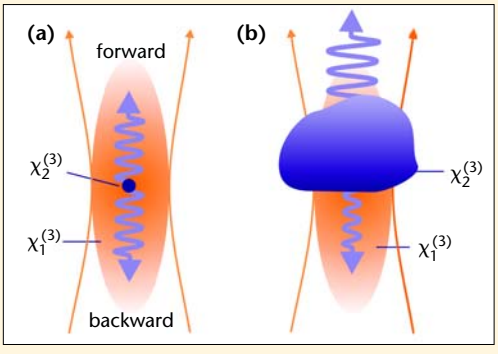
Phase Matching Revisited

Studies of signal generation in Epi-CARS microscopy have added a new dimension to the conventional interpretation of phase matching. In the backward direction, the waves are heavily phase mismatched, which results in destructive interference of the signal. Yet certain sample geometries lead to detectable signals in the Epi direction. Two important cases are sketched below. In (a), a small particle with a nonlinear susceptibility $\chi^{(3)}$ different from its surroundings in the focal volume is shown; because of its subwavelength dimensions, destructive interference is incomplete and radiation is emitted in both the forward and backward directions. This mechanism allows small features inside cells to be detected against a large nonresonant background.



A second mechanism that leads to a signal in the Epi-direction is illustrated in (b): a thick object exhibits a different $\chi^{(3)}$ in the upper half of the focal volume compared to the lower half. Because of this breaking of the $\chi^{(3)}$ symmetry in focus, destructive interference is incomplete; this results in detectable signals in the Epi-direction. This mechanism can be understood as back reflection on the interface introduced by the $\chi^{(3)}$ difference. Such reflection is large when the $\chi^{(3)}$ of the object is vibrationally resonant. Resonant $\chi^{(3)}$ back reflection facilitates imaging of thick objects such as those encountered in tissues. [See also Cheng et al., J. Opt. Soc. Am. B **19**, 1363 (2002).]