CARS microscope designed for video-rate chemical imaging of live tissue

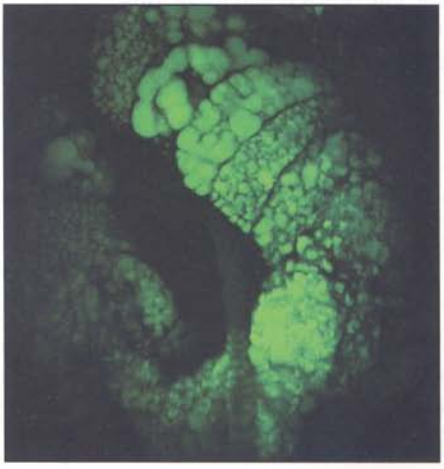
by Kevin Robinson, Contributing Editor

Coherent anti-Stokes Raman scattering could one day be used to study and diagnose diseases, including cancer and atherosclerosis, and skin disorders.

The ability to combine an image of a cell with information about its chemical components is an important quest for research. Medical imaging has made significant advances in the past decade. In the US, MRI and CT imaging are routine at even the smallest hospitals, and a combination of PET technology and CT provides clinicians with images of anatomy along with information about tumor composition and spread.

Several techniques can deliver a degree of chemical sensitivity, including MRI with contrast agents, fluorescence imaging with various fluorescent labels, and Raman and infrared microscopy. Unfortunately, MRI is not particularly sensitive and is limited by spatial and temporal resolution. Fluorescent labels don't have these limitations, but few of them are approved for human use. Researchers have used confocal Raman microscopy for tissue imaging, but the method produces weak signals that require long data acquisition times, high laser powers or both. Infrared microscopy has low spatial resolution because of the long wavelengths involved.

As a result, surgical biopsy remains the gold standard for clinical tissue analysis. However, in most cases, biopsies sample only questionable tissue that requires sectioning and staining before it can be evaluated. Using an imaging technique that is noninvasive and chemically sensitive could eliminate several steps, which would speed up the process for diagnosing diseases. In addition, in situ optical imaging would allow doctors to sample a much larger area if necessary.



Coherent anti-Stokes Raman scattering microscopy can create chemically sensitive images on the cellular scale. This 3-D reconstruction of a mouse sebaceous gland was imaged by tuning the microscope to CH_2 symmetric stretching vibration, which is abundant in lipids. The image reveals that the crescent-shaped sebaceous gland surrounds a hair shaft and comprises multiple cells, each filled with numerous micron-size CH_2-rich sebum reservoirs. Images courtesy of Conor L. Evans.

Coherent anti-Stokes Raman scattering (CARS) is a nonlinear optical process related to Raman scattering. Every molecule has Raman vibrational bands, frequencies that, if excited, will cause the molecule to emit a Raman signal. To create a CARS signal, researchers probe a molecule with two specially tuned laser beams — the "Stokes" and the "pump." Because the two lasers are not at the same frequency, they create a third "beat" frequency. Researchers tune the beat frequency so that it matches the Raman vibrational frequency, causing the molecule in question to emit a strong anti-Stokes signal, which is orders of magnitude stronger than a conventional Raman signal.

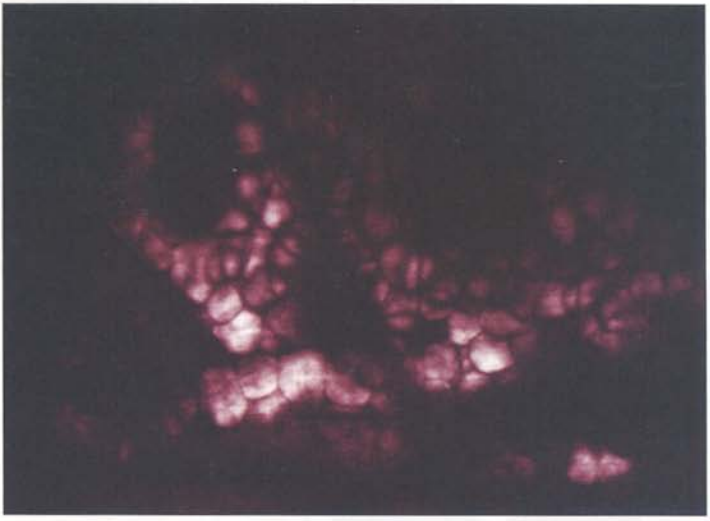
Because the CARS process is nonlinear, the signal is generated only in the laser focal volume, created in this instance by a high numerical aperture water immersion lens from Olympus. To generate an image, researchers raster scan the spot over the tissue. As with confocal imaging, optical sectioning is possible by adjusting the depth of the spot in the sample.

X. Sunney Xie is a researcher in a lab at Harvard University in Cambridge Mass., that has been working on CARS microscopy for several years. He said that the technique has matured into a powerful method for imaging live cells. In particular, his group has successfully used an epidetection scheme. Epidetection involves detecting the backscattered CARS signal, which propagates opposite to the direction of the incoming laser beams. He said the system can use the epi signal, which is only a small portion of the forward-scattered signal, and get very sharp contrast for small objects in a living cell against the cell's background.

For tissue imaging, the conventional wisdom held that CARS signals from a sizable object in a tissue propagate in the forward direction — with the direction

of the incoming laser beams. According to Conor L. Evans, the lead author of a paper on CARS microscopy in the Nov. 15 issue of *PNAS*, the method seemed unlikely to be useful for imaging live tissue because it's rather difficult to put a detector in the forward direction. However, the work of Evans and colleagues Eric O. Potma, Mehron Puoris'haag, Daniel Côté, and principal investigators Xie and Charles P. Lin at Massachusetts General Hospital in Boston, showed that using epidetection and samples of thick tissue, they could detect a backscattered CARS signal that was much higher than expected.

The team's work determined that the large CARS signal is due to highly efficient backscattering of the signal in tissue greater than 150 µm thick. This, however, doesn't affect the resolution of the image. "The signal is not distorted because it is still generated only at the focal spot of the laser beams," Evans explained. "Once generated, CARS photons leave the focal spot, propagate in the forward direction and can then be backscattered due to multiple scattering events. Unlike images taken with cameras, CARS microscopy images are built up by detecting the intensity of the CARS signal at a particular point in the sample."



The CARS microscope allowed the researchers to view small adipocytes of the subcutaneous layer of mouse skin. These are found at a depth of nearly 100 µm in skin.

New laser source

The combination of a reliable new laser source and a video-rate microscope made this imaging power possible. Video-rate imaging is crucial to the group's goal of producing a clinical instrument. "We want to acquire images quickly because movements of the animal or patient blur the images," Xie said. With video-rate imaging, patients don't have to hold perfectly still. "Even more importantly, the video rate allows study of fast dynamic processes, such as blood flow," he added.

To allow video-rate CARS scanning, the researchers modified a Vivascope confocal microscope by adding a polygon mirror from Lincoln Laser Co. of Phoenix. They used a 10-W Nd:vanadate laser from High-Q Laser Production GmbH in Hohenems, Austria, operating at 1064 nm with a 7-ps, 76-MHz pulse train created by mode-locking the laser with a semiconductor saturable absorber mirror.

They split 10 percent of the beam off to use as the Stokes beam. The rest of the beam pumps an optical parametric oscillator (OPO) from APE of Berlin. The OPO has a periodically poled KTP crystal that allows tuning in the 1560- to 1860-nm range. By frequency doubling the beam, they can tune from 780 to 930 nm. The pump beam also is adjusted for temporal overlap using a delay stage before it is combined with the Stokes beam and sent to the microscope.

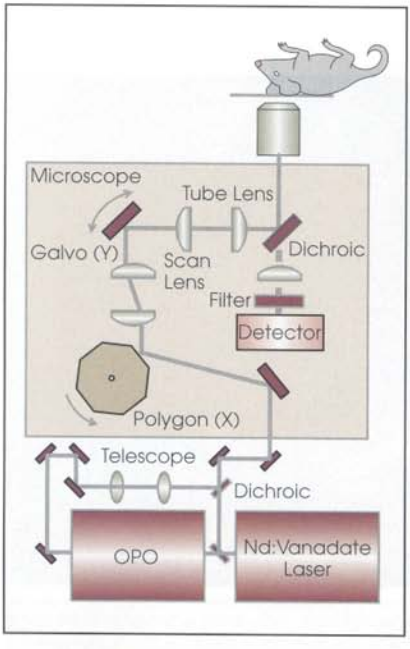
Xie said that the laser system is far more stable than a previous system that used two mode-locked Ti:sapphire lasers for the pump and Stokes beams. "The jittering between the two pulse trains was large, resulting in instability of the CARS signal," he explained. "We needed a stringent environment with air conditioning and a floating optical table in order to maintain stability of operation." The OPO source keeps the two beams perfectly synchronized optically even without air conditioning or a floating table. This stability, Xie said, is key to making such a system work in a hospital.

For Y-axis scanning, the investigators used a galvanometric mirror from General Scanning. To focus the combined Stokes and pump beams to a diffraction-limited spot, they used a 60×, 1.2-NA objective from Olympus. They combined the objective with a 40-mm focal length tube lens. To filter out the excitation light, they placed a dichroic mirror from Chroma Technology in the optical train and passed the signal through three bandpass filters centered around the anti-Stokes wavelength. For detection, they used a Hamamatsu red-sensitive photomultiplier tube, whose analog signal was digitized with a digital conversion board from National Instruments. The group also used a manual 3-D stage supplied by Newport for moving the subject to select an area for sampling.

Better contrast in vivo

The scientists conducted in vivo imaging of skin tissue of a mouse ear using the CARS microscope. Scanning from 100 µm up to 20 µm, they observed the variety of lipids present in the layers. Their images correlate the structure of the skin tissue with lipids, ceramides, cell nuclei and fat-producing cells. They could distinguish between the sebaceous glands and the adipocytes, which have similar spectra in some ranges.

In an additional experiment, they applied mineral oil to the skin of live mice



The combination of a new stable laser source and a video-rate confocal scanning microscope head allows researchers to create a video-rate coherent anti-Stokes Raman microscope, which they hope will one day offer doctors and hospitals a noninvasive way to diagnose a variety of disorders, including cancer. Reprinted with permission of PNAS.

and tuned the CARS microscope to the oil's CH_2 stretch vibration. Imaging the area over 20 minutes, they watched as the mineral oil penetrated the stratum corneum but did not go deeper than the epidermis.

This result has sparked interest in the technique by skin care companies looking to evaluate their products, Xie said. Similarly, CARS microscopy can be used to monitor the tissue penetration and cellular uptake of topically applied drugs.

Lin added that they are particularly excited about using the technique to help develop better and safer therapeutic agents.

"CARS microscopy provides a route to label-free chemical imaging of tissue, which would be beneficial for almost all biomedical imaging studies and techniques," Evans said. "The most obvious application is dermatology. Since CARS microscopy is extremely sensitive to lipids and fats, there are applications in cardiovascular health, such as the study of atherosclerosis."

These epidetection experiments also serve as a proof-of-principle for a CARS endoscope, Xie said. The endoscope would have simultaneous excitation and detection by optical fibers and could be used for surgical intervention. In fact, his group has been working on CARS endoscopy. Meanwhile, it is using CARS microscopy to study cellular lipid metabolism as well as improving the technique's sensitivity to image drug distribution in cells and tissues. □