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Laser tricks without labels

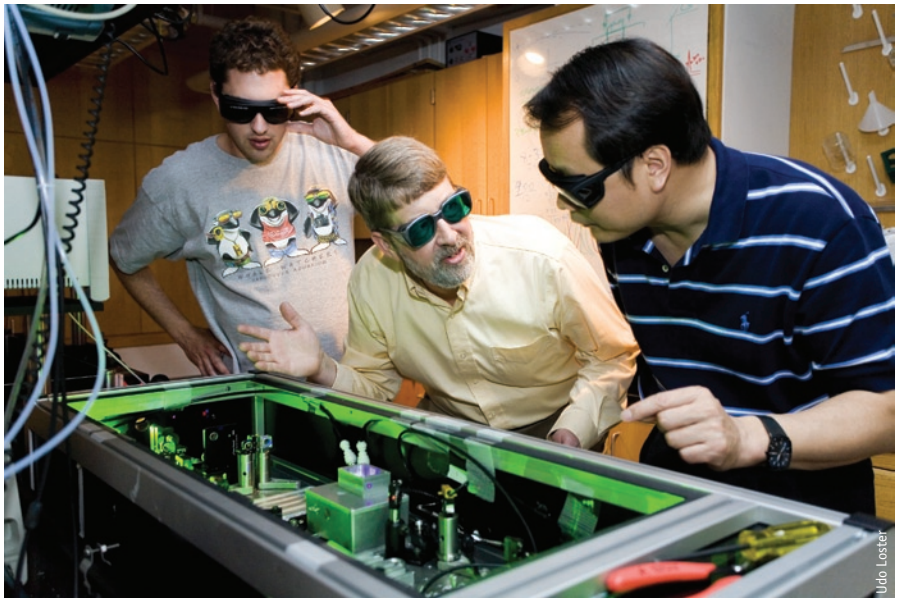
Monya Baker

Nonlinear optical microscopy lets researchers see chemical composition in living cells and organisms.

A couple years ago, Annika Enejder confronted confusing results from her studies of lipid storage in the roundworm *Caenorhabditis elegans*. Fluorescence microscopy images very clearly showed a decrease in signal from lipid droplets when the worms were treated with statins, a widely prescribed class of cholesterol-lowering drugs. But simultaneous experiments using another type of microscopy, which visualizes lipids directly, showed no such thing. In fact, coherent anti-Stokes Raman scattering (CARS) microscopy identified lipid droplets where they could not be observed with fluorescence techniques.

What had happened was that worms fed with the commonly used fluorescent dye Nile red processed it as a toxin: instead of being localized to lipid droplets, the dye was sequestered in intestinal lysozome-like granules interspersed among lipid droplets. In fact, the dye can be misleading in other ways: statins themselves seem to affect either its staining or fluorescence. "There are so many artifacts that you have to take into account when using fluorophores," says Enejder, a scientist at the Chalmers University of Technology in Göteborg, Sweden.

No one can deny the power of fluorescent probes and molecular stains to see the inner workings of cells, but such labels have considerable drawbacks. Delivering labels can be a problem, particularly for whole organisms. Some labels work only on dead cells; others damage cells or perturb the very processes they are intended to study. Label-free microscopy offers a way to investigate living cells while eliminating a slew of possible artifacts. Though some techniques rely on endogenous fluorophores (**Box 1**), most eschew fluorescence all together, along with the well-known problem of photobleaching.



Harvard University's Brian Saar, Gary Holtom and Sunney Xie (left to right) are developing new techniques and applications of nonlinear microscopy.

Instead of detecting photons emitted from excited fluorophores, these alternate techniques detect subtle changes in light as it is absorbed or altered by biological samples, relying on the nonlinear optical phenomena observed when high-intensity light moves through matter. In essence, pulses of laser light can be used to 'see' chemical composition: the C-H bonds of lipids, the amide bonds of proteins, the reduced or oxidized states of certain biomolecules, the regularly repeating units of microtubules or collagen.

Of course such techniques have their own limitations: whereas fluorescence labeling can often allow discrimination of single molecules, label-free techniques are less sensitive and specific. All but the most common substituents tend to be hidden in signals generated from a few abundant species. "What is nice is that you don't need any labeling; you can just start imaging," explains Kees Jalink, a biophysicist at the Netherlands Cancer Institute, "but what is not so nice is that the signal is weak, you need a lot of power to irradiate a cell and may only get the coarsest of details."

Many modes of nonlinearity

In addition to CARS, some of the other techniques that can be done without adding labels include two-photon absorption, second-harmonic generation (SHG) and stimulated Raman scattering, each with its own detection capabilities and requirements (**Table 1**). The spread of such technologies to biologists, however, does not occur at light speed. Expensive lasers must be coupled to microscopes; short pulses of light must be precisely aimed, coordinated

BOX 1 OPTICS FOR MEDICINE

Researchers like Watt W. Webb at Cornell University are using fluorophores that are naturally present in unmanipulated cells. He and colleagues at several institutions are comparing diseased and normal tissues throughout the body, looking for differences in both fluorescence spectra and in the structures of tissues revealed by imaging with endogenous fluorophores. One advantage of label-free imaging techniques for medicine is that there is no need to deliver foreign molecules into people, and so no expensive requirement to convince regulators that such molecules are likely to be harmless. Results so far are encouraging, Webb says: "they look amazingly diagnostic."

"The problem is almost nothing interesting in you fluoresces," says Warren S. Warren of Duke University, who has developed an alternate technique, called two-photon absorption. Instead of detecting fluorescence, he hunts for the much weaker signal of absorption, light that is taken out of the laser pulse. This can distinguish between, say, different forms of hemoglobin and melanin and be useful for evaluating whether a mole is benign or malignant. Sunney Xie of Harvard University has developed a similar technique called stimulated emission microscopy.

Webb, Warren and Xie are some of a growing number of physicists who hope that pathologists working for hospitals across the world will soon start putting away their stains and swapping out their light microscopes for ones based on more sophisticated optics. Xie, Webb and others are independently working on new types of endoscopes for *in vivo* diagnostics that rely on advanced optics. The debate is over which techniques will win out. Webb, who introduced multiphoton fluorescence microscopy 20 years ago, believes that neither absorption nor light scattering provides sufficiently distinct signals compared with the potential for endogenous fluorescent techniques, and Warren makes a similar argument in favor of absorption. What the researchers agree on is that methods using unmanipulated, living cells can provide better data than stained, fixed tissue slices. "All of these methods give the pathologist a better tool for diagnosis," says Warren; "that's low-lying fruit to get into clinical practice pretty quickly."

and shaped; and detection devices must be optimized to home in on signals and discard background. "It's not easy to do the assembly without a lot of expertise; these instruments are pretty finicky," says Robin F.B. Turner, who holds positions in the chemistry and engineering departments at The University of British Columbia. And just setting up the equipment is not enough. "You have to make adjustments on a day-to-day basis," adds Turner.

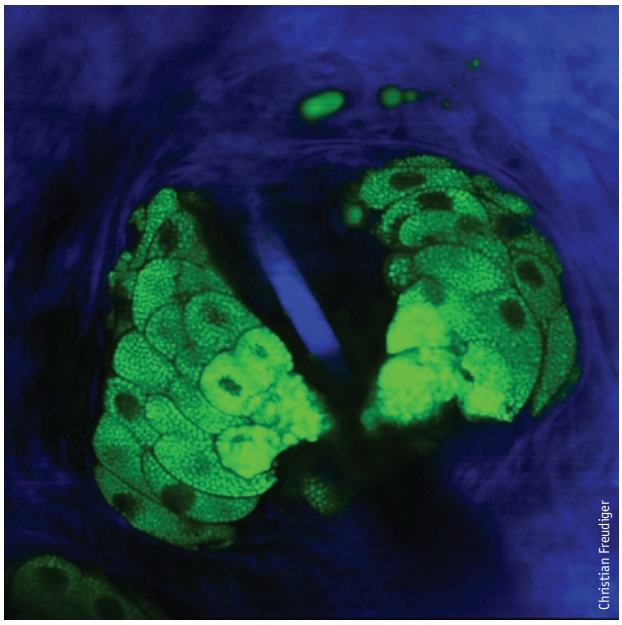
Turner says he has good reason for tackling these techniques: he wants to know how stem cells' composition changes as they differentiate into other types of cells, and there is no better way to answer his questions. "The reason we use Raman and CARS is because we can do that nondestructively," he says. Other measurements destroy cells, creating isolated snapshots of different cells at given points in time; such data are not particularly useful for heterogeneous cultures containing spontaneously differentiating cells, Turner says, adding, "we want to follow cells as they grow."

Such advantages apply to organisms as well. Harvard University's Gary Ruvkun, for example, is conducting RNA interference screens in *C. elegans* to probe thousands of genes for their role in lipidogenesis and using a microscopy technique called stimulated Raman scattering (SRS) to monitor the results.

Ruvkun's collaborator is Sunney Xie, also of Harvard University. About ten years ago, Xie made a big splash by describing CARS microscopy, a technique that boosts signals from a phenomenon known as spontaneous Raman scattering, in which chemical bonds in a sample shift the wavelength of light passing through it[1]. Early implementations of Raman microscopy required powerful lasers and sometimes required exposure times as a long as a day. Xie and colleagues showed CARS could work in living cells. The feeble Raman signal coming from cells could be amplified

by using two laser beams whose frequency difference is tuned to the vibrational frequency of the molecular bonds to be imaged. "It provides orders of magnitude more sensitivity than spontaneous Raman," says Xie. But CARS has drawbacks. It typically focuses on just one part of the broad Raman spectrum at a time, restricting the amount of information collected; it also produces a high background signal. In practical terms, these limitations mean that most applications of CARS involve the detection of lipids, whose high concentrations of carbon-hydrogen bonds create a strong characteristic signal.

Xie's enthusiasm has shifted to SRS, which Xie developed with lab members Wei Min and Christian Freudiger and reported late in 2008 (ref. 2). "In CARS, the peak is shifted," explains Xie; "that means we cannot use the wealth of Raman literature to assign chemical species." In contrast, he says, SRS eliminates the background signal of CARS by using an incredibly fast and precise modulation of the lasers. Not only does this produce the same spectrum as that obtained in classical Raman spectroscopy, but its signal is orders of magnitude stronger and requires much less time to detect than non-amplified Raman signals. Even better, Xie says, the signal from SRS rises linearly with the number of vibrating bonds, making the technique quantitative. Applications include real-time observations: the absorption of retinoic acid into the skin, for example, has applications for drugs and cosmetics. The ability to monitor how either acids or enzymes remove lignin from plant cell walls could be used to produce biofuels more efficiently.



A lipid-rich sebaceous gland wraps around a protein rich-hair. Image taken with stimulated Raman scattering, showing lipids in green and proteins in blue.

These proof-of-principle studies have grown from Xie's collaborations with researchers at Pfizer and Harvard University. Xie goes so far as to predict that SRS will supersede CARS, but other researchers are not so sure. SRS requires mixing and understanding signals from multiple light sources and overlapping spectra can be hard to deconvolute. Turner says he has tried using SRS to look at nucleic acids in solution and decided to stick with the techniques already used in his lab. With those, he can distinguish RNA from DNA in cells. Whereas a Raman microscope might be slow, he says, "it would be quite an undertaking to apply SRS and use it to advance our knowledge as fast as plain-Jane Raman."

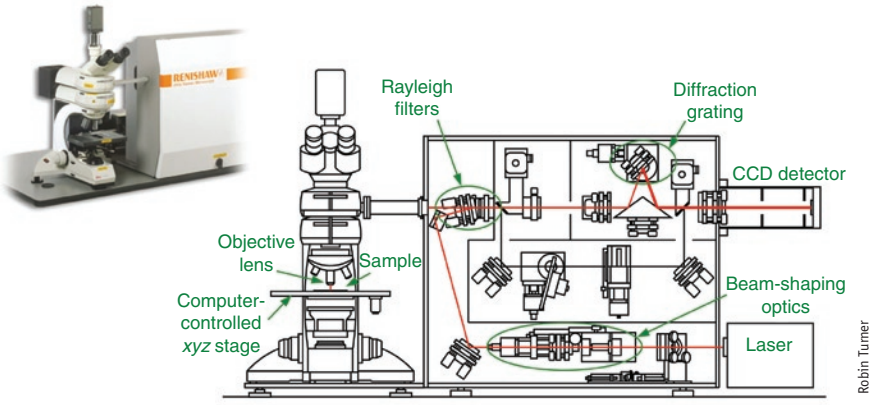
Something to buy, something to share

Xie predicts SRS adoption will spread once it has been built into a commercially available system, an advance that he believes will happen later this year; both Zeiss and Leica are reported to have licensed the technology last year. But, as an example from fluorescence microscopy shows, the spread of a technology can be slow. The first commercial multiphoton microscope was introduced in 1996; a survey in 2003 found that 66% of biological research studies using multiphoton microscopy still used custom-built systems[3]. Today, commercial multiphoton microscopes are commonplace.

In October 2009 Olympus announced plans to offer a femtoCARS module that can be added to multiphoton microscopes, ten years after Xie's publication. In January of 2010, Newport Corporation introduced its wavelength-extension unit that can be attached to a laser and multiphoton microscope to support CARS, SHG and other forms of imaging. Leica will reportedly roll out its own prod-

Table 1 | Selected label-free nonlinear optical techniques

Microscopy technique	Excitation method	Signal measured	Examples of commonly visualized molecules
Fourier transform infrared spectroscopy[4]	Broad-spectrum infrared radiation	Absorption	Nucleic acids, carbohydrates, lipids and proteins
Spontaneous Raman[5]	Continuous wave, monochromatic laser beam	Shifts in frequency of inelastically scattered light	Nucleic acids, carbohydrates, lipids and proteins
Coherent anti-Stokes Raman[6]	Synchronized laser pulses of different wavelengths	Light at a frequency higher than the two incident beams	Lipids and water
Stimulated Raman scattering[2]	Synchronized laser pulses of different wavelengths	Intensity change of one of the two incident beams	Lipids, water, proteins and small-molecule drugs
Second harmonic generation[7]	Pulsed light of a single frequency	Scattered light of double the excitation frequency	Collagen, microtubles and regular protein fibers
Two-photon absorption[8]	Shaped laser pulses of the different wavelengths	Absorption	Different states of hemoglobin and melanin
Stimulated emission[9]	Synchronized laser pulses of different wavelengths	Increase of light intensity of the lower frequency beam	Chromoproteins



Microscope and optics set up for spontaneous Raman imaging. Other techniques require a more complex setup. CCD, charge-coupled device.

uct later this year. Olympus product manager Yiwei (Kevin) Jia says he was helping research groups get started with CARS even before the femtoCARS module was announced; this add-on, set up to detect lipids, will make getting started much easier. If the spread of commercial products for CARS imitates that of multiphoton microscopy, sales will take off in a few years, he says. Right now, though, most researchers doing CARS microscopy are doing so out of physics labs using systems they built themselves.

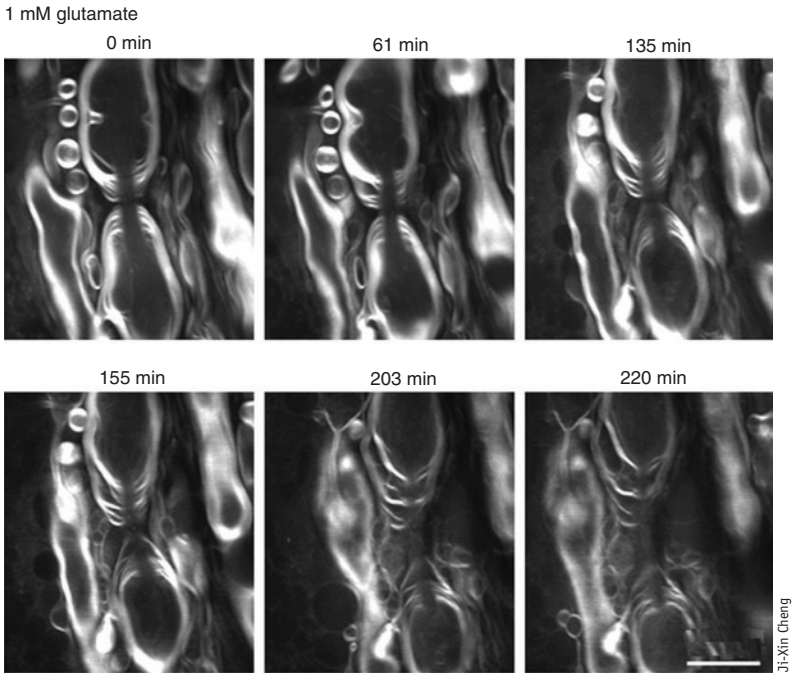
Still, these researchers are collaborating with biologists. At Purdue University, biomedical engineering professor Ji-Xen Cheng uses CARS to quickly find lipid bodies within a cell and then, using the same laser source, switches to confocal Raman for a finer analysis of chemical composition within that area. Recent work on human prostate tumor cells showed that regions previously considered to be composed of fat are, surprisingly, oxidized fatty acids. The next step is to determine whether such fatty acids can be used as an indicator of how aggressive the cancer is likely to be. In other work, Cheng has developed a platform to collect signals simultaneously from CARS, which sees lipid, and from a technique called sum frequency generation, which can visualize certain protein fibers. This allowed Cheng and his collaborators to study how lipid-rich immune cells embed themselves in the collagen matrix found in the walls of blood vessels—observations that should lead to insights about how plaques form in atherosclerosis. Separately, Cheng and colleagues monitored the myelin sheath of neurons in mouse models of multiple sclerosis and pinpointed exactly where on the axons damage occurs. "Before," Cheng says, "there was no way to visualize the myelin at this single-cell resolution in live tissues."

Myelin, with its densely packed lipids, is particularly suited for CARS microscopy, says Jalink. Other applications of label-free microscopy face a high bar. Researchers who work regularly with lasers could probably figure out a way to adopt such techniques, he says, adding, "technically, it's completely doable, but what is the reason for going through this somewhat complicated, expensive process if I can get the information another way?"

As techniques become established, researchers can apply them to new uses. Rafael Yuste at Columbia University is using optics to measure the voltage of neurons. SHG imaging relies on a hyper-scattering of light caused by a very regular arrangement of molecules with a strong induced dipole moment or a requisite type of charge distribution. Yuste is particularly interested in such molecules in cell membranes of neurons, across which there is an electric field. As the second harmonic signal is directly proportional to the strength of the electric field, it automatically reports the voltage.

The problem is that few appropriate molecules have been identified that can work well for this purpose. To get to the cutting edge, Yuste says, "you need to scan the whole spectrum carefully, looking for potential second harmonic endogenous chromophores." The lack of such resources, he says, comes from the fact that the development of such techniques comes from intersections of disciplines, requiring work on the periphery of researchers' expertise, often with less funding and support in researchers' own departments.

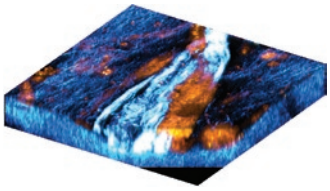
Indeed, researchers like Enejder believe that moving across disciplines can help researchers uncover a wealth of problems that can be addressed only with label-free nonlinear microscopy. Though trained as a physicist, Enejder transferred to her



Label-free CARS imaging of the same axon in live spinal tissue shows that damage to myelin can be induced by glutamate excitotoxicity. Scale bar, 10 μm.

university's biology department where it is easier to learn what researchers are having trouble visualizing and how nonlinear optics might help. Researchers who are closeted in physics departments can continue technical developments, she says, but they may not know what biologists hope to see: "I don't have any problem with that at all. I see applications everywhere."

And while such communication is certainly important so, too, is willingness to attempt experiments that defy physicists' previous experience. In one bioengineering project aimed at producing elastic blood vessels, Enejder and colleagues wanted to monitor the growth of muscle cells seeded into a cellulose matrix. Along with CARS, Enejder and colleagues used SHG to watch the seeded cells and found to their delight that they could monitor how the seeded cells made contact with the cellulose network and began to lay down collagen fibers, a crucial ability for defining optimal parameters for tissue engineering. Though plant-produced cellulose fibers in paper are invisible to SHG, those secreted by bacteria have the kind of regular pattern that does generate an SHG signal, Enejder explains, adding, "you can't rely on different reports of what should be visualized. It is far more reliable to just try it yourself."



Annika Enejder

A cell-seeded scaffold shown with overlaid images obtained with CARS (muscle cells in orange) and SHG (cellulose and collagen fibers in blue) microscopy. Volume shown is 100 × 100 × 16 μm.

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SUPPLIERS GUIDE: COMPANIES OFFERING NONLINEAR OR LABEL-FREE IMAGING EQUIPMENT

Company	Web address
Amplitude Systemes	http://www.amplitude-systemes.com/
Andor Technology	http://www.andor.com/
Applied Precision	http://www.appliedprecision.com/
Bitplane	http://www.bitplane.com/
Bruker Optics	http://www.brukeroptics.com/
CDHC Optics	http://www.cdhcorp.com/
Chroma Technology	http://www.chroma.com/
Cobolt AB	http://www.cobolt.se/
Coherent	http://www.coherent.com/
Conoptics	http://www.conoptics.com/
DeltaNu	http://www.deltanu.com/
FEMTOLASERS Produktions	http://www.femtolasers.com/
Fianium Ltd	http://www.fianium.com/
Hamamatsu Photonics	http://sales.hamamatsu.com/
High Q Laser Innovation	http://www.highqlaser.at/
HORIBA	http://www.horiba.com/
JASCO, Inc	http://www.jascoinc.com/
Kaiser Optical Systems	http://www.kosi.com/
Lambda Solutions	http://www.lambdasolutions.com/
Lambert Instruments	http://www.lambert-instruments.com/
Leica Microsystems	http://www.leica-microsystems.com/
Menlo Systems	http://www.menlosystems.com/
Nanophoton	http://www.nanophoton.jp/
NBL Imaging System	http://www.nbl.com.cn/
Newport Corporation	http://www.newport.com/
Nikon	http://www.nikon.com/
NKT Photonics	http://www.nktphotonics.com/
Olympus	http://www.olympus-global.com/
Omega Optical Inc.	http://www.omegafilters.com/
Onefive GmbH	http://www.onefive.com/
Perkin Elmer	http://www.cellularimaging.com/
PicoQuant	http://www.picoquant.com/
Point Source	http://www.point-source.com/
Princeton Instruments	http://www.princetoninstruments.com/
Prior Scientific	http://www.prior.com/
Renishaw	http://www.renishaw.com/
RGBLase	http://www.rgblase.com/
Rowiak	http://www.rowiak.de/
Spectro Physics	http://www.spectro-physics.com/
Stanford Research Systems	http://www.thinksrs.com/
Thermo Scientific	http://www.thermo.com/
Thorlabs	http://www.thorlabs.us/
Time-Bandwidth Products	http://www.tbwp.com/
Tokai Hit	http://tokaihit.com/
Universal (HK) Techology Company	http://www.universalhkco.com.cn/
Varian Inc	http://www.varianinc.com/
Visitron Systems	http://www.visitron.de/
WITec	http://www.witec-instruments.de/
Zeiss	http://www.zeiss.com/