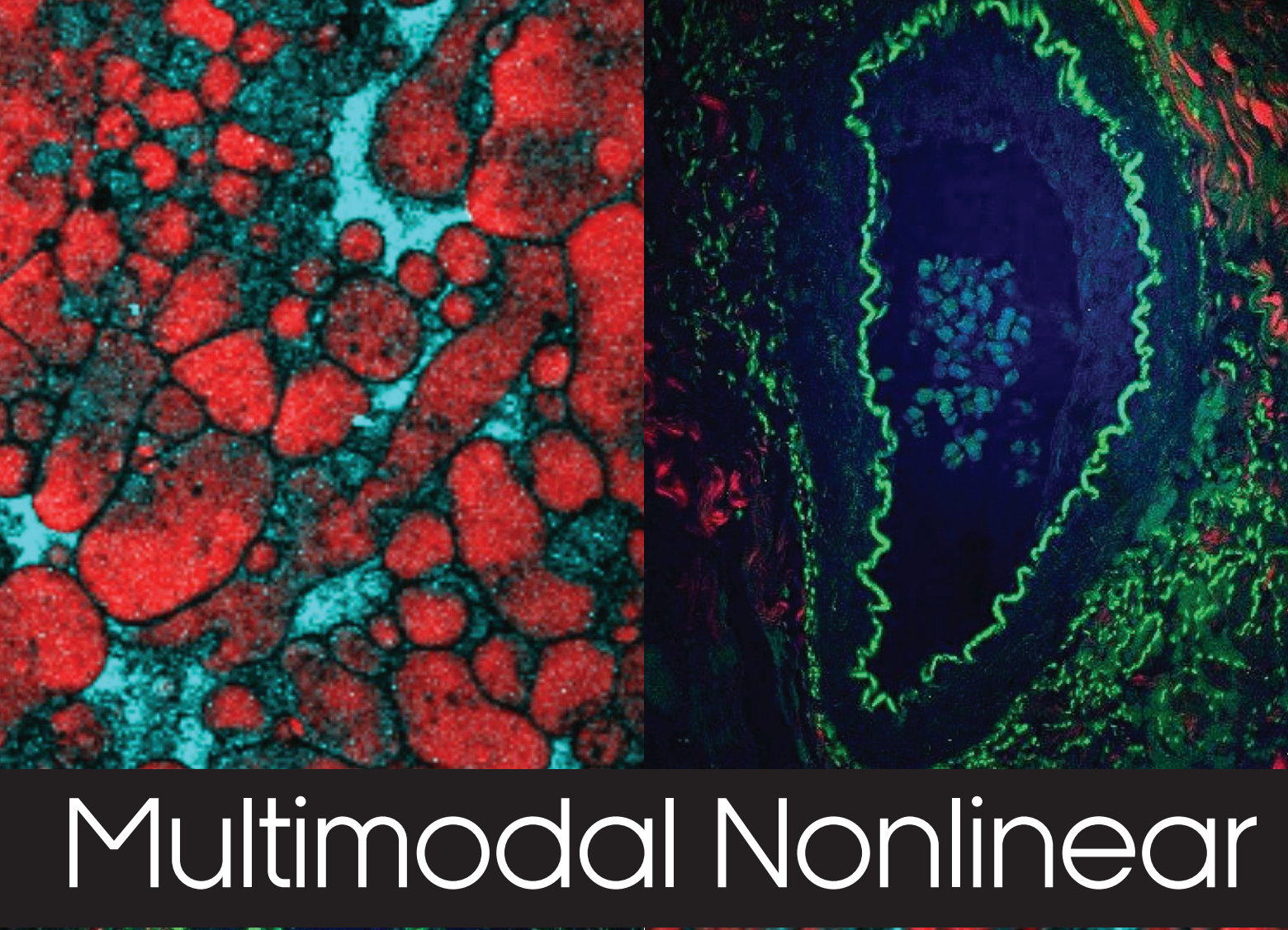
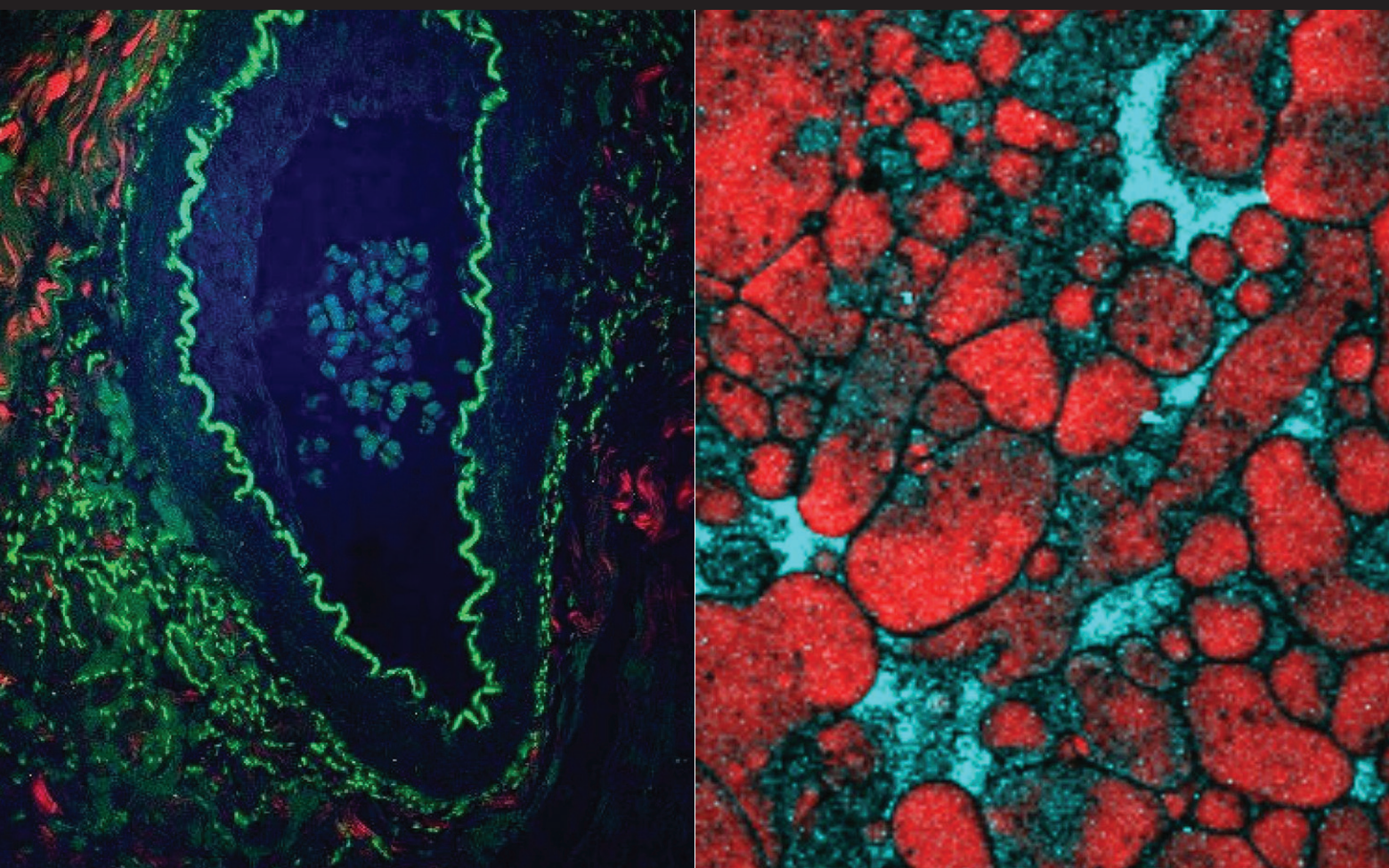


Multimodal Nonlinear



From Biophotonics to Geophotonics

Optical Microscopy

Nonlinear optical spectroscopies such as CARS and SRS offer rapid, label-free, chemical-specific imaging. And the ability to simultaneously deploy multiple modes of nonlinear imaging on a single sample is creating some striking new views of natural materials.

Marco Andreana and Albert Stolow

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Linear optical microscopy techniques, such as transmission or laser fluorescence microscopy, are ubiquitous in medical research and diagnostics. And, since nature did not color-code biological materials for our viewing pleasure, these methods typically rely on exogenous stains or fluorophores, such as organic dyes and quantum dots, to provide high contrast when imaging with visible light.

But this "optical labeling" can cause problems in studying dynamic, living systems: the label might interfere with complex biochemical pathways, adhere to the wrong target, or be metabolized or transported away. Moreover, scattering of visible light can limit penetration depths in turbid media. In laser scanning microscopy, exposure times to focused laser light must be limited to avoid irreversible damage from heating, photobleaching and photoxicity.

The widespread commercial availability today of short-pulse lasers opens up a range of nonlinear optical methods of imaging.

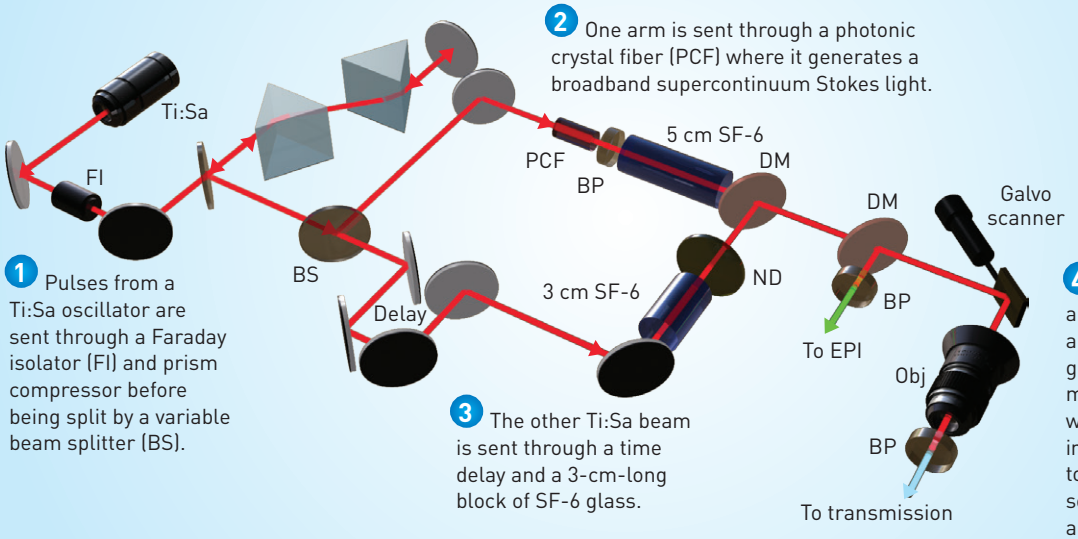
Fortunately, the widespread commercial availability today of short-pulse (ps and fs) lasers—which can deliver high intensities at low energies—opens up a range of nonlinear optical (NLO) methods of imaging. NLO microscopy can image samples without the use of exogenous labels, by leveraging a variety of well-established NLO spectroscopies, such as those that use Raman vibrational resonances. And putting more than one of these NLO techniques together in a "multimodal" imaging setup has created a powerful new approach for applictions in biology, materials science, and most recently, geology and natural resources.

Surveying NLO imaging techniques

In NLO imaging, two or more photons interact within a medium, generating signals that depend nonlinearly on laser intensity. There can be resonant enhancement of these generated signals (as with linear spectroscopy); however, the nonlinear nature of the interaction means that the resonances can appear at differing orders of interaction, allowing for more than one type of contrast mechanism in

A MULTIMODAL IMAGING SETUP

Multimodal CARS microscopy brings together multiple NLO imaging techniques using variable chirping of a single fs light source. The setup shown here allows simultaneous imaging of a single sample using CARS, SHG and TPEF.



1 Pulses from a Ti:Sa oscillator are sent through a Faraday isolator (FI) and prism compressor before being split by a variable beam splitter (BS).

2 One arm is sent through a photonic crystal fiber (PCF) where it generates a broadband supercontinuum Stokes light.

3 The other Ti:Sa beam is sent through a time delay and a 3-cm-long block of SF-6 glass.

4 The two beams are recombined and routed into a galvo-scanning microscope whereupon three independent detectors collect CARS, second harmonic and fluorescence (epi) signals.

Illustration by Phil Saunders/Adapted from M. Andreana and A. Stolow

the imaging. There are also nonresonant NLO responses that still generate bright signals. And, often, several NLO methods can be used in parallel within the same sample—a multimodal approach.

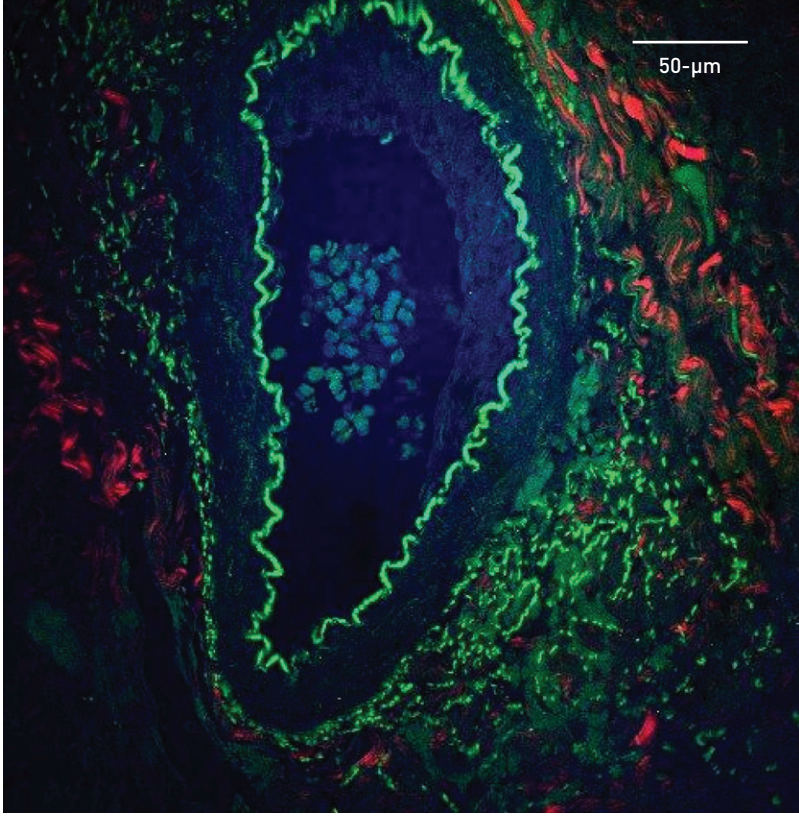
NLO imaging techniques can be classified as parametric, in which the state of the probed molecule is unchanged, and nonparametric, in which the probed molecule is left in a different state after the imaging. Here is a quick overview of some of the most common methods.

Two-photon excitation fluorescence (TPEF), a nonparametric technique, relies on electronic resonances within the sample. Information on very local variations can be obtained from the fluorescence spectrum and the fluorescence lifetime. Because electronic cross-sections are commonly large, the measurement of TPEF signals is quite easy, and even single molecules can be detected and tracked.

Second-harmonic generation (SHG), a parametric second-order NLO process, involves two photons of the same frequency, interacting at the sample to create a single photon having twice the frequency. (The general case of SHG is sum frequency generation, or SFG, in which the two incident photons do not have the same frequency.) Because SHG phase matching (see below) cannot take place within centrosymmetric (e.g., isotropic) media, the focal volume must include areas where centrosymmetricity is locally broken. Examples where SHG works particularly well would include interfaces between different media, or materials that are inherently asymmetric at the molecular level.

Third-harmonic generation (THG) is analogous to SHG, except that it involves three rather than two photons, making it a third-order nonlinear process. THG, unlike SHG, does not require broken inversion symmetry; however, in focused laser beams, the phase change upon propagation through the focus tends to cancel forward-propagating THG signals in homogeneous materials. That makes THG imaging attractive for applications that require sensitivity to edges or interfaces.

Coherent anti-Raman scattering (CARS), a parametric, third-order nonlinear process, is one of two NLO imaging techniques based on Raman vibrational spectroscopy. CARS commonly involves two input beams, called the pump beam and the Stokes beam. The pump frequency is greater than the Stokes frequency, with the difference frequency between the two beams tuned to match



APPLICATION: BIOPHOTONICS

Multimodal CARS image of an unstained rabbit arteriole. A 50-µm thick section of arteriole, tangentially cut to the lumenal side, reveals lipids (red zones, CARS), collagen (blue zones, SHG) and smooth muscle elastin (green zones, TPEF). All three channels were obtained simultaneously from a single sample.

vibrational resonances in the molecule of interest. The generated signal resulting from those resonant interactions, called the anti-Stokes or CARS signal, has a higher frequency than the pump. Varying the frequency difference between the input beams allows different molecular vibrations to be probed. The technique requires no chemical labels because the contrast comes from the molecules naturally occurring within the sample. That has made CARS microscopy an attractive and increasingly popular technique for label-free, molecule-specific imaging.

Stimulated Raman scattering (SRS) is a nonparametric process. Like CARS, it employs pump and Stokes beams at separate frequencies, with the difference frequency tuned to the molecular vibration of interest. When the frequency difference between pump and Stokes corresponds to an intrinsic molecular vibration, the optical power of the pump is transferred to the Stokes via stimulated emission to an excited molecular vibration. The power transfer is measured either as stimulated Raman gain in the Stokes or as stimulated Raman loss in the pump. These signals are small and require sensitive signal-recovery techniques such as those based on modulation transfer and lock-in amplifier detection.

Nonetheless, SRS microscopy, like CARS microscopy, is gaining considerable interest in the scientific community.

An important difference between image formation from coherent signals (such as those from SHG and CARS) and incoherent signals (such as those from TPEF and SRS) involves "phase matching." For coherent NLO processes, the signal at the detector is a forward-propagating field that contains all interferences (phases) between microscopic signals generated at different points within the macroscopic laser focal volume. Thus the signal at the detector is not necessarily the numerical sum of all the individual responses within the focal volume—the way in which the local phases add up matters. For incoherent processes, there is no phase relationship between the microscopic signals emitted from different points, and these signals directly reflect the sum of all emitters within the focus. This is one factor that makes image formation in parametric NLO microscopy more complex than in incoherent NLO microscopy.

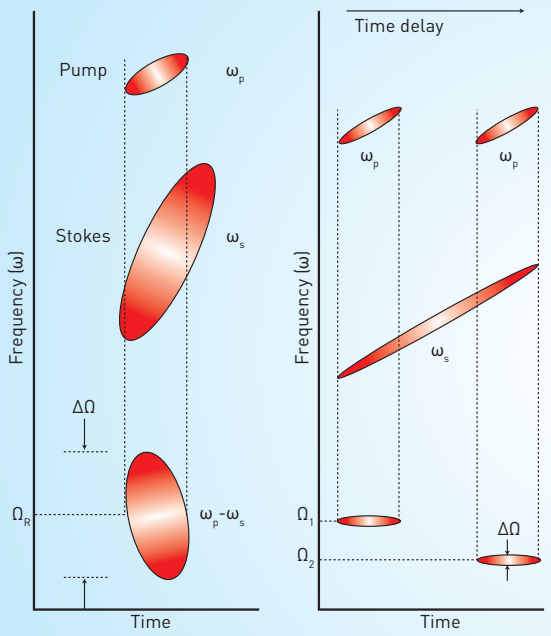
CARS and SRS have drawn considerable attention, owing to the interest in using NLO microscopies to "fingerprint" chemicals within a sample.

CARS and SRS: Toward molecular fingerprinting

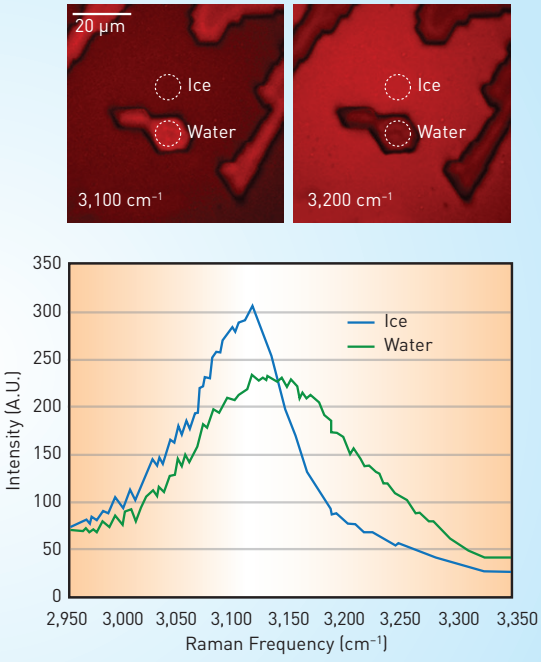
NLO Raman spectroscopies such as CARS and SRS have drawn considerable attention, owing to the increasing interest in using NLO

RAPID IMAGING USING CARS MICROSCOPY

Spectral-focusing (chirped-pulse) CARS microscopy simultaneously allows for user control of the spectral resolution and for the fastest of CARS spectral scanning.



Time-frequency plots showing relationship among chirp, spectral resolution and spectroscopy. Near-transform-limited pulses show as ellipses with vertical major axes (left); strongly chirped pulses result in elongated ellipses (right). The degree of chirp directly controls the difference frequency between pump and Stokes pulses and, thus, the spectral resolution ΔΩ. The frequency difference Ω_R rapidly probes different Raman modes (Ω_1 and Ω_2).



(Top) A dynamic mixture of ice and water is rapidly imaged using CARS microscopy, with contrast obtaining from the slightly different Raman resonances of water versus ice. (Bottom) The associated CARS spectra for the water and ice regions.

Illustration by Phil Saunders

microscopies to "fingerprint" chemicals within a sample using characteristic molecular resonances. For large biological molecules, methods based on electronic spectroscopy, such as TPEF, often do not differentiate between molecules. Other nonresonant NLO imaging processes such as SHG, SFG and THG derive contrast from spatial variations of the nonlinear electric susceptibility. As these are coherently summed over the focal volume, it can be challenging to determine to source of the contrast.

In contrast, vibrational spectroscopies, such as infrared absorption and Raman scattering, can identify specific molecular groups and are a long-established analytical method of fingerprinting molecules. Infrared absorption does not work well in microscopy of biological samples, owing to both the long wavelengths (and, hence, poor spatial resolution) and the strong absorption of infrared light by water, which is everywhere in biological systems. Raman scattering techniques, in contrast, typically use visible light to obtain vibrational information, making them a far better candidate for molecular fingerprinting.

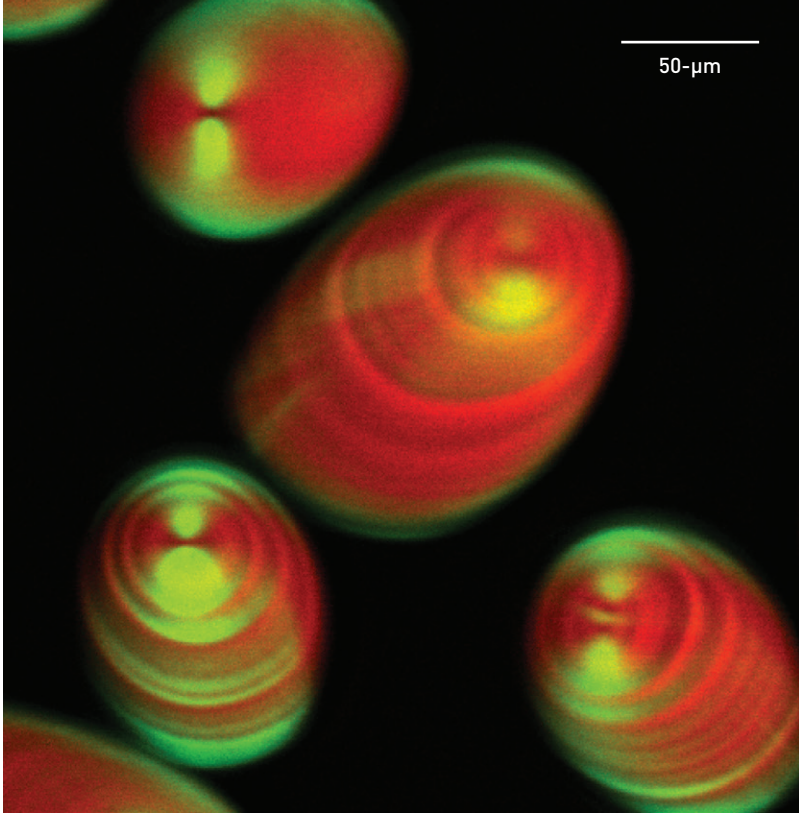
Making it multimodal

NLO microscopy uses well-established laser scanning microscopy platforms, and advances in the field have largely relied on advances in ultrafast lasers and in simple implementations of NLO spectroscopy. Yet, because of the differences between the various NLO imaging processes—differences in optimal wavelength, spectral and temporal pulse width, pulse repetition rate, peak power and spatial-resolution requirements—there is no ideal, universal short-pulse laser source for all NLO microscopy applications. How can we create a multimodal layout bringing multiple NLO imaging techniques (including Raman techniques) to bear on a single sample? One answer lies in variably "chirping" the laser pulses so as to tune the effective linewidth to the mode of interest.

NLO Raman microscopies based on CARS and SRS, for example, require the input of two short optical pulses whose difference frequency must be readily tunable to the linewidth of the Raman mode of interest. The bandwidth of the input pulses constitutes a crucial variable in this tuning. Initially, it was thought that fs laser pulses, 10 to 100 times spectrally broader than ps pulses, would favor nonresonant background signals over the resonant signals, thereby reducing contrast. On the other hand, the narrow bandwidth of ps pulses, while it matches particularly well the narrow Raman linewidths in the so-called fingerprint region (approximately 5 cm^{-1}), is spectrally too narrow for efficient imaging of water (O-H resonance, nearly 400 cm^{-1}) or lipids (C-H resonance, around 100 cm^{-1}).

Ideally, therefore, users would like a two-color laser system with adjustable bandwidths, allowing optimization for a given sample. And it turns out that, for CARS and SRS microscopy, stretching fs pulses in time—chirping the pulses—until they have ps duration is equivalent to using ps transform-limited (TL) pulses of the same duration and energy. Furthermore, the degree of linear chirp is an adjustable parameter that users can optimize to enhance signals and contrast in multimodal NLO microscopy. TPEF, SHG and THG signals, for example, increase with intensity and therefore benefit from shorter (fs) pump pulses. In contrast, CARS and SRS benefit from matching the bandwidth of the difference frequency to the Raman linewidth of interest.

How do we create a layout for accomplishing this kind of multimodal imaging using a fs light source? A simple, robust implementation appears in the diagram on p. 44. In this setup, a single Ti:Sa (or fiber) oscillator produces near-infrared pulses (as short as 50 fs at 80 MHz repetition rate) that are split into a pump beam and, passing through a photonic crystal fiber (PCF), the red-shifted Stokes beam. The two beams



APPLICATION: MATERIALS SCIENCE

Multimodal CARS microscopy of individual potato starch grains—around 65% amylopectin and 20% amylase starches. CARS (red, C-H stretch at 2,880 cm^{-1}) and SHG (green) bands show alternating hard crystalline layers and soft, semi-crystalline layers of amylopectin radiating outwards from the central core.

are variably filtered and delayed, and passed through blocks of high index (SF-6) glass with lengths selected to match the desired chirps, before being recombined and sent to the microscope. By using three detectors with appropriate filters, CARS/SRS, SHG and TPEF images are recorded simultaneously, permitting rapid multimodal CARS imaging, with Raman shifts ranging from 850–4,000 cm^{-1}. The phase-matched CARS and SHG signals

Multimodal NLO microscopy—with its ability to perform rapid, label-free, chemical-specific imaging—has matured into a flexible tool that is finding many new applications.

are collected in the forward direction with a condenser, whereas the TPEF signals are collected in the backwards direction through the objective lens (epi-detection).

A simple example of "spectral focusing" using chirped pulses in CARS/SRS microscopy is the figure on p. 46, which shows how the degree of chirp determines the effective linewidth. The method yields a spectral resolution (~8 cm^{-1}) comparable to that achieved with TL ps pulses. In this simple single-oscillator+PCF implementation, the spectrum of the Stokes light is much broader than that of the pump, which allows for very rapid (ms) and facile tuning across the Raman spectrum, simply by changing the time delay between pump and Stokes pulses. The result: rapid, label-free, hypespectral imaging of a dynamic system (in this case, water-ice equilibrium).

Expanding applications

Multimodal NLO microscopy—with its ability to perform rapid, label-free, chemical-specific imaging—has matured into a flexible tool that is finding many new applications. Among them:

Biophotonics. Applications of multimodal NLO microscopy to medicine and biology have received the most attention, owing to the potential for label-free imaging of tissue structure and chemical composition, low phototoxicity and good depth penetration. Imaging the structure and composition of complex tissue samples can visualize disease-related changes and provide direct comparisons with histopathology, allowing investigation of dynamic process on large, intact tissue samples at subcellular spatial resolution.

The example on p. 45 shows label-free multimodal NLO microscopy applied to atherosclerosis research: Within an image of a rabbit artery, atherosclerotic plaques show as blue due to collagen (SHG), elastin in the artery wall is green (TPEF), and lipid bodies are imaged as red (CARS). This kind of image can help characterize the relative plaque load in the artery and differentiate between healthy and diseased tissue.

Materials science. Applications in materials science generally and biomaterials (such as starch or bone) in particular have emerged and gained traction in recent years. Carbohydrate materials such as cellulose, a starch, are the most abundant biomaterials on the planet. Starch grains are composed of crystalline and amorphous layers. The image on p. 47 shows a multimodal NLO image of potato starch grains, with simultaneous red (CARS) and green (SHG) imaging depicting those layers.

These grains, which are insoluble in water at room temperature, will suddenly swell and disintegrate as they are heated in water: the amylose and amylopectin structures are lost and a viscous solution is formed. Interestingly, multimodal CARS microscopy is fast enough to allow for real-time imaging (video) of this process, observing in detail how the crystalline and amorphous regions break down as water enters the grain. These kinds of real-time observations of process-induced changes of starch grains in particular and condensed carbohydrate systems in general are of significant interest in food sciences and pharmacology.

Geophotonics. Geology, Earth sciences, and natural-resources research constitute a novel application area for multimodal NLO

imaging—a new discipline we call geophotonics. The correlation of a sample's chemical state and mineralogical distribution with its geologic history is important for understanding transport processes within the Earth. Extractive industries want to know the microscopic, colloidal composition of mining ore slurries, and the petroleum industry has an obvious interest in the distribution of organic materials within rock.

Recently, for example, we have studied fluid inclusions (high-pressure gases and liquids trapped in rock) using multimodal NLO microscopy. In this case, SHG imaging reveals grain boundaries and local crystallinity, whereas CARS imaging identifies (in a nondestructive, noninvasive manner) the chemical composition of the fluid inclusion, including the local pressure and density.

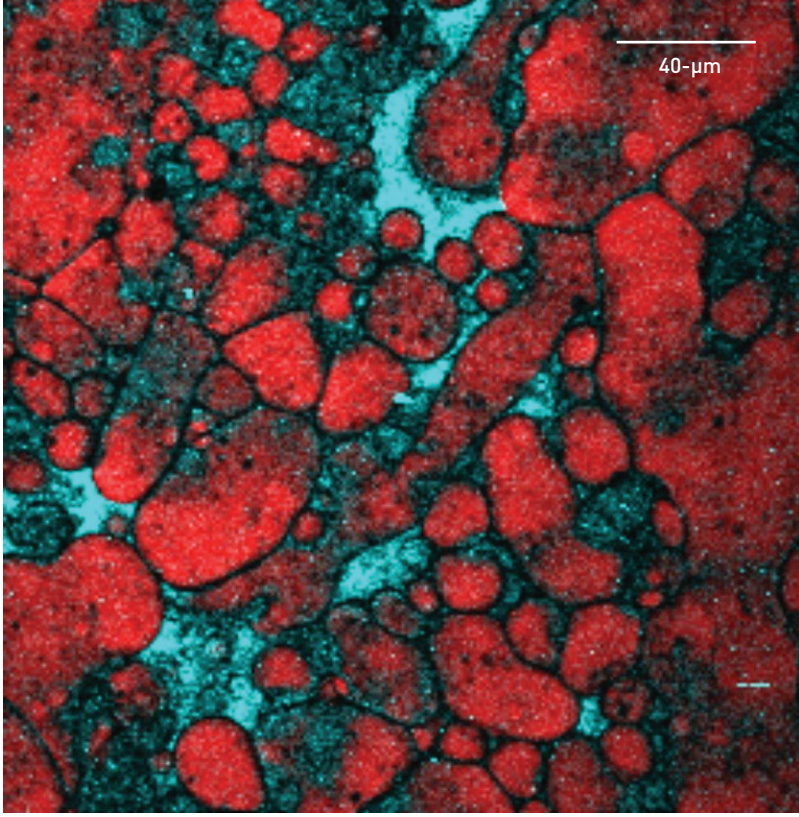
Another opportunity for geophotonics application, shown in the figure at right, involves supporting the bitumen water-based extraction processes (WBEP) used in the energy industry to produce heavy oil for refineries. WBEP can allow for bitumen recovery rates of more than 90 percent from high-quality ores, but extraction from lower-quality ores, as well as tailings remediation, remain important technical challenges. Label-free, molecule-specific multimodal NLO imaging of heavy hydrocarbon slurries clearly depicts the spatial distribution of hydrocarbons (red), water (blue) and kaolinite clays (grey)—fundamental data of great interest to both industry and regulators.

Future prospects

The increasing commercial availability of turnkey laser systems has opened up these and other applications of multimodal NLO imaging in science, medicine and industry. Most laser oscillators used for NLO microscopy, however, remain expensive and sensitive to environmental changes. But recent technical developments are bringing compact, stable and high-power ultrafast fiber laser systems to market; indeed, all-fiber-based CARS microscopy of live cells was demonstrated in 2009.

Such developments will certainly hasten the transition of NLO imaging from the laboratory into the hospital, clinic or factory. The spectral characteristics of the noise, which can reduce the effective contrast in multimodal NLO microscopy, remain a key issue, and future designs will need to focus on minimizing these effects.

Another area of future development will be the continued miniaturization of multimodal NLO imaging instruments. In medicine, for example, compact operating room NLO microscopes or endoscopes will become important tools for in vivo tissue imaging and characterization, reducing the time required for biopsy. In materials, pharmaceuticals or ore processing, compact online NLO instruments will provide rapid, chemical-specific feedback for process optimization. The efforts over the past thirty years on short-pulse laser oscillator design could well have far-reaching implications for science, health and industry. OPN



APPLICATION: GEOPHOTONICS

Complex slurry of heavy hydrocarbons (red, C-H stretch at 2,880 cm^{-1}), water (blue, O-H stretch at 3,200 cm^{-1}) and kaolinite clay particles (gray), such as typically found in bitumen ore process samples, can be rapidly imaged in a chemical-specific, label-free manner using multimodal CARS microscopy.

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