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**“Invisible”
molecules glow
with new label-free method**

Compiled by BARBARA GOODE AND PETER GWYNNE

LABEL-FREE IMAGING

Stimulated emission microscopy makes nonfluorescing molecules glow

"It is truly important work," says Cornell University Professor Chris B. Schaffer of stimulated emission microscopy, described in the October 22 issue of *Nature*. The approach, pioneered by Wei Min and Sijia Lu and their colleagues under the direction of Harvard University Professor Sunney Xie, exploits the stimulated emission phenomenon, first described by Albert Einstein and the basis for LASER.

This brand new method enables imaging of molecules that absorb, but do not fluoresce at sensitivity levels "orders of magnitude higher than for spontaneous emission or absorption contrast," explain the researchers. Not only does it enable three-dimensional optical sectioning and allow the use of nonfluorescent reporters for molecular imaging, but according to Xie, "It allows spectroscopic identification of molecules in living organisms and is free from the complication of light scattering by the sample. This opens many new possibilities for biomedical imaging, such as label-free mapping drug distributions and blood vessels in tissues." Indeed, the *Nature* paper demonstrates a variety of applications.

Xie explains the approach by pointing out that we experience color primarily because of the mechanism of absorption contrast, whereby the intrinsic energy level of a molecule causes it to absorb specific colors of light—leaving us to see the color of the remaining light. Under an optical microscope, absorption is too weak to measure because each specimen contains just a few molecules. ("The signal is much weaker than those in a conventional

UV-vis spectrometer, the sensitivity limit of which is about one part per 10,000 light attenuation," he notes). Also, light scattering complicates the absorption signal. "For these reasons, fluorescence microscopy has been the dominant contrast mechanism for optical microscopy," says Xie, who is famous for his work in single-molecule imaging, including the development of coherent anti-Stokes Raman (CARS) microscopy and stimulated Raman spectroscopy (SRS).

The setup

The Harvard team discovered that while non-radiative decay dominates spontaneous emission for chromophores with undetectable fluorescence, stimulated emission can compete—and in fact can become the dominating light decay pathway—when the stimulation field is designed with appropriate energy and timing.

In their system, the incident excitation and delayed stimulation pulse trains are spatially overlapped and focused onto the common focal spot in the sample. A modulator switches the intensity of the excitation beam on and off at 5 MHz. The spectrally filtered stimulation beam is detected by a large area photodiode, and demodulated by a lock-in amplifier to create the image contrast while raster scanning the collinear exciting and stimulating beams (see Fig. 1).

The researchers' setup consists of two femtosecond (fs) optical parametric oscillators (Coherent/APE) synchronously pumped by a femtosecond mode-locked 76-MHz Ti:sapphire laser (Coher-

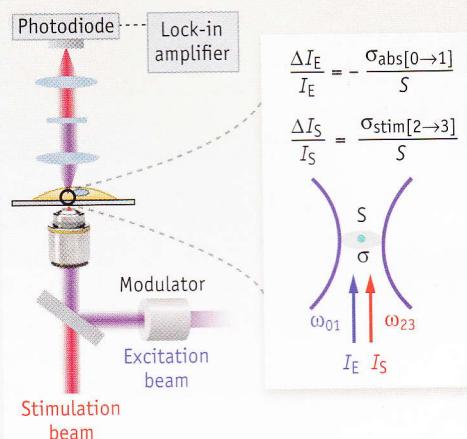


FIGURE 1. The relative energy gain or loss of the stimulation and excitation beam, respectively, for a single chromophore at the laser focus (area of S), is given by the equations (inset).

ent). Two independent frequency-doubled outputs from these two optical parametric oscillator signal waves, in the wavelength range of 560 to 700 nm with pulse widths around 200 fs, serve as either the excitation or stimulation pulse trains. A pulse compressor consisting of a pair of SF11 prisms controls the pulse width. Collinear excitation and stimulation beams are combined and focused with a high numerical aperture (NA51.2) objective onto a common focal spot. The temporal delay between the synchronized excitation and stimulation interpulse is adjusted to between 0.2 and 0.3 ps. The intensity of the excitation beam is modulated by an acousto-optical modulator (Crystal Technology) at 5 MHz. A condenser with NA50.9 is used to collect the forward propagating stimulation beam, which is spectrally filtered before being detected by a photodiode.

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Schaffer, known for his work on blood imaging and nonlinear microscopy, notes that among the molecules visible through this method are many important in biology, "including hemoglobin and cytochrome-c, which play a critical role in oxygen transport and metabolism." (Xie adds melanin and retinal to that list.) And, Schaffer says stimulated emission microscopy makes it "possible to image these molecules with sub-micrometer resolution inside thick tissues" and with a sensitivity level approaching that of fluorescence methods, "which enable detection of single molecules."—Barbara G. Goode

REFERENCES

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IN VIVO IMAGING

Optical molecular imaging: closer to clinical

Optical molecular imaging continues its steady march toward the clinic as demonstrated by a number of talks at the World Molecular Imaging Conference (September 23–26, Montreal, QC, Canada). At the meeting's kick-off keynote, Nobel Prize winner Roger Tsien stressed that surgery and radiation therapy offer key opportunities for optical molecular imaging. His group has developed synthetic molecules called activatable cell penetrating peptides (ACPP) that selectively accumulate in diseased tissue. Brighter and smaller than fluorescent proteins, these molecules help visualize small metastases in the lung and are useful for surgical guidance.

"We have found that molecular fluorescence image guid-

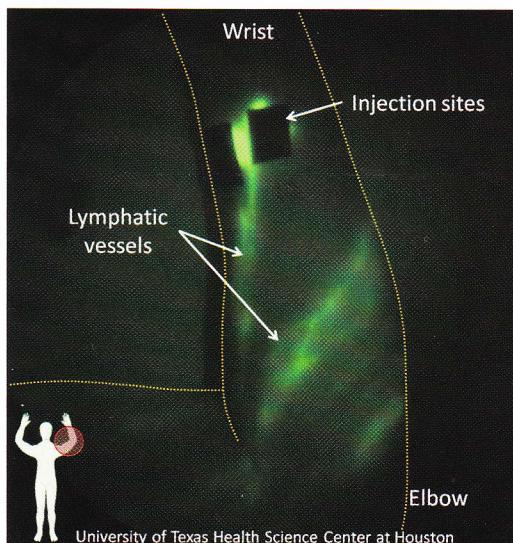


FIGURE 1. Using the near-infrared fluorophore indocyanine green, researchers at The University of Texas Health Science Center-Houston can image the human lymphatic system. In this case, six intradermal injections of 25 µg ICG in 100 µL of saline highlight lymphatic vessels in a patient's arm.

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