It's free imaging — label-free, that is

Label-free imaging lets labs 'see' their samples less invasively than other techniques.

Vivien Marx

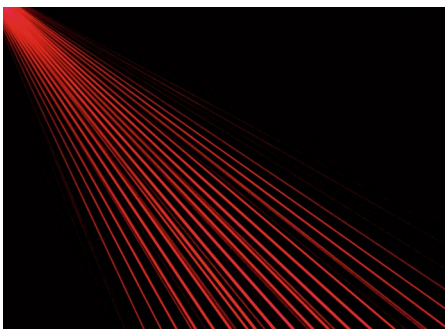
Under a microscope, cells might reveal themselves to an observer. Many labs use labels and fluorescence microscopy to, for example, track the movement of protein A in a particular cell type. Chemical or fluorescent labels are routine in cell biology and the choice is wide. Labels are specific and labs can use them to multiplex, says Vahid Sandoghdar from the Max Planck Institute for the Science of Light. Yet the use of fluorescence is all too often a struggle against photobleaching, blinking and saturation, which limits brightness.

Other drawbacks, says Angelika Unterhuber, a researcher at the Medical University of Vienna, include the fact that dyes or fluorescent proteins can interfere with cell function. And labs might battle spectral overlap when multiplexing, "which makes it difficult to discriminate between the labeled structures or cells." To do label-free imaging, experimenters can use photons and the scatter of light from a sample, or they can use sound and bounce-back of sound from a sample. When imaging, instead of using a standard linear optical imaging technique such as laser-scanning microscopy, they can use non-linear imaging, a process in which the signal does not grow proportionally to the incident light intensity[1–3].

One-photon imaging is probably better for a thin or relatively transparent specimen. "Non-linear imaging really shines for deep imaging in scattering tissue," says Chris Xu from Cornell University. So, even without fluorescent labels, labs can resolve tissue and cell structures such as membranes or organelles. None of these methods are perfect, says Xu, but they are advancing. "I think we are slowly approaching a situation, where various methods are mature enough for applications," and they can be combined with one another, says Sandoghdar. Solving real biomedical problems with optical microscopy is an exciting prospect for him.

Lasers are key

Lasers are the "engines" of multiphoton microscopy advances, whether in label-free or contrast-agent-based imaging, says Xu. "The laser is the crucial part in non-linear label-free imaging," says Unterhuber. Needs will vary between label-free techniques but



Most label-free optical techniques call for femtosecond or picosecond pulses in the near-infrared region. Credit: J.A. Bernat Bacete/ Moment/ Getty

most call for femtosecond or picosecond pulses in the near-infrared (NIR) region. The harvested signals with these methods tend to be small, which drives an experimenter's choice of laser. It has to deliver low noise levels and be quite stable, she says. Labs traditionally used titanium sapphire lasers, also called Ti:sapphire lasers, that emit in the NIR range. They can be broadly tuned and generate the fast pulses needed in non-linear label-free imaging[4]. Until recently, these lasers, fabricated as solid-state lasers, were both bulky and expensive, she says. Much technology development has taken place to cut costs, increase stability of the laser signal and increase the depths to which a beam can penetrate a sample. Fiber lasers are more compact, cheaper and potentially more robust, says Xu, "but their penetration in research labs is still very limited." Perhaps, he says, more labs will use fiber lasers "when they are able to provide more flexibility in output wavelength." Fiber lasers now work at longer wavelengths, such as 1,300 or 1,800 nanometers, says Unterhuber, and continuing laser development is needed in non-linear optical imaging (NLOI).

Scatter happens

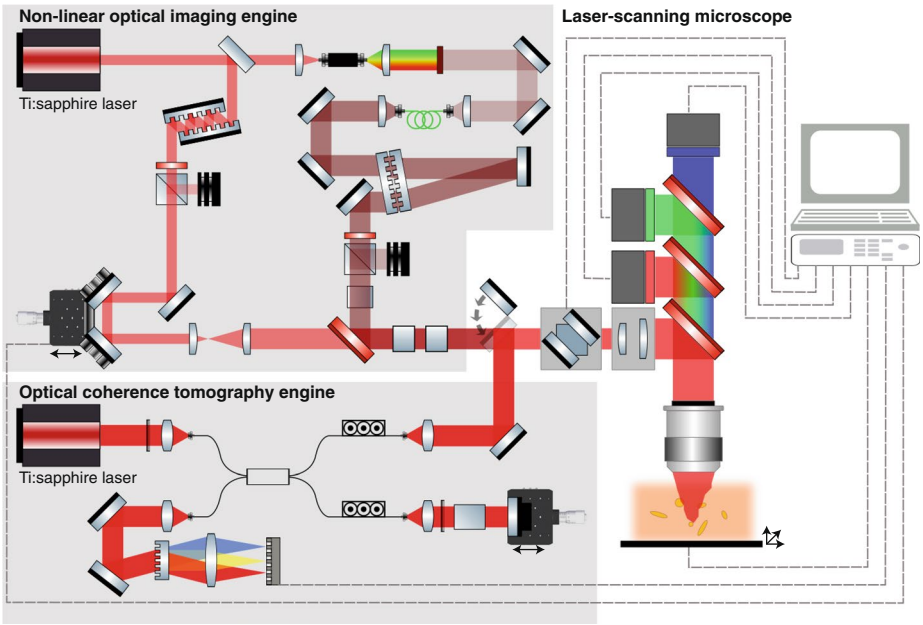
To obtain an informative signal with an NLOI technique of choice, labs face a balancing act: between the wavelength of the applied laser and the kind of scattering and absorption their sample generates. "In the near-infrared window in biological tissue, the main limitation is absorption of blood, melanin and water," says Unterhuber. The scattering coefficient of tissue decreases monotonically as the wavelength increases, but the absorption will vary greatly in the visible and NIR spectrum, she says.

It's helpful if experimenters keep the local absorption minima for water in mind: 800, 1,050, 1,300 and 1,800 nm. These windows in imaging experiments allow tissue penetration up two millimeters. They are used in optical coherence tomography (OCT), in which a sample scatters NIR light and the image is reconstructed from the scatter pattern. OCT, says Unterhuber, allows labs to non-invasively obtain a cross-sectional tomographic visualization of live tissue, for example. The resolution approaches that achieved in histology but, instead of surgery, it's optical 'excision'. And contrast is achieved by photons coming off a sample in different ways. Non-linear microscopy techniques require objectives with high numerical aperture, a factor that determines how much light enters the objective and shapes its ability to capture detail. The techniques also differ in terms of the contrast levels that can be achieved through imaging. "It is essential that you chose the right technique to investigate the right sample," she says.

Getting some glow

Two-photon excited fluorescence (TPEF) can leverage endogenous fluorescence for studying a range of biochemical changes. It has a number of advantages over single-photon fluorescence. With TPEF, two photons are absorbed at the same time, they excite a fluorophore and there is subsequent emission of a blue-shifted photon. A single-photon process requires an incident photon be in either the ultraviolet spectrum or the blue–green region, whereas TPEF needs only low-frequency photons that are typically in the infrared spectral range, says Unterhuber. "With single-photon fluorescence microscopy, separating the excitation and emission wavelengths can be problematic," she says.

Given that the energy difference between the two frequency bands is small, "filter selection can be delicate," she says. The bands are so close together that one filter might either pick up the second signal or cut



Researchers at the Medical University of Vienna have built a system that combines several non-linear optical techniques. Adapted from ref. [3], RSC.

it completely, such that it cannot be picked up in another detection channel.

Second harmonic generation (SHG) microscopy is a frequency-doubling technique[5]. The incident light sent to a sample is coherent: two photons of the same frequency are used with the photons in 'lock-step', in phase with one another. The signal that comes back from a sample is one combined photon with twice the energy and half the wavelength of the two, incident photons. The signal — the second harmonic — is coherent and detectable. Spatial information comes from raster scanning the sample. The technique works well for noncentrosymmetric molecules, such as the collagen in the extracellular matrix, says Unterhuber. Structures such as biological membranes are also noncentrosymmetric.

Third harmonic generation (THG) microscopy[6] involves a non-linear coherent scattering process, like SHG, that results from phase matching and the summation of light waves. In THG microscopy, three photons are directed at a sample. They are converted into one photon emission with one third the wavelength of the excitation photons and around three times the energy of the incoming individual photons. The approach is used to study structural interfaces, which might be water–lipid interfaces or the interfaces between water and proteins, intra and extracellular membranes, or extracellular matrix structures. Red blood cells have a strong THG signal and this imaging approach can be used to characterize microcirculation in model organisms. THG is not easy to do in living tissue with a Ti:sapphire laser. The photon tripling leads to photon emission in the ultraviolet range, in which damage due to ionizing radiation becomes a major issue, says Unterhuber.

As Bettina Weigelin and her colleagues at Radboud University Medical Center point out[6], THG is versatile "because it does not require a specific asymmetry of the structure to be imaged." Its signal intensity depends on size and organization of the structures at the micrometer scale. Any wavelength can be used in THG. What is optimal is a "trade-off between tissue-scattering and absorption." Longer wavelengths, higher than 1,350 nm, lead to more absorption by water and limits penetration into tissue. Some labs have found that, in brain tissue samples, a wavelength of around 1,750 nm delivers "optimal" data. With THG, no energy from incident light is absorbed, it's scattered. There is no photo bleaching or reactive oxygen species, but the high illumination can cause tissue damage. Phototoxic molecules can result from the concurrent two and three-photon autofluorescence excitation or there can be heat damage. Labs will want to choose wavelengths carefully, the scientists note. Wavelengths of 1,200–1,300 nm "do not seem to generate photodamage" and are suitable for long-term time-lapse imaging.

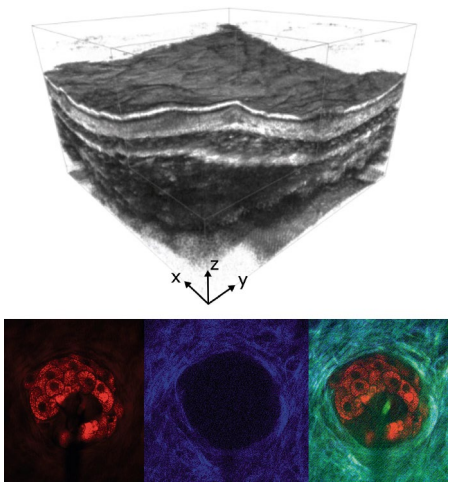
"The laser is the crucial part in non-linear label-free imaging," says Angelika Unterhuber.

Credit: Med. Univ. of Vienna

Vibrations speak

Raman microscopy or infrared microscopy use incident light to capture tell-tale vibrational signatures of chemical bonds in a sample's molecules, rather than photon-based excitation. It's a rather weak effect, though, which is where CARS comes into play — that is, coherent anti-Stokes Raman scattering. CARS enhances the Raman signal[7] and molecular vibration is detected with blue-shifted photons.

As Unterhuber explains, CARS involves lasers used at two wavelengths, at so-called 'pump' and 'Stokes' frequencies. Together, they stimulate a sample by the way the incoming frequencies mix. What results is anti-Stokes emission, where photons are emitted with a lower wavelength than the incident light. It's a response that occurs when the difference between the two incoming frequencies is equal to molecular resonant frequencies. The signal depends on the number of illuminated molecules and the type of molecule being targeted. CARS, says Unterhuber, is used to capture



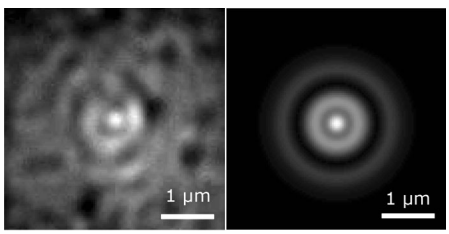
Mouse ear tissue is optically sectioned with optical coherence tomography (top); the sebaceous gland is imaged in the same system with CARS (lower left), SHG microscopy (middle) and CARS + SHG + two-photon fluorescence (right). Adapted from ref. [3], RSC.

molecular fingerprints, such as to determine if a certain chemical species is present in a sample. CARS is, for example, used to study lipids such as in the context of metabolism[8]. It leverages certain areas of a lipid molecule, such as the C–H stretching region, which has a characteristic vibrational mode and one that can also be used to distinguish proteins and DNA.

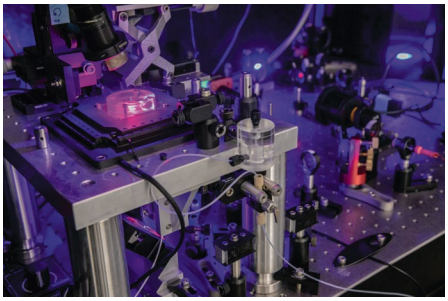
Even though the technique is label-free, "it is fair to ask," note Aaron Streets, from the University of California, Berkeley, and colleagues[2], whether CARS microscopy can be harmful to the probed system. They point to studies showing that lipid-droplet organelle transport in live cells was performed with CARS while avoiding photo damage. There are limits and standards related to the power levels that can be applied to living tissue, says Unterhuber. Many cell viability studies have been performed, "but phototoxicity is always an issue that should be considered," she says. CARS is as specific as Raman imaging, but it can involve a more complicated laser system than classic Raman and it can be limited to a "handful" of chemical bonds, says a scientist who wishes to remain anonymous. OCT allows labs to image several millimeters into a sample, such as tissue, and is used in ophthalmology to image retina structure based on a scattering pattern of incident light and computed 3D tissue reconstruction. It's an in vivo imaging technique that lacks molecular contrast, says Unterhuber.

Scatter, interfere

When Sandoghdar first got involved in microscopy through near-field optics, single-molecule fluorescence microscopy was established but, he says, "still very new and exciting," as labs experimented with extracting data from single fluorophores. "Although one learned some things about the biology of the sample, the approach was not satisfactory for a laser physicist like me who was used to having full control over



Live HeLa cells imaged with iSCAT. Left, an imaged gold nanoparticle is attached to a protein at the cell membrane. Right, the computationally processed image. Adapted from ref. [13], Springer Nature Ltd. Credit: MPI for the Science of Light



With iSCAT, light scattered from a nanoparticle in a sample interferes with a laser beam reflected by the sample. Credit: MPI for the Science of Light

his measurements." He followed single-molecule imaging without entering the field. He had begun using gold nanoparticles as plasmonic nanoantennas to study near-field effects. Such nanoparticles were also used in electron microscopy. But the size of the particles, around 40 nm in diameter, made them too big to be a viable biolabel.

The team's interferometric scattering microscopy (iSCAT)[9,10] made it possible to detect particles as small as five nanometers across on a glass slide but, he says, "it took us nearly another 10–15 years to apply this to real biological samples." These samples include naked viruses, lipid bilayers and proteins as small as 50 kilodaltons. They have also used the approach to label and track protein motion in live cells. With interferometric scattering, laser light is focused on the back focal plane of a microscope objective, which coinncides with the back focal plane of the imaging lens. The iSCAT approach applies scattering and interference: light scattered from a nanoparticle in a sample interferes with a laser beam reflected by the sample. The group developed algorithms to analyze the point spread functions of the captured signal.

Getting iSCAT to work with individual proteins is tough because the signal is one thousand times smaller than from a virus, and much smaller than the intensity fluctuations that originate from the illumination and other sources. Identifying and imaging a nanoparticle on a busy scattering background such as a cell "is a huge challenge," says Sandoghdar. "We employed some tricks from image processing and machine learning to distinguish the point spread function of a gold nanoparticle." Now, he sees many possibilities, such as studying viruses without needing a gold label, he says. iSCAT is a technique that can be used in a label-free fashion or via a scattering label. "In any event, it is fluorescence-free." It's potentially also a protein characterization technique; iSCAT contrast can be used to measure protein size. This concept is one his former postdoctoral fellow Philipp Kukura, now with his own lab at Oxford University, has leveraged to develop optical mass spectrometry.

In principle, the sensitivity of iSCAT and related methods is not limited, says Sandoghdar, but the team has bumped up against technical issues such as the dynamic range of existing cameras. The plan is to push iSCAT to enable tracking of single particles inside cells and tissues. For that, the scientists will be using more "optical tricks," he says, and machine learning to decipher the signal of a nanoparticle over a large scattering background. Among the group's future plans: "For example, we expect to document the whole life cycle of a single virus in the cell," he says. It is promising to explore how to combine iSCAT and fluorescence to co-localize different entities and events.

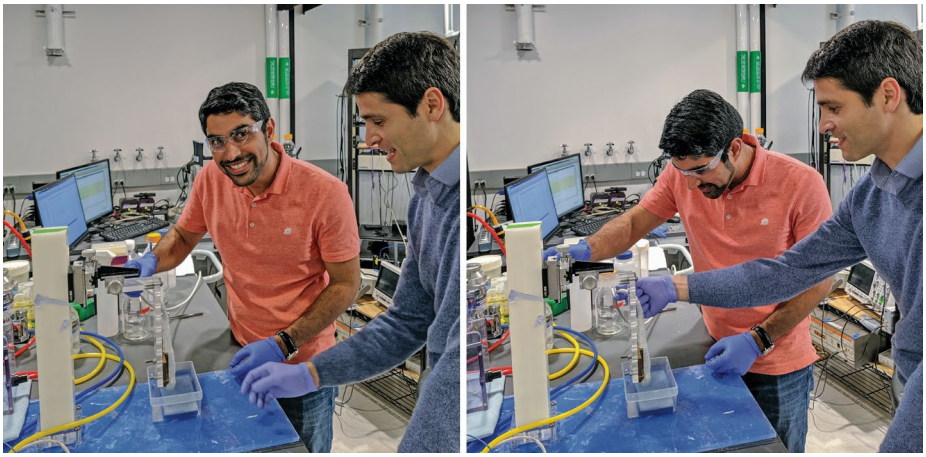
Multi-modality modes

A number of labs work on multimodality platforms to balance strengths and weaknesses of each technique. In biomedicine, some labs choose to combine TPEF and SHG microscopy, where experimenters discern the SHG signal based on the incident frequency and know that the corresponding TPEF signal is red-shifted. "Thus, both signals are easily separated spectrally," says Unterhuber. Labs might also use TPEF of intracellular coenzymes such as flavin adenine dinucleotide and pyridine nucleotides, which provide intrinsic contrast and let scientists assess cell metabolism. Some labs combine techniques.

Unterhuber, Wolfgang Drexler and others have developed a way to give OCT molecular sensitivity[3]. They integrated multiple modalities — SHG, CARS and TPEF — such that there is no interference and the techniques complement one another. The researchers believe the modular approach can be used with laser scanning microscope systems, super-resolution or structured illumination, among others. With OCT and NLOI, they imaged part of a mouse ear: OCT reveals the skin layers and CARS, combined with SHG, reveals structural and molecular aspects of the sebaceous gland. Speaking generally and not about the aforementioned system, Xu says that combining different techniques lets labs gain more information from a sample, "Some combinations are more convenient than the others, depending on the hardware requirement."

Bubble vision

Photoacoustics involve photonics and acoustics. Short laser pulses lead to acoustic pulses. This energy, once absorbed, can be converted into



Using ultrasound, Caltech researcher Mikhail Shapiro (right), postdoctoral fellow Arash Farhadi (left) and colleagues use bacterial vesicles to render gene expression visible in mammalian cells. Credit: Caltech

thermoelastic expansion, says Unterhuber, which leads to a detectable pressure wave. Acoustic waves suffer much less scattering and absorption than light, which makes them suitable in some biomedical applications, and may make it the technique of choice instead of optical means. Photoacoustic imaging can image deep into tissue as long as there is absorption, says Unterhuber. It reaches deeper than fluorescence microscopy in those instances. Absorption of photons can occur due to endogenous molecules, which might be hemoglobin or melanins or exogenously delivered contrast agents. "Since blood usually has orders of magnitude higher absorption than surrounding tissues, there is sufficient endogenous contrast for photoacoustic imaging to visualize blood vessels."

Acoustics can be harnessed to use ultrasound for cellular imaging. Mikhail Shapiro from the California Institute of Technology, postdoctoral fellow Arash Farhadi and their colleagues borrowed genes from bacteria to render gene expression visible in mammalian cells using ultrasound[11,12].

Many microorganisms have air-filled protein nanostructures that evolved to keep them afloat in aquatic environments. The scientists engineered mammalian cells to produce such vesicles, which served as ultrasound contrast agents. In the lab's experiments, the team inserted microbial genes from *Bacillus megaterium* so their mammalian cell genomes had 'acoustic reporters.' The cells produced these vesicles, which enabled a view of gene expression in a tumor xenograft.

The signal was mainly from the tumor perimeter, says Shapiro, where most of the blood vessels and nutrient supply lines are. "That's something that you would have never been able to see with optical imaging," he says. With light-based techniques, this tumor microstructure is shrouded in visual noise, "whereas with ultrasound we could see it very clearly."

To distinguish the vesicle signal from the background tissue, the team sequentially imploded the vesicles. An ultrasound pulse with high enough pressure leads the vesicles to collapse and emit a sound wave. "That creates a stronger signal than you would get from a normal scattering interaction," says Shapiro. The next pulse of ultrasound can no longer detect the imploded vesicle. "You see this pattern of signal that goes from low, to high, to low that corresponds to the presence of the gas vesicles," he says. The rest of the tissue lacks this response.

The mammalian cells the team tested were not damaged either by producing the vesicles and having them collapse; it's not a violent affair, it's more like "collapsing with a whimper," says Shapiro. The technique will, he says, hopefully, be a way to help cell biologists study function in vivo in the animal models of their choice. One can use it to image processes over time, such as to follow development. The cell is also making what is needed for imaging: as it continues through its cell cycle, divides, differentiates and migrates, it can continue to make these vesicles.

It's not quite label-free imaging but the method is non-invasive. Shapiro draws inspiration from the advances with fluorescent and luminescent proteins, and calls Nobel laureate and green-fluorescent-protein developer Roger Tsien his "scientific hero."

Unlike fluorescent proteins encoded by one gene, nine genes are involved in vesicle production and they need to be expressed properly, at the appropriate ratios, and their products need to find one another and assemble into vesicles. "That's challenging," he says.

To get the complete genetic program for producing vesicles into the cell, the team uses in vitro transfection with three different plasmids. The cell line needs to produce enough vesicles to be visible with ultrasound. The coding regions of the needed genes could fit on a viral vector, but the scientists control the stoichiometry with two copies of some genes and that exceeds what a viral vector can carry. "But we're working on more compact ways of stringing the genes together that could fit onto a viral vector," he says.

For labs seeking to try the method, they would need an ultrasound imaging system that can be programmed and "then we can send them a script that they can run on their system that would make it possible for them to see the signals," he says. He hopes this approach can help to build a new field of biomolecular ultrasound, which sits at the intersection of chemical engineering, biology, physics and computation. In many ways, this intersection is also where many optical label-free methods sit. ❐

Vivien Marx
Technology editor for Nature Methods.
e-mail: v.marx@us.nature.com

Published online: 21 November 2019
https://doi.org/10.1038/s41592-019-0664-8

References

1. Webb, W., Zipfel, W. & Williams, R. *Nat. Biotechn.* **21**, 1369–1377 (2003).
2. Streets, A., Li, A., Chen, T. & Huang, Y. *Anal. Chem.* **86**, 8506–8513 (2014).
3. Andreana, M. et al. *Photochem. Photobiol. Sci.* **18**, 997 (2019).
4. Xu, C. & Wise, F. W. *Nat. Photon.* **7**, 875–882 (2013).
5. Cox, G. *Biophys. Rev.* **3**, 131 (2011).
6. Weigelin, B., Bakker, G.-J. & Friedl, P. *J. Cell Sci.* **129**, 245–255 (2016).
7. Cheng, J.-X. & Xie, S. *J. Phys. Chem. B* **108**, 827–840 (2004).
8. Folick, A., Min, W. & Wang, M. C. *Curr. Opin. Genet. Dev.* **21**, 585–590 (2011).
9. Taylor, R. & Sandoghdar, V. *Nano Lett.* **19**, 4827–4835 (2019).
10. Young, G. & Kukura, P. *Annu. Rev. Phys. Chem.* **70**, 301–322 (2019).
11. Farhadi, A. et al. *Science* **365**, 1469–1475 (2019).
12. Bourdeau, R. et al. *Nature* **553**, 86–90 (2018).
13. Taylor, R. et al. *Nat. Photon.* **13**, 480–487 (2019).