MICROSCOPY

Good vibrations for super-multiplexed imaging

Researchers optimize stimulated Raman scattering microscopy and combine it with a new panel of Raman-active dyes to enable 24-color bioimaging.

Fluorescence microscopy has been an invaluable tool for understanding complex biological systems, but it is largely limited to studying a few factors at a time. This limit is determined by the number of colors that can be imaged simultaneously, because fluorophores often have broad, overlapping spectra. Even the most sophisticated methods are limited to imaging around ten fluorophores at once. Methods that can improve multiplexing are needed to help researchers understand complex processes as they occur in cells.

Wei Min at Columbia University and Lu Wei, a postdoctoral fellow in Min's laboratory, sought to develop a multiplexed imaging method that would bypass the barriers imposed by spectral overlap in fluorescence microscopy. For this, they turned to nonlinear Raman imaging: specifically, stimulated Raman scattering (SRS) microscopy. In Raman imaging, spectral peaks are roughly 100 times narrower than fluorescence peaks, which makes colors much easier to distinguish. In SRS microscopy, samples are illuminated with two aligned beams (the pump and Stokes beams). Fine tuning of these beams at multiple wavelengths stimulates molecular vibrations that cause intensity changes in both beams, and these changes can be measured to provide contrast and generate a multicolor image.

Unlike fluorescence microscopy, where fluorescent molecules are excited near their absorption maxima, conventional SRS microscopy uses laser energy levels that are nonresonant, or far below the electronic absorption of molecules. Consequently, conventional SRS is limited to millimolar detection sensitivity. "We were curious what would happen if we brought electronic resonance to the nonlinear Raman imaging," recalls Min.

This curiosity led them to develop electronic preresonance SRS (epr-SRS), a technique that made it possible to get strong SRS signals from dyes like Atto740 with very low background

SENSORS AND PROBES

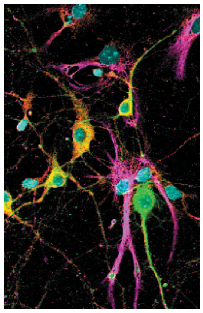
CRISPR'S PAPER TEST

A spot of CRISPR protein on paper provides a powerful diagnostic assay.

In the spring of 2016, the research teams of Feng Zhang at the Broad Institute and Eugene Koonin at the National Center for Biotechnology Information reported that a CRISPR–Cas protein they had characterized in a publication several months earlier can target and cleave RNA rather than DNA. Besides its unusual targeting activity, the bacterial protein, known as C2c2 or Cas13a, was shown to cleave endogenous RNA indiscriminately—an altruistic immune response to viral infection that triggers cell death. Zhang and graduate students Omar Abudayyeh and Jonathan Gootenberg saw an opportunity in this collateral activity. "C2c2 cuts RNA and then goes completely crazy and constitutively active," says Abudayyeh. "And that's basically a sensor."

Around the same time, Jim Collins and his group at the Massachusetts Institute of Technology published a low-cost diagnostic for Zika virus that combines isothermal RNA amplification with sequence sensors known as toehold switches. Collins was looking for directions to take the freeze-dried paper-based approach; and when Zhang's team reached out, the two groups started devising ways to combine the technologies to exploit Cas13a for nucleic acid sensing and diagnostics.

The platform they developed, named SHERLOCK for "specific high sensitivity enzymatic reporter unlocking," involves Cas13a, a short CRISPR RNA (crRNA) to guide Cas13a to its target, and a quenched fluorescent RNA reporter that is released by collateral cleavage upon Cas13a sensing. The rambunctious nuclease activity of Cas13a makes the assay very sensitive, and the researchers improved this sensitivity to the femtomolar range by selecting a more active Cas13a from a bacterial sister species. Adding an initial isothermal amplification step to the sample DNA or reverse-transcribed RNA, followed by *in vitro* transcription, increased sensitivity to a remarkable attomolar or single-molecule range.



Multicolor epr-SRS imaging in cultured hippocampal neurons. Adapted from Wei *et al.* (2017).

signal. They found that epr-SRS was around 1,000 times more sensitive than conventional SRS. These results, which show that epr-SRS approaches the sensitivity of confocal fluorescence, surprised Min, although he notes they are consistent with the literature.

Based on these promising findings, the researchers developed epr-SRS microscopy and imaged dye-labeled DNA in fixed mammalian cells. They saw that their method produced images that corresponded well with images obtained by fluorescence microscopy but offered much higher chemical selectivity. They also observed low photobleaching.

They then extended the method for 'super multiplexing'. For this, they developed a set of twenty near-infrared dyes, called the Manhattan Raman scattering (MARS) dyes, based on the xanthene dye scaffold. These dyes were easily separable using epr-SRS microscopy, and they were used for sixteen-color imaging of dye-labeled cells. The researchers also demonstrated eight-color imaging in hippocampal neurons as well as live-cell imaging in mammalian and bacterial cells.

Epr-SRS microscopy can be implemented on commercial SRS and coherent anti-Stokes Raman scattering microscopes, and this should facilitate broad uptake of the method. According to Min, "the future challenge is probably on the labeling side rather than on the imaging side," as "immunohistochemistry of using a large number of antibodies can sometimes be nontrivial."

Min also notes that the research was multidisciplinary, requiring scientists with expertise in spectroscopy, microscopy, chemistry and biology to work closely together. He says that future work will involve expanding the palette of available dyes and making them more compatible with labeling living cells. The work as a whole represents a major step forward for highly multiplexed imaging and should encourage more biologists to consider Raman microscopy.

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RESEARCH PAPERS

Wei, L., *et al.* Super-multiplex vibrational imaging. *Nature* **544**, 465–470 (2017).

The researchers showed that SHERLOCK can detect Zika genome fragments directly from clinical serum and urine samples and that it functions in a 'one-pot' reaction. The assay also works when the components are freeze dried and spotted on paper, and it requires only a simple incubation for isothermal amplification. In its tests with clinical samples and with freeze drying, "C2c2 turned out to be remarkably robust," says Collins.

The CRISPR system lends inherent specificity to the SHERLOCK assay, which was further tweaked to attain single-nucleotide discrimination. Cas13a tolerates a single mismatch in the crRNA target sequence, but not two; by engineering an artificial mismatch into the crRNA spacer sequence, the scientists demonstrated SHERLOCK's ability to distinguish single-nucleotide polymorphisms in Zika and human genomes.

Many robust diagnostic tools already exist, but there is a constant need for improvement, the team says. "A challenge for the field is developing rapid, inexpensive point-of-care devices that can be used by healthcare personnel," notes Collins. Gootenberg believes that SHERLOCK is unique in how it combines many favorable diagnostics properties into a single assay. In addition to being sensitive and specific, the assay is inexpensive, portable and flexible; it is very easy to program a crRNA for a new target. "In a matter of days we can go from concept and design to a working test that is reliable," says Abudayyeh. "It could be deployed easily in the field or very quickly in the clinic," Gootenberg adds.

The teams are exploring routes to develop and distribute the technology, and Zhang notes that the reagents have been made freely available to the academic community via the Addgene plasmid repository. This work once again illustrates the benefits of exploring the diversity of proteins that have evolved to confer bacterial immunity.

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RESEARCH PAPERS

Gootenberg, J.S. *et al.* Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* **356**, 438–442 (2017).