In vivo imaging: Raman-style

The patient lies unconscious on the operating table, brain exposed through a hole in the skull. The tumor – a spherical glioblastoma perhaps 6 mm in diameter – is nothing more than a slightly yellowish opacity on an otherwise pale-pink background.

The surgeon excises the tumor in quarters. He pauses after each step to track his progress using a custom-built imaging system that renders the tumor bright fuchsia. Once the first quadrant is cut away, the tumor takes on the appearance of a pink Pac-Man in mid-chomp. Removing another piece leaves half a pie. Soon, only a sliver of pink remains, and then nothing.

To the naked eye, the operation appears successful. But the surgeon's imager can see what his eyes cannot, rogue cancer cells that have wandered away from the bulk of the tumor to infiltrate the surrounding tissue in the mouse's brain.

Fortunately, the patient was a mouse, and the surgical suite was in a biomedical research lab. Yet the case illustrates one of the biggest patient fears when it comes to tumor removal – leaving a small, unseen piece of cancer behind. Little can allay that fear at the moment. But new imaging techniques based on the so-called Raman effect could eventually alter the emotional calculus. The question is, will they live up to their potential?

A case for Raman

To understand the Raman effect, consider for a moment the physical properties of light that make it possible to read this issue of *BioTechniques*.

To read a magazine, watch a movie, or marvel at the beauty of your partner's face, light from the object being viewed must pass into your eyes. If that object is a light source, it can be viewed directly. Otherwise, the light that reaches your eyes is reflected off of the object's surface.

In general, when light bounces off an object, the reflected light is of the same wavelength as the incident light, and the reflection is said to be “elastic.” Physicists describe this behavior as the Rayleigh effect, and it explains why things around us appear to have different colors: If you shine a white light on a red billiard ball, most of the light will be absorbed, except the red fraction of the incident light,

Sanjiv Sam Gambhir, who is the Director of the Molecular Imaging Center at Stanford University, is applying Raman spectroscopy approaches to in vivo animal imaging.

which bounces toward you. Thus, you see a red billiard ball.

In 1928 physicist Chandrasekhara Venkata Raman, working at Calcutta University, observed that photons can bounce off an object with different energies (and therefore different wavelengths) than the incident light – a discovery that won Raman the 1930 Nobel Prize in Physics.

“Inelastic” Raman reflections are exceedingly rare, amounting to perhaps one in every million reflected photons. Yet each material has a characteristic Raman signature, a fact that is exploited today in chemical and physical science labs that use the effect to probe chemical bonds and the molecular composition

of materials. The Raman effect also is increasingly being harnessed for in vitro analyses in biological labs and occasionally, for direct in vivo imaging.

Most in vivo imaging, though, is done using either the medical modalities, such as x-ray or MRI, or more popularly, fluorescence or bioluminescence. Yet neither optical modality is optimal. For one thing, tissues tend to absorb and/or scatter visible light, leading to a diminished signal — a problem compounded by tissue autofluorescence. Tissues are also largely transparent to near-infrared (NIR) wavelengths, an effect you can see for yourself by shining a flashlight behind your hand and watching the color of light that passes through. However, there are relatively few dyes available in this region of the spectrum, making multiplexing tricky. Plus, fluorescent dyes are notoriously unstable, often “bleaching out” long before an experiment is complete.

“There is no one Swiss army knife of imaging that can do everything,” says Sanjiv Sam Gambhir, the Virginia and D.K. Ludwig Professor of Cancer Research, Chair of Radiology, and Director of the Molecular Imaging Program at Stanford University. Each modality has its strengths and weaknesses. The strengths of Raman imaging are sensitivity and high multiplexing levels. Raman signals are sharper than fluorescent spectra, meaning they can be multiplexed more easily. They also don't bleach out, allowing for repeated and long-term imaging and can be both excited and detected in the NIR range.

But Raman has its limitations — particularly, when it comes to the very low number of photons produced. In the 1970s, researchers discovered that if Raman-active molecules were coupled to certain metallic nanostructures, dramatic signal amplification could result — in some cases up to 15 orders of magnitude. This effect was termed “surface-enhanced Raman spectroscopy” (SERS). Still, another 35 years would elapse before two research labs, working

independently, would combine these discoveries and make Raman imaging a viable choice for biological labs.

Picture Perfect

The first paper, published in January 2008, came from the lab of Shuming Nie, the Wallace H. Coulter Distinguished Faculty Chair in Biomedical Engineering at Emory University (1). Led by research assistant professor Ximei Qian, a physical chemist-turned-biomedical engineer, Nie's team prepared SERS-active particles by coating 60-nm colloidal gold with Raman-active compounds, such as malachite green or diethylthiatricarbocyanine iodide, and then with a polyethylene glycol shell spiked with a molecule that recognizes EGFR on the surface of cancer cells. When the team injected those particles into the tail veins of nude mice harboring a human head-and-neck tumor expressing EGFR, the particles concentrated in the tumor over 4 to 6 hours, an effect the research team observed by illuminating with a 785-nm NIR laser coupled to a Raman spectrometer.

The second paper, published in April 2008, came from Gambhir's lab. (2) An MD/PhD from UCLA who did his thesis work in applied mathematics, Gambhir helped establish the field of molecular imaging, the union of traditional imaging modalities with the molecular specificity of antibodies, receptors, and pro-substrates. "Imaging tends to have a lot of mathematics because of image reconstruction and modeling of how imaging agents behave in the body," he says.

In the mid-2000s, Gambhir was testing nanoparticulate quantum dots for their use in vivo. Frustrated by their limitations, notably in terms of cytotoxicity and multiplexing, he began looking for other potential imaging strategies. "By going into the literature we said, 'oh, there may be a way to make a parallel technology work that doesn't rely on quantum dots but still uses nanoparticles. And, because they may not be toxic, maybe we should try them.'"

Whereas quantum dots are made of toxic semiconductor materials like cadmium and selenium, SERS-active compounds are made mostly of silver, gold, and platinum. Traditional SERS substrates are planar surfaces. But like Nie, Gambhir's team opted for gold nanoparticles – specifically, an off-the-shelf formulation comprising a gold core, Raman reporter, and silica shell.

The team imaged whole mice by raster scanning their NIR laser over the animal's body. They used a modified Renishaw InVia Raman microscope – basically

Subcutaneous injections of four surface enhanced Raman scattering nanoparticle flavors (with unique spectral profiles) can be successfully detected and unmixed into the correct spectral channel using Raman spectroscopic mapping at 785 nm. This mapping measurement covers an area of 20 mm x 4 mm and was performed in just 2 minutes, due to the high sensitivity of this novel molecular imaging technique.

a confocal microscope coupled to a Raman spectrometer, which was further customized for small animal imaging with new optics and a computer-controlled stage. A complete scan took 20–25 minutes, Gambhir says, compared to 1 minute using fluorescence and bioluminescence imagers. Each point was imaged for 3 seconds, and dedicated software converted the spectral peaks into colored pixels. The resulting image is like a red-green-blue JPEG, with each color channel mapping to a single Raman reporter.

Gambhir also tested a non-metallic option, single-walled carbon nanotubes (SWNT) used either as-is or coupled to a targeting peptide. Carbon nanotubes, as Gambhir notes in his article, "show an intense Raman peak produced by strong electron–phonon coupling that causes efficient excitation of tangential vibration in the nanotubes quasi one-dimensional structure upon light exposure." Simply put, the unique electronic structure of a SWNT allows it to act as both Raman reporter and SERS amplifier. A 2010 study led by Hongjie Dai at Stanford University showed that SWNTs bearing different C13/C12 ratios produce distinct Raman signals, meaning the nanotubes may be multiplexed – an important consideration in biological imaging.

In its earlier 2008 study, Gambhir's team demonstrated that Raman imaging with nanoparticle probes could also support multiplexing. They injected four reporters at separate sites in the mouse body, plus a mixture of the four at a fifth site. The resulting image shows the injection sites as a series of low-res pixels in varying shades of primary colors — green, red, yellow, and blue, while the fifth injection site appears

purple, thanks to an abundance of red and blue particles.

The team next injected these particles into tail veins of live mice. When imaged two hours later, Raman-active particles could be located without knowing where they were *a priori* (they had accumulated in the liver). When peptide-conjugated SWNTs were injected into mice bearing a human tumor, they homed to the tumor over 24 hours, whereas non-peptide-conjugated nanotubes did not. Critically, these particles could be detected even when there were very few of them to find. In its 2008 paper, Gambhir's team could see as few as 600 particles. Today, he can measure at close to the 10-attomolar range. The technique is "exquisitely sensitive," he says.

Raman on the ward

An obvious application for in vivo Raman imaging would be in oncology, where Raman's (or more properly, SERS's) "exquisite" sensitivity could help a physician ensure that a tumor is truly gone.

In 2011, Nie and Qian used their SERS nanoparticles and a handheld Raman probe to test blood samples for the presence of circulating tumor cells (CTC), those rare tumorigenic seeds that can lead to metastases wherever they land (3). The technique was sensitive enough to detect as few as one CTC per milliliter of whole blood.

For his part, Gambhir in 2012 described a tri-functional nanoparticle useful in magnetic resonance imaging, photoacoustic imaging, and SERS (4). The new particle, called an MPR (MRI-photoacoustic imaging-Raman) nanoparticle, is a 60-nm gold structure coated with a thin layer of trans-1,2-bis(4-pyridyl)-ethylene (a Raman reporter) and then again with a

silica shell, and finally with gadolinium, making it MRI-responsive. When the team injected these particles into the tail veins of mice harboring a human glioblastoma, the particles accumulated in the tumor via the so-called “enhanced permeability and retention” (EPR) effect, a disruption of the blood-brain barrier, and could be detected using any of the three modalities. This was the tumor that was resected from that mouse brain earlier in the story, and Raman played a critical role in its detection and removal.

“The Raman part was very important because it let us see individual tumor cells that would have otherwise been totally missed,” Gambhir says — a development that, if replicated in humans, could dramatically improve long-term prognoses.

Of course, to achieve such benefits, physicians and researchers need access to user-friendly Raman equipment. “Technology based on Raman scattering is fairly specialized, so it requires more instrumentation than fluorescence,” says Nie. Indeed, Nie notes that relatively few groups have the capacity to use Raman for in vivo imaging, his and Gambhir’s being among the most prominent.

“It’s a concept foreign to the biological sciences community,” Nie says. But the development of “cheap devices” and off-the-shelf SERS particles could help, he says.

Off-the-shelf SERS particles do exist; Gambhir uses them in his studies. But new designs are also in development. Washington University mechanical engineer Srikanth Singamaneni recently described a new SERS particle design dubbed BRIGHT — “bilayered Raman-intense gold nanostructures with hidden tags.” (5) Unlike the core-shell structures Nie and Gambhir have used, BRIGHTs have a gold core, a thin Raman label shell, and then a gold coating, resulting in particles that produce some 20-fold brighter signals than traditional particles according to Singamaneni — at least in vitro.

On the hardware front, Nie’s group has developed a dedicated handheld Raman spectroscope called a SpectroPen, and tested it in mice. The device, being commercialized through an Emory spinoff company called SpectroPath (for which Nie is Chief Scientific Consultant), is a pen-sized gadget that will allow surgeons to identify tumor margins following SERS particle injection based on a beeping sound, similar to a contractor locating wall studs with a stud-finder.

Gambhir’s lab is developing a fiber-optic endoscopy add-on that enables imaging of SERS particles in, for instance, the colon wall of human patients. According to Gambhir, the tool allows multiplexed detection of up

to 10 different Raman reporters over a wide working distance, which is critical when dealing with the uneven contours of living tissue. The lab, in collaboration with GE, is also working on a high-speed, dedicated Raman imager, called SARI (small animal Raman imaging).

The result of all this development work could be in vivo imaging orders of magnitude better, in terms of both sensitivity and multiplexing, than currently achievable with optical modalities. That’s not to say fluorescence and bioluminescence are going anywhere. “The advantage of fluorescence is you can get fluorescence from small molecules; you don’t have to inject gold nanoparticles,” says Gambhir. “Also, there are already instruments available for it.” Plus, Raman can really only be used for relatively shallow imaging, up to 1-cm deep. “You could go into a mouse skull, but not into a rabbit or human skull,” he says, meaning human brain imaging, for instance, would have to be carried out in the operating theater.

In the end, says Gambhir, there’s room enough for multiple modalities in clinical imaging. “There are advantages and disadvantages for fluorescence and bioluminescence and Raman. There’s not a single clear winner.” Well, other than patients, of course.

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