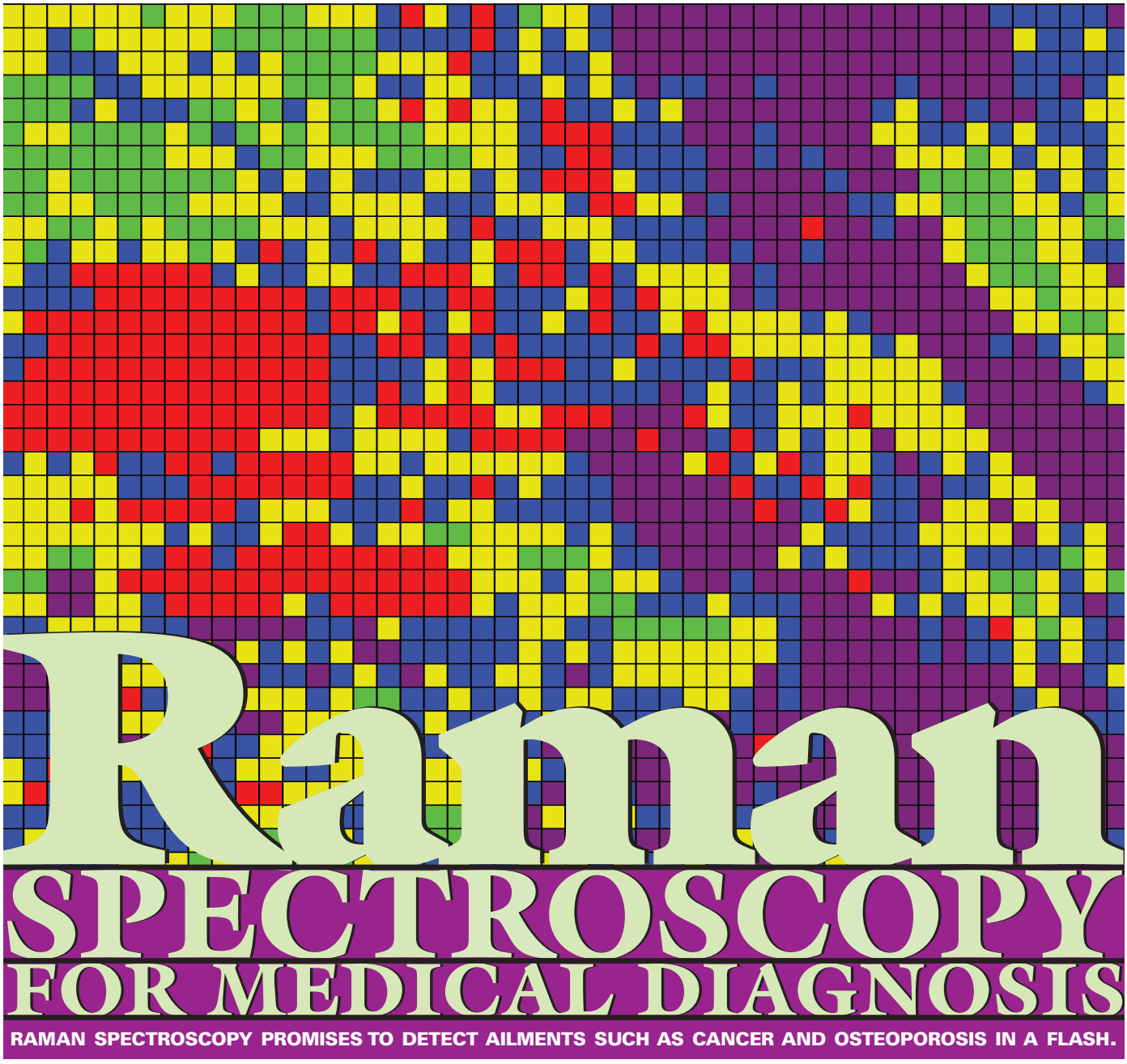


Raman SPECTROSCOPY FOR MEDICAL DIAGNOSIS

RAMAN SPECTROSCOPY PROMISES TO DETECT AILMENTS SUCH AS CANCER AND OSTEOPOROSIS IN A FLASH.

It's difficult to believe that once upon a time, the medical thermometer was a stunning innovation in clinical diagnostics. Thermometers were initially developed in the 16th century but weren't put into routine medical use until 1866, when Thomas Allbutt designed a 6-in. portable version to use with patients (*1*). The thermometer was the first scientific instrument to make the leap from benchtop to bedside, and a myriad of other technologies have followed. Raman spectroscopy, the latest to attempt the jump, has launched itself over the abyss, but it hasn't quite yet touched down on the other side.

"Understanding of how Raman works, for tissue especially, has come far," says Anita Mahadevan-Jansen of Vanderbilt University. But she notes that "it's still a difficult field", with just a handful of researchers worldwide who persist in studying the topic.

So why bother? Advocates of tissue Raman spectroscopy say that it has the potential to one day provide doctors and patients with immediate, biopsy-free diagnoses of many forms of cancer, as well as play a role in detection of other medical conditions as varied as simple dental cavities and osteoporosis.

Jennifer Griffiths

"I see it as the thing that gives you the best answer as to what's going on in the tissue, because it's purely based upon the molecules present in the cells," says Nicholas Stone of Gloucestershire Royal Hospital (U.K.). That sentiment is echoed by other tissue Raman researchers, who all radiate excitement about the potential of this up-and-coming technique.

What Raman can do

The idea behind any Raman tissue experiment is simple: tissue of one type (A) has a different chemical makeup than tissue of another (B). Raman spectra of tissues A and B will be linear combinations of the spectra of each of their respective Raman-active components. If the ratios of those components are different in the two tissues, their Raman spectra

will be different. How different they are depends on which tissue types are being compared. Often the spectra of the two populations look very similar to the naked eye, and many research hours have been poured into mathematically deconvoluting and giving clinical meaning to those spectra.

To set up a model for a tissue comparison, researchers might start with a mixture of tissue samples that have been classified (e.g., cancerous vs noncancerous) by the gold standard of histopathology. They would take spectra of all those samples and apply one of several statistical methods to extract the subtle spectroscopic differences between the two classes of tissue. These differences are written into an algorithm that can then be used to classify an unknown tissue sample.

One of the hottest areas of research in tissue Raman is identifying cancerous versus noncancerous tissue from the brain, cervix, bladder, and other organs (Figure 1). Most of the work to date has been ex vivo, but Raman researchers say that their ultimate goal is to move into the body. "The direct advantage of Raman is that you don't need to do anything with your tissue," says Gerwin Puppels of Erasmus University Medical Center (The Netherlands). "The only thing you do is shine light on the tissue and collect the light that comes back," he adds; this makes Raman nondestructive and safe to use on real patients.

One application could be the use of Raman during surgery to identify the margins of a tumor. Today, surgeons often walk a fine line in deciding how much tissue to remove. Remove too much and they may damage the organ; remove too little and the tumor may recur, necessitating more surgery. A biopsy can take hours or days, whereas Raman could give an answer in seconds.

It's not only soft tissue that can be classified by Raman spectroscopy. Bone and tooth enamel have relatively strong Raman signals, and both are receiving attention from scientists in the tissue Raman community. Currently, the risk of osteoporotic bone fracture is assessed by a technique called dual-energy X-ray absorptiometry (DXA), which measures absorption of X-rays by calcium. According to Michael Morris of the University of Michigan, only 50–70% of the fracture risk is predicted by DXA. What DXA doesn't measure is "bone quality", a general term that includes the chemical makeup of bone.

Morris and colleagues use Raman to detect the minerals and collagen fibrils that make up bone. "What we bring to the table are very clear and very unambiguous measurements of bone chemistry that include cross-linking [of collagen] and measures of mineral maturity," Morris says. They have found a clear correlation between bone chemistry and osteoporotic fracture risk ex vivo (*2*), and they say that they will be moving their studies in vivo shortly.

Meanwhile, other researchers are interested in the oral health applications of Raman spectroscopy. "Dental is one area that has been overlooked quite a lot," says Lin P'ing Choo-Smith of the National Research Council Canada Institute for Biodiagnostics. Caries (the clinical term for dental decay) generally show up on dental X-rays, but some types, especially the early ones, are difficult to see by traditional means.

Researchers had to take a slightly different approach in detecting caries with Raman. Enamel is made up of crystalline rods of the mineral hydroxylapatite that are oriented so that the long axis is perpendicular to the surface of the enamel. When caries develop, some of that structure is lost, and researchers found that they could detect the shift in orientation by polarized Raman spectroscopy (*3*). "Polarized Raman is sensitive not only to biochemistry but also to structural orientation," explains Choo-Smith. "It turned out to be very useful for our applications," she adds.

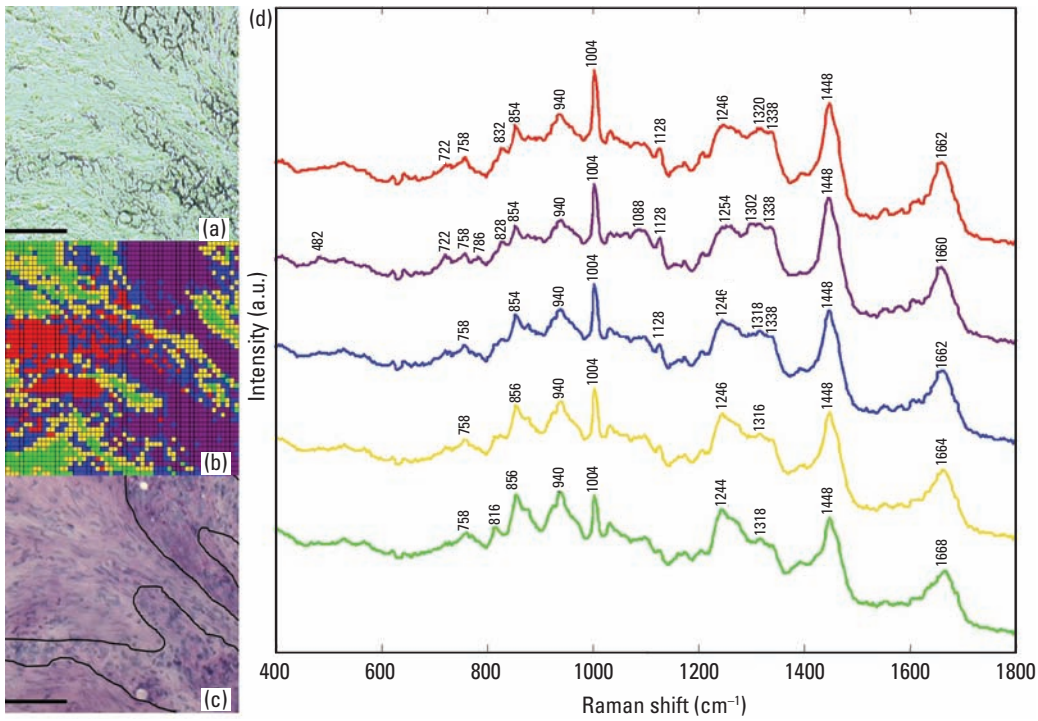


FIGURE 1. (a) Unstained section of bladder tissue. (Scale bar = 100 μm.) (b) Color-coded Raman spectroscopic map; purple represents tumor, red represents muscle, green represents collagen, and blue and yellow represent areas of transition between tissue types. (c) Same section as in (a), but histologically stained. (d) Averaged spectra of the clusters in (b). (Adapted from de Jong, B. W. D.; et al. *Anal. Chem.* **2006**, *78*, 7761–7769.)

Better together?

Despite all this promise, Raman by itself still has its limitations. Raman scattering only occurs in a small fraction of in-

cident photons, so getting a good S/N takes time. "It's such a weak effect that to gather signal, you usually have to dwell on a tissue . . . for at least a few seconds—preferably a minute—in order to get a really good Raman spectrum," says Andrew Berger of the University of Rochester. For example, the typical area imaged in a Raman tissue spectrum is on the order of square micrometers; to scan over a significant area of tissue could take hours—and could try the patience of patient and doctor alike. Many researchers think that Raman will really shine at the fine-diagnosis level, that is, looking only at the spots that have been tagged by some other scanning technology.

To find those suspicious spots, many researchers are coupling other spectroscopy methods, such as optical coherence tomography (OCT) or fluorescence, with Raman for a two-tiered scanning approach. These more traditional techniques have been used in medical imaging for years, but typically they can only pick out the gross features of tissue. "But they are very good at surveying large areas and helping you zoom in on a small area where you could then use the Raman, and I think that's really the likely way forward," Stone says.

Choo-Smith has used OCT to zero in on possible carious spots in enamel (*4*). OCT is good at picking out potential problems but results in lots of false positives. For example, some people have teeth that are hypocalcified. "It's just a genetic malformation and nothing that needs to be treated," Choo-Smith explains. "When we look at it with OCT, we see a bit of the same scattering as you would see in caries, so a clinician might be misled," she says. Raman, however, is not fooled because the two conditions look very different chemically.

Berger points out that a two-tiered approach isn't really all that different from what happens in the clinic now. "That's what doctors are currently doing; they're going in there with white-light endoscopes and looking around," he says. The difference is that now a suspicious patch is biopsied, whereas with Raman, the doctors could simply touch the spot with a fiber-optic probe and get a detailed biochemical assessment in seconds. Of course, to do that, they would have to *have* a probe.

The probe problem

Problems with probe design may be the biggest technical stumbling block to using Raman in vivo. Fiber-optic probes for transmitting light have been available since the 1970s, but most are not appropriate for collecting Raman spectra, because the fused silica that makes up the fibers generates its own Raman signal. Sending light down a typical optical fiber is like sending it through a 3-m-thick wall of quartz, with each thousandth of a nanometer generating Raman signal. "So what you're doing is you're hitting the tissue not only with the excitation light but also with this fused-silica junk," says Michael Feld of the Massachusetts Institute of Technology. "And then, to make it even worse, the light which hits the tissue and gets reflected back can go into the collection fibers and generate more of the quartz noise," he adds. It's a problem that has been solved in larger-diameter probes by using a complex series of optical filters, but that hasn't helped researchers who, for example, need catheter-sized probes for studying arterial plaque in vivo.

"Most of the commercially available probes are very large—a centimeter or larger, obviously bigger than you can put into a coronary artery," says Jason Motz of Harvard Medical School. Several researchers reminisced about a company called Visionex that used to produce small-diameter fiber-optic Raman probes

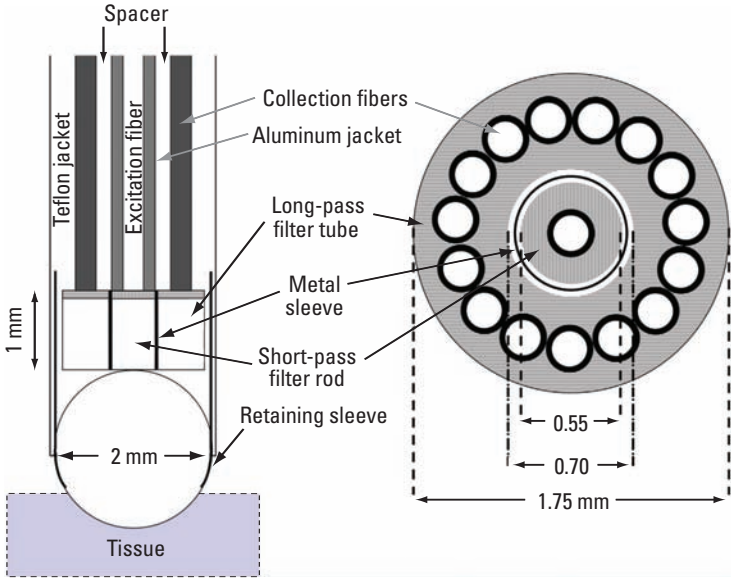
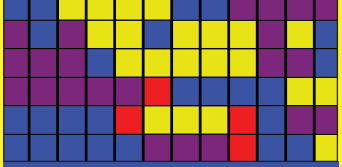


FIGURE 2. Schematic of the Feld and Motz Raman probe tip, showing a longitudinal view at left and a transverse cross section at the fiber–filter interface at the right. (Adapted with permission. Copyright 2004 Optical Society of America.)

but has gone out of business. The Visionex probes that are still in existence are highly valued. "They're floating around out there, guarded like Christian relics or something," says Berger.

Until another company steps up and starts mass-producing uniform fiber-optic Raman probes, researchers are largely left to design and piece together their own. Each research group has its own design strategy. "Some just try to miniaturize the complicated optical layout of the thicker probes. Others try to simplify the probe," says Reiner Salzer of Dresden University of Technology (Germany). Each approach has its pros and cons. High background noise may be a problem with a simpler optical probe design, but on the other hand, "the more components you have in the probe, the more problems you may experience," according to Salzer.

Several groups indicated that they have probe designs that may become commercialized in the near future, but most declined to discuss them for proprietary reasons. Feld and Motz have published one design that has been particularly successful (Figure 2); it allows the researchers to characterize arterial plaques and breast cancer tumor margins, both during real surgeries (*5*, *6*). Their probe, which is 2 mm in diameter, consists of a delivery fiber surrounded by a ring of collection fibers (*7*). The delivery fiber has a microoptic filter on the tip that cuts out most of the fused-silica noise before the excitation beam hits the tis-



Raman spectroscopy has the potential to provide an immediate, biopsy-free diagnosis of many forms of cancer.

sue. The collection fibers are also fitted with filters that cut off the reflected excitation light yet allow the Raman signal to pass back to the detector. The probe "required a lot of careful study and physics and special design," says Feld. "Now that we have that working . . . we can go into any part of the body that we want."

Recently, a new approach to probe design has been introduced by Puppels and colleagues (*8*). Rather than filter out the noise generated by silica, their probe avoids generating that noise altogether by using light in a different region of the spectrum. Most researchers measure signal in the "fingerprint region" (400–2000 cm^{-1}) because that range is particularly rich in spectroscopic information. Puppels's group has instead measured Raman signal in the high-wave-number (HWVN) region (2000–4000 cm^{-1}), in which silica noise is minimal.

The HWVN region has been overlooked, according to Puppels, because its peaks are broad and seem to contain less information than those of the fingerprint region. "But if you look at it closely, you see that there's a lot of fine structure there," he says. His group found that they could distinguish among different types of brain tissue ex vivo by using a greatly simplified single-fiber probe that the researchers think will eventually be useful to physicians. Though the HWVN region may not yield useful diagnostic information for every type of tissue, Puppels believes that "for any application this works for, there is no reason to make it more complicated."

Clearly the probe problem is one that has yet to be completely resolved. Whether complex microoptics or simply shifting the measured wavelength is the ultimate answer, these advances have definitely propelled tissue Raman much closer to the clinic.

Romancing the MDs

Many tissue Raman researchers also believe that they need to get physicians on board with their technique before it will take off. Much discussion in the community revolves around the best way to transition Raman into a medical setting. Richard Mendelsohn of Rutgers University points out that tissue Raman researchers may want to take cues from another form of spectroscopy that has successfully made the jump to the clinic: "The NMR people have solved this problem," he says, referring to the now-routine clinical use of magnetic resonance. As many researchers see it, gaining acceptance from physicians has two aspects: proving that Raman is as good as or better than the current gold standard, and making Raman technology so routine and easy that clinicians reach for it first when making a diagnosis.

The first aspect is a little tricky because it turns out that in this case, the gold standard of histopathology is a bit tarnished. "A lot of our work has been really understanding where the gold standard fails," says Stone. Tissue Raman researchers write their analysis algorithms on the basis of histopathological identification of specimens, so failure of the gold standard means problems with Raman identification, as well. "If there's a problem with your gold standard, you're never going to be able to prove that you're as good or better," says Mahadevan-Jansen. This problem may ultimately slow down the acceptance of Raman spectroscopy in the clinic, because researchers will likely have to carry out long trials to prove by outcome that their technique is better.

The second aspect to winning clinician acceptance involves smoothing over a lot of the complexities of tissue Raman spectroscopy. Researchers have spent years working out the intricate algorithms and instrumentation to make tissue Raman work, but clinicians don't want to see a spectrum and interpret it; they just want an answer. "All the background calculations should be done very, very quickly in real time so that the surgeons or clinicians can see exactly that if they place the probe on the tissue, as we routinely do on skin lesions, they get the information," says Ganesh Sockalingum of the University of Reims (France). "It should be an aid to the clinicians or surgeons, not a handicap to what they're doing every day," he adds.

And then, of course, many details that may not become apparent until Raman is moved into the clinic will need to be worked out. Mahadevan-Jansen relates her experience measuring Raman spectra in a hospital setting: "Any time we have a patient room where there's a window, we end up picking up a lot of noise. Even if the person's head is facing the window and we are looking at her cervix, we still end up picking up strange bands from sunlight," she says. "So we don't do studies in rooms with windows anymore."

Moving into the clinic

So what will it take to routinely put Raman into those windowless rooms? The answer is largely determined by exactly what kind of tissue is being analyzed.

Many researchers believe that routine clinical analysis of skin can be done today. Skin analysis largely sidesteps the probe problems discussed above, and recently, River Diagnostics, the first company dedicated solely to Raman spectroscopy of this application, was founded by Puppels. "What we're hoping is that by making the company successful, we'll make this whole field successful and give it a boost—show others that it's really possible," says Puppels.

Raman analysis of other tissues is on a much faster track to routine medical use than the thermometer, which took two centuries to make it to patients. Mendelsohn points out how far the field has already come: "When we started this in the late 1960s and early 1970s, you couldn't do anything with these technologies of any medical significance," he says. "Now, how the technology has improved—it's stunning!"

Jennifer Griffiths is an associate editor of Analytical Chemistry.

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