ELECTRONICALLY REPRINTED FROM JUNE 2015

Spectroscopy®

Solutions for Materials Analysis

Molecular Spectroscopy Workbench

Current Uses of Raman Microscopy in Biomedical Studies

There is growing interest in using Raman as a spectroscopic probe of biological systems based on its high information content, its compatibility with an aqueous environment, and the spatial resolution that is consistent with physical optics (as good as ~0.5 µm). Sampling hardware innovations in recent years such as cell sampling in a lab-on-a-chip and progress in the design of Raman endoscopes are enabling new realms of measurement. The integration of multivariate analysis into Raman mapping has enabled information to be teased out that might not be seen by the human eye. In addition, there are nonlinear techniques such as coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) that provide images more rapidly. For those who are contemplating jumping into this arena, this column will summarize where the field is now and what might be some pitfalls.

Fran Adar

In spite of the potential of Raman spectroscopy to provide an intrinsic means of studying biological systems, it is the complexity of the systems studied in combination with the complexity of the Raman spectra of large molecules (1) such as proteins, carbohydrates, nucleic acids, and membrane lipids that has prevented application of the technology to anything other than purified systems until recent years. After selecting this topic for the current column I realized that I have already written a column on this topic (2). But the field continues to evolve rapidly, and based on many talks that I heard at the International Conference on Raman Scattering (ICORS) 2014 (Jena, Germany), SciX 2014 (Reno, Nevada), and Pittcon 2015 (New Orleans, Louisiana), I felt it would be appropriate to revisit the topic. What is curious is that when I was hired in 1978, it was believed that the fields of biomedical and pharmaceutical research were expected to be fertile ground for development of applications of Raman instrumentation. On the other hand, based on my understanding of these fields from five years spent in the Biophysics Department at the University of Pennsylvania Medical School, I was quite skeptical that anything practical could be done, except for purified materials, or cases where the interest was in studying pigments experiencing resonance Raman (RR) phenomena, such as hemeproteins and carotenoids. However, the developments in instrumentation and data processing algorithms have changed all that. For a start, the microscope enables the study of single cells, subcellular structures, and histological sections. Current multichannel instruments acquire spectra in one-tenth to one-hundredth the time previously required. Availability of excitation wavelengths between 1.1 µm and 200 nm provide a means to customize the experimental conditions. Powerful computers enable application of multivariate statistical algorithms to large data sets that tease out information, often not obvious

to the human eye. This was all true in 2009, but the work done over the last six years shows that the potential continues to expand. First, I will discuss some instrumentation innovations that are beginning to provide breakthrough capabilities, and then some areas where clinically relevant results have been demonstrated.

Laser Trapping

Laser trapping of particles is a phenomenon known for quite a while (3) in which the photonic pressure gradient is able to balance gravitational forces resulting in static suspension of particles in space for examination, including measurement of Raman spectra. As early as 2005, single bacterial cells in solution were identified by their Raman spectra (4). More recently, a group at Cornell University exploited subwavelength liquid-core slot waveguides to overcome the limitations of examining particles smaller than a few hundreds of nanometers that cannot be trapped in available optical fields in diffraction-limited systems (5). In these devices, a slot waveguide with widths between 40 and 200 nm is immersed below a microfluidic channel at 90° to the direction of flow. As the fluid flows over the waveguide, particles from the fluid are attracted to and trapped in the waveguide. These authors were even able to demonstrate trapping of 48-kilobase λ-DNA in a 60-nm slot waveguide. Detection of the particles is by fluorescence tagging. These devices have been commercialized more recently, using a 785-nm laser for trapping, and for simultaneous excitation of Raman spectra that are recorded on a microscope (6).

Lab-on-a-Chip and Cell Sorting

Microfluidic devices allow for the capability to manipulate small volumes of fluids spatially, temporally, and chemically. For example, Raman was used by a group at the University of Bordeaux to study interdiffusion (7) and droplet formation in a microchannel (8).

More relevant to the topic here,

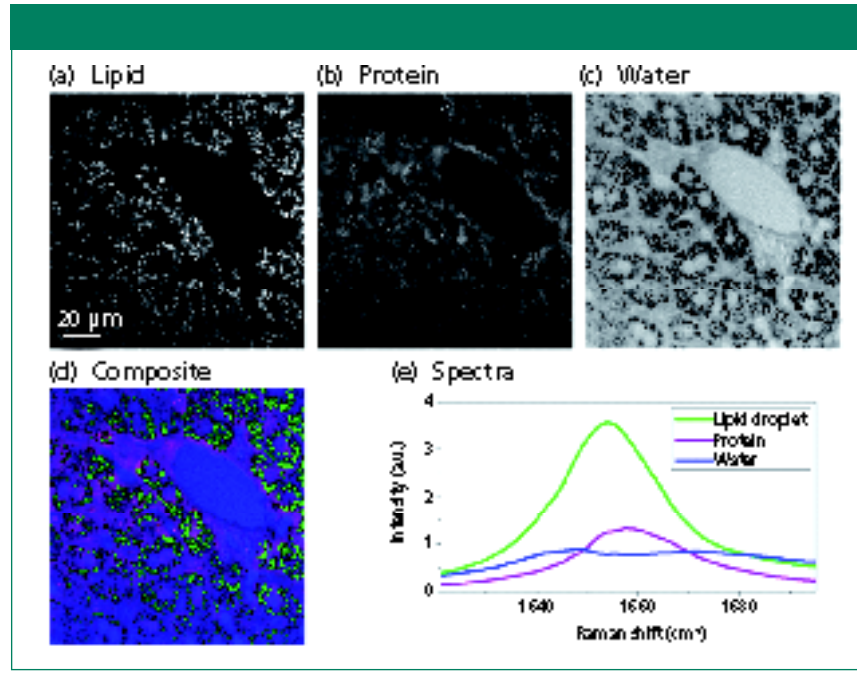

Figure 1: Hyperspectral SRS imaging and MCR analysis of a mouse liver tissue. (a–c) Concentration maps of lipid droplets, proteins, and water produced by MCR analysis of SRS images. Scale bar: 20 µm. (d) Composite image: green, lipid droplets; purple, protein; and blue, water. (e) MCR resolved spectra. Adapted with permission from reference 34.

Michael Ramsay's group used fluorescence signals to assay acute cytotoxicity both statically and dynamically (9). The test system described involved incubating cells with detergents that would disrupt cell membranes causing cell death. The status of the cells was monitored by measuring fluorescence signals that respond to cell status. In another example from the same year, Balagadde and colleagues (10) reported a microreactor designed to prevent biofilm formation by controlling the chemistry and movement of *E. coli* bacteria in the reactor. A synthetic population control circuit regulated cell density using a quorum sensing feedback mechanism allowing optical observation of morphological changes over hundreds of hours. But in the cases of both of these studies, optical detection was with fluorescent signals from exogenous molecules.

In 2009, Raman spectra of single cells were recorded so that phenotypic heterogeneity could be characterized as a means to understand cellular response to changing environmental conditions (11).

In 2011 Huang and colleagues (12,13) reported Raman-activated cell sorting (RACS) using a microfluidic device. While such a device has numerous applications in biology (such as linking gene expression to physiological states and phenotypes), the authors demonstrated its capabilities by growing bacteria in ^{13}C-enriched substrates. The goal was to determine the metabolic state of the bacteria. The authors pointed out that for technical reasons it has not been possible to cultivate many bacterial species in bulk, so a method to cultivate them in a bioreactor and then characterize single microorganisms can add to the elucidation of their biochemistry (for example, formation of biofilms, toxicity, and metabolic state). Differentiation of bacteria was accomplished by recognizing Raman spectral shifts because of the incorporation of ^{13}C from isotopically enriched substrates. Cell sorting can be accomplished with laser-tweezers (4,8) or microfluidics. These authors also explored ways to accelerate spectral acquisition of single organisms to accel-

erate the separation of individual organisms; they tested resonant Raman scattering (RRS), coherent anti-Stokes Raman scattering (CARS), stimulated Raman scattering (SRS), and surface-enhanced Raman scattering (SERS).

In 2013 Dochow and colleagues published a description of "Raman-on-chip" which used fiber Bragg gratings (FBGs) (see the next section), for laser-free detection of the scattered light (14,15). A near-infrared (NIR) laser to trap a particle, a Raman-excitation laser, and three fibers with FBGs for collection were all mounted in a planar array, and the light collected by the FBGs was easily transferred to an imaging spectrograph. Spectra of solutions of urea and nicotine down to the millimolar range were acquired in 10 s with 700 mW at 785 nm. Spherical particles of polystyrene were also tested to determine if the additional scattering would produce a strong overwhelming background, which it did not.

Raman Endoscope

Fiber-optic devices have been under construction for remote Raman measurements for more than 20 years. The original designs used a 6x1 fiber bundle. The central fiber delivered the laser beam and the surrounding fibers collected the Raman light. The central fiber was bifurcated from the others so that the laser could be launched into it, and the other fibers were then aligned in a line to match the spectrograph slit. The sizes of and spacings between the fibers were engineered to optimize efficiency as was the tip design and focusing optics. Optical filters had to be mounted at the sampling end to eliminate the silica Raman signal generated in the delivery fiber, and the unshifted laser signal in the Raman light. Fabrication and mounting of small filters to accomplish these tasks is time-consuming and expensive. While miniaturization of such a design for an endoscopic application is possible in principle, the cost and requirement for disposability makes it impractical.

The concept of a totally new design based on in-line FBGs to act as a notch filter in the collection path (that is, the grating reflects the laser and is therefore used in transmission), described at ICORS in Jena last summer, was previously published (14,15). But because the fabrication of FBGs requires single mode fibers 4–10 µm in dimension, the efficiency of collection of the Raman light is immensely reduced. To optimize collection efficiency, 19 single-mode cores were grouped hexagonally in a 125-mm-diameter fiber; by optimizing the relationship between the core diameter and the core spacing (pitch), core coupling was avoided. Note that the simulations made in designing the FBG characteristics included temperature changes (room temperature to body temperature) as well as bending-induced changes, so that a final design would be practical for endoscopic purposes. A final Raman probe was fabricated by mounting six such multicore single-mode fibers (MCSMF) around a 125-µm laser fiber. The probe was tested by measuring porcine brain whose spectrum showed characteristics of protein and lipid. Although the signal shown is credible, it is not yet of the quality required for endoscopic work, and efforts are ongoing to improve the performance.

Bacterial Infection

When an individual presents in a doctor's office or hospital emergency room with an infection, a course of antibiotic treatment has to be initiated immediately. However, the choice of antibiotic is critical so that it will be effective in treating the particular organism. In addition, as organisms develop resistance to particular antibiotics, more potent antibiotics have to be chosen and the antibiotics tend to become less benign as their potency increases. Currently, to make the best selection of antibiotic, the patient's blood (or other secretion) has to be cultured for up to three days to determine the best course of therapy. During this time the patient is treated with a broad spectrum antibiotic in the hopes that the disease will not progress until a better course of treatment is identified.

Several academic groups have been measuring Raman spectra of individual bacteria and showing that the spectra can be used to first differentiate species, and then strains (16–18). Because the spatial dimensions of a bacterium are commensurate with the laser focus, it is assumed that a Raman spectrum acquired with a microscope represents the entire organism. As an example of what can be done, Ute Neugebauer of Jena gave a talk at ICORS (19) that described direct identification of bacteria in urine on a small sensor chip, work that has been published (20). Urine samples were injected and then focused at the center of the chip using electric fields. Principal component analysis (PCA) training and prediction enabled differentiation between two common bacterial strains, one susceptible to and one resistant to vancomycin, a common antibiotic used for treatment. In a second example, Neugebauer showed that PCA scores of bacteria sequestered in cells enabled differentiation from extracellular bacteria; this is an important observation for detecting bacteria in chronic infections that would be silent in terms of disease, but could potentially be activated during immune weakness or could infect a second individual.

Note that characterization of bacteria can also be relevant in other areas such as food safety and cosmetic manufacture.

Raman Imaging of Diseased Tissue

In my 2009 article (2), I already noted that Raman images were being produced from hyperspectral cubes using multivariate techniques. Reference textbooks (21,22) as

well as review articles (23) have appeared. The article by Tu and Chang (23) does not show many Raman images but does discuss some of the algorithms used to decompose the spectra, which are essential for constructing meaningful images. Figure 3 in that article, however, does show how a nondescript image constructed using CH intensity was vastly improved when hierarchal cluster analysis (HCA) was applied. Another article that can be useful includes an extensive table of analytical bands where a frequency shift is associated with a specific type of motion of a particular functional group (24). It is this type of information that will enable the association of Raman images to particular disease states. For example, diseased tissue may contain lipid material. The factor used to produce the loadings in the image will give insight into the type of species in this material. Is the lipid saturated? Is it free fatty acid or esterified? Is there phosphate? Is there eicosanoid in the lipid? Which one? Can the lipid head group be identified? If spectra of the suspected pure species are available, the composite spectra can be constructed to estimate the composition (25). Bridging the gap between spectral interpretation and disease can be found in other reviews (26,27), which also include literature references to studies of metabolic changes and disease states. Ultimately it is "the collective characterization and quantification of pools of biological molecules that translate into the structure, function, and dynamics of an organism or organisms" (28) that one would hope that the Raman microscope could provide. In their work on bacterial identification, Huang and Spiers (29) have realized the importance of developing a systematic informatics for the emerging "omics" technologies. Here is what they say in their abstract:

> Raman spectroscopy of single bacteria provides an OMIC-like view of the chemical status of individual cells, reporting on metabolism, cell stress and growth, and is likely to become a significant tool in environmental and medical microbiology. We advocate the early development of integrated data models and informatics frameworks, in parallel with the development of Raman hardware and experimental protocols, in order to maximize the benefits of this emerging OMIC technology to the research community.

Ultimately, the goal is to extract biochemical information from the Raman spectra and images. What Huang is saying for microbiological systems is equally valid for the imaging of tissue systems.

Coherent Raman Imaging

Raman mapping, in combination with multivariate analysis, is known to provide useful images. But because of the weakness of the Raman process, the acquisition of these images is slow. Over the past 10 years or so, Sunney Xie's group at Harvard has been developing methods to acquire Raman images using multiphoton processes. These processes have been known for quite some time, but with the availability of evolving laser technology, implementation possibilities have multiplied. While it is really beyond the scope of this column to review the field, our intent is to comment on work that has been done that is showing the evolution of the field and its potential. At a minimum we can say that this technology, which includes both CARS and SRS, uses two laser beams that intersect at the sampling position, and whose difference in energy corresponds to the Raman frequency of an analytical vibration. In 2006 Richard Mathies's group published a review of the use of femtosecond stimulated Raman spectroscopy for following temporal processes of biochemical reactions (30). For example, his measurements can follow reorganization of visual pigments that occurs as part of the light detection process. But Mathies's review does not deal with Raman imaging. As early as 1999 Xie and colleagues (31) demonstrated the ability to record CARS images, which showed promise because of the higher sensitivity over spontaneous Raman imaging. However, from the beginning, it was recognized that CARS suffers from interference from nonresonant backgrounds that distorts spectral line-shapes and causes difficulty in interpretation. More recently Xie's group reported SRS of biological tissues, noting that the problems encountered with CARS were eliminated because of the absence of the interfering background continuum and because the intensity scaled with the amount of material present (32,33). To prevent laser damage to the biological samples of interest, a high-repetition-rate picosecond pulse train was used, but the peak power was lowered by three orders of magnitude (relative to previous work). The Stokes beam was a mode-locked Nd:YVO_4 laser (1064 nm fixed wavelength) and the pump beam was a synchronously pumped, tunable optical parametric oscillator (OPO). To boost the signals ($\Delta I_P/I_P$ and ($\Delta I_S/I_S$) over the laser noise, a high-frequency, phase-sensitive detection scheme was implemented. The biomedical applications that were demonstrated included imaging distributions of omega-3 fatty acids and saturated lipids in living cells, brain and skin tissues as well as using lipid contrast and drug delivery through the epidermis. CARS images were shown for some of the examples, and demonstrate the superior behavior provided by SRS.

But after demonstrating the possibilities of SRS for vibrational and chemical imaging, it became clear that a significant drawback was that the images were being created one-Raman-shift-at-a time. In their 2014 review of the field, Zhang and colleagues (34) described various methods that have been developed for spectrally multiplexing the acquisition of SRS images. One can now use well-known multivariate techniques to resolve chemical species whose spectra overlap, making the selection of isolated analytical wavelengths impossible. Figure 1, reproduced from reference

34 (Figure 7) clearly illustrates contrast between lipid, protein, and water, even though the three species have overlapping bands centered between 1640 and 1660 cm^{-1}. This image could not be constructed without the hyperspectral cube over this range and chemometrics algorithms.

Summary

Writing this column installment has enabled me to think about the developments that have caught my attention during the last year: developments in instrumentation, bacterial identification, and Raman imaging for diagnosis of disease and metabolic conditions. But there are interesting developments that I haven't even mentioned: single molecule detection, optical super-resolution (for which the Nobel Prize was recently awarded), identification of cancer biomarkers, interactions of nanoparticles with cells for imaging, diagnosis, cancer therapy, targeted drug delivery, and targeted gene delivery. Another topic that I considered including here was the design of SERS particles and substrates. Our understanding of the origin of the large electric fields giving rise to the enhancements is improving immensely so that one might expect that analytical use of SERS is on the horizon. But I will leave this topic to my next column. It will also be a second installment of a topic already considered!

References

(1) J. De Gelder, K. De Gussem, P. Vandenabeele, and L. Moens, *J. Raman Spectr.* **38,** 1133–1147 (2007).

(2) F. Adar, *Spectroscopy* **24**(3), 26–31 (2009).

(3) A. Ashkin, J.M. Dziedzic, J.E. Bjorkholm, and S. Chu, *Opt. Lett.* **11,** 288–290 (1986).

(4) C. Xie, J. Mace, M.A. Dinno, Y.Q. Li, R.J. Newton, and P.J. Gemperline, *Anal. Chem.* **77,** 4390–4397 (2005).

(5) A.H.J. Yang, S.D. Moore, B.S. Schmidt, M. Klug, M. Lipson, and D. Erickson, *Nature* **457,** 71–75 (2009).

(6) http://www.opfluid.com/#!nanoparticle-identification/c1ygk

(7) J-B Salmon, Al Aidari, P. Tabeling, L. Servant, D. Talaga, and M. Joanicot, *Appl. Phys. Lett.* **86,** 094106 (2005).

(8) G. Cristobal, L. Arbouet, F. Sarrazin, D. Talaga, J.L. Bruneel, J. Joanicot, and L. Servant, *RSC/Lab Chip* **6,** 1–8 (2006).

(9) C.R. Poulsen, C.T. Culbertson, S.C. Jacobson, and J. M. Ramsey, *Anal. Chem.* **77,** 667–672 (2005).

(10) F.K. Balagadde, L. You, C.L. Jansen, F.H. Arnold, and S.R. Quake, *Science* **309,** 137–149 (2005).

(11) A. Hermelink, A. Brauer, P. Lasch, and D. Naumann, *Analyst* **134,** 1149–1153 (2009).

(12) M. Li, J. Xu, M. Romero-Gonzalez, S.A. Banwart, and W.E. Huang, *Curr. Opin. Biotechnol.* **23,** 1–8 (2011).

(13) W.E. Huang, A.D. Ward, and A.S. Whiteley, *Environ. Microbio.l Rep.* **1,** 44–49 (2009).

(14) I. Latka, S. Dowchow, C. Krafft, B. Dietzek, H. Bartelt, and J. Popp, *Proc. SPIE* 8087 80872D, 80872D-8 (2011).

(15) S. Dochow, I. Latka, M. Becker, R. Spittel, J. Kobelke, K. Schuster, A. Graf, S. Bruckner, S. Unger, M. Rothhardt, B. Dietzek, C. Krafft, and J. Popp, *Opt. Express* **20**(18), 20156–20169 (2012).

(16) W.E. Huang, M. Li, R.M. Jarvis, R. Goodacre and S.A. Banwart, in *Advances in Applied Microbiology Vol. 70* (Academic Press, Burlington, Massachusetts, 2010), pp. 153–186.

(17) M. Harz, P. Rosch, and J. Popp, *Cytometry, Part A* **75A,** 104–113 (2009).

(18) J.F.M. Almarashi, N. Kapel, T.S. Wilkinson, and H.H. Telle, *Hindawi Spectroscopy* **27**(5–6), 361–365 (2012).

(19) U. Neugebauer, "Spectroscopic Detection of Infection," presented at ICORS, Jena, Germany, 2014.

(20) U. Neugebauer, S. Kloß, U-C. Schroder, P. Rosch, and J. Popp, *Optic&Photonik* **4,** 36–39 (2013).

(21) *Vibrational Spectroscopy for Medical Diagnosis*, M. Diem, P.R. Griffiths, and J.M. Chalmers, Eds. (Wiley, Chichester, UK, 2008).

(22) *Biomedical Vibrational Spectroscopy,* P. Lasch and J. Kneipp, Eds. (Wiley-Interscience, Hoboken, New Jersey, 2008).

(23) M.S.Q. Tu and C. Chang, *Nanomedicine: Nanotech. Biol. and Med.* **8,** 545–558 (2012).

(24) Z. Movasaghi, S. Rehman, and I.U. Rehman, *Appl. Spectrosc. Rev.* **42,** 483–541 (2007).

(25) BioRAD KnowItAll, http://www.bio-rad.com/en-us/product/knowitall-raman-id-expert.

(26) D. Naumann, *Appl. Spectrosc. Rev.* **36**(2,3), 239–298 (2001).

(27) D.I. Ellis and R. Goodacre, *The Analyst* **131,** 875–885 (2006).

(28) http://en.wikipedia.org/wiki/Omics.

(29) W.E. Huang and A.J. Spiers, *OMICS: J. Integr. Biol.* **10**(2), 238–241 (2006).

(30) P. Kukura, D.W. McCamant, and R.A. Mathies, *Annu. Rev. Phys. Chem.* **58,** 461–488 (2007).

(31) A. Zumbusch, G.R. Holtom, and X.S. Xie, *Phys. Rev. Lett.* **82,** 4142–4145 (1999).

(32) C.W. Freudiger, W. Min, B.G. Saar, S. Lu, G.R. Holtom, C. He, J.D. Tsai, J.X. Kang, and X. S. Xie, *Science* **322,** 1857–1861 (2008).

(33) C.W. Freudiger, W. Min, B.G. Saar, S. Lu, G.R. Holtom, C. He, J.D. Tsai, J.X. Kang, and X.S. Xie, *Science* **322,** 1857–1861 (2008).

(34) D. Zhang, P. Wang, M.N. Slipchenko, and J. Cheng, *Acc. Chem. Res.* **46,** 2282–2290 (2014).

Fran Adar is the Principal Raman Applications Scientist for Horiba Scientific in Edison, New Jersey. She can be reached by e-mail at fran.adar@horiba.com

For more information on this topic, please visit: www.spectroscopyonline.com/molecular-spectroscopy-workbench-column