

Raman microscopy: Taking chemical microscopy to the next level

Barbara Foster

Although Raman microscopy preceded infrared microscopy commercially by more than five years, infrared microscopy has dominated the market for the past 20 years. Now easier to use and more accessible to the general analytical laboratory population, Raman microscopy is coming into its own.

Raman extends chemical fingerprinting to aqueous samples, as well as those with N-H functional groups, and expands traditional materials applications such as polymer analyses, multilayer film measurements, and corrosion and contamination studies into the realms of biology, biotechnology, and drug discovery. The microscopy component greatly simplifies sample location and provides ancillary image-based information. Innovative tools such as the LabRam™ (Jobin Yvon, Edison, NJ) merge Raman

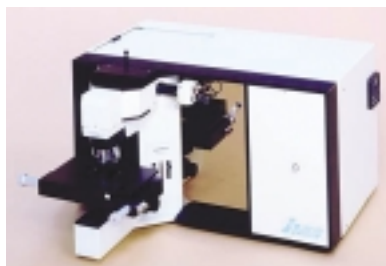


Figure 1 LabRam Raman microprobe with integrated Olympus microscope.

[ATR] and the Schwarzschild all-reflecting optics) needed for imaging and measuring in the IR. For biological and living cell applications, an inverted model is now available. Both approaches retain common contrast enhancement techniques, including fluorescence, differential interference contrast (DIC), and phase.

The standard system has two detectors: a charge-coupled device

mentary FTIR domain, Jobin Yvon adds the IlluminatIR™ (SensIR Technology, Danbury, CT).¹

Depending on its configuration, LabRam's output ranges from a microscope image to a variety of Raman, FTIR, and/or fluorescence spectra. Spectral data can be collected and displayed as single line scans or full chemical spectral maps, reminiscent of energy dispersive X-ray spectroscopy (EDX) or wavelength dispersive spectroscopy (WDS) maps from the world of electron microscopy. The microprobe can measure from areas as small as 1 μm in the XY direction and depths as shallow as 2 μm.

The power of Raman mapping

Raman maps are built from the spectra collected at each point in an image, obtained by one of three different methods. Global imaging is conceptually the simplest and fastest, providing an XY map of intensities at a particular wavelength. However, it can miss fine detail and tends to have poor contrast due to lack of confocality and the presence of significant background spectra.

Point-by-point imaging couples the mobility of a motorized scanning stage with the high numerical aperture of the 100× microscope objective to take the full spectrum at each XY point. While it provides better spectral and spatial resolution as well as full spectra, it is much slower. The patented "confocal line scanning technique" (Jobin Yvon) provides the best solution by taking advantage of the linear entrance slit in the spectrometer and the short dimension of a two-dimensional array detector. This approach yields faster scans with higher spectral resolution and lower background interference.

Now easier to use and more accessible to the general analytical laboratory population, Raman microscopy is coming into its own.

with microscopy, making it possible to switch quickly from conventional light or confocal imaging modes to Raman, fluorescence, and even FTIR spectroscopy.

The microRaman interface

Figure 1 shows a typical LabRam Raman microprobe with an integrated Olympus BX microscope (Olympus Corp., Melville, NY). The most common configuration is built around an upright stand fitted with a mixed set of conventional microscope optics (typically 10× and 100× objectives) and IR optics (attenuated total reflection

(CCD) video camera for standard digital imaging, and a spectrometer with a CCD spectral detector for Raman analyses. For multilayer applications, a built-in confocal system and motorized focus drive enable spectra collection from well-defined Z planes in the sample, and a motorized scanning stage can be added for point-by-point or confocal line scanning imaging of a 2-D region.

Routine Raman analyses use a helium-neon (HeNe) laser for illumination, but other lasers are available that expand the spectral range from the near IR to the UV. To extend the system into the comple-

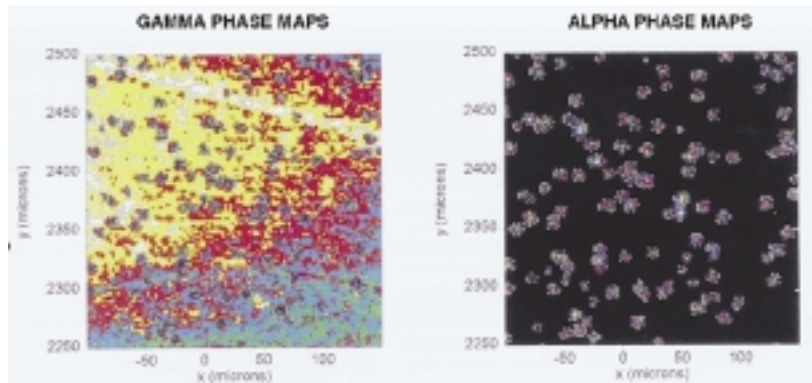


Figure 2 Alpha (R) and gamma phase (L) after 15 min at 1100 °C.

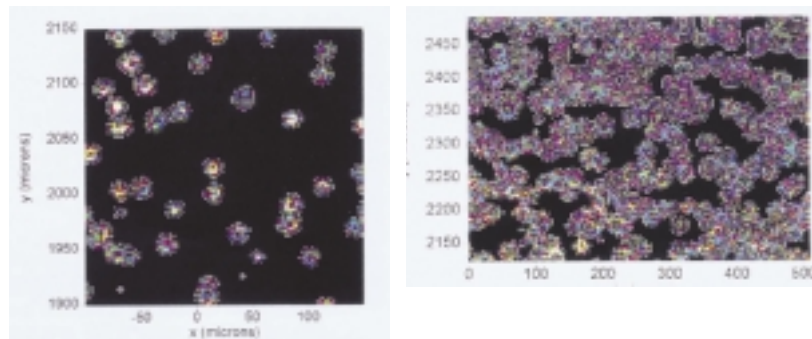


Figure 3 Growth of alpha stage at (L) 30 min and (R) 60 min.

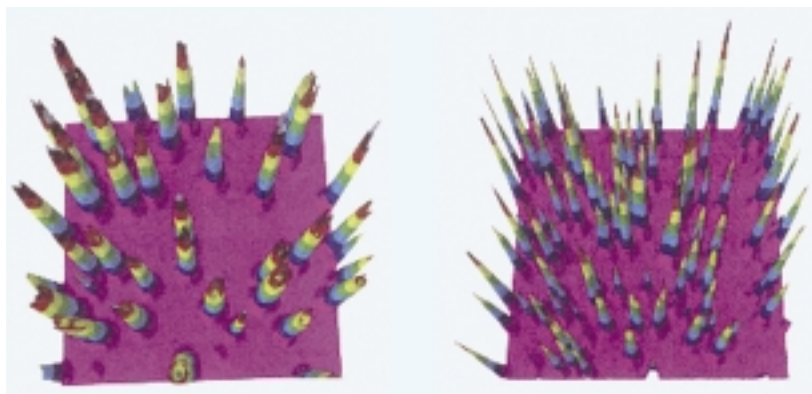


Figure 4 Three-dimensional intensity maps after (R) 15 min and (L) 30 min.

Raman maps: New insight into kinetics and processes

Metal oxides have such unique Raman spectra that it is often easy to differentiate areas due to one crystalline polymorph versus another, as well as determining the degree of hydration of the metal cation, quickly and nondestructively. This combination of information affords a rare perspective into the oxidation mechanism. In particular, knowing which oxida-

tion and hydration complexes are present at various depths provides clues as to how surfaces can be better protected against corrosion, erosion, and ultimate failure.

For example, three samples of a NiAl superalloy were heated to 1100 °C for 15 min, 30 min, and 60 min.² This type of alloy often contains chromium as a trace impurity. During oxidation, unique crystalline structures form for the alpha and gamma phases of Al₂O₃. Each phase

affects the fluorescence spectrum of the oxidized chromium contaminant differently, providing a tool for mapping the location of that phase.

Using a multichannel detector, multiple spectra are collected simultaneously. The data are plotted as pseudo-colored maps, with sequential colors corresponding to changing spectral intensity. *Figure 2* demonstrates the complementary nature of the two Raman maps: The alpha phase (R) nucleates at discrete points within the continuous gamma phase (L). Over time, the alpha phase grows at the expense of the gamma polymorph (*Figure 3*).

Figure 4 illustrates an interesting and, to date, unexplained phenomenon. By extending the 2-D Raman maps into 3-D intensity plots, a fine structure emerges for the islands of the alpha phase. Initially, they are sharp cones with the maximum intensity in the center of the cone. However, at some

Raman maps are built from the spectra collected at each point in an image, obtained by one of three different methods.

point between the 15- and 30-min readings, the center of the cone collapses. The maxima now form a raised ring around a hollow core.

In addition to showing the distribution of one phase within another, the Raman spectra also map the strain experienced by the alpha phase as those domains grow in size, providing a noncontact analysis that can predict the failure of structures such as turbine engines.

Raman plus confocal: A multilayer solution to securing forensic evidence

Differentiating one component from another in a multilayer system has long been a serious chal-

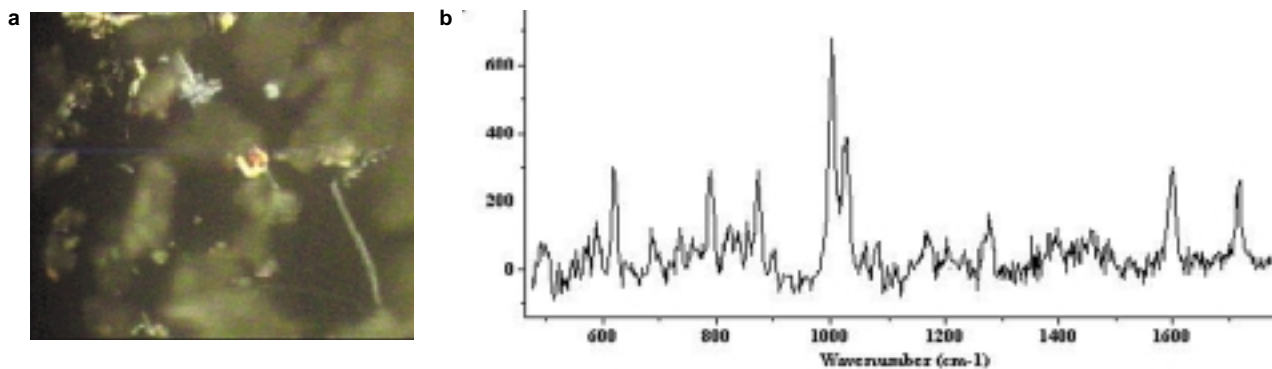


Figure 5 a) Crossed polar image of unknown substance from drug raid. b) Raman spectrum, confirming cocaine.

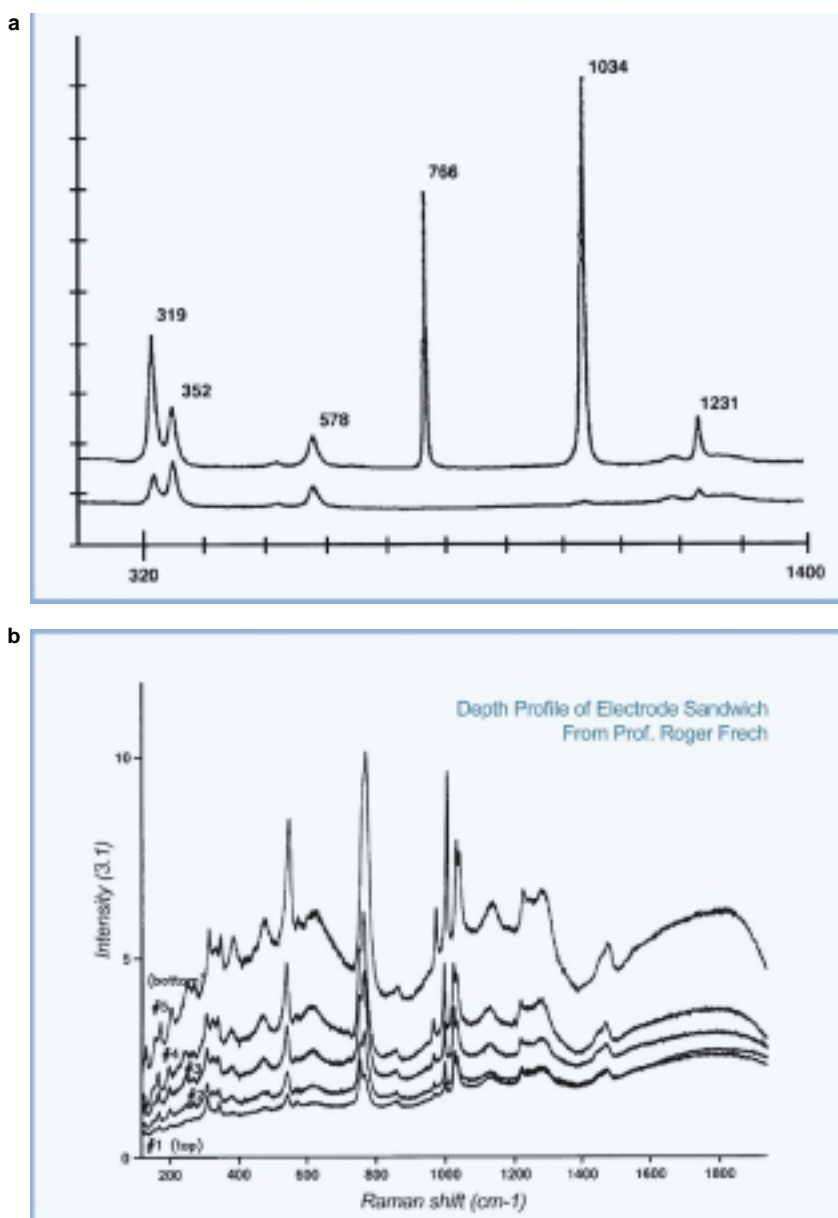


Figure 6 a) Spectrum: triflate in water. b) Spectra of Li⁺:triflate complex at varying depths.

challenge for microscopists. Combining Raman with confocal microscopy not only solves that problem, but also resolves issues such as measuring the thickness of each layer and observing processes that occur in specific layers.

In a simple example, Raman confocal helped maintain the security of critical evidence in a drug case. Traditionally, an unknown sample seized in a drug raid would first be sprinkled on a slide and placed under a light microscope for routine polarized light imaging, then moved to a Raman spectrometer for chemical analysis. *Figure 5a* shows the microscopic image of such a mixture, observed between crossed polars. Notice that some of the crystals appear bright yellow against the darker matrix of the cutting agent. The Raman spectrum of those crystals (*Figure 5b*) confirms that they are cocaine.

However, removing a powder from its original container always leaves an opening for challenge from the defendant's lawyer. In at least one instance, LabRam solved that problem. Using its confocal mode, the system imaged through the sealed plastic bag found at the drug scene, to the level of the drug. A variable aperture pinhole at the entrance to the spectrometer defined the depth of the sampled area and the intensity of the acquired signal. The resulting spectrum confirmed the presence of cocaine, without potential for contamination or dispute, according to Deborah Cole and Tom Brettel of the New Jersey State Police.

continued

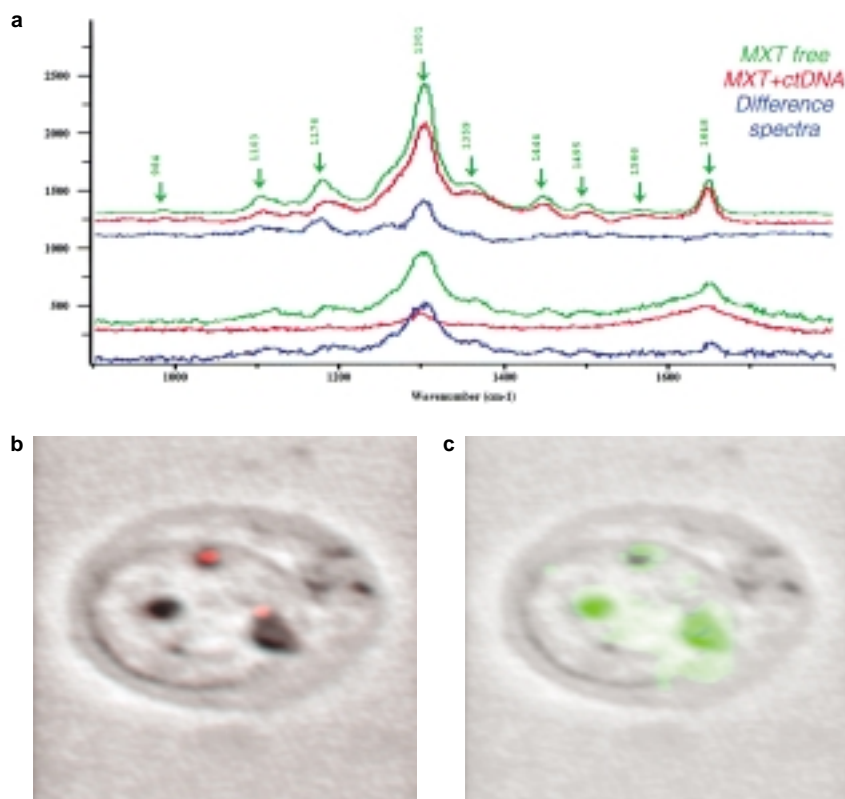


Figure 7 a) Raman spectra of free versus bound MXT. b) Raman map of cell showing free MXT entering cell. c) Raman map showing locations of MXT bound to DNA.

Microbatteries: Analyzing processes at changing depths

In a more complex situation, Raman confocal elucidated the charge transfer in a microbattery of the type used in microelectronics. In these batteries, lithium ions conduct charge by diffusing from the electrode through a polymer layer. Until recently, understanding and measuring the diffusion process has been a challenge. However, the ability of Raman confocal to acquire spectra at very specific depths tracked the process very precisely.³

To mimic the construction of a battery, a layer of poly(ethylene oxide), was cast on a 20- μm -thick vanadium oxide ceramic substrate. PEO is transparent in the visible range. The polymer was doped with two ions: Li^+ , the mobile cation, and the anion triflate (trifluoromethanesulfonate). Li^+ ions can form coordination complexes with five oxygen atoms from the triflate and/or oxygen from the host polymer.

Triflate spectra were acquired at different depths in the polymer at specific times. The standard focus knob on the microscope was used to set the depth. Although this process is valid for information

Knowing which oxidation and hydration complexes are present at various depths provides clues as to how surfaces can be better protected against corrosion, erosion, and ultimate failure.

collected from surfaces, the refractive index of the polymer (typically on the order of 1.5) creates both optical depth and spectral artifacts. Simple mathematical corrections adjust for real depth as well as for any changes in spectral intensity due to alterations in optical path for the excitation light from the laser and for scatter and absorption in the polymer layer.

Figure 6 clearly illustrates the results. The reference spectrum of the uncoordinated triflate in

water shows characteristic strong singlet peaks at 766 nm and 1034 nm (*Figure 6a*). As the Li^+ forms complexes with the triflate's oxygen atoms, these peaks split (*Figure 6b*). The two components of the doublet change in intensity as a function of distance to the polymer/electrode interface, presumably due to differences in the coordination complexes with or without Li^+ .

New answers for cell biology and the pharmaceutical industry

By far the most active, current application for Raman is the pharmaceutical industry. Applications include identity testing, characterization of molecular structure resulting from chemical reactions, crystallization, formulation, and dissolution testing.

In addition to the characterization of the pharmaceuticals themselves, there are questions about how a drug candidate reacts with a living cell. Early answers came from flow cytometry. Unfortunately, this approach only tells how many cells interacted with the drug. It lacks specificity as to mechanism and location. In contrast, Raman confocal studies show exactly where the drug is located, as well as when it arrives (intracellular kinetics), the

exact nature of the drug:target interaction, and the concentration of the drug in specific subcellular features.

Prof. Michel Manfait (University of Reims, France) has been using Raman confocal to study how the drug Mitoxantrone (MXT) binds to nuclear DNA.⁴ Because the bound species produces a different fluorescent spectrum than the nonbound, the Raman microscope can map the active location of the drug.

Figure 7a shows three spectra. The green line in each is the spec-

trum of the free species of the drug MXT, while the red line is the spectrum of the drug after it has bound to DNA. The blue line is the difference between the two spectra. The lower set of spectra illustrates a resonance effect between the incoming laser light and the colored agent in

Raman microscopy differentiates layers and measures thicknesses that previously required cross-sectioning or were not possible because of a lack of contrast.

the cell (resonance Raman spectra [RRS]), while the upper set illustrates the enhancement derived from the use of special gold particles (surface-enhanced Raman [SERS]).

In Figure 7b and 7c, the corresponding RRS and SERS maps

overlaid the microscopic image of a human cell affected by leukemia, showing the exact location of the active drug.

The spectra were collected in confocal line scan mode and then deconvolved to isolate the unique contributions from the two species and separate the MXT information from the cell's background fluorescence. This sophisticated processing and imaging was only possible because the Raman confocal could collect full spectra at each point in the image.

Conclusion

Raman microscopy solves many challenges faced by microscopists. It differentiates layers and measures thicknesses that previously required cross-sectioning or were not possible because of a lack of contrast. It images surfaces, but adds the capability of optically mapping processes, tracking both physical and chemical changes. Now easier to use, faster, and more flexible, Raman microscopy is having a major impact on research, process analysis, and quality control.

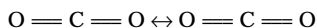
References

1. Foster B. Focus on microscopy: From light microscopy to molecular analysis at the touch of a button. *Am Lab* 2001; 33(22):42-9.
2. Clarke D, Freck R. Observing oxidation kinetics on an aluminum alloy surface with fluorescence mapping. *Jobin Yvon appl note* 5, Sept 1995. Samples provided by Prof. David Clarke, Univ Calif at Santa Barbara, Materials Dept.
3. Microscopic measurement of diffusion. *Jobin Yvon tech note*, Nov 1995.
4. Chourpa I, Manfait M. Pharmacokinetics on living cells. *Jobin Yvon appl note* RA5, in press.

Ms. Foster is President, Microscopy/Marketing & Education, 125 Paridon St., Ste. 102, Springfield, MA 01118, U.S.A.; tel.: 413-746-6931; fax: 413-746-9311; e-mail: bfoster@mme1.com.

How does Raman work?

Microscopists involved with fluorescence will find many similarities in Raman. Raman uses monochromatic laser as an excitation source. As in fluorescence, when a photon hits a Raman-susceptible molecule, it causes a transition. In fluorescence, the photon is absorbed, causing bonding electrons to jump to a higher energy state. In Raman, the photon bounces off susceptible electrons in the molecule, causing that section of the molecule to vibrate. A wooden ball mounted on the end of a spring is a good model. After a Raman excitation, the spring extends and compresses.



In fluorescence, a small amount of energy is absorbed as heat, and then the remaining energy is emitted as light when the electron returns to ground state. This process (high energy in, low energy out) is called a Stokes' shift and permits spectral separation of the incoming excitation from the emitted fluorescence.

Similarly, in Raman, the energy released after the light/matter interaction is decreased by the energy absorbed to cause the molecular vibration; this is another Stokes process.

$$\Delta v = v_{\text{laser}} - v_{\text{Raman}}$$

The pattern of the resulting spectral bands provides a fingerprint of the material under observation. The spectra are plotted in wave numbers (waves/cm or cm^{-1})* and provide either chemical identity and/or information about physical properties such as strain in solid materials, pressure of gases, or crystallinity and/or orientation in polymers.

*To convert from wave numbers to the more conventional microscopy notation of wavelength (μm): $v (\text{cm}^{-1}) = 10,000/\lambda (\mu\text{m})$. Normal green light (500 nm) = 20,000 cm^{-1} ; Raman operates in the 0-4000 cm^{-1} shift range.