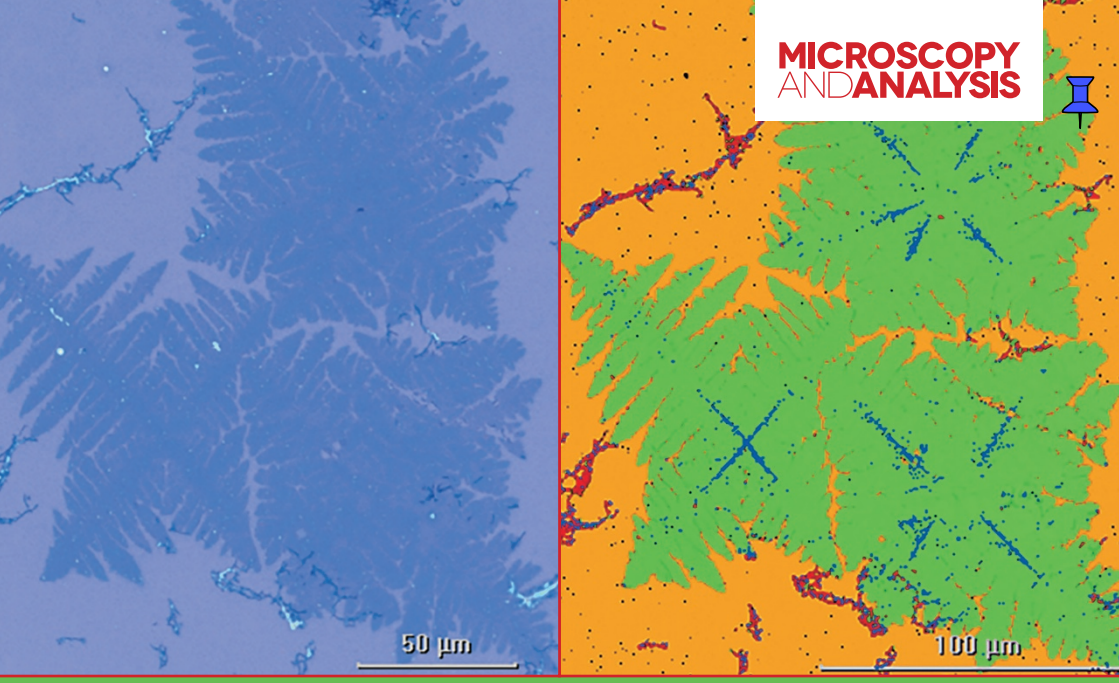

MICROSCOPY AND ANALYSIS

Raman Microscopy

Essential Knowledge Briefings

First Edition, 2017

thermo scientific

Front cover. Left: graphene was grown using chemical vapor deposition on the surface of a copper substrate, then transferred to a silicon substrate. The visual image shows the dendritic (fern-like) growth pattern. Right: the Raman image, collected using the Thermo Scientific™ DXR™2xi Raman Imaging Spectrometer, shows the silicon (orange), single layer graphene (green), defect regions (blue) and multi-layer graphene (red). The graphene sample was generously provided by Michael S Arnold and Robert M Jacobberger, Department of Material Science and Engineering, University of Wisconsin, Madison, WI, USA.

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Microscopy EKB Series Editor: Dr Julian Heath
Spectroscopy and Separations EKB Series Editor: Nick Taylor

CONTENTS

About Essential Knowledge Briefings

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INTRODUCTION

The absorption and scattering of sunlight by matter is a phenomenon known to all on an everyday basis. It is the reason why the sky and seas are blue, why plants are green, why rainbows have their brilliant colors and why sunglasses protect your eyes. Some of these everyday phenomena involve elastic scattering – scattering with no loss of energy of the emitted light. But sometimes the light that is emitted is of a different wavelength because it has lost some of its energy; this is called inelastic scattering.

The Raman effect is one form of inelastic scattering of light and is the basis of a powerful form of non-destructive chemical analysis called Raman spectroscopy. By measuring the degree of energy loss of light, the analyst can identify specific chemical bonds in a sample and obtain a chemical fingerprint of the molecules in the sample, thus allowing its identification. Furthermore, by performing Raman spectroscopy on a microscope the analyst can also reveal the spatial distribution of molecular components within the sample at micrometer or even nanometer resolution. This is Raman microscopy or Raman microspectroscopy and is the subject of this EKB.

This EKB starts with the discovery of elastic and inelastic scattering and outlines the physics of the interaction of light and molecules. It then goes on to explain how these principles are incorporated into modern light microscopes to allow Raman imaging or Raman microspectroscopy. This is followed by a detailed explanation of the main ways in which researchers use Raman microscopy, from preparing samples for analysis to obtaining Raman spectra and maps. The EKB also discusses

practical issues that need to be considered when working with Raman microscopy, how to solve potential problems, and concludes by looking forward to potential future developments.

HISTORY AND BACKGROUND

When light interacts with matter of any kind it is either transmitted, absorbed or passes onwards in a process we call scattering. Plants look green because the blue and red wavelengths of white sunlight are absorbed and only green light is reflected to our eyes. In contrast, the sky appears blue because shorter-wavelength blue light is scattered more strongly by the gas molecules and tiny particles in the Earth's atmosphere than are longer wavelengths (green and red) so that mostly blue light reaches our eyes.

When there is no loss of energy of the scattered light the process is called elastic or Rayleigh scattering, named after the English physicist Lord Rayleigh (John William Strutt, 1842–1919). Rayleigh scattering is inversely proportional to the fourth power of wavelength, which is why blue light is more strongly scattered in the sky than red.

During the interaction of light and matter, some of the molecules can begin to vibrate, taking some energy from the incident light. The remainder of the incident light scatters away inelastically. The loss of energy increases the wavelength of the scattered light as these two parameters are inversely related.

The discovery of inelastic scattering began almost a century ago with the work of Adolf Smekal, who in 1923 postulated that when light hits a molecule some of the scattered light should be of a different color (frequency) than the incident light. This was confirmed experimentally in 1928 by the research of two Indian

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Energy $E = h\nu$ where h is Planck's constant (6.63×10^{-34} joules.second) and ν is frequency (cycles per second).

Wavelength $\lambda = c / \nu$ where c is the speed of light (3.00×10^{8} meters per second).

Figure 1. Sir Chandrasekhara Venkata Raman 1888–1970. A copy of a portrait by Homi Bhabha, the famous Indian theoretical physicist who also had a very considerable reputation as a painter. Image held in the archives of the Tata Institute of Fundamental Research, Mumbai

physicists, Chandrasekhara Raman (Figure 1) and Kariamanickam Krishnan. They were investigating the transmission of monochromatic (single wavelength) light from a mercury arc lamp through solids, liquids and gases. By using a spectrograph that dispersed the transmitted light onto a photographic plate they noticed a few extra lines of different wavelengths next to the main unaltered signal from the arc lamp, indicating that the scattering process had caused a loss of energy of some light. This is now called the Raman effect or Raman scattering, and for his work Raman received the Nobel prize in physics in 1930.

By the end of the 1930s, Raman spectroscopy had become a mainstream method for non-destructive chemical analysis, though was mostly used by research spectroscopists for the analysis of bulk samples. But it took another 40 years before Raman microspectroscopy was established.

The first reported use of Raman spectroscopy on a light microscope was from Delhaye and Dhamelincourt in 1975, working at the University of Lille in France. An instrument based on their work called the MOLE (molecular optics laser examiner)

was commercialized by the French company Jobin Yvon in the same year. In 1977 Jobin Yvon released an improved version called the Raman microprobe.

Since then the proliferation of lasers, better optical filters and more sensitive charge coupled device (CCD)-type detectors has allowed the growth of Raman microscopy and there are now over 10 major players in the Raman microscopy market who supply instruments of differing specifications for different applications.

The principle of Raman spectroscopy is based on the interaction of light or photons with vibrations (the stretching and bending of bonds) or phonons (the vibrations of crystal lattices) in molecules. Raman scattering is a function of the polarizability of the bond, or how easy it is to move electrons within the molecule. In general, bonds that have a weak dipole moment often have a high polarizability and the vibrational modes of the bond are Raman active because light polarizes the cloud of electrons around atomic nuclei.

Figure 2, called a Jablonski diagram, schematically shows what happens during both elastic and inelastic scattering. In elastic (Rayleigh) and spontaneous Raman scattering, the incident light boosts the molecule into an extremely short-lived virtual state. If the molecule returns directly to the ground state then the light is elastically scattered – no energy is exchanged. This is Rayleigh scattering. Most photons are Rayleigh scattered, however on very rare occasions (about one in ten million collisions) a change in the polarizability of the bond causes the photon to donate some of its energy to the molecule, leaving it in an excited state. Therefore the scattered photon has a lower energy or frequency; this is called Stokes scattering, one component of the Raman effect.

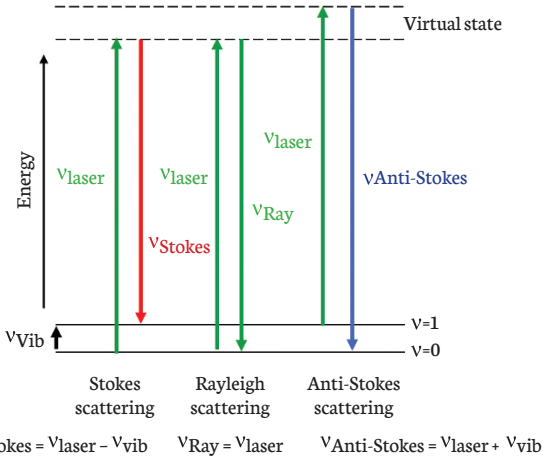

Figure 2. Jablonski diagram showing energy levels in Stokes, Rayleigh and anti-Stokes scattering. (© Thermo Fisher Scientific, used by permission)

If the molecule is already in an excited state before the light strikes (due to thermal or other excitation), then relaxation adds this energy to the scattered light, resulting in a higher-energy scattered photon. This is called anti-Stokes scattering. In spontaneous Raman, the anti-Stokes lines are far less intense because most molecules are in the ground state, though there are techniques that can stimulate anti-Stokes lines (such as coherent anti-Stokes Raman spectroscopy [CARS]).

The Stokes and anti-Stokes photons are shifted relative to the Rayleigh line, called the Raman shift. The degree of Raman shift is specific for the type of intramolecular bond; for example when a molecule containing hydrocarbon bonds (C–H) is illuminated by 780nm laser light, Raman scattered light at 1018nm is emitted, a shift of 238nm. The exact amount of shift can vary according to factors such as the bond's environment and applied stress. (Note that spectroscopists prefer to describe the Raman response using units

called wavenumbers, which are the centimeter reciprocals of wavelength, as these units are directly proportional to energy.)

The efficiency of Raman scattering is proportional to $^1/_{\lambda^4}$ so it is enhanced at shorter wavelengths of incident light; this equates to an intensity decrease of 15-fold going from blue to an infrared laser. This will be discussed more in the section on fluorescence.

Knowing how much energy the molecules absorbed provides the analyst with information about the chemistry and physical state of the sample such as the types of covalent bonds, the electronic state, the crystallinity and the degree of stress present in the sample. This is the basis of Raman spectroscopy and Raman microscopy.

Two major advantages of Raman imaging are its independence of excitation wavelength, which allows the experimentalist to choose a wavelength suitable for the sample under study, and the ability to probe large biochemical compounds or structures, such as cells or tissues, without the need for markers.

A Raman spectrum is a graphical representation of the Raman shifts and their relative intensities. It can be thought of as a fingerprint of the sample comprising overlapping sets of the Raman shifts produced by each of the different molecular species in the sample. With the help of a database of Raman spectra the analyst can extract specific set of peaks (shifts) and identify the molecular make-up of the sample.

Some of the most useful functional groups within molecules for chemical identification purposes are the single and double

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$\nu/(cm^{-1}) = 10^7/\lambda$ in nanometers.

Thus green light at 500nm has a wavenumber of 20 000cm^{-1}.

carbon–carbon bonds (C–C, C=C), hydrocarbon (C–H, CH_3 *etc*), carbonyl (C=O) and amide (N–H) bondings that are found in a huge variety of materials and biological samples. So for example CH_2/CH_3 stretching vibrations are seen at a Raman shift of about 3000cm^{-1} and carbon–oxygen double bonds at 1550cm^{-1}.

The number one use of Raman spectroscopy – like its sister technique infrared microspectroscopy – is to identify materials. Raman microscopy has a higher spatial resolution than infrared and can operate through water, glass and even, with depth profiling, down into a material. Raman also requires minimal sample preparation and, again like infrared, provides an answer fast. Raman provides low wavenumber information (far-infrared) quite easily, permitting analysis of inorganic materials with ease. And Raman is hypersensitive to environmental differences and stress. Polarization in Raman additionally provides insights into molecular symmetry and surface alignment.

Raman microscopy has applications in almost every field of scientific research, from art conservation to zoology. The use of Raman knows no bounds, literally: the ExoMars rover due to be launched in 2018 will carry a Raman spectrometer for the *in situ* identification of both organic and inorganic compounds in the search for life forms on Mars.

IN PRACTICE

Raman microscopy can be performed on any standard upright or inverted light microscope equipped with the usual complement of optics and accessories for high-resolution widefield or confocal imaging.

These may include *inter alia* a motorized stage, low (*eg* 10x, 0.25NA) or high power (*eg* 100x 1.4NA oil immersion) objectives and a digital camera to take images of the sample. For Raman spectroscopy four additional components are required: a laser, sets of optical filters, a spectrometer or spectrograph, and a detector. A shroud (or class 1 laser safety enclosure) to exclude extraneous light and avoid operator exposure to laser light is also standard on most commercial Raman microscopes.

For thick samples, confocal optics are used to allow optical sectioning through planes in the sample, a technique called depth

Figure 3. A modern Raman microscope – the Thermo Scientific™ DXR™2xi. This dispersive Raman system uses an Olympus microscope and is equipped with interchangeable lasers and optics enabling operations at 455, 532, 633 or 785nm

profiling. Essentially, the excitation and collection optics focus on the same point, and the stage can be raised to allow that point to 'dive' into the sample resulting in a Raman signal from below the surface.

The design of the motorized stage is important as this determines the rate at which the sample can be scanned and spectra collected. Standard systems use stepper-motor driven stages, while high-speed imaging systems require optical encoder-based stages. The main difference is that the former counts x and y steps from some preset point while the latter reports its absolute location at each instant, and can scan without stopping – an essential feature for ultrafast data collection.

Sample holders are determined by the type of sample under study and its environment – gaseous, aqueous, solid powders or films each require slightly different handling. Further, environmental chambers, such as those from Linkam or Instec, provide control over the temperature, pressure and gas environments.

The basic optical pathway for a Raman microspectrometer is shown in Figure 4.

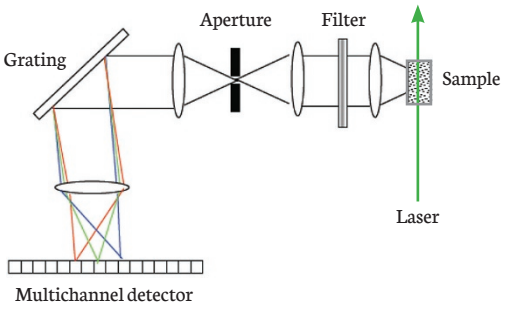


Figure 4. The basic optical path for a Raman microspectrometer. The scattered light is directed through a filter that eliminates the Rayleigh light. The Raman signal then passes the entrance slit or aperture and strikes a dispersive element, shown here as a simple grating. The dispersed light is then directed onto a multichannel detector

Light from the laser is directed through the objective lens and focused in a small spot on the sample. The sample is scanned under the laser beam using the motorized stage: the operator can then choose to do spot scans (called point and shoot), line scans or a 2D map or image scan of the sample. At each point or pixel of the scan, all the light reflected from the sample is collected by the lens and passes to the spectrometer where the Rayleigh scatter is blocked by an edge filter (preventing detector overload) allowing only the Raman scattered light to be dispersed into its separate wavelengths and detected by a detector (CCD camera).

Most commercial Raman microscopes can be configured with one or more lasers, which may be gas-based (*eg* He–Ne) or solid-state (diode lasers, such as Ti:sapphire, Nd:YVO_4). While each laser emits essentially a single frequency, the varied lasers enable users to select from a wide range of excitation wavelengths from ultraviolet (UV), through visible (Vis) to the near infrared (NIR). The Raman system is equipped with Rayleigh filters and spectrographs specific to the laser choice, to allow fast switching of wavelengths during an experiment. The multiple laser offerings provide the needed trade-off between Raman efficiency and fluorescence, as discussed in the Problems and Solutions section of this EKB.

Optical filters are placed between the sample and the spectrometer to reject the excitation wavelengths from the laser and allow through only the Raman scattered emission wavelengths. These filters are of the edge or notch types that allow transmission of only light of longer wavelengths or of a specific bandwidth, respectively.

Raman spectrometers operate in one of two modes: dispersive or interferometric (also called Fourier transform [FT]). The choice

of mode is largely determined by the sample, as the long wavelength lasers used in FT-Raman (typically 1064nm) allow for avoidance of fluorescence (see the Problems and Solutions section).

In a dispersive system, the Raman scattering enters through an entrance slit, aperture or confocal pinhole and falls on a grating. Dispersive spectrometers contain a ruled or holographic diffraction grating that separates the Raman signals into its component wavelengths. Most gratings are optimized for a specific set of wavelengths so they must be changed when a different laser frequency is used. The number of grooves per mm determines the dispersion characteristics. A high number of grooves per mm (lines per mm) results in a high dispersion and thus a high resolution. It is often useful to switch between two or more gratings: one that covers the full Raman spectrum (50–3600cm^{-1}) and a high-resolution grating that delivers approximately 1cm^{-1} spectral resolution over a smaller range.

The original detectors in Raman spectrographs were photographic plates. The first digital systems relied on photon counters coupled to photomultiplier tubes, a vacuum tube technology with high sensitivity, but only good for operations in scanning modes. Modern dispersive spectrometers use CCD detectors, discussed in detail below. Figure 5 shows the spectrograph and detector assembly of the Thermo Scientific DXR2 spectrometer, from the entrance slit to the detector. In contrast, the FT-Raman units use an InGaAs NIR detector or a Ge cryogenic detector.

Lens-based spectrographs use an on-axis lens-system instead of mirrors to guide light through the spectrometer. The lenses are optimized for a certain spectral range and can therefore provide optimal Raman signal transmission (typically as high as 60–70%),

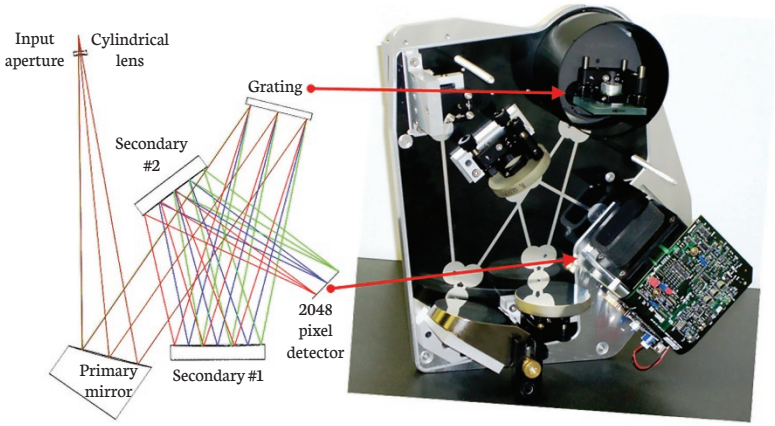


Figure 5. The actual light path is more complex than shown in Figure 4. Multiple mirrors control the spatial and spectral integrity of the signal. The spectrograph module of the Thermo Scientific DXR2 Raman microscope is shown on the right

spectral resolution and imaging capability. Therefore lens-based spectrometers are preferred for the generation of high-quality Raman images.

In FT-Raman spectrometers the Raman scatter from each point in the sample passes through a Michelson interferometer, producing an interferogram on the detector. The FT mathematics convert this time-domain signal into the standard wavelength or wavenumber spectrum.

Most modern dispersive Raman spectrometers use CCDs – the chips found in scientific grade cameras – as the detector. For highest resolution and speed an electron multiplying CCD (EMCCD) can be used. CCDs have a large wavelength response, typically extending from 400nm up to 1000nm, making them suitable for all lasers except NIRs (at 1064nm). CCDs are slower, being limited by read-out noise, whereas EMCCDs are faster and limited only by shot noise. Both can be used in the back-illuminated versions,

increasing sensitivity. EMCCDs have five-times better signal-to-noise ratios at very low light levels – the kind found when doing very fast Raman imaging (about 50 photons per second).

There are two resolution specifications for Raman microscopes: spatial and spectral. Spatial resolution defines the smallest sampling points while spectral resolution defines the ability to separate discrete wavelengths (neighboring signals) of the Raman signal. In CCD systems, the spectral resolution is determined by the dispersive element, the size of the CCD elements and other optical parameters.

The spatial resolution is determined by the optics of the microscope, which are diffraction limited, restricting the best resolution to 250nm (or half the wavelength of the imaging light). But the Raman signal is collected from the area illuminated by the laser and at best the beam can only be focused to a spot about 0.5µm in diameter.

In-depth profiling, confocal optics permit a depth resolution (z) of around 2µm to be achieved.

Once the Raman signals have been collected and processed they are then displayed on a computer monitor in either graphical or map form. The former shows the Raman shifts, *ie* how much energy has been lost by scattering, on the abscissa (x-axis, but note that, unconventionally, units go from highest energy on the left to lowest on the right), and on the ordinate (y-axis) the intensity is reported in counts per second or arbitrary units. The spectrum of benzene is shown in Figure 6; there are two very intense peaks due to the C–C and C–H symmetric stretches.

In a Raman map or image, there are four or five dimensions of data involved: x and y locations (in some cases, z as well), plus

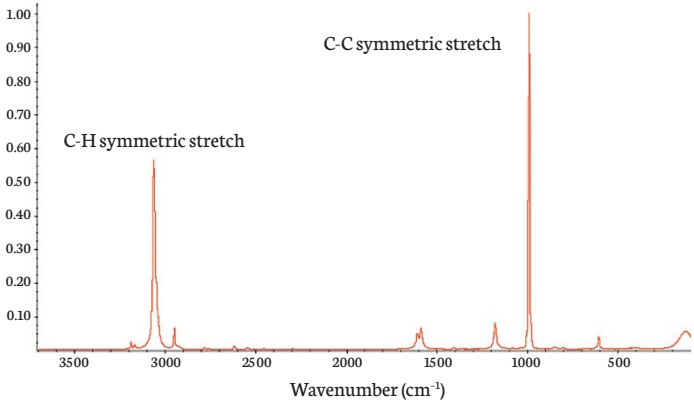


Figure 6. Raman spectrum of benzene (C_6H_6) showing C–H and C–C bond stretching

frequency and intensity. Visualization involves fixing one or more variables and then color coding the response. For instance, the user can choose a specific wavelength and color code the intensity over the x-y plane, or they can select a reference spectrum and generate a correlation map. Multiple targets can be color-coded simultaneously, as seen in the tablet map shown in Figure 7, allowing homogeneity and even relative concentrations to be calculated.

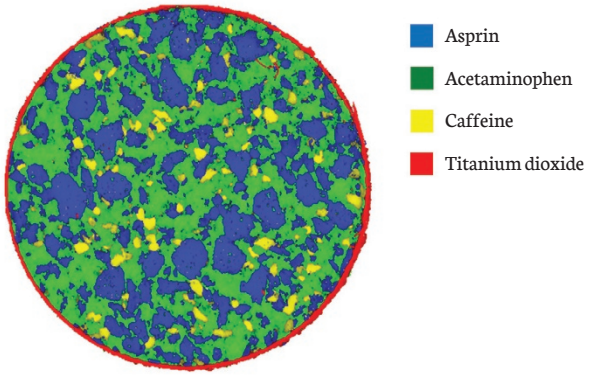


Figure 7. Raman map of a pharmaceutical tablet showing constituents

For Raman mapping there are several options:

- Spot mapping *ie* measurement of non-linear points
- Line mapping – measurement of points on a linear x-y axis
- Area mapping – measurement of points in a 2D area
- Hyperspectral mapping, which is the ultimate in Raman imaging. Here a full Raman spectrum is collected at each pixel in the sample and used to reconstruct a 4D or 5D data cube.

Similarly, by using confocal microscopy it is possible to collect a Raman signal at different depths (focal points) inside the sample, *ie* in the z direction, into the sample. You can do a single point, line maps or area maps combined with depth profiling (see Case Study 2).

The next step is to identify what chemical and molecular components of the sample give rise to the detected spectra. To do this requires a custom database or commercial library of Raman spectra and sophisticated software to interrogate the spectra and extract characteristic signatures of specific molecules from among the many spectral patterns present.

Libraries of Raman spectra of almost every inorganic and organic chemical, mineral and macromolecule known to man have been published by academic and national institutes and by commercial organizations (see the Further Information section). These libraries are used as databases to allow searching for patterns in any Raman spectrum that reveals what materials give rise to the observed Raman shifts. Pattern recognition in Raman microscopy often involves multivariate analysis methods.

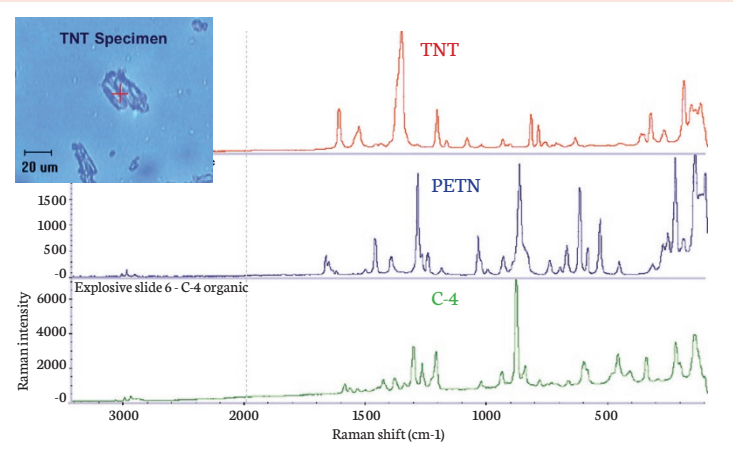
With large numbers of samples to analyze, the speed of data acquisition and processing is of prime importance in Raman

imaging labs. The Thermo Scientific DXR2xi Raman imaging microscope with its latest technologies in EMCCD detectors, automated x-y-z stages, software, and data acquisition and control is a modern system capable of extremely fast imaging with rates of up to 600 spectra per second over areas as large as 4x3 inches at submicrometer spatial resolution.

CASE STUDY 1. Forensic analysis of explosives

Raman microscopy is a very useful technique for the analysis of trace materials in forensic samples. The ability of Raman to identify materials through library searching relies on spectra that are molecular fingerprints – these can be compared to databases for identification. The advantages of Raman microscopy in forensics are its non-destructive nature, the need for little or no sample preparation, and most importantly the ability to analyze through containers, preserving the chain of evidence.

Many interesting materials appear in forensic investigations, including inks, drugs (legal and not), paints, fibers and even explosives. Small particles, and even residues from explosions and fires, can be analyzed using Raman microspectroscopy. The work shown in the figure below, collected by the applications team at Thermo Fisher Scientific, used a Raman microscope with a 785nm laser and standard collection optics to investigate explosives in forensics samples.



Trace residue analysis by Raman microscopy. Figure from Thermo Fisher Scientific, used by permission

The figure shows how successful this can be. The image shows 20μm TNT particles from which Raman spectra were collected. The spectra reveal TNT (2,4,6 trinitrotoluene), PETN (pentaerythritol tetranitrate), and C-4 (RDX: 1,3,5-trinitroperhydro-1,3,5-triazine).

CASE STUDY 2. Confocal Raman analysis of mineral inclusions in diamonds

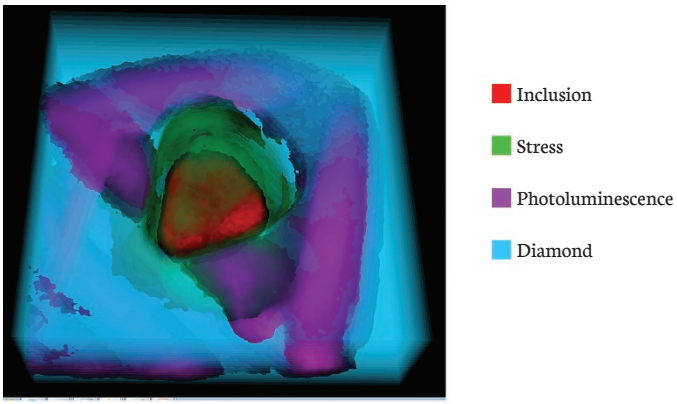
Raman depth-profiling permits mineral inclusions to be investigated while still encapsulated in the matrix, thus avoiding potential material loss or contamination during extraction. This is particularly important with inclusions in diamonds because of the hardness of the matrix. Polishing or grinding to expose inclusions is time-consuming, costly and destructive. Even if the primary goal is to probe the inclusion, there is also additional information contained in the surrounding, not to mention the value of the gem itself.

Dr Robert Heintz of Thermo Fisher Scientific collected the data using a Thermo Scientific DXR2xi Raman imaging microscope equipped with a 532nm laser, fast stage and EMCCD detector to investigate the identity and effect on the matrix of an inclusion in a diamond.

A series of planar images (optical 'cuts') were collected at different focal (z) positions. The planar images were then stacked to give a 3D image of the inclusion and surrounding environment. The figure below shows Raman data for the region around an inclusion (shown in red). Identification of the inclusion was straightforward, by simply choosing spectra from one of the cuts through the inclusion. The inclusion is clearly a silicate, and the best spectral match was to omphacite ($(Ca,Na)(Mg,Fe,Al)Si_2O_6$). Omphacite is rare but is known to be associated with diamond in diamond deposits. The inclusion peaks appear slightly shifted relative to the library spectrum, but omphacite is known to have a variable composition.

Raman spectroscopy of minerals also reveals the spatial distributions of physical phenomena. Stress can shift Raman peaks, as is well known for diamonds. The Raman imaging revealed a stress pattern in the surrounding diamond near the inclusion, seen in the green area of the figure. A shift to a higher wavenumber indicates compressive stress. By using the wavenumber

shift as the basis for generating Raman images it is possible to get a 3D spatial distribution of the stress around the inclusion. In addition, photoluminescence in diamonds is commonly observed, due to crystal defects such as substitution of carbon for another element, like nitrogen. Many different photoluminescence peaks have been observed for diamond and the specific 613nm peak observed in the spectra around the inclusion (shown in violet) is consistent with a previously reported photoluminescent peak in diamond.



Raman imaging goes beyond just identification. It can be used for visualizing physical properties revealed in the Raman spectra. The colors show different types of information and images from a single data set. Figure from Thermo Fisher Scientific, used by permission

PROBLEMS AND SOLUTIONS

Fluorescence

A major problem in Raman microscopy is fluorescence of the sample, which can severely mask the Raman signals. If the incident photon has sufficient energy, electrons in the sample are excited to higher states of energy and on relaxation to their ground state they emit light of longer wavelengths than that of the incident light. So together with the Raman signals, these fluorescence wavelengths will be able to pass the edge filters that are designed to block the incident light. Critically, fluorescence is orders of magnitude more efficient than Raman scattering, so a strong fluorophore will overwhelm the Raman signals.

There are two solutions to this problem: first, you can deal with it during spectral processing. There are proprietary fluorescence rejection or correction algorithms (companies such as Thermo Fisher Scientific, Horiba, and JASCO include these in their spectra management software) that can effectively remove or minimize fluorescence regardless of the laser wavelength used. Secondly, the excitation frequency can be chosen to minimize fluorescence. The energy of the laser can be reduced so as not to promote fluorescence, *ie* there is insufficient energy to pump the electrons to higher energy levels. Since fluorescence has very specific excitation and emission parameters, the user can often select an incident laser line for Raman imaging that does not cause fluorescence in the sample. Typically, longer, *ie* more red, laser lines over 700nm do not cause fluorescence. This is why FT-Raman microscopes, with their 1064nm lasers, do not suffer from this problem. The trade-off is that the efficiency of the Raman scattering decreases rapidly with lower energy excitation. Other

methods, such as UV lasers and stimulated Raman experiments, are beyond our scope.

Sample degradation

Lasers produce very intense beams of light that are capable of causing damage to the sample. It is important to select a laser power (wattage) that does not cause any changes in the sample during the investigation. At worst the laser could cause burning of the sample but the damage is usually more subtle. Samples can show a change in phase due to recrystallization, and living cells are very sensitive to phototoxicity and their macromolecules to radiolysis.

Fortunately in most cases this is not a problem: although Raman scattering intensity is inversely proportional to wavelength, the spectra (peak locations) are not wavelength dependent and so the investigator can often simply reduce laser power or change to a longer wavelength to avoid this type of sample damage.

Anecdotal reports of samples being destroyed or even catching fire are common, usually resulting from high laser power hitting a deeply colored material, such as ink on a tablet.

Brightness of lasers

There are two ways of looking at the suitability of lasers for Raman microscopy (other than the obvious wavelength dependencies already noted): power (wattage) and brightness. Power is simply the output of the laser. Brightness describes the power density at the sample. Lower power lasers can often deliver more brightness at the sample by using a smaller beam divergence resulting in greater Raman emission. The tight focus of these lasers and small spot sizes also contribute to high spatial resolution (Table 1).

Comparing two 780nm laser options

Laser power	150mW	14mW
Beam size	5x5mm	1mm diameter
Spot size w/ 100X objective	8 microns	1.6 microns
Power density at sample	3mW/μm²	7mW/μm²

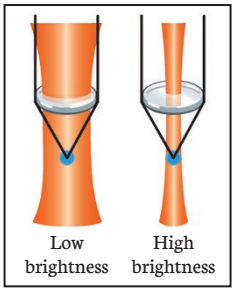


Table 1. Less is more! Comparing two 780nm laser options for Raman. Table from Thermo Fisher Scientific

FT versus dispersive Raman? The pros and cons

In Raman microscopy the analyst is faced with a choice between dispersive and FT systems. So how do you choose which is better for your samples?

Critically, because the laser wavelength is longer, the spatial resolution of FT-Raman is lower and is typically not much better than 5μm.

Raman microscopes use a multichannel CCD detector, with extremely low intrinsic noise and a high quantum efficiency. This results in dispersive Raman being shot noise limited, whereas the FT-Raman's detector is noise limited. The benefits for dispersive Raman are far higher sensitivities (of the order of 100 times) and far lower detection limits than FT-Raman.

FT-Raman should be used when the samples fluoresce at shorter laser wavelengths or are likely to contain minor impurities that may fluoresce.

Because it uses the FT technique, FT-Raman allows you to measure all the Raman wavelengths simultaneously and offers superior wavelength accuracy due to the internal calibration inherent to an interferometer.

Three types of sample are not suited to FT-Raman analysis: these are aqueous phase samples because they may strongly absorb both the exciting laser radiation and the Raman scattered light at 1064nm; samples at temperatures over 250°C because intense black body emission can mask the Raman signal; and black samples because they can strongly absorb, heat up, and produce intense background emission, or even degrade.

Widefield or confocal modes?

When a laser beam strikes a sample, if it is sufficiently transparent, the whole depth of the sample is irradiated and Raman signals are generated from the entire thickness of the sample. If the sample is thin this is not a problem. But if it is thick and especially if it displays any inhomogeneity such as different layers in a multilayer film or from inclusions within the sample matrix, then the Raman signals will be mixed from the differing regions of the sample. The solution is to use confocal optics. As the name suggests, a selected region or plane of the sample is confocal or is conjugate with the plane of an aperture in front to the spectrometer/detector. In this way, only light that is from a specific plane enters and is sampled. Since the Raman signal is most intense at the focal point of the excitation, confocal systems capture spatially resolved data from the highest intensity Raman scattering region.

Sample dependent parameters

Raman scattering is sensitive to the intrinsic anisotropy or polarization of the sample, hence its orientation on the microscope stage during the investigation may be significant. Examples of orientation-specific samples include carbon nanotubes (CNTs),

polymers and most crystalline materials (lithium niobate is used as a polarization standard for this reason). If the laser light is polarized, *ie* all the light waves have the same plane of oscillation, the Raman signal will be strongest when the sample's intrinsic polarization is in the same plane. This can be determined by rotating the sample under the laser beam until the Raman signal is maximal.

In addition, Raman signals are sensitive to the environment of the sample. For instance, the large carbonyl band of acetone broadens and red-shifts (to lower frequency) when water is added due to hydrogen bonding. Small changes in the stress on diamonds also cause peak shifts.

Amplifying the Raman signal: surface enhanced Raman

Sometimes the Raman signal from a sample is so low that there is great difficulty in detecting it. Here scientists have come up with a couple of tricks to help. In 1974 Fleischmann, Hendra and McQuillan, working at Southampton University, in the UK, were measuring Raman spectra of pyridine on an electrochemically roughened silver surface and found that the signal was much higher than expected. In 1977, a surface-plasmon-polariton-enhanced mechanism for this effect was proposed by van Duyne *et al* and this explanation has now become widely accepted. This technique is now called surface enhanced Raman spectroscopy (SERS). Signal enhancements by SERS of 10^4 to 10^6 times and even up to 10^{14} times have been reported. The metals exhibiting the largest SERS enhancements are silver, gold and copper.

WHAT'S NEXT?

At a recent analytical conference, it seemed every aisle had at least one vendor showing new aspects of Raman systems. This surge in use has been driven largely by technological advances, such as new lasers, spectrometers and detectors. A major trend in the last 10 years has been the rapid evolution of Raman spectroscopy from a technique used only by spectroscopy experts into a tool used by scientists and engineers to solve problems. Compact, enclosed systems, such as the Thermo Scientific DXR2 family of spectrometers, provide the flexibility to analyze particles or image entire tablets, with huge applications in the field of carbon-materials, such as nanotubes and graphene. Substantial growth in the handheld or portable markets has been driven by this same innovation in components.

The ability of the Raman laser and Raman scatter to be directed by glass optics (rather than the mirrors and salt plates needed for infrared) allows novel access in multimodal analyses, where Raman is combined with other techniques. Three examples are Rheometry-Raman, X-ray photoelectron spectroscopy (XPS)-Raman and atomic force microscopy (AFM)-Raman.

Rheometry involves the measurement of the shear storage modulus G' and shear loss modulus G" of a material. Essentially, G' relates to the elastic response, while G" relates to the viscous response. As an epoxy cures, it passes from being a viscous fluid (higher G") to an elastic solid (high G'). By coupling a Raman spectrometer into the rheometer, a relationship between the physical parameters and the underlying chemistry can be constructed. Figure 8 shows G', G" and the Raman measurements for the freezing of a polymer (the experiment started hot and cooled, right to left

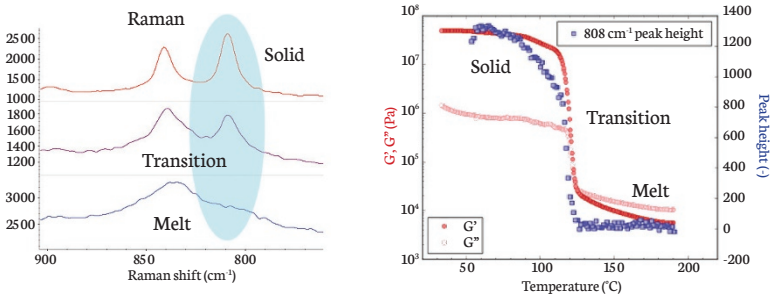
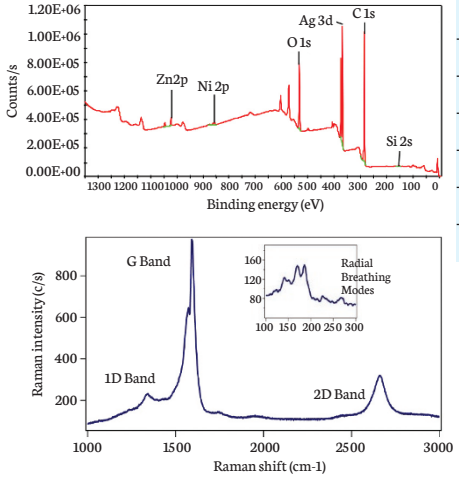


Figure 8. Correlation of shear storage modulus G' and shear loss modulus G" and corresponding Raman shifts during the melting of a polymer. Figure from Thermo Fisher Scientific, used by permission

on the plot). There is a clear appearance of a crystalline peak in the Raman showing the onset and rapid progress of the solidification, matched with changes to G' and G".

XPS studies surface chemistry at the atomic level. A beam of X-rays strike the surface, causing the emission of photoelectrons; the energy of those electrons directly relates to the atom emitting them. Raman, as we have seen, probes molecular information. Figure 9 shows a combined XPS-Raman study of single-walled carbon nanotubes (SWCNTs). Raman is highly sensitive to the differences between SWCNTs and multi-walled CNTs; the response here is clearly SWCNTs. The XPS reveals other metallic residuals, including silver, zinc and nickel (silicon is the substrate) not seen by the Raman. They are from the process used to grow the SWCNTs. The mutually supportive data shows the utility of the combination.

AFM has grown to be a power tool in materials research. A sharp tip – only a few nanometers in size – is scanned across the material and responds to surface topography of the sample. This provides a very high resolution view of the surface, down even to the atomic level. There are two ways to combine this with



Name	Atomic %
C	76.0
O	16.8
Ag	4.9
Si	1.3
Zn	0.5
Ni	0.4

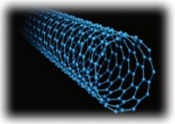


Figure 9. Correlation of XPS and Raman data from SWCNTs. Figure from Thermo Fisher Scientific, used by permission

Raman spectroscopy – co-localized and tip-enhanced Raman spectroscopy (TERS).

In co-localized AFM-Raman, the Raman beam straddles the tip and standard backscattering geometry is used to collect the Raman signal. In this configuration, the Raman signal is limited to a spatial resolution between 250 and 500nm, depending upon the system.

AFM can also be used in a way similar to SERS. An AFM tip made from silver or gold is used and a significant enhancement in signal results in the vicinity of the tip. TERS enables the Raman signal to be obtained at the same spatial resolution as the AFM signal. The main difficulty with TERS appears to be in preparation of the tips, which do not last long (due to oxidation and general degradation) and the less rigid structure of silver and gold. Work is proceeding with other metals in an attempt to overcome this limitation.

FURTHER INFORMATION

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