

Confocal Raman Microscopy: A Clinical and Biological Tool

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Confocal Raman microscopy analysis of biological samples allows several advantages over standard dispersive Raman techniques.

Scientists in the fields of biology and biochemistry are familiar with some of the more common forms of spectroscopic analysis, such as absorption and emission spectroscopy. The former measures the amount of light absorbed at a given wavelength to provide information about a sample's structure, and the latter produces information re-

garding a substance by measuring the amount of light of a particular wavelength that is emitted.

Raman spectroscopy — which measures the light scattered by a substance over a defined range of wavelengths when excited by a laser of a particular wavelength — offers significant advantages over other forms of spectroscopy. It does

not require any sample preparation, samples are not destroyed, water bands are usually small and easily subtracted, and Raman spectra usually contain sharp bands that are characteristic of the specific molecules in the sample.

In biological samples such as cells and tissue, infrared spectra often show broad spectral features that can offer information regarding cellular components. However, Raman spectra also provide more detailed information on the constituents of the specific components. This allows good specificity for qualitative analysis and for discrimination among similar materials. Because the intensity of the bands in a Raman spectrum are proportional to the concentration of the molecules that give rise to them, it can be used for quantitative analysis.

Confocal Raman microscopy has the added advantages of providing chemical information with microscopic resolution, allowing specific spatial analysis of regions within a bulk sample, offering depth profiling and improving rejection of fluorescence.

A brief history

Raman spectroscopy has been around since the beginning of the 20th century. Sir C.V. Raman is credited with discovering the scattering effect in 1928, when he observed it in a sample mixture of water and alcohol that was excited by

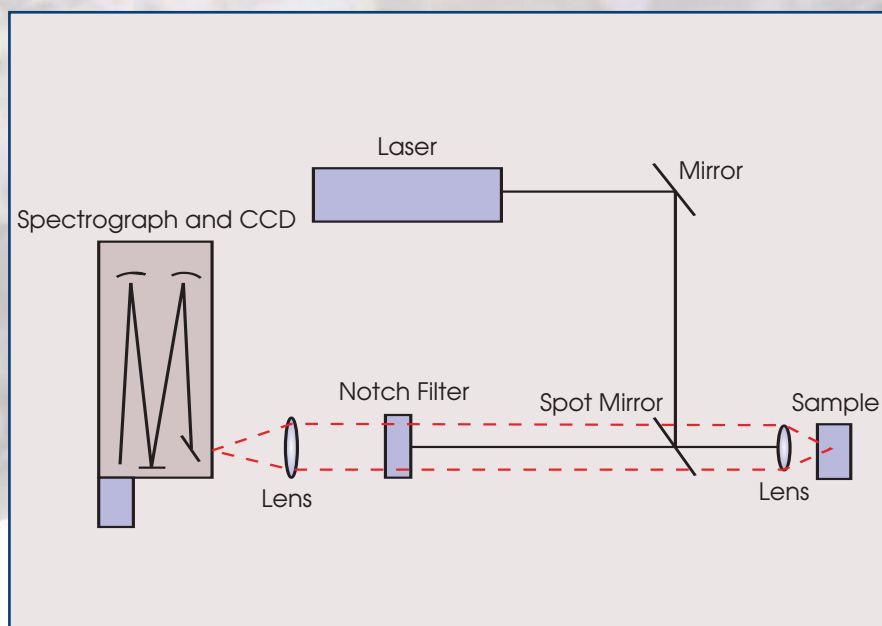


Figure 1. A simple Raman setup consists of a notch filter, a spectrograph, a laser and a CCD detector.

monochromated sunlight while using the eye as a crude detector.^{1,2} Soon after these initial experiments, a relationship between the excitation wavelength and the scattered wavelengths was discovered.³

The differences between the incident and scattered wavelengths (Raman shifts) were related to the vibrational energies of molecular bonds. It was realized that Raman spectroscopy was a probe of the vibrational energy levels within a molecule and, thus, complementary to infrared absorption spectroscopy.

At that time, there was an increased interest in Raman spectroscopy because it provided molecular information more easily than infrared absorption spectroscopy and was the only way to measure low-frequency vibrations. However, it was overshadowed during the 1940s, when advances in infrared instrumentation resulted in the first commercially available instrument for infrared absorption, making IR easier to use and more readily available than Raman spectroscopy.

By the 1950s, photomultiplier tubes (point detectors) had replaced photographic film for the detection of Raman scattering. The first commercially available Raman instrument was introduced in 1953.

It was not until the development of the laser, with its superior power and monochromaticity, that a renaissance occurred in Raman research. In the 1970s, the development of multichannel detectors, which allow large numbers of wavelengths to be viewed simultaneously, resulted in another large step forward in Raman instrumentation.⁴

Raman and biology

The 1990s saw the final stages in the modernization of instrumentation with the development of holographic notch filters⁵ and of compact diode and diode-pumped lasers, and with the integration of confocal microscopy into the technique. Holographic notch filters made the instrumentation much more compact because a single filter replaced multiple dispersion stages as a means of filtering the intense laser light and separating it from the weak inelastically scattered Raman effect. The addition of confocal microscopy has brought Raman spec-

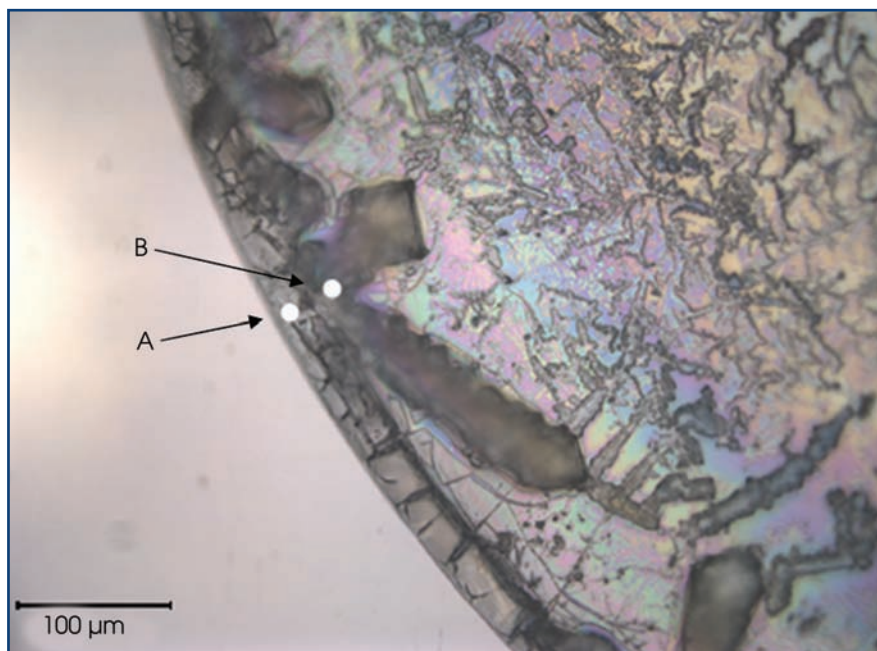


Figure 2. Investigators placed a 5- μ l droplet containing 20 μ M of lysozyme in a phosphate buffer onto a slide. After the protein segregated from the buffer, confocal Raman spectroscopy helped elucidate where pure lysozyme (A) and phosphate salt (B) remained.

troscopy firmly into the realm of biological analysis.

At its simplest, Raman spectroscopy is performed by focusing laser light of the required excitation wavelength onto a sample. The scattered light is collected and focused onto the entrance of a spectrograph, which separates the light into individual wavelengths using a dispersion grating. A notch filter usually is placed in the optical path of the spectrograph to reduce the amount of Rayleigh scattered (reflected) light from the laser entering the spectrograph, and a CCD detector mounted to the spectrograph detects and measures the Raman shifts (Figure 1). The distance from the laser line at which Raman lines can be measured, the number of excitation wavelengths used, and the resolution will increase the complexity and cost of the system.

One of the principal obstacles in using Raman spectroscopy for biological applications is the presence of a lot of background noise caused by fluorescence from contaminants or background matrices in the sample. There are many methods of overcoming fluorescence, but the most popular one is to use an excitation wave-

length that does not excite fluorescence. For this reason, most biological applications use either UV or near-IR lasers.

In laser spectroscopy labs, resonance Raman spectroscopy with UV excitation is widely used for probing the structure of proteins and other biological molecules. It achieves Raman enhancement by exciting a molecule near its transition state. Yet it does not lend itself to use outside a highly specialized environment because of the high cost of suitable UV lasers and because it is difficult to use notch filters in this wavelength region.

Near-IR wavelengths have become preferred for biological applications because of the low cost of diode lasers and the fact that the longer wavelengths also can avoid fluorescence. It is relatively easy to integrate these compact lasers with notch filters, a spectrograph and a CCD to provide a dedicated Raman instrument. Most modern commercial instruments used for biological applications also integrate a confocal microscope because it offers the added advantages of being able to use both visible and near-IR excitation while avoiding fluorescence, increasing spatial resolution and allowing comprehensive

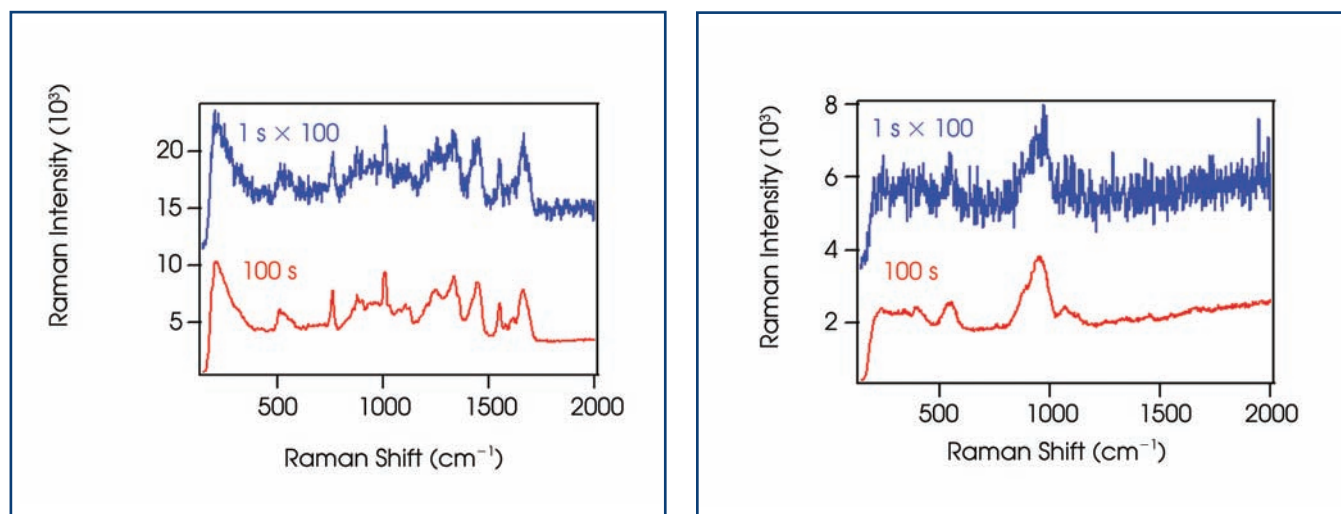


Figure 3. Raman spectra taken from points A (left) and B (right) of Figure 2 contain either pure lysozyme or only phosphate salt.

mapping of a sample. It also provides a familiar interface for biologists.

In confocal Raman microscopy, also called Raman microspectroscopy, the microscope objective focuses the incident laser radiation onto a point in the sample. The Raman scatter is collected by the objective and coupled into the spectrograph through a pinhole aperture. The aperture enables confocal detection: Raman scatter obtained from outside the focal point is out of focus at the aperture and, thus, not detected.

Because the laser is focused to a very small point, the laser flux in the sample is high enough to quench fluorescence in a much shorter time than in conventional Raman spectroscopy. Also, excited electrons that might cause fluorescence can migrate to molecules with lower energies so that a molecule outside the sampling area excited by the laser may emit the fluorescence photon, which would miss the pinhole aperture. This occurs because electrons within the sampling area will naturally migrate to lower-energy molecules, which are found outside the area.

Applications

Several types of confocal Raman microscopes are commercially available, ranging from the open modular type systems that can be reconfigured to accommodate a wide variety of experiments and applications to dedicated turnkey systems with sophisticated software for biological applications.

Raman spectroscopy has become a popular analytical method for many biolog-

ical applications, and as the instrumentation evolves toward becoming a dedicated biologist's tool, the variety and scope of applications will only widen. Raman microspectrometry involves almost no sample preparation, and it has better axial and spatial resolution than conventional microscopy. This makes it possible to perform extremely detailed analysis of cells in their natural state.

Microscopic resolution allows the chemistry of individual cells to be investigated and mapped images to be generated. These images can contain full spectral information at each pixel so that the distribution of components within the cell can be visualized based upon their Raman signature. This is extremely valuable to researchers because biochemical changes can be observed during a cell's life cycle or when a cell becomes damaged or cancerous. Using confocal Raman, the changes in a variety of cell types, including bacteria and eukaryotes, can be monitored over time, and comparison between healthy and diseased tissue states can be easily analyzed.

One challenge faced in analyzing cells in their natural state is that they often will move around within a given matrix. Researchers at the University of East Carolina in Greenville, N.C., have solved this problem by combining a confocal Raman system with optical tweezers that can immobilize the cells.⁶

Researchers at Purdue University in West Lafayette, Ind., are using Raman microscopy for proteomic analysis of femtomole quantities derived from micro-

liter volumes of micromolar protein solutions.⁷ They have used a dropped coating deposition method, in which a small volume of a dilute solution of protein (and buffer) is deposited on a slide, consisting of a highly polished stainless steel coated with nanometer Teflon coating (Figures 2 and 3).⁸ □

Meet the author

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