A Coherent White Paper

Raman in Life Sciences: Bacterial Phenotype & Inhibition Analysis

Abstract

Raman scattering can provide similar compositional spectroscopic information to Fourier Transform Infrared (FTIR) absorption but without the complexities of using infrared sources, optics and detectors. And, unlike FTIR, Raman is particularly well-suited to aqueous samples making it a useful tool for in vitro, and potentially in vivo analytical applications in life sciences. Moreover, Raman's wavelength flexibility and its compatibility with glass optics mean it can be combined with techniques such as fiber optics and microscopes for imaging and remote analysis. New technology from Ondax (now Coherent) has recently extended the analytical power of Raman spectroscopy from the traditional "chemical fingerprint" frequency region associated with vibrational resonances to the low frequency (THz) domain, providing unique information about phase (degree of crystallinity, polymorphism, etc.) effects, phonon scattering, etc.. In this whitepaper, we discuss why there is fast growing interest in Raman in life sciences, and examine some typical lasers used in this field. We then look in detail at an application at the Leibniz Institute of Photonic Technology (Leibniz-IPHT), where Raman is being used both to analyze bacterial pathogens responsible for infections and to quantify their resistance to antibiotics, potentially enabling patient-specific optimization of drug choice and dosing.

Raman Basics

The Raman effect is a type of inelastic light scattering. When light from a monochromatic source is incident on a sample with polarizable molecules or crystals, a small portion of the molecules are left in a different quantum state and the scattered light is frequency shifted by the difference between the initial and final states.

A laser is virtually always used as the light source for Raman for two reasons. First, many lasers are naturally monochromatic. And secondly, lasers provide the high intensity required for the weak Raman effect: typically < 10^{-6} of incident photons are Raman shifted. Most commonly, Raman involves red (Stokes) shifts of the incident light, but anti-Stokes Raman can be combined with pulsed lasers to enable stimulated Raman techniques such as Coherent Anti-Stokes Raman Scattering (CARS) spectroscopy and microscope imaging.

Historically, Raman was used to provide data based on vibrational resonances, the so-called chemical fingerprint. It thus provides much the same compositional information as infrared absorption. However, Raman can be performed at a wide range of wavelengths – see lasers for Raman below – so it can be implemented using wavelengths that efficiently transmit through water and glass. Plus, the Raman signals are at wavelengths where they can be conveniently

detected by low-noise devices such as photomultiplier tubes (PMTs) and CCD/CMOS cameras. Consequently, Raman is increasingly used to conduct chemical fingerprint measurements remotely via fiber in industrial applications, or in the laboratory in combination with a microscope or telescope that provides spatially resolved information without the use of labels.

Holographic Filters – Raman Renaissance

For many years, Raman was limited by the challenge of separating the weak Raman signals from a stronger background of scattered excitation light. The Raman signals are spectrally shifted (Stokes or anti-Stokes) relative to the scattered laser light so some type of wavelength dependent filtering can be used. However, because of the low Raman intensity, this required a cut-off filter and a monochromator. This situation completely changed with the advent of holographically generated notch filters based on photosensitive gels. These served to provide very high blocking efficiency – multiple orders of magnitude – over a very narrow band of wavelengths centered on the laser wavelength, while providing transmission approaching 100% at other wavelengths. Together with CCD and then CMOS cameras, this enabled compact and efficient experiments as well as integrated spectrometers and microscopes.

In the past decade, engineers at Ondax (now Coherent) pioneered the development of patented next generation filters based on Volume Holographic Grating (VHG) technology using glass, rather than gels, as the substrate. Compared to earlier gel filters, these provide higher extinction ratios, greater environmental stability, and much sharper cut-on/cut-off characteristics. In addition to improving the signal-to-noise performance of Raman in the chemical fingerprint region, this technology extends the range of traditional Raman spectroscopy down into the low frequency (low wavenumber) spectral range and beyond into the anti-Stokes region, where important structural details – including lattice or polymer structures, crystal orientation, spin waves, and phonon modes – can be clearly discerned. Since these vibrational energies correspond to molecular transitions and vibrations in the 5 cm^{-1} to 200 cm^{-1} range (equivalent to 0.15 to 6.0 THz), the term "THz-Raman®" is used to describe this new spectral region and the associated instrumentation (Figure 1).

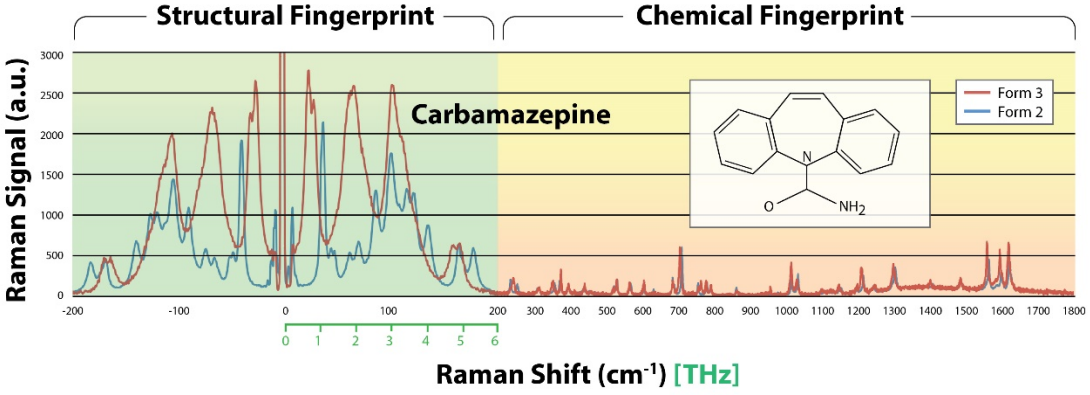

Fig. 1 THz-Raman spectra of Carbamazepine show the additional Structural Fingerprint, which provides a clearer differentiation of the polymorphic forms. (Excitation wavelength 785 nm.)

This same VHG filter technology has also enabled a new cost-effective method of stabilizing laser diodes for use as Raman excitation sources.

Lasers for Raman

Coherent produces three different categories of lasers that are well suited to Raman, as well as ultrafast lasers for non-linear Raman methods used in life sciences, e.g., Coherent Anti-Stokes Raman Scattering (CARS) and Stimulated Raman Scattering (SRS).

Single frequency CW visible lasers. Raman has long been performed using visible lasers, initially with ion lasers, then first generation solid state (DPSS) lasers, and now optically pumped semiconductor lasers (OPSL). Two of the most commonly used wavelengths are 488 nm and 532 nm. In Raman spectroscopy, spectral features are measured relative to the laser wavelength (frequency), so it is important that the laser produce spectrum-narrowed output and the output wavelength is stabilized to a narrow frequency range much smaller than spectrometer resolution. Depending on the application, a few tens of milliwatts of output power is required. This need is met with the low-noise Sapphire SF series which provide a choice of 488 and 532 nm outputs with powers ranging from 20 to 150 mW.

Stabilized visible and NIR laser diodes. In addition to scattered laser light, some samples – particularly organic and biological materials – emit fluorescence which acts as background noise. The probability of fluorescence has a highly non-linear dependence on $1/\lambda$, so longer (near infrared) laser wavelengths are often preferred for these samples. However, there are trade-offs –Raman intensity also scales as $1/\lambda^4$ and there is the need to ensure that Stokes shifted signals are within the detection range of silicon based detector arrays and cameras. For this reason, the 780-800 nm window has become the excitation wavelength of choice for many organic and biological samples. Single-frequency laser diodes are available such as the ultra-compact SureLock series from Coherent, where a VHG filter acts to form an external cavity. These unique lasers are also available at visible wavelengths, again with powers in the tens of milliwatts range.

***Figure 2**. Coherent provides a wide range of laser powers and wavelengths to match very type of Raman spectroscopy and microscopy. Shown (left to right) are Coherent suggest Sapphire, Genesis, and Innova FreD lasers.*

CW ultraviolet lasers. In terms of quantum mechanics, the Raman scattering process involves a virtual (non-stationary) excited state at an excitation energy determined by the laser photon energy. If this virtual transient state is close to a real excited state, then there is a massive increase in the Raman scattering probability, i.e., Raman signal intensity. This is called Resonance Raman. In practical terms, for many samples this means the use of a deep ultraviolet laser. In addition, at UV wavelengths the entire range of Raman signals is so small that it does not extend into the fluorescence spectral region, enabling fluorescence free Raman in many samples. For these applications, frequency-doubled gas lasers still provide the simplest route to low-noise single frequency performance at the requisite power levels. A standout example is the Coherent INNOVA® FreD series which deliver single-frequency output at a choice of wavelengths, including 229 nm, 244 nm and 257 nm.

Ultrafast lasers for stimulated Raman. For completeness we should also mention tunable ultrafast lasers that are used for stimulated Raman spectroscopy and imaging methods such as CARS and SRS. These so-called four-wave techniques require pulses at two different wavelengths. This need is met by Coherent's Chameleon Discovery series that deliver a fixed output at 1040 nm and a software tunable output covering 680 nm to 1300 nm

Bacterial Analysis Application at Leibniz-IPHT

The Leibniz Institute of Photonic Technology (Leibniz-IPHT) is a non-university research facility in Jena, Germany. Professor Jürgen Popp is the Director of Leibniz-IPHT and explains, "Our overarching mission can be described as 'Photonics for Life.' We are focused on providing light-based solutions that address challenges in life sciences and medicine. Importantly, our work is not just limited to pure research; Leibniz-IPHT spans fundamental research through to application-oriented procedures, instrumental concepts, and demonstrators to solve challenges in medicine, health, safety, and the environment. We don't just stop at patents or published papers, we cover 'Ideas to Instruments.' Our light-based solutions are then spun-off as commercial entities or licensed to established bio-instrumentation manufacturers. We are well-positioned for this work because we combine vertical integration – e.g., we have our own fiber drawing facility and our own clean room – with extensive collaboration with other groups in Jena, which is a well-known geographic center for photonics excellence."

One of Popp's personal areas of research is to investigate and develop the use of Raman – specifically Raman microspectroscopy – to provide faster analysis technology for bacteria associated with human infections. This research covers both identification of the bacterial species as well as performing antibiotic susceptibility testing (AST). The latter approach enables determining the level of resistance towards different antibiotics by varying their concentration. As a result the so-called minimum inhibitory concentrations (MIC) are obtained. The goal is a fast diagnosis of the disease followed by narrowband antibiotic treatment, rather than broadband antibiotic treatment which unnecessarily kills symbiotic bacteria and contributes to the overall increase in highly resistant bacteria strains. This aim fits well with a key current trend in medicine – personalized medicine based on superior analysis followed by highly targeted and effective treatment.

One important focus of the work concerns urinary tract infections (UTI). Popp explains that his group selected UTI because they represent the single most common group of infections, with some estimates that up to 50% of all women worldwide experience at least one UTI episode over their lifetime. The current gold standard for clinical analysis of UTI is a laboratory urine culture, with a minimum diagnosis time of 24 hours.

Why Raman? Popp explains, "Using Raman microspectroscopy, we can target and analyze individual bacterial cells. For a statistically valid result we only need to investigate 50-100 isolated bacteria, which can be easily found in urine samples of UTI-patients. Accordingly, this eliminates the time-delay and complexity of a lab culture. As the Raman microspectroscopic identification of bacterial cells is a label-free approach, sample preparation is further simplified. Another subtle but important advantage is that Raman looks at the compositional information – proteins, lipids, etc. – so it is a phenotype measurement, rather than the current tests based on genotype markers. Furthermore, by using Raman to distinguish between replicating (i.e., vital) and non-replicating cells, we can simultaneously determine the minimum inhibitory concentration (MIC) for various antibiotics. For example, within our current chip system, we implemented 20 reaction chambers, which allows micro-Raman analysis of bacteria treated with four concentrations of four distinct antibiotics. However, Raman spectra of chemically complex and naturally varying entities like live bacteria cannot be analyzed just in terms of a few peak heights as in other chemical samples of more simple composition. So, an essential part of the method we are developing entails so-called deep learning using reference samples for training and chemometric-type fitting and modeling."

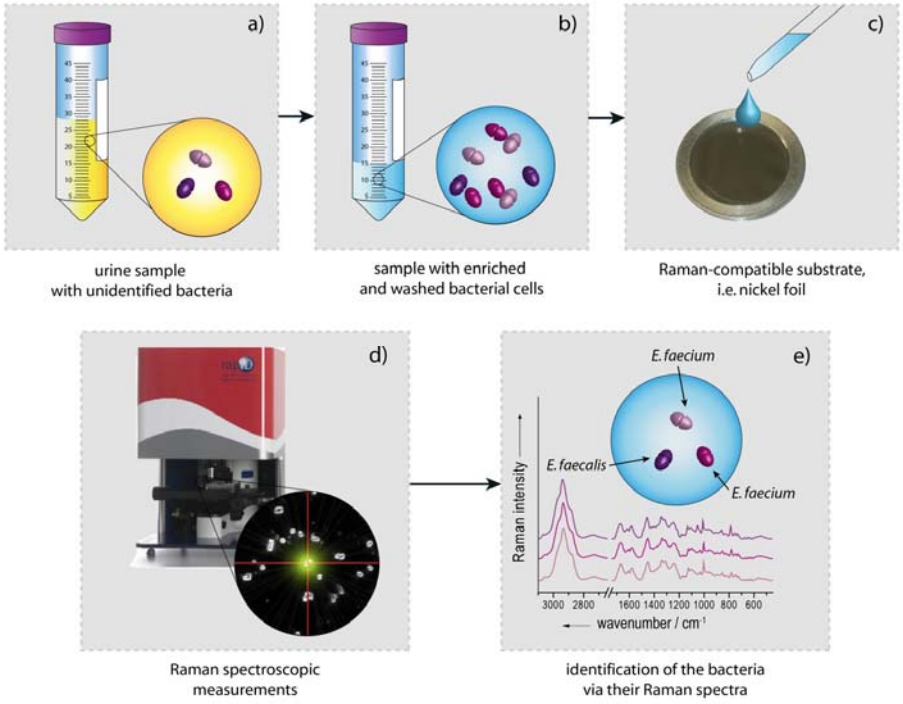

Figure 3. *Workflow for the Raman spectroscopic identification of single bacterial cells from urine samples.*

Most of the data have been recorded using a Raman microscope developed in collaboration with RapID (now mibic Berlin, Germany) configured with a 100X objective. The laser is focused through the objective and backscattered Raman is detected by a single stage monochromator equipped with a TE-cooled CCD camera. For investigating bacterial samples an excitation wavelength of 532 nm is the most common choice. However, for certain applications 785 nm or 244 nm (for Resonance Raman) might be better suited.

In one recent study, the first step was to acquire spectra of pure samples of 11 bacterial species that are commonly associated with UTI– see figure 3. The bacteria were extracted from urine samples by centrifuging and transferred onto a nickel foil for subsequent Raman spectroscopic characterization. Popp's group has investigated several methods for automating the optical targeting of single cells. For example, a smart imaging system ensured that spectra were only recorded for bacteria sized particles within the microscope field of view. An alternative method ideally suited for measuring several cells at once in liquid environment involves dielectrophoresis using four diagonal electrodes – see figure 4. Due to the electric field the cells experience polarization and will be accumulated in the region with maximum field strength.

A typical data processing routine Popp's group uses for converting the raw spectral data into a classification model proceeds as follows: A minimum of two single spectra for each target (i.e. one cell) are used to eliminate any cosmic spikes. The spectra are then calibrated regarding the wavenumber using a same-day recording of a Raman reference spectrum of acetaminophen. The background of each spectrum is eliminated with a modified commercial clipping algorithm. Key wavenumber regions are chosen (e.g., between 3100 – 2650 cm^{-1} and 1750 − 450 cm^{-1}) for the subsequent chemometric analysis performed using support vector machine (SVM) methodologies as well as principal component analysis (PCA).

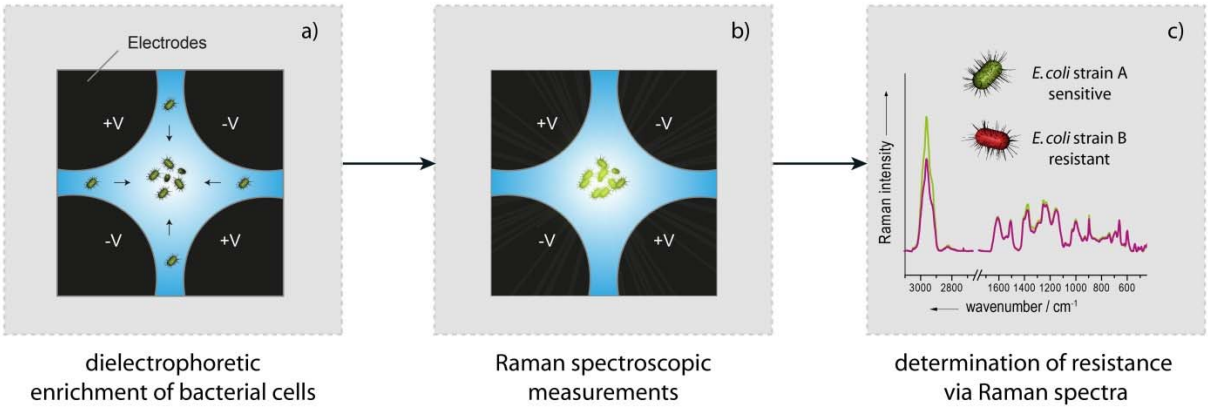

Figure 4. *Workflow for the Raman spectroscopic assessment of antibiotic susceptibility for bacteria.*

For the study concerning the UTI pathogens, a classification model was established using a total of 2952 single cell spectra. Overall, an accuracy of 92.1% was achieved for classification. For validation, the model was then tested on 429 independent single cell samples. 422 of them were identified correctly, yielding a sensitivity of 98.4 % and a specificity of 99.2 %. Due to this

promising result, the model was further challenged with spectra acquired from 10 urine samples of patients with confirmed UTI. For each sample the model assigned the same species as the reference method (VITEK 2 by biomerieux: cultivation based microbial identification system). This is a remarkable result, as many of the patients already had received an antibiotic treatment and this parameter hadn't been included in the model.

In another study Popp's group used a similar Raman technique to measure the effects of the antibiotic ciprofloxacin on 13 clinical E. coli isolates of varying resistance levels. They found that a good concordance could be established between the MIC values obtained by the Raman measurements after only <90 minutes of exposure to ciprofloxacin, and the MIC values from the gold standard broth microdilution assay which takes up to 12 hours.

Popp concludes, "After many years of research, we believe that Raman and related spectroscopic techniques are becoming promising tools in the medical arsenal of clinical tools. At Leibniz-IPHT, we are excited that our research is playing an active role in this translation process."

Future Research – Low Frequency THz-Raman – Emerging Field

Unlike the chemical fingerprint region most commonly studied in Raman, the low wavenumber region (THz-Raman) provides data about lower frequency excitations in condensed matter such as phonon scattering, and can thus be used to detect subtle phase differences such as two polymorphs of the same pharmaceutical, or differences in the orientation of nanodomains and microcrystals. For example, THz Raman spectroscopy modules from Coherent provide a "crystallinity meter," to observe a crystalline to amorphous transition (and vice versa) in real time. When coupled with a microscope, these modules can be applied for microspectroscopy, to assess phase effects and changes in specific locations, i.e., individual cells. Popp's group has recently acquired one of these Raman modules to expand their work regarding infectious diseases and other clinical applications, for example by combining THz data with chemical fingerprint spectra to develop chemometric models with even higher sensitivity and accuracy.

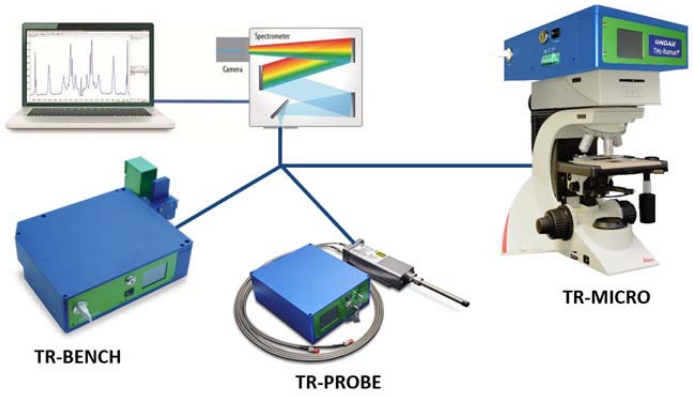

Figure 5. *Coherent now offers a series of integrated modules and probes to enable the performance of any Raman spectrometer or microscope to be extended to the THz frequency range.*