Chemical Imaging Using Molecular Spectroscopy – Providing Answers to Fundamental Biomedical Questions at the Nanoscale.

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ABSTRACT

Recent and future major developments in molecular and micro-analysis for nanomaterial and biomedical applications will be discussed and explained in terms of our vision of how instrumentation will need to perform and operate in the future to meet the challenging measurements required for nanomaterial research and development.

Keywords: Raman, AFM, SEM, spectral imaging, biomolecular imaging

INTRODUCTION

As leaders in Raman and fluorescence spectroscopy for over two decades HORIBA Jobin Yvon have been at the cutting edge of major breakthroughs in many key areas of instrument development and materials analysis. The history and philosophy of our instrument design including the latest hybrid approach of combining two or more techniques in one instrument for enhanced material characterization will be discussed. These hybrid combined microanalysis techniques include Raman/AFM, Raman/SEM, Raman/FT-IR and Raman/PL/CL.

Recent instrumental advances have allowed biomolecular imaging to probe molecular properties of biological materials at the nanoscale. Biomolecular imaging simultaneously combines nanomaterial distribution, morphology and size measurements with molecular identification and conformation – in other words it answers most of your material characterization questions at the same time. This fundamental understanding of what determines the physical and chemical properties of biomaterials at the nanoscale provides researchers with the capability for true "intelligent design".

These breakthrough studies include direct imaging of cells without the need for staining or tagging (which will often result in missed information or misinformation), the determination and/or the prediction of the nanostructure failure mechanism in bone and artificial joints and the characterization of single-walled nanotube diameter and chirality. The significance and direct applicability of this type of research is becoming thoroughly established in the biomedical, material and physical sciences.

With the concentration of research in nanotech, biotech and new energy alternatives – much of which in the future will also be directed towards nanotechnology, it is the intention HORIBA Jobin Yvon to continue to develop suitable instrumentation and software to enable and accelerate this research. Already we have developed or are developing instrumentation to allow Raman spectroscopy to provide direct molecular imaging of cancer, the location and monitoring of drug activity within tissue and at the celluar level, in-vivo spectroscopic guidance during surgery, in-situ studies during electronic and other physical perturbations of single nanotubes and drug interaction and delivery when combined with nanotubes.

INSTRUMENTATION AND APPLICATIONS

Raman microscopy is a coupled device of Raman spectrometer and a microscope, providing molecular identification and information about inter and intra-molecular interactions and changes at the spatial resolution of the microscope. The microscope, ultimately, can be any type – e.g. an optical microscope or a scanning microscope – with unique advantages for each type.

Automated Raman microscope

A Raman microscope with an optical microscope is a well established technology and available commercially from multiple manufacturers (Figure 1).

Figure 1. LabRAM ARAMIS IR^2, a commercially available, fully automated Raman microscope combined with an optical microscope and FT-IR

The spatial resolution can reach 200 nm, depending on the excitation laser wavelengths and the objective lens. Confocality is one of the unique advantages of an optical microscope enabling axial discrimination. Depth profiling of a transparent multi-layer sample (e.g. polymer) provides

information on the thickness and order of each layer as well as the chemical composition.

Raman spectral imaging

The first step of Raman spectral imaging is to record a Raman map. There are roughly three hardware approaches in recording Raman maps – point mapping, line scanning and global imaging.

Point mapping is the most often used method in the industry because Raman maps acquired achieve the highest spatial resolution and image quality. The laser illuminates a point on the sample to record a Raman spectrum, and the sample is moved along both X- and Y-axis. While point mapping has suffered the long standing criticism of being a slow technology, due to breakthrough improvements in hardware and software, the speed of Raman mapping has increased a great deal in the recent years and has become suitable for routine analysis. A few examples include piezo electronic stage with 1 nm precision, ICCD and EMCCD with single photon detection, and kinetic or blast mode for the fast data transfer.

Details of line scanning designs are different for each manufacturer. In essence, the method illuminates a line on the sample, and uses each row of CCD to record a Raman spectrum from each point on that line. The sample is then moved in only one direction, X- or Y-axis. Since the light is 'shared' by multiple points on the line, the laser power or acquisition time per line need to be increased. The speed is increased compared to point mapping because the number of lines is only the square root of the number of points.

Global imaging requires diffusing laser beam to illuminate a finite area. The Raman signal is generated from all points within the area. Unlike point mapping and line scanning methods that use monochromator or FT to discriminate spectral points, global imaging methods use LCTF (liquid crystal tunable filter) to discriminate the spectral points. The spectral resolution and range, therefore, is determined by a particular LCTF design, which, so far, is inferior to monochromators or FT methods. Also, the method collects one spectral point at a time. In other words, to achieve 1000 spectral points, which is a typical number of spectral data points collected with a monochromator based instrument, LCTF must be tuned 1000 times.

All three method produce 3 dimensional data (2 spatial axes and 1 spectral axis). Since all data presented in this paper is recorded with point mapping technique, the raw data format will be called a Raman map.

The second step of Raman spectral imaging is to process a Raman map to create Raman spectral or chemical images. The contrast in Raman spectral or chemical images is a result of spectral analysis of Raman maps and based on chemical difference in the sample. One of the major advantages of Raman spectral imaging is that without staining, tagging or extracting, the chemical composition is readily identified from the Raman spectra.

Since a Raman map consists of a large number of Raman spectra, it fast becomes impractical or impossible to examine every single spectrum within a map. Therefore, it is very important to develop data processing strategies to distill all scientifically accurate information into a few Raman spectral or chemical images. Especially for a sample that is spatially and chemically complex, univariate analysis (monitoring the intensity of a spectral band that is unique to one component in the sample) falls short, and it is necessary to perform multivariate analysis. Many mathematical and statistical algorithms have been developed for this purpose. Examples include classic least square analysis, K harmonic clustering, linear discrimination analysis, principal component analysis and partial least square analysis.

However, these mathematical and statistical algorithms are ultimately numeric analysis with, most often, no chemical constraints. Variance from non chemical sources such as noise, instrument response, fluorescence background, etc., influences the results. Therefore, it is imperative to maintain 'good' spectral quality to reduce these interferences and retain raw data to verify the analysis results.

Nanotubes Raman spectral imaging

Group theory predicts 15 to 16 Raman active modes. In actuality there are only 6 or 7 intense Raman active modes for any given tube chirality. Two most important Raman bands for carbon nanotubes (CNTs) are the ring breathing mode (RBM) and tangential mode (TM).

The RBM shows the most characteristic changes with CNT properties. For single wall carbon nanotubes (SWCNTs), RBM frequencies (Raman shift, cm^{-1}) are correlated linearly with the reciprocal of tube diameter (d_t). This relationship for an isolated SWCNT is shown in Equation 1 [1]. SWCNTs that are not isolated are subject to inter-tube interactions which affect the RBM frequency by 6-14 cm^{-1} increase.

$$\text{RBM peak position } (cm^{-1}) = 224/d_t \quad (1)$$

The TM corresponds to the stretching mode of the -C-C- bond in the graphite plane, and is sometimes called the G mode. The frequency of TM is ~ 1580 cm^{-1}. The D mode is located ~ 1340 cm^{-1} and is an expected mode in Multi Wall NanoTubes (MWNT). For SWCNTs, they represent defects.

When the excitation laser wavelength matches the energy bandgap between valance and conduction bands, resonance Raman spectrum is observed. The energy bandgap is dependent on tube diameter and chirality, and lasers of different wavelengths meet the resonance conditions with different species of SWCNTs. In resonance conditions, the Raman signal is highly enhanced and it is even possible to record the Raman spectrum of an isolated CNT, even if the size of an isolated CNT (~ 1 nm)

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is much smaller than the typical laser spot (~ λ). Raman spectra provide the information on its structural and electronic (n,m) properties.

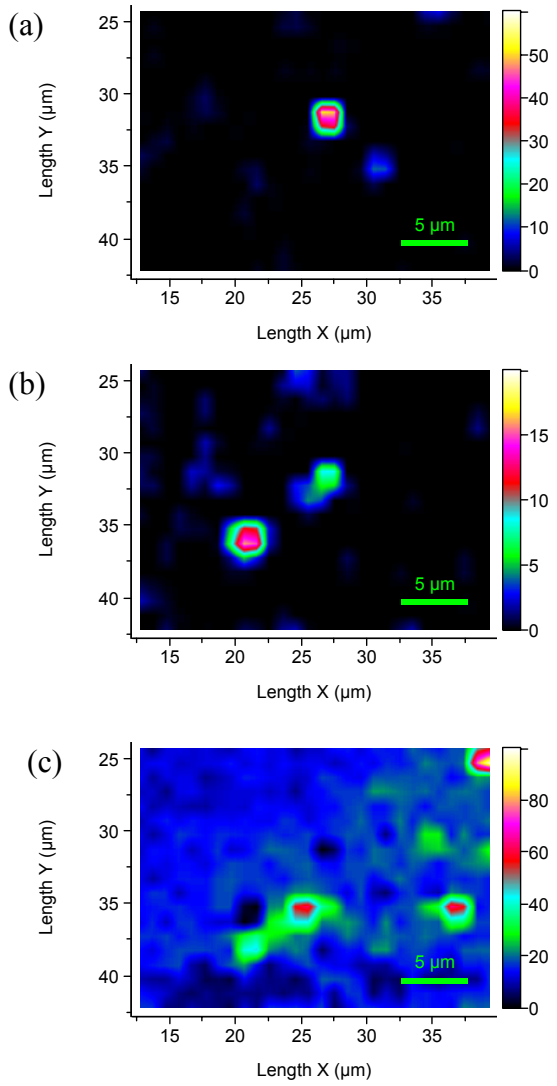

Figure 2. Raman intensity maps between (a) 157.8 – 161.7 cm^{-1} (b) 165.6 – 169.5 cm^{-1} (c) 262.9 – 270.6 cm^{-1} highlighting different CNT species.

Most often, nanotubes are fabricated 'in mass' and many different types can be present within a single sample. Raman spectral imaging over an area doped with nanotubes provides the details to where and what types of nanotubes are present.

A Raman map of SWCNTs doped on silicon substrates was recorded. Intensity maps at different RBM frequencies highlight SWCNT islands of different diameters (Figure 2).

Hybrid Raman microscopes

An advantage of a Raman microscope with an optical microscope is the ability to combine other spectroscopic techniques such as FT-IR and PL (photoluminescence) to perform multiple spectroscopic measurements from the same spot of the sample.

Combining Raman and PL spectroscopy can be accomplished by relatively minor modifications to the optical design. For example, PL signals of nanotubes fall in the near-IR region. Therefore, a detector that is sensitive to the NIR region such as InGaAs detector is added to the Raman microscope. At times, different types of filters and/or beam splitters that are more effective for PL experiment are also added.

Due to its complimentary nature to Raman spectroscopy, FT-IR unit is a very attractive addition to a Raman microscope. HORIBA Jobin Yvon incorporates the IlluminatIR series (Smiths Detection, Danbury, CT) to the LabRAM series of Raman microscope to share the microscope and optical path to the sample. This enables both Raman and IR measurements from the same spot (SameSPOT technology), whether it is a single measurement or a mapping measurement.

One of the latest breakthroughs in Raman microscopy is to couple Raman spectrometer with a scanning microscope such as AFM (atomic force microscope) or SEM (scanning electron microscope). Hybrid instrument between AFM and Raman are already available. (Figure 3).

Figure 3. LabRAM HR NANO, a commercially available Raman microscope with an AFM

One critical point that must be noted is that this particular instrument is designed to record near field Raman utilizing surface enhanced Raman scattering (SERS) phenomenon, as well as recording normal Raman spectrum and AFM topography, separately. Out of various designs to record near field signal with AFM or SNOM, illuminating the AFM tip from a side angle was developed into the first commercial unit due to its versatility in sample selection. Instead of preparing the sample with SERS substrate, the AFM tip is coated with silver or gold to provide surface enhancement field and brought in touch with the sample. The near field signals from the nano volume where the AFM tip touches are enhanced and, for the right sample, overwhelm the far field signals that are mixed in. Since the enhancement field is provide by the AFM tip, this is called tip enhanced Raman scattering (TERS).

This opened the possibility of coupling a Raman spectrometer with other scanning microscopes such as SEM (scanning electron microscope), STM (scanning tunneling microscope), SPM (scanning probe microscope) and TEM

(transmission electron microscope). Recently, Raman spectrometer and SEM has been successfully coupled and offered by multiple manufacturers by collimating the excitation laser beam path with the electron beam path of SEM.

CL (cathodluminescence) requires an electron beam source, therefore is relatively easy to couple with SEM first. Using the electron beam source from SEM, the CL signals (photons) are separated from SEM signals (electrons) with an ellipsoidal mirror and guided to the spectrometer.

After Raman and SEM are coupled, it naturally followed to add CL capacities to the combined instrument. The development is actively under progress.

REFERENCES

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