Nondestructive, High-Resolution Materials Characterization with the Confocal Raman-AFM

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Materials research, biomedical research, and semiconductor manufacturing can all benefit from nondestructive, high-resolution methods of analysis. As most materials are heterogeneous, it is important to not only acquire high resolution topographic information, but also to identify the chemical composition of samples. A combination of high resolution microscopy with chemically sensitive spectroscopy combined in one instrument allows the detailed characterization of samples with different analytical techniques. When individual instruments are used, returning to a previously surveyed sample area can be very time consuming if not impossible without surface markers.

The Techniques

In terms of chemical composition, Raman spectroscopy has become widely used for the characterization of materials [1, 2]. The primary advantage of the Raman Effect lies in its ability to detect specific molecular vibrations. In Raman spectroscopy the sample is irradiated with light from a laser source and consequently the molecules are excited from their ground state to an unstable virtual state. Immediately after excitation, the molecules return to either their original vibrational level (elastic Rayleigh scattering) or to the first energy level (Raman Stokes scattering). The difference in energy between the elastically scattered photons and the Raman shifted photons is caused by the excitation (or annihilation) of a specific molecular vibration. This energy shift is characteristic and therefore a fingerprint for the type and coordination of the molecules involved in the scattering process. As an example, Fig. 1 shows the Raman spectrum of poly-ethylene teraphtalate (PET). The correlation of the Raman-active bands to their molecular vibrations are summarized in Table 1 [3]. By comparing the position and intensity of Raman lines, qualitative and quantitative information about the composition of materials can be achieved. However, in most spectroscopy setups, spatial resolution is very poor because the exciting laser spot diameter is on the order of 100 µm. Optical microscopy, on the other hand, is capable of providing spatial resolution down to 200 nm using visible light excitation.

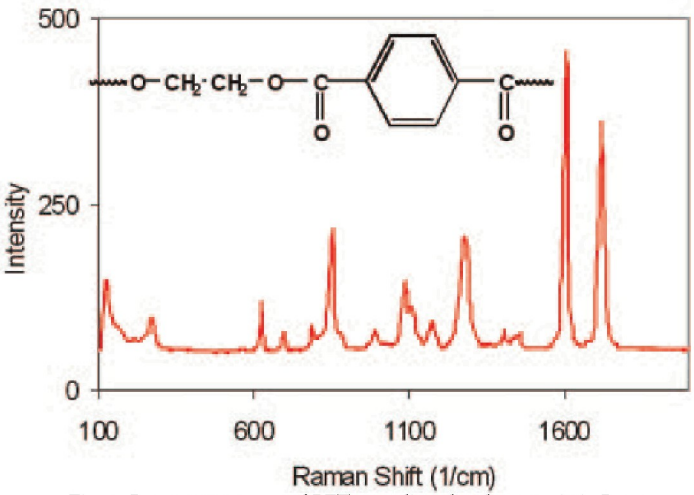


Fig. 1: Raman spectrum of PET revealing the characteristic Raman active bands.

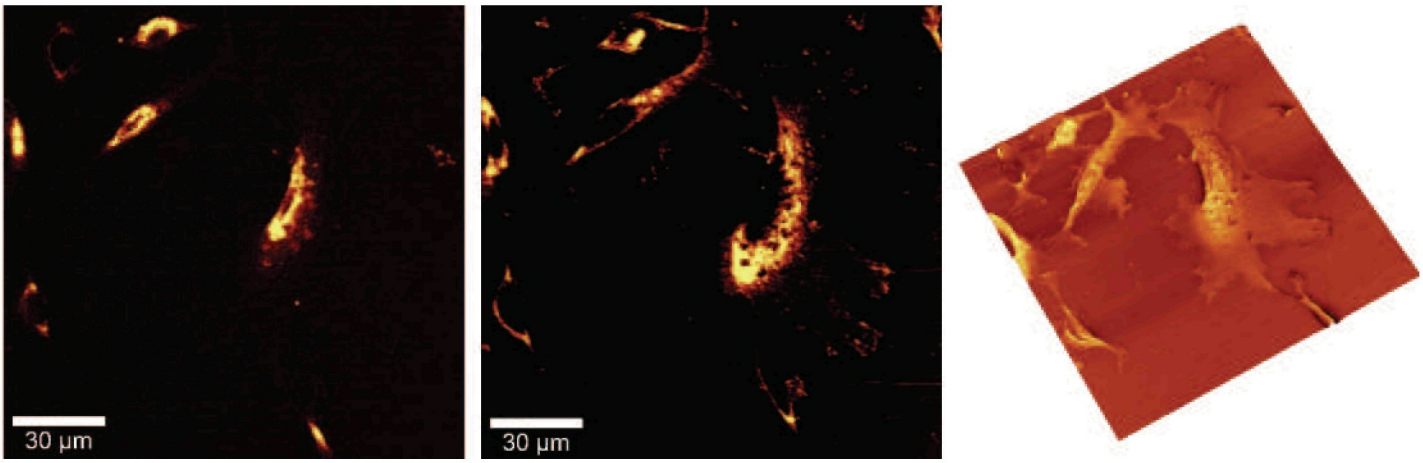


Fig. 2: Confocal microscopy images of HeLa cells: focal plane 2 µm (a) and 1 µm (b) above the supporting substrate; 3D stack of 15 confocal images (c).

Table 1: Raman active bands of PET and their associated molecular vibrations

Raman band (cm^{-1})	Molecular Vibration
626	Ring Mode 6b
857	Ring CC and C(O)-O stretching
1096	Ring CC, C(O)-O and ethylene glycol CC stretching
1119	C(O)-O stretching and ethylene glycol CC stretching
1295	C(O)-O stretching
1615	Ring Mode 8a
1730	C=O stretching

In a Confocal Microscope, the light from the sample is detected through a pinhole placed in the back focal plane of the microscope [4]. This setup detects only light from the focal plane, while out of focus light is strongly rejected, thus providing depth resolution and

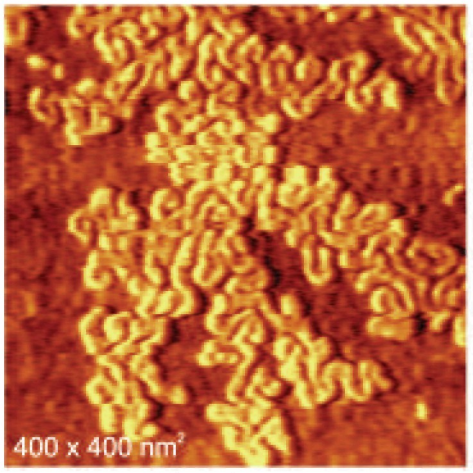


Fig. 3: High resolution AFM images of macromolecules of fluoroalcane.

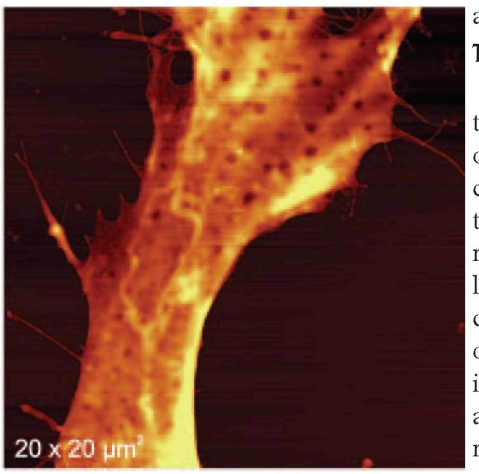


Fig. 4: AFM image of the growth cone attachment of a HeLa cell to the substrate.

a strongly reduced background signal. Images are recorded point by point and line by line by scanning the sample through the excitation focus. With this technique, the specimen can be analyzed in segments along the optical axis and even a depth profile, or a 3D image, can be generated. Fig. 2 shows confocal images of a HeLa cell. HeLa cells are human epithelial carcinoma cells that are cultured for scientific uses such as: studies of cellular effects arising from drugs and radiation, development of a polio vaccine *etc*. Fig. 2a shows a confocal image taken at z = 2 µm from the supporting surface and Fig. 2b at z = 1 µm. In Fig. 2c, an image stack is shown. This image consists of 15 confocal images recorded at different focal depths and stacked into a 3D image. The total height of the image is 2.5 µm.

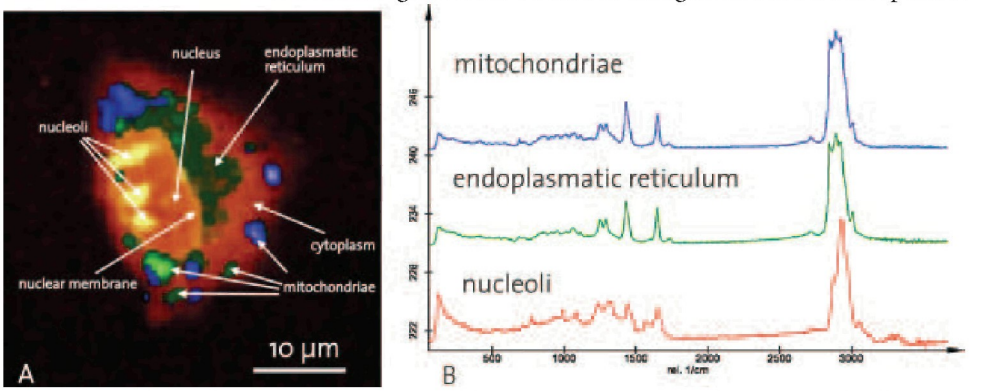


Fig. 5: Chemical composition of a living cell imaged in Raman spectral imaging mode (a) and characteristic spectra (b).

To overcome the optical resolution limits arising from the diffraction of light, a new imaging technique became necessary. A breakthrough in the imaging of surfaces was achieved with the introduction of the Atomic Force Microscope in 1986 [5]. In this technique, the interaction forces between a surface and a sharp tip mounted on a cantilever are employed to trace the topography of samples. This relatively new technique can provide spatial information parallel and perpendicular to the surface with resolution on the order of 1 nm. Single molecules can be visualized as shown in Fig. 3. In addition to topographic high-resolution information, local material properties can also be investigated by analyzing the tip-sample interaction forces.

The Confocal Raman-AFM (CR-AFM)

The Confocal Raman–AFM combines the three techniques mentioned above in one instrument, enabling the nondestructive characterization of materials with respect to their chemical composition and high resolution 3D surface topography without laborious sample preparation. The materials can be analyzed under ambient conditions or in a liquid environment. For all measuring techniques, the sample is mounted on a piezoelectric scan table with typical scan ranges of 100x100 µm² and 20 µm *z*-movement. Capacitive feedback control guarantees the linearity of the piezo-scanner in all three dimensions. With the Confocal Raman Microscope (CRM), it is possible to obtain Raman spectra from extremely small sample volumes (down to 0.02 µm³) and to collect high resolution Raman images. In the Raman spectral imaging mode, a complete Raman spectrum is acquired at every image pixel and the images are generated by analyzing spectral features (sum, peak position, peak width, *etc*.). As a Raman image consists of several thousand pixels (spectra), the integration time per spectrum must be as short as possible. Therefore, special care has been taken to optimize the optical throughput and sensitivity of the setup. By simply rotating the microscope turret, the CRM is converted into an AFM. A special objective that allows for the mounting of an AFM cantilever was designed, allowing the same sample area to be imaged at the highest resolution.

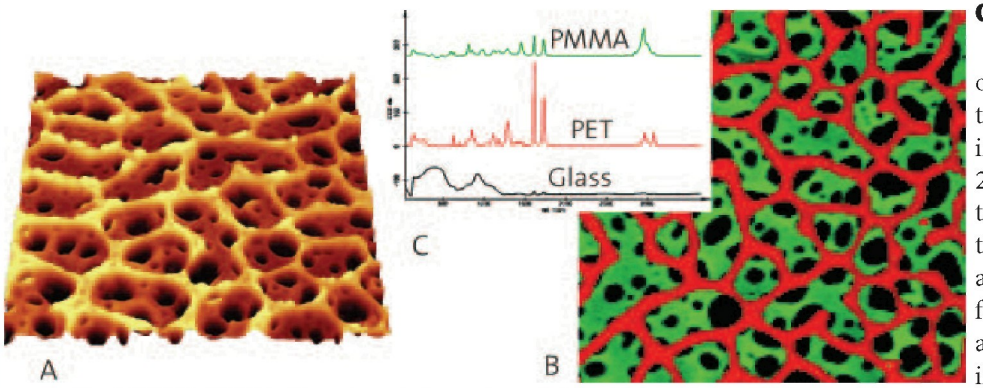
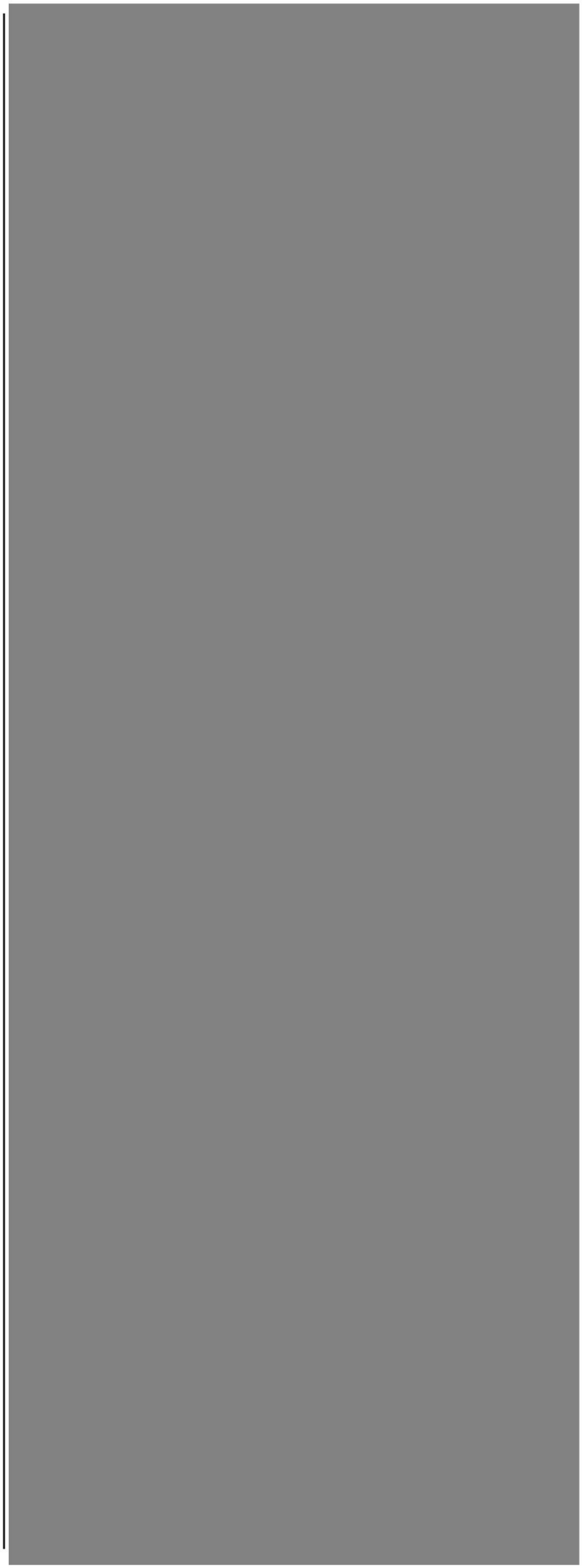


Fig. 6: PMMA-PET thin film spin coated on a glass substrate: high resolution AFM image (a), color coded Raman spectral image (b), and characteristic Raman spectra of components (c).

CR-AFM Applied to Biology

Imaging of living cells in their physiological surroundings without damaging them is a highly sought-after capability in life science. As already shown in Fig. 2, Confocal Microscopy is an appropriate technique for gathering information about the shape and size of cells. A more detailed analysis of the cell surface can be performed by rotating the microscope turret and converting the Confocal Microscope into an AFM. Fig. 4 shows the high resolution surface topography of the HeLa cell



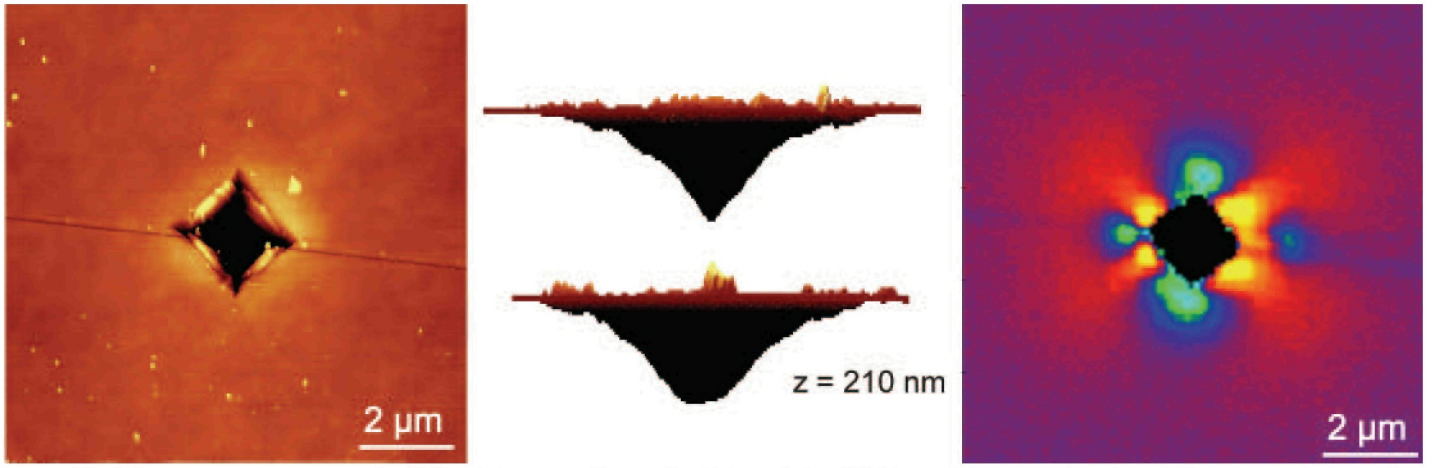


Fig. 7: Stress measurements around a Vickers indent on a silicon wafer: high resolution AFM image (a), cross sections through the asymmetric shape of the indent (b), and stress map (c).

growth cone attached to the surface. This AFM image reveals many more details of the cell surface than the optical image, allowing for the study of sub-membrane structures on the cell surface.

Nevertheless, microscopic studies alone do not provide any information about the chemical composition of the cell. Confocal Raman Microscopy reveals the distribution of complex chemical compounds such as proteins and lipids in cells. Fig. 5a shows a color-coded Raman spectral image of a living epithelial rat cell in its physiological surroundings. To obtain such an image, no labeling of the cell is required. Characteristic Raman spectra of the cell are represented in Fig. 5b. By analyzing characteristic peaks of the spectra, the components of the cell can be distinguished with a resolution of approximately 270 nm. The nucleus of the cell consists mainly of amino-acids, the mitochondriae are composed of phosphorilated proteins, and the endoplasmatic reticulum is responsible for the protein biosynthesis. Variations in the C-H stretching bands at 2800 – 3000/cm, the protein characteristic bands at 1450/cm and 1660/cm, and in the characteristic bands for lipids at 1070/cm, 1300/cm and 1440/cm can be observed while imaging and allow the identification of the various components in the cell.

CR-AFM Applied to Materials Research

The development of advanced polymer materials requires detailed information about the physical and chemical properties of these materials on the nanometer scale. However, some details of the phase separation process in polymers are difficult to study with conventional characterization techniques due to the inability of these methods to chemically differentiate materials with good spatial resolution, without damaging, staining, or preferential solvent washing. The CR-AFM provides insight in terms of both high resolution topographic information and the distribution of chemical species within the polymer. Fig. 6a shows a 20 x 20 µm^2 AFM image of a thin film of the polymer blend PMMA-PET spin-coated onto a glass slide. Three topographic levels become visible in the AFM image: a netlike structure with an average height of 170 nm, a film with an average height of 80 nm, and the dark holes within the film. The data acquired with the Raman spectral imaging mode from these polymer blend films (Fig. 6b) reveal that the netlike structure corresponds to PET, whereas the lower film is the PMMA phase. The dark topographic holes correspond to the plain glass substrate. The spectra of the corresponding materials are shown in Fig. 6c.

In addition to the identification of different chemical components within a material, slight changes in the crystallographic structure that lead to stress in materials can also be detected with Raman spectroscopy. The stress field around a Vickers indent in a silicon wafer and its topographical shape was analyzed with the CR-AFM. The Vickers indent was performed with a force of 50 mN, resulting in a pyramidal hole. The shape of the pyramidal hole was characterized with the AFM as shown in Fig. 7a. The Vickers indent has the following geometric characteristics: base dimensions 2 x 1.7 µm^2, depth 210 nm. The cross sections along the short (top) and long (bottom) sides of the Vickers pyramid are shown in Fig. 7b, revealing the asymmetric shape of the indent. The Raman image is obtained by just rotating the turret from the AFM objective to a standard optical microscope objective. The same sample area was imaged in Raman spectral imaging mode. The relative shift of the 520/cm Si Raman line was analyzed and is shown in Fig. 7c. The stress field extends more than 5 µm from the center of the Vickers indent and mirrors the geometry of the Vickers pyramid. The tensile strain appears at the edges of the pyramid, while the compressive strain appears at the flat sides.

Summary

The ability to study samples with respect to their topographic structure at the highest resolution and to link these fine structures to the chemical composition of the materials, provides new perspectives in material characterization. Heterogeneous materials can be analyzed without labeling, staining, or preferential solvent washing. The topographic features observed on the nanometer scale with the AFM can be directly linked to the chemical information obtained with the Confocal Raman Microscope. The materials can be analyzed under ambient conditions or in a liquid environment without laborious sample preparation. ■

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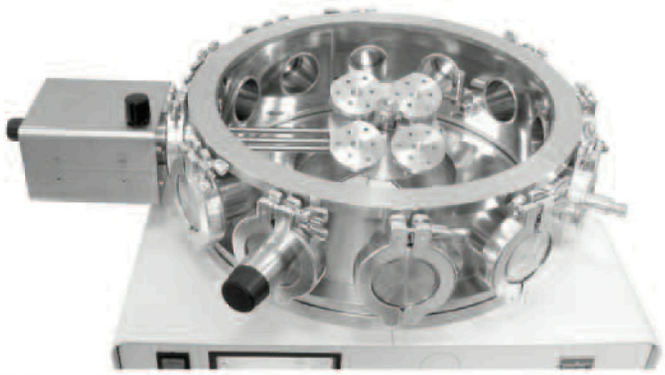

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