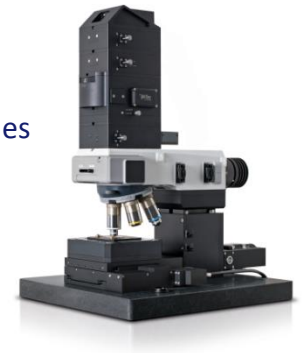
Whitepaper

Five criteria for high-quality Raman microscopes

An introduction to correlative Raman techniques including application examples



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90 years ago Chandrasekhara Venkata Raman and Kariamanickam Srinivasa Krishnan first documented “A New Type of Secondary Radiation”, which then became known as the Raman Effect [1]. Raman spectroscopy is based on this effect and is used for qualitative and quantitative analysis of the chemical components and molecules of a sample. It is a non-destructive method that requires little, if any, sample preparation.

Nevertheless, Raman spectroscopy long remained a technique that was only performed in special laboratories. In recent years, however, it has been increasingly losing its outsider status. The reason for this is the development of the confocal Raman microscope, with which not only individual Raman spectra, but also complete images generated from thousands of spectra can be acquired [2]. Through continuous development, commercially available Raman microscopes are also becoming more user-friendly. For example, modern software interfaces guide the user through the Raman measurement and the subsequent data analysis, while automated opto-mechanical components can allow higher-end systems to self-align and self-calibrate.

There are several key factors that can be used as criteria for determining the quality of confocal Raman microscopes (Figure 1).

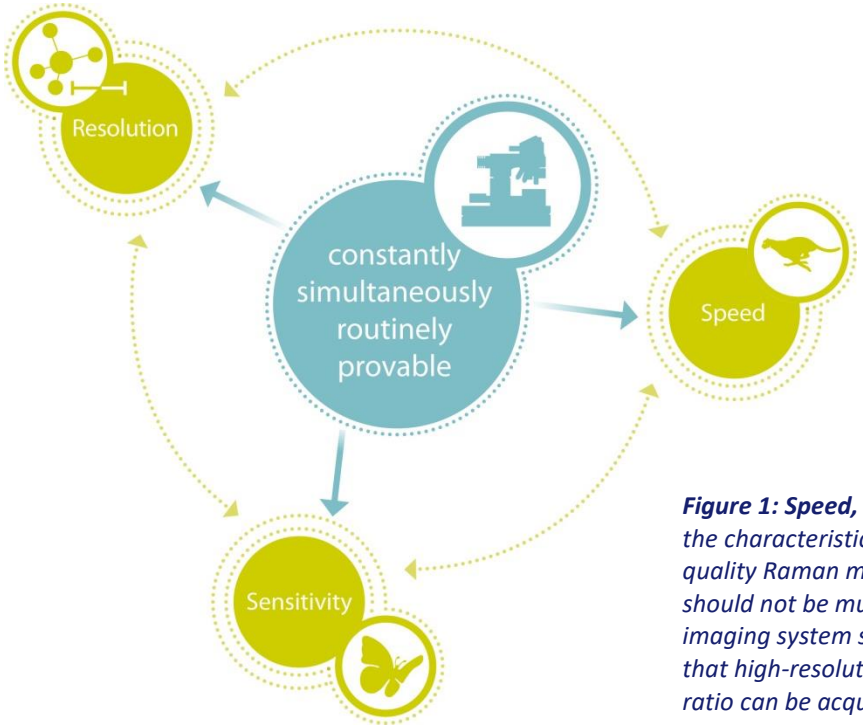

Figure 1: Speed, sensitivity and resolution *are some of the characteristics that can be used to identify a high-quality Raman microscope. These three characteristics should not be mutually exclusive. Ideally, the Raman imaging system should be configured in such a way that high-resolution images with a high signal-to-noise ratio can be acquired in a short period of time.*

1) Speed

While in the past exposure times of minutes to hours were common for acquiring single Raman spectra, today these times are generally fractions of a second to less than one millisecond. In one second more than 1000 Raman spectra can be recorded. Thus a Raman image can be generated within a few minutes (Figure 2). To achieve this acquisition speed, the Raman imaging system should be equipped with optimized optics and an EMCCD camera.

High acquisition speeds are particularly important for measurements on sensitive and valuable samples in which the excitation energy must be as low as possible. Time-resolved investigations of dynamic processes can also benefit from rapid Raman spectral acquisition. Operating costs can also be reduced by shorter analysis times concurrent with increased data rates. Having a high system speed is also advantageous for time-critical work.

2) Sensitivity

The signal sensitivity of a system is critical for the quality of the results and is especially important when weak Raman signals are to be detected. In order to achieve the best possible sensitivity, a confocal beam path, i.e. using a diaphragm aperture, must be employed to eliminate light from outside the focal plane to increase the signal-to-noise ratio. The entire Raman imaging system should also be optimized for high light throughput. This includes a spectrometer that ensures throughput of over 70% and is designed for measurements with low light and signal intensity. CCDs optimized for spectroscopy, which exhibit more than 90% quantum efficiency in the visible range, are most commonly used as detectors. Finally, the use of almost lossless photonic fibers ensures efficient light and signal transmission.

3) Resolution

The resolution of a Raman system is comprised of both spatial and spectral resolution.

The spatial resolution includes the lateral resolution (x- and y-directions) and the depth resolution (z-direction). The spatial resolution is determined by the numerical aperture of the objective used and the excitation wavelength. The higher the numerical aperture and the smaller the excitation wavelength, the higher is the achievable lateral resolution. In addition, a confocal microscope produces images with a higher contrast because the background signal is reduced. The smaller the aperture of a confocal microscope, the higher its resolution is. In a confocal Raman microscope, a lateral resolution of about 200 - 300 nm and a depth resolution below 1 µm can be achieved. A confocal microscope can also create optical sections from different focal planes, which can be used with transparent samples for depth profiles and 3D images (Figure 2).

Spectral resolution defines the ability of a spectroscopic system to separate Raman lines near one another. Symmetric peaks in the spectrum are ensured by a spectrometer design that operates free of coma and astigmatism. The grating used, the focal length of the spectrometer, the pixel size of the CCD camera and the size of the aperture also affect the spectral resolution. At room temperature, the width of the Raman lines is typically greater than 3 cm^{-1}, but some applications (gases, low temperature or stress analysis) may require significantly higher resolution.

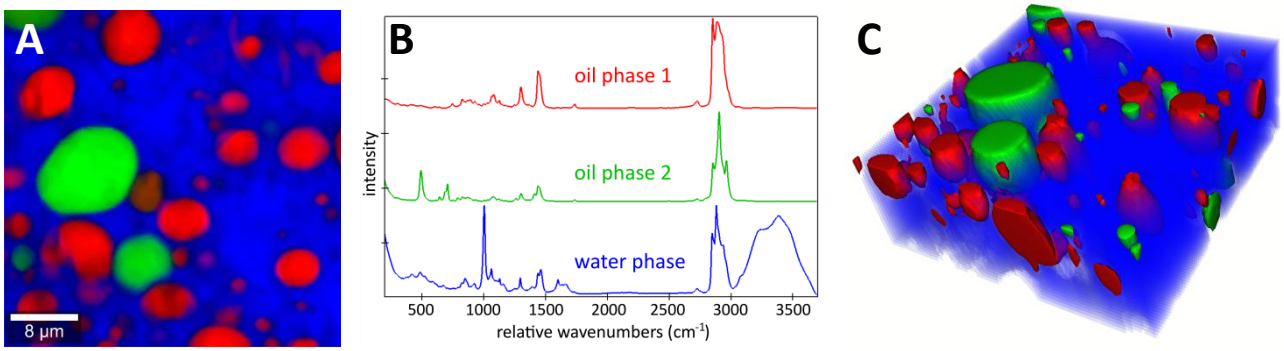

Figure 2: Confocal Raman imaging measurement of a moisturizing hand cream. *The emulsion consists of a water phase (blue) and two oil phases with different moisturizing ingredients dissolved in them (red and green).* ***A:*** *2D Raman image recorded in about 5 minutes and color-coded according to the Raman spectra in B. 40 x 40 µm²; 200 nm per pixel; 5 ms per spectrum.* ***B:*** *Raman spectra of the three detected components.* ***C:*** *3D Raman image generated from 15 2D images recorded at different focal planes. 200 x 200 x 15 pixels; 40 x 40 x 15 µm³; 5 ms per spectrum.*

4) Modularity and upgradeability

The introduction of Raman microscopy into laboratories places new demands on commercially available systems. These requirements can sometimes appear contradictory: easy operation with diverse functionality, a wide range of applications with optimized sensitivity, low cost and high performance. In order to offer users a Raman system tailored to their individual requirements, it is particularly important that systems have a modular design that can be adapted to new conditions through being reconfigured or upgraded. A system can be optimized for specific requirements by individually combining suitable lasers, filters, lenses, spectrometers and detectors. With such a customized Raman imaging system (Figure 3) the user is able to obtain meaningful Raman images, perform 3D volume scans and create depth profiles (Figure 2).

5) Combinability

Confocal Raman microscopy can be combined with other microscopy techniques. By using different methods and correlating the data, the user attains a more comprehensive understanding of the sample. Common examples of correlative microscopy techniques are Raman-AFM (AFM = Atomic Force Microscopy), Raman-SNOM (SNOM = Scanning Nearfield Optical Microscopy) and Raman-SEM (SEM = Scanning Electron Microscopy). In order to correlate the data of these disparate technologies, the exact same sample location must be examined by each approach. If different instruments are to be used, finding this sample location can be very difficult and time-consuming. This is made much easier with a hybrid system that combines the different analysis methods in one instrument so that the sample can remain in place during all measurements. Some applications of correlative Raman microscopy are presented below.

Figure 3: The confocal Raman imaging microscope alpha300 R (WITec GmbH): *modular and upgradable for adapting to new requirements.*

Application examples for correlative Raman microscopy

Raman and profilometry

The combination of a confocal Raman microscope with a profilometer module for focus stabilization allows rough or inclined surfaces to be examined [3, 4]. During Raman analysis, the examination area is kept constantly in focus by the simultaneously acquired profilometry data. This also compensates for thermal shifts and enables long-term measurements. The application example in Figure 4 shows the analysis of a microstructured silicon sample. The chemical image of the Raman measurement was overlaid onto the topographic profile measurement.

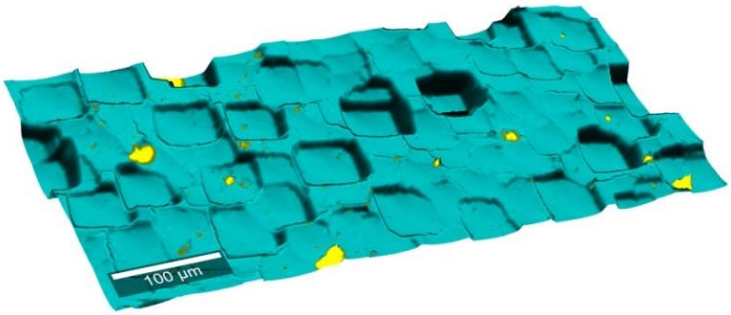

Figure 4: Topographic Raman image of a silicon microstructure. *The height difference was up to 9 µm. Turquoise: silicon; yellow: impurities that exhibited fluorescence.*

Raman and fluorescence

Fluorescence microscopy has been a widespread imaging method for the analysis of biological cells and organisms for decades. Samples are stained with fluorescent dyes or organisms are genetically engineered to express fluorescent proteins. The fluorescence signal is usually much stronger than the Raman signal. Nevertheless, correlative Raman fluorescence measurements are possible with an appropriate system. Figure 5 shows a Raman fluorescence image of a live cell culture of eukaryotic cells. An inverted confocal Raman microscope was used to examine the cells in their aqueous cell culture medium in the Petri dish. The cell nuclei were stained with the fluorescent dye DAPI. An excitation wavelength of 532 nm was used for the Raman measurement. In the correlative Raman fluorescence image (Figure 5A), the nuclei are shown in blue (recorded with fluorescence microscopy), the nucleoli in green and the endoplasmic reticulum in red (recorded with Raman microscopy). The corresponding Raman spectra are shown in Figure 5B.

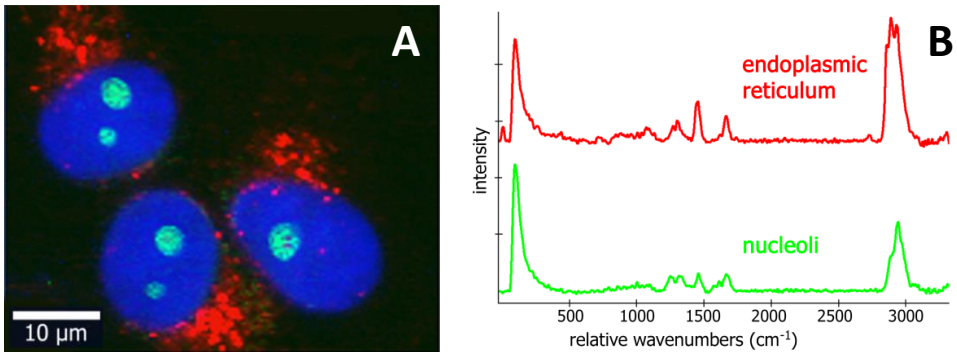

Figure 5: Correlative Raman fluorescence image of eukaryotic cells in cell culture. A: *DAPI-stained nuclei (blue) were recorded by fluorescence microscopy and endoplasmic reticulum (red) and nucleoli (green) by Raman microscopy.* ***B:*** *Associated Raman spectra. Sample courtesy of Claudia Scalfi-Happ (ILM, Ulm, Germany).*

Raman and AFM

The combination of Raman microscopy, which provides information about the type and distribution of molecules in a sample, and the high-resolution AFM technique, which determines the surface characteristics of a sample, enables the visualization of both chemical and morphological properties.

Here the analysis of a 1:1:1 mixture of polystyrene (PS), 2-ethylhexyl acrylate (EHA) and styrene-butadiene rubber (SBR) is shown. For this, a correlative Raman-AFM microscope was used, in which Raman microscopy and AFM technologies are fully integrated. The measurement with AFM in intermittent AC mode documents the topography of the polymer mixture (Figure 6A). The simultaneously-recorded phase image provides information on the viscosity and elasticity of the individual components of the polymer mixture (Figure 6B). The confocal Raman image (Figure 6C) shows that PS (red) and EHA (green) are present separately. SBR (purple) partly mixes with EHA (mixture shown in blue). By correlating the Raman image with the AFM image, the chemical information can be linked to the structural information (Figure 6D).

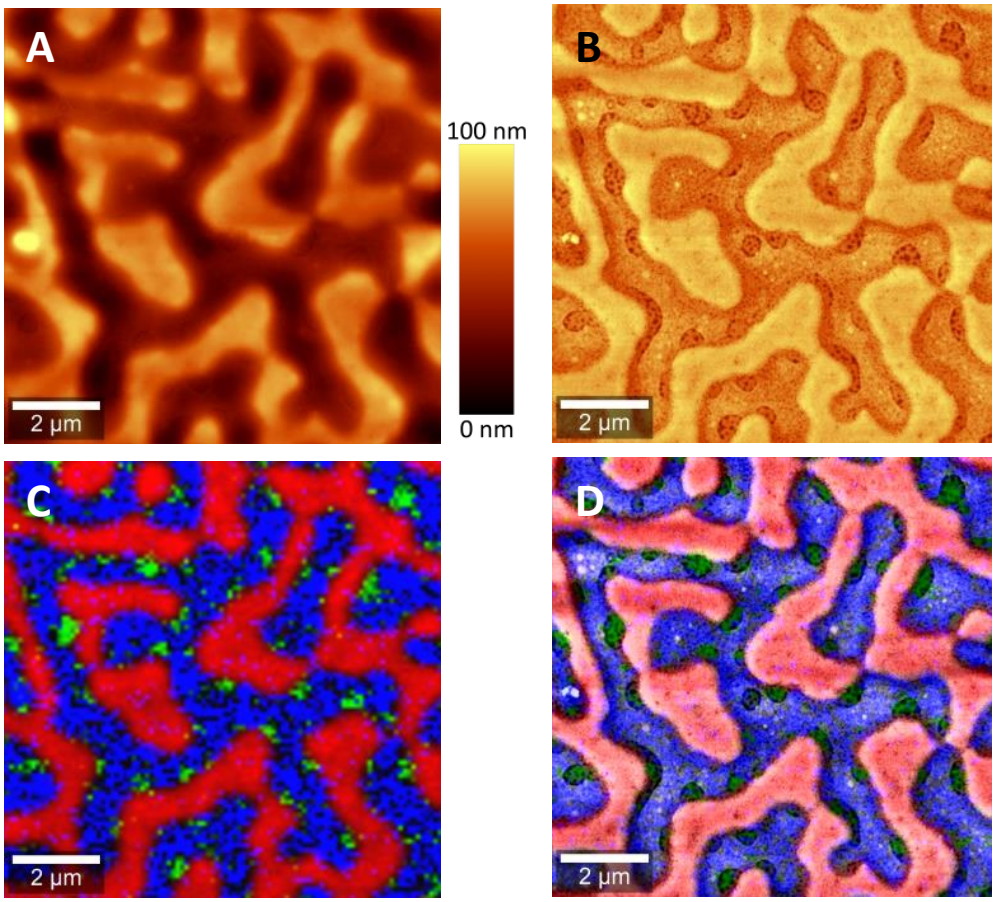

Figure 6: Correlative, high-resolution Raman-AFM microscopy of a 1:1:1 mixture of polystyrene (PS), 2-ethylhexyl acrylate (EHA) and styrene-butadiene rubber (SBR). *Both measurement technologies are combined in the WITec alpha300 RA microscope.* ***A:*** *The topography of the polymer mixture was determined with AFM in AC mode.* ***B:*** *The phase of the AFM image shows the fine structure of the compound.* ***C:*** *A color-coded, confocal Raman image, generated from the Raman spectra, shows the distribution of the polymers PS (red), EHA (green), SBR (purple) and SBR-EHA mixture (blue).* ***D:*** *The correlative Raman-AFM image links the structure and distribution of the different polymers.*

Raman and SEM

Scanning electron microscopy (SEM) is a well-established method for structural surface analysis. By combining Raman imaging with SEM in a correlative microscope it is possible to combine results of SEM structural analysis with chemical and molecular information from confocal Raman microscopy [5]. The sample is placed in the vacuum chamber of the electron microscope. Both analysis methods are then carried out automatically at the same sample location. The obtained SEM and Raman images can then be superimposed. This combination is referred to as Raman Imaging and Scanning Electron (RISE) microscopy.

In Figure 7 a structure several atoms in thickness comprised of graphene layers was analyzed by correlative Raman-SEM microscopy. The Raman image consists of 22,500 spectra with 50 ms recording time per spectrum. While structural features are visible in the SEM image, the number of graphene layers and their different orientations can be analyzed in the Raman image. This is not possible with SEM alone.

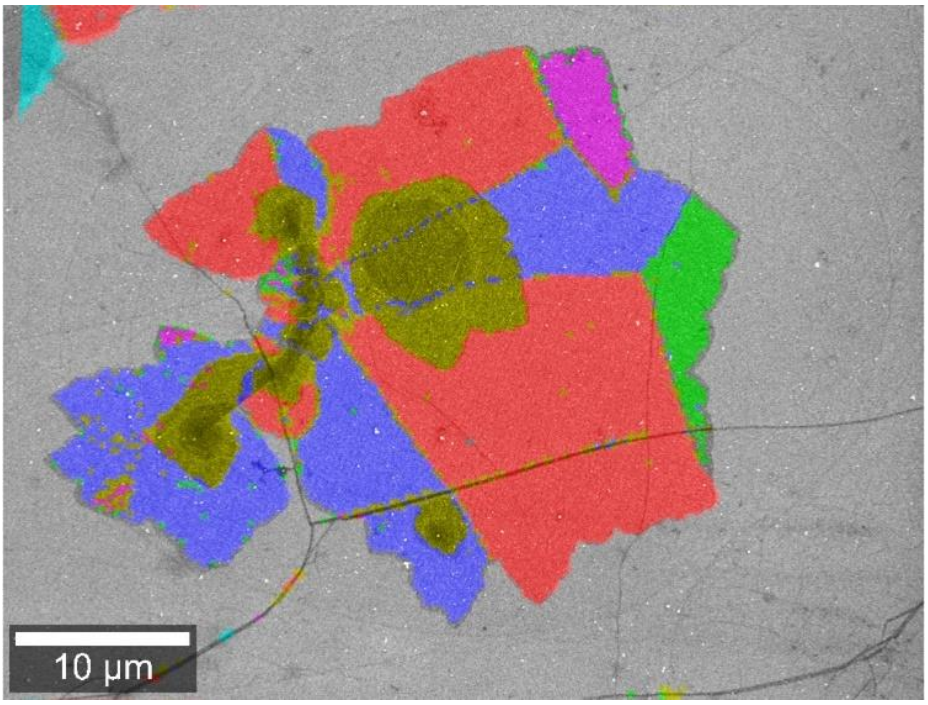

Figure 7: Correlative RISE (Raman-SEM) microscopy image of a multilayer graphene flake. *The colors represent different stacking orders, twist angles and layer numbers in the graphene and could be identified by Raman analysis.*

Raman particle identification and characterization

High-resolution investigations of particles are of great interest in many fields of application such as environmental science, pharmaceutical research and many others. Combining a particle analysis tool with the fast, label-free and non-destructive Raman imaging technique makes it possible to find, classify, and identify particles in a sample according to their size, shape and chemical characteristics. The physical and molecular attributes of the particles in a sample may be correlated and qualitatively and quantitatively evaluated. Figure 8 shows the results of particle analysis carried out on a cosmetic peeling cream sample. Figure 8A shows the overlay of an optical bright-field image with the corresponding confocal Raman image. The chemical analysis revealed anatase (a form of titanium dioxide) and boron nitride particles in an oil matrix (Raman spectra shown in Figure 8B). For a more detailed particle analysis, about 4000 particles were identified automatically by targeted acquisition of their Raman spectra. Quantifying the molecular sample components revealed that anatase and boron nitride particles were present in similar amounts (Figure 8C). The size distribution of these two particle types further showed that boron nitride particles are statistically smaller than anatase particles (Figure 8D). In extended analyses the chemical characteristics of particles could also be linked to parameters such as perimeter, bounding box, Feret diameter, aspect ratio, circular equivalent diameter and many others. This illustrates the potential for comprehensive investigations of particles in many fields of application.

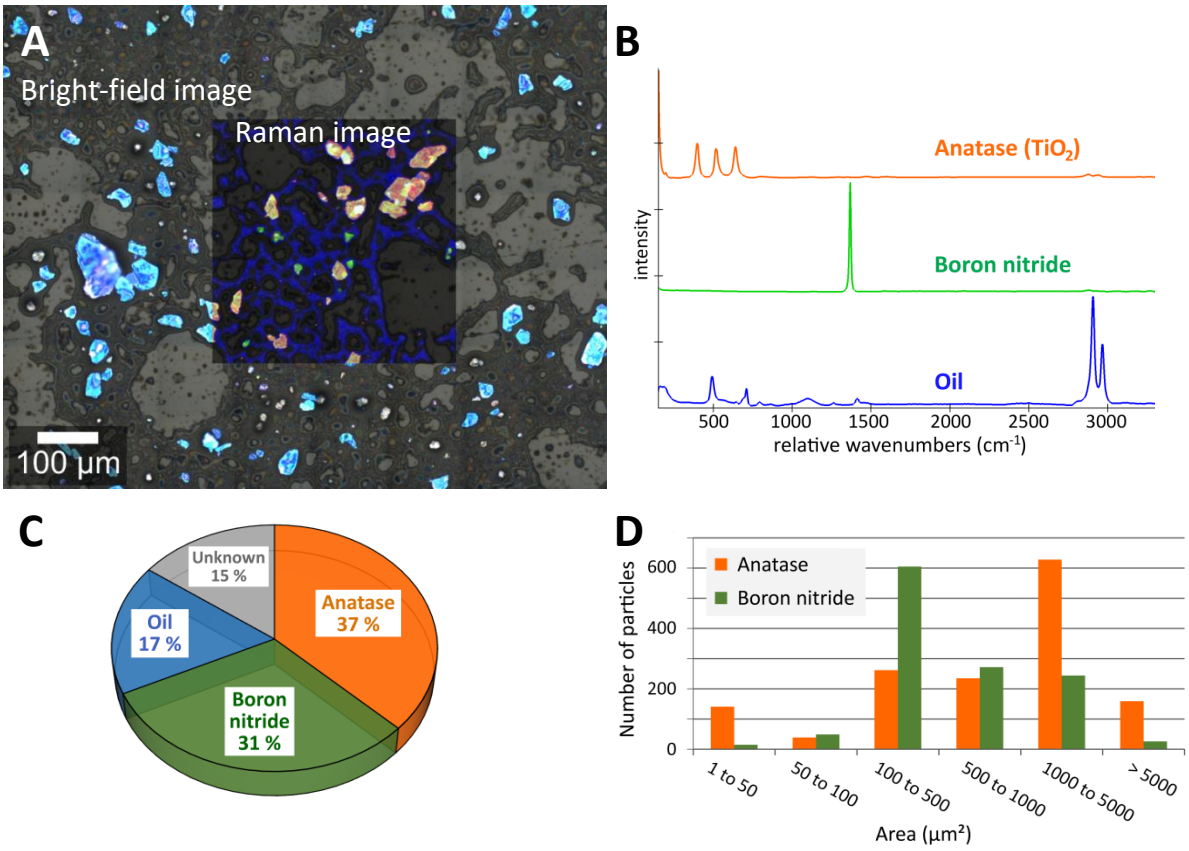

Figure 8: Particles in a cosmetic peeling cream sample. A: *Bright-field image overlaid with the confocal Raman image (color-coded according to the spectra in B).* ***B:*** *Corresponding Raman spectra of the sample's molecular components anatase (orange), boron nitride (green) and oil (blue).* ***C:*** *Pie chart of the quantitative compound distribution in the sample. In total, 3941 particles were measured, including some oil droplets.* ***D:*** *Size distribution of the anatase (orange) and boron nitride (green) particles, quantified by their projection areas.*

Summary

Raman microscopy enables qualitative and quantitative analysis of chemical components in a sample without requiring specialized sample preparation or risking damage to the sample. High-quality Raman microscopes should feature high speed and sensitivity as well as high spatial and spectral resolution, all at the same time. Modular and upgradable systems additionally make it possible to react flexibly to changing experimental requirements. The combination of Raman imaging with other techniques, such as AFM, profilometry, SEM, fluorescence microscopy or particle analysis, can yield additional information on the sample that would not be available with only one technique. This is illustrated by numerous examples from diverse fields of application.

Literature

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About WITec

WITec GmbH pioneered 3D Raman imaging and correlative microscopy and continues to lead the industry with a product portfolio that offers speed, sensitivity and resolution without compromise. Raman, AFM and SNOM microscopes, select combinations thereof, and WITec-developed Raman-SEM (RISE) instruments can be configured for specific challenges in chemical and structural characterization through a modular hardware and software architecture with built-in capacity for expansion. Research, development and production are located at WITec headquarters in Ulm, Germany, and the WITec sales and support network has an established presence in every global region. In September 2021, WITec became a member of the Oxford Instruments Group, bringing technology leadership in Raman microscopy to its extensive portfolio of businesses.