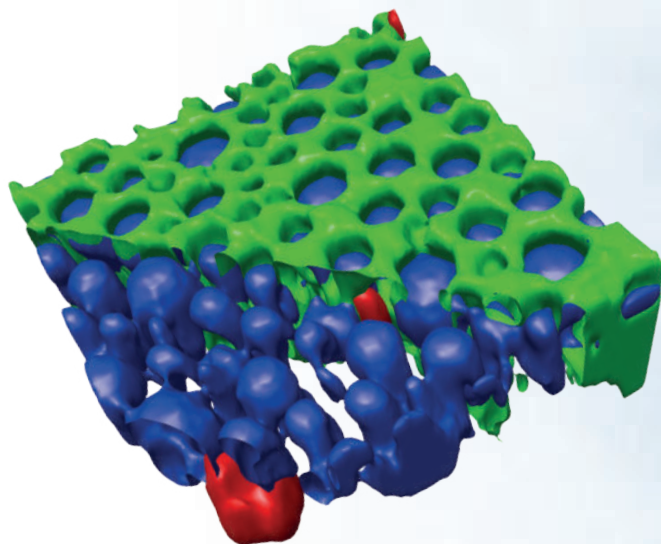
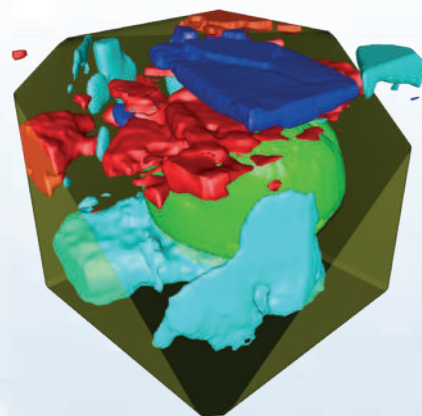
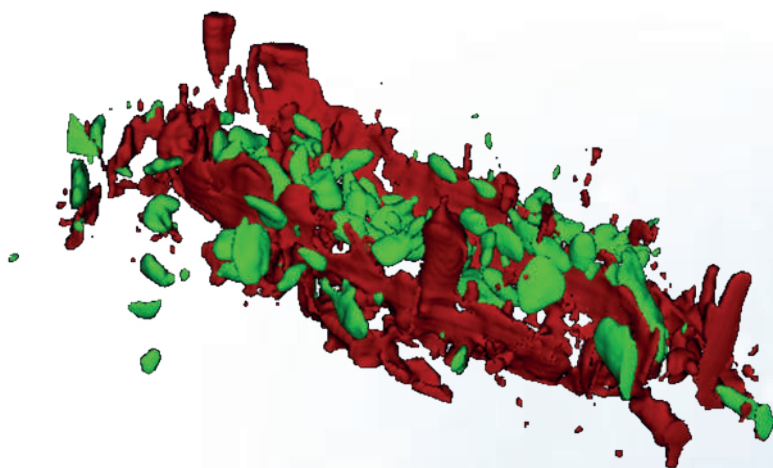


3D Raman Imaging

Best practice examples from various fields of application

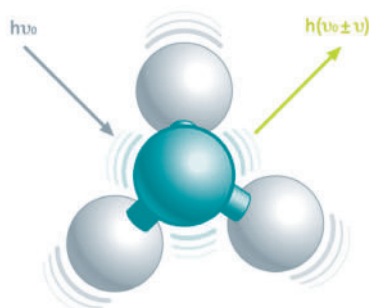


Confocal Raman microscopy is a powerful technique for high-resolution, non-destructive and label-free chemical analysis. It is well-suited for three-dimensional investigation of samples from various fields of application.

The Raman principle

The Raman effect is based on the inelastic scattering of light by the molecules of gaseous, liquid or solid materials. The interaction of a molecule with photons causes vibrations of its chemical bonds, leading to specific energy shifts in the scattered light. Thus, any given chemical compound produces a particular Raman spectrum when excited and can be easily identified by this individual “fingerprint.”

Raman spectroscopy is a well-established, label-free and non-destructive method for analyzing the molecular composition of a sample.



Raman imaging

In Raman imaging, a confocal microscope is combined with a spectrometer and a Raman spectrum is recorded at every image pixel. The resulting Raman image visualizes the distribution of the sample’s compounds. Due to the high confocality of WITec Raman systems, volume scans and 3D images can also be generated.

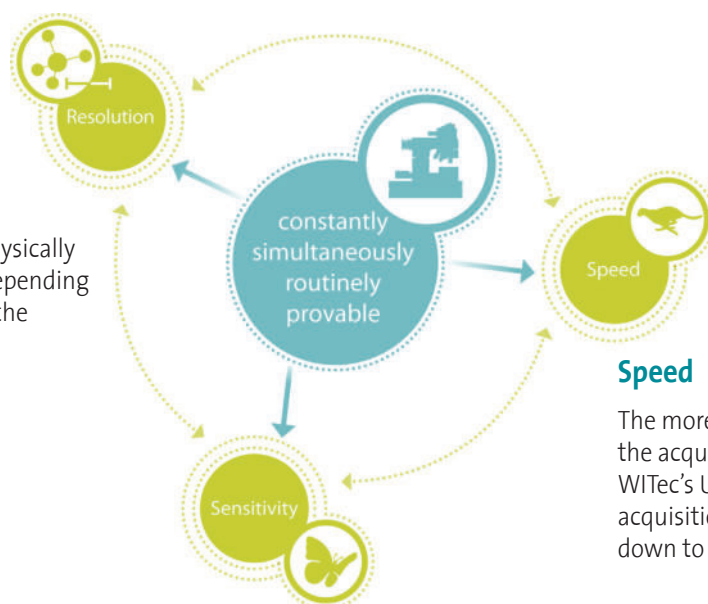
No need for compromises

The Raman effect is extremely weak, so every Raman photon is important for imaging. Therefore WITec Raman imaging systems combine an exceptionally sensitive confocal microscope with an ultra-high throughput spectrometer (UHTS). Precise adjustment of all optical and mechanical elements guarantees the highest resolution, outstanding speed and extraordinary sensitivity - simultaneously!

This optimization allows the detection of Raman signals of even weak Raman scatterers and extremely low material concentrations or volumes with the lowest excitation energy levels. This is an unrivaled advantage of WITec systems.

Resolution

Lateral resolution is physically limited to ~200 nm, depending on the wavelength of the incident light.



Speed

The more sensitive a system is, the shorter the acquisition time for a single spectrum. WITec’s Ultrafast Raman Imaging reduces acquisition times for single Raman spectra down to well below 1 ms.

Sensitivity

A high confocality increases the signal-to-noise ratio by reducing the background. With the UHTS Series, WITec developed lens-based, wavelength-optimized spectrometers with a spectral resolution down to 0.1 cm⁻¹ relative wavenumbers.

3D confocal Raman imaging

2D Raman imaging visualizes the distribution of chemical compounds, e.g. on the sample's surface or in a focal plane within the sample (x-y plane), while 3D Raman imaging enables more complex chemical analyses of component distribution throughout a volume. This is particularly advantageous for investigations of bulky samples, complex emulsions or mixtures, geological specimens and living organisms [1-6]. This application note presents a collection of best practice examples from various fields of research, such as pharmaceuticals, food science, semiconducting materials and geology.

Pharmaceutical emulsion

Figure 1A shows a large-area, high-resolution 2D Raman image of a pharmaceutical emulsion. For more details, a zoom-in image of the indicated area is shown as well. As a complete Raman spectrum was acquired at each pixel, the image results from evaluating 4,194,304 Raman spectra. For image generation the raw data was processed by applying a cosmic ray filter and a constant background subtraction. The characteristic spectra of the sample were then identified and a basis analy-

sis was performed. The resulting Raman image (Figure 1A) is color-coded according to the spectra of the three identified components (Figure 1B). The emulsion consisted of water droplets containing an active pharmaceutical ingredient (API, blue) in an oil-matrix (green). In addition to the water/API and oil phases, a third component was found (red), which was present in only a low concentration. Their distributions can be evaluated in more detail from a volume scan.

In order to generate a 3D image, confocal 2D Raman images from 50 successive focal planes were acquired by automatically scanning through the sample along the z-axis. The resulting image stack was then combined into a 3D representation of the sample's chemical composition (Figure 1C). This 3D Raman image of the pharmaceutical emulsion contains the information of a total of 2 million Raman spectra and can be used to assess the volume of the droplets.

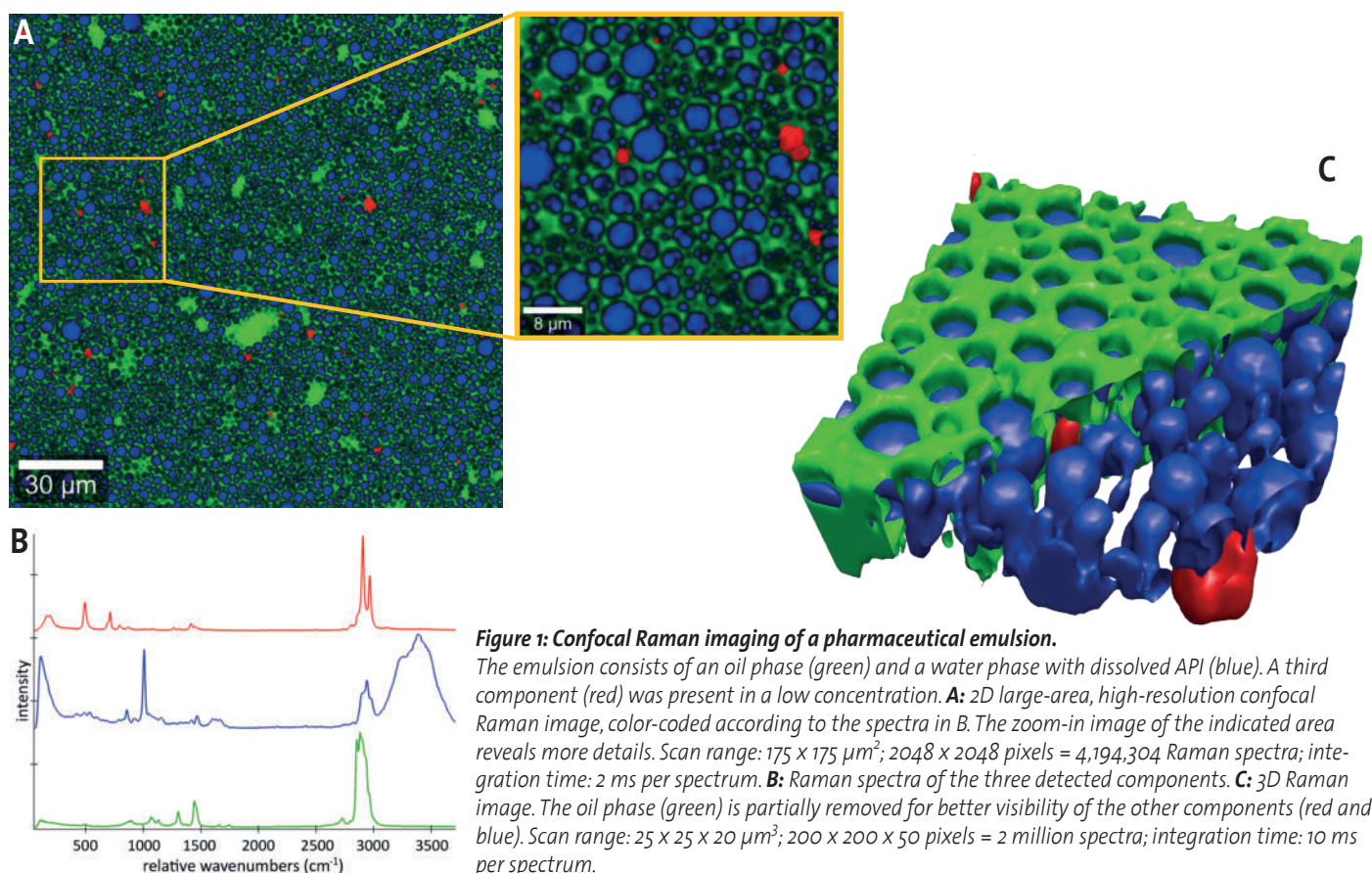


Figure 1: Confocal Raman imaging of a pharmaceutical emulsion.

The emulsion consists of an oil phase (green) and a water phase with dissolved API (blue). A third component (red) was present in a low concentration. **A**: 2D large-area, high-resolution confocal Raman image, color-coded according to the spectra in B. The zoom-in image of the indicated area reveals more details. Scan range: $175 \times 175 \mu\text{m}^2$; 2048×2048 pixels = 4,194,304 Raman spectra; integration time: 2 ms per spectrum. **B**: Raman spectra of the three detected components. **C**: 3D Raman image. The oil phase (green) is partially removed for better visibility of the other components (red and blue). Scan range: $25 \times 25 \times 20 \mu\text{m}^3$; $200 \times 200 \times 50$ pixels = 2 million spectra; integration time: 10 ms per spectrum.

Banana pulp

Pressed banana pulp mixed with water was investigated with an alpha300 Ri inverted confocal Raman microscope. Figure 2 shows a 3D representation generated from a stack of 45 2D Raman images and a total of 6,075,000 Raman spectra. Starch grains are displayed in green while the cell wall components are shown in red.

Figure 2: 3D Raman image of squashed banana pulp. Starch grains (green) and cell wall components (red) are shown. Scan range: $300 \times 200 \times 90 \mu\text{m}^3$; $450 \times 300 \times 45$ pixels = 6,075,000 Raman spectra; integration time: 34 ms per spectrum.

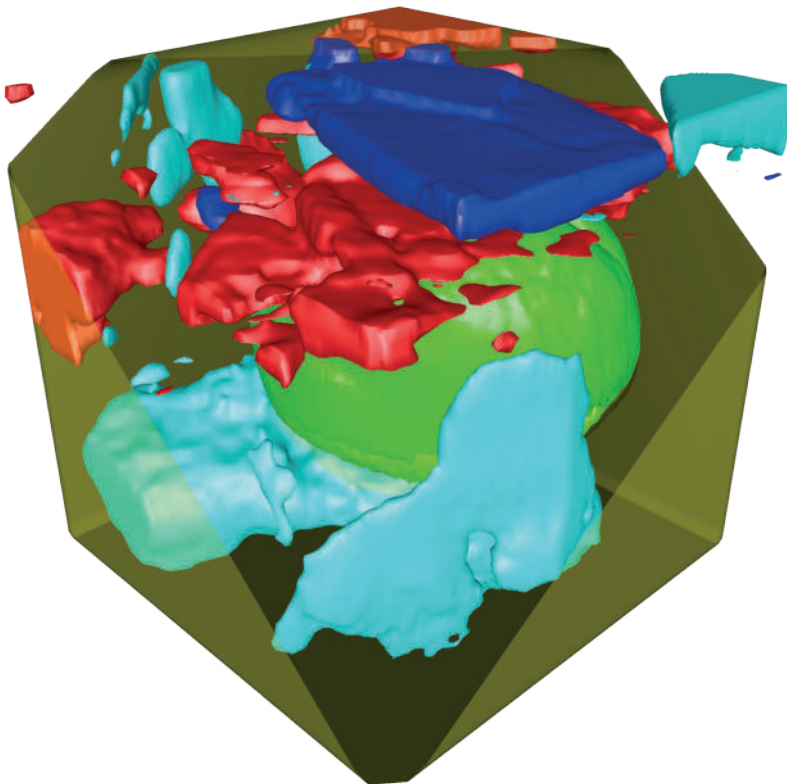
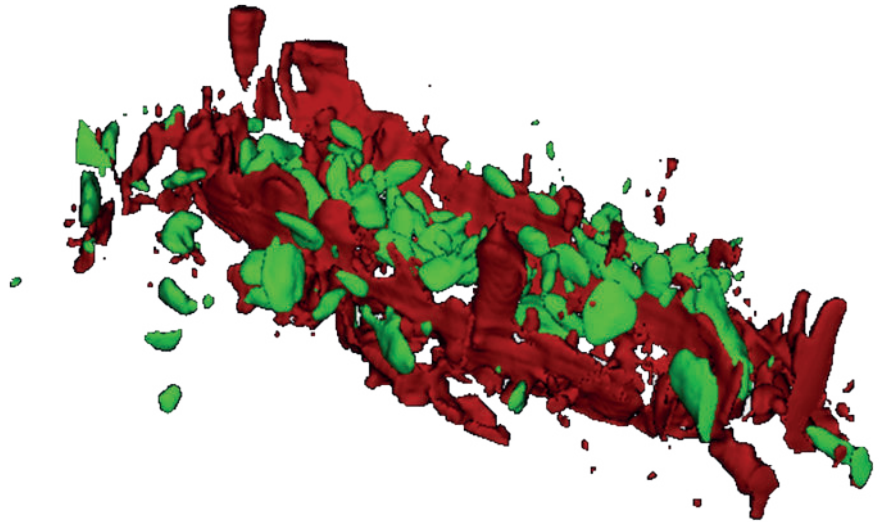


Figure 3: 3D confocal Raman image of pollen in honey. The liquid honey phase (yellow) surrounds a pollen grain (green) and different crystalline honey phases (red, blue, cyan). Scan range: $50 \times 50 \times 50 \mu\text{m}^3$; $150 \times 150 \times 50$ pixels = 1,125,000 Raman spectra; integration time: 2 ms per spectrum.

Honey

A sample of natural honey was investigated. For the 3D image, 50 individual 2D Raman images (x-y scans) were acquired from different z-positions through the sample. In a first processing step the main sample components were identified by the cluster analysis function of the WITec Software Suite. As biological samples tend to show a strong fluorescence background signal, an automatic background subtraction filter was also applied. The data was further processed and optimized for 3D visualization by applying the basis analysis algorithm for spectral demixing. The resulting signal intensity values were used to generate the 3D image stack from the 50 2D scans in which the identified phases are color-coded (Figure 3). A pollen grain (green) is surrounded by different crystalline phases of the honey (red, blue and cyan) and the liquid honey phase (yellow). The complete 3D image consists of 1,125,000 Raman spectra, which were recorded in less than 40 minutes.

Gallium nitride crystals

3D Raman imaging can be used for non-destructively monitoring the crystal quality of semiconducting materials, as strain and crystallinity influence the shape of Raman spectra. Here it was applied to gallium nitride (GaN) grown on a patterned sapphire substrate that featured evenly-spaced hexagonal pits. A depth scan in the x-z plane was performed to get a cross-section along a line of these pits (Figure 4A). Then, 20 2D scans in the x-y plane (Figure 4B) were recorded at different focal planes and a complete 3D representation was generated from this set (Figure 4C).

Evaluation of the recorded Raman spectra revealed three distinct areas in the sample (Figure 4A-C). High-quality GaN was present mainly at the surface of the sample (red). The GaN material directly above the substrate's pits was characterized by lower crystal quality (blue), indicated by strong broadening of the A₁(LO) Raman peak. An enhanced fluorescence signal was detected around the walls of the substrate's pits (green).

As strain leads to Raman peak shifts, strain fields in the sample volume were visualized by quantifying and color-coding the position of the E₂^{high} peak for each image pixel (Figure 4D). The strain fields became narrower towards the surface, again indicating that the GaN crystal quality was higher at the surface than close to the substrate. However, the overall differences in peak position, and thus in the crystal strain, were quite small (Figure 4D). They could be detected due to the high sensitivity and resolution of the UHTS300 spectrometer connected to the alpha300 Raman microscope.

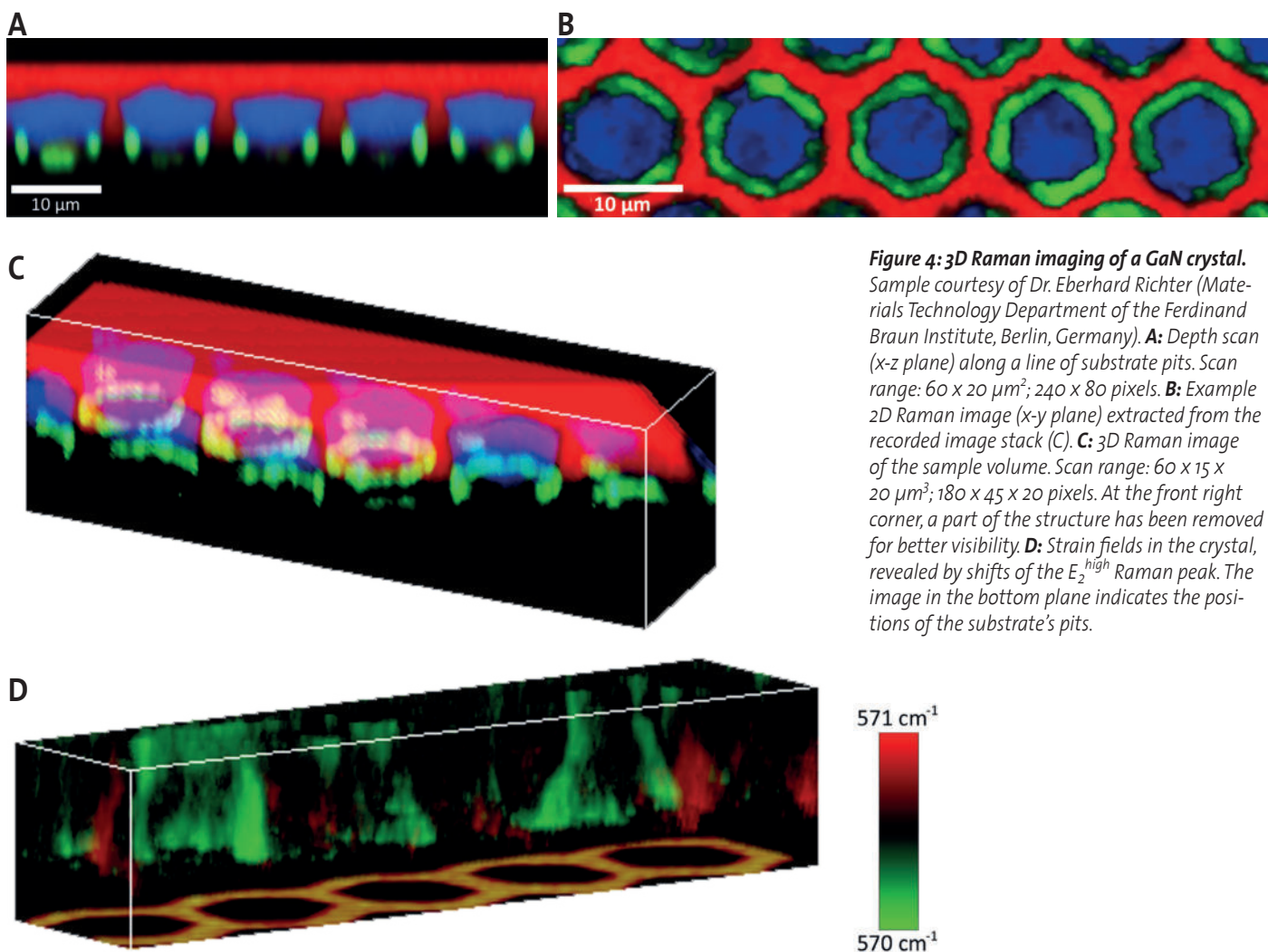


Figure 4: 3D Raman imaging of a GaN crystal. Sample courtesy of Dr. Eberhard Richter (Materials Technology Department of the Ferdinand Braun Institute, Berlin, Germany). **A:** Depth scan (x-z plane) along a line of substrate pits. Scan range: 60 x 20 μm²; 240 x 80 pixels. **B:** Example 2D Raman image (x-y plane) extracted from the recorded image stack (C). **C:** 3D Raman image of the sample volume. Scan range: 60 x 15 x 20 μm³; 180 x 45 x 20 pixels. At the front right corner, a part of the structure has been removed for better visibility. **D:** Strain fields in the crystal, revealed by shifts of the E₂^{high} Raman peak. The image in the bottom plane indicates the positions of the substrate's pits.

Fluid inclusions in geological samples

Fluid inclusions in rock samples are quite common but vary widely in their dimensions. Using 3D Raman imaging, the composition of many geological samples can be characterized without damaging them. Here a fluid inclusion in garnet was investigated. The 3D Raman image (Figure 5) revealed the distribution of garnet (red), calcite (green) and mica (cyan) surrounding a water inclusion (blue).

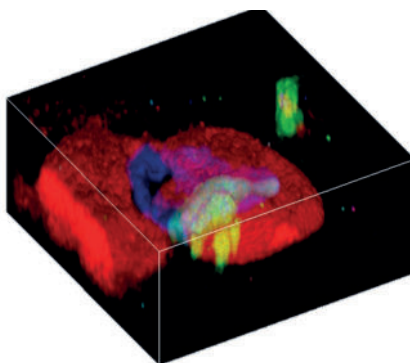


Figure 5: 3D confocal Raman image of a fluid inclusion in garnet. Water inclusion (blue) in garnet (red), calcite (green) and mica (cyan). Scan range: $60 \times 60 \times 30 \mu\text{m}^3$.

Asbestos fibers in mineral matrices

Asbestos refers to a group of six silicate minerals which form fibers. Due to its flexibility, strength and heat-resistance, asbestos was a very popular building material. However, its use is now banned in many countries because all asbestos forms are highly carcinogenic. Therefore the detection of asbestos fibers is necessary in materials intended for construction, such as stone from quarries, and also the previously installed materials in old buildings. Their detection is often challenging because the fibers' diameters are usually only a few hundred nanometers [6].

Together with the BRGM, the French national geological survey office, we recently demonstrated the utility of combined 3D Raman imaging and scanning electron microscopy (SEM) for identifying and characterizing asbestos-like fibers in different rock samples [6]. 3D Raman measurements revealed the shape of the fibers

within the mineral matrices and identified the chemical composition of the fibers and the matrix [6]. Such a 3D analysis allows for the calculation of the aspect ratios and volume fractions of the asbestos fibers. Figure 6 shows actinolite asbestos fibers (red) in an orthoclase (blue) and calcite (green) matrix [6]. With an integration time of 100 ms per spectrum, the entire 3D image was acquired in only about 2 hours.

The measurements were performed using a combined Raman Imaging and Scanning Electron (RISE) microscope (WITec and Tescan). Thus, SEM and Raman images could be obtained from the same sample position, which enabled the correlation of the structural information from SEM with the chemical information from Raman imaging [6]. More information on RISE microscopy can be found in our Application Note [7].

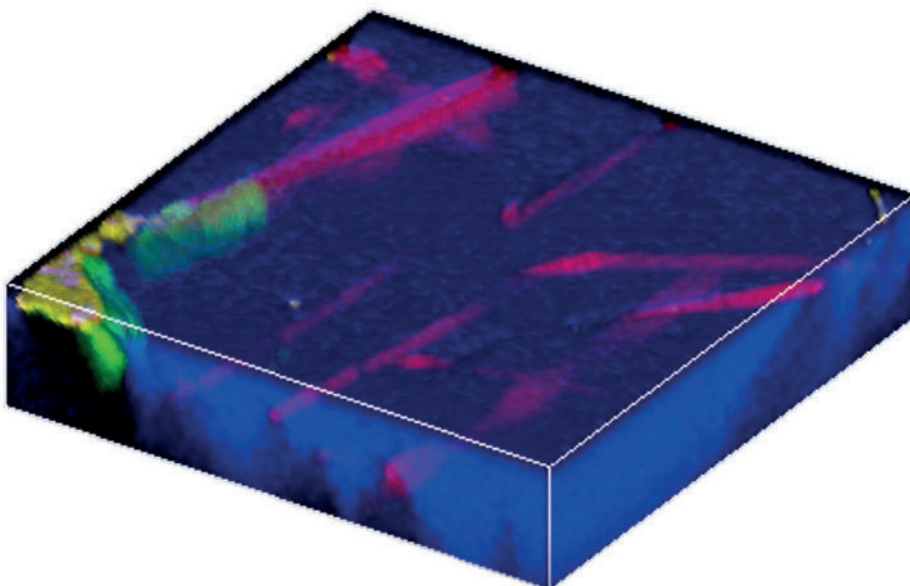


Figure 6: Volume analysis of asbestos fibers in a mineral matrix [6]. Actinolite asbestos fibers (red) are surrounded by a matrix comprised of orthoclase (blue) and calcite (green). Image size $60 \times 60 \times 20 \mu\text{m}^3$.

Emulsion with CCl_4

Carbon tetrachloride (CCl_4) was emulsified with an alkane, water and oil and Raman imaging revealed the distribution of these ingredients. Three different components were identified in the mixture according to their specific Raman spectra (Figure 7A): the alkane (green), water (blue) and a mixture of CCl_4 and oil (yellow). The Raman images visualize the spatial distribution of these components in one image plane at high resolution (Figure 7B) and in a recorded sample volume (Figure 7C).

CCl_4 can serve as a reference sample for evaluating the spectral resolution of a Raman spectroscopy system. The characteristic band at 460 cm^{-1} should be clearly resolved into three peaks at room temperature, as can be seen in Figure 7A (inset). The data was recorded with an alpha300 *apyron* fully automated microscope, which allows for fast Raman imaging at high spatial and spectral resolution due to its consistently optimized optical throughput.

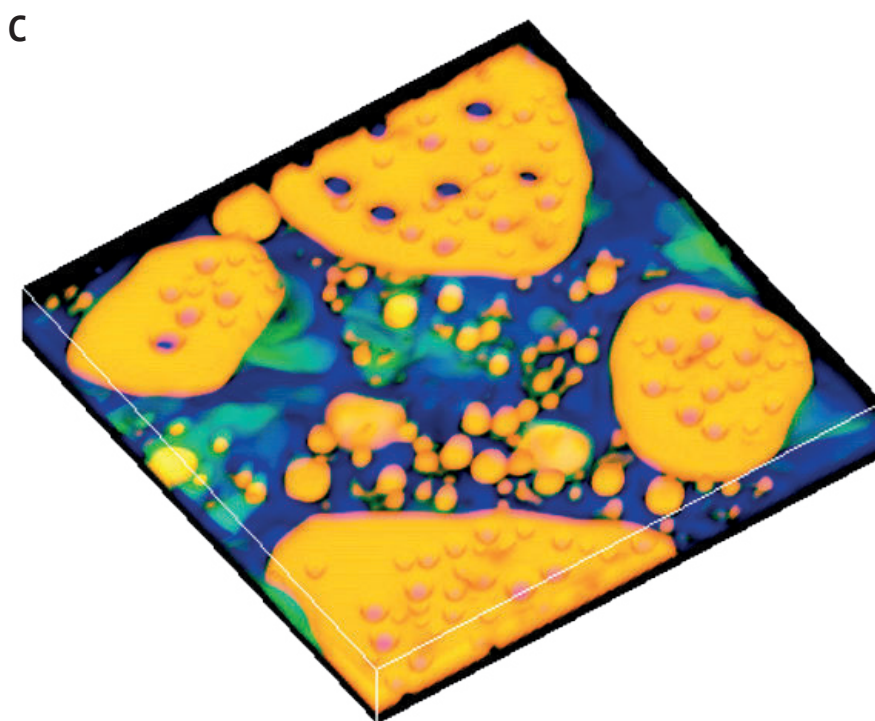
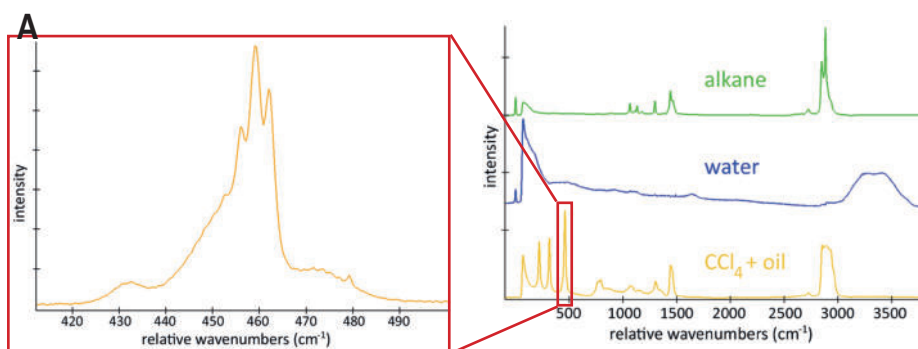
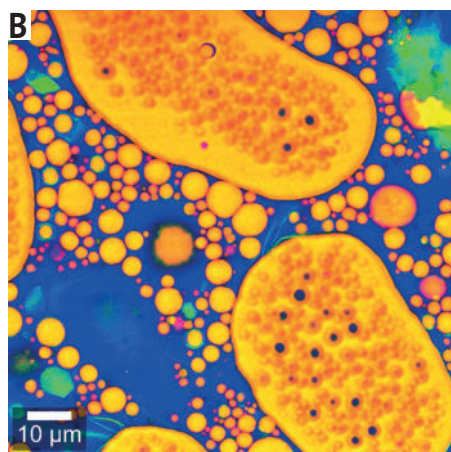


Figure 7: Confocal Raman image of a CCl_4 emulsion. **A:** Raman spectra of the identified components alkane (green), water (blue), CCl_4 in oil (yellow). The zoom-in image of the CCl_4 spectrum shows that the peak at 460 cm^{-1} is clearly resolved. **B:** Example high-resolution 2D Raman image (100 nm per pixel), color-coded according to the spectra in A. **C:** 3D confocal Raman image of the emulsion. Scan range: $200 \times 200 \times 20$ pixels, $100 \times 100 \times 10\ \mu\text{m}^3$; integration time: 60 ms per spectrum.

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WITec Microscope Series



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