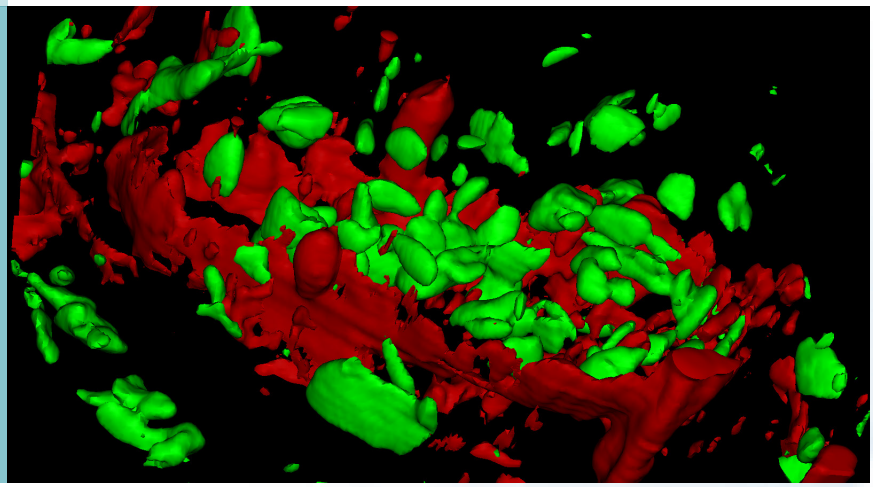
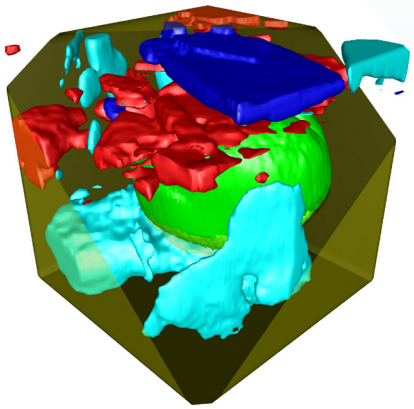
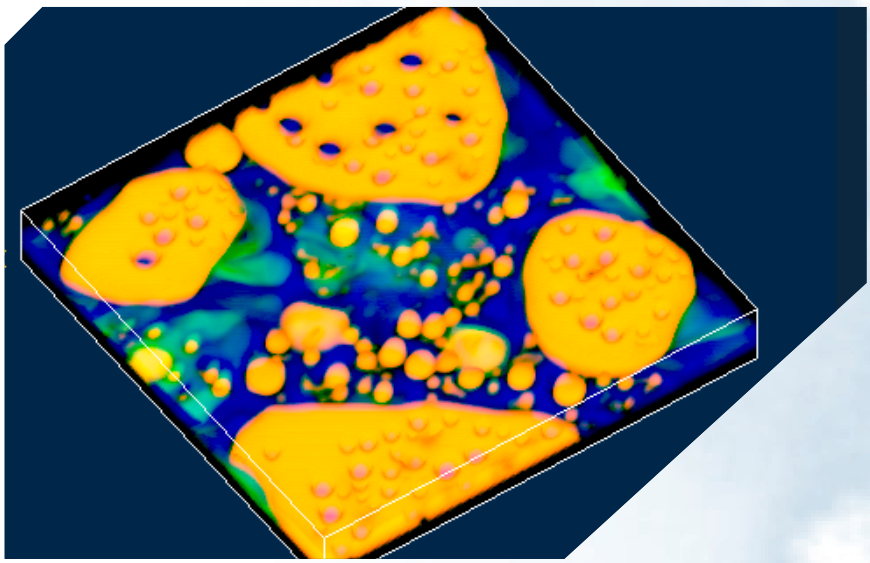
APPLICATION NOTE

3D Raman Imaging

Best practice examples from various fields of application







WITec GmbH, Lise-Meitner-Str. 6, 89081 Ulm, Germany
Tel. +49 (0) 731 140 700, Fax +49 (0) 731 140 70 200
info@WITec.de, www.WITec.de

application note

Lise-Meitner-Straße 6 · D-89081 Ulm, Germany
Tel. +49 (0) 731 140 700 · Fax. +49 (0) 731 140 70200
www.witec.de · info@witec.de

3D Confocal Raman Imaging

The Principle of Confocal Raman Microscopy for Chemical Characterization and Imaging

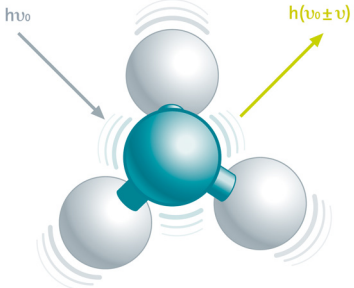
Confocal Raman imaging is a microscopy technique used for the identification and imaging of chemical and molecular compounds. This non-destructive, non-invasive and label-free method is used in various fields of application such as nanotechnology, materials and surface research, geological and environmental sciences and life sciences and pharmaceutics.

The Raman effect (or Raman scattering) is based on the inelastic scattering of light interacting with molecules in a sample. This interaction causes vibrations in the chemical bonds, leading to a specific energy shift in the scattered light that is visible in its spectrum. The Raman spectrum is as unique for each chemical compound as a fingerprint and can be detected and identified by Raman spectroscopy.

For confocal Raman imaging a Raman spectrometer is combined with a confocal optical microscope. The microscope facilitates morphological characterization and establishes the spatial distribution of chemical components within a sample. High-resolution confocal Raman systems acquire the information of a complete Raman spectrum at every image pixel and achieve a lateral resolution at the diffraction limit. The confocal microscope setup is furthermore distinguished by a high signal-to-noise ratio and enables the generation of 3D Raman images and depth profiles. Additional sample characteristics such as the relative amount of a specific component, stress and strain states, or crystallinity can be further analyzed and imaged.

Benefits:

- Identifies a chemical „fingerprint“ of the investigated compounds
- Is non-invasive, non-destructive, label-free
- Requires minimal, if any, sample preparation
- Is insensitive to water
- Can be used for imaging



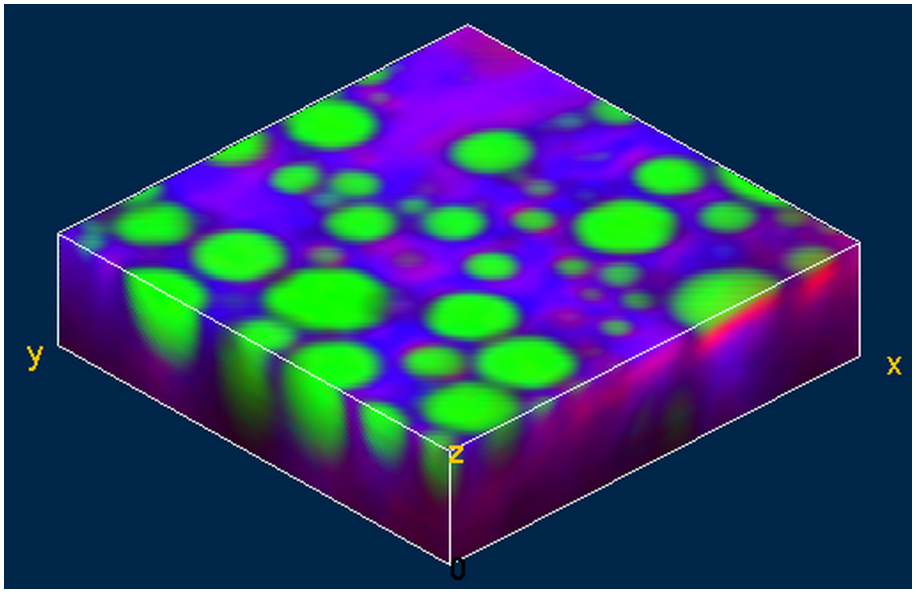
Raman effect: light interacting with chemical bonds leading to inelastic light scattering.

Introduction

2D Raman 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 analysis of the components distribution in three dimensions (x-y-z-plane). This is particularly advantageous for the investigation of comprehensive or bulky samples, complex emulsions or mixtures, geological specimens and components in living organisms [1-5].

3D Raman Imaging Method

In order to generate 3D images, confocal 2D Raman images from different focal planes are acquired by automatically scanning through the sample along the z-axis. After data acquisition, evaluation and processing (WITec Software Suite), the data from the image stack is used to generate the 3D image.



Example 3D Measurement of an Oil-Water-Emulsion.

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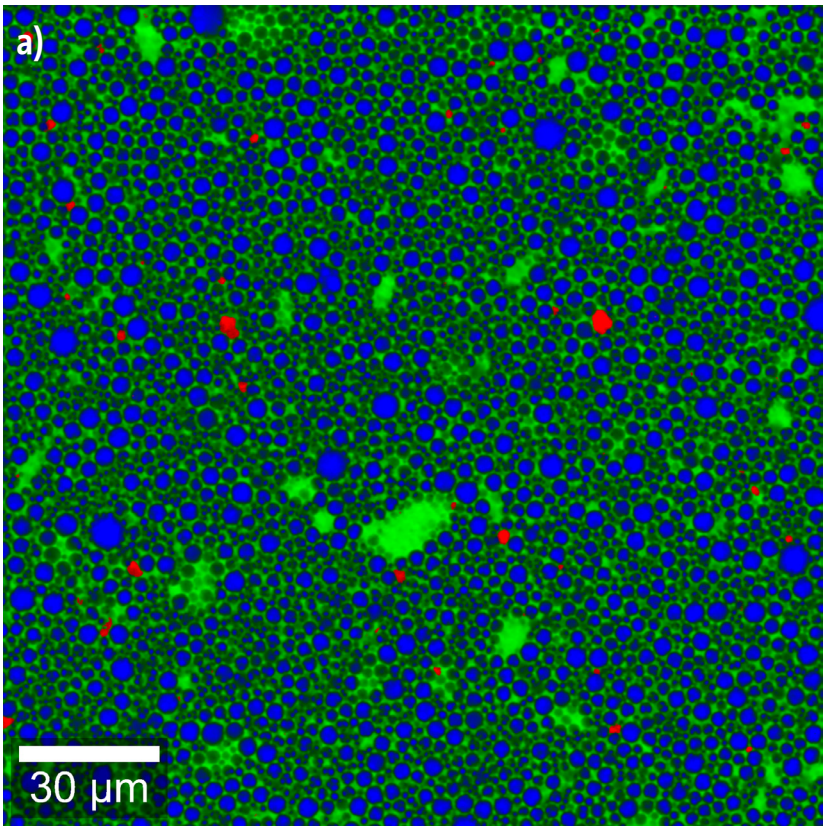
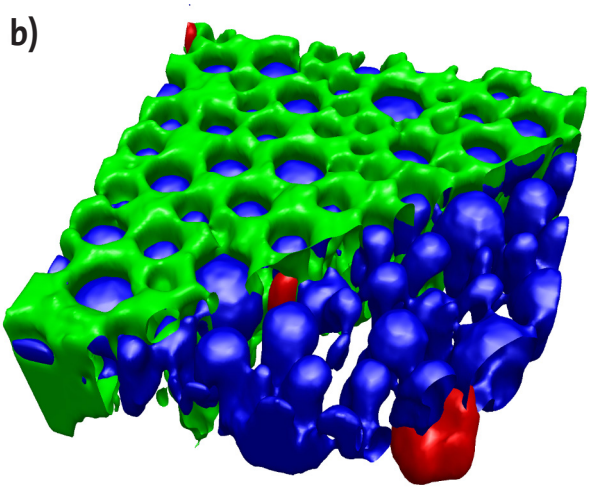
Lise-Meitner-Straße 6 · D-89081 Ulm, Germany
Tel. +49 (0) 731 140 700 · Fax. +49 (0) 731 140 70200
www.witec.de · info@witec.de

Application Examples

Pharmaceutical Emulsion

In the first example a pharmaceutical emulsion was investigated. The active pharmaceutical ingredient (API) is dissolved in water. In Figure 1a a large-area, high-resolution image of the emulsion is shown. The image scan range is 180 x 180 µm² with 2048 x 2048 pixels. At each image pixel a complete Raman spectrum was acquired, therefore the large image is the result of an evaluation of 4,194,304 Raman spectra. The integration time per spectrum was 2 ms. 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 analysis was performed in order to relate the detected spectra to known spectral information to demix them. In the resulting color-coded images the water- and API- containing phase is presented in blue, and the oil-matrix is displayed in green. In addition to the distribution of the known materials, silicone-based impurities could be visualized (red in the images). To investigate the volume of the impurities of the emulsion from Figure 1a in more detail, a 3D scan was performed. For that a volume of 25 x 25 x 20 µm³ was analyzed with 200 x 200 x 50 pixels. At each image pixel a complete Raman spectrum with 10 ms integration time per spectrum was generated. The image stack contains the information of a total of 2 million Raman spectra (Figure 1b).





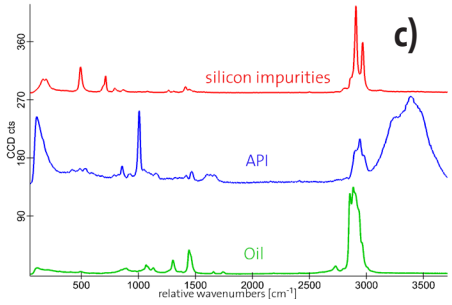


Figure 1: a) Large-area, high-resolution confocal Raman image of a pharmaceutical emulsion. Scan range: 180 x 180 µm²; 2048 x 2048 pixels = 4,194,304 Raman spectra. Blue: active pharmaceutical ingredient; Green: Oil; Red: Silicon impurities. b) Confocal 3D Raman volume image. The green parts are partially removed in the image to clarify the identification of the red impurities. Scan range: 25 x 25 x 20 µm³, 200 x 200 x 50 pixels = 2,000,000 Raman spectra, Integration time per spectrum: 10 ms. c) Corresponding Raman spectra.

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www.witec.de · info@witec.de

Plant Pulp

For 3D Raman imaging a pressed piece of banana pulp mixed with water was investigated with the inverted confocal Raman microscope. The scan range was 300 x 200 x 90 µm³ with 450 x 300 x 45 pixels. At each pixel a complete Raman spectrum was acquired resulting in a total of 6,075,000 Raman spectra. The integration time per spectrum was 34 ms. The image shows a 3D compilation of the Raman image stack. The starch grains are displayed in green while the cell wall components are shown in red (Figure 2).

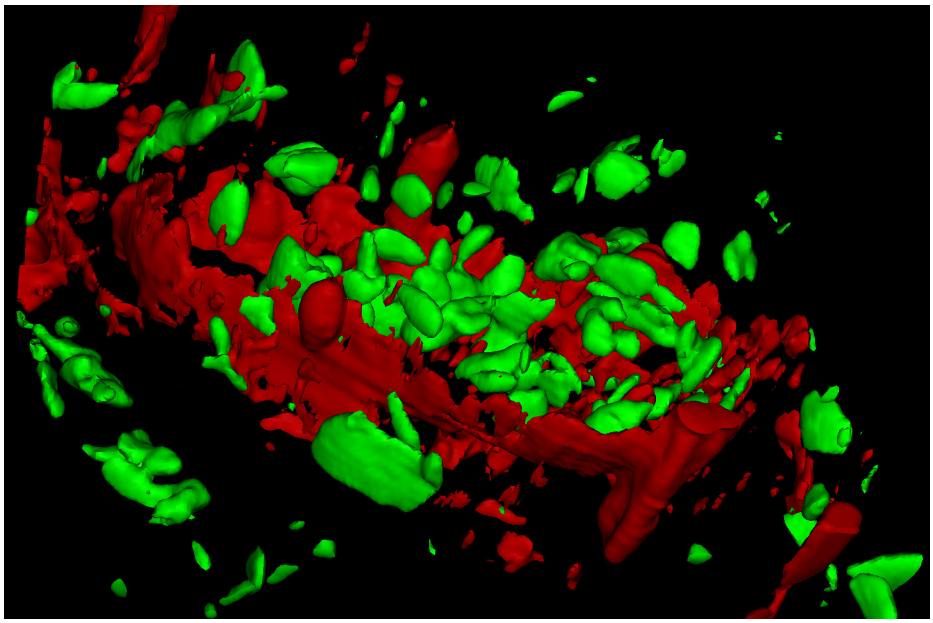

Figure 2: 3D confocal Raman image depiction of banana pulp. Shown are starch grains (green) and cell wall components (red). Scan range: 300 x 200 x 90 µm³, 450 x 300 x 45 pixels = 6,075,000 Raman spectra; Integration time: 34 ms/spectrum.

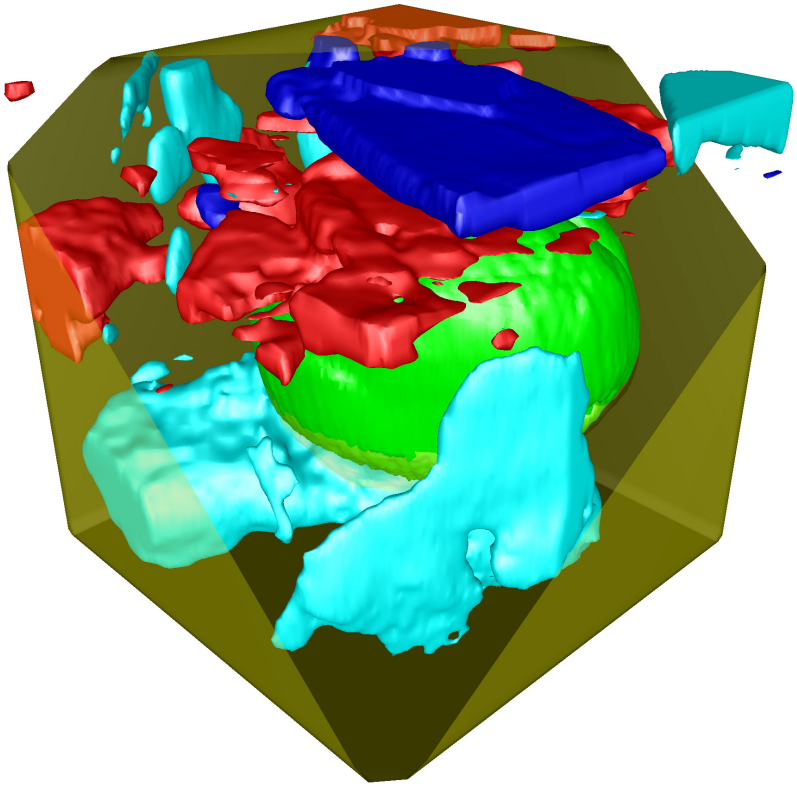

Figure 3: 3D color-coded Raman image of pollen in honey. Green: Pollen; Red, Blue, Cyan: Different crystalline phases in the honey; Yellow: Honey. Image parameters: volume size: 50 x 50 x 50 µm³; 150 x 150 x 50 = 1,125,000 spectra; Integration time per spectrum: 2 ms.

Honey

A sample of natural honey was investigated. For the 3D image, 50 individual 2D Raman images (x-y scans) were generated from different z-positions through the sample. The scan range for each 2D image was 50 x 50 µm² with 150 x 150 pixels. 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 additionally applied in order to remove varying background signals from the data set. 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. The color-coded 3D image has a volume of 50 x 50 x 50 µm³ and displays a pollen grain shown in green surrounded by different crystalline phases of the honey in red, blue and cyan and the liquid honey phase displayed in yellow (Figure 3).

WITec focus innovations
Lise-Meitner-Straße 6 · D-89081 Ulm, Germany
Tel. +49 (0) 731 140 700 · Fax. +49 (0) 731 140 70200
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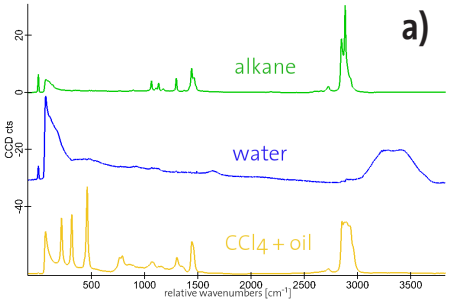
Emulsion with CCl_4

Carbon tetrachloride (CCl_4) was emulsified with an alkane, water and oil. For 3D Raman imaging of the emulsion a 3D scan was obtained with 200 x 200 x 20 pixels and 100 x 100 x 10 µm³ scan range. The integration time per spectrum was 0.06 s. The spectroscopic data were analyzed and three different "endmembers" were determined according to their specific Raman spectra. The green spectrum is specific to the alkane, the blue curve shows the water spectrum and the yellow spectrum represents the mixture of CCl_4 and oil (Figure 4a). A 3D Raman image was then generated from the data set of 2D images (Figure 4b). The volume image shows the three-dimensional distribution of the emulsion components, with the CCl_4 clearly dissolved in the oil phase (Figure 4c).



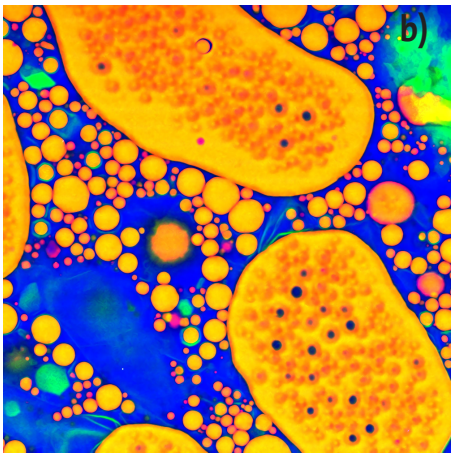
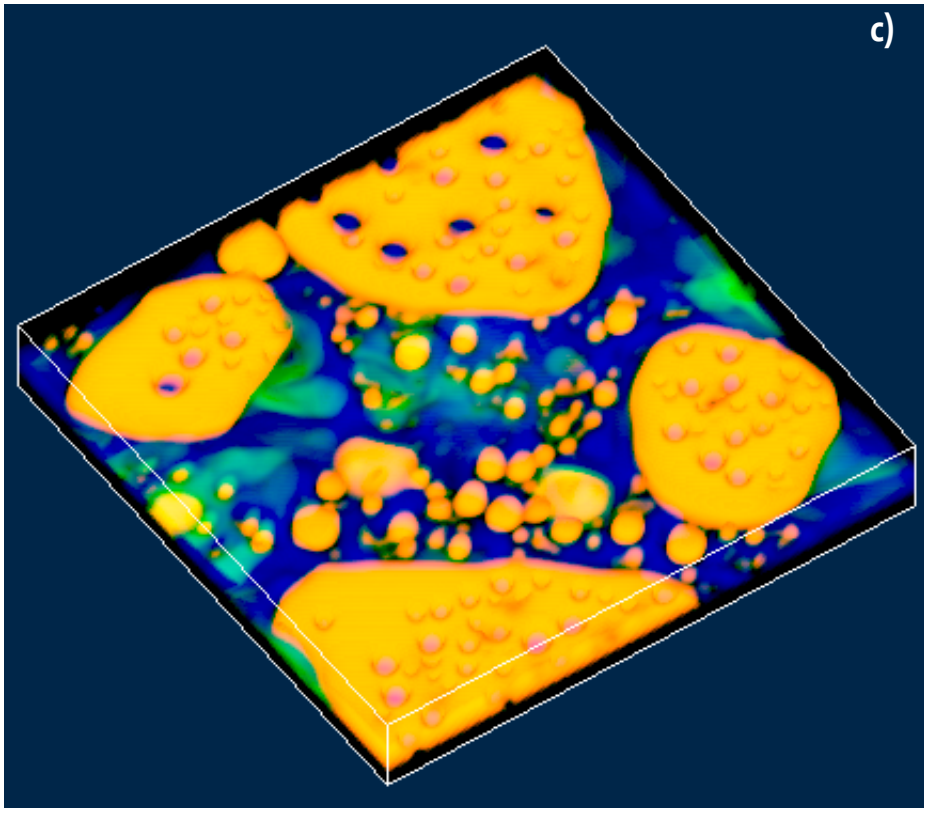


Figure 4: a) Analyzed Raman spectra of the emulsion. b) Example 2D image c) Three-dimensional confocal Raman image of the emulsion: Alkane (green), water (blue) and CCl4 + oil (yellow). Image parameters: 200 x 200 x 20 pixels, 100 x 100 x 10 µm³ scan area; 0.06 s integration time per spectrum, 532 nm excitation laser.



Bulky Geological Samples

Fluid inclusions in rock samples are quite common but vary widely in their dimensions. In the following, a fluid inclusion in garnet was observed. The scan range was 60 x 60 x 30 µm³. The fluid inclusion (water) is displayed in blue, the garnet in red, calcite in green and mica in cyan (Figure 5).

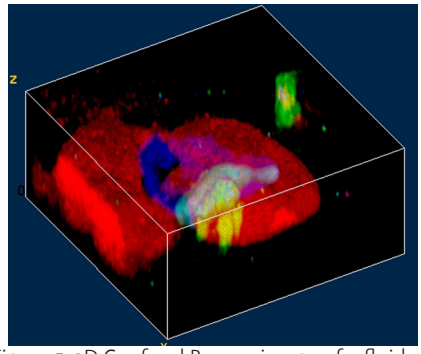


Figure 5: 3D Confocal Raman image of a fluid inclusion in garnet

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Lise-Meitner-Straße 6 · D-89081 Ulm, Germany
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www.witec.de · info@witec.de

WITec Product Portfolio

The WITec product portfolio includes imaging systems for Raman, AFM and SNOM analysis as single technique solutions as well as correlative imaging configurations (e.g. Raman-AFM, Raman-SEM).
All WITec microscopes are high-quality modular systems with exceptional optical throughput, unparalleled signal sensitivity and outstanding imaging capabilities.
Their various specifications range from advanced though budget-conscious microscopes to high-end instruments at the very cutting edge of available technology.
The common thread throughout is that all systems are based on the same hardware and software architecture. Whenever required it is possible to simply upgrade any system, even the most basic, with additional features and equipment, allowing our customers to keep pace with future challenges.