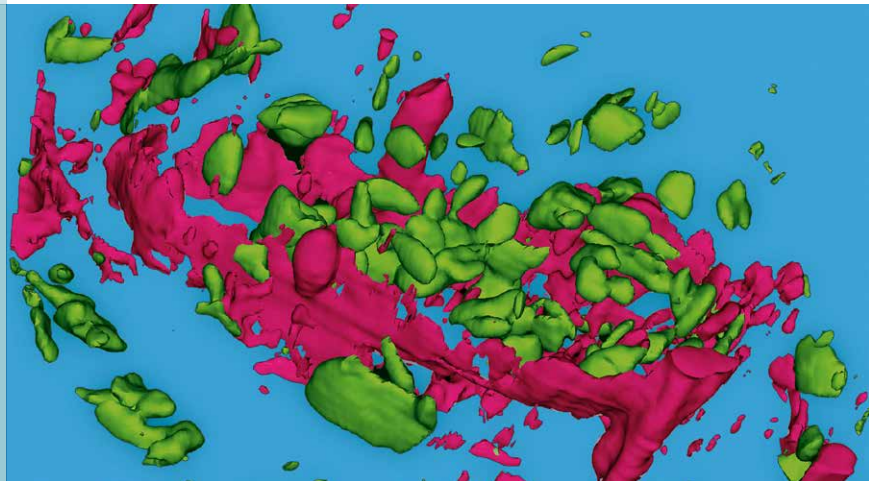
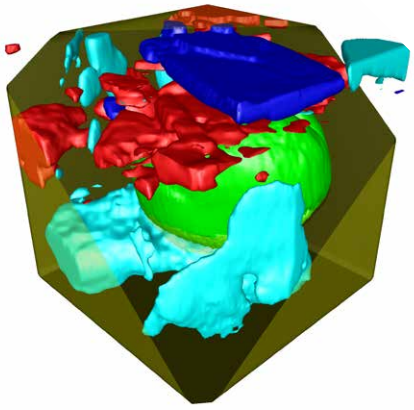
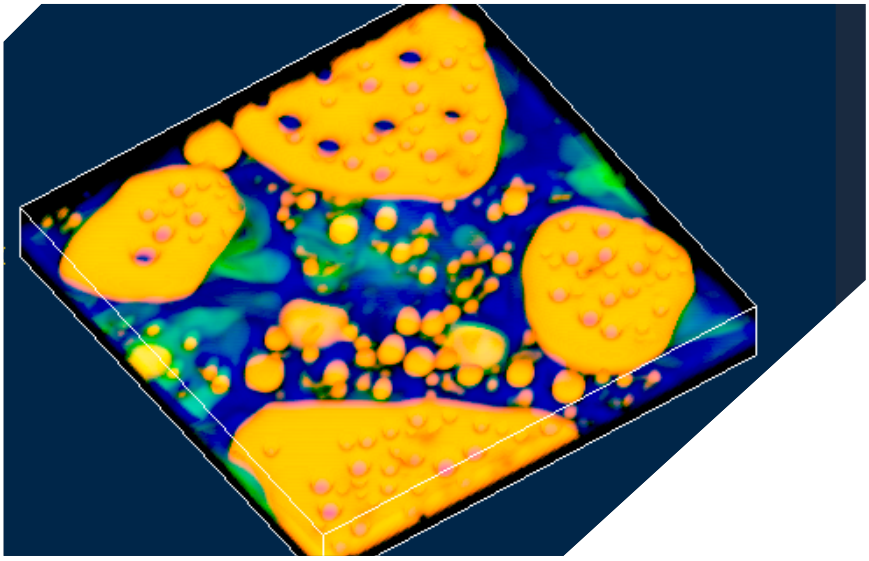
APPLICATION NOTE

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.

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application note

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3D confocal Raman imaging

The principle of confocal Raman microscopy for chemical characterization and imaging

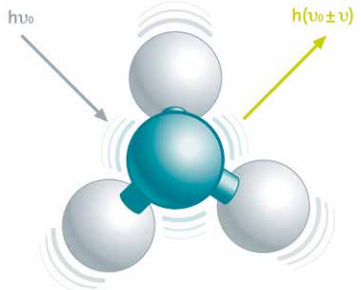
Confocal Raman imaging is a microscopy technique for identifying and imaging of chemical and molecular compounds. This non-destructive, non-invasive and label-free method is used in various fields of application such as nanotechnology, materials and surface research, geological, environmental and life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

Raman spectroscopy

- 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

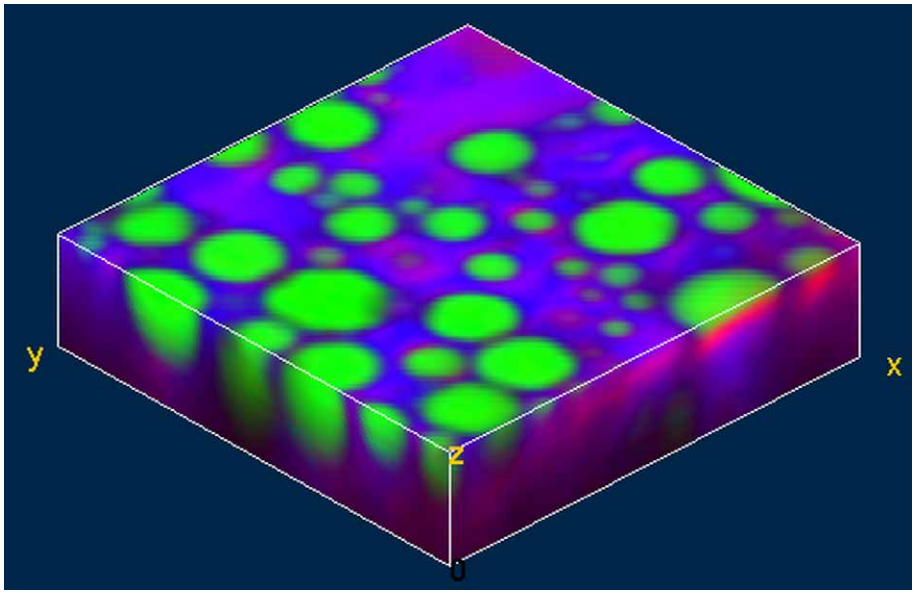


Raman effect: light interacting with chemical bonds leads to inelastic light scattering.

3D Raman imaging

2D Raman microscopy 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 and z). 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].

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, such as the example on the right. Applications from various fields will be illustrated in the following.



Example 3D measurement of an oil-water-emulsion.

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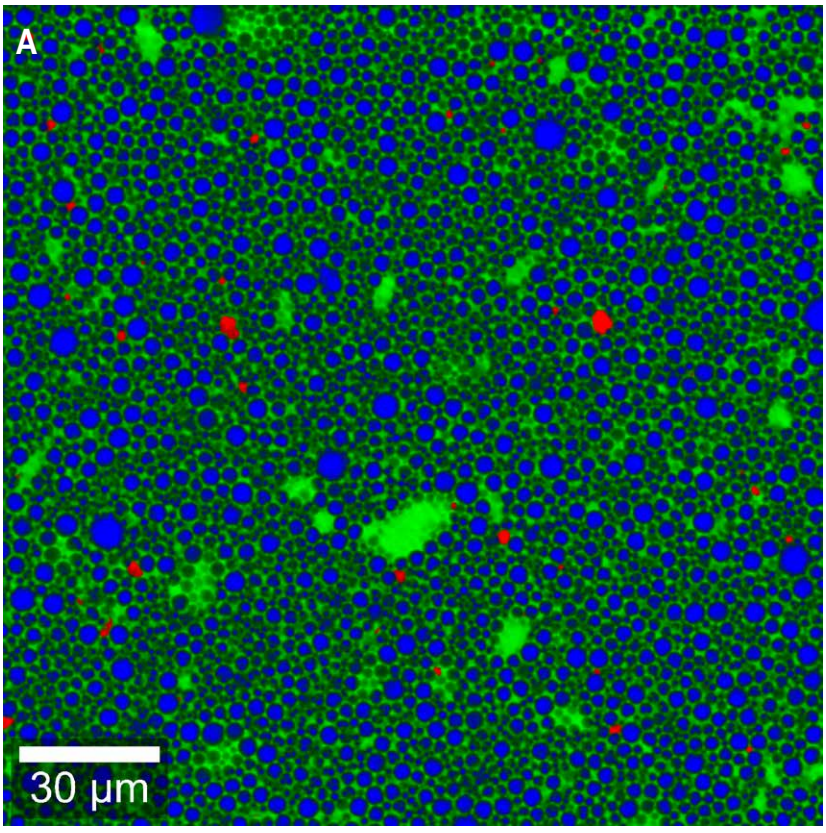
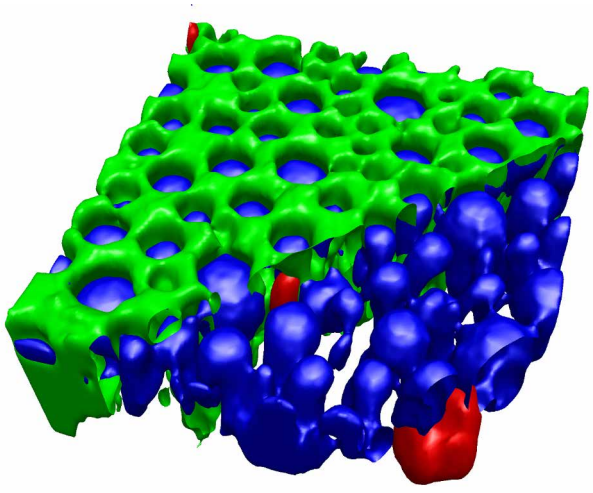
Application examples

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. As a complete Raman spectrum was acquired at each pixel, the image results from evaluating 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 image (Figure 1A) 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 were found (red). Corresponding Raman spectra are shown in Figure 1C.

To investigate the volume of the impurities of the emulsion in more detail, a 3D scan was performed, covering a volume of 25 x 25 x 20 µm³ 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 thus contains the information of a total of 2 million Raman spectra (Figure 1B).





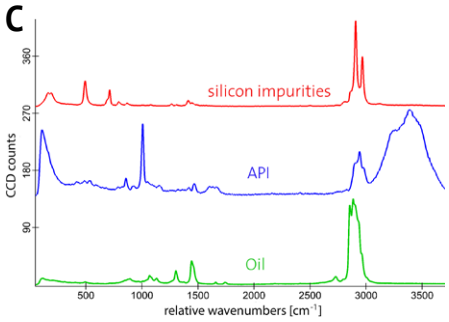


Figure 1: Confocal Raman image of a pharmaceutical emulsion.
(A) *2D large-area, high-resolution confocal Raman image. Active pharmaceutical ingredient dissolved in water (blue); oil (green); silicon impurities (red). Scan range: 180 x 180 µm²; 2048 x 2048 pixels = 4,194,304 Raman spectra, integration time per spectrum: 2 ms.* ***(B)*** *Confocal 3D Raman volume image. The oil phase (green) is partially removed in the image for better visibility of the silicon impurities (red) in the water and API containing phase (blue). Scan range: 25 x 25 x 20 µm³, 200 x 200 x 50 pixels = 2 million Raman spectra; integration time per spectrum: 10 ms.* ***(C)*** *Corresponding Raman spectra.*

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Banana pulp

For 3D Raman imaging a pressed piece of banana pulp mixed with water was investigated with the inverted confocal Raman microscope alpha300 R*i*. Figure 2 shows a 3D compilation of 45 Raman image stacks, resulting from 6,075,000 Raman spectra. Starch grains are displayed in green while the cell wall components are shown in pink.

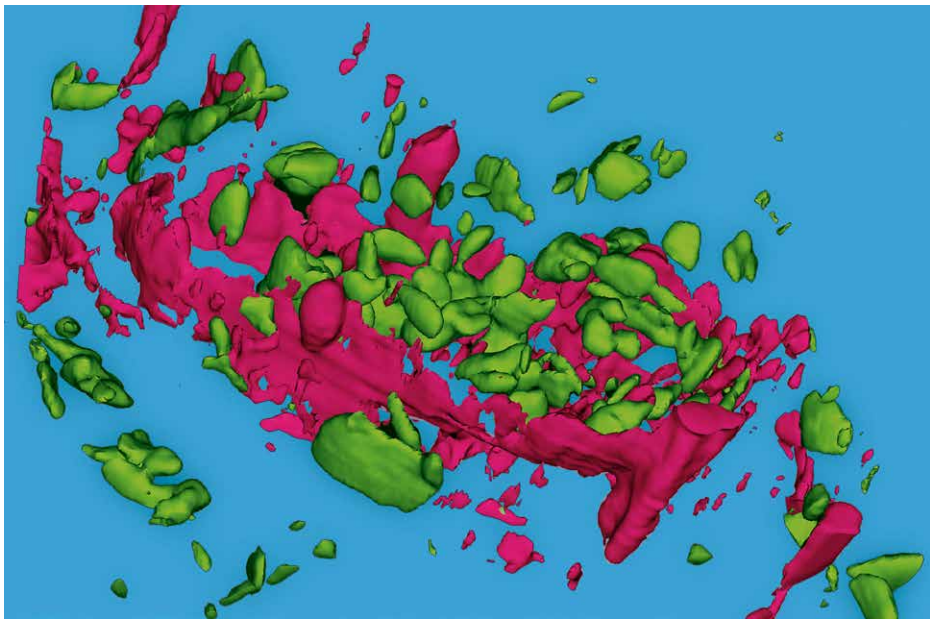

Figure 2: 3D Raman image of banana pulp.
Starch grains (green) and cell wall components (pink) are shown. Scan range: 300 x 200 x 90 µm³, 450 x 300 x 45 pixels = 6,075,000 Raman spectra; integration time: 34 ms/spectrum.

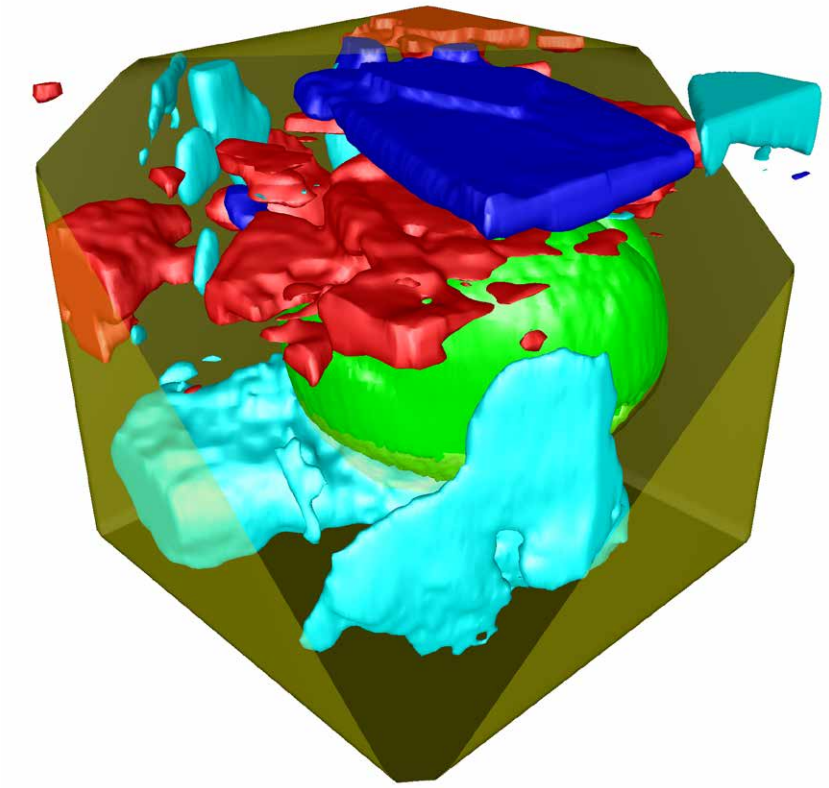

Figure 3: 3D confocal Raman image of pollen in honey.
The liquid honey phase (yellow) contains a pollen grain (green) and different crystalline honey phases (red, blue, cyan). Image parameters: volume size 50 x 50 x 50 µm³; 150 x 150 x 50 pixels = 1,125,000 Raman spectra; integration time per spectrum 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 complete 3D image in Figure 3 consists of 1.125 million Raman spectra, which were recorded in less than 40 minutes.

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 from the 50 2D scans. The color-coded 3D image (Figure 3) displays a pollen grain (green) surrounded by different crystalline phases of the honey (red, blue and cyan) and the liquid honey phase (yellow).

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Emulsion with CCl_4

Carbon tetrachloride (CCl_4) was emulsified with an alkane, water and oil and a 3D Raman image was recorded. The spectroscopic data were analyzed and three different components were determined according to their specific Raman spectra (Figure 4A): the alkane (green), water (blue) and a spectrum representing the mixture of CCl_4 and oil (yellow). Figure 4B shows an exemplary 2D Raman image and the 3D Raman image was generated from 20 such 2D scans. The volume image (Figure 4C) shows the three-dimensional distribution of the emulsion components, with the CCl_4 clearly dissolved in the oil phase.

Bulky geological samples

Fluid inclusions in rock samples are quite common but vary widely in their dimensions. A water inclusion in garnet was investigated. The 3D Raman image in Figure 5 reveals the fluid water inclusion (blue) surrounded by garnet (red), calcite (green) and mica (cyan).

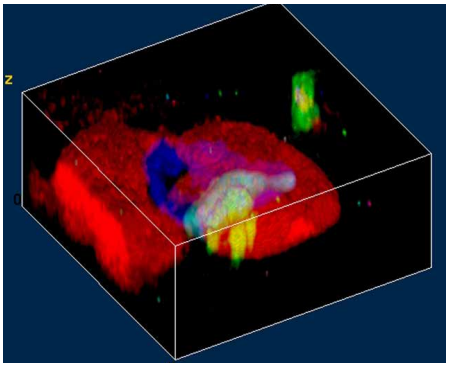
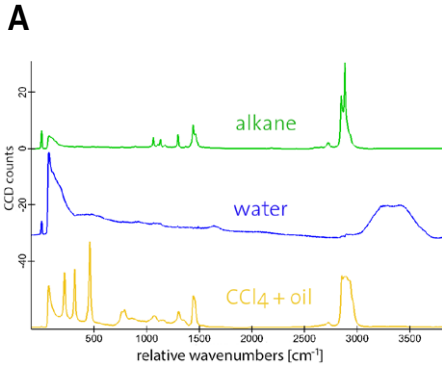
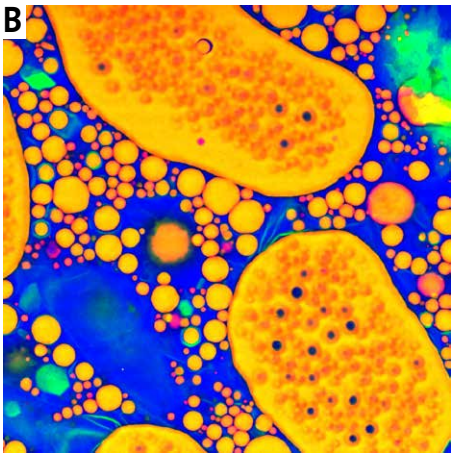


Figure 5: 3D confocal Raman image of a fluid inclusion in garnet. *Water inclusion (blue) in garnet (red), calcite (green), mica (cyan). Scan range: 60 x 60 x 30 µm³.*





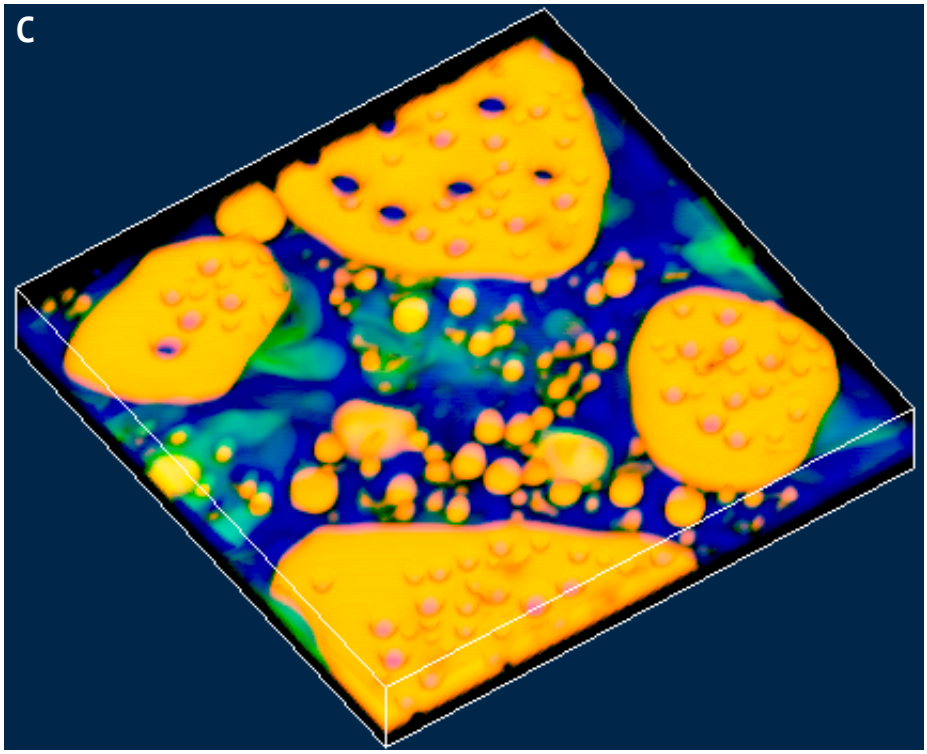


Figure 4: Confocal Raman image of a CCl_4 emulsion.
(A) *Analyzed Raman spectra.* ***(B)*** *Example 2D image.* ***(C)*** *3D confocal Raman image of the emulsion: alkane (green), water (blue), CCl_4 + oil (yellow). Image parameters: 200 x 200 x 20 pixels, 100 x 100 x 10 µm³ scan area; 60 ms integration time per spectrum, 532 nm excitation laser.*

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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.