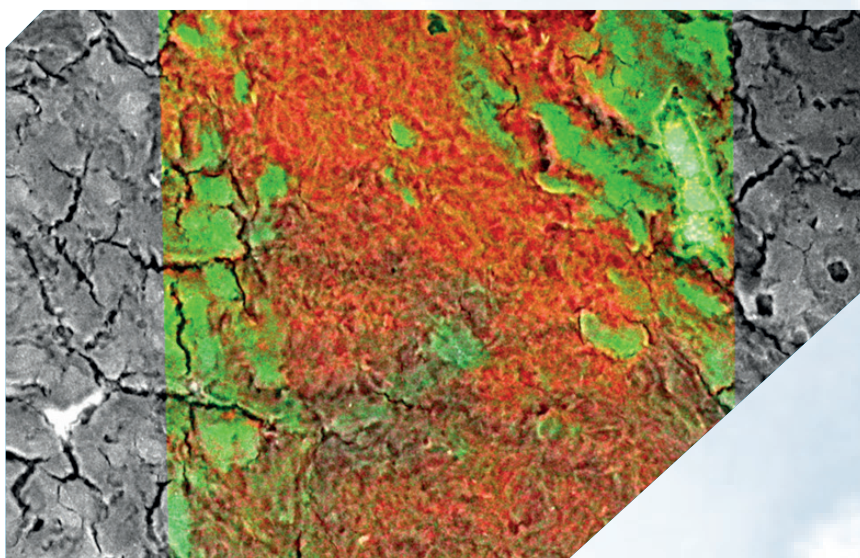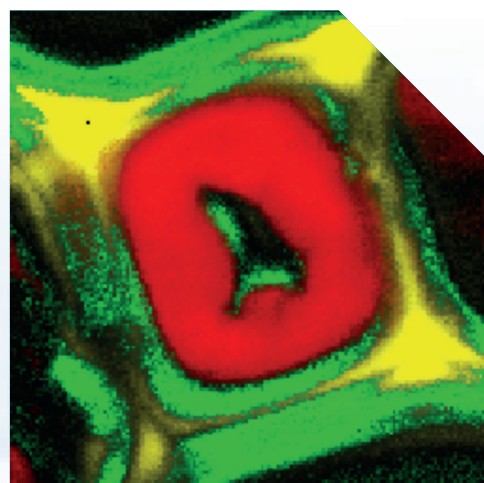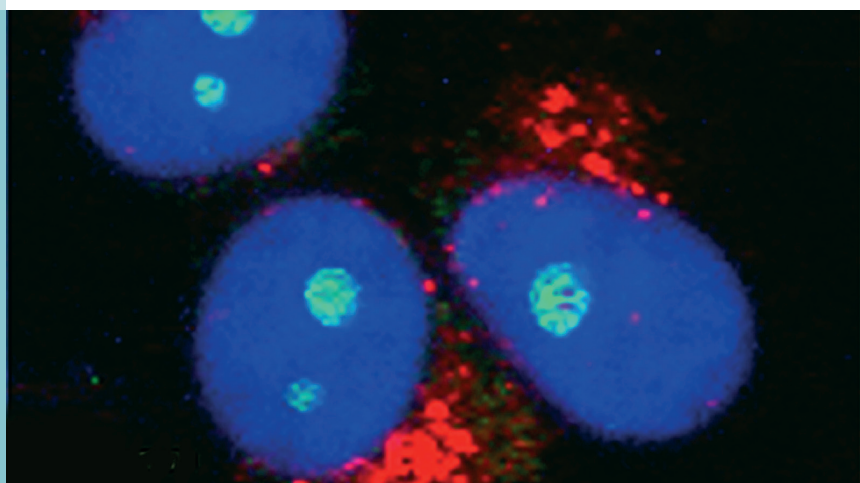# Confocal Raman Imaging and Correlative Techniques in Life Sciences

Confocal Raman microscopy is a promising technique for high-resolution, non-destructive and label-free analysis of cells and their main components. It is ideally suited for correlative imaging.

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.
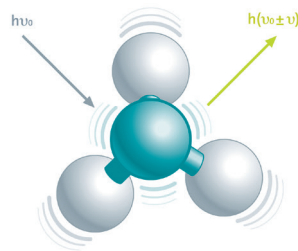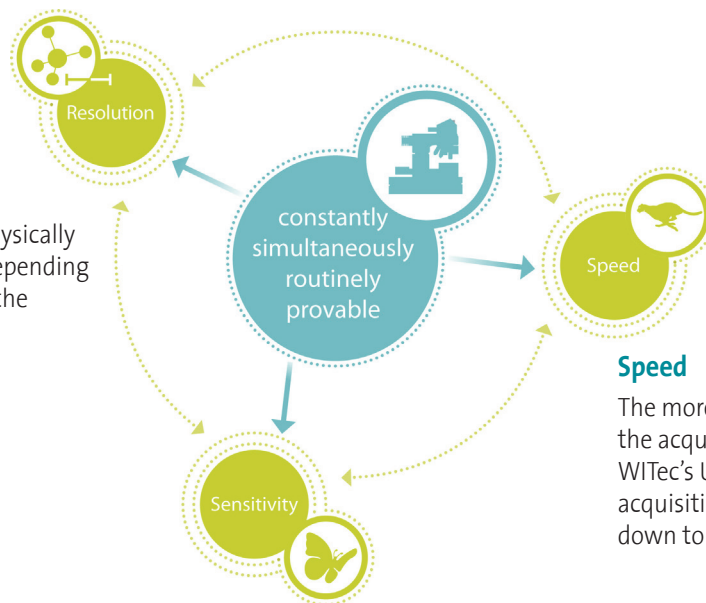
Raman imaging

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.
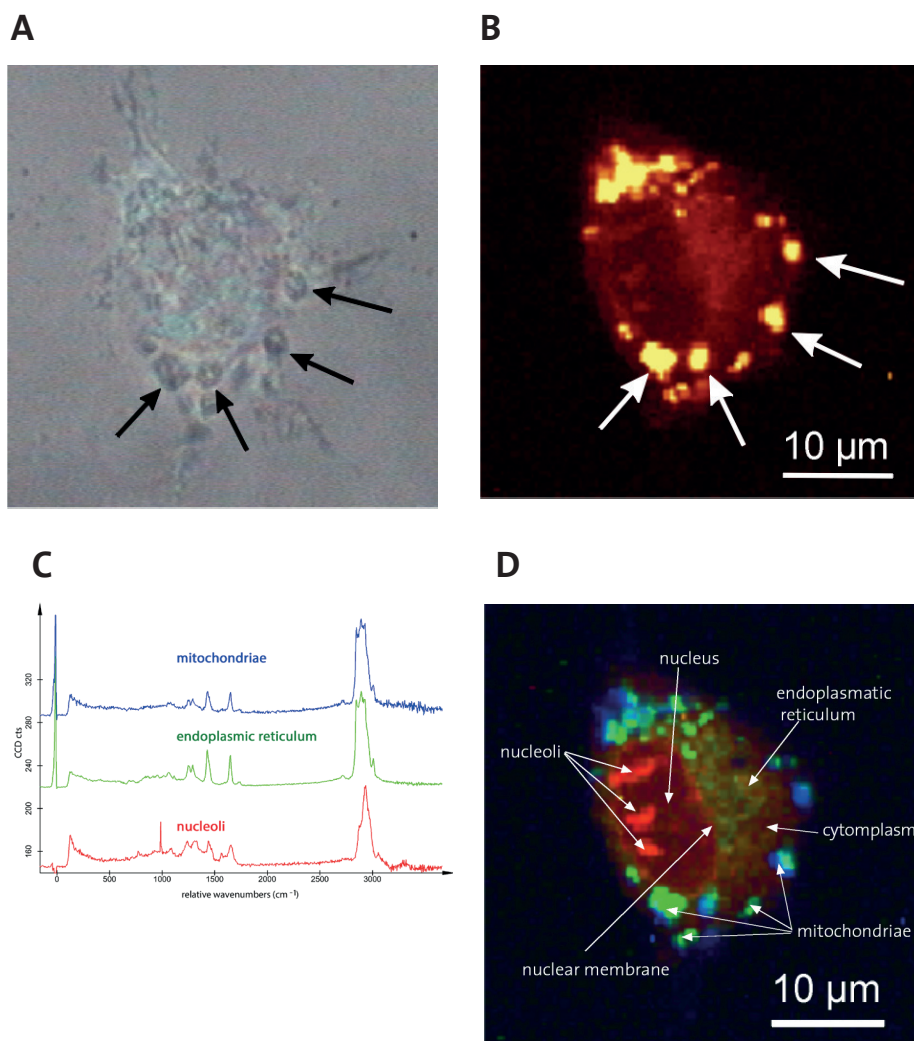
Sensitivity

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.

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Confocal Raman imaging of living cells

Being a non-destructive microscopy technique, confocal Raman imaging is well suited for the study of living cells in their physiological surroundings without damaging them.

Here, epithelial rat cells were investigated with a WITec alpha300 R Raman microscope system. After a suitable cell was identified (A), complete Raman spectra were acquired at every pixel of the image (scan range: 40 x 40 µm², 100 x 100 pixels, 10,000 spectra). The integral intensity of the C-H stretching band was used to generate a Raman image of the cell (B).

Of all spectra obtained, three, shown in (C), were identified as being typical for mitochondria (blue), endoplasmic reticulum (green) and nucleoli (red). Based on these spectra, a Raman image was generated with the corresponding colors (D). The endoplasmic reticulum and other different parts of the cell are clearly visible. Even the nuclear membrane is well-defined. Thus, using confocal Raman imaging, a "color-labeled" image can be created without dyeing the sample.

For extensive coverage of Raman imaging in life sciences see the book

Confocal Raman Microscopy

DOI 10.1007/078-3-319-76380-5,
Springer Int. Publishing, 2018.

A

B

10 µm

C

D

nucleus

endoplasmatic reticulum

nucleoli

cytomplasm

nuclear membrane

mitochondriae

10 µm

mitochondriae

endoplasmic reticulum

nucleoli

CCD cts
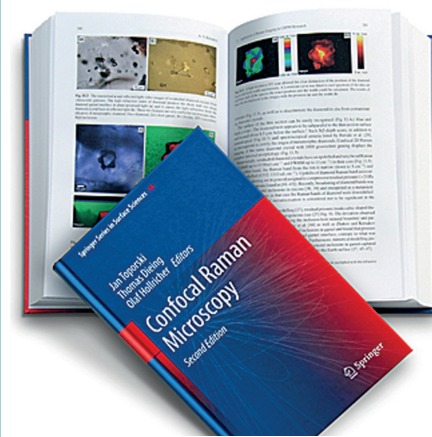
relative wavenumbers (cm⁻¹)

Figure 1: *Raman imaging of a living rat cell*

(A) Video image. (B) Intensity in the C-H stretching band (2800 cm⁻¹ – 3000 cm⁻¹). (C) Determination of basic spectra from mitochondria (blue), endoplasmic reticulum (green) and nucleoli (red). (D) Color-coded Raman image generated from the basic spectra.

Sample courtesy of Angelika Rück, ILM, Ulm, Germany.

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Raman imaging reveals components of atherosclerotic human aorta

Healthy and diseased tissues differ from each other with regard to cellular components. Raman spectral imaging can be used to identify and locate these differences. This intriguing capability of Raman imaging has been demonstrated extensively, i.e. in studies on cellular differentiation (*Nat. Chomm.* 8, 148843, 2017, *J. Chemometrics* 22, 42973, 2018), mitosis, apoptosis and tissue regeneration (*Regenerative Med.* 2, 12-21, 2017) and in many models of diseases such as cancer (*Anal. Chim. Acta* 909, 91-100, 20169, hypertension (*Analyst* 140, 2178-2184, 2015), inflammation and adiposis (*Sci. Rep.* 7, 1-10, 2017; *Analyst* 143, 5999, 2018)

Here (Fig. 2) we present data from a study by Amanda You and colleagues at the Imperial College London (*Sci. Adv.* 3, e1701156, 2017).

To elucidate the differences between calcification and atherosclerosis the scientists have conducted an extensive Raman study on nonatherosclerotic and atherosclerotic human aorta.

Raman spectral data of cross sections through the blood vessels revealed several characteristic Raman bands. Using spectral demixing nine main components could be identified by their typical Raman spectra (A): elastin, collagen, cholesterol and its esters, triglycerides, ß-carotene and two mineral species: apatite and whitlockite.

These spectra were used to image the distribution of the respective components in cross sections of a healthy and an atherosclerotic aorta. Classic histological dyes were used as controls.

Changes in composition such as high concentrations of triglycerides and cholesterol ester and lack of ß-carotene in the atherosclerotic aorta could be detected in atherosclerotic plaques and also at distant loci of the aorta. Despite limitations such as missing of biochemical compounds "We have unearthed significant new insights into the characterization of aortic tissue through the use of Raman spectroscopy," conclude the scientists who conducted the study.
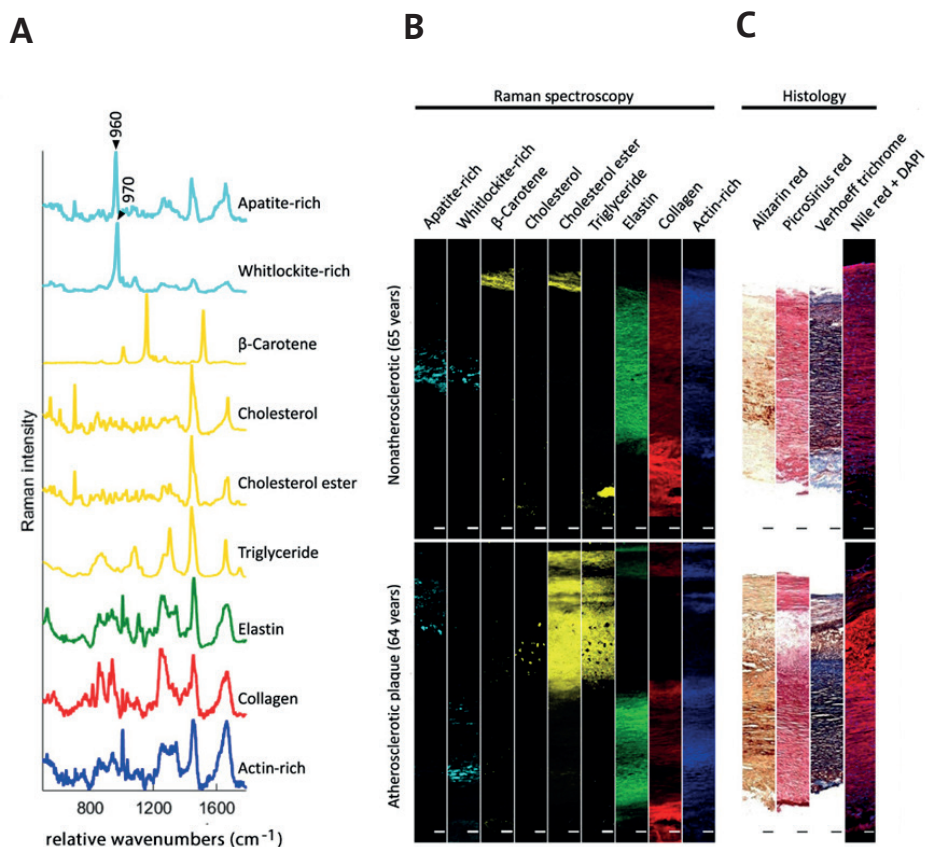
Figure 2: Raman imaging of calcified, artherosclerotic aorta

Demixed Raman spectra for nine major components of aortic tissues (A) and representative images from nonatherosclerotic and atherosclerotic (plaque) aorta (B) along with associated histology (C). Scale bars= 100 µm.

Modified from Amanda You et al., Sci. Adv. 3, e1701156, 2017

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Identification and discrimination of cells with Raman imaging

Recent studies have shown that Raman microscopy can be used to discriminate between malignant and healthy cells.

As an example, we present data from a study performed by Halina Abramzcyk and Beata Brozek-Pluska (*Analyst* 140, 2134-2143., 2015).

They examined healthy and cancerous human breast cell tissue of a patient with an alpha300 R confocal Raman microscope. They found the main difference in the concentration of carotinoids (Fig. 5): healthy tissue contained a substantial amount of carotinoids (A), whereas they were almost absent in tumor tissue (B). Carotinoids were also absent in the breast ducts of cancerous tissue, monounsaturated fatty acids were reduced, but the amount of saturated lipids and sphingomyelin increased. This molecular pattern could serve as a marker for breast cancer diagnosis.

Raman signatures were also identified for medullablastoma cells (*Child's Nervous System* 34, 2425-2430, 2018), brain cancer (*Spectrochim. Acta* A 188, 8-19, 2018) and allows monitoring angiogenesis during cancer development (*Spectrochim. Acta A* 198, 338-345, 2018). These and other studies may pave the way for "Raman cytology" in cancer diagnosis.

Raman cell studies are not restricted to tumor tissue. Surmacki and colleagues demonstrated that Raman imaging is sensitive and specific enough to identify different living human lung cell lines in culture (*Sci. Rep.* 8, 12604, 2018). Others also showed that this technique is suitable to iffderentiate other cell types, i.e. CHO host from producer cell lines (*Biotechnol. J.* 12, 2017).

A

C

B

D
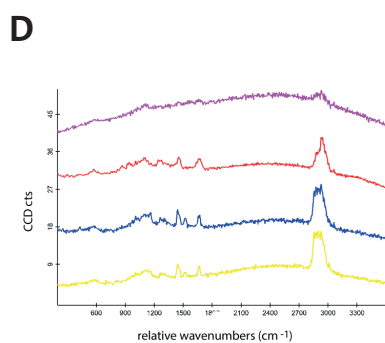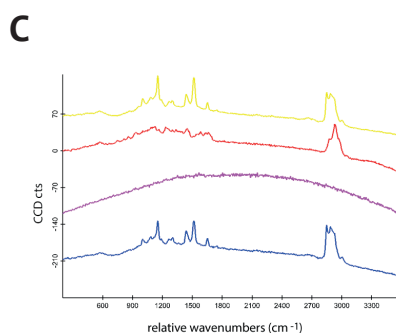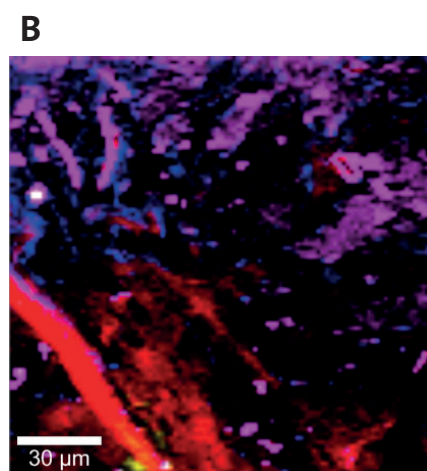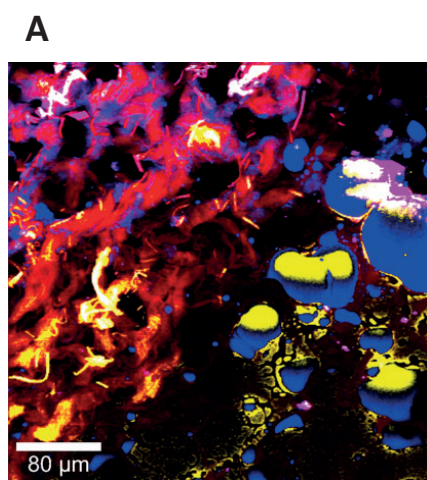
Figure 3: Raman analysis of tumor tissue

Breast tissue from the margin of the tumor mass (A) and tumor mass (carcinoma ductale G3 infiltrans mammae) (B).

Based on the Raman spectra (C, D) color-coded Raman images were developed: carotinoids (yellow) could only be identified in healthy tissue and was missing in tumorous tissue. Color codes for spectra (C, D): yellow: carotinoids (Raman bands 1490 – 1580 cm⁻¹), red: proteins (2900 – 3010 cm⁻¹), purple: fluorescence, blue: lipids (2850 – 2950 cm⁻¹)

Images courtesy of Halina Abramczyk and Beata Brozek-Pluska, Laboratory of Laser Molecular Spectroscopy, Lodz University of Technology, Poland.

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Yes you can! Combine fluorescence microscopy with confocal Raman imaging

The use of fluorescent markers is a well-established and widely-used technique in life sciences for locating and imaging specific molecules. Raman microscopy, however, allows the investigation of all the molecular components of a sample, not only those prepared with fluorescent markers. This is despite the fact that fluorescence is generally regarded as the worst enemy of Raman imaging. Why? Because the Raman effect is a very weak effect, fluorescence is 100 to 1000 times stronger. So, can you acquire a Raman image from a fluorescing sample?

Yes, you can!

Here we demonstrate that Raman imaging is possible on cells that have also been dyed with DAPI (Fig. 4).

DAPI is a fluorescent molecule that specifically stains nuclei of eukaryotic cells (A). By means of specific Raman spectra (C), the endoplasmic reticulum (red) and nucleoli inside the nuclei (green) were identified. An overlay of the confocal Raman image and the DAPI stain is shown in (B). These images were acquired with the confocal, inverted alpha300 R*i* Raman microscope, which is well suited for imaging samples in aqueous solutions.
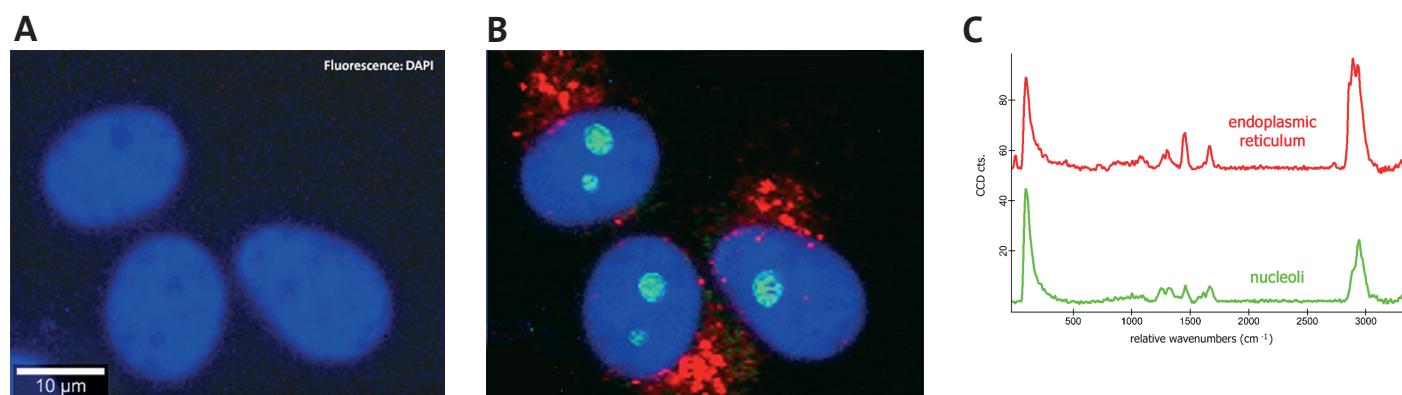
Figure 4: Correlative Raman – fluorescence microscopy imaging of eukaryotic cells

(A) Nuclei were stained with DAPI (blue). (B) Endoplasmic reticulum (red) and nucleoli (green) were identified by their Raman signals. (C) Raman spectra corresponding to nucleoli (green) and endoplasmic reticulum (red).

Sample courtesy of Claudia Scalfi-Happ ILM, Ulm, Germany.

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Another experiment was carried out on Chinese hamster ovary (CHO) cells that were fixed on a microscope slide (Fig. 5). The cells, identified via video imaging (A), expressed the green fluorescent protein (GFP) in their mitochondria (B).

The same cells were analyzed in confocal Raman imaging mode. A Raman spectrum was acquired at every pixel of the image, summing up to 14,000 spectra. In the color-coded Raman image the cell body, the cytoplasm (green), the nucleus (yellow) and the nucleoli (pink) can be clearly distinguished (C). Note that GFP fluorescence did not interfere with Raman imaging.

In post-processing, the Raman spectra were subjected to cluster analysis. For this procedure, the data was automatically compared and similar spectra were grouped into one cluster each. The spectra are plotted in (D). Thus, a simplified Raman image could be developed (E) in which the cytoplasm is shown in blue, the nucleus in red, nucleoli in green and lipids in yellow.
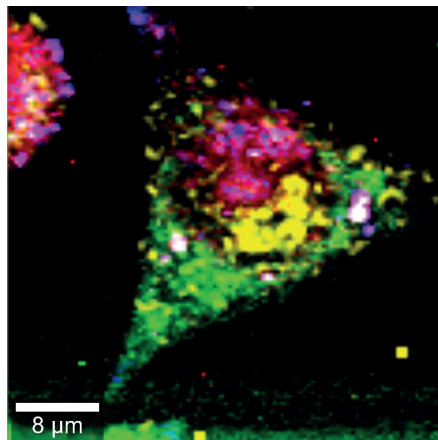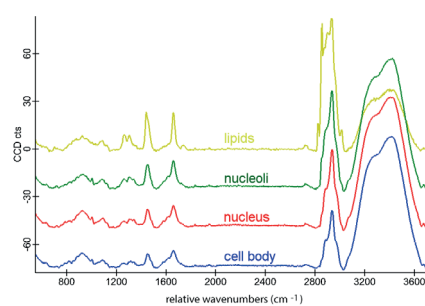
A
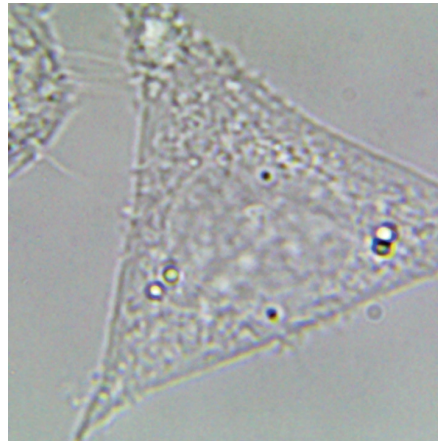
B
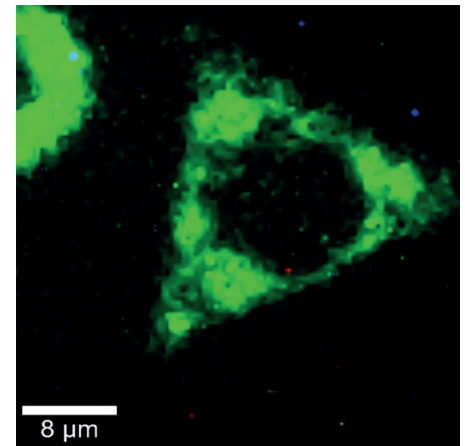
C

Figure 5: Correlative fluorescence and Raman microscopy of eukaryotic cells
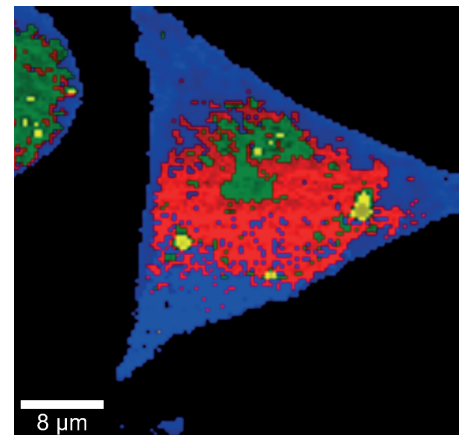
From a culture of Chinese hamster ovary (CHO) cells, a single cell was chosen as seen in the video image (A). The cells stably expressed GFP in the mitochondria (B). Raman spectra were then acquired at every pixel of the sample (not shown) and from them a color-coded Raman image was generated (C). Furthermore, a cluster analysis of the Raman data was performed (D): spectra that identify proteins are shown in blue, those of the nucleus are in red, nucleoli in green and lipids in yellow. The cluster analysis is shown in (E).

Images courtesy of Claudia Scalfi-Happ ILM, Ulm, Germany.

D

E

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Studying the life of macrophages

The monitoring of living cells is essential for unraveling dynamic processes in cells and tissues. Raman studies showing the uptake of lipids into macrophages in real time were successfully performed by the group of Jürgen Popp at the Leibniz-Institute of Photonic Technology in Jena.

Macrophages take up low density lipoproteins (LDL) for recycling and removal. If their export capabilities are overextended, the cells store lipids and develop into foam cells. These foam cells contribute to cardiovasular diseases such as athereosclerosis. With real time Raman imaging it was possible to follow the dynamics of lipid uptake into macrophages observed over a period of more than 35 hours, revealing striking cell-to-cell-heterogeneity (*J. Biophotonics* 10, 1-10, 2017).

In previous studies, the uptake of lipids into macrophages was studied on fixed cells. Oleic acid was labelled with deuterium. For quantification of its uptake, C-D stretching vibrations at 2050 – 2275 cm⁻¹ were evaluated. The images clearly show that the lipid concentration increases over time and that the fatty acid is stored in droplets (Fig. 6). The appearance of lipid droplets is a hallmark of foam cell formation.
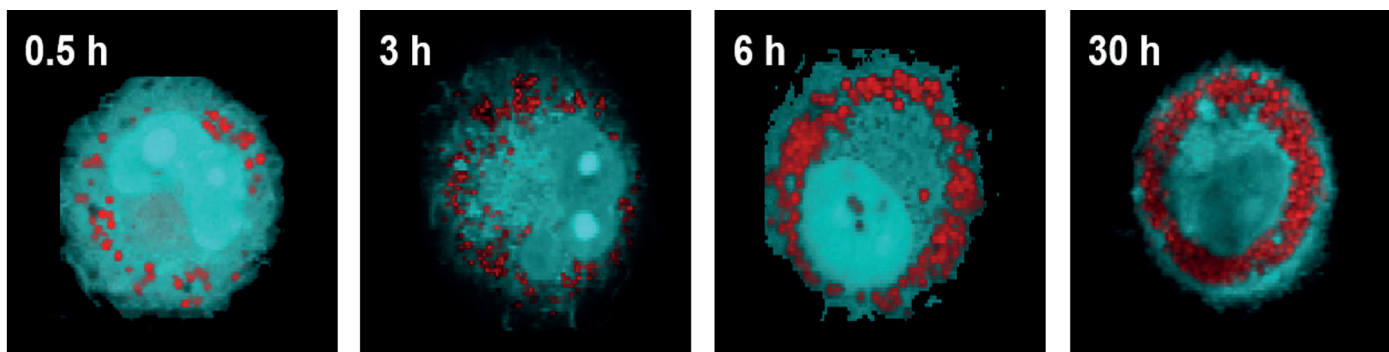
Figure 6: Raman imaging of macrophages

Raman images of macrophage cells aquired by integrating over the 2401 cm⁻¹ C-D stretching band of deuterium-marked oleic acid (red) and the C-H stretching band at 2800 - 3020 cm⁻¹ (blue). After 30 hours of incubation time, the cells store excessive lipids and develop into foam cells.

Images courtesy of Christian Matthäus, Leibniz Institute of Photonic Technology, Jena, Germany.

## Bacteria in the focus of Raman microscopy

Raman imaging has also proven very useful in examining the microbial world. With this technique, bacteria species can be distinguished and their physiological status and growth as well as their response to drug treatment can be analyzed.

Here, a strain of *Bacillus cereus* was investigated (Fig. 7). The video image (A) shows a part of the bacterial colony. Raman microscopy (B) distinguishes between vegetative cells without (yellow) or with the storage polymer polyhydroxybutyrate PHP (green) from spores (magenta). The generative cells can only develop after the bacteria have grown to the stationary phase and accumulated enough PHB.

The fact that all three components can be easily detected highlights the diagnostic power of confocal Raman imaging for identifying phenotypic heterogeneity at a single-cell level.
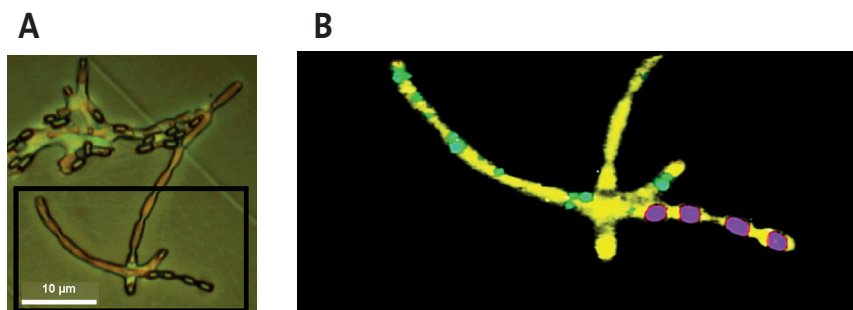
Figure 7: Raman imaging of Bacillus cereus

(A) Video image of growing Bacillus cereus. (B) Raman image from the region indicated by the rectangle in (A) showing vegetative cells without PHB in yellow, with PHB in green and spores in purple.

Images courtesy of Antje Hermelink, Robert-Koch-Institute, Berlin, Germany.

3D Raman imaging of a cell

In this study provided by Katarzyna Majzner and colleagues Raman imaging was used to three dimensionally investigate the heterogeneity of single endothelial cells and to establish the size, volume, shape and biochemical composition of their cellular organelles.

The ability of confocal Raman imaging to generate 3D maps without disrupting the spatial integrity of the cell provides unique insight into biochemical architecture and cellular processes of the endothelium.

3D confocal Raman imaging can be carried out over subsequent measurements of several sample layers. It can also be used e.g. for the early diagnosis of cancer by detecting subtle biochemical changes in cells and tissues associated with cancer development and progression or for morphological analysis of tumors.

An alpha300 R confocal Raman microscope with a 60x water-immersion objective for cells was used for this study. All spectra were baseline corrected using a 3rd degree polynomial and the routine procedure for removal of cosmic rays was applied. The Image J processing program was applied to obtain 3D pictures of cells and tissues.

The results of this and similar studies (*Analyst* 138, 603-610, 2012; *Nature Comm.* 8, 14843, 2017) document that 3D Raman imaging is a label-free method that provides unique insight into the three-dimensional distribution and localization of molecular components of a cell.

A

4,0 µm   3,2 µm   2,4 µm   1,6 µm   0,8 µm   0 µm

B

relative wavenumbers (cm $^{-1}$)
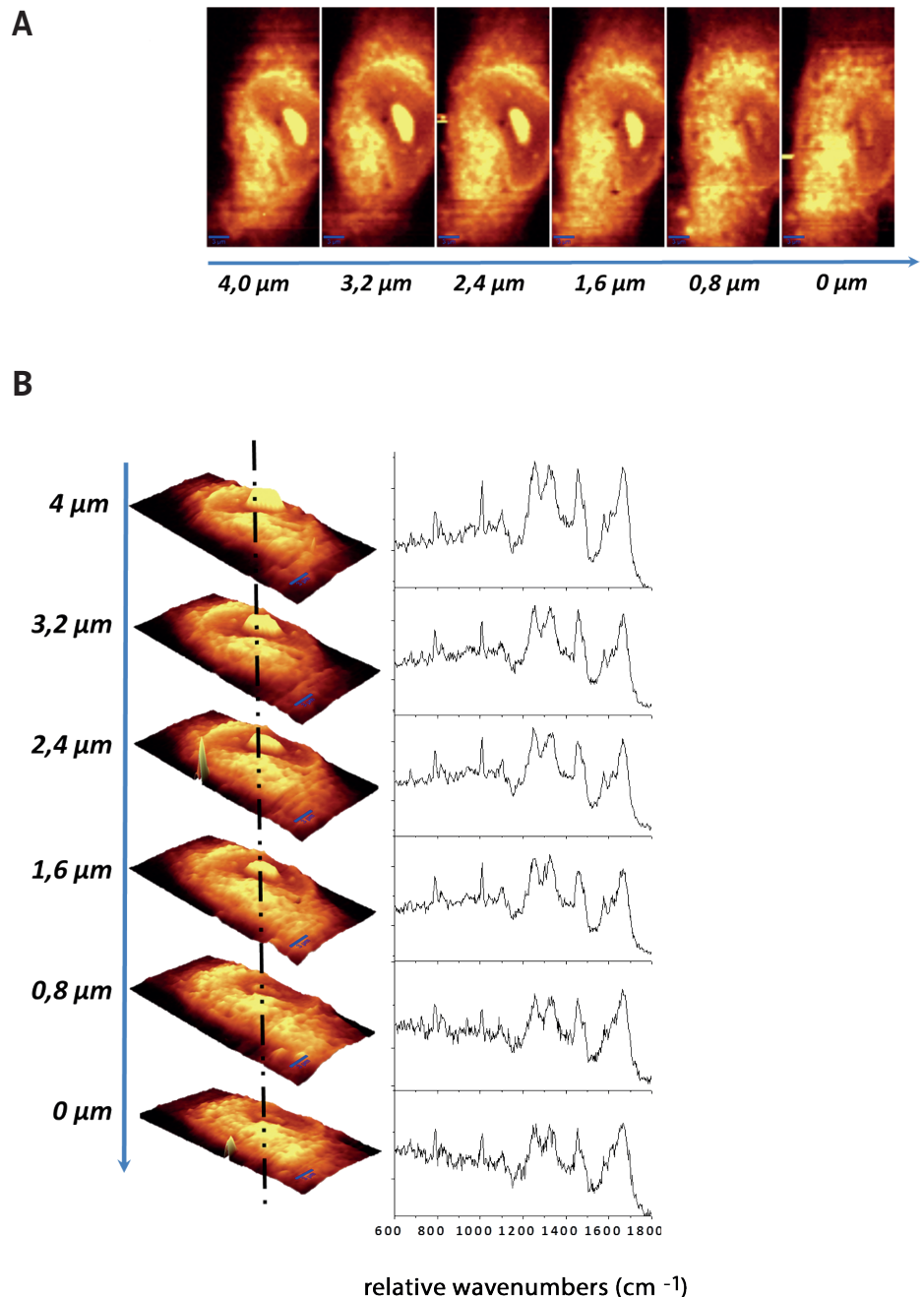
Figure 8: Confocal Raman imaging-stack of an EA.hy 926 endothelial cell

(A) 2D Integration maps over the CH stretching vibration (2800–3020 cm^{-1} range) at different z-positions with corresponding single spectra (B) extracted from the same point in the maps (dotted line).

*Images courtesy of Katarzyna Majzner and Malgorzata Baranska, Faculty of Chemistry, Jagiellonian University, Ingardena 3, 30-060 Krakow, Poland.*

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Characterization of woody plant cells

The cell walls of woody plant tissues are complex, multi-layered structures. Their characteristic feature is the development of secondary cell layers. These layers strengthen and thicken the primary cell wall on which they are based after the cell has reached its final size and shape. Primary cell walls consist primarily of carbohydrates (cellulose, hemicellulose and pectin). Secondary cell walls incorporate more carbohydrates. Cellulose is deposited as stiff fibrils that are embedded in more ductile hemicellulose and lignin. Ordinarily, secondary cell walls contain three layers, S1 – S3, that vary in thickness and chemical composition.

The S2-layer, which accounts for 75 - 85 percent of the total secondary cell wall thickness, is most important for the mechanical stability of the plants' stems and branches. The middle lamella is attached to the adjacent primary cell walls. It ensures the adhesion of a cell to adjacent cells.

Here (Fig. 9) confocal Raman microscopy was used to illustrate the molecular composition of tension wood in hybrid black poplar (*Populus nigra* x *Populus deltoides*). Tension (reaction) wood is produced in response to environmental influences such as wind. It develops in young wood, before the secondary cell wall is completed. It consists of the historically-named gelatinous layer or G-layer and a small S2-layer.

In this experiment, a cross section (20 µm thick) was investigated in water using the alpha300 R confocal Raman microscope by integrating over defined spectral areas. The intensity of the aromatic lignin band (1550 – 1650 cm⁻¹) that is characteristic for lignin is highest in the cell corners and the compound middle lamella, and lower in the S-layers (A). The G-layer can be identified by integrating over the C-H stretching Raman band (2810 – 2940 cm⁻¹) that identifies cellulose as the main component of this layer (b). Lignin could not be detected in this layer. The S-layers and the G-layer show an intense Raman band
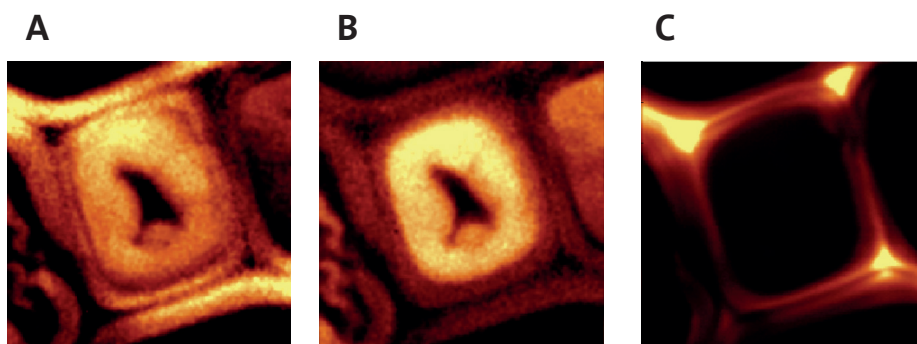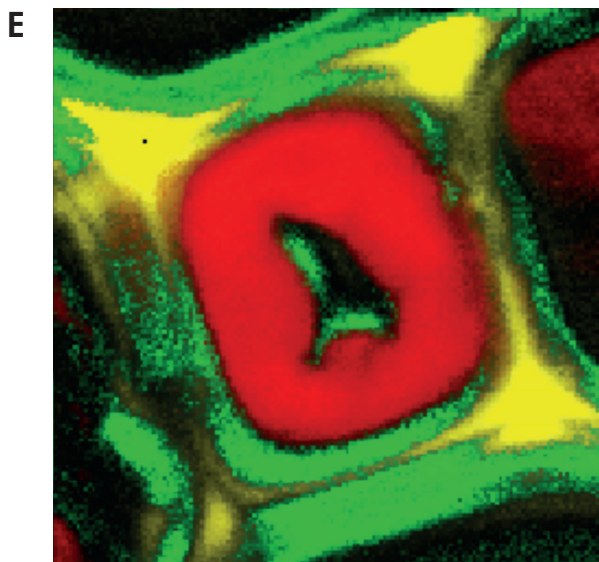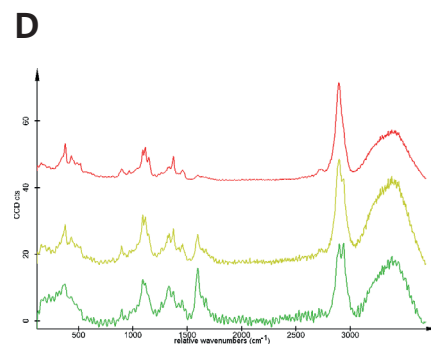
Figure 9: Raman imaging of young wood of Populus nigra x Populus deltoides in water

(A-C) Raman images of a cross section (14 x 14 µm²) generated by integrating over different Raman lines. Intensities of (A) the aromatic lignin band (1550 – 1650 cm⁻¹), (B) the C-H stretching band (2810 – 2935 cm⁻¹) and (C) the C-C/C-O stretching bands (1026-195 cm⁻¹). Raman spectra that are typical for the components of secondary cell walls (D) were used to create a color-coded Raman image of the section (E), indentifying the G-layer (red), the S2-layer (green) and the middle lamella and cell corners (yellow).

at 1026 - 1195 cm⁻¹, which is typical for carbohydrates.

Three spectra that can distinguish the layers (D) were used to develop a color-coded Raman image of the wood cell (E).

Detailed Raman analyses of various woody tissues were published by Nothburga Gierlinger and colleagues (*Planta* 227, 969 - 980, 2008; *Plant J.* 61, 854 - 861, 2010; *Tree Physiol.* 38, 1526-1537, 2018, *Nano Lett.* 20, 2647-2653, 2020).

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RISE™ microscopy: Correlative Raman imaging and scanning electron microscopy

Confocal Raman imaging identifies and localizes the molecular compounds of a sample. Scanning electron microscopy (SEM) reveals the morphology and surface structure of the object under investigation from the millimeter range down to atomic resolution.

The combination of both techniques in one instrument enables the correlation of chemical and structural features. The Raman measurements are performed within the vacuum chamber of an SEM. A precise translation stage automatically transfers samples from one measuring position to the other, thus streamlining the workflow and drastically improving ease of use. We named this type of correlative analysis RISE™ microscopy.

Here we show the application of RISE™ microscopy on three biological samples: a cross-section of hamster brain (Fig. 10), the surface of a pearl (Fig. 11) and a polished cross-section of an abalone shell (Fig. 12).
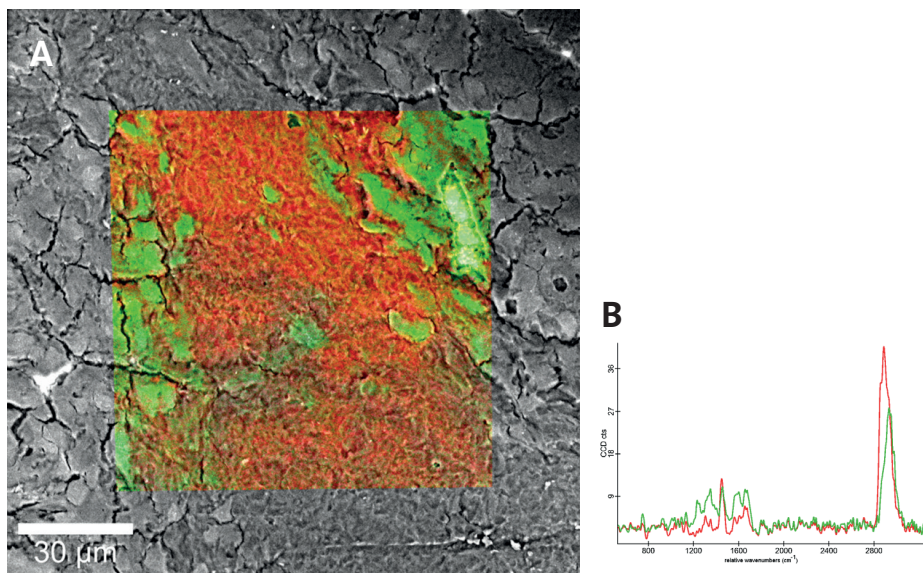
Figure 10: Hamster brain imaged with RISE™ microscopy

(A) RISE image of the brain tissue sample. In the color-coded Raman image the white brain matter is shown in green and the gray matter in red. (B) Raman spectra reveal the different spectral characteristics of the white and gray matter of a hamster brain slice.
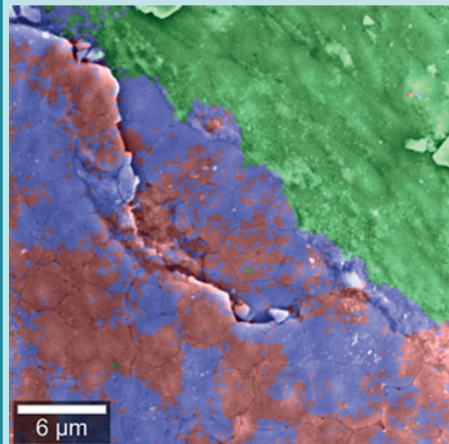
**Figure 11: RISE™ microscopy image of a pearl**

Confocal Raman imaging indicates that the pearl consists of two polymorphs of calcium carbonate, namely aragonite (blue, red) and vaterite (green). Structural features were imaged with SEM. The correlation of the information of both techniques results in the RISE™ image.
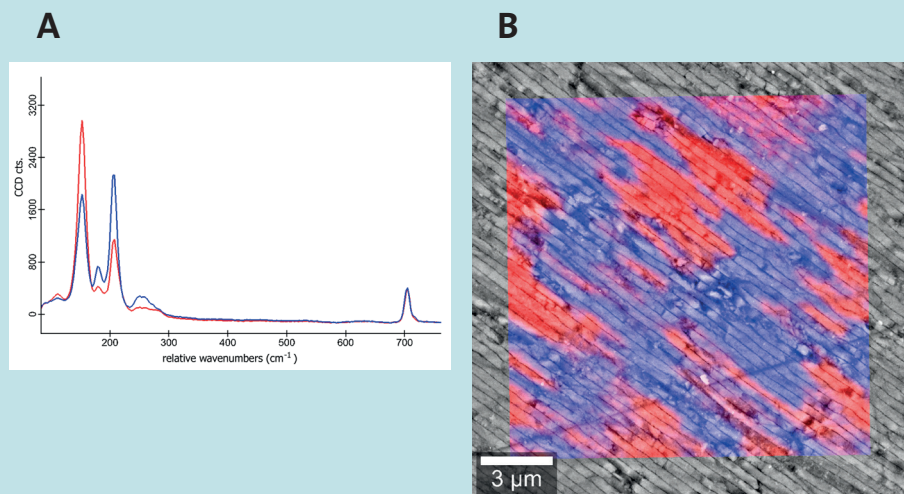
Figure 12: RISE™ microscopy image of an abalone shell

Mother-of-pearl consists of several crystal forms of aragonite. They can be distinguished by their Raman spectra (A): the different crystal orientations are shown in blue and red. The RISE™ image (B) illustrates how the anisotropy is correlated with the tructural features of the material.

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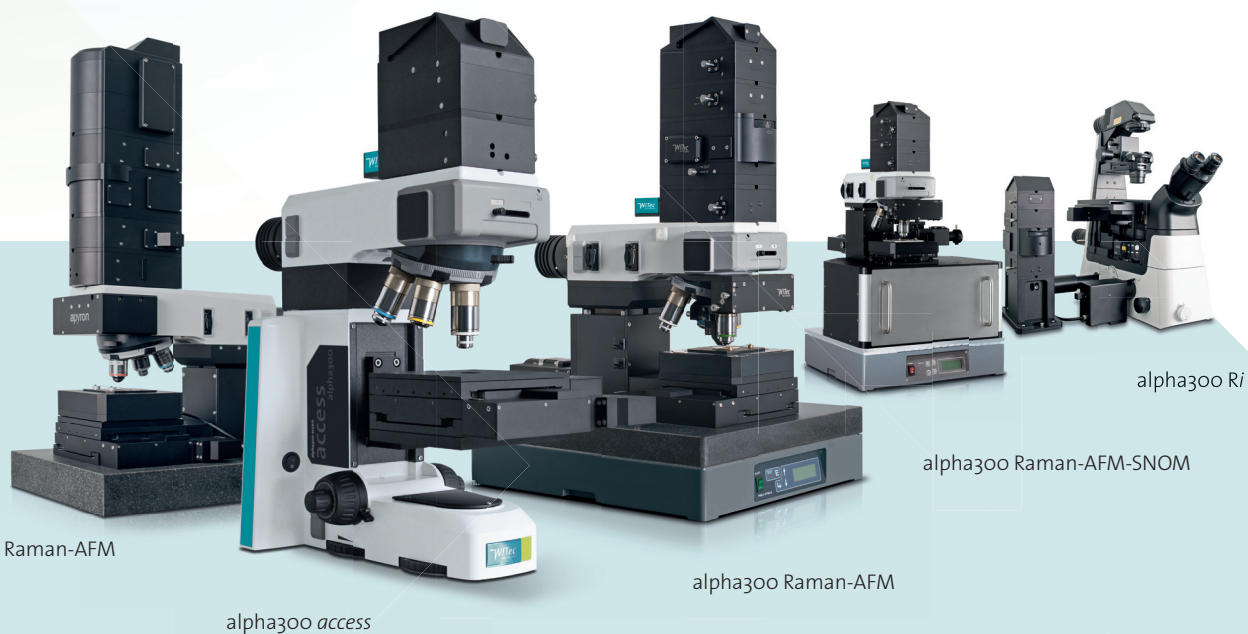
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automated Raman-AFM

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alpha300 Raman-AFM

alpha300 Raman-AFM-SNOM

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