APPLICATION NOTE

Raman Imaging: A powerful tool for the label-free characterization of single cells and bacteria
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With confocal Raman imaging the molecules of a sample can be chemically identified and their distribution can be imaged three-dimensionally. These benefits are gaining more and more recognition in biological and medical research and confocal Raman microscopy (CRM) is becoming a more frequently used method for answering crucial questions in life sciences. CRM is used for measurements in liquids and live cell imaging, solid samples and soft tissues. Through various examples this application note describes possible uses of confocal Raman microscopy and correlative techniques.

**Working Principles**

**Confocal Raman Imaging**

The Raman effect is based on light interacting with the chemical bonds within a sample. This causes a specific energy shift in the backscattered light which appears in a unique Raman spectrum through which the molecular components of a sample can be detected. The confocal Raman imaging technique combines Raman spectroscopy with a confocal microscope. This allows the spatial distribution of the chemical components within the sample to be imaged.

High-resolution confocal Raman microscopes acquire a complete Raman spectrum at every image pixel and achieve a lateral resolution diffraction limited only by (circa \( \lambda/2 \) of the excitation wavelength).

A confocal microscope setup is furthermore characterized by an excellent depth resolution and facilitates the generation of 3D Raman images and depth profiles. Confocal Raman microscopy can be coupled with correlative microscopy techniques such as fluorescence microscopy, electron microscopy and atomic force microscopy.

**Confocal Raman Microscopy**

The outstanding sensitivity of WITec’s optical systems reduces acquisition times for single Raman spectra down to well below 1 ms and enables FAST RAMAN IMAGING® and time-resolved micro-Raman spectroscopy with unparalleled resolution. Detecting signals from weak Raman scatterers or extremely low material concentrations or volumes with the lowest excitation energy levels is the unrivaled advantage of WITec systems. All Raman spectra are collected and processed by the WITec software suites, which also provide for post-processing and in-depth analysis of spectral data.
Raman imaging on fluorescent cells

Fluorescence microscopy is a well-established method for the investigation of biological samples. Relevant to fluorescence experiments, the expression of certain genes is linked to the presence of fluorescent proteins (GFP, for example) or can be revealed by fluorophore-coupled antibodies against certain proteins.

Raman microscopy enables the investigation of the molecular and chemical components of a sample. Therefore, all components of a cell can be investigated and not only those that exhibit fluorescence. Nevertheless, it is sometimes challenging to identify an area of interest in Raman microscopy. In this case, fluorescence can help to locate a specific region prior to the Raman analysis. As fluorescence emissions interfere with the Raman signal, it is important to select fluorophores that are compatible with the Raman investigations.

Fixed cells on a microscope slide were studied with the alpha300 Fluorescence-Raman microscope. Cells containing Green Fluorescence Protein (GFP) in their cytoplasm were identified in fluorescence mode (Figure A) using a Nikon 40x (NA = 0.6) air objective. The same cells were then imaged with the integrated video camera (Figure B) before being imaged in confocal Raman imaging mode with a ZEISS 63x (NA = 1.25) oil immersion objective. The sample was excited with a 532 nm diode laser. A Raman spectrum at every pixel was acquired (Scan range: 43 x 43 µm², 120 x 120 pixels, 14,000 spectra). With the color-coded Raman image, the cell body, cytoplasm, nucleus, and the nucleoli can be clearly distinguished (Figure C). In further analysis, the Raman spectra obtained from the measurement shown in Figure C were analyzed by cluster analysis. With this Post-processing technique, the Raman spectra are automatically compared and similar spectra are grouped into several clusters. From this analysis, a reduced and simplified Raman image can be displayed (Figure D) in which proteins are shown in blue, the nucleus is shown in red, the nucleoli are shown in green, and lipids are shown in yellow. The corresponding Raman spectra are shown in Figure E.

(A) Fluorescence image of the GFP-containing cells with excitation from above

(B) Video image from above

(C) Color-coded Raman image of the GFP-containing cell.

(D) Color-coded cluster analysis image

(E) Corresponding Raman spectra of the four clusters.

Image courtesy of Dr. Claudia Scalfi-Happ, ILM, Ulm, Germany
Investigating the intracellular components

Confocal Raman microscopy was used to image intracellular components of macrophages. Macrophages take up low-density lipoproteins (LDL) for recycling and removal. If their export capabilities are overextended, macrophages store lipids and develop into foam cells. These foam cells contribute to cardiovascular diseases such as arteriosclerosis. The subcellular lipid distribution of an in vitro THP-1 monocyte cell model was analyzed. The monocytes were differentiated into macrophages, incubated with deuterium- labeled lipids and examined with an alpha300 R confocal Raman microscope with a 60x NA=1.0 water immersion objective, an excitation wavelength of 488 nm, and 5 mW laser power at the sample. For the lipid uptake quantification of each cell, the C-D (2050-2275 cm\(^{-1}\)) to C-H (2800 – 3020 cm\(^{-1}\)) ratio of the Raman scattering intensities were evaluated. Thereby the C-D stretching vibrations were used as markers for intracellular lipids, while the C-H stretching vibrations reflected the general density of the cellsw. A complete Raman spectrum was acquired at every image pixel. The WITec Project software was used for data evaluation and processing. For further information please refer to the figurecaptions.

(A) Raman image of a macrophage cell incubated with 400 µM of oleic acid for 3 hours, generated from the C-H stretching intensities. The image was recorded with 488 nm excitation using a 60x NA=1.0 water immersion objective at a step size of 0.5 µm. A Raman image reconstructed using a spectral unmixing algorithm, which decomposes the data set of the image into its most dissimilar spectral components is shown in (B). The associated spectral information is plotted in (C). Clearly visible are the Raman signals that originate from the C-H stretching vibrations around 2104 cm\(^{-1}\).

Raman images of macrophage cells incubated with 400 µM of oleic acid for different periods of time. After 30 hours macrophages store excess lipids and develop into foam cells.

*Images courtesy of Dr. Christian Matthäus, Leibniz-Institute for Photonic Technology, Jena, Germany*
3D confocal Raman imaging of endothelial cells

In this study Raman imaging was used to three-dimensionally study the heterogeneity of single endothelial cells and to define the size, volume, shape and biochemical composition of the cellular organelles. The ability of confocal Raman imaging to construct 3D maps without disrupting the spatial integrity of the cell provides a unique insight into the biochemical architecture and cellular processes of the endothelium. 3D confocal Raman imaging can be carried out through 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 a tumor.

An alpha300 R confocal Raman microscope with a 60x water immersion objective for cells and an excitation wavelength of 532 nm was used for this study. The data acquisition was controlled by the WITec Project software package. All spectra were baseline-corrected using a polynomial of degree 3 and the routine procedure for removal of cosmic rays was applied. The Image J processing program was used to generate 3D pictures of cells and tissues. For further information please refer to the figure captions.

Confocal Raman imaging-stack of an EA.hy.926 cell. Integration maps over the $\nu_{\text{C-H}}$ (2800 – 3020 cm$^{-1}$ range).

Images courtesy of Prof. Malgorzata Baranska, Faculty of Chemistry, Jagiellonian University, Ingardena 3, 30-060 Krakow, Poland
Bacteria studies

It has been reported that Raman microscopy can be used to classify bacteria by their individual Raman spectra down to the substrain level. A high-resolution Raman imaging system also allows a single bacterium to be evaluated in terms of metabolic products, the presence of drugs or intra- and inter-cellular heterogeneity. In the following study, Legionella Bozemanii and Bacillus Cereus were investigated with an alpha300 R confocal Raman imaging system at the single cell level.

Legionella Bozemanii

Legionella strains can produce Poly-ß-hydroxybutyric acid (PHB) in response to physiological stress, which serves as an energy storage molecule that can be detected with Raman spectroscopy. In this experiment a Raman image was acquired with a scan range of 25 x 25 µm². The blue area in the color-coded Raman image corresponds to the integral intensity of the C=O ester stretching band at 1726 cm⁻¹. The red area depicts the cell body imaged using the protein amide I band at 1662 cm⁻¹.

The corresponding Raman spectra are shown below. The Raman image clearly reveals that the bacterial cells can contain different levels of PHB. Cells that contained little or no PHB could be differentiated from cells with a very high concentration.

(A) Raman image of Legionella Bozemanii and corresponding Raman spectra (B). Vegetative cells (red) and PHB (blue).