application note

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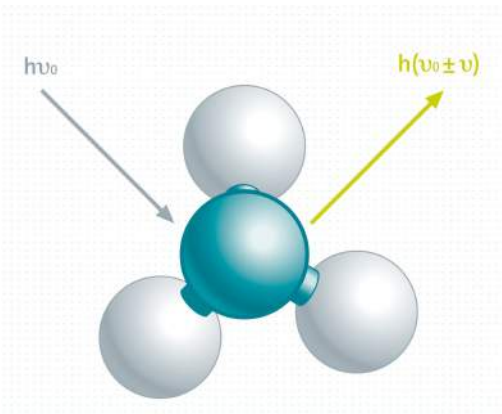
WITec
focus innovations

Topographic Confocal Raman Imaging of Archaeological Samples

In archeological research, various specimens such as paintings, mummies, antique objects or fabrics are regularly investigated. Those samples are often unique and require specialized treatment when analyzed. This application note introduces topographic confocal Raman Imaging as a flexible, non-invasive and non-destructive tool for the chemical and molecular characterization of archaeological specimens.

Confocal Raman Imaging

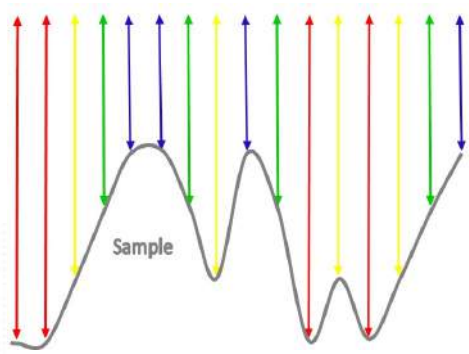
A Raman spectrum shows the energy shift of the excitation light (laser) as a result of inelastic scattering by the molecules in a sample. The excitation light excites or annihilates vibrations of the chemical bonds within the molecules which results in an energy shift of the photon scattered from this molecule. Different chemical species consist of different atoms and bonds, so each molecule can be easily identified by its unique Raman spectrum. As only molecular vibrations are excited (or annihilated), Raman spectroscopy is a nondestructive technique. In Raman imaging the Raman spectra are collected with a high-throughput confocal microscope/Raman spectrometercombination. A high-sensitivity CCD camera connected to a powerful computer and software system is used to detect the Raman signal. With specialized software tools the imaging capabilities can be expanded even further. For example, it is possible to generate images by integrating over selected spectral areas, determining the peak width, peak position or by even more sophisticated procedures such as the fitting of complete spectra or cluster analysis.



Raman Principle

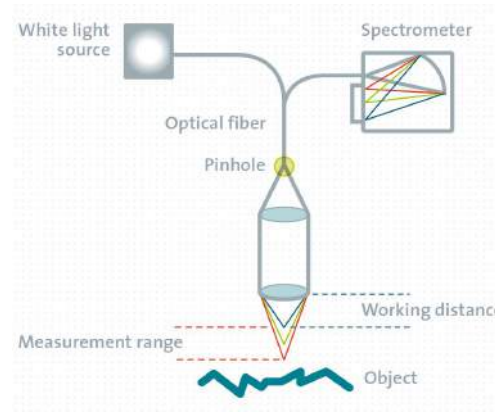
TrueSurface® Microscopy

The key element of this novel imaging mode is a topographic sensor that works using the principle of chromatic aberration. With this non-contact, purely optical profilometer technique it is possible to trace a sample's topography and follow it in a subsequent Raman measurement, thus remaining in focus throughout. For profilometry a white light point-source is focused onto the sample with a hyperchromatic lens assembly: A lens system with a good point mapping capability, but a strong linear chromatic error. Every color has therefore a different focal distance. The light reflected from the sample is collected with the lens and focused through a pinhole into a spectrometer. As only one color is in focus at the sample surface, only this light can pass through the confocal pinhole. The detected wavelength is therefore related to the surface topography. Scanning the sample in the XY plane reveals a topographic map of the sample. This map can then be followed in a subsequent Raman image so that the Raman laser is always kept in focus with the sample surface (or at any distance below the surface). The results are images revealing chemical and/or optical properties at the surface of the sample, even if the surface is rough or inclined.

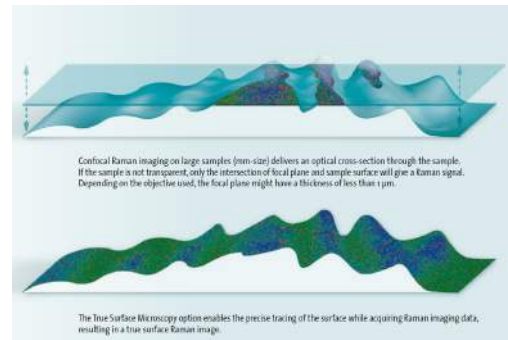


TrueSurface®: Each color corresponds to a certain focal distance.

Integrated TrueSurface® Microscopy



TrueSurface® Principle



Above (without TrueSurface®): When imaging rough surfaces in confocal imaging mode only parts of the sample are in focus. Below (with TrueSurface®): Following the topography in confocal imaging mode enables the surface to always stay in focus.

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Introduction

Raman spectroscopy has long been employed as a spectroscopic tool for materials characterization in the field of cultural heritage. Its non-invasive and non-destructive nature make it an attractive candidate for both material constituents and damage assessment and monitoring. However, specific requirements associated with objects of interest such as the impossibility of sampling as well as frequent autofluorescence associated with ageing related degradation products, made the extraction of useful Raman information extremely difficult.
In this application note it is shown how to overcome some of the mentioned challenges by applying the most advanced Raman imaging techniques. The sampling issue was addressed by applying a large-area imaging approach in correlation with the state-of-the-art profilometric measurement. This patent-pending method was recently developed by WITec. In such a way large objects (in terms of dimensions) can be automatically scanned and surface chemical images acquired.
The fluorescence problem is also an important one and cannot be easily solved. Recent advances in surface-enhanced Raman spectroscopy (SERS) enable the extraordinary enhancement of the Raman signal that can be seen above the autofluorescence. However, the limitation of the enhancement to only some types of the chemical compounds makes SERS specific to a limited number of applications. Here we suggest another approach to address the fluorescence issue: It combines the collection of the non-resonant Raman signal with a diffraction-limited spatial resolution. This approach allows the spatial separation of fluorescence affected degradation products and fluorescence-free intact particles.

Materials and Methods

All results were acquired with a WITec alpha500 R microscope for large-area confocal Raman imaging. Furthermore an Ultra-High-Throughput Spectrometer UHTS300 with a 600g/mm grating (BLZ 750nm) in combination with a 785 nm excitation laser, a deep depletion CCD camera, and a 100x NA 0.9 air objective was used. The TrueSurface® topographic confocal Raman imaging extension was directly integrated with the objective turret, which allows the acquisition of topographic and Raman data without any sample repositioning.
A piece of an ancient wall painting served as the specimen for all investigations. The fragment, part of a private collection, displays several layers of original polychrome paintings in relatively precarious state of preservation. The sample is shown in Fig. 1 with the areas of interest that have been characterized indicated by the arrows. For the investigation of the area of interest 1 the sample was not prepared prior to the measurements. Due to the absorption of the sample the laser light is unable to penetrate deep through the layers. Therefore a small section of the sample was additionally prepared and examined in order to generate vertical depth-profiles of the sample layers in the z-direction.

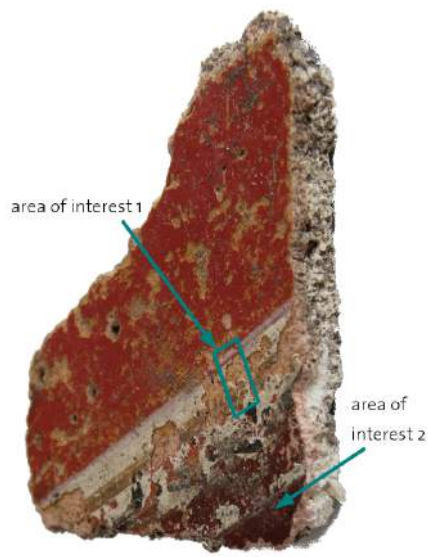

Fig. 1: Photograph of the sample with the areas of interest labelled.

Results

Investigation of the Area of Interest 1:
It is clear from Fig. 1 that the area of interest 1 shows a strong topography and can therefore not be measured by conventional confocal Raman microscopy. Therefore TrueSurface® confocal Raman imaging was used to gather the information from this area. In this mode the topography is first recorded using an optical profilometer. By simply rotating the objective turret the system is changed to Raman mode and the recorded surface can then be used to ensure that the sample always stays in focus. Prior to the measurement a white light stitching image was recorded to locate the area of interest. The white light image is shown in Fig. 2, the area from which the TrueSurface confocal Raman imaging was consecutively recorded is marked with a black rectangle.

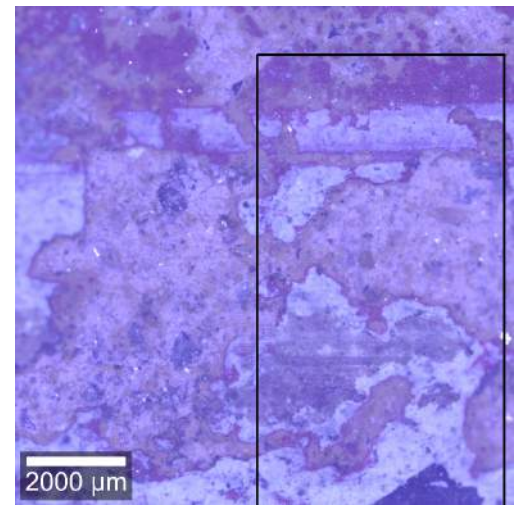

Fig. 2: White light stitching image of the area of interest 1 of the sample.

Fig. 3 shows the sample's topography recorded over an area of 5mm x 10mm with the TrueSurface® profilometric sensor. Please note that a change of over 500 µm in the focus position was observed. This variation in height would normally prevent measurements in standard confocal Raman imaging mode due to the surface being frequently out of focus.

Following the recording of the surface a large area confocal Raman image was recorded using the topographic information to trace the z-position. The scanned area in Raman imaging mode was again 5 mm x10 mm with 150x300 Raman spectra recorded. The integration time per spectrum was 1s.
The most prominent red painting layer in the analyzed area could easily be identified as cinnabar (HgS). Cinnabar is a precious red pigment characteristic of the Roman period. Minium, a ground version of cinnabar, was considered the Romans' most valuable pigment and it was used

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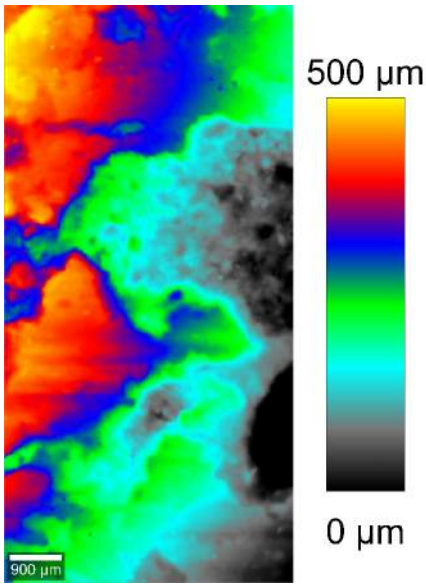
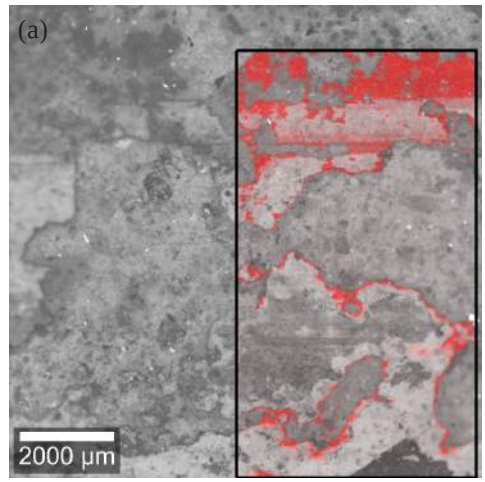


Fig. 3: Topography of the sample as recorded using the TrueSurface® extension of the alpha500 R.

in many ambitious works, including some Pompeiian masterpieces, and demonstrated great wealth. In fact, the price of Spanish minium used in Pompeii was capped by law at 150 sesterces per kilogram to stop it rising out of all proportion (Pigment Compendium: A Dictionary and Optical Microscopy of Historical Pigments By Nicholas Eastaugh, Valentine Walsh, Tracey Chaplin, Ruth Siddal 2008, Elsevier).The location at which cinnabar was detected by confocal Raman imaging is overlaid in red onto the white light image in Fig. 4a. By comparing the location of the cinnabar with the red painted areas shown in Fig. 4b, it can be clearly seen that the distribution of cinnabar is closely correlated. Furthermore an appearance of cinnabar can be detected at the edges of the layers indicating that a cinnabar-based preparation had been applied which evinces the importance and richness of the analyzed fragment.

In addition to cinnabar, other components of the investigated area can be identified by their unique Raman spectra. Fig. 5 shows the primary spectral components of the Raman analysis. Thus calcite, carbon black, and an unknown phase can be additionally detected.



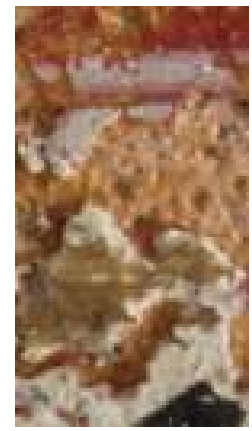


Fig. 4: a) White light image of the area of interest 1 with the locations at which cinnabar pigment could be found overlaid in red. b) Photograph of the area of interest 1.

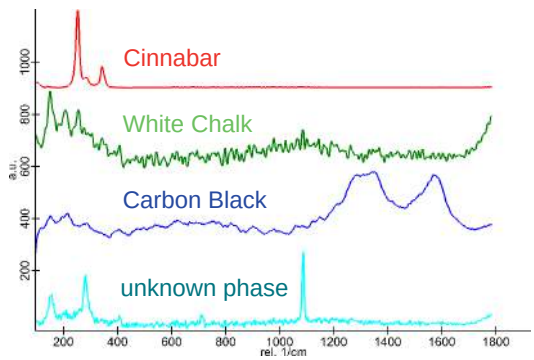


Fig. 5: Spectral components Raman analysis. The spectra could be identified as: Red = Cinnabar; Green = White Chalk (CaCO3, Calcite); Blue = Carbon Black; Turquoise = unknown phase.

Analyzing the entire data set allowed the generation of a color-coded image which could then be overlaid onto the topography as shown in Fig. 6. The colors of the areas correspond to the colors of the spectra shown in Fig 5.

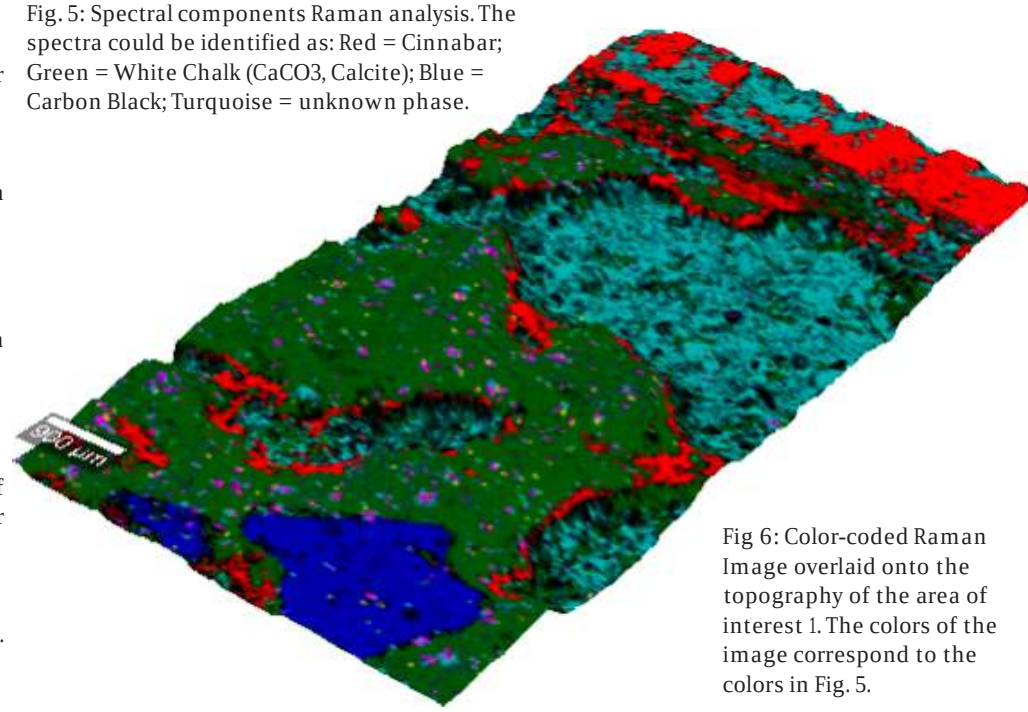


Fig 6: Color-coded Raman Image overlaid onto the topography of the area of interest 1. The colors of the image correspond to the colors in Fig. 5.

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Investigation of the Area of Interest 2:
Next the area of interest 2 was investigated. Here a distinctly different red color can be seen compared to the bright red color investigated in area of interest 1. This observation could be confirmed by the spectral analysis. The Raman spectra shown in Fig. 7 identifies red ocher as the main chemical component of the dark red color of area of interest 2. For comparison the spectrum of cinnabar is also shown in Fig. 7.

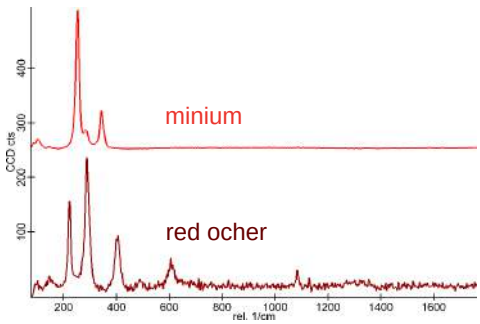

Fig. 7: Spectra of the bright red color (identified as minium) and of the dark red area (identified as red ocher)

For the generation of a confocal Raman image of area of interest 2 an area of 70 µm x 70 µm was measured with an integration time of 133ms per spectrum. The resulting Raman image consists of 70 x 70 pixels with the information of a complete Raman spectrum at every image pixel. The color-coded Raman image was overlaid onto the white light image as shown in Fig. 8. The colors correspond to the colors of the spectra shown in Fig. 9 with ocher crystals shown in red and calcium carbonate binder in blue and green, indicating a high level of fluorescence.

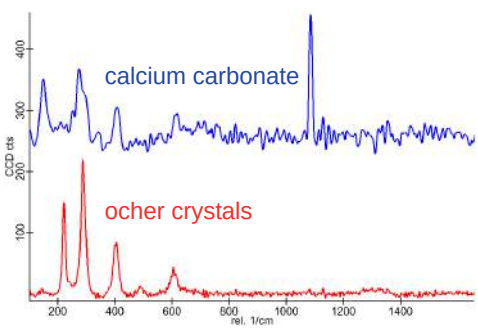

Fig. 9: Spectra extracted from the confocal Raman image scan on area of interest 2. The colors correspond to the colors shown in Fig. 8.

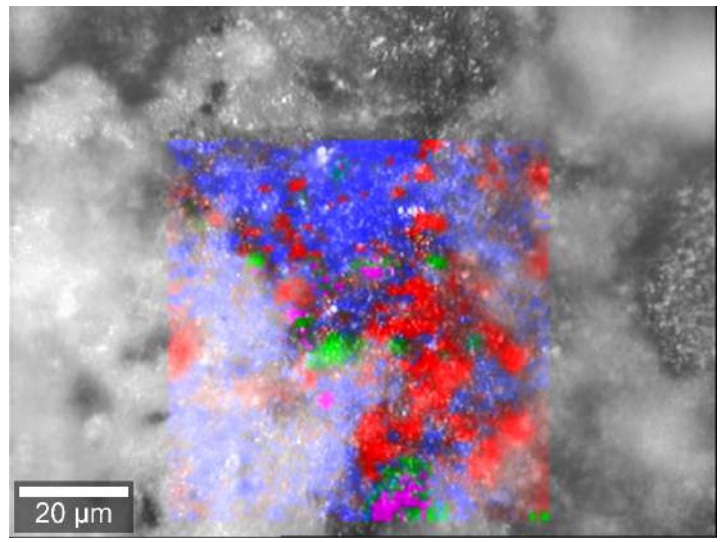

Fig. 8: Color-coded Raman image overlaid on the white light image of the area. Color coding: Ocher crystals - Red; Calcium carbonate binder mixed with ocher - Blue; High level of fluorescence - Green.

Cross Section Measurements
As outlined earlier, the penetration depth of the laser light into the material is very limited. For this reason cross sections were prepared. The cross sections allow the visualization of the succession of individual layers in z-direction (vertically). Therefore a small sampling is necessary and the fragment taken is embedded in a resin then polished perpendicular to the plane of the painting surface. In this way a suitable specimen cross section is obtained for observation of the individual layers by confocal Raman microscopy.
Fig. 10 shows the overlay of the combined Raman image onto the white light image. The Raman image was recorded with 175 µm x 175 µm (175x175 pixels). The integration time was 133ms per spectrum and the light was collected through a pinhole with a diameter of 100 µm. The gray frame in Fig. 10 shows the zoomed area that was recorded in a second scan. The colors of the overlay image correspond to the colors of the Raman spectra in Fig. 11. The spectra in Fig. 11 show calcite characteristic profiles in two different configurations/orientations (green and blue spectra). The minium is again identified as the red pigment used (red spectrum) as well as a very weak signal of amorphous carbon originating from a thin layer at the surface (yellow color).

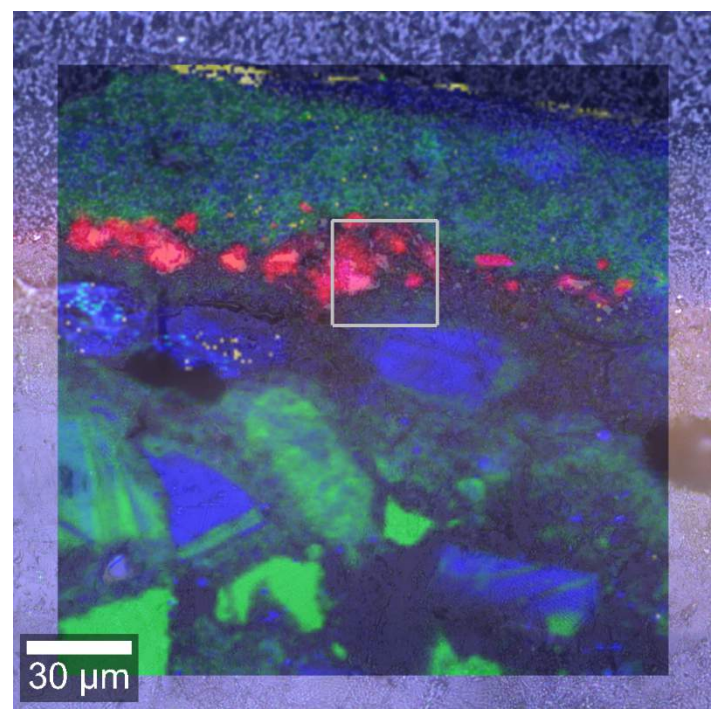

Fig. 10: Color-coded confocal Raman image overlaid on a white light image of a cross section of the sample. The colors correspond to the colors of the spectra in Fig. 11. The frame indicates the area at which a consecutively second scan was performed.

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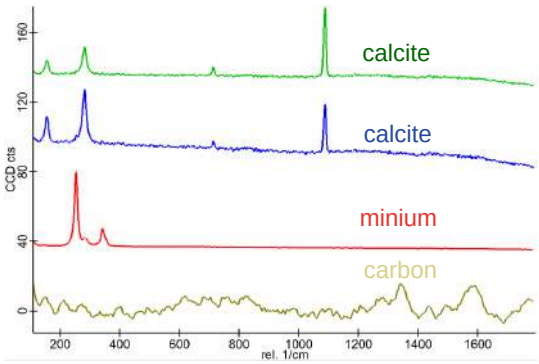

Fig. 11: Spectra extracted from the confocal Raman image scan shown in Fig. 10. Green and blue: calcite in two different configurations/orientations; Red: Minium; Yellow: Amorphous carbon.

With a high resolution scan (marked with a gray frame in Fig. 10) the area at which the minium color particles can be prominently seen was imaged again. Fig. 12 shows the combined color-coded Raman image and Fig. 13 the corresponding spectra. In this area a further component of unknown origin (blue color) could be extracted from the spectra using spectral de-mixing. From the image it can be seen that the color particles (in Red) cluster into larger and smaller agglomerates and the smallest detectable in the image are on the order of half a micrometer (marked with an arrow in Fig. 12)
which corresponds to the resolution limit for a 785nm laser used in conjunction with a 100X NA 0.9 objective ($0.51^{*}\lambda / NA \approx 445nm$ [FWHM]).

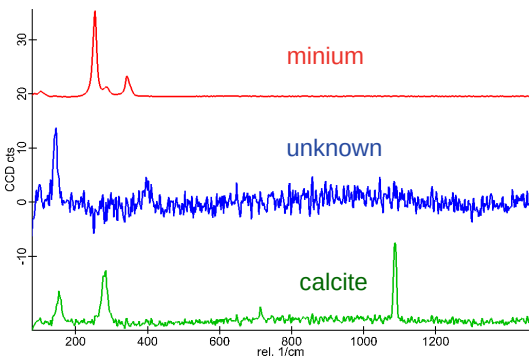

Fig. 13: Raman Spectra extracted from the zoomed scan of the cross section. Red: Minium; Blue: Unknown origin; Green: Calcite

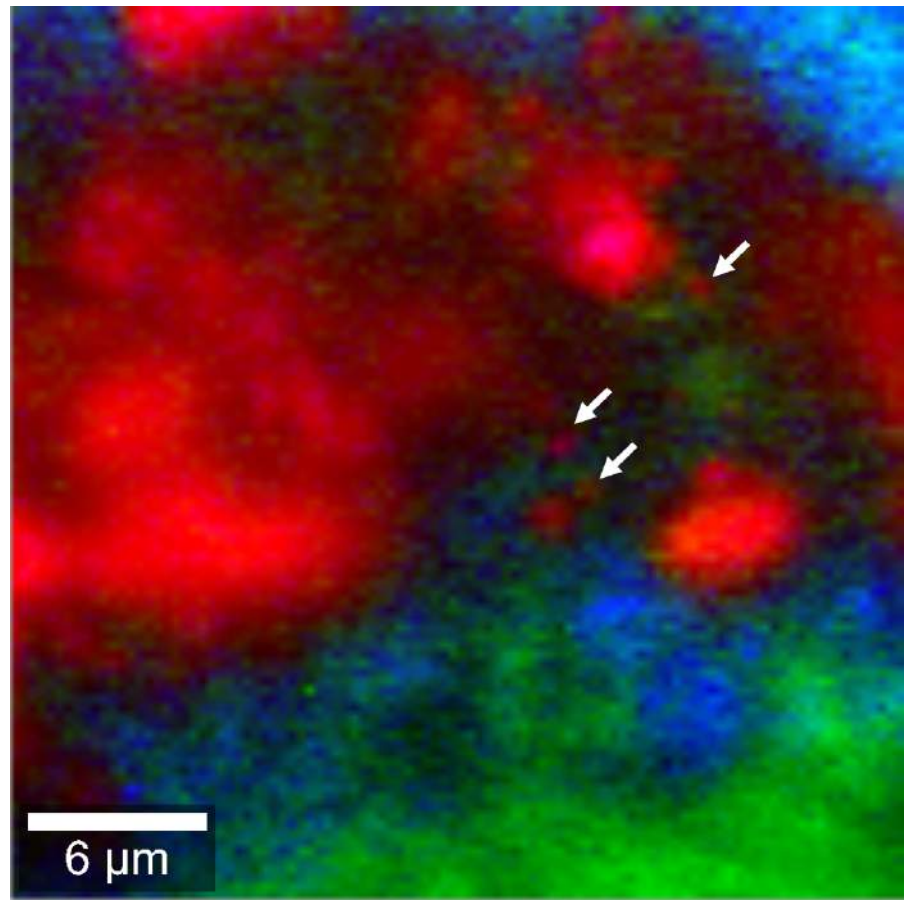

Fig. 12: Combined Raman image of the zoomed scan of the cross section. The colors correspond to the colors of the spectra shown in Fig. 13. The arrows mark the small agglomerates on the order of half a micrometer.

Conclusion

The recent instrumental developments in Raman microscopy enable remarkable new applications in archaeological sciences. The possibility given by TrueSurface® of non-invasively and non-destructively studying large areas of ancient objects with irregular surfaces is a breakthrough in the field of cultural heritage research. It further allows investigation of extremely precious samples *in situ* without the need for any sampling or sample preparation. When sampling is possible, the sub-micron resolution large area imaging of a cross section further improves the quality of the analytical output and allows detailed characterization of primary material constituents on the micron scale. In conclusion, confocal Raman imaging is a valuable tool for the identification of minute quantities of a material that can provide the archaeologist, conservator and other researchers with invaluable information regarding provenance, manufacturing technology, state of preservation, and in some instances, authenticity.

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Dr. Admir Masic

The application note was compiled in collaboration with Dr. Admir Masic

Dr. Masic is an independent researcher in the Department of Biomaterials at the Max Planck Institute of Colloids and Surfaces in Potsdam, Germany. His current research focus is the development of innovative spectroscopic methodologies, mainly based on Raman imaging.

Selection of Publications in the Field of Archaeology

R. Schutz, L. Bertinetti, I. Rabin, P. Fratzl, A. Masic, Quantifying degradation of collagen in ancient manuscripts: the case of the Dead Sea Temple Scroll. Analyst 138, 5594-5599 (2013); (10.1039/c3an00609c).

M. Janko, R. W. Stark, A. Zink, Preservation of 5300 year old red blood cells in the Iceman. Journal of the Royal Society, Interface / the Royal Society 9, 2581-2590 (2012)10.1098/rsif.2012.0174).

M. Janko, A. Zink, A. M. Gigler, W. M. Heckl, R. W. Stark, Nanostructure and mechanics of mummified type I collagen from the 5300-year-old Tyrolean Iceman. Proceedings. Biological sciences / The Royal Society 277, 2301-2309 (2010)10.1098/rspb.2010.0377).