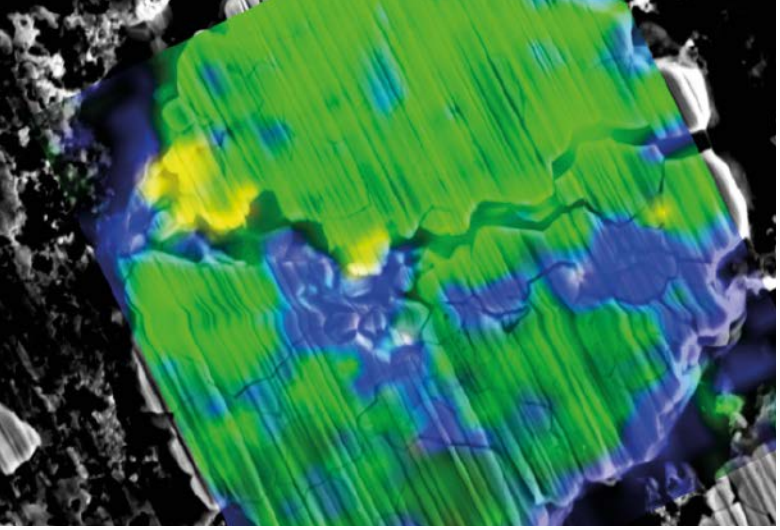
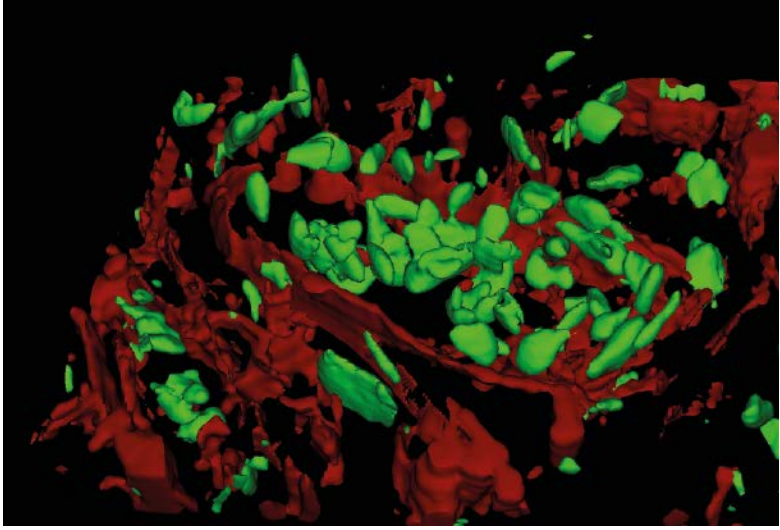
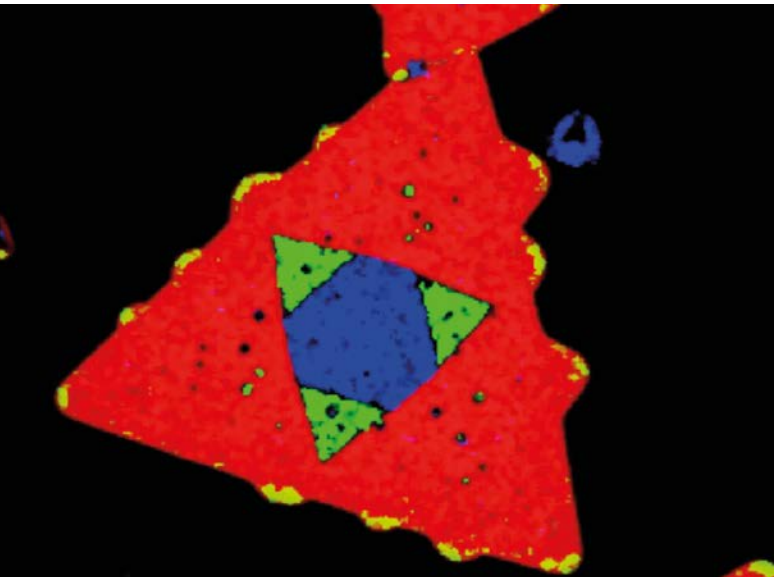
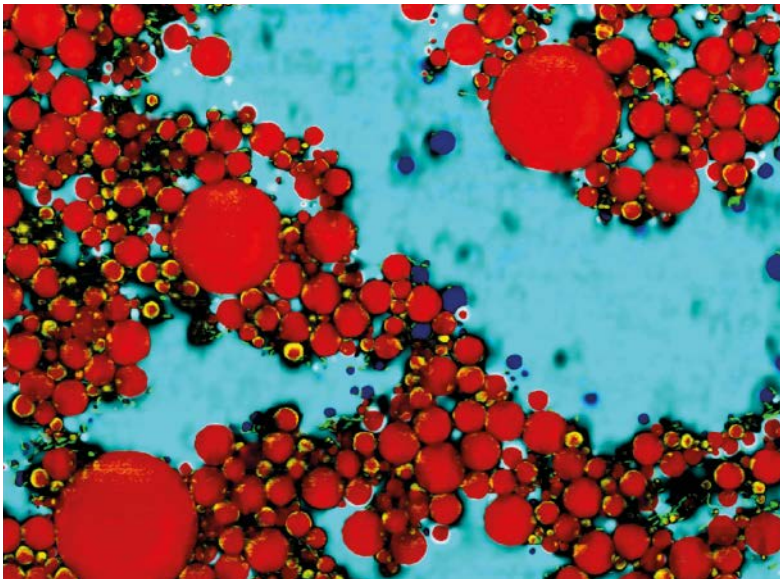
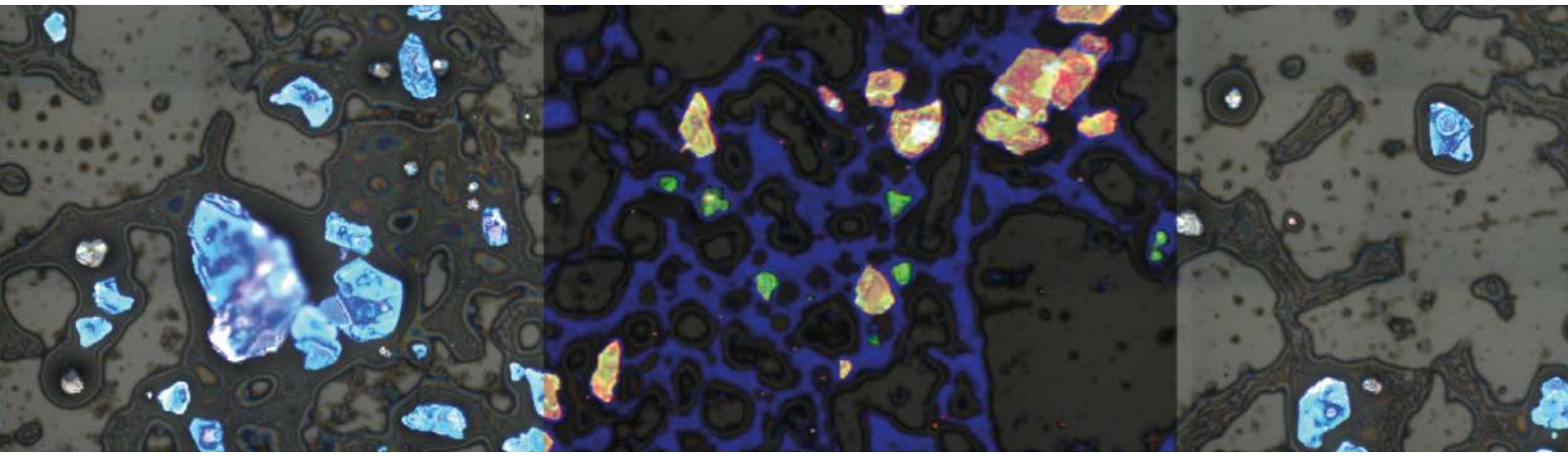



Raman Microscopy

A Comprehensive e-Guidebook

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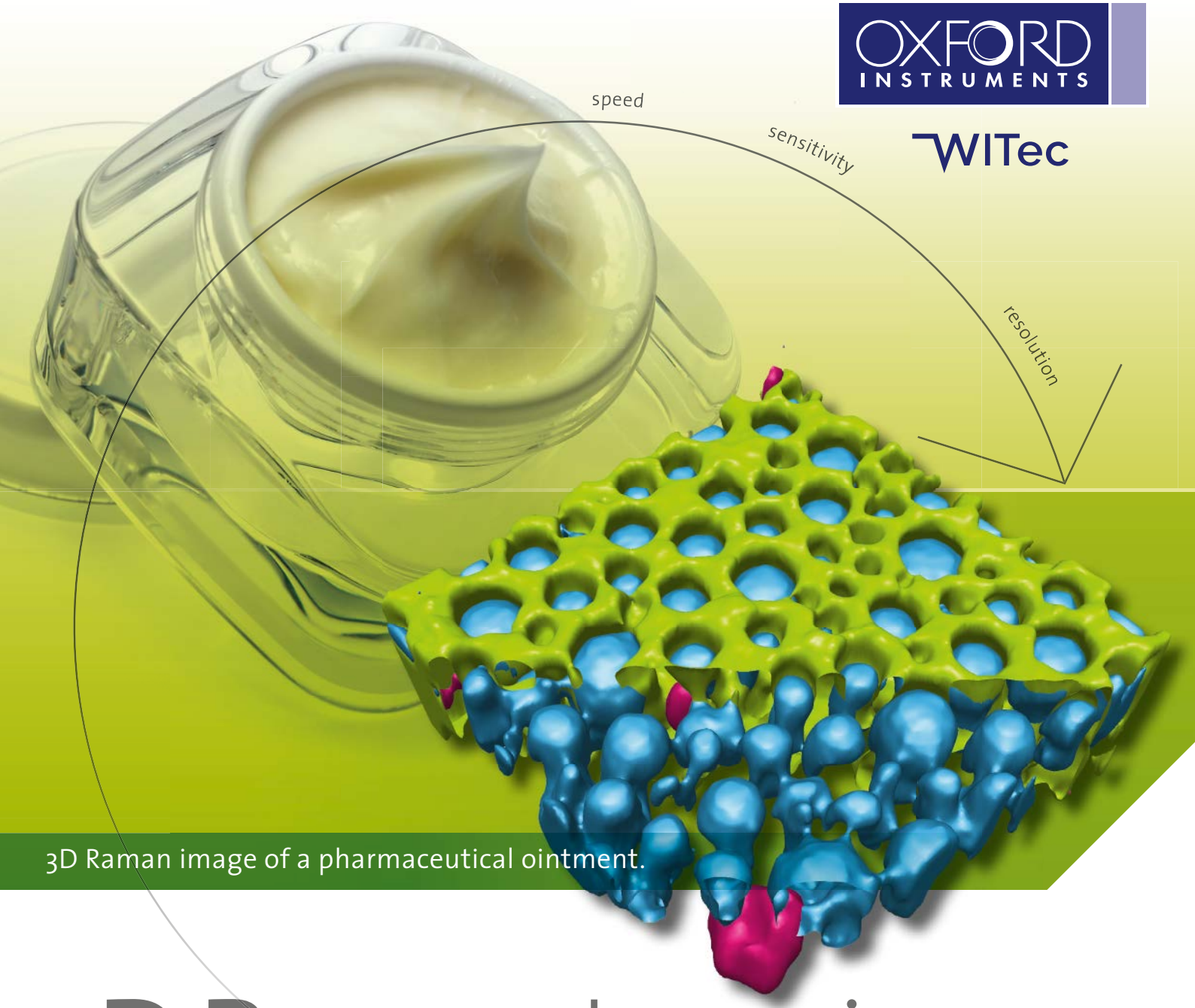

3D Raman image of a pharmaceutical ointment.

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Dear Reader,

Wiley and WITec are pleased to present this article collection on Raman microscopy. The Raman microscope is a laser-based instrument for performing Raman spectroscopy. With the help of Raman microscopy, different samples can be analyzed and various scientific questions can be explored. The resolution and areas of application of this analytical technique have been continuously developed in recent years.

A wide variety of samples can be analyzed, such as rocks and meteorites, semiconducting materials, cosmetics samples, eukaryotic cellular structures, micro- and nanoplastics, lithium-ion batteries and more.

For imaging, Raman microscopy can be combined with techniques such as electron microscopy or fluorescence microscopy and others in correlative applications.

In this e-book you will find a broad overview of Raman microscopy and a selection of possible applications. In addition to the application notes from WITec, we interviewed four researchers who use Raman microscopy in their research projects and asked them about their experiences with this technology. Enjoy reading this article collection and learning about the advantages of using Raman microscopy in the lab.

Dr. Birgit Washburn
Editor-in-chief Imaging & Microscopy
Wiley

Contents

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Editor-in-Chief
Dr. Birgit Washburn

Introduction

Several different things can happen when light hits any kind of material: some wavelengths of light pass straight through, some are absorbed, some are reflected and some are scattered. Whether a specific wavelength is absorbed, reflected, scattered or passes straight through depends on how it interacts, or doesn't, with the molecules making up the material. This means that exactly how a specific wavelength of light interacts with a molecule can reveal information about it, including its identity, which is the basis for most forms of spectroscopy.

Infrared spectroscopy, for example, identifies molecules from the specific wavelengths of infrared light they absorb, as most molecules absorb at infrared wavelengths. But molecules can also be identified from the way in which they scatter specific wavelengths of light, as a result of inelastic collisions between photons of light and the molecules.

Most of the scattered light will be at the same wavelength, and thus frequency, as the incoming light (known as Rayleigh scattering), but some of the scattered light will be at shorter wavelengths and higher frequencies than the incoming light (known as anti-Stokes scattering), and some will be at longer wavelengths and lower frequencies (known as Stokes scattering). This was discovered by the Indian physicist Chandrasekhara Raman in 1928 and is thus called Raman scattering.

This effect occurs when collisions with photons cause changes in the vibrational and rotational energy levels of the molecules. An increase in the frequency of the scattered light represents a loss of molecular energy, while a decrease in the frequency represents a gain of molecular energy. As a consequence, the wavelength at which Raman scattering occurs, together with the difference between the incoming wavelength and the scattered wavelength, is different for different molecular bonds, depending on their energy levels, and can be used to identify molecules. This is the basis for Raman spectroscopy.

Because scattering is a rather weak effect compared to absorption or reflection, and because Raman scattering is less common than Rayleigh scattering, Raman spectroscopy is not as inherently sensitive as infrared spectroscopy, but it has certain other advantages. For a start, because Raman scattering occurs with shorter wavelengths of light (visible and near-infrared) than infrared spectroscopy, Raman micro-spectroscopy has a higher spatial resolution. The latest instruments can reveal molecular information for regions with lateral dimensions of less than 300nm, compared with 10µm for infrared spectroscopy. Also, water molecules display very weak Raman scattering, but absorb strongly at infrared wavelengths, meaning water doesn't interfere much with Raman spectroscopy, which is useful when analyzing biological specimens.

To enhance Raman scattering as much as possible, Raman spectroscopy utilizes a laser to irradiate samples with intense light, meaning a high density of photons. If the laser is focused onto a tight spot, which is then scanned across a sample, Raman spectroscopy can be used to build up a picture of the molecular composition over an entire specimen at a resolution below approx. 300nm. This also means Raman spectroscopy can be combined with various forms of microscopy, from normal light microscopy to electron microscopy, to produce Raman microscopy, in which molecular information is overlaid on top of a magnified image of the specimen.

Raman spectroscopy is usually combined with microscopy by attaching two optical fibres to the microscope: one delivers light from the laser to the specimen, while the other captures scattered light from the specimen and delivers it to a spectrometer. When combined with confocal microscopy, the light from the laser is usually passed through the microscope objective to focus it on the specimen. A pinhole is used

to block any out-of-focus scattered light from regions outside of the area being probed, thus reducing background noise and allowing 3D imaging. The raw spectral data is processed by software to identify the molecules present at each analysis point, and the different molecular information can be displayed as different colours on the magnified image.

Over the years since its discovery, scientists have learnt a great deal about Raman scattering, and many fields of applications can benefit from the technique. It has been applied to a wide range of specimens – biological, geological, environmental and synthetic – from cells to rocks to hand cream to battery electrodes.

A successful analysis of a specimen with Raman spectroscopy first requires finding the optimum wavelength for probing the specimen. Shorter, higher energy wavelengths are generally better as they produce more scattering and allow for higher resolution, but can damage more fragile specimens, such as biological specimens. They can also induce autofluorescence from the proteins in biological specimens, which can swamp the Raman scattering, making it impossible to detect.

For biological specimens, a wavelength of 785nm – in the near-infrared – is a good compromise, especially as infrared light passes further into biological tissue than visible wavelengths. For tougher non-biological materials, however, visible wavelengths are often ideal.

In conclusion, the properties of Raman microscopy render it useful for an ever-increasing range of diverse applications. The following articles in this e-book illustrate this very well, from improving the performance of rechargeable batteries, through detecting nanoplastics and environmental change, to forensic analysis and studies of cellular processes and materials synthesis.

Automated Raman Microscopy

New Developments in Optimization and Flexible Operation

Damon Strom[1] and Thomas Dieing[1]

Raman microscopy, long used by specialists in purely scientific research, is being employed as a routine analysis tool in an ever-growing range of fields. Automation has been the key to making the technique easier to use while still offering the full benefits of its analytical power and sensitivity. Developments that introduce self-alignment, modular optical components and remote operation will change what is expected of a fully automated Raman imaging system. The following overview describes these technologies and presents several examples of their application.

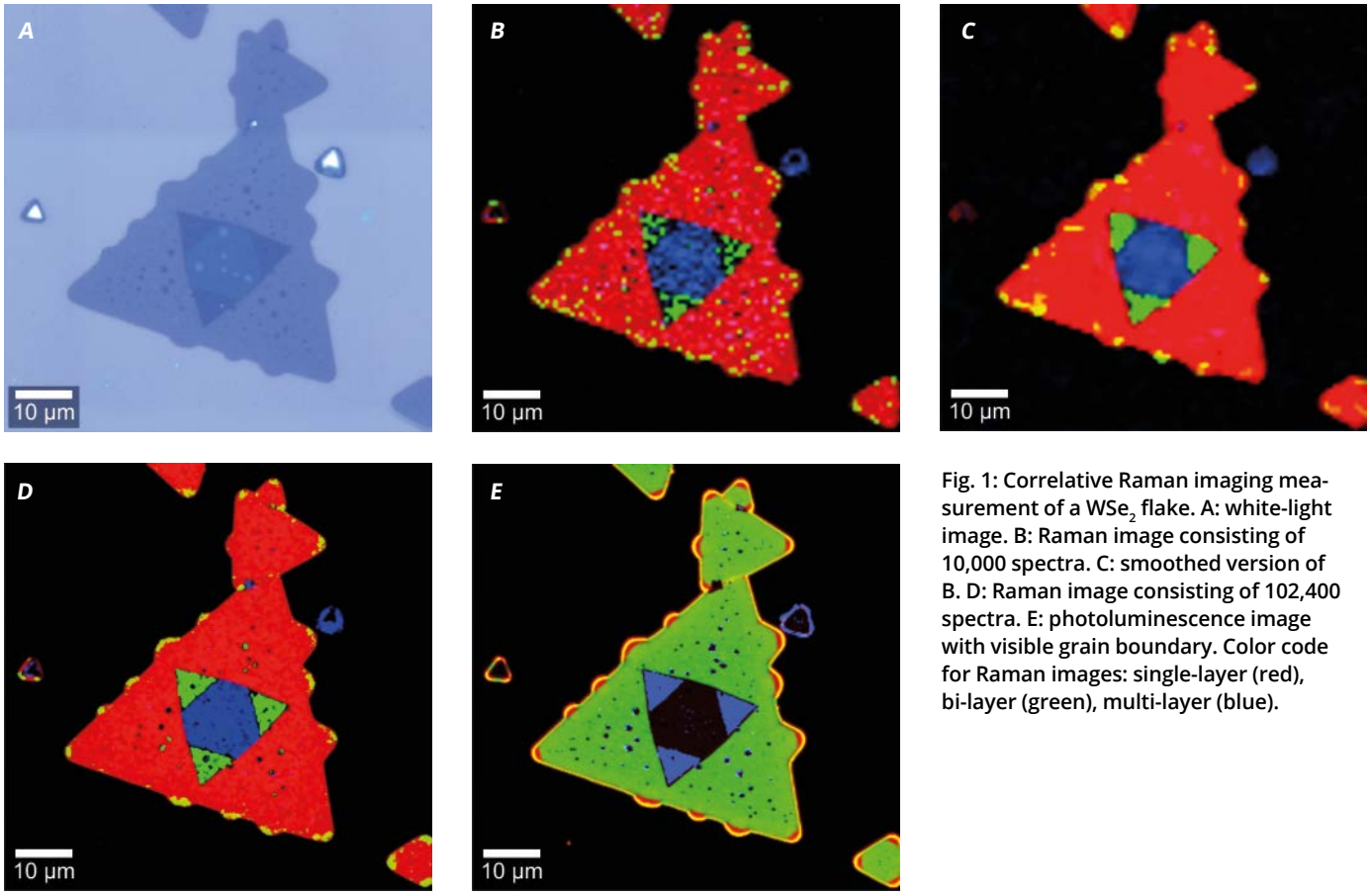

Fig. 1: Correlative Raman imaging measurement of a WSe_2 flake. A: white-light image. B: Raman image consisting of 10,000 spectra. C: smoothed version of B. D: Raman image consisting of 102,400 spectra. E: photoluminescence image with visible grain boundary. Color code for Raman images: single-layer (red), bi-layer (green), multi-layer (blue).

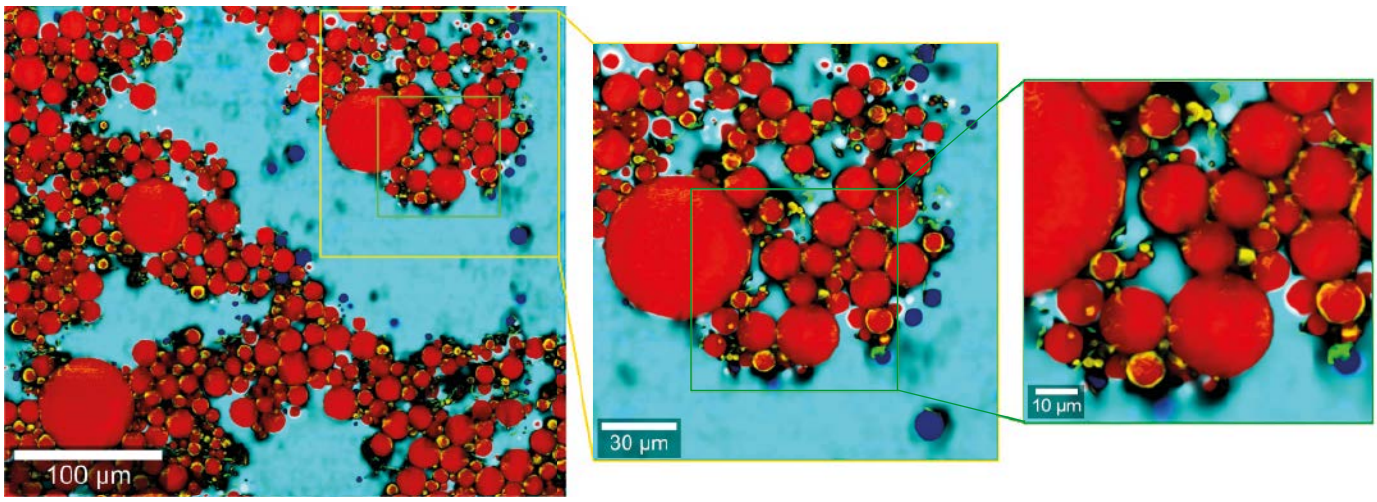

Fig. 2: Large-area Raman image and zoomed-in views of a moisturizing shower gel with increased oil content. Cyan and blue: aqueous phases; red: oil; yellow: emulsifier. 250 nm/pixel, 10 ms per spectrum acquisition time. 532 nm excitation laser, 100x oil-immersion objective with NA = 1.25.

Confocal Raman Imaging Microscopy

Raman microscopy is a non-destructive and label-free technique that relies on the Raman effect, in which light scattered by molecules exhibits a distinct shift in energy due to molecular vibrations. It characterizes materials by these unique shifts, which are visible in their Raman spectra. The method's spatial resolution is limited only by physical law and it can be applied to very small sample volumes and low material concentrations.

Confocal Raman microscopy uses a beam path geometry that strongly rejects light from outside the focal plane to increase sensitivity and enable 3D measurements. Raman imaging acquires a complete Raman spectrum at each pixel to visualize the distribution of sample components.

Self-Alignment and Self-Calibration

Advanced Raman imaging experiments often include many optical elements in their beam paths. A fully automated instrument controls all the individual pieces from one integrated software suite, and records their settings with each measurement.

Developments in opto-mechanical components now enable systems to self-align and self-calibrate. This increases the sample turnover rate and optimizes performance for every experimental setup. Software-driven routines ensure the consistency and repeatability of results while also substantially reducing the researcher's workload by requiring less user input and eliminating potential sources of error.

An automatically aligned and calibrated Raman microscope should always be capable of a lateral resolution under 300 nm and a depth resolution of less than 900 nm with 532 nm excitation. A spectral resolution down to 0.1 cm^{-1} relative wavenumbers at 633 nm excitation and acquisition times faster than 1 ms per spectrum should also be regularly achievable.

Modular Optical Components

Automated Raman microscopes designed around standardized optical modules with an integrated software environment can incorporate new capabilities as they are introduced and be configured as an experiment requires or reconfigured as requirements evolve.

The modules necessary for self-optimization as described above include a calibration source that can validate and calibrate spectrometer gratings, an output coupler that maximizes signal throughput to a spectrometer, and motorized iris diaphragms that adjust the beam path for optimum contrast and homogeneity in white-light imaging.

The latest multi-laser input coupler technology can adjust the optical elements for each wavelength. It can also configure the beam path to perform measurements with methods complementary to Raman microscopy while remaining at the same sample position. Excitation laser wavelengths can be chosen to best produce the Raman effect, or to generate or avoid photoluminescence from the sample.

Remote Operation

Remote operation through automated components allows Raman imaging measurements in environmental enclosures such as glove boxes. This has great utility in semiconductor research and life science, among other disciplines. Raman microscopes that can align and calibrate themselves can be controlled from another location entirely. Only the mounting of the sample on the microscope stage requires physical interaction. This delivers the full capability of a laboratory instrument from anywhere, including home offices.

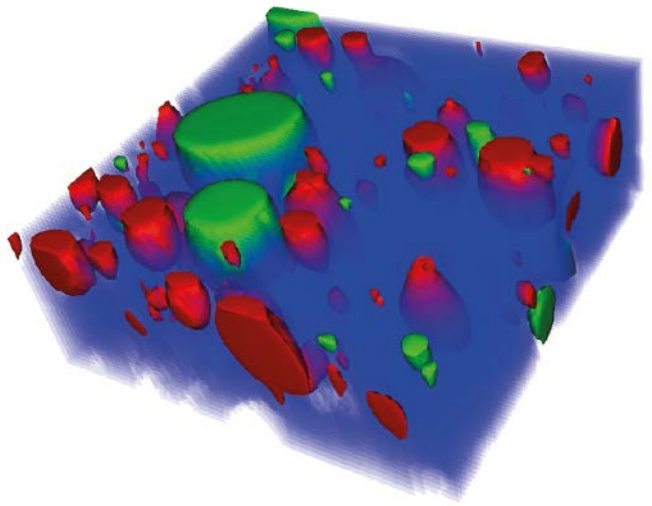

Fig. 3: 3D Raman image of a hand cream. Blue: water; red: oil; green: one of the hand cream's moisturizing ingredients dissolved in oil. Scan range: 40 x 40 x 15 µm³.

Application Examples

The following measurements were performed with a WITec alpha300 *apyron* automated Raman imaging microscope equipped with several UHTS ultra-high throughput spectrometers optimized for different wavelengths. After the sample was put in place, every step of each measurement was performed completely through the Suite FIVE integrated software and EasyLink handheld controller.

Correlative Raman Measurement of Tungsten Diselenide

The speed and sensitivity of a Raman microscope that has been optimized through automation is demonstrated with the analysis of a tungsten diselenide (WSe_2) flake (fig.1). Different layers in the flake are visible in the white-light image (A). In approximately 2 minutes, a clear and detailed 75 x 75 µm² Raman image of 10,000 spectra was recorded (B). The flake shown consists of single-layer (red), double-layer (green) and multi-layer (blue) areas. The same measurement after smoothing is shown in (C). A measurement acquired in about 17 minutes of more than 100,000 spectra produced an even sharper image (D). The increased signal to noise ratio was achieved by reducing the pixel size from 750 nm (B) to 230 nm (D). The photoluminescence image (E) shows the same structures as the Raman image and even the grain boundary between the larger and the smaller flake is visible. The integration time was 6 milliseconds per pixel for all measurements.

Raman Image of Moisturizing Shower Gel

Raman imaging microscopy can be used to visualize different phases and the boundaries between them in liquid samples. In this investigation, a moisturizing shower gel had its oil content increased with olive oil. Its phase boundaries were located and the chemical identities of the components in the emulsion were identified using Raman spectroscopy. Visualizing the boundaries between emulsifiers, surfactants and moisturizers is essential in their development (fig.2).

3D Raman Image of a Hand Cream

Automated confocal Raman microscopes feature exceptional lateral and depth resolution. This can be clearly seen in a 3D Raman measurement of hand cream. 15 images of 200 x 200 pixels each were acquired in rapid succession along the z-axis to form a 3D image stack (fig.3).

Conclusion

Investigations of a semiconducting material and cosmetics samples showed the analytical capability of an instrument that requires minimal user input. For the tungsten diselenide flake, white-light, Raman, and photoluminescence measurements were carried out at the same sample position with the beam path automatically configured for each method. The benefits of self-optimization routines were also vividly demonstrated by the spatial resolution attained in measurements on liquid samples.

The automation of Raman microscopy will continue to broaden its appeal. The advantages that it initially offered, in user-friendliness and the rate at which samples can be measured, will be supplemented by new advances. Self-alignment and self-calibration, modular optical components, and the ability to be operated remotely have revised the definition of fully automated Raman microscopy.

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Analyzing Materials for Semiconductor Devices with Raman Microscopy

Prof. Dr. Georg S. Düsberg and Oliver Hartwig are working at the Institute of Physics at the University of the Bundeswehr in Munich, Germany. Here they are covering materials science and process technology used to fabricate conventional and innovative semiconductor devices. In particular they deal with the integration of functional materials for new applications and systems. Focusing especially on the synthesis of 2D materials, the group is interested in the development of reliable processes to produce high quality materials. They are investigating the growth and properties of a large variety of 2D materials such as graphene, transition metal dichalcogenides (TMDs) such as MoS_2, $MoSe_2$, WS_2 and WSe_2 as well as the noble metal dichalcogenides such as $PtSe_2$, PtS_2, $PdSe_2$ and PdS_2.

Prof. Dr. Georg S. Düsberg

Oliver Hartwig

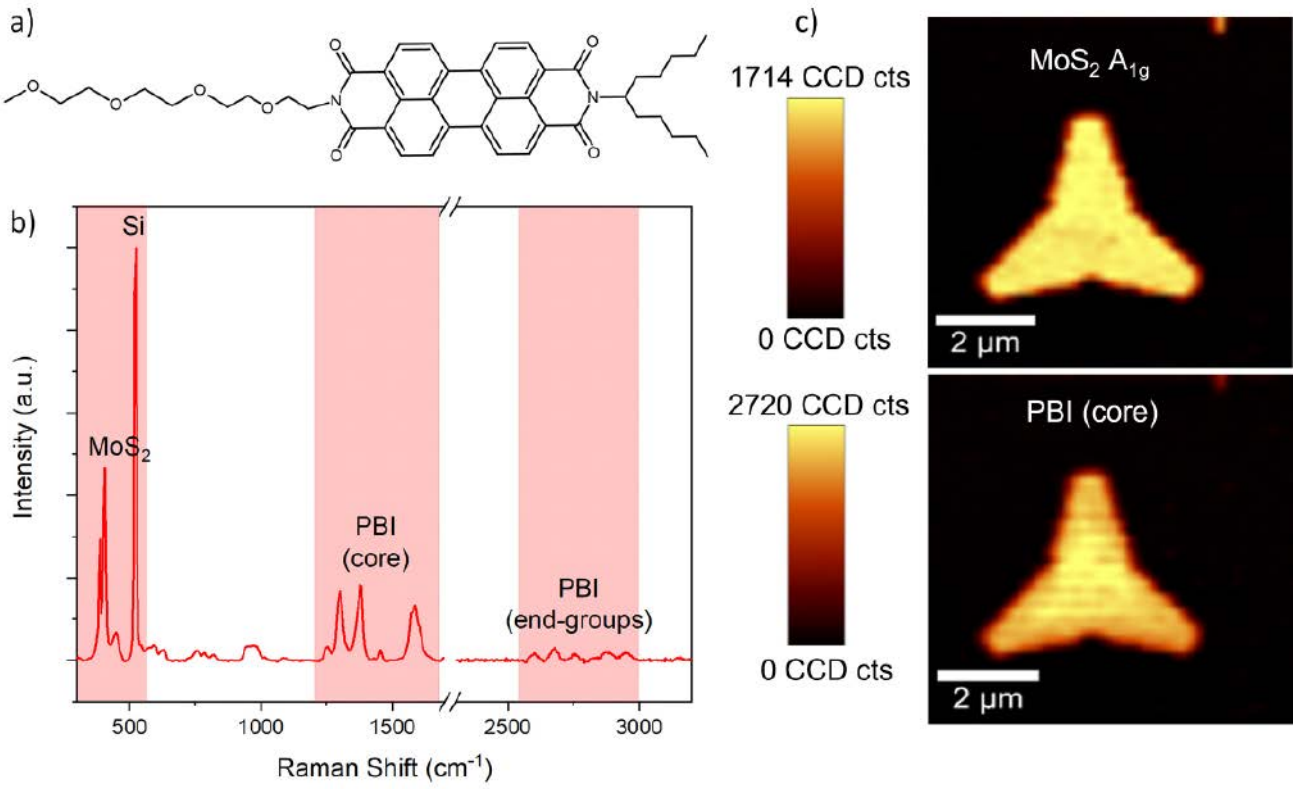
The group also develops electrical devices based on these materials, such as sensors and transistors. In doing so, they take advantage of the extraordinary properties of these novel materials. This enables the production of smaller and more accurate sensors, which are tested and characterized in the laboratories. These include optical sensors, but also pressure and gas sensors.

Which scientific questions have you been able to answer in the past using Raman microscopy?
The synthesis of 2D materials is still under development and is always in need of quality control. Raman spectroscopy is the measurement of choice to investigate most 2D material growth processes. By obtaining Raman spectra of the sample surface, we can characterize the abundance and quality of the grown materials. Even monolayers can be characterized efficiently with Raman spectroscopy. Especially Raman imaging allows to assess the quality of the growth over larger areas in a reasonable time.

Thus, we get insights to which process parameters affect the synthesis in beneficial ways. This yields a better understanding of the materials themselves. Raman spectroscopy also allows us to probe materials' properties which are linked to the material specific Raman modes. Most Raman spectra of the investigated materials exhibit multiple modes with different excitation probabilities. These can depend on the laser polarization and orientation of the sample with respect to the laser. The energies and relative intensities of the characteristic modes of some TMDs vary substantially with increasing crystal layers, starting at monolayer [1, 2].

Further processing steps such as doping or functionalization can also be evaluated by Raman spectroscopy. For example, monolayers of organic molecules can be detected. At UniBW M we characterize thin layers of perylene, which stick preferably to MoS_2 and graphene rather than to SiO_2 [3]. Here we have obtained new insights into the formation of self-assembled monolayers of perylenes, which promotes the application of these materials as sensor materials.

Which devices or technologies do you use in your laboratory? Which methods can be combined well with Raman microscopy?
Our laboratories offer a wide spectrum of analytical devices and methods which we need to characterize and optimize our processes for material synthesis. Probing the optical



Non-covalent functionalization of layered 2D materials is an essential tool to modify and fully harness their optical, electrical, and chemical properties. Illustrated is the Raman characterization of selectively self-assembled monolayers (SAMs) of perylene bisimide (PBI) on MoS_2. (a) Chemical structure of PBI derivative, (b) average Raman spectrum of the PBI deposited on as-grown CVD MoS_2 on SiO_2, with peak assignment in the red regions, (c) corresponding Raman peak intensity maps of PBI (core) and MoS_2 (A_{1g} mode) regions [4].

response of the materials using photoluminescence, absorption and reflection measurements combines well with Raman since the same microscope and light sources can be used. With these optical methods the lateral resolution is confined by the diffraction limit, so the investigation of structural properties down to the nm-range is often performed by AFM-scans. This provides a complementary measurement. With our Atomic Force Microscope (AFM) we also have successfully used the available AFM-IR option to highlight the assembly of the perylene layers [3]. Structural analysis is also done by using SEM scans.

In addition, we have the possibility to do a chemical analysis by X-Ray Photoelectron Spectroscopy (XPS) to understand the crystal composition and examine the samples for impurities. We also have the option to assess the abundance of elements and compounds with ToF-SIMS providing sensitivity down to ppb-concentration levels.

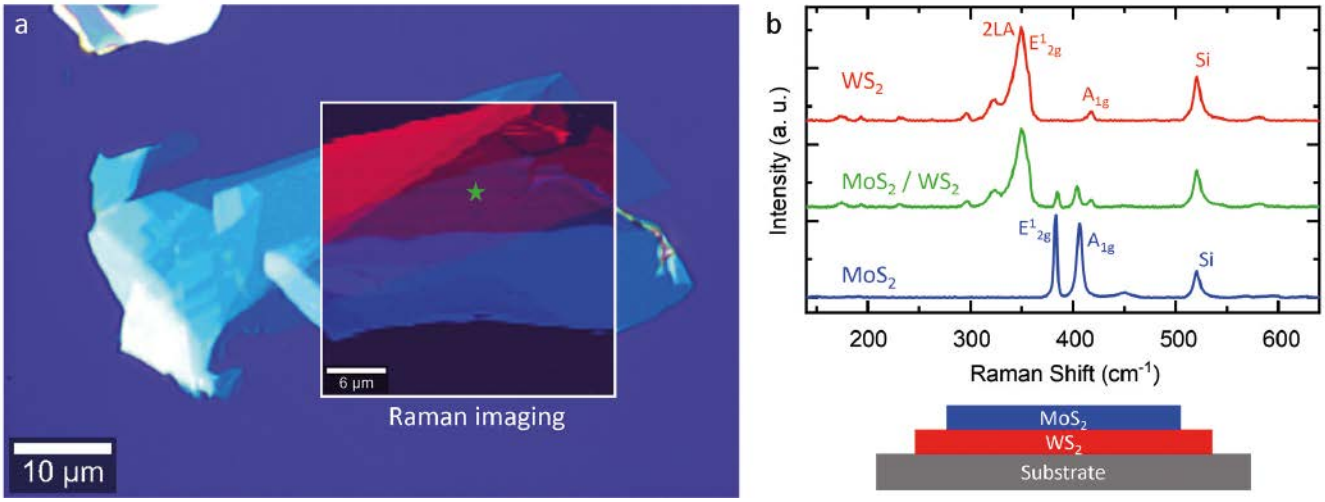
In your opinion, what are the advantages of using Raman microscopy?
As an optical analytical method Raman spectroscopy is fast and contactless. As such it is the method of choice to quickly become familiar with our samples after the processes.

It is also non-destructive, scalable and doesn't require complex sample preparation or vacuum atmosphere. Raman spectroscopy allows definition of metrics to reliably characterize 2D materials with high throughput. This has the advantage of evaluating large batches of samples, which can be compared to reference materials.

Through our research we learned that based on the observed Raman modes other material properties such as film thickness or strain can also be derived and, thus, quantified. With a state-of-the-art Raman microscope other optical phenomena such as photoluminescence can also be probed all on wafer scale.

With its relatively straightforward operation principle, Raman spectroscopy also can be taught to students or employees in a reasonable amount of time.

Which scientific questions would you like to investigate with the help of Raman microscopy in the future?
Our research focuses on new low dimensional materials for electronic devices. We therefore investigate the synthesis and processing of numerous novel types of 2D materials and heterostructures. Some of those are not yet or just sparsely analyzed by Raman spectroscopy and their exciting optical properties are not known yet. Here, the experimental verifica-



The ability to stack single crystal layers of different 2D materials to van der Waals heterostructures allows formation of composite materials which do not occur naturally and offer unique properties. (a) Optical microscope image of a stacked MoS_2/WS_2 heterostructure with Raman imaging data as overlay. The Raman data shows the spatial distribution of the integrated 2LA+E^1_{2g} mode intensity of WS_2 (red) and the integrated E^1_{2g} mode intensity of MoS_2 (blue). (b) Raman spectra of monolayer WS_2 (red), monolayer MoS_2 (blue) and the heterostructure (green). The Raman spectrum of the heterostructure was obtained at the position marked by the green star where the monolayers of both materials overlap, as shown by the scheme on the right [2].

tion of theoretically predicted properties are of major interest. Also discovering and describing potential links of the Raman modes to other material properties is pursued. This would allow additional potentially more complex analytical methods to be bypassed.

Collaborations with industry and other partners show the demand to create and define benchmarks and figures of merit to compare fabricated devices and sensors. Reliable quality assessment of the plethora of materials and devices synthesized in labs is a major goal. The use of Raman spectroscopy will help gain insights into functionalization, doping and tailoring 2D materials and heterostructures.

Which work in the field of Raman microscopy has particularly impressed you?

As pointed out we are interested in creating and using defined metrics which can be measured by Raman to assess quality and other properties of 2D materials. In particular the work of Aline Amorim Graf, University of Sussex, titled: "Raman metrics for molybdenum disulfide and graphene enable statistical mapping of nanosheet populations" [4] is thoroughly put together. She and colleagues have developed new metrics correlating size and thickness of both liquid- and mechanically-exfoliated MoS_2 nanosheets. Profound studies like this facilitate the standardization of characterization of 2D materials, further helping research and industry to advance in these fields.

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Georg S. Duesberg graduated in Physical Chemistry from the University of Kassel, Germany in 1996. He gained his PhD at Max-Planck-Institute for Solid State Research, Stuttgart from 1997 – 2001. From 2001 – 2005 he worked at the Infineon AG, in the Corporate Research Department in Munich, followed by two years in the Thin Films Department at Qimonda AG, Dresden. In 2007 Georg Duesberg became Assoc. Prof. in the School of Chemistry of Trinity College Dublin, Ireland and a Principal Investigator at the National Research Institute CRANN. In 2017 Prof. Duesberg took on the Chair for Sensortechnologies at the Universität der Bundeswehr Munich, Germany. Georg Duesberg has co-authored more than 250 publications with more than 35000 citations (H-index 82, Google) and has filed more than 25 patents. His research focuses on the synthesis, characterization and devices fabrication of low-dimensional structures.

Oliver Hartwig started his study of Physics in 2011 at the Karlsruhe Institute of Technology. He obtained his undergraduate degree in 2015. In the same year he moved to Munich to continue his degree in physics at the Technical University of Munich. In 2018 he graduated with a master thesis titled: Towards on-Chip Photon Generation and Detection Using Two-Dimensional Materials Coupled to Plasmonic Waveguides. With his research interest already focusing on materials research he then started his PhD at the Universität der Bundeswehr Munich with the Group of Prof. Georg S. Duesberg. Here, Oliver focuses on the synthesis and characterization of 2D materials.

Find, Classify and Identify Microparticles with Raman Imaging

Comprehensive Tools for Automated Analysis

Dieter Fischer[1], Franziska Fischer[1], Josef Brandt[1], Lars Bittrich[1], Klaus-Jochen Eichhorn[1], Harald Fischer[2], Olaf Hollricher[2], Karin Hollricher[2], Miriam Böhmler[2]

High-resolution measurements of particles are of great interest in many fields of application. Combining confocal Raman microscopy with particle analysis tools makes it possible to find, classify and identify particles almost entirely automatically.

Pollen, dust, flour, metal flakes and pigments in paints, titanium dioxide in sunscreen and toothpaste, fat crystals in food emulsions – these and many more substances in our daily lives contain or consist of microparticles. Recently, the public and scientific community have directed their attention toward microplastic particles in the environment [1, 2].

The definition of a microparticle is not universally agreed upon. According to the International Union of Pure and Applied Chemistry, microparticles are smaller than 100 micrometers and larger than 0.1 micrometer [3]. Other definitions range from 1 micrometer to 5 mm in size [4]. Confocal Raman microscopy is ideally suited to finding, classifying and identifying microparticles because not only does it yield images with a resolution down to 300 nm, but with Raman vibrational spectroscopy the chemical components of a sample can be identified [5]. It is a nondestructive method that requires little, if any, sample preparation. A Raman microscope can generate high-resolution images that show both the structural features and distribution of molecules within a sample. However, Raman spectroscopic imaging is not yet widely applied to microparticle analysis.

The challenge in Raman microparticle analysis lies in automating the detection of individual particles and classifying those of interest by size or shape before determining their chemical compositions [6, 7]. For such analyses, a comprehensive and integrated software solution is essential [8, 9, 10]. Here, using the examples of microplastics in an environmental sample and microparticles in a cosmetic peeling cream, we present the application of the open source program GEPARD [8] and the commercial solution ParticleScout [9].

Microplastics in Environmental Samples

Increasing plastic manufacturing, low recycling rates, the degradation of plastic during the utilization phase and the degradation of plastic waste inevitably produces millions of tons of microplastic particles (MP) every year that find their way into rivers, the sea, sediments and air. MP originating from packaging and the food web are already found in food and drink. To assess possible effects of MP on the health of humans and animals, it is necessary to quickly and reliably monitor their abundance, size, size distribution and chemical composition. This can be done by a combination of optical particle recognition and Raman microscopy. The complex and difficult problem of determining these data from real environmental samples is shown with a sludge sample from a wastewater treatment plant. 50 grams of the sludge sample were pretreated and purified and then filtered in a two-stage fil-

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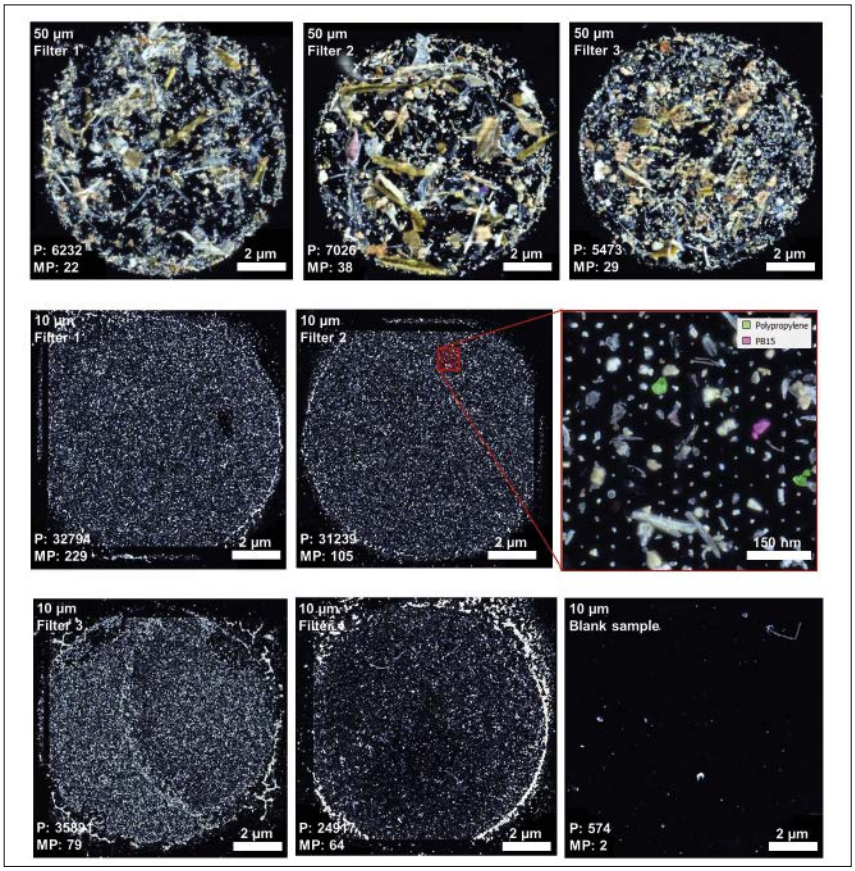

Fig. 1: Particles from a wastewater treatment plant sludge sample (50g) distributed on 7 filters and a blank sample filter (Explanation and results in the text).

tration process using 7 silicon filters: 3 filters with 50 µm and 4 filters with 10 µm pore size (fig.1) [10]. An optical particle fragmentation was done with the GEPARD program [8]. We identified 143572 particles and fibers from all 7 filters. A blank sample from our laboratory is also included in figure 1.

After this optical localization of the particles on all filters, Raman analysis was performed with an alpha300 R microscope (WITec GmbH) and their chemical composition identified using the TrueMatch Raman database management software (WITec GmbH). 566 MP particles (118 from these are dyes) were identified, of which 371 were definitely from the sludge sample. All others (parafilm, PV23, PTFE) could have resulted from sample preparation and were therefore discarded. The extension of the 10 µm filter 2 in figure 1 shows a detailed view of the result, which displays the MP particles in color. The distribution by polymer type and particle size is shown in figure 2. The majority of the MP particles were between 10 and 100 micrometers in size. The bulk plastics PP, PE and PET and some dyes are most frequently represented as MP particles.

Particles in Cosmetics

The ParticleScout software (WITec GmbH) [9] enables highly automated, quick and straightforward analysis of microparticles on the basis of a confocal white light image of the sample and Raman microscopy. Localization, classification and identification of its chemical compounds are easily carried out with this analysis tool, as demonstrated here with a cosmetic peeling cream sample. For imaging, an alpha300 R microscope equipped with ParticleScout was used (fig. 3). First a survey of a large area was generated by the stitching together of optical bright field images by an automated routine. Focus stacking yielded more sharply defined particle outlines. 3941 particles were located and categorized according to their physical shape and size using Boolean filters. As conventional Raman imaging of large areas would also include much of the empty space surrounding the sparsely distributed particles, the software automatically records spectra of identified particles only, thus greatly accelerating the workflow of the measurement.

Using the seamlessly-integrated TrueMatch software, the Raman data were processed and the components could be identified by referencing the Raman database information. The chemical analysis revealed anatase (a mineral form of titanium dioxide) and boron nitride particles in an oil matrix (Raman spectra in fig. 3B). Titanium dioxide in cosmetics is currently under debate in the European Union with regard to its toxic effects [12]. Further evaluation of the results determined the quantitative prevalence of the molecular sample components in the particles (fig. 3C) and also the distribution of chemical compounds correlated to particle size (fig. 3D). In extended analyses, particles could also be linked to parameters such as area, perimeter, bounding box, Feret diameter, aspect ratio, equivalent diameter, spherical equivalent volume and others. As particle classification, image processing and analysis of Raman

spectra are executed within one platform, ParticleScout offers an effective solution for automated, comprehensive investigations of particles.

Conclusion

The presented measurements of microparticles carried out using a confocal Raman microscope were largely automated and illustrate the potential of the featured analysis tools for comprehensive investigations in many fields of application. The particle fragmentation program and particle analysis software integrated with the Raman database management component have the speed and sensitivity necessary for both high sample throughput and precise particle characterization.

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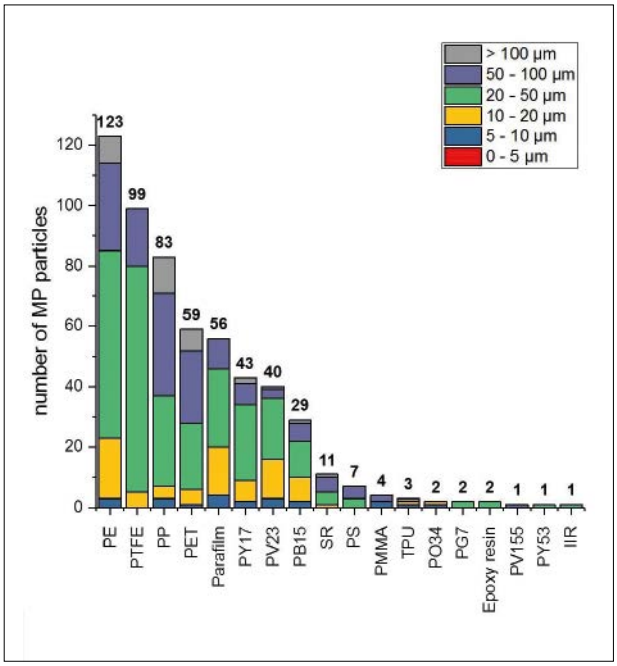

Fig. 2: Size and polymer types of MP particles in a sludge sample from a wastewater treatment plant

PE	Polyethylene
PTFE	Poly (tetra-fluoroethylene)
PP	Polypropylene
PET	Poly (ethylene terephtalate)
Parafilm	Parafilm
PY17	Pigment Yellow 17-based
PV23	Pigment Violet 23
PB15	Pigment Blue 15
SR	Silicone rubber
PS	Polystyrene
PMMA	Poly(methyl-methacrylate)
TPU	Thermoplastic polyurethane
PO34	Pigment Orange 34
PG7	Pigment Green 7
Epoxy resin	Epoxy resin
PV155	Pigment Yellow 155
PY53	Pigement Yellow 53
IIR	Isobutylene isoprene rubber

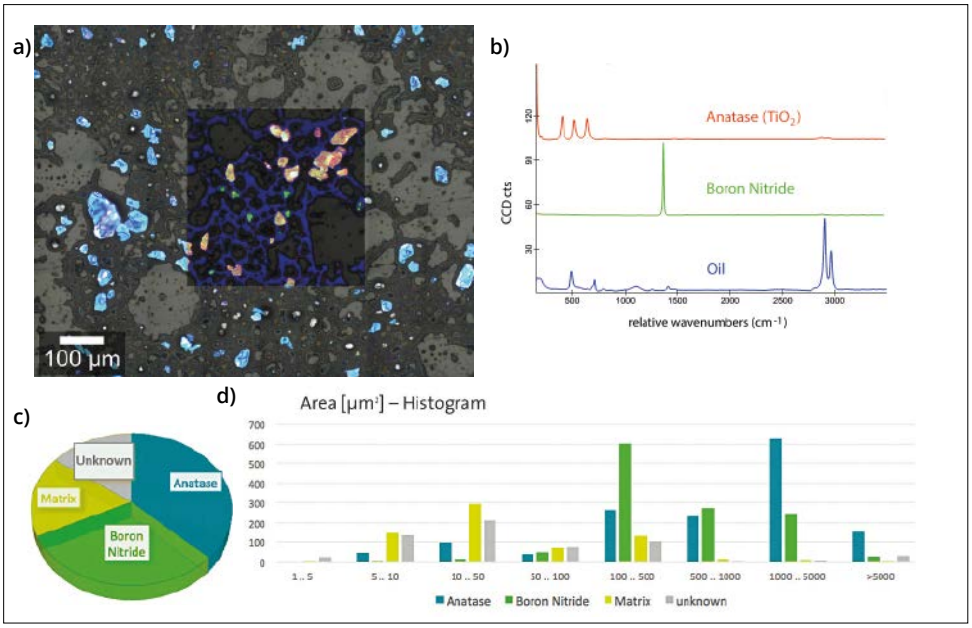

Fig. 3: Particles in a cosmetic peeling cream. A: Optical bright field image overlaid with the confocal Raman image. B: Corresponding Raman spectra of the molecular components in the sample. C: Pie chart of the quantitative compound distribution in the sample. D: Graphical representation of the correlation between chemical characteristics and particle size.

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Correlating Raman Microscopy with Different Techniques to Analyze Nanoplastic Particles

Dr. Harald Fitzek is working as a senior scientist in the group of Hartmuth Schröttner at the FELMI-ZFE-Graz consisting of the Institute of Electron Microscopy and Nanoanalysis (FELMI) at the Graz University of Technology, Austria and the Graz Centre for Electron Microscopy (ZFE). From a methodical point of view the main focus of the research group is the correlation of Raman microscopy with scanning electron microscopy and energy dispersive x-ray spectroscopy (SEM-EDS). At this point the group have moved past the experimental challenges of doing both techniques on the same sample and are looking at the application of machine learning tools to get as much information as possible out of a combined dataset of light microscope images, SEM images, a Raman map and an EDS map. In addition, the correlation of several techniques, with different contrast mechanisms and interaction volumes, into a single dataset poses surprising fundamental problems about which technique is "the most correct", which they are trying to address.

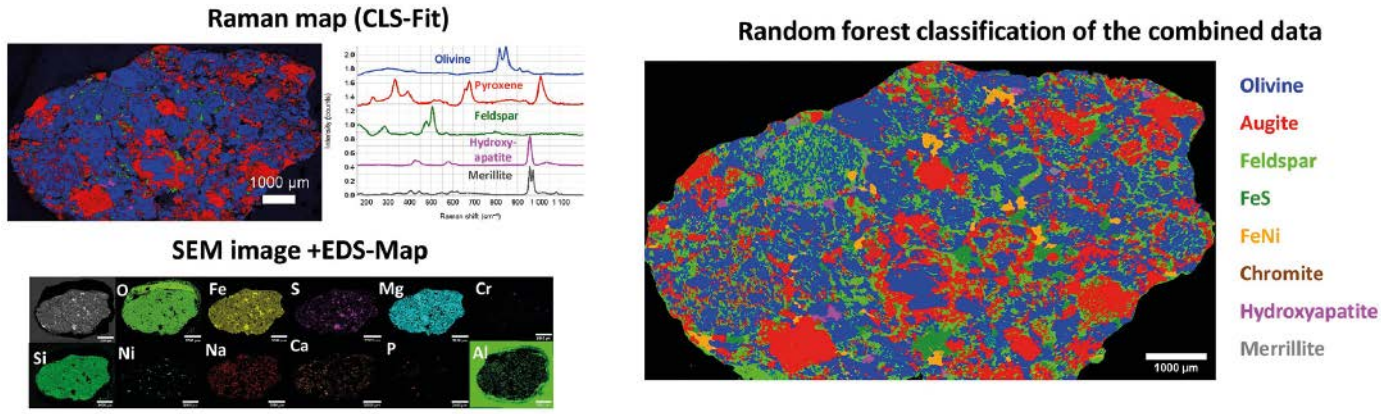
From an application point of view the most interesting question they are looking at currently is the detection of micro- and especially nanoplastics. Raman microscopy is one of the main microscopic techniques to detect microplastics at the moment, but for nanoplastics, Raman microscopy struggles as particle sizes go well below the resolution limit of light microscopy. Here the group is trying to push the limits of Raman microscopy by applying their novel correlation methods.

Which scientific questions have you been able to answer in the past using Raman microscopy?
That is a difficult question to answer concisely, let me give you two examples involving different aspects of our work.

Perhaps the most interesting example from an application point of view is when we helped to explain the details of the energy storage mechanism in iodine/carbon based batteries and super capacitors [1]. We designed an in situ experiment that allowed us to monitor both the carbon electrode and the polyiodides deposition during electrochemical cycling. Combined with complimentary results from our colleagues (especially in situ SAXS), this allowed us to give a detailed account of the interaction of carbon and iodide during energy storage. The main challenge in this example was both designing the experiment and understanding minute changes in the Raman spectra of carbon correctly.

The most interesting example from a methodical point of view is when we did a whole sample mapping with both Raman and SEM-EDS of a piece of the Chelyabinsk meteorite [2]. This gave us the opportunity to both analyze a unique sample and develop a protocol for correlative whole sample measurements. We managed to give a uniquely detailed analysis of the Chelyabinsk meteorite fragment. The main challenge in this example was to optimize the measurement procedure and data treatment, in order to get a unified interpretation out of several separated datasets.

Which devices or technologies do you use in your laboratory? Which methods can be combined well with Raman microscopy?
In addition to a standalone Raman system we also use a RISE (WITec) combined with a Sigma 300 SEM (Zeiss)/X-Max 80 EDS (Oxford) as a correlative SEM-EDS-Raman system. In addition, we have other SEMs and TEMs, as well as AFM, XRD,



Correlative whole sample SEM-EDS-Raman mapping of a fragment of the Chelyabinsk Meteorite. *On the left-top:* Raman mapping of the sample evaluated using a CLS-fit. *On the left-bottom:* SEM (BSE) image and EDS mapping (evaluated as at-%) of the same sample. *On the right:* The measurements on the left are combined into a single data set and simultaneously evaluated using a random forest classification, in order to get a classification of all phases that is not possible with only Raman or SEM-EDS. This figure is a composition of figures from [2] under CC BY 4.0 license.

infinite focus microscopy and IR microscopy. Another key to high-quality Raman microscopy is sample preparation, where we use a variety of techniques such as grinding/polishing, microtomy and Broad Ion Beam cutting.

The best method to combine with Raman microscopy of those is probably SEM-EDS, because (low vacuum) SEM is even more versatile in terms of sample material/preparation than Raman microscopy. In addition, in terms of analytical potential EDS and Raman nicely cover each other's blind spots: organics (EDS) and metals (Raman). Both XRD and IR can be a helpful addition to Raman mappings in cases where the interpretation of the Raman spectra is not straightforward.

In your opinion, what are the advantages of using Raman microscopy?

From my point of view, as somebody who also does a lot of measurements for academic or industry partners, the big advantage of Raman microscopy is versatility. Raman microscopy is versatile in terms of the materials that can be measured (pretty much anything except pure metals/metal alloys). It is highly versatile in terms of sample preparation. You can measure point spectra on basically anything you can fit into the microscope without preparation, and mappings can be generated from anything that can be prepared with a reasonably flat surface. It is also highly versatile in terms of sample size/lateral resolution range. We have measured oversampled mappings of small features with a 200–300 nm step size and whole sample mappings of cm-large samples with an appropriately coarse step size. Finally, Raman microscopy is a highly versatile tool for in situ measurements, since you can easily measure through glass or water. We once had a visiting PhD student who, for a quick and dirty proof-of-principle measurement, designed a workable in situ electrochemistry setup in one day out of everyday lab equipment.

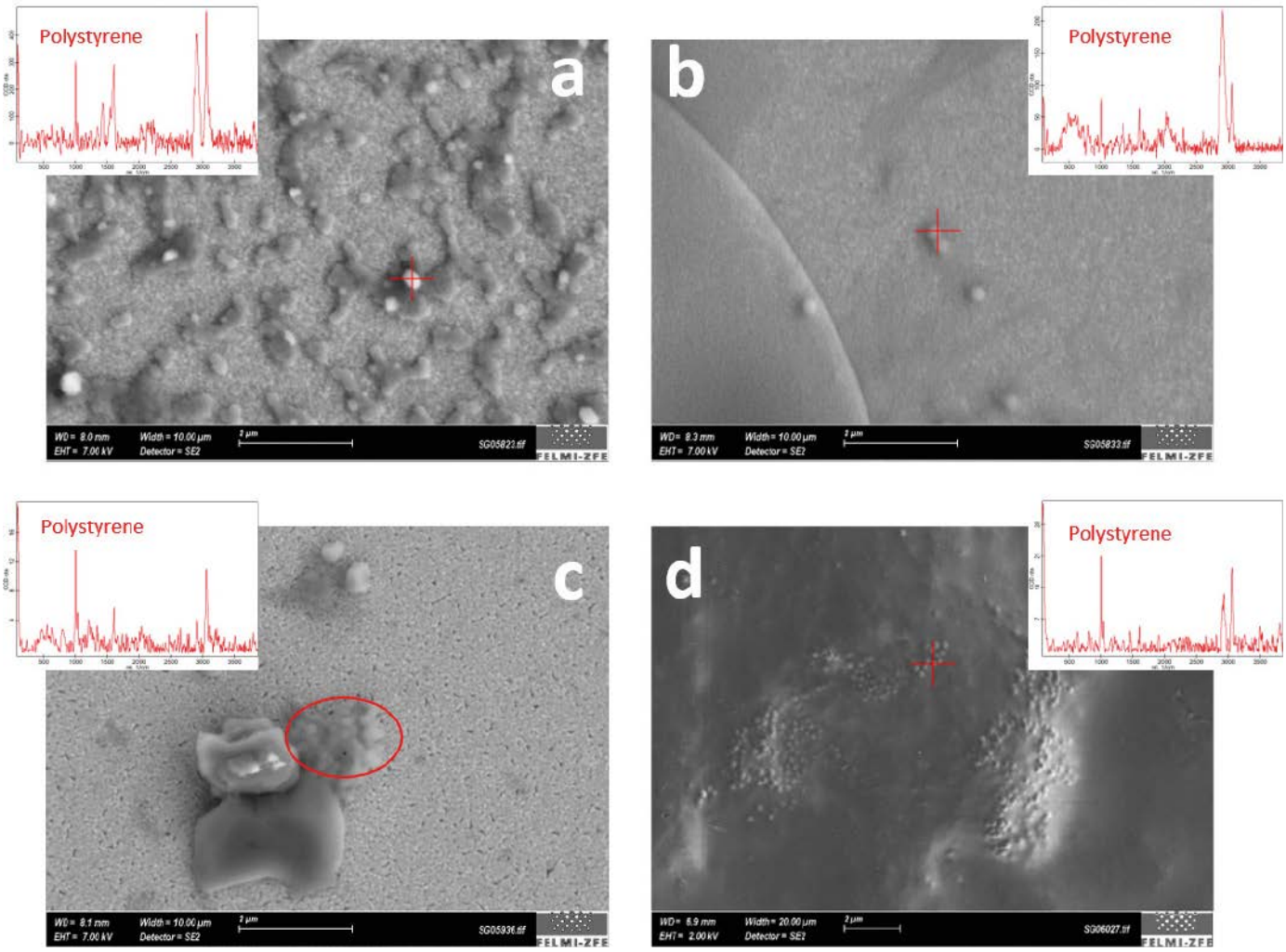
Which scientific questions would you like to investigate with the help of Raman microscopy in the future?

The most immediate question we hope to do more work on in the coming years is the detection of nanoplastic particles. We have already shown that we can detect particles in the 100 nm range using a combination of SEM, to find the particles, and Raman with long integration times, to analyze the composition, under ideal circumstances [3]. We now want to improve the sample preparation/pretreatment so we can get a limit of detection similar to the ideal pure water sample in most liquids. This would make this approach a remarkable addition to the analytical techniques currently available for micro- and nanoplastics. In addition, we hope to include the detection of even smaller particles in the future by adding either correlative TERS or AFM-IR.

Another interesting question we would love to work on is to use image segmentation of, for instance, the light microscope image, though any image correlative to the Raman mapping would work, in order to optimize the mapping pattern of the Raman mapping. This could potentially greatly speed up the Raman mapping. Note that the mapping speed often is a major weakness of Raman microscopy. In addition it might be possible to, though admittedly somewhat artificially, improve upon the resolution/quality of the Raman mapping.

Which research work in the field of Raman microscopy impressed you in recent years?

Given my obsession with the versatility of Raman microscopy, I am eagerly following the recent developments in time-gated Raman spectroscopy [4]. Fluorescence is the biggest problem, if you are applying Raman microscopy to a large variety of samples. Unfortunately, since we do not have a time-gated Raman I cannot give you an estimation from experience how much difference it would make in practice, but I think it would often improve the quality or reduce the effort of the measurement.



Correlative SEM-Raman measurements of polystyrene beads (200 nm diameter) in various liquids. The beads were mixed in at different concentrations to test the limit of detection. A droplet of each liquid was applied to an Au-coated glass slide and evaporated. a) distilled water (bead concentration $2 \cdot 10^4$ µg/L; high concentration test sample), b) distilled water (bead concentration $2 \cdot 10^{-3}$ µg/L; limit of detection), c) Fleur de Sel dissolved in distilled water (bead concentration 20 µg/L; limit of detection), d) amniotic fluid (bead concentration 200 µg/L; limit of detection). This figure is reproduced under CC BY 4.0 license from [3]

In addition, I find the recent progress in the application and development of TERS [5] highly interesting. TERS has been a very specialized technique until recently and is currently transitioning, or perhaps has already finished the transition, into a widespread and promising method.

Finally, I would like to mention the instrumental developments in Optical Photothermal Infrared microscopy [6], which is trying to bring the sampling advantages of Raman microscopy to IR microscopy, whilst also allowing for perfectly correlated Raman and IR mappings.

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Harald Fitzek started his venture into the world of Raman microscopy in 2014 with his PhD thesis on "Surface Enhanced Raman Spectroscopy" at the Institute of Electron Microscopy and Nanoanalysis (FELMI) at the Graz University of Technology. This thesis set the tone for his future work, with the combination of experimental and theoretical/computer methods being central. After finishing his PhD thesis in 2018, he became a senior scientist at the Graz Centre for Electron microscopy (ZFE). There he has the main responsibility for Raman microscopy with a focus both on fundamental research and applied cooperation with industry.

Switching to a New Perspective

Expanding Analysis Options through Inverted Confocal Raman Imaging

Wolfram Ibach[1], Andrea Richter[1], David Steinmetz[1], Karin Hollricher[1], Damon Strom[1], Harald Fischer[1], Sonja Breuninger[1]

Introduction

Confocal Raman imaging is a well-established technique for investigating the chemical and molecular compositions of solid and liquid samples. Its varied fields of application in academia and industry range from materials and surface sciences to environmental and geo sciences to life sciences and pharmaceutics. The array of measured sample characteristics requires versatile and adaptive analyzing capabilities. For confocal Raman microscopy, a conventional upright microscope geometry is usually used, where the microscope stage is located beneath the objective and the sample is observed from above. However, some samples are not suitable for examination with an upright microscope as they require a specific environment, such as biological cells in culture, or due to large sample dimensions. In these cases, an inverted microscope setup in which the microscope stage is located above the objective and the sample is observed from below is preferable for confocal Raman imaging. Thus inverted confocal Raman imaging opens up new opportunities for the investigation of aqueous or bulky samples. In the following, we will introduce the inverted confocal Raman imaging technique and provide some application examples.

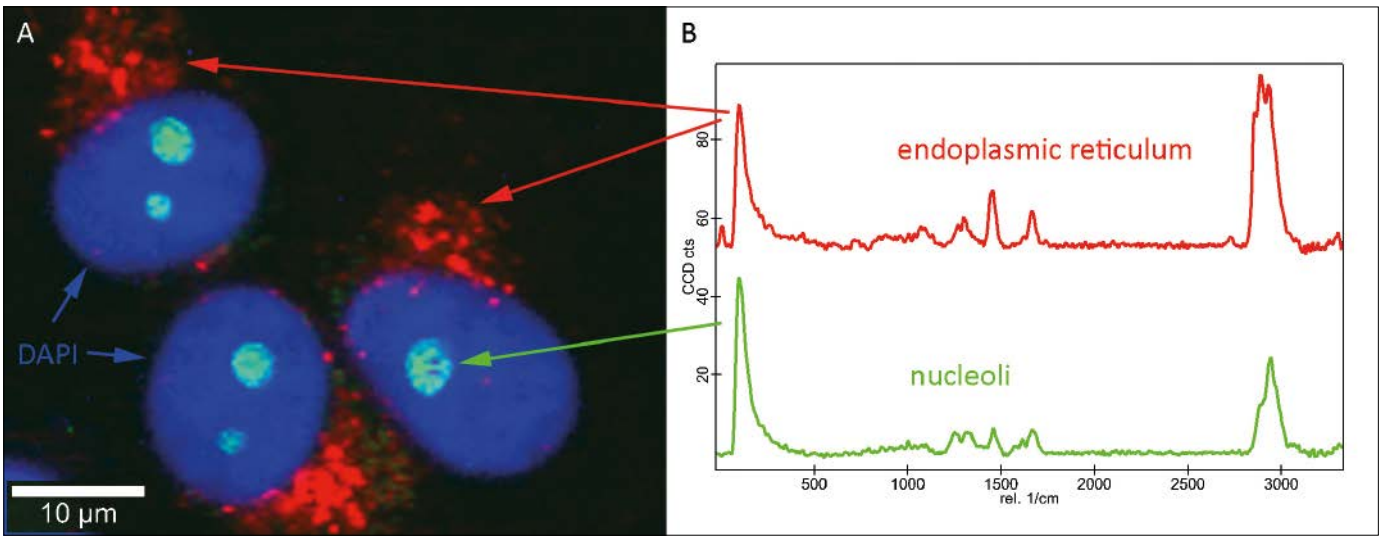

Fig. 1: (A) Correlative fluorescence and confocal Raman image of adherent eukaryotic cells in aqueous solution. Scan range: 50 x 40 µm², 150 x 120 pixels; integration time: 0.2 s/spectrum. The correlative image shows the nucleus (blue) imaged by fluorescence microscopy and the nucleoli (green) and endoplasmic reticulum (red) imaged by confocal Raman microscopy. (B) Corresponding Raman spectra of the nucleoli (green) and the endoplasmic reticulum (red).

Methods

Confocal Raman Microscopy

The Raman effect is based on light interacting with the chemical bonds of a sample. Due to vibrations in the chemical bonds this interaction causes a specific energy shift in the backscattered light which appears in a unique Raman spectrum that can be detected by spectroscopic methods. Hereby the chemical and molecular compounds can be identified and additional sample characteristics such as the relative quantity of a specific component, stress and strain states, crystallinity or polymorphic structures can be further analyzed. The confocal Raman imaging technique combines Raman spectroscopy with a confocal microscope. Thus the spatial distribution of the compounds within a sample can be visualized. High-resolution confocal Raman microscopes acquire the information of a complete Raman spectrum at every image pixel and achieve a lateral resolution at the diffraction limit (approx. $\lambda/2$ of the excitation wavelength). A confocal microscope setup is furthermore characterized by an excellent depth resolution and enables 3D Raman images and depth profiles to be acquired. The Raman technique is non-destructive, non-invasive, and contact- and label-free [1, 2].

Inverted Confocal Raman Imaging

In inverted confocal Raman microscopy, samples are viewed and investigated from below. This provides the great advantage that most samples can be investigated without conflicting with elements of the microscope setup such as the microscope turret. Large, bulky samples can be accommodated by placing them on the stage of the inverted microscope without specialized sample preparation. Liquid samples can be placed on the fixed plane of the stage for quick and repeatable measurements. The sample stage also facilitates the mounting of e.g. environmental enclosures. As with conventional confocal Raman microscopy, combinations with other techniques such as fluorescence, DIC and phase-contrast are also possible with the inverted microscope.

Application Examples

Correlative Fluorescence and Raman Imaging of Eukaryotic Cells

Fluorescence microscopy is a standard method for the investigation of cells and tissues in life sciences. In combination with Raman imaging, its analytical utility can be greatly extended [3, 4, 5]. By choosing a suitable laser wavelength, signal overlap between the fluorescence and Raman signals can be successfully avoided. Inverted confocal Raman imaging also enables the investigation of living cells in controlled environmental conditions and aqueous solutions. For the correlative fluorescence-Raman application example, a WITec alpha300 R*i* inverted confocal Raman imaging microscope was used. Eukaryotic primate kidney cells (Vero ATCC CCL-81)

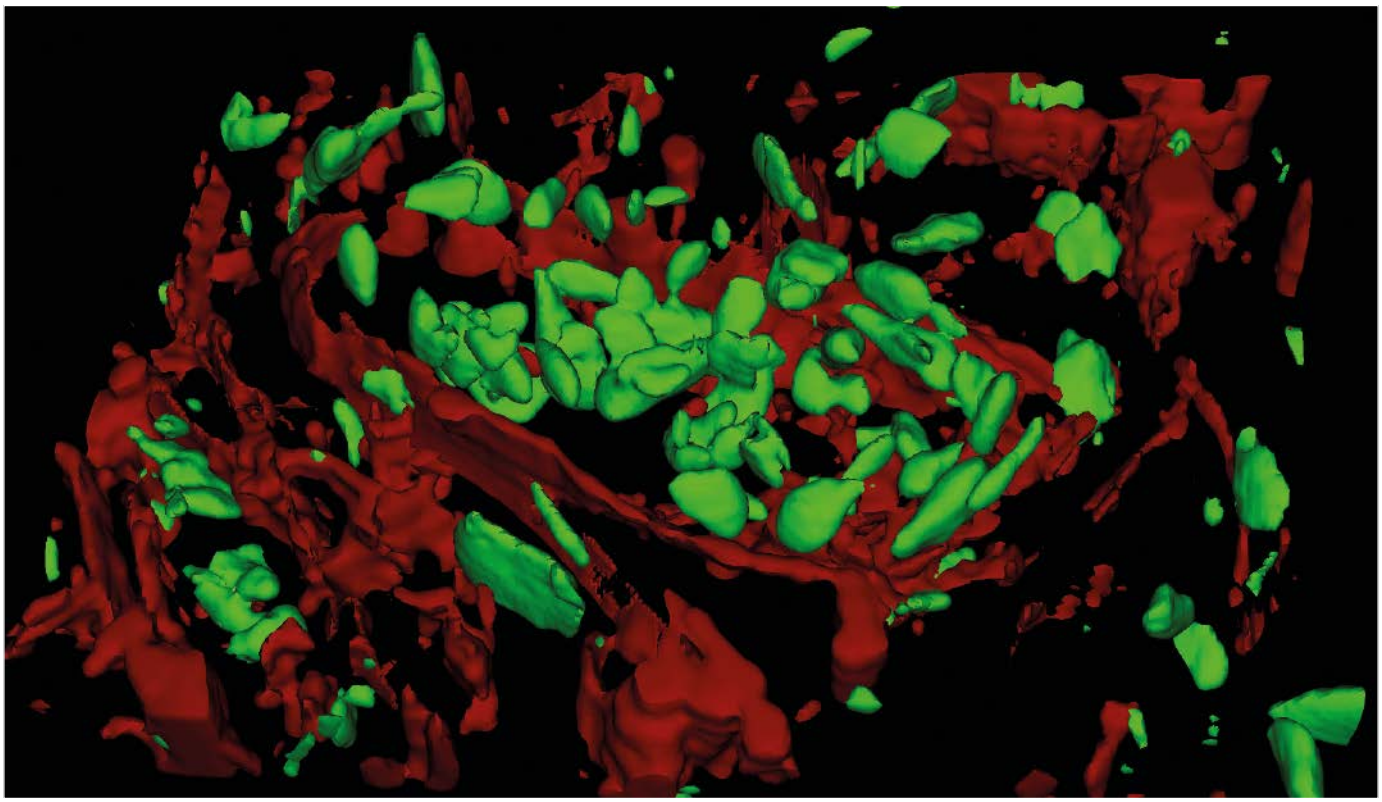

Fig. 2: 3D confocal Raman image reconstruction of banana pulp. Shown are starch grains (green) and cell wall components (red). Scan range: 300 x 200 x 90 µm³, 450 x 300 x 45 pixels = 6,075,000 Raman spectra; Integration time: 34 ms/spectrum.

were grown in a petri dish. The adherent cells were stained with DAPI to mark the nuclei and examined in aqueous solution under the microscope. For inverted confocal Raman imaging a laser wavelength of 532 nm was used and an image of 50 x 40 µm² with 150 x 120 pixels was generated. At each image pixel a complete Raman spectrum was acquired. The acquisition time was 0.2 s/spectrum. The resulting correlative Raman-fluorescence image shows the nuclei (blue) imaged by fluorescence microscopy and the nucleoli (green) and endoplasmic reticulum (red) imaged by confocal Raman microscopy (fig. 1). Please note that the fluorescence dye does not interfere with the outcome of the Raman results.

3D Confocal Raman Imaging of Plant Pulp

A confocal microscope setup delivers an excellent depth resolution that enables the acquisition of information from below the surface. Hence depth profiles and 3D Raman images can be generated. 3D Raman images are a valuable tool in providing information about the dimensions of objects or the distribution of a certain compound throughout the sample [6, 7]. In order to generate 3D images, confocal 2D Raman images from different focal planes are acquired by automatically scanning throughout the sample in the z-direction. The 2D images are then combined into a 3D image stack. 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 reconstruction of the Raman image stack. The starch grains are displayed in green while the cell wall components are shown in red (fig. 2).

Conclusion

In the article the versatility of inverted confocal Raman imaging was shown by application examples from the fields of food and life sciences. Through correlative microscopy techniques, such as fluorescence microscopy, the analyzing potential of the inverted confocal Raman microscope can be further enhanced. Additionally, imaging techniques such as depth profiles and 3D volume scans can provide information about the distribution of the chemical information throughout the sample. In conclusion, chemical and molecular analyses are made accessible for a broader range of applications and samples by employing inverted confocal Raman microscopy.

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Establishing a Live Monitoring System for Organ-on-Chip Using Raman Spectroscopy

Dr. Håkon Høgset is currently working with Professor Stefan Krauss at the Hybrid Technology Hub at the University of Oslo, Norway. Here the group is working to develop organ-on-a-chip technology for use in disease modelling and to study developmental processes and organogenesis.

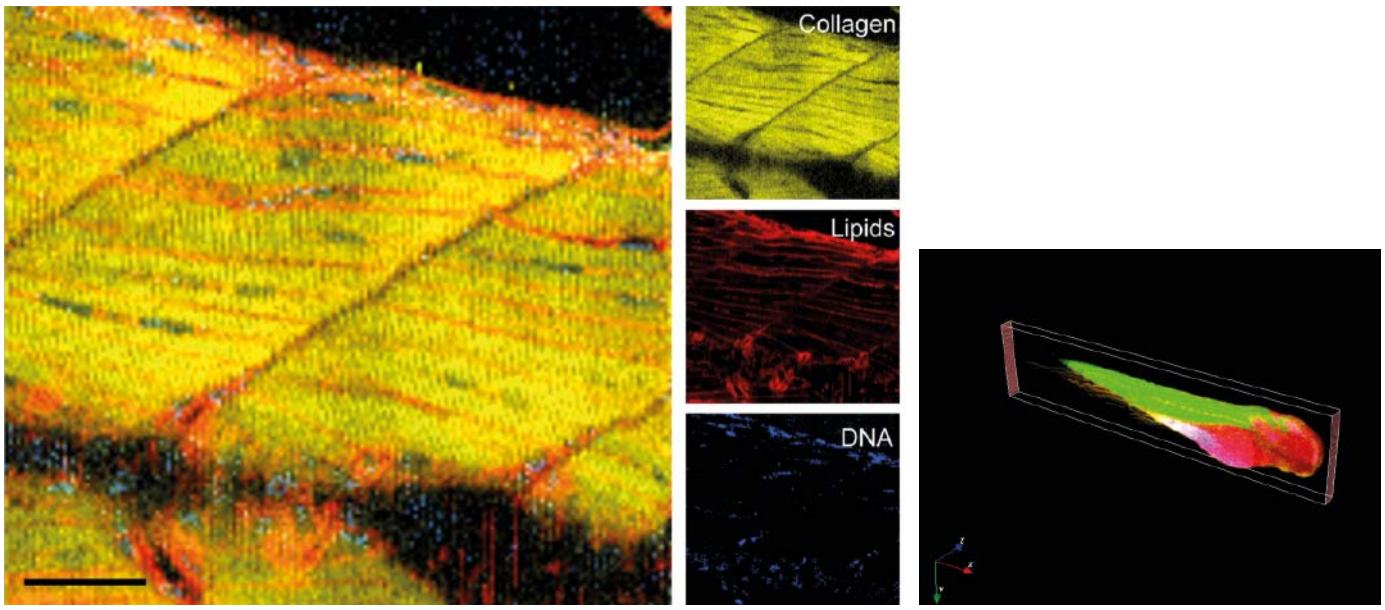
His research focuses on implementing Raman spectroscopy to monitor cellular processes (e.g. cell differentiations, toxicological effects) directly on the chips. The long-term goal is to enable a live monitoring system for organ-on-chip using Raman spectroscopy.

Which scientific questions have you been able to answer in the past using Raman microscopy?
I got interested in Raman microscopy during my PhD, which I did in Professor Molly Stevens' group at Imperial College London. Before that, I was working on disease modelling in zebrafish. Learning that Raman spectroscopy could be used to derive comprehensive metabolic profiles from cells, I wanted to see whether Raman microscopy could be used to probe the metabolic changes inside the zebrafish [1]. We tested this both in a live wound healing model and in a bacterial infection model.

Which devices or technologies do you use in your laboratory? Which methods can be combined well with Raman microscopy?
Currently, we are using confocal Raman microscopes and are now focused on Raman spectroscopic imaging. However, we are interested in implementing other Raman techniques in the future such as SRS or SERS. I think Raman microscopy can be combined well with many different methods depending on the question, but I think it is always a good idea to use some correlative method that also contains positional information, such as spatial transcriptomic, DESI-MS or fluorescence microscopy. Such combinations make it easier to interpret and understand the Raman spectroscopic data sets, which is not always straightforward.

In your opinion, what are the advantages of using Raman microscopy?
For me the biggest advantage of Raman microscopy is that you can get multivariate biochemical information in 3D. This is a quite exceptional feature. It is also an important advantage that Raman microscopy is label-free. Raman spectroscopy in general is also very good for deriving lipid profiles and this advantage also extends to Raman imaging and this method is therefore well suited to study processes involving lipid metabolism.

Which scientific questions would you like to investigate with the help of Raman microscopy in the future?
I am currently very interested in developing Raman microscopy for disease modelling and cell differentiation for use in organ-on-a-chip technology. One of the main applications of Raman in general has been for diagnostics and I think Raman analysis could be an important tool in advanced *in vitro* disease modelling, such as organ-on-a-chip, especially because Raman microscopy is non-destructive and can be applied to live specimen, allowing repeated measurements. These fea-



Left: Confocal Raman image of zebrafish embryo muscle tissue. Collagen-rich regions are displayed in yellow, DNA-rich regions are displayed in blue, and lipid-rich regions are displayed in red.
Right: 3D scan of whole zebrafish embryo at 3 days post fertilization. Green displays the protein content, red displays the lipid content and magenta displays the carorenoid component of the embryo.
The images were obtained using a confocal Raman microscope (alpha300R+, WITec, GmbH, Germany) with a 532 nm laser light source and ×63/1.0 NA water-immersion objective lens (W Plan-Apochromat, Zeiss, Germany)
(Image credit: [1] Høgset H. *et al.:* Nat Commun 11, 6172 (2020) doi: 10.1038/s41467-020-19827-1)

tures could be very important when using for instance patient derived samples where it may be necessary to study the effect of a treatment over time and where starting material may be scarce.

Which research work in the field of Raman microscopy has particularly impressed you in recent years?
There is a lot to choose from! I have been really intrigued by the development and applications of the Raman Carbow tags developed by Fanghao Hu *et al.* [2] in the Wei Min lab over the last few years. Two other areas of Raman microscopy I am paying close attention to is development of non-linear Raman microscopy techniques and especially SRS, and the development of machine learning approaches for Raman analysis and to improve the throughput. With regards to the latter, I see a lot of potential in using a strategy like the DeepeR that was recently published by Conor Horgan *et al.* [3] and I would really like to test that framework in the future too.

Håkon Høgset was born in Oslo, Norway. He did his BSc in Molecular Biology at the University of Oslo and his MSc in the group of Prof. Gareth Griffiths at the University of Oslo. After his PhD in the research group of Prof. Molly Stevens at the Imperial College London, he is now a Marie Curie Fellow at the University of Oslo at the Hybrid Technology Hub supervised by Prof. Stefan Krauss. His research interests are Raman spectroscopic imaging, cell biology, developmental biology, zebrafish, stem cells, regenerative medicine.

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Looking into Batteries with Raman

Correlative RISE Microscopy Studies of Li-Ion Cells

Karin Hollricher[1], Damon Strom[1], Ute Schmidt[1]

Understanding structure-composition-property-performance relationships is fundamental in developing more powerful, long-lived and affordable Li-ion batteries. RISE microscopy is an extremely useful technology for investigating these features.

Ever since Alessandro Volta invented the voltaic pile, the first electric battery, research on generating electricity from chemical reactions has continued and led to the development of many energy storage designs, culminating in lithium-ion batteries (LIBs) [1]. Significant improvements to LIBs resulted from the introduction of new cathode materials and the replacement of liquid electrolytes by solid materials. Anodes usually consist of graphite and amorphous carbon. Cathode materials used in commercial LIBs include $LiCoO_2$ (LCO), $LiMn_2O_4$ (LMO), $LiNi_{0.84}Co_{0.12}Al_{0.04}O_2$ (NCA), $LiNi_xCo_{1-x-y}Mn_yO_2$ (NCM/NMC), and $LiFePO_4$ (LFP). Cobalt-free batteries such as spinel-structured $LiNi_{0.5}Mn_{1.5}O_4$ (LNMO) cells recently became a focus of research as they avoid requiring this expensive element [2].

Non-destructive Raman imaging microscopy can visualize structural and chemical information acquired from the battery's internal components such as their molecular composition, grain fractures, the formation of solid electrolyte interphase (SEI) layers and degradation processes at the electrodes [3, 4].

Here we document changes of new and used electrodes with correlative Raman imaging and scanning electron (RISE) microscopy. High-resolution scanning electron microscopy (SEM) enables the detailed analysis of the electrodes' ultrastructure and energy-dispersive X-ray spectroscopy (EDX) detects most of their incorporated elements. Lithium itself evades EDX detection because it is too light. However, Li-containing molecules are identifiable by their Raman spectra, which can reveal changes in their localization and concentration. Raman spectroscopic imaging can also differentiate polymorphic variations of molecules such as amorphous carbon and graphite, which EDX is not able to do.

All high-resolution Raman measurements shown here were performed using a WITec alpha300 confocal Raman microscope integrated with an SEM system to enable the quick and easy correlation of ultrastructural and chemical properties of the sample. An alpha300 microscope can also operate as a stand-alone, remotely controlled instrument that provides the opportunity to carry out the entire process of delicate sample preparation and Raman imaging within the controlled gaseous environment of a glove box.

We examined two type 18650 Li-ion batteries, one in its initial condition, while the other cell had been cycled over 480 times, resulting in state of health of approximately 64%. Cross sections were prepared under an argon atmosphere in a glove box. For RISE microscopy the WITec Raman system was attached to a Zeiss scanning electron microscope equipped with an EDX detector. The SEM-EDX measurement of the new battery reveals that the cathode consists of Co/Ni (pink) and Mn-rich parts (cyan) (fig. 1a). The separator and the anode are not visible, as the polymers and the carbon molecules cannot be distinguished from each other with this method. However, Raman imaging can visualize graphite (cyan) and amorphous carbon (blue) in the anode and amorphous carbon and lithium with manganese oxides LMO (red) in the cathode (fig. 1b). The separator is built up from a layer of polyethylene (PE) (green) between two layers of polypropylene (PP) (yellow). All of the molecules mentioned were identified by their Raman spectra (fig. 1c). Note that the polymorphs of carbon and polymers can only be differentiated by Raman analysis. During

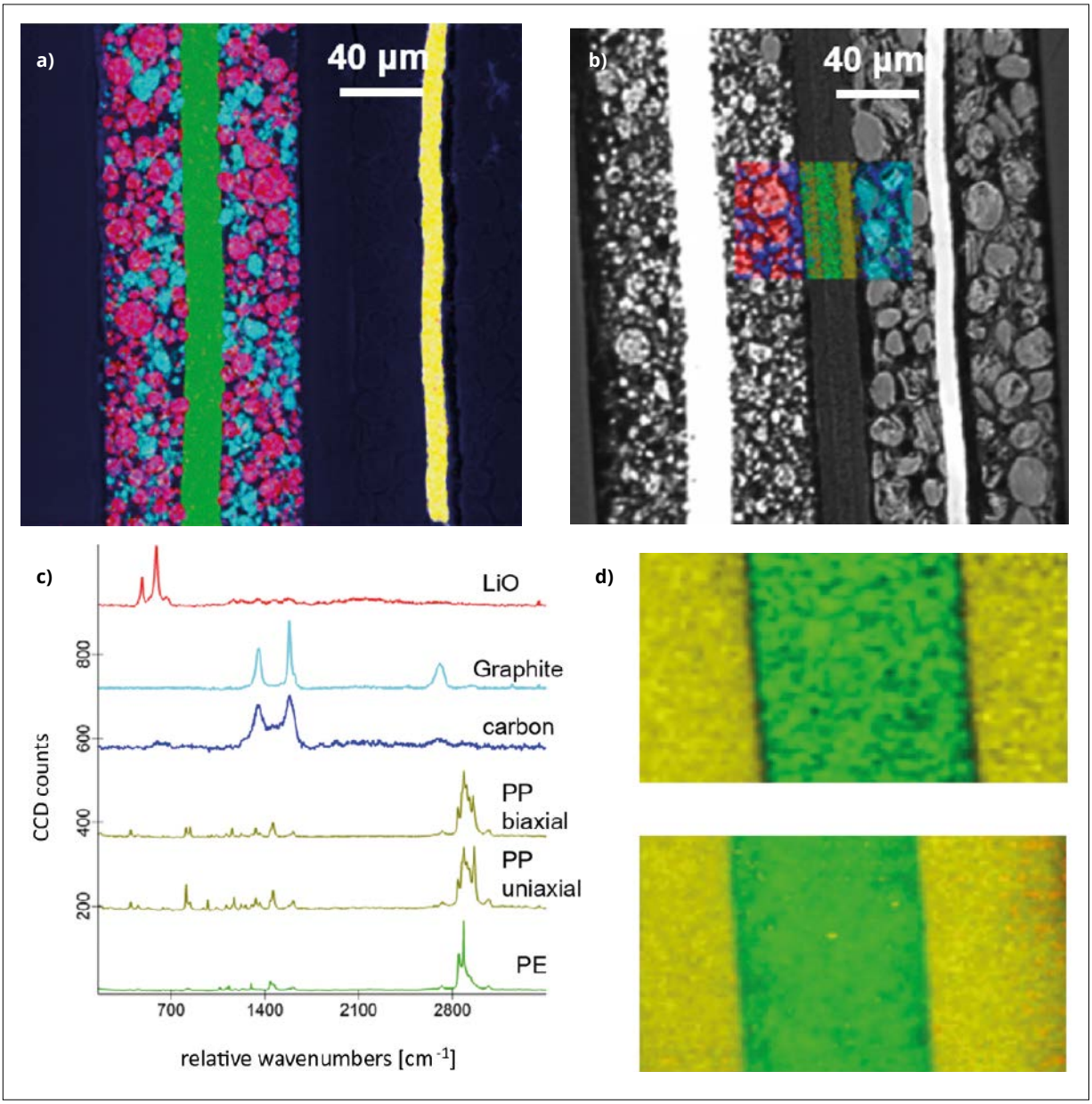

Fig. 1: Raman microscopy and SEM-EDX mapping investigation of 18650 cell LMO batteries. (a) SEM-EDX image of a cross section. The cathode consists of Co/Ni-rich regions (pink) and Mn-rich parts (cyan). The separator and the anode cannot be differentiated (black). (b) The Raman image was overlaid on the white-light image. The anode consists of graphitic (cyan) and amorphous carbon (blue), the separator is made up of polypropylene (yellow) and polyethylene sheets (green), and the cathode is comprised of LMO (red) and amorphous carbon (blue). (c) Raman spectra of the battery's components. Colors as in (b). (d) Raman images of the separator before (above) and after (below) cycling. The process induced changes in structure of the polypropylene sheets. Sample courtesy of Timo Sörgel and Gerhard Schneider, Aalen University of Applied Sciences, Germany.

cycling the separators' polymers undergo molecular deterioration (fig. 1d). While the outer layers of the separator of the new battery include only uniaxial PP, the polymer chains change their directions during cycling, appearing as bi-axial PP in the used battery. It has been described that changes in the composition of separators influence significantly the performance of a Li-ion battery [5,6].

We then performed analyses of NMC batteries that underwent fast recharging (fig. 2). Rapid charging of empty batteries is in great demand in the automotive sector, yet it impairs the batteries' performance. The investigated NMC cell was subjected to 400 cycles, leading to a 40% loss of capacity. Changes in performance are often a result of inhomogeneous degradation in battery electrodes. This local deterioration of microstructure in NMC battery electrodes subjected to fast charging and long-term cycling was studied using a Raman system integrated with a Tescan SEM that includes a focused ion beam (FIB). Cross sections were created with the FIB for imaging.

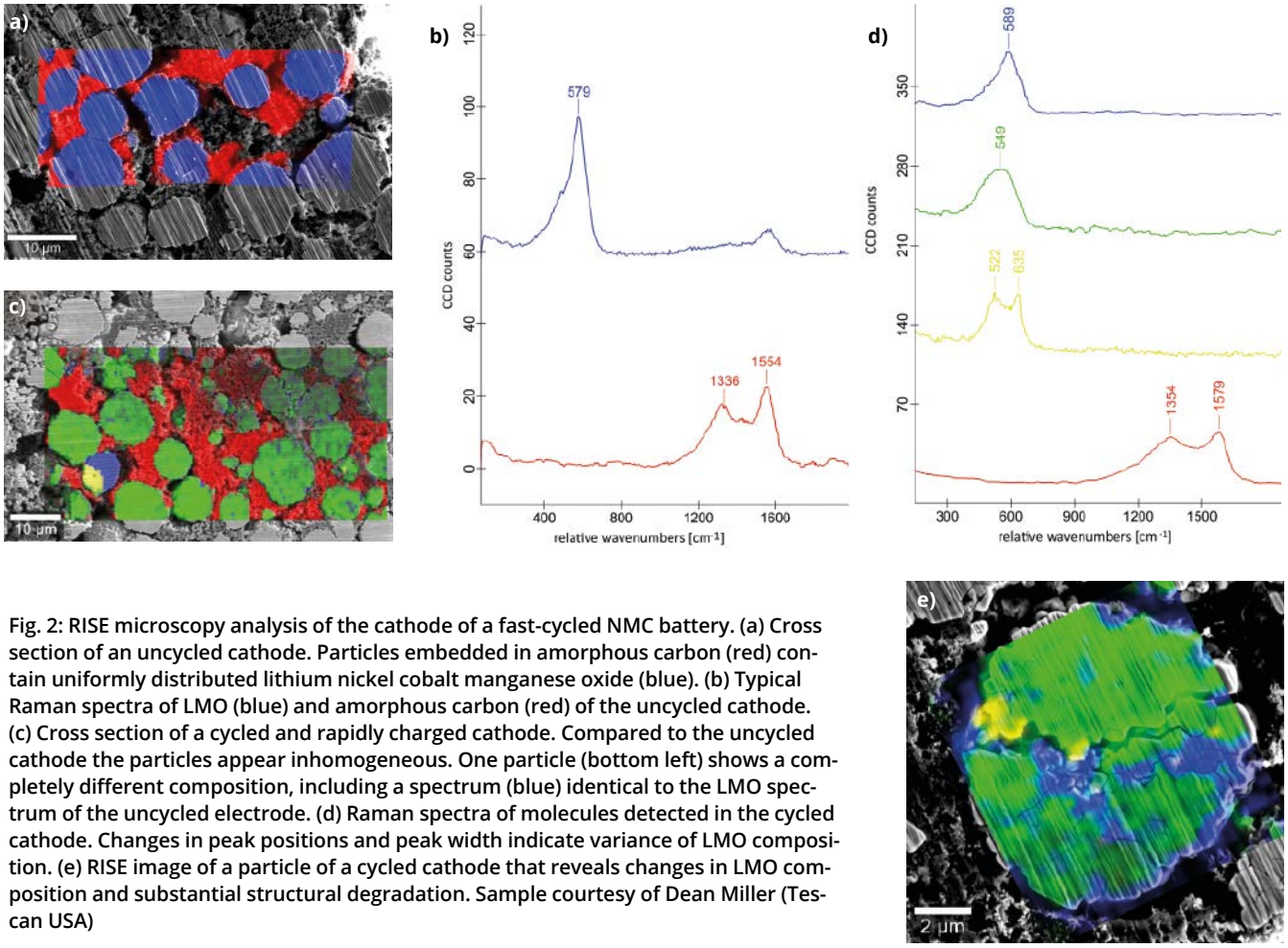

Fig. 2: RISE microscopy analysis of the cathode of a fast-cycled NMC battery. (a) Cross section of an uncycled cathode. Particles embedded in amorphous carbon (red) contain uniformly distributed lithium nickel cobalt manganese oxide (blue). (b) Typical Raman spectra of LMO (blue) and amorphous carbon (red) of the uncycled cathode. (c) Cross section of a cycled and rapidly charged cathode. Compared to the uncycled cathode the particles appear inhomogeneous. One particle (bottom left) shows a completely different composition, including a spectrum (blue) identical to the LMO spectrum of the uncycled electrode. (d) Raman spectra of molecules detected in the cycled cathode. Changes in peak positions and peak width indicate variance of LMO composition. (e) RISE image of a particle of a cycled cathode that reveals changes in LMO composition and substantial structural degradation. Sample courtesy of Dean Miller (Tescan USA)

In the RISE image of the new, charged cathode its particles appear to consist of uniform LMO (fig. 2a). The components were identified by their typical spectral peaks around 580 cm^{-1} (LMO, blue), 1300 cm^{-1} and 1550 cm^{-1} (amorphous carbon, red) relative wavenumbers (fig. 2b). Rapid cycling induced significant changes in lithiation of the particles as indicated by changes in the Raman spectra (green) (fig. 2c). Raman peaks have broadened and shifted (fig. 2d). The Raman data reveals local variations even at the single particle level. In figure 2c one particle is characterized by two spectra, one of which corresponds to the LMO spectrum of the native electrode, indicating that this particle might not have participated in the cycling process. Lorentzian fitting of the spectral peak positions of another particle also shows a high level of inhomogeneity and degradation in the form of cracks (fig. 2e). More significant cracking was detected near the separation membrane (not shown).

Conclusion

The integration of Raman microcopy with SEM provides an excellent opportunity to explore the compositions of electrodes on the nanoscale. The RISE data shown here depicts a history of deterioration in cathode particles that may be at least one of the reasons for the loss of performance and reduction in lifetime of Li-ion batteries.

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Studying Ocean Acidification Processes Using Raman Microscopy

Dr. Thomas Becker manages the Scanning Probe Microscopy Facility of the School of Molecular and Life Sciences at Curtin University in Perth, Australia. The scanning probe microscopy facility is a multi-user facility accessible to researchers from many different disciplines. Current research projects are concerned with the measurement of residual pressure within mineral inclusion, understanding the relationship between zinc homeostasis and cognitive function during natural ageing, zircon (U-Th)/He thermochronometry, spectroscopic techniques for imaging oxidative stress, chemical and physical properties of latent fingermarks, geochemical and crystallographic studies of molluscs, distribution and identification of microplastics, investigation of steel corrosion and corrosion inhibition, differentiation of carbon nanomaterials. Apart from industrial and academic research projects conducted by PhD students or postdoctoral researchers, the research group also implement the use of confocal Raman microscopy into teaching and research projects for undergraduate students, giving the students the opportunity to experience working with Raman micro-spectroscopy.

Which scientific questions have you been able to answer in the past using Raman microscopy?
Within our facility we are collaborating with many researchers from various disciplines, allowing us to be part of a team to answer a variety of interesting scientific questions.

In the area of marine biology, we have been part of a research project investigating variations in shell microstructure and composition utilizing a combined crystallographic, spectroscopic and geochemical approach with respect to geographical (environmental) factors and biological factors, producing valuable data on molluscan shell geometry from southwestern coastal regions of Australia [1]. For bivalve shells to be reliably used as proxies for paleoenvironmental reconstructions, it is necessary to characterize the organic as well as the inorganic crystalline material in order to take all influencing factors into account. The findings of this study provided further insights into the use of molluscs as proxies for reconstructing marine climates. Thin sections of bivalve shells were analyzed with confocal Raman measurements to reveal the subtle differences between the different polymorphs of $CaCO_3$, aragonite and calcite, present in the shells. It was found that the two different species of shells, although sampled from the same location and thus having experienced the same environmental conditions, showed differences in the chemical composition and the way some trace elements were incorporated, indicating a species dependent composition.

Ocean acidification due to the invasion of anthropogenic CO_2, which dissociates into carbonic acid and decreases the pH of seawater, reduces the concentration of carbonate ions in seawater. Coral polyps build calcium carbonate skeletons, the building blocks of massive coral reef structures, which act as protection of shorelines and are home to a significant concentration of biodiversity. A critical aspect for the calcification process of coral is the extraction of calcium ions and carbonate ions from the seawater to grow aragonitic $CaCO_3$. In a related but different project to the above we investigated methods to improve the understanding of the biomineralization process of corals and their sensitivity to environmental changes such as ocean acidification through quantifying the saturation state of aragonite (Ω_{Ar}) within the calcifying fluid of corals by Raman spectroscopy [2]. For the first time we present a technique to quantify Ω_{Ar} using Raman spectroscopy to relate Ω_{Ar} to the Raman peak width. The mea-

surements were initially performed with abiogenic aragonites precipitated under a range of various chemical compositions and temperature treatments and it was found that the Raman peak width clearly depends on Ω_{Ar}, without being confounded by other factors such as temperature, supporting the use of Raman spectroscopy as a proxy for coral calcifying fluid Ω_{Ar}.

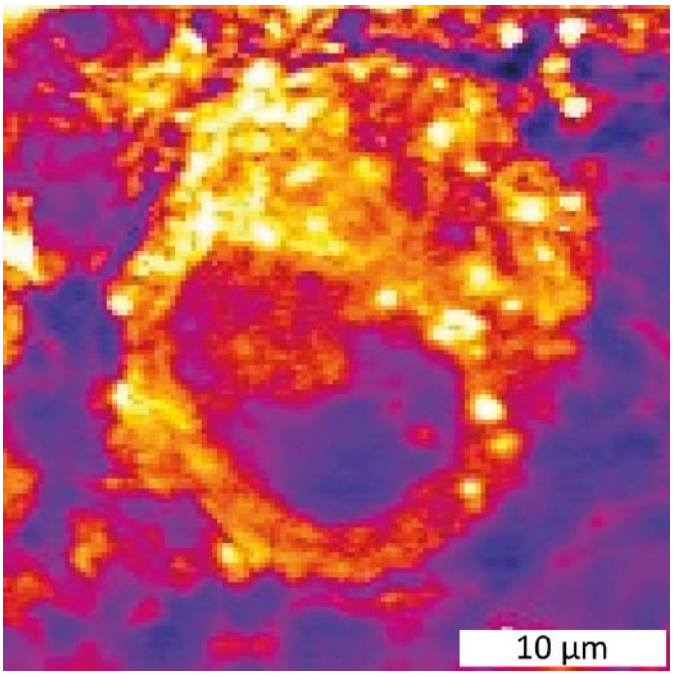
As part of a forensic research project, we have investigated the chemical composition of latent fingermarks, trace quantities of cellular debris and skin secretions, which are transferred when the finger contacts a surface. Detection methods, for example dusting powders or wet chemical techniques, then can reveal and visualize the unique fingermark, which represents a significant form of crime scene evidence. Complementary sub-micron spatial resolution confocal Raman microscopy and sub-micron pixel resolution ATR-FTIR microscopy demonstrated that latent fingermark droplets constitute complex chemical compositions of both sebaceous (lipophilic) and eccrine (hydrophilic) materials [3]. The study contributes significantly to the fundamental understanding of the chemical composition of fingermarks and its effect on the sensitivity and robustness of current fingermark detection methods in forensic investigations. In contrast to previous theories that natural fingermarks are primarily comprised of eccrine material, this study showed that sebaceous material is abundantly present in natural fingermarks. This information about the chemical composition of latent fingermarks is vital for the development of successful visualization techniques.

Which devices or technologies do you use in your laboratory? Which methods can be combined well with Raman microscopy?

Our facility houses a range of Scanning Probe Microscopes which are being employed for surface characterization including topography imaging and quantification of mechanical and electrical properties of samples using atomic force microscopy as well as investigating conductance of individual molecules using scanning tunnelling spectroscopy. The laboratory also houses a WITec alpha300 SAR+ system, combining confocal Raman micro-spectroscopy with scanning probe microscopy and scanning near field optical microscopy, which is equipped with a temperature-controlled sample stage. Further instrumentation in the facility includes a dynamic light scattering/ZetaSizer system and a contact angle goniometer. Other analysis techniques such as NMR, chromatography techniques and electron microscopy are available within the department or on campus at Curtin University. The combination of confocal Raman microscopy and scanning probe microscopy as it is available in our alpha300 system allows a detailed and comprehensive analysis of surfaces and thin films and coatings. The combination of scanning electron microscopy (SEM) and confocal Raman microscopy would be particularly useful for geological projects in our facility. In a number of projects in which our facility is involved, confocal Raman only constitutes a part of the analysis and the samples are analyzed with a suite of complimentary techniques, such as IR spectroscopy. A problem that is likely to be encountered in this case is that the samples need to be transferred between different instruments and analyzing the same location can be challenging.

In your opinion, what are the advantages of using Raman microscopy?

One of the main advantages of using Raman microscopy is the fast and relatively easy operation of modern Raman microscopes to chemically identify species in a sample with high spatial resolution. The fast acquisition time and high spatial resolution of today's instruments allows for detailed studies on the microscopic scale. The ease of use of the instruments these days enables even novice users to conduct meaningful measurements. Minimal sample preparation and non-destructive measurements are further advan-



Sub-micron resolution confocal Raman microscopy has been applied to study sub-cellular biochemical changes that occur in the brain during health and disease. This image depicts a purkinje neuron from the cerebellum of a rat brain and was used in a study to develop correlative Raman microscopy and immunohistochemistry on the same tissue section, in order to study how lipid metabolism and lipofuscin accumulation may impact brain ageing as neurodegeneative disease (WITec alpha300 SAR+; courtesy of Ashley Hollings).

tages of Raman microscopy, especially if other techniques are employed on the same sample as well.

Which scientific questions would you like to investigate with the help of Raman microscopy in the future?
One of the current research topics within our facility is the investigation of microplastics, their identification and their distribution in both environment but also living organisms, and the effect the microplastics have.

Another scientific research question we are hoping to answer with Raman microscopy is the development of a robust technique to analyze mixtures of materials and quantitatively determine the relative amounts of the individual components of these mixtures.

We are further interested in reversible hydrogels, which we have investigated in great detail with regards to the formation and dissolution of the fibrous network within the gel using atomic force microscopy. These hydrogels have potential applications for example in tissue engineering, personal care products and drug delivery. While we were able to analyze these gels in term of physical properties, we are further interested to monitor for example the drug release under controlled environmental conditions, for which Raman microscopy would be a suitable technique.

Which research work in the field of Raman microscopy has particularly impressed you in recent years?
I am particularly impressed by the research work in the field of microplastics as well as the developments in the automation of the measurements analyzing large samples of microplastics. The enormous amount of microplastics present in the environment and the pathway of these microscopic plastic pieces through and into living organisms such as fish and livestock and ultimately humans, and their effect on these organisms are highly interesting and of significant concern.

Dr. Thomas Becker studied Physics at the University of Ulm in Germany and obtained his PhD from the University of Twente (The Netherlands) in 2005. In the same year he started at Curtin University in Perth, Australia, where he manages the Scanning Probe Microscopy Facility of the School of Molecular and Life Sciences and is teaching units in the Chemistry curriculum. His research interests focus on sample characterization with scanning probe microscopy techniques and confocal Raman microscopy. He is involved with a number of university based research projects from different disciplines such as corrosion research, hydrogels, and health sciences as well as with collaborations with industry.

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Raman Microscopy Evolution

From Specialist Favourite to Mainstream Standard

The near future will likely see Raman imaging microscopy complete its evolution from a specialized tool to a commonplace analytical technique. The overall number of fields that employ it, and the variety of its applications within those disciplines, should both show steady increases. The primary factors set to drive this expansion are advances in user-friendliness and experimental flexibility.

Chemical characterization enabled by Raman spectroscopy became practical with commercially-available solid-state photonics devices in the 1970s and 80s. Lasers provided stable, monochromatic light sources in a range of wavelengths for sample excitation. Charge-coupled devices offered the sensitivity to detect signals from weak Raman scatterers and to acquire large numbers of spectra very quickly. Though they remained primarily used by those familiar with spectroscopy, such as physicists, chemists and materials scientists, these technologies started the first wave of adoption of Raman-based analysis.

In the 1990s, increasingly powerful personal computers and precise piezo-driven sample scan stages made it possible for Raman instruments to acquire a complete spectrum at each measurement point and compile them to generate an image. Raman imaging microscopy was introduced to the marketplace by WITec GmbH in 1999. Once scientists saw visualizations of sample component distributions 3-dimensionally in high resolution (Fig. 1), demand for the nondestructive, label-free technique spread rapidly.

Today Raman imaging can be found far beyond its initial user base, in fields as diverse as archaeology, quantum device research, forensics, pharmaceutical development, environmental science and many others. While the method continues to be embraced for its precision and speed, strides in accessibility and versatility are the keys to its widening appeal.

High-resolution Raman imaging experiments involve many components working in concert to extract the most from each measurement. This was at times intimidating for users new to the technique. The introduction of advanced control software and motorized optical elements has succeeded in making even complex investigations easily manageable.

Experimental workflow configuration can now be accomplished through a graphical user interface and every setting can be stored along with the acquired data. Automated components remove the necessity of manual fine adjustment and

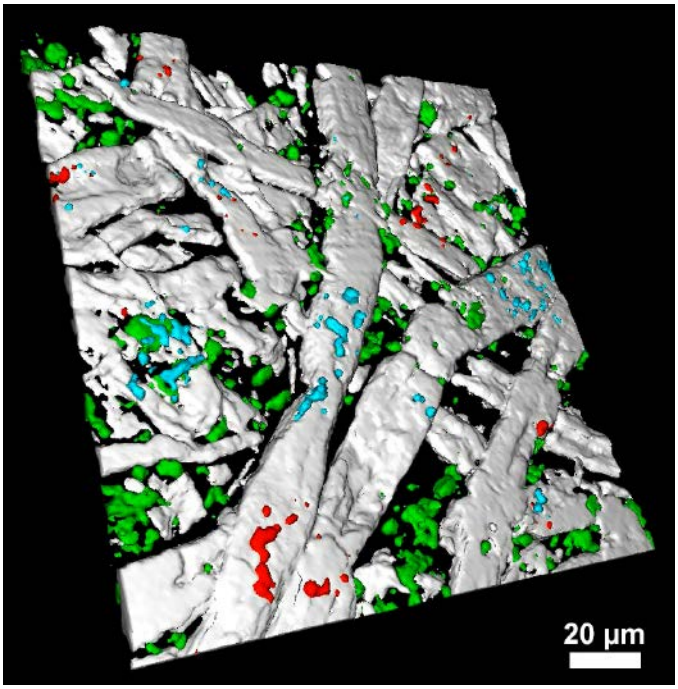

Fig. 1: 3D Raman image of paper with surface additives

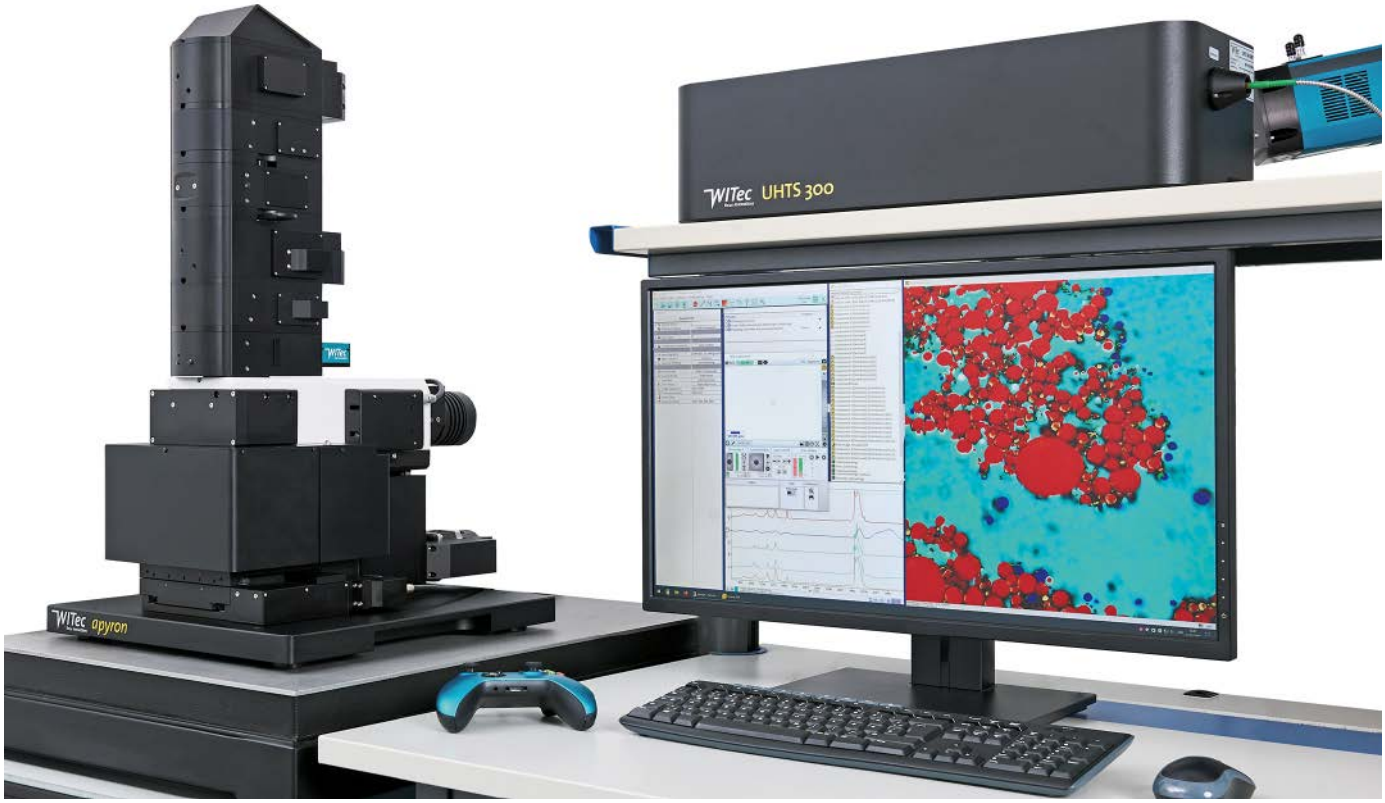

Fig. 2: Automated Raman imaging system WITec alpha300 *apyron*

calibration while guaranteeing the reproducibility of results and eliminating potential sources of error. With these developments (Fig. 2), the full analytical power of Raman imaging can be placed in the hands of researchers anywhere, from university groups and multi-user facilities to industrial quality control teams.

The path ahead for Raman microscopy will be enabled by its further integration with complementary techniques and controlled sample environments. Advanced modular Raman microscopes have recently been integrated within scanning electron microscopes (SEMs). The foremost among these instruments, WITec's RISE microscopes (Fig. 3), feature energy-dispersive X-ray spectroscopy (EDS) and a common vacuum chamber for all measurements. Now, leading-edge, non-linear methods including second- and third harmonic generation (SHG, THG) have joined the list of correlative Raman imaging options, and are especially useful for exploring low-dimensional materials such as transition metal dichalcogenides (TMDs).

Advanced image processing algorithms can use white-light images to generate a map of very large numbers of microparticles in a sample. This map can then automatically guide a measurement to acquire a Raman spectrum from each for chemical identification, a process that can be expedited by using Raman spectral database software. Tools that fea-

Fig. 3: RISE Microscopes: Fully-integrated Raman Imaging and Scanning Electron Microscopy

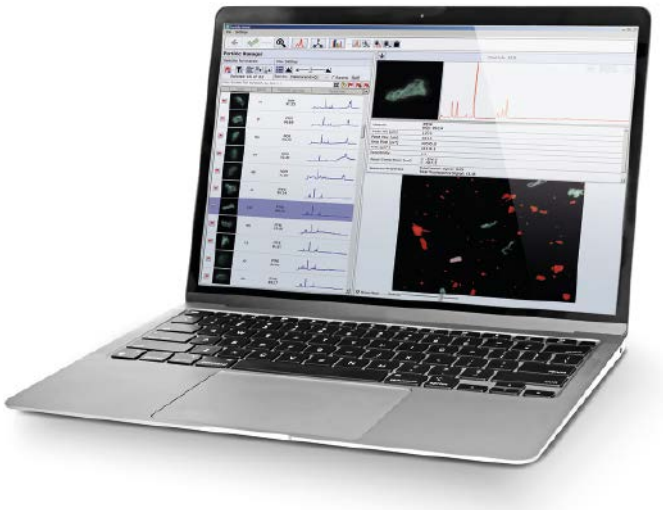

Fig 4: ParticleScout is an advanced tool for particle analysis with Raman

ture these elements, such as WITec's ParticleScout (Fig. 4), are proving invaluable in analyzing microplastic pollution in environmental samples. As Raman-based particle analysis offers exceptional resolution and the ability to perform investigations of aqueous solutions, it has a distinct advantage over conventional methods.

Modular Raman microscopes can also lend their chemical sensitivity to experiments conducted in cryogenic chambers and environmental enclosures. Cryogenic Raman microscopy can reveal intricate details of exotic materials as quantum effects become visible near absolute zero. Fully automated Raman instruments can be placed in glove boxes for evaluations of hazardous samples or those produced with processes such as chemical vapor deposition.

The journey of Raman spectroscopy – from Nobel Prize-winning experiment, to expert's choice for chemical characterization, through popular visualization method to becoming a standard analytical technique – has been a series of steps toward fulfilling its initial promise. Now that everyone can access its benefits for characterizing materials, the research community is devising novel ways to apply it to emerging challenges. Raman microscopy, in all its myriad forms, looks ready to become a ubiquitous sight in laboratories around the world and across the full range of scientific inquiry.