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EMRP European Metrology Research Programme
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1 Executive Summary

Introduction
Raman spectroscopy (RS) is an important technique used by a range of EU industries. However, the accuracy and reliability of RS is limited by a lack of a metrology (measurement science) infrastructure, constraining the technique's potential application. This project developed metrological techniques for RS, including practical and modelling methods to trace Raman measurements to their SI unit definitions, standardised reference materials, and methods to display results as 3D images. These achievements have transformed RS into a robust quantitative technique which is being adapted to a range of new, and previously unattainable applications, such as the real-time imagining of dynamic processes within living cells.

The Problem
Raman spectroscopy is the preferred tool for the full in-situ chemical and structural identification of species at higher spatial resolution. However, Raman spectroscopy is only used as a qualitative tool in the pharmaceutical, healthcare, biotechnology, nanotechnology, medical technology and forensic science sectors to identify and map the distribution of substances in 2D or 3D, because before this project the technique had no underlying metrological infrastructure, such as standardised methods to trace RS measurements to their SI unit definitions. Without this infrastructure the full potential of RS was constrained, preventing the further refinement and development of the technique required for the creation of innovative products and services throughout a number of EU industries.

The Solution
In response to this problem, we set out to develop a measurement infrastructure for Raman spectroscopy, to allow it to transition from a qualitative to a quantitative technique. This involved:

(a) Developing a scheme to measure the sampling volume in confocal Raman spectroscopy enabling quantification of substances at different depths; **(b)** Developing quantitative surface-enhanced Raman spectroscopy (SERS); **(c)** Proposing schemes to improve comparability and reproducibility of tip-enhanced Raman spectroscopy (TERS); **(d)** Increasing the speed of Raman measurements using non-linear Raman spectroscopy such as stimulated Raman (SRS) and coherent anti-Stokes Raman scattering (CARS); **(e)** Validating the experimental observations using low-cost graphics processing unit (GPU) enabled simulations.

As a result of the project, the following outcomes have been achieved:

(i) The first Consultative Committee for Amount of Substance (CCQM) study to quantify the amount of substance in ambient conditions using Raman spectroscopy enabled by the confocal volume reference standard; **(ii)** use SERS to quantify down to femto-molar concentrations; **(iii)** compare the enhancement factor and spatial resolution in TERS between different instrumentations and laboratories; **(iv)** Real time visualisation of living cells and tissues to study diffusion of active molecules; **(v)** Software has been developed with a user interface to model different optical configurations.

Impact
The project has created a metrology infrastructure to address specific standardisation issues. The methods developed in this project have enabled us to initiate intercomparison studies through CCQM and Versailles Project on Advanced Materials and Standards (VAMAS). These are enabling CE marking of instrumentations required for clinical trials for diagnosis of diseases such as cancer.

The video-rate Raman imaging microscope at the National Physical Laboratory, UK, is being used to develop measurement solutions for healthcare and pharmaceutical companies. This facility has provided measurement services worth more than £50k and its continued use in skin care product testing could generate an estimated 20 % cost saving for a UK based skin health company across the product development life cycle.

The chemometric software developed, for large-scale Raman data analysis, is a powerful new tool for analysing Raman images of complex surfaces. The software is being further developed for implementation by a commercial instrument manufacturer.

Some of the issues in the batch production of TERS tips were resolved in this project through new tip designs and production methods. One such method has been adopted for commercial production by the Natural and

Medical Sciences Institute, a medical science institute at the University of Tübingen, with the aim of producing and selling standardised tips.

In future, the instrumentation, protocols, procedures and methodologies developed during this project will continue to create impact in a range of fields including the fundamental understanding of biological processes and early diagnosis of diseases which will contribute to save millions of lives and costs and faster and safer product development. All these will increase uptake of Raman instrumentation which has been growing steeply in the last 10 years.

2 Project context, rationale and objectives

Context

In the last three decades, there has been a steep rise in the publication of research involving Raman scattering, yet to metrologists Raman spectroscopy is a relatively new technique. Raman scattering has a unique place as a label-free, non-destructive, contact-less, fast, optical method for the identification, quantification and mapping of the distribution of chemicals and micro-to-nano-scale structures in 2D or 3D in an ambient air or in an aqueous medium.

There has been a surge in the use of Raman spectroscopy in the last two decades and a number of novel techniques have evolved which can:

- Increase the sensitivity *e.g.* surface enhanced Raman scattering (SERS), coherent anti-Stokes Raman scattering (CARS), stimulated Raman scattering (SRS), and
- Increase spatial resolution *e.g.* tip-enhanced Raman scattering (TERS).

However, the metrology for enhanced Raman techniques has never been addressed in a structured way. This has led to a lack of traceability and underpinning metrology tools, which in turn has led to inadequate standards.

Inadequate current standards: Currently instrument manufacturers and existing users do not use the standards that are available as these do not cater to their needs. This is because they are not fit for purpose. Other requirements, that are currently not met, include a strong need to develop calibrated reference materials as a Raman transfer standard and the need to underpin traceable quantification through the development of standard methods for defining the degree of confocality in terms of confocal volume, spatial resolution and depth resolution.

Inadequate accuracy of commercial instruments: Currently different Raman instruments from different manufacturers can generate different results, and often even those from the same manufacturer suffer from this problem. This means there can be no comparison between laboratories and frustratingly even within the same laboratory. This is due to the lack of standard methods, calibrated reference samples and traceability. This means that currently Raman spectroscopy is only suitable for qualitative measurements that compare results from a single instrument.

This project aimed to significantly advance the state of the art in traceable Raman spectroscopy, developing a number of standards where none currently existed, and improving the repeatability and sensitivity of a number of methods and techniques.

Recently TERS has been applied extensively to various fields of science and technology including electrochemistry, surface science, and quantitative and qualitative analysis. The applications to trace assays of biological molecules are of interest because TERS is a highly sensitive analytical tool in laboratory medicine as well as in basic research of biological sciences. A particular advantage is that TERS allows spectroscopic identification and imaging in a completely label free manner. Recent advances in instrumentation, sample handling techniques, and theoretical studies have also enabled observation of TERS signals from a single molecule.

The major advantage of TERS is that it provides at least ~10 times higher spatial resolution than micro-Raman spectroscopy. Therefore the calibration of spatial resolution is important. In order to determine the spatial

resolution without applying a deconvolution method, the dimension of the sample needs to be smaller than that of the tip. The sample should also have a strong Raman cross-section because the sample volume under the TERS probe is of the order of 10^{-8} μm^3. Single wall carbon nanotubes (SWCNT) are strong Raman scatterers, and have diameters ~1 nm, which is at least 10 times the diameter of the TERS tip.

This project aimed to use optical contrast rather than the enhancement factor for comparing different tips and also for determination of the enhancement factor. The optical contrast is the ratio of Raman signal intensities that are acquired with the tip in close proximity to the sample, so the near-field signal (STM tunnelling feedback, AFM contact or semi-contact feedback) and the signal detected with the tip retracted from the sample. The sizes of the sources of near-field and far-field signals also need to be known, as these often rely on estimated numbers. The quality of a TERS image (the amount of blurring of the resolution due to diffraction-limited far-field contribution) directly depends on the optical contrast. This project aimed to develop prototypes of reference TERS samples that can be produced by industry and which can ultimately become available to TERS research labs, users, and industry.

The overall goal of this project was to develop a measurement infrastructure for Raman spectroscopy, to allow it to transition from a qualitative to a quantitative technique. To achieve this, objectives 1 and 2 developed methods to trace RS measurements to their SI unit definitions (mole and metre respectively). Objective 3 developed a method to display RS measurements as 3D images. Objective 4 established mathematical models to underpin measurement traceability. Objective 5 developed measures of uncertainty in RS measurements, and methods to ensure quantitative RS measurements were repeatable.

1. Establishing traceability to the mole by conventional Raman scattering and surface-enhanced Raman scattering (SERS)
2. Establishing spatial and depth resolution measurements traceable to the metre (target uncertainty sub-micrometre resolution in the XY plane) using Tip-Enhanced Raman Scattering (TERS).
3. Developing methods for 3D chemical imaging at high speed using multi-photon Raman scattering, Stimulated Raman Scattering (SRS) and Coherent Anti-Stokes Scattering (CARS)
4. Investigating light-matter interactions in Raman scattering using finite difference time domain (FDTD) calculations, to establish mathematical models to underpin traceable measurements
5. Improving the repeatability of Raman measurements and establishing robust uncertainty budgets for Raman spectroscopy.

3 Research results

The project addressed the overall aims and requirements as discussed in previous sections, the underpinning technical achievements are discussed in this section.

3.1 Objective 1: Establishing traceability to the mole by conventional Raman scattering and surface-enhanced Raman scattering (SERS)

3.1.1 Reference specimens for Raman depth profiling and laboratory comparison (PTB, NPL, INRIM, Inmetro)

Depth profiling is used in a wide range of applications including coating, thin films, tissues and organisms, and Raman spectroscopic measurement provides easy and quick measurements. The influence of the measurement set up on the depth resolution is rarely addressed. It is obvious that the confocal volume in these measurements depends on the optical properties of the material as well as on the depth of probing. Although there has been some work reported before, before this project there were no standard reference samples available for researchers and instrument manufacturers to investigate the degree of confocality of an instrument at different depths.

Some of the key achievements of this work were accomplished in the area of standardisation for Raman depth profiling by means of reference specimens. Two different types of test specimens were prepared and distributed among the partners:

a) Single layer polymer specimens made from different polymer materials having thicknesses of 13 µm (polyethylene terephthalate, PET), 25 µm (polystyrene, PS) and 50 µm (polymethyl methacrylate, PMMA). These specimens are intended to provide a link between the true thickness of a layer and the apparent thickness as determined by evaluation of Raman depth profiling data.

Linking the thickness of the test specimens to the SI-unit metre was achieved using a calibrated measurement system based on mechanical probing with an inductive length measurement system operated at PTB. The expanded measurement uncertainty (k=2) associated to measured thickness values h in the range 10 µm ≤ h ≤ 200 µm is 0.1 µm.

b) Multilayer polymer specimens made by alternated stacking of the three single-layer materials PET, PS and PMMA. The aim of analysing these kinds of test specimens was to extend the comparison towards samples of certain practical relevance, i.e. having a larger thickness and consisting of different chemical compounds.

The reference specimens were tested by five participating NMIs (PTB, NPL, INRIM, INMETRO, NIST) employing Raman instruments from different manufacturers but under similar or even identical experimental conditions. Particularly those parameters which have a substantial influence on the outcome of Raman in-depth measurements namely the excitation laser wavelength (532 nm) and the microscope objective magnification and numerical aperture (NA) (100x, NA 0.9) were commonly used by all participants.

For the PS layers in the single layer specimens, having a true thickness of (25.72 ± 0.1) µm, the measured apparent thickness values are between 17.5 µm and 21 µm. Hence, experimental depth-profiles appear to be narrowed compared to the true thickness of the polymer films as measured by an independent reference method. This mismatch between apparent and true layer thickness occurred also for the other two materials under study and is attributed to differences in the refractive index between object (glass/polymer, $n \approx 1.4$ - 1.6) and the space between objective and the object (air, $n \approx 1$). As a result, Raman scattering originates from a region deeper below the surface than the nominal z-point. A mathematical modelling based on refraction of light can be used to correct for this deviation.

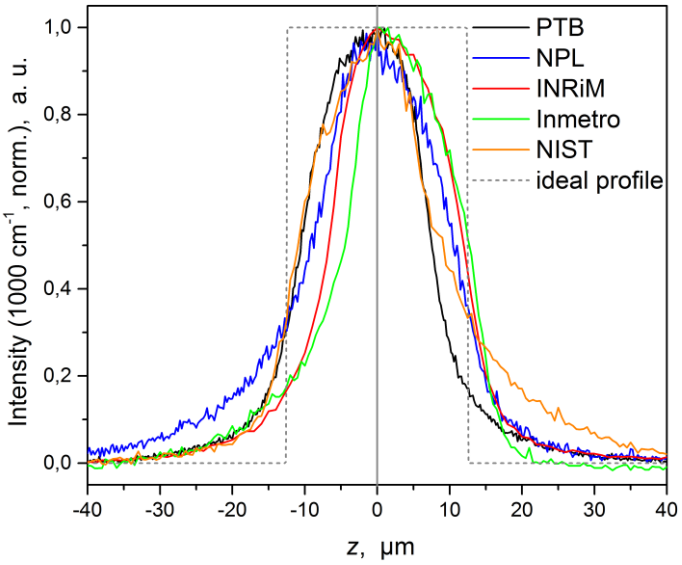

Figure 1.Typical depth-profiles obtained for a test specimen made of single layer PS (25 µm), plotted in the direction from below the sample (-*z*) through the layer towards the air (+*z*). Intensities were normalised with respect to the maximum intensity value (*z*-point) and centered on the x-axis.

On the other hand, measurements on identical test specimens result in similar shaped profiles obtained by the participants (Figure 1) and led to a narrow range of measured apparent thickness values. The differences in profile shape which are mainly reflected by a lack of symmetry (skewness) indicating an improper adjustment of the laser beam path within the instrument. Hence, measurements of the test samples might also point to the fact that a re-adjustment of the optical components is required.

In practical applications of Raman depth profiling such as quality control, the samples to be checked commonly consist of multiple chemical compounds. Therefore, multilayer test specimens consisting of stacked polymer films were analysed within the laboratory comparison for a more authentic representation of "real world samples". The profiles obtained for a specimen consisting of three PET (13 µm) and two PS (25 µm) films stacked alternately demonstrates the gradual change in confocal volume and the vanishing ability for detecting the scattered light as soon as the laser focus penetrates into the sample (Figure 2).

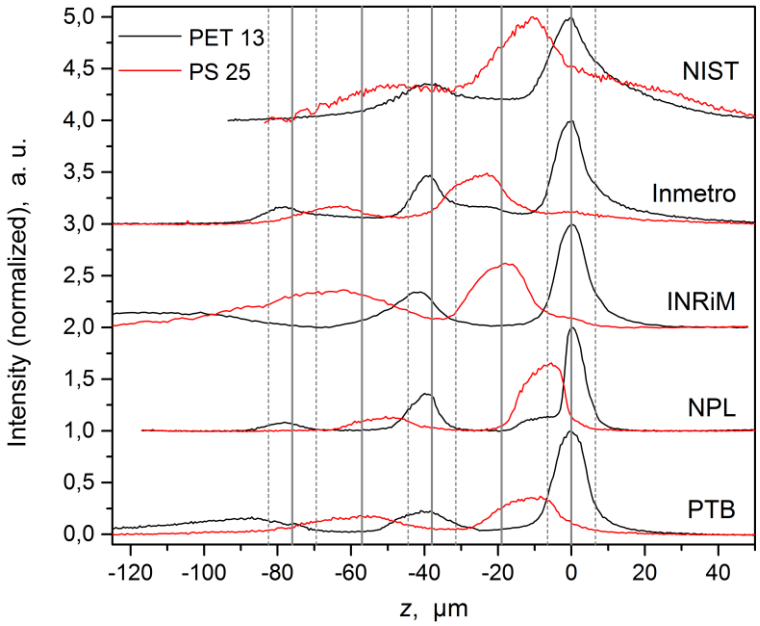

Figure 2. Typical depth-profiles obtained from each participant for a multilayer specimen consisting of the layer sequence PET-PS-PET-PS-PET. The intensities were normalized with respect to the maximum intensity value of the first PET layer and the profiles were then shifted on the *x*-axis to $I_{max,\ PET,1} = 0$ for better comparability. The solid lines indicate the positions of the central plane and the dotted lines indicate the borders of each individual layer in an ideal stack.

In practice, this causes a decreasing ability to resolve the layers from each other and to estimate their extensions with increasing depth. Therefore, corrections have to be applied to the data to give a more realistic estimate of the true extensions of the individual components.

Figure 3: Screenshot of the Matlab toolbox (“Evalmap”) developed at PTB for the evaluation of Raman depth profiling data. Lorentzian functions are calculated in batch mode for a full set of spectra and can be evaluated in terms of various fitting parameters such as intensity, FWHM, or Raman shift to generate thickness profiles of the probed layer.

PTB as the organising NMI was concerned with the evaluation of the data provided by the participants. To ensure the highest degree of consistency and comparability of the intercomparison results, a standardised

evaluation protocol was developed and applied to each set of spectra, thus preventing data evaluation from being influenced by laboratory- or user-specific interactions. Various routines for spectra pre-treatment and fitting of compound-specific Raman bands were implemented such as a Matlab toolbox (EVALMAP).

The variety of output quantities provided by the evaluation of the fitted Lorentzian curve offers some alternative measures to be used for the generation of depth-profiles besides using the maximum intensity (Figure 3). For example, Raman shift or FWHM define a more sharply delineated interval but require a threshold value to be defined on the basis of the true layer thickness.

This work has been identified for further interlaboratory study through VAMAS and the goals will be to develop an international standard method for depth profiling using Raman spectroscopy.

3.1.2 Isotope dilution surface enhanced Raman Spectroscopy (ID-SERS) for large biomolecules (INRIM, PTB, INMETRO)

One of the most challenging tasks in biomarker quantification for monitoring the states of a disease is the quantification of large molecular biomarkers. For example, protein biomarkers (ß-Amyloid, Tau protein) are the most important hallmarks for monitoring the progress of Alzheimer's disease.

Possible approaches for the primary measurement procedure based on isotope dilution involve (a) direct isotope substitution of specific atoms in the protein, (b) direct attachment of a Raman reporter in a natural and an isotopically labelled form to the protein and (c) attachment of natural and isotopically labelled Raman reporter molecules to antibody-functionalised nanoparticles (NPs).

The first approach requires an isotopically labelled protein which is, in most cases, either not available or extremely expensive.

For the realisation of the second approach, a procedure has been applied that involves linking a SERS-active marker to the protein. Here, haemoglobin (Hb) was used as the target protein and the Raman reporter is based on Rhodamine 6G (R6G) with a molecular modification that allows the establishment of a selective link to the lysine entities of the Hb protein. The marker was available in a natural and a deuterium (d4)-enriched isotopologue that led to separate Raman bands at 600 cm^{-1} and 612 cm^{-1}, respectively (see Figure 4). The Hb-d4-R6G complex serves as the spike, a known amount of which is added to a blood sample containing the Hb-R6G complex resulting from an unknown Hb concentration. Then, the true Hb concentration in the sample can be calculated by evaluating the intensity ratio of the two R6G-bands. Due to the high Raman scattering efficiency of the reporter molecule, a limit of detection in the low nmol L^{-1} range was achieved, thus providing an excellent basis for the quantification of extremely low analyte concentrations.

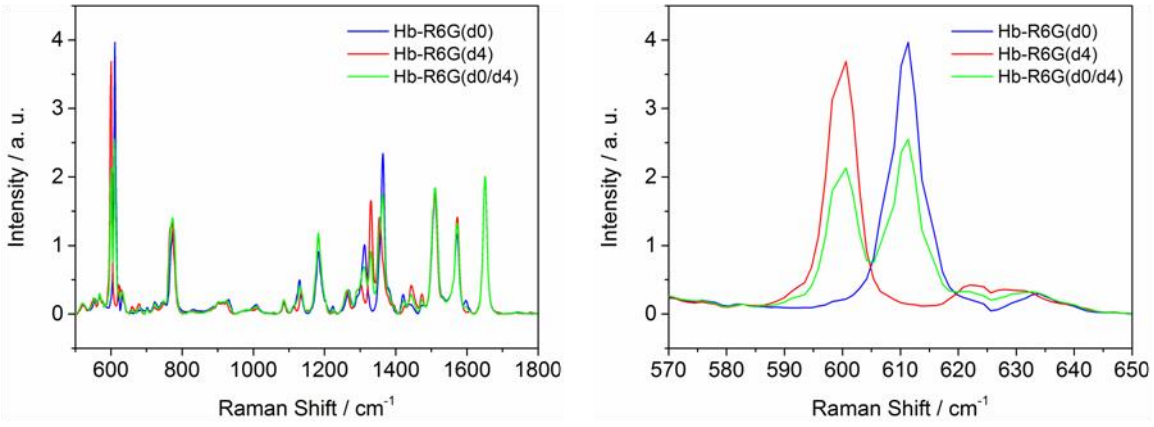

Figure 4: SERS Spectra of the two individual complexes of Haemoglobin and the two R6G-isotopologues compared to the spectrum obtained from an equimolar mixture. The corresponding Hb concentration was 8 nmol L^{-1}.

The third possible approach was regarded to be highly challenging and could not be completed within the lifetime of the project. The procedure involves the development of a so-called ID-SERS assay based on a specific protein-antibody interaction (Figure 5). This highly selective coupling reaction shall be realised while the biomolecules are attached to metallic or magnetic nanoparticles. Again, detection of the coupling reaction and quantification of the protein is achieved with the help of two isotopologues of a Raman reporter. Here, the Raman reporter is linked to the nanoparticle-aggregate which simultaneously serves as the SERS-active medium.

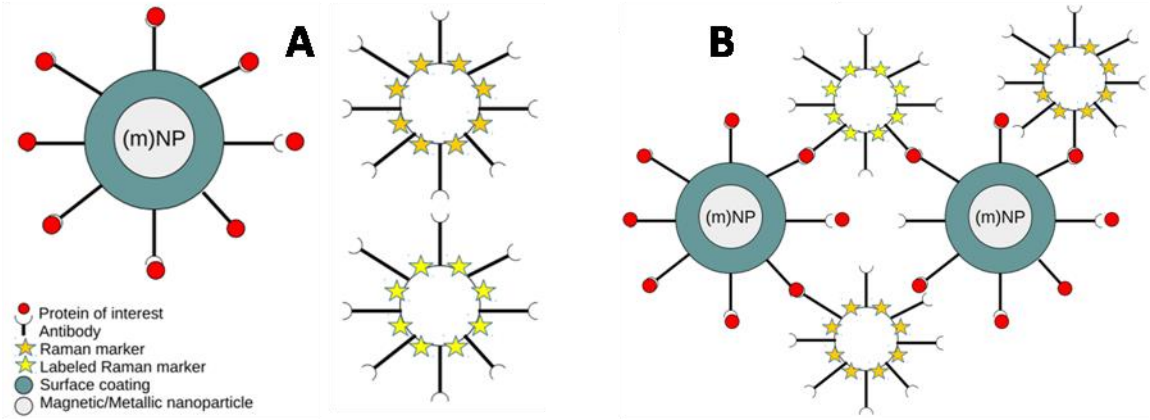

Figure 5: Functionalised metallic/magnetic nanoparticles acting as the building blocks for the SERS-based sandwich assay (A) and the coupling scheme of the nanoparticle entities for identification of the protein-antibody interaction and for protein quantification via evaluation of the SERS intensity ratio obtained for the two isotopologues of the Raman reporter (B).

The advantage of this approach is that cross reactions can be avoided by choosing appropriate antibodies and that the NP/protein/antibody-adduct can be separated from a complex matrix such as blood serum with the help of magnetic nanoparticles. Simultaneously, it must be ensured that nanoparticle coverage with the reporter compound must be stable over time. This shall be achieved by adopting well-established preparation protocols that allow for the formation of core-shell nanoparticles. In such systems, the reporter-molecules are attached directly to the surface of the metallic core and then covered with a protective layer that prevents desorption of the reporter and simultaneously forms the basis for bio-functionalisation.

This approach will be further developed beyond the lifetime of the project by PTB and collaborators. As soon as the technical feasibility of the working scheme has been validated for a well-characterised model system, a procedure to quantify a relevant protein biomarker in human serum will be worked out in the following step.

3.2 Objective 2: Establishing spatial and depth resolution measurements traceable to the metre (target uncertainty sub-micrometre resolution in the XY plane) using Tip-Enhanced Raman Scattering (TERS)

3.2.1 Metrology for Tip-Enhanced Raman Spectroscopy (INRIM, NPL, REG(ETHZ) and NMI)

Reproducible TERS tips

The TERS tip is the most important element in a TERS instrument. However, only approximately 30 % of the TERS tips that are currently made actually work. Besides, TERS tips made by conventional methods are known to undergo both rapid chemical and mechanical degradation, which strongly limits their use from a couple of hours to a few days, depending on the application. Furthermore, most TERS measurements suffer due to a lack of consistency of the TERS tips. It is still very difficult to interpret TERS spectra for a multitude of reasons such as unknown TERS “selection rules”, lack of reference spectral data, frequency dependence of the enhancement, tips aspect ratio, coating reproducibility related to the nature of the tips as well as the

theoretically predicted enhancement which is up to 10^8 times higher and so is not always reproducible in experimental measurements.

During the project, novel methods of tip manufacturing were investigated. Since most instruments use commercially available cantilever tips as starting tips, which are later modified by thin film deposition techniques, significant emphasis was given to this method of TERS tip preparation. Reproducible TERS tips were thus fabricated depositing different noble metals, i.e. silver or gold with thicknesses in the nanometre range, by highly controlled techniques such as electron gun physical vapour deposition. Protective coatings were also deposited on top of the metal layers to avoid tip degradation, as shown in Figure 6. The main parameters affecting TERS measurements were studied, such as roughness of the film, thickness of the film, oxide thickness of the cantilever tip underneath and the size of the tip. The tips were then distributed to the partners to carry out measurements for inter-laboratory studies.

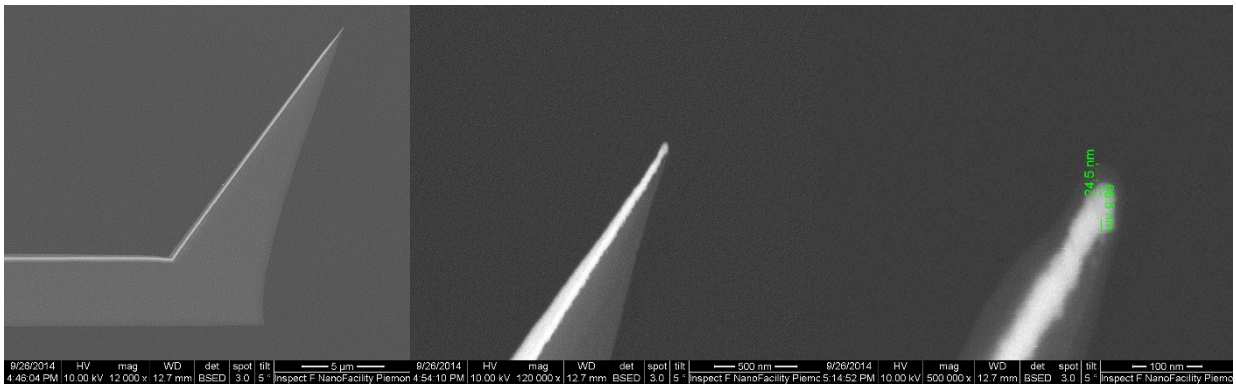

Figure 6: Images of the TERS tips that were produced in the project obtained with an electron microscope (SEM) at increasing magnifications. On the left, the brighter layer represents the noble metal, which can be deposited by electron gun physical vapour deposition with an high degree of reprodubibility on commercially available tips of different geometries. The achieved uniformity of the thin film is evident in the central image, showing the cone area surrounding the tip apex. The tip apex is further protected from mechanical and chemical degradation by a thinner dielectric coating visible on the right image magnification.

Some of the tips produced during the project were shown to survive for 5 months after their fabrication, which was an unprecedented and unexpected result. The increase in the plasmonic lifetime of these tips was achieved via storage inside a nitrogen glovebox environment with an extremely low (< 1 ppm) concentration of oxygen and moisture (See, “Extending the plasmonic lifetime of tip-enhanced Raman spectroscopy probes”, N. Kumar, S. J. Spencer, A. J. Wain, D. Imbraguglio, A. Rossi, B. M. Weckhuysen, D. Roy, *Phys. Chem. Chem. Phys.* 2016, **18**, 13710-13716) The total yield of enhancing tips was increased up to 90 %, much beyond the original target planned at 75 %.

3.2.2 Reference sample for TERS contrast and spatial resolution (INRIM, NPL, REG(ETHZ))

The optical contrast is one of the most important parameters in TERS. It results from a series of tip-in and tip-out measurements and it allows us to characterise the quality of TERS tips. However, the values are currently measured incorrectly as they also take into account contributions to the measured signals coming from multiple reflections between the tip and the sample. This is the most likely reason which leads to inconsistent results when different measurements on equivalent samples are compared. In order to resolve this issue, which is critical in view of developing metrology for TERS, a reference sample based on a bilayer structure was developed by NPL. The sample has a top layer of ≈ 50 nm on top of a bottom layer of ≈ 350 nm with different characteristic Raman features. This structure ensures that during the TERS measurements the Raman signal from only the top layer is actually enhanced by TERS, whereas the Raman signal from both top and bottom layers is enhanced due to reflection from the tip-shaft (Fig. 7b). The Raman signal from the bottom layer is then used to eliminate the contribution of reflection in the measurements, allowing accurate characterisations of TERS tips.

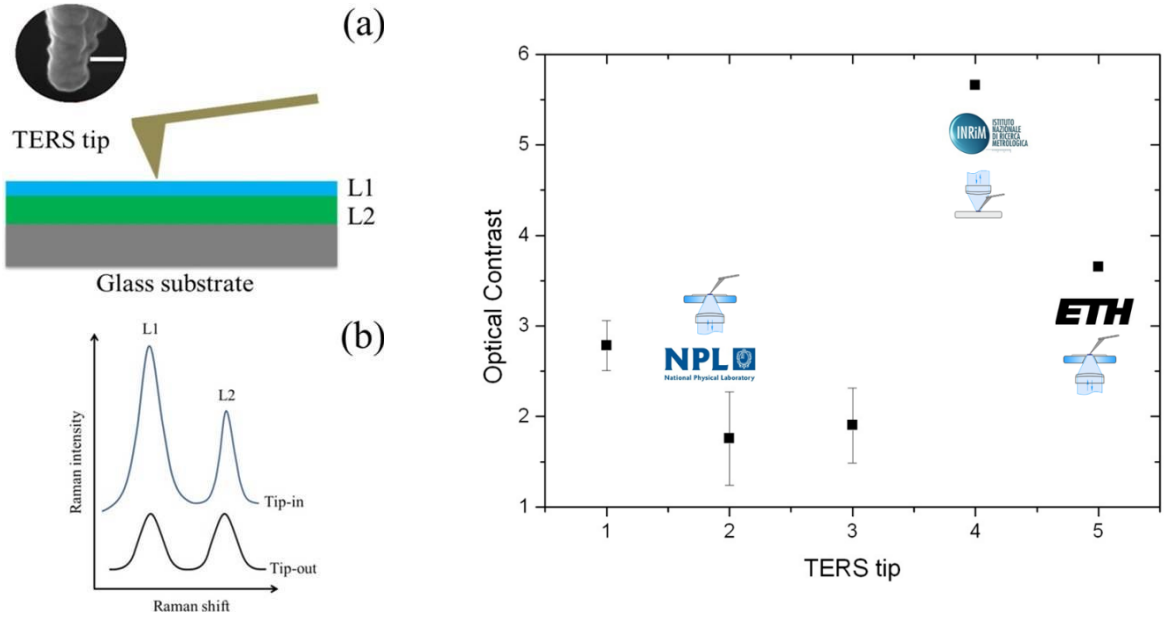

Figure 7: Inter-laboratory comparison on accurate measurements of TERS optical contrast using the bilayer reference sample developed at NPL (a). Compared to a monolayered structure, the bilayered sample allows the extraction of the real near-field contribution of the top layer from the total Raman signal of top and bottom layers (b). Correct inter-lab comparisons were enabled (right graph), the reference sample was tested with different TERS configurations/geometries: both inverted/transmission mode (NPL, ETH) and upright/reflection mode (INRIM)

During the project, reference samples with bi-layered structures were fabricated and detailed procedures for accurate measurements and correct evaluation of the optical contrast were developed. Samples and measurement protocols were distributed to the partners for inter-laboratory studies. The final results showed comparable values in terms of optical contrasts from different technological systems although some differences due to the particular configuration used for the experiment were found and highlighted. Accurate measurements allow accurate comparisons between tips, thus enabling the development of more and more performing TERS tips.

The major advantage of TERS is that it provides higher spatial resolution than micro-Raman spectroscopy. Therefore, the calibration of spatial resolution is of primary importance. In order to determine such parameters directly, namely without applying more complex deconvolution methods, the lateral dimension of the reference sample used for the measurement needs to be very small, even smaller than that of the TERS tip. A good candidate reference sample should also have strong and specific Raman signals to give reference for the optical contrast, one of the characteristic quantities used in TERS to assess the degree of enhancement. The sample volume under the TERS probe achieved is indeed of the order of 10^{-6} μm^3. Single-walled carbon nanotubes (SWCNTs) fulfil these requirements, and are well-known, studied materials in Raman spectroscopy. They can have diameters close to even 1 nm, which is ~ 10 times the diameter of a sharp TERS tip, but lengths in the micrometre range for easy detection by Raman spectroscopy. For these reasons, SWCNTs were selected as reference samples for measuring the spatial resolution and the optical contrast in TERS.

Since SWCNTs may occur in a variety of forms, depending on the method used for their fabrication, certified samples from NIST were chosen as starting materials for the fabrication of the reference samples. These SWCNTs have particularly well controlled diameters and defined lengths. An immobilisation procedure was developed in order to isolate only a few SWCNTs on different supports. The samples produced were distributed among the laboratories, these operate different TERS configurations, and an inter-lab comparison (Figure 7) on measurements of spatial resolution and optical contrast was undertaken by NPL, INRIM and REG(ETHZ). Figure 8 reports the most representative results of the work by showing parameters estimated from chemical images of an isolated SWCNT. The image obtained in high resolution TERS mode is compared with that obtained by conventional Raman spectroscopy.

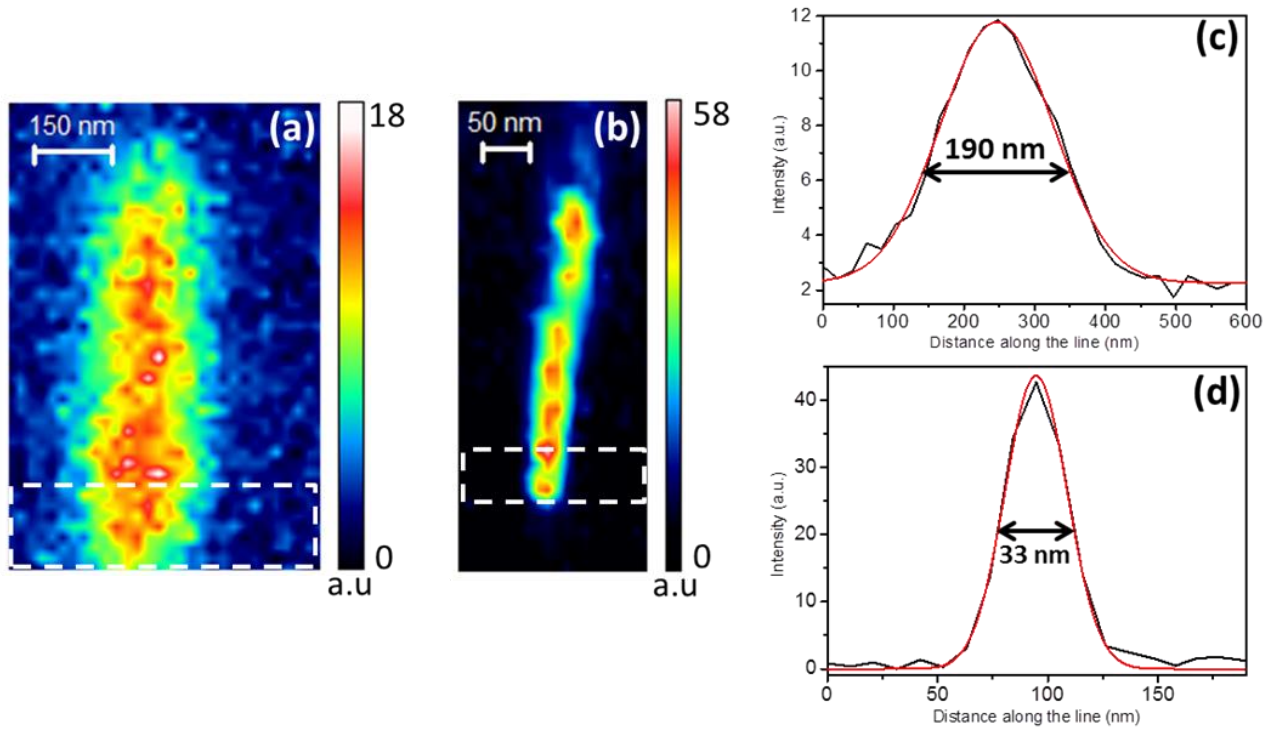

Figure 8: Chemical map of the same isolated SWCNT obtained by conventional confocal Raman spectroscopy (a) and TERS (b). The spatial resolution improves from 190 nm (c) down to 33 nm (d), respectively.

The final reference values established for the spatial resolution and the optical contrast in TERS using the SWCNTs samples were equal to (33 ± 1) nm and (7 ± 3) nm, respectively. Using these values, therefore, the SWCNTs samples can be used to calibrate all types of TERS instrument irrespective of the particular configuration used.

3.2.3 Localisation of biological macro-molecules on surfaces using TERS (INRIM, NPL and REG(ETHZ))

This field of application of TERS is quite new. Although some pioneering work with biological samples exists, there is no general agreement on the results reported in the literature, due to the complexity of the measurements and repeatability related issues. Biomolecules are indeed soft materials which can easily degrade under tight focusing of laser light such as in TERS, they may burn or stick to the tip, giving rise to inconsistent signals. On the other hand, in so far as chemical mapping is concerned, TERS would provide spatial resolution unattainable by other techniques. In this project, several biomolecules of interest were probed in order to obtain chemical information of biological structures at the nanoscale. In this project some biomolecules were engineered and modified to give a specific TERS response, whereas others were studied due to their importance for various clinical conditions, including cardiac disease and cancer. Figure 9 reports one of the best results obtained from sectioned cells at NPL. The nucleus is visualised in green colour, the surrounding cytoplasm in red. High resolution TERS imaging was obtained in the region delimited by the dashed square in the Raman map obtained through conventional confocal Raman spectroscopy.

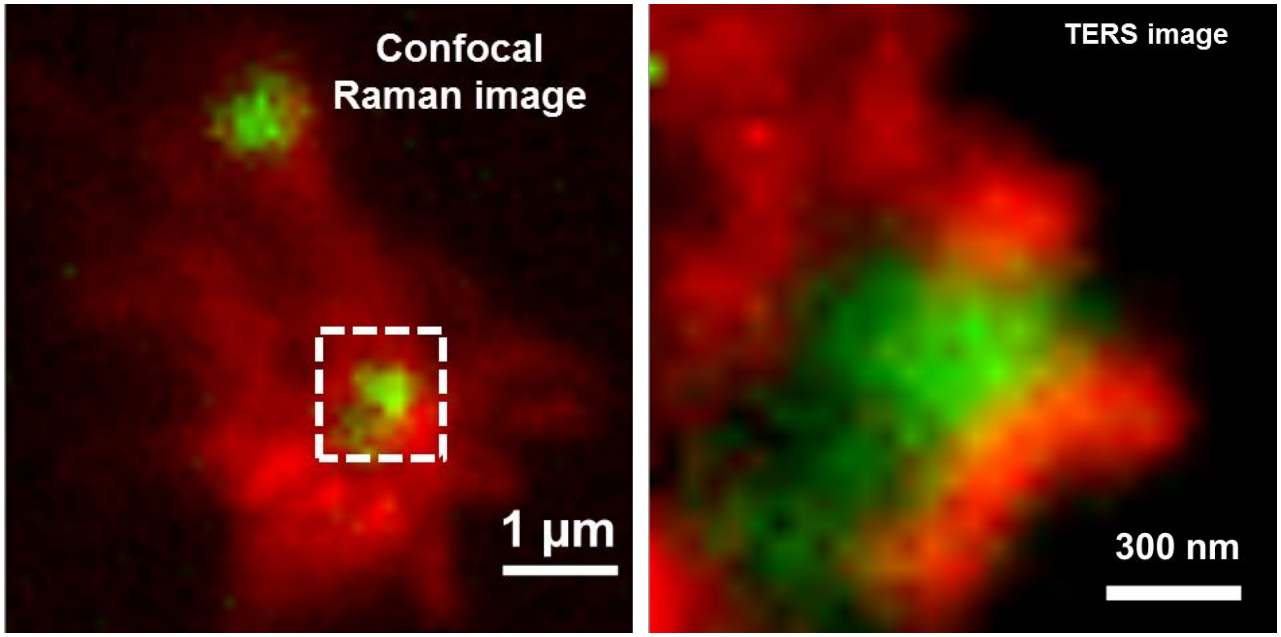

Figure 9: Raman imaging of sectioned cells obtained by conventional confocal Raman spectroscopy (left) and TERS (right).

Nucleus and cytoplasm are the two basic structures of eukaryotic cells. The ability to chemically distinguish one from the other, at high resolution by TERS, opens the door to the spectroscopic study of smaller subunits such as organelles, along with that of their specific functions within the cell.

3.3 Objective 3: Developing methods for 3D chemical imaging at high speed using multi-photon Raman scattering, Stimulated Raman Scattering (SRS) and Coherent Anti-Stokes Scattering (CARS)

3.3.1 Video rate Raman imaging set up to investigate metrology parameters (NPL, REG(KCL) and IISc)

High time resolution in imaging enables the measurement of dynamic phenomena and visualises the processes that are not possible by other means. Multiphoton Raman provides the opportunity to increase the speed of Raman imaging and gives an insight into the dynamic processes. Metrology parameters such as signal to noise ratio, spatial resolution in a mirror scanning system have not previously been investigated systematically. We have observed that the spatial resolution in a mirror scanning system depends on the location of an object in the sample.

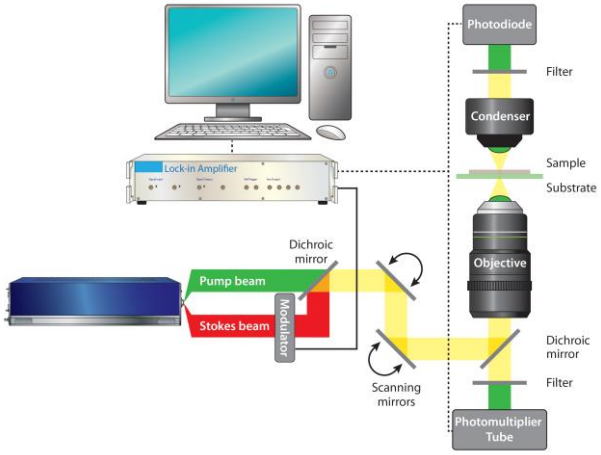

Figure 10: Schematic diagram of the coherent Raman imaging system used in this investigation.

Due to the multi-wavelength nature of Stimulated Raman scattering (SRS) microscopy, chromatic aberrations contribute significantly to the spatial resolution, confocal volume and signal intensity of a particular microscopy setup. The wavelength range typically used for SRS microscopy is 700 – 1200 nm. The requirement of diffraction limited performance across large fields of view (400 – 700 µm) places significant demands on the imaging optics. This issue is further compounded by the fact that 'home built' coherent Raman instruments typically employ poorly telecentric scanning systems (Figure 10) composed of two closely spaced galvanometer mirrors. The resultant movement of the beam at the back aperture of the objective leads to significantly non-uniform intensity across the scan field and a reduction in resolution.

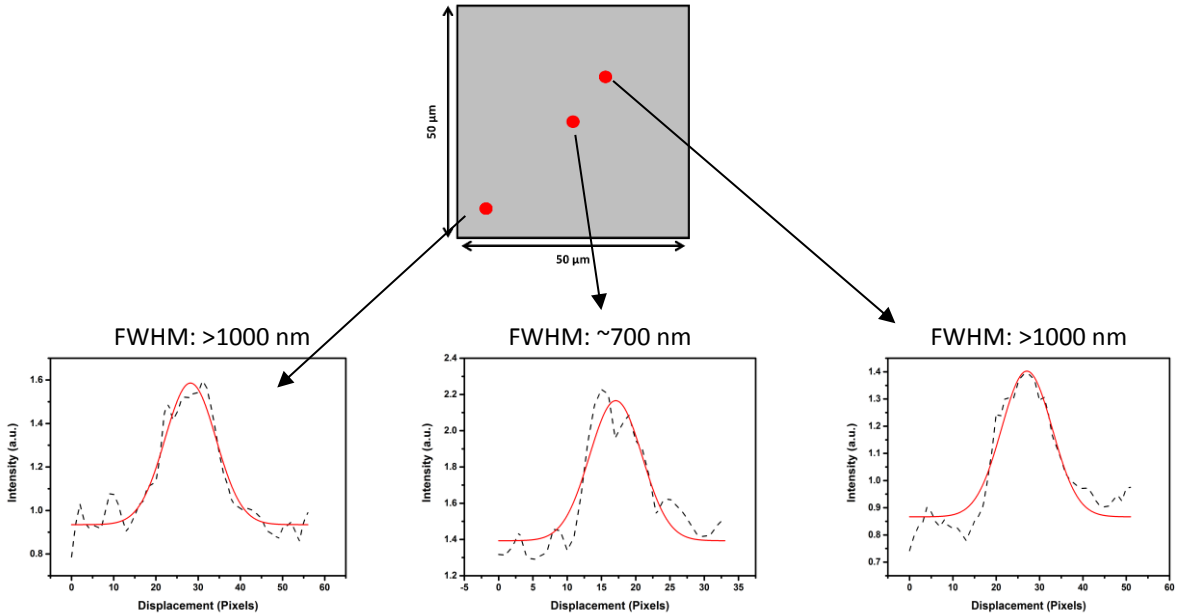

Figure 11: Variation of spatial resolution (FWHM of 100 nm polymer beads) within a field of view in a mirror scanning SRS microscope.

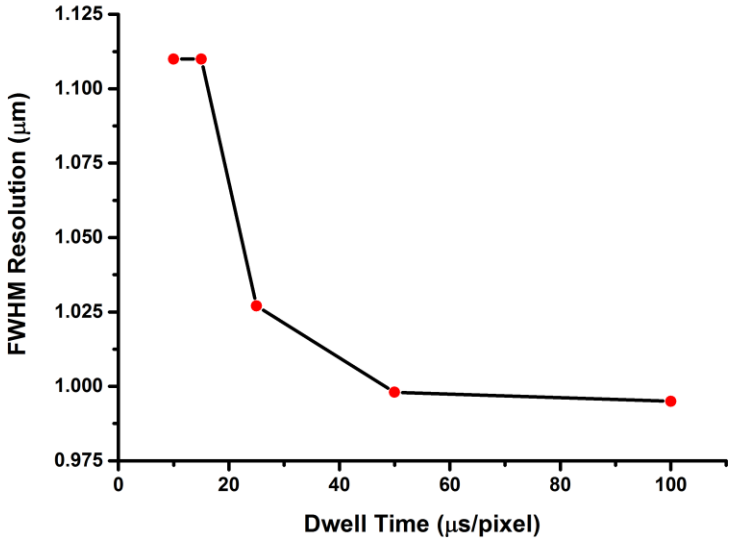

Figure 12: Variation of full width half maxima of a 100 nm polymer bead with imaging speed.

It is clear from the results presented in this report that the location of an object in the field of view can have a significant effect on the observed resolution of the system. It is also noted that this deviation in resolution is not uniform across the scan field, and is hard to predict and correct for.

A proposed solution to this issue is the implementation of a parabolic scanning unit. Parabolic mirrors are attractive for multiphoton imaging applications as they are immune to chromatic effects present in lens based scanning systems. Calculations show that, with the appropriate choice of scan and tube lenses, this setup should give close to diffraction limited performance across the whole field of view for IR corrected 20x or 60x lenses.

3.3.2 High speed Raman imaging of living cells (NPL)

Scientists involved in pharmaceutical drug discovery need a quantitative imaging technique that will locate the drug, the target, and ideally their interactions in a living cell. Currently, mass spectrometry is widely used for drug imaging in frozen cells in ultrahigh vacuum; however, quantification of such measurements is very difficult. In this project we aimed to quantify the amount of drug substance in living cells using advanced Raman scattering techniques such as stimulated Raman scattering (SRS) that will use near-infrared beams to image the cells with <1 µm spatial resolution without hampering the regular cellular functions. Furthermore, spectroscopic Raman technique can be used to confirm the measurements and co-localisation of the drug and the target from the Raman spectra of these species. The technique facilitates early detection of failure of a drug saving significant cost, and accelerate assessment of efficacy of drug uptake. An example image of a lypophilic drug, amiodarone, localised with lipid globule is shown in Figure 13.

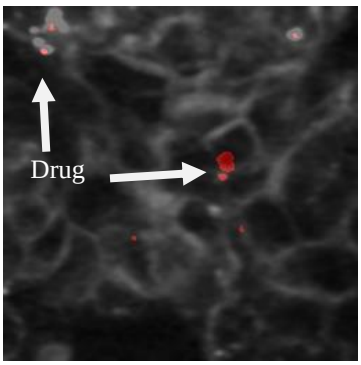

Figure 13: Lipid (white) and drug (red) distribution in a cluster of living cells measured at NPL using SRS microscopy. Cell membranes are visible as white boundaries.

Another study conducted in this project was on skin tissue to investigate the localisation of chemical species of interest such as oil, drug molecules or medium. Due to the ability of imaging at high speed, dynamic processes such as permeation through inter and intracellular channels, and crystallisation of active ingredients due to segregation from the media can also be studied. This capability is now being used to assist in developing skin-health products. An example SRS image showing the distribution of lipids in a section of untreated porcine tissue is shown in Figure 14.

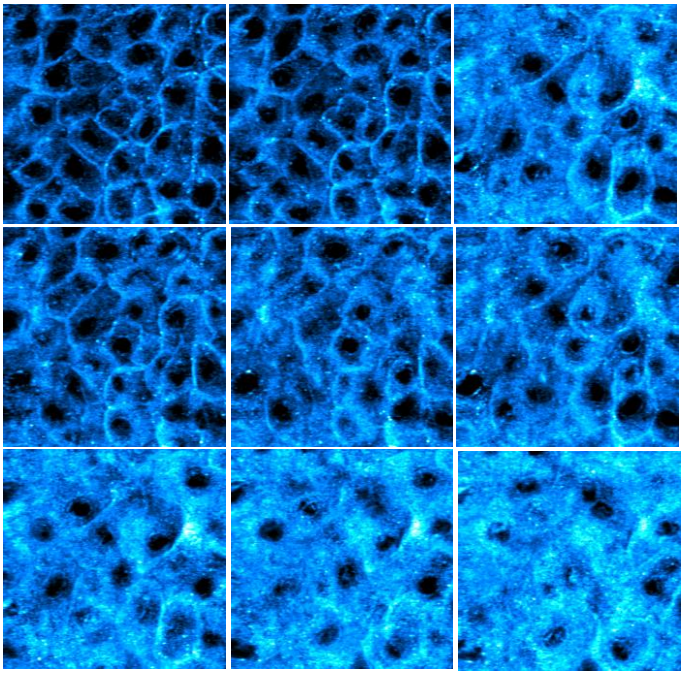

Figure 14: SRS images of pig-skin (size 80 µm ×80 µm) at different depths from the stratum corneum region towards the basal layer.

3.3.3 Confocal volume measurement reference method (NPL, KCL)

For quantification of the concentration of a substance traceable to the mole, it is essential to measure (a) the amount of substance (number of molecules) using an intensity vs concentration calibration curve, and (b) the volume of the probe i.e. confocal volume traceable to the SI unit. Many research groups make their calibration curve, however, there is no straightforward way of measuring the confocal volume in confocal Raman microscopy.

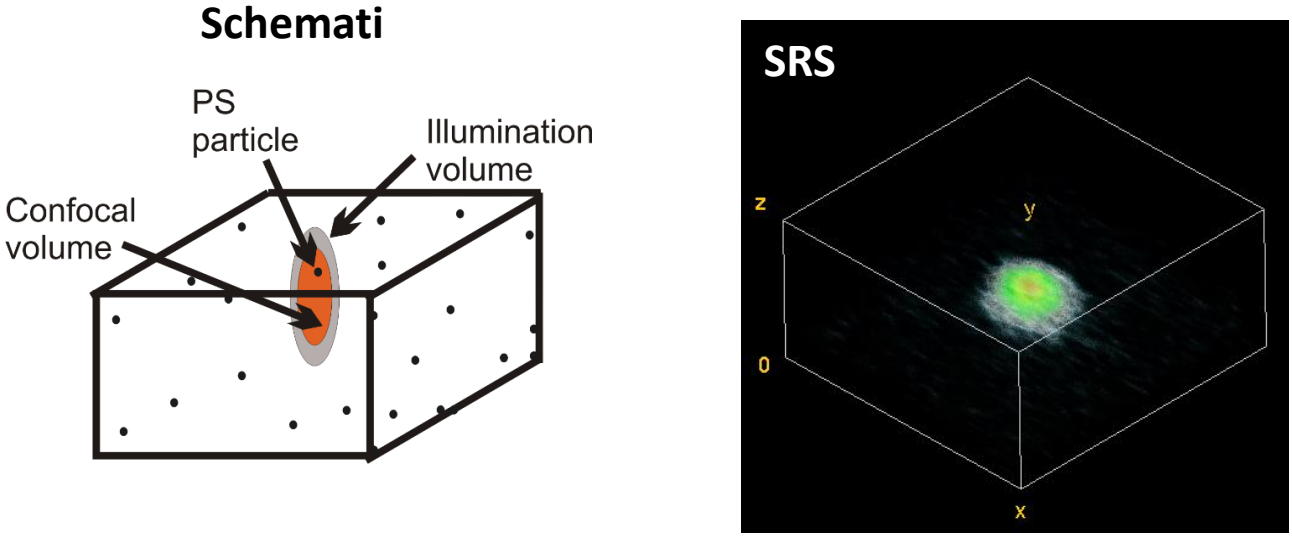

Figure 15: Schematic of a confocal volume measurement sample (left) and an example of a confocal volume measured using SRS microscopy. 100 nm polystyrene beads (NIST reference sample) were used for the measurements.

From the 3D image stack obtained in NPL, we can calculate the confocal volume probed by our SRS microscope. Using the image stack presented above (Figure 15), this value is calculated to be 0.451 f with uncertainties of <10% arising from image analysis, variance in the size of the bead and the accuracy of the stage.

3.3.4 3D modelling software package (CMI, NPL, PTB, INRIM and REG(ETHZ))

Raman scattering is a complex process influenced by many factors such as local sample morphology and chemical composition. Besides experimental development, it is therefore important to develop the theoretical and numerical means for the characterisation of all the phenomena connected with local field interactions with micro and nanostructure. The project concentrated on creating a set of tools for numerical modelling of light propagation in various geometries related to Raman measurements. For most of the calculations a fast graphics card based Finite Difference in Time Domain solver was adapted and further developed. In particular we have built numerical approaches to include different imperfections, like surface roughness and to be able to treat such roughness in a statistical way. This is an important step for estimating the uncertainty related to measurements in real conditions, where different structural imperfections, impurities and inhomogeneities significantly affect the measurement data.

The following experimental conditions were considered during modelling and related software development:

- ✓ SERS substrates efficiency and its relation to the shape of morphological features forming the substrate.
- ✓ Micro Raman focal volume changes while scanning on inhomogeneous samples.
- ✓ TERS enhancement factors for tips made out of different materials and layers.
- ✓ Effects of probe and surface roughness on TERS enhancement factors.
- ✓ Performance of TERS probes on samples developed within the project (e.g. bilayer reference sample).

For all these calculations, spectral dependences of the appropriate quantities were obtained using a graphics card based high performance computing device or a conventional high performance computing system. The software used for all the calculations was an open source package Gsvit (http://gsvit.net, illustrated in Figure 16). Besides general improvements in the software performance, the following numerical algorithms and approaches were developed or implemented:

- Surface roughness generation based on simulated ballistic deposition with limited relaxation.
- Piecewise linear recursive convolution approach for metals treatment.
- Tetrahedral mesh, Gwyddion surface and structured mesh VTK format loading.
- Computational model preparation from scanning probe microscopy (SPM) data for simulated scanning.
- Focused source handling infinitely extending layers and suitable for scanning.

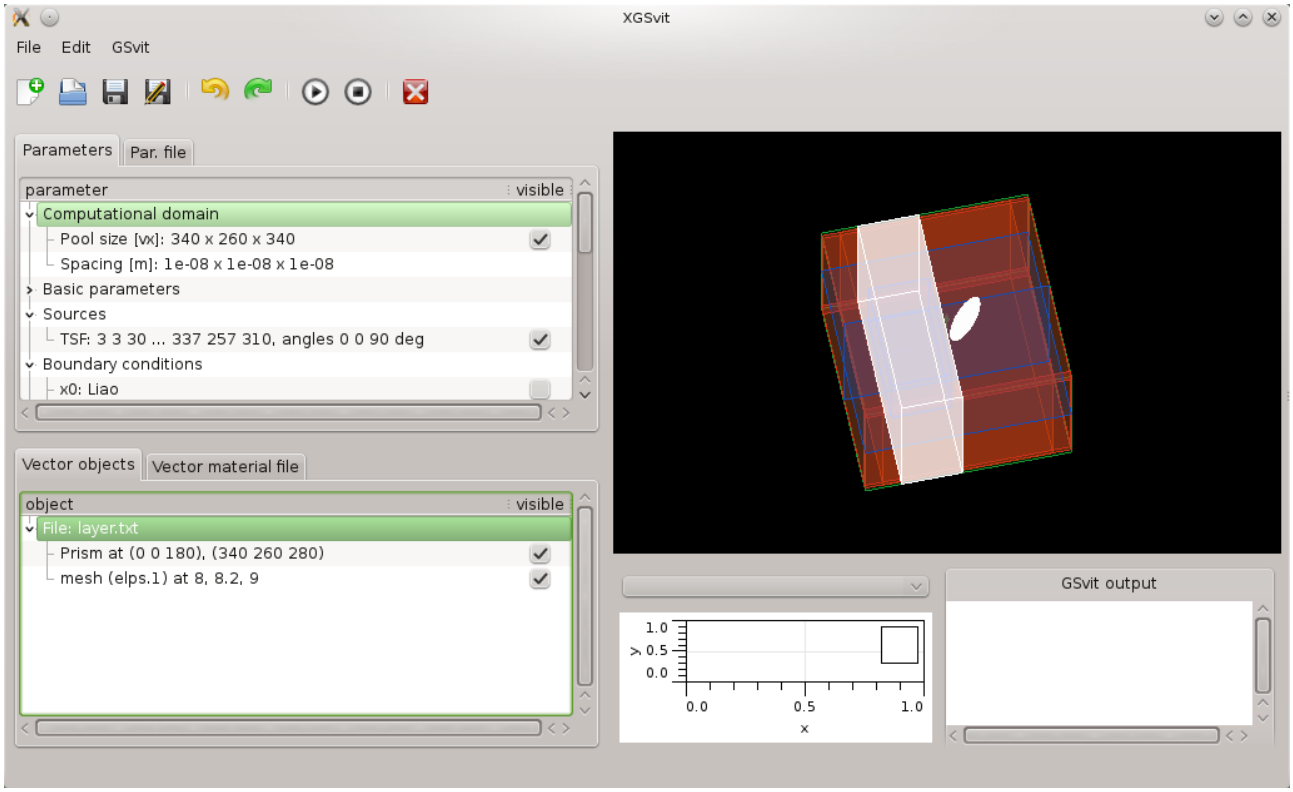

Figure 16: Snapshot of user interface from GSVIT software.

3.3.5 Confocal volume calculation (CMI)

As an example of the problems solved, in Figure 17 there is a set of simulations for confocal volume calculation for a focused beam illuminating various surfaces. As we can scan with the focused beam and create virtually any sample composition, we can map the confocal volume changes across the sample. The significance of this capability is in measurement of inhomogeneous samples such as a cell where different organelles have different refractive indices and therefore distorts the confocal volume which in turn adds uncertainty in quantification.

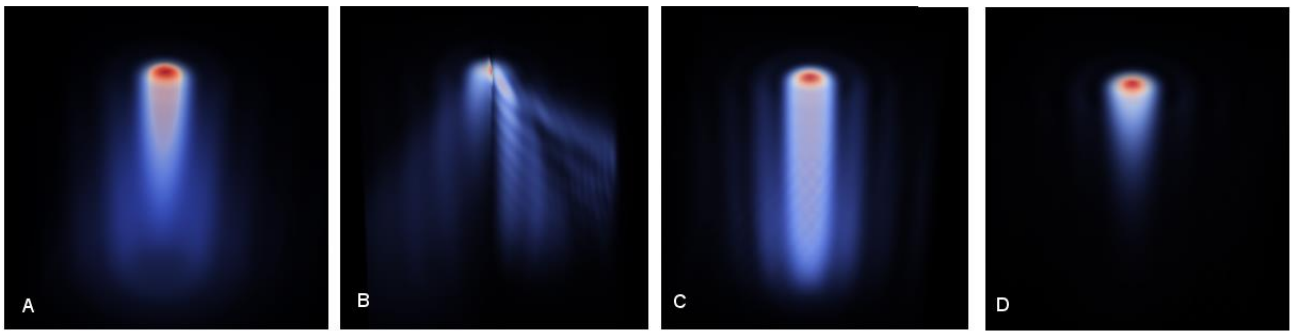

Figure 17: Time averaged absorption of a focused beam hitting different media: A) low refractive index low absorption half space, B) edge of a glass cube in low absorption half space, C) glass cube, D) highly absorbing medium. Note that the false colour scale is not the same, for better visibility.

3.4 Objective 4: Investigating light-matter interactions in Raman scattering using finite difference time domain (FDTD) calculations, to establish mathematical models to underpin traceable measurements

3.4.1 Near-field interaction in TERS measurements: (CMI, INRIM, NPL, REG(ETHZ))

The aim of this task was to study the composition and geometries of different TERS tips, including material or geometrical imperfections, and their effects on field enhancement. This was undertaken to support TERS experiments carried out.

To simulate electromagnetic field distribution in the sample or tip-sample region we used a Finite Difference in Time Domain (FDTD) method, which is a universal tool used in many different areas of numerical electromagnetics. FDTD is able to solve Maxwell equations in a small volume only (each side corresponding to a few tens of wavelengths as a maximum), however it can treat many different materials, sample geometries, incident and boundary conditions.

Most of the calculations were performed to support TERS experiments. An ideal numerical model for a TERS experiment interpretation is shown in Figure 18; it includes different media and imperfections. To approach the computation capabilities to such a model we have developed tools for roughness treatment, metallic materials handling and scanning with a focused beam. Numerical experiments were used in particular to assist with prediction of experimental results at different configurations and probe/sample geometries, like the bilayer sample performance shown in Figure. 19.

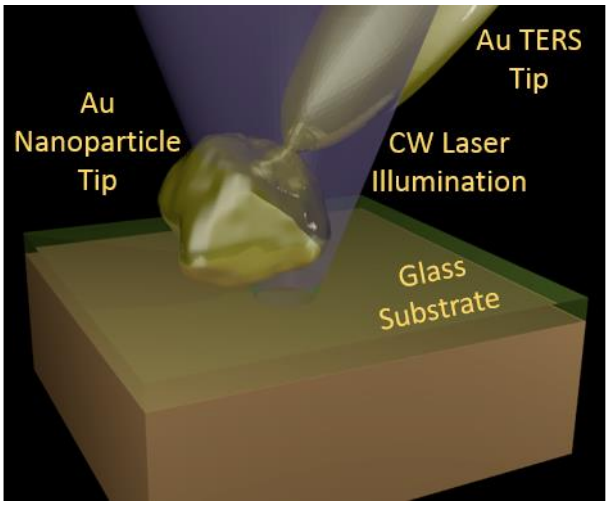

Figure 18: Simulation of a TERS tip in contact with a gold nanoparticle illuminated by a continuous wave laser. The gold nanoparticle resides on a glass substrate

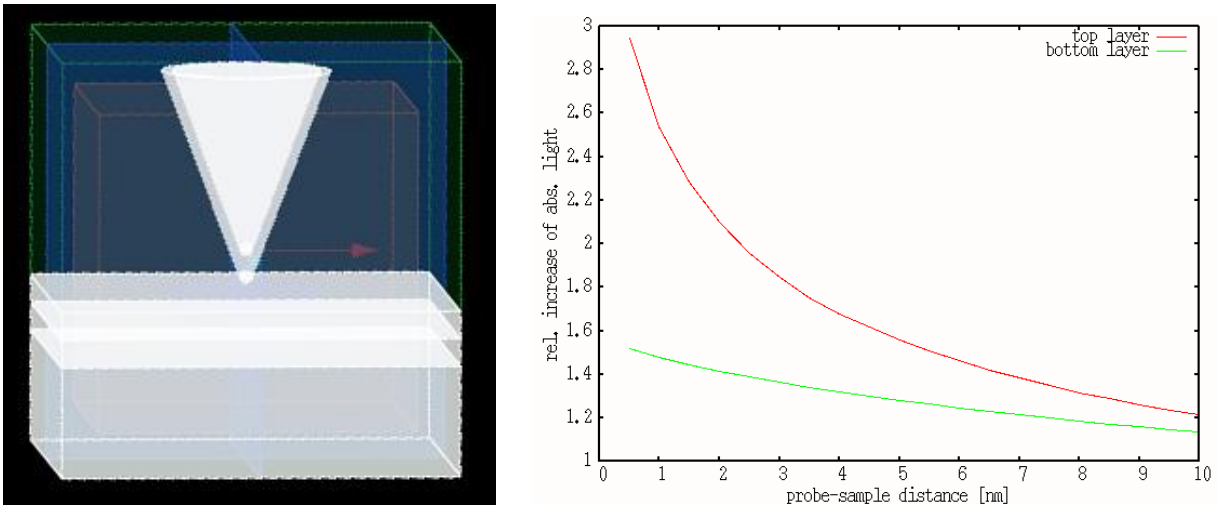

Figure 19. (Left) numerical model for bilayer sample calculations, (right) field enhancement dependence on probe-sample distance.

The simulations were carried out by CMI, but there was a strong collaboration between CMI (numerical modelling and software development), NPL and INRIM (experimental setups and measurements); none of the results could be obtained without this collaboration as this shared expertise had to be used to combine the experimental and numerical approach.

3.5 Objective 5: Improving the repeatability of Raman measurements and establishing robust uncertainty budgets for Raman spectroscopy (NPL)

Stimulated Raman scattering (SRS) microscopy holds significant potential as a robust and traceably quantitative chemical spectroscopy tool. In addition to the determination of the confocal volume (3.3.3) required to relate the signal observed to the number of molecules of a species present in the sample, a robust understanding of the sources of uncertainty within the SRS signal itself is also crucial.

To evaluate the uncertainty in the SRS signal obtained from a sample, an uncertainty budget was constructed for the SRS microscope at NPL. This uncertainty budget takes into account the key sources of uncertainty arising in a single channel SRS system. For the SRS microscope established at NPL, these sources of uncertainty where determined as; laser intensity noise, thermal drift of the laser delay line, tuning/temperature instability of the optical-parametric oscillator, current/voltage noise of the photodiode detection circuit and non-chemically specific non-resonant background signals arising from other non-linear optical processes in the focal volume. For the measurement of the polystyrene bead confocal volume reference sample, the expanded uncertainty (k=2, 95 %) was determined to be less than 10 %.

For the non-linear optical microscope at NPL, the largest source of uncertainty was the laser intensity fluctuation and OPO stability, however, there is scope to improve these parameters with better thermal control of the laboratory. Although this uncertainty budget is tailored specifically to the instrument at NPL, it provides a platform which can be adapted to other non-linear optical microscopy systems employing similar detection schemes to NPL's non-linear optical microscopy system.

3.6 Summary

As a result of the project, a new measurement infrastructure on Raman spectroscopy is now in place in European NMIs (NPL, CMI, INRIM, PTB), and strategic collaborations have been developed with external NMIs (e.g. Inmetro).

This project has set up new capabilities on:

- Video rate Raman imaging in NPL. The real time spectral imaging capability of this technique will enable better understanding of the kinetics of drug diffusion in biological systems such as cells and tissues. Initial work carried out in this area has already attracted significant interest within the tissue imaging field.
- New TERS instrumentation in INRIM. This forms a unique national capability and places INRIM at the forefront of nano-optics research in Italy.
- Low cost simulation capability in CMI. This is a huge step forward in bringing immensely powerful Finite-difference time-domain (FTDT) simulations to everyday users. Typically these simulations have been limited to dedicated supercomputing clusters. This new software, which runs on ordinary desktop PCs, will be an incredibly powerful computation tool for not only people working in TERS but across the field of optics
- Quantitative measurements in PTB. Uncertainties around the values measured by Raman depth profiling have been a major limitation in establishing it as a robust technique for the analysis of industrially relevant samples such as multi-layered polymer structures and tissue samples. The reference samples, software tools and uncertainty budgets established by PTB will enable confidence in Raman spectroscopy as a robust tool for depth profiling organic materials.

A commercial partner is in the process of commercialising TERS probes developed in the project.

Reference samples and methods have been developed for:

- confocal volume,
- depth profiling,
- enhancement factor and
- resolution measurements

These will accelerate further growth in application of Raman spectroscopy in materials science, clinical diagnosis and nanotechnologies.

Strong momentum has been gained for the first time on developing standards for Raman spectroscopy. A CCQM study is in progress and new projects for VAMAS studies are being proposed. All the NMIs interested in Raman spectroscopy are together in this journey to establish the quantitation and reduce uncertainties.

4 Actual and potential impact

Dissemination of results

At the beginning of the project awareness of metrology in the fields of Raman spectroscopy was very limited. Significant effort has been made to bring this awareness through conference presentations and publications. 15 papers have been published in international journals and 43 conference presentations have been delivered. Effort was focused on bring this awareness through conference presentations and metrology sessions at the largest international conference on Raman spectroscopy (ICORS). These sessions have been attended by more than 50 people and increased the awareness of metrology in Raman spectroscopy. The project consortium led the metrology discussion in the following conferences:

- International Conference on Raman Spectroscopy, Bangalore, 2012
- International Conference on tip-enhanced Raman Spectroscopy, Zurich, 2013
- International Conference on tip-enhanced Raman Spectroscopy, Rio, 2014
- International Conference on Raman Spectroscopy, Jena, 2014

- International Conference on Tip-enhanced Raman spectroscopy, Osaka, 2015
- SPM workshop in the Czech Republic, 2015

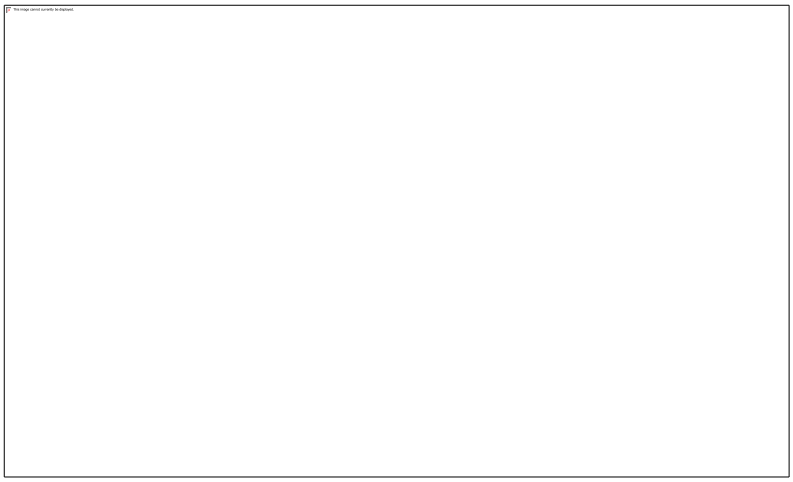

Figure 20: Attendees of the International Conference on Tip-enhanced Raman Spectroscopy (TERS) in Osaka 2015

In addition to this, an article was written by NPL on the subject of Quantitative Raman spectroscopy in 'The Analytical Scientist' a well-read online trade journal relevant to the industry.

Impact on standardization

Before this project there were no internationally accepted standards for Raman spectroscopy. The project has created the infrastructure for Raman metrology research through collaboration between European and international partners. Towards the end of the project, 13 metrology institutes and 4 instrument manufacturers (Figure 21) have come together to continue to address the key metrology issues in Raman spectroscopy. Representative institutes include NPL (UK), INMETRO (BR), NIST (USA), INRIM (IT), PTB (DE), KRISS (KOR), NMIJ (JP), NRC-CNRC (CA), NMI (CN), IMRE (SG), CENAM (MX), Thales Group (FR). Instrument manufacturers include Renishaw, Horiba, Thermo Fisher, Bruker Optics.

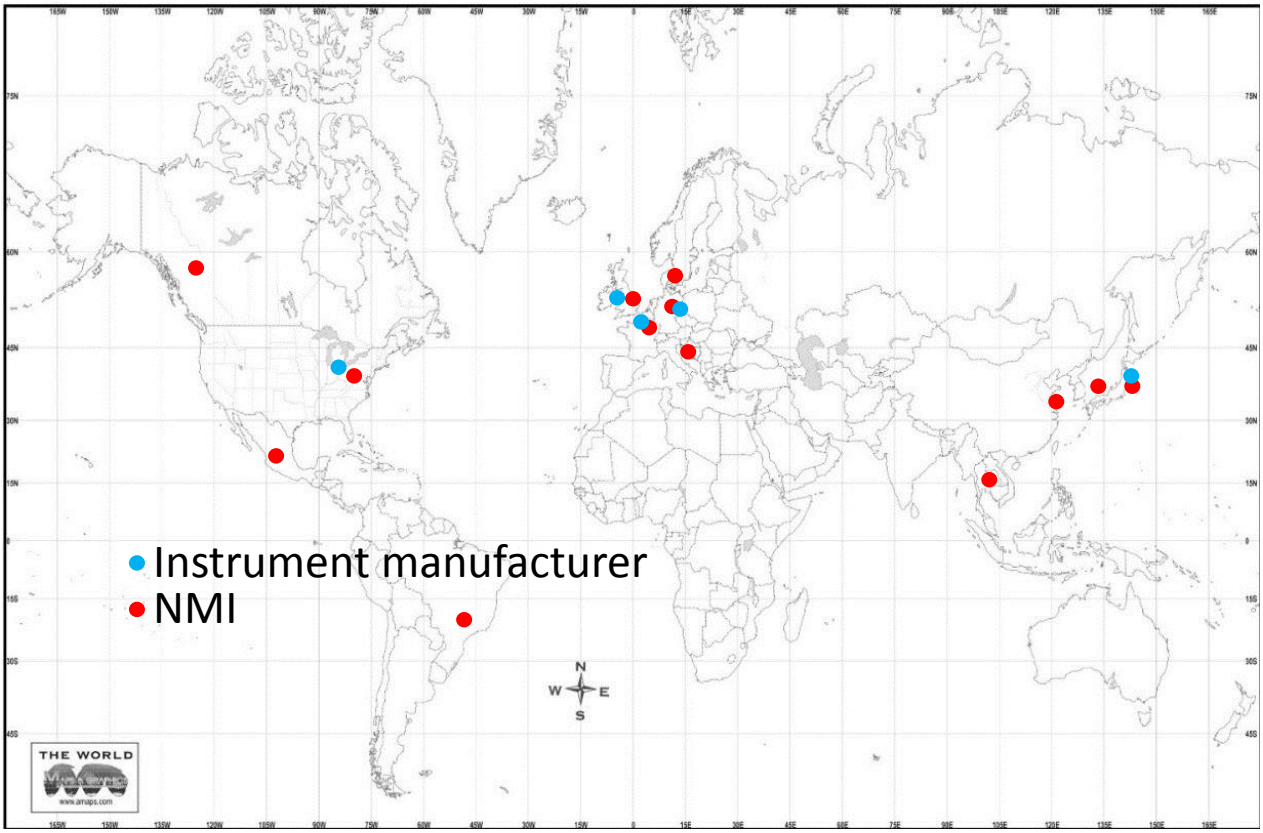

Figure 21: International initiative to develop standards on Raman spectroscopy in collaboration with instrument manufacturers

Although there are not yet any internationally accepted standards for Raman spectroscopy, 5 potential standards (reference standards and methods) have been identified for prenormative study with the aim of developing ISO standards. A BIPM Consultative Committee (CCQM) pre-pilot study is in progress.

A number of prenormative studies has been planned to carry out through CCQM and VAMAS.

- Confocal volume measurement in Raman spectroscopy has already started as a preliminary study within CCQM. The outcome of this study will be facilitating quantification of substance traceable to the mole.
- The study outcome on Raman depth profiling has also triggered follow-on activities in this area and the consortium members have decided to continue co-operation for the development of a confocal volume standard for Raman microscopy. In the framework of the metre convention (CCQM working group on surface analysis, SAWG) a pilot study on 3D Raman imaging will be organized and carried out.
- Progress beyond the present state of the art has been achieved in the quantification of large biomolecules by means of ID-SERS. A distribution to the wider community will be achieved via presentation of the results in the CCQM working group on protein analysis (PAWG) in which consortium members (PTB) are engaged. Validation of this methodology by participation in international comparison schemes will lead to the acceptance of the method as a higher-order reference measurement procedure followed by integration in the publicly accessible JCTLM database on laboratory medicine and in-vitro diagnostics maintained by the BIPM.
- A new Technical Work Area (TWA) is being formed in VAMAS to carry out the prenormative studies of existing in-house standards used by instrument manufacturers.

Early impact

The methods developed in this project have ensured that RS is currently the most attractive and viable label-free technique for quantitative imaging of molecules within their native environments, and work has already begun to adapt RS to new applications.

The video-rate Raman imaging microscope at NPL, UK, is being used to develop measurement solutions for healthcare and pharmaceutical companies. For instance, the facility is being used to investigate UV damage of samples of living-skin-equivalence to assess the stability of a topical cream formulation in its native state, and drug uptake in living cells. A UK skin health company has estimated that their uptake of this technique could generate a 20 % cost saving throughout the development cycle of a new skin care product. Since the beginning of the project, the facility has provided measurement services worth more than £50k.

The greatest barrier to the more widespread adoption of TERS is the lack of reliable and clearly defined TERS tips. Some of the issues in the batch production of TERS tips were resolved in this project through new tip designs and production methods. One such method has been adopted for commercial production by the Natural and Medical Sciences Institute, a medical science institute at the University of Tübingen, with the aim of producing and selling standardised tips.

The chemometric software developed for large-scale Raman data analysis, is a powerful new tool for analysing Raman images of complex surfaces. The software can process millions of spectra with a standard desktop PC, and is being further developed for implementation by a commercial instrument manufacturer.

Potential future impact

Before this project RS measurements were qualitative. But the techniques and infrastructure developed here allow RS to be used to make quantitative measurements of target molecules, traceable to SI unit definitions. This accomplishment will enable RS to be refined and adapted to develop new products and capabilities in a range of industries and scientific fields. In the short-term, the techniques developed are currently being used to develop skincare products, commercialise the production of TERS tips, and to develop new software and new measurement capabilities throughout Europe. Over the longer term we anticipate that the use of RS will proliferate further, into new areas of research and new industries, from the identification of microbes in soil samples in environmental science, to detecting the presence of burgeoning tumour cells in the fight against cancer. The use of RS for generating real-time images of processes occurring within biological samples, such as living cells, is especially valuable, and demonstrates the potential power of the technique, and gives an insight into its potential future uses, from medical diagnostics to exploring the interactions between biology and nanotechnology.

5 Website address and contact details

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6 List of publications

1. The Use of Raman Spectroscopy to characterize the carbon materials found in Amazonian anthosoils. J. Ribeiro-Soares, LG Cancado, NPS Falcao, Erlon HM Ferreira, CA Achete, A Jorio **Journal of Raman Spectroscopy** 16/10/2012, DOI: 10.1002/jrs.4191
2. "Tip-enhanced Raman Spectroscopy –An Interlaboratory Reproducibility and Comparison Study. Carolin Blum, Lothar Opilik, Joanna M. Atkin, Kai Braun, Stefan B. Kämmer, Vasily Kravtsov, Naresh Kumar, Sergey Lemeshko, Jian-Feng Li, Karol Luszcz, Teimour Maleki, Alfred J. Meixner, Steve Minne, Markus B. Raschke, Bin Ren, Jan Rogalski, Debdulal Roy, Bruno Stephanidis, Xiang Wang, Dai Zhang, Jin-Hui Zhong and Renato Zenobi. **Journal of Raman Spectroscopy (JRS)** January 2014, Volume 45, pp 22-31 DOI: 10.1002/jrs.4423
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4. Spectral Interferometric Implementation with Passive Polarization Optics of Coherent Anti-Stokes Raman Scattering. Bradley Littleton, Thomas Kavanagh, Frederic Festy and David Richards **Physical Review Letters** September 2013, Volume 11, pp 103902-0 to 103902-05 DOI: 10.1103/PhysRevLett.111.103902
5. Validation of isotope dilution surface-enhanced Raman scattering (IDSERS) as a higher order reference method for clinical measurands employing international comparison schemes. Sabine Zakel, Stefan Wundrack, Gavin O'Connor, Bernd Güttler, Rainer Stosch **Journal of Raman Spectroscopy** September 2013, Volume 44, Issue 9, pages 1246–1252. DOI: 10.1002/jrs.4349
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9. Nanoscale optical spectroscopy: an emerging tool for the characterization of graphene and related 2-D materials. Andrew Pollard, Naresh Kumar, Alasdair Rae, Sandro Mignuzzi, Weitao Su, Debdulal Roy **J. Mat. NanoSci.** 2014, 1(1), 39-49
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12. Multimodal optical characterisation of collagen photodegradation by femtosecond infrared laser ablation. Manickavasagam, A., Hirvonen, L., Melita, L. N., Chong, E. Z., Cook, R., Bozec, L. & Festy, F **Analyst** (2014) 139 (23), 6135-6143
13. Autocorrelation measurement of femtosecond laser pulses based on two-photon absorption in GaP photodiode. Chong, E. Z., Watson, T. F. and Festy, F **Applied Physics Letters** (2014) 105 http://dx.doi.org/10.1063/1.4893423
14. Multiphoton luminescence imaging of chemically functionalized multi-walled carbon nanotubes in cells and solid tumors. Rubio N, Hirvonen L, Chong EZ, Hassan H, Wang JTW, Bourgognon M, Kafa H, Al-Jamal W, McCarthy D, Hogstrand C, Festy F and Al-Jamal KT **Chemical Communications** (2015) 51, 9366-9369
15. An MMP-inhibitor modified adhesive primer enhances bond durability to carious dentin. Almahdy, A., Koller, G., Festy, F., Bartsch, J.W., Watson, T.F., Banerjee, A **Dental Materials** (2015), DOI:10.1016/j.dental.2015.03.003