Final Publishable JRP Summary for NEW02 Raman
Metrology for Raman Spectroscopy

Overview

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.

Need for the project

Over the last three decades the use of RS has flourished, and today the technique is used in a range of European industries, including pharmaceuticals, biotechnology, nanotechnology, healthcare and medical technology. Although the use of RS is widespread, 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.

RS is used to identify specific molecules in samples, such as proteins that indicate the presence of cancer in biopsies, or pollutant chemicals in air samples. RS is popular as the technique identifies molecules quickly, including molecules that alternative techniques find difficult to identify; is non-destructive, the sample is left undamaged and can be used again; and is label-free, RS doesn't require the use of additional label molecules to identify targets, which can affect the sample and compromise results. Although the technique has considerable advantages, it was used qualitatively rather than quantitatively, to identify the presence of a target molecule, but not amounts of that molecule. If the appropriate measurement infrastructure were provided, RS could be developed from a qualitative to a highly-effective quantitative technique.

Scientific and technical objectives

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.

EMRP
European Metrology Research Programme
Programme of EURAMET

The EMRP is jointly funded by the EMRP participating countries within EURAMET and the European Union

Results

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

Methods were needed to trace RS measurements to the definition of the mole, the SI unit of the amount of a substance. Key to this was the development of a standardised procedure for Raman depth profiling, the identification of target molecules at depth below the surface of a sample. The objective aimed to develop such methods for conventional RS, and for surface-enhanced Raman scattering (SERS), an RS method with the highest chemical detection sensitivity.

A standardised depth profiling procedure was established by developing reference samples, and then reference procedures to calibrate measurements for these samples. Measurements could be made with nearly identical results if only a few key experimental parameters were kept constant. This outcome demonstrated that SI-traceability and comparability of Raman depth-profiling results can be achieved if suitable reference standards are made available to Raman instrument manufacturers and end users.

This objective was then achieved through the development of two different approaches for quantifying amounts of biomolecules:

1. Direct labelling, by attaching a Raman active molecule (reporter), in a natural and isotopically labelled form, to the biomolecule. Due to the high Raman scattering efficiency of the reporter molecule, a limit of detection in the low nmol L^{-1} range was achieved, providing an excellent basis for the quantification of extremely low target molecule concentrations.

2. Indirect labelling, by attaching a reporter molecule with an antibody-joined magnetic nanoparticle to the target molecule. The advantage of this approach is that the magnetic nanoparticles can be used to separate the target molecules from complex samples, such as blood serum. A measurement method was successfully developed for determining amounts of target molecules, including a specified detection limit.

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)

TERS combines Raman spectroscopy with atomic force microscopy (AFM) to offer nanometre-scale spatial resolution for RS. To ensure 2D spatial/distance measurement in TERS are sufficiently accurate, techniques were needed to trace measurements to the SI base unit of length, the metre. Before this project, TERS suffered from a lack of defined and reproducible tips (the probes used to analyse samples), as tips could not be produced consistently to the required dimensions and with the required properties. Key to this objective therefore, was the development of a reliable method to produce high-quality tips with defined properties.

Bi-layered reference samples were fabricated with defined optical and spatial properties, confirmed via inter-laboratory comparisons. The performance of various TERS tips could be assessed with the reference samples to identify the limitations of the different tips, to provide guidance on how tip quality could be improved. This new measurement capability enabled tips to be accurately assessed and manufacturing problems to be identified and resolved, such as the tip radius and chemical and physical robustness. As a result, the yield of useable tips fabricated in the project was improved to 90%, far beyond the planned target of 75%. One of the project's improvements in batch production is being adopted for commercialisation.

The new TERS capability found that the resolution of TERS measurements were limited by the size of the TERS tips (smaller tips produced finer resolution images). To establish the dimensions of the tips with sub-nanometre accuracy, a measurement method was created using single-wall nanotubes. This procedure, couples with high-quality tips, allowed sub-nanometre spatial and depth measurements to be made, traceable to the meter.

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)

Multi-photon RS techniques, such as SRS and CARS, have the potential to become quick and accurate diagnostic tools, as they are highly-sensitive approaches for fast, label-free mapping of chemicals in three dimensions in biological samples. The aim of this objective was to realise the potential of multi-photon RS by developing methods to make measurements quantifiable.

The result was achieved through the development of a stimulated Raman scattering microscope that was capable of providing high-speed, label-free quantitative images from biologically relevant samples. A chemometric software tool was developed for the analysis of a large (>1M) numbers of Raman spectra for chemical imaging. The technique was used to make successful quantitative measurements, measuring chemical concentrations to within an error of 7%, including the measurement of drug concentrations in cells (the actual dosage of a drug a patient receives is partly determined by how efficiently cells absorb the drug molecules. This was previously notoriously difficult to determine with any speed).

Software was also developed for SRS and CARS to display Raman imaging as video (at a frame-rate of a few frames per second). This powerful video-rate tool allows real-time images to be produced of chemical and biological processes such as phase separation, permeation and diffusion of molecules within living cells. This capability has since been used to study oxidative stress in living skin tissue, the distribution of drugs (ibuprofen) and permeation of propylene glycol (PG) in skin, and drug metabolism.

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

RS measurements are influenced by a range of factors, such as local electromagnetic fields, and the dimensions and chemical composition of samples. In addition to developing experimental techniques, numerical approaches were also needed that could model and account for the effects of all variables that influence measurements – the aim of this objective was to develop modelling methods to support the accuracy and traceability of the methods developed in objectives 1 – 3, with a focus on biological samples.

A set of numerical tools were successfully developed to support TERS and micro-Raman measurements, based on Maxwell's equations solver (FDTD technique). Software for the simulation of scanning by a micro Raman instrument has also been developed.

5. Improving the repeatability of Raman measurements and establishing robust uncertainty budgets for Raman spectroscopy

The goal of this objective was to ensure that quantitative RS measurements can be made repeatedly and reliably across academic fields and industries with established levels of uncertainty. Reference techniques were developed from the results of objectives 1 to 4, and reference samples upon which the techniques can be calibrated.

Four standardised quantitative techniques were developed for both commercially available and emerging RS methods. Three inter-laboratory studies were conducted. Two new reference samples have been developed for quantitative RS measurements, including a confocal volume standard, and a depth-profiling standard for confocal Raman spectroscopy. A BIPM Consultative Committee (CCQM) pilot-study is being conducted on the confocal volume standard.

Overall, the project results improve the levels of confidence in quantitative, label-free measurements using RS techniques of biomolecules, and other substances, at concentrations ranging from nano-molar to milli-molar, and spatial resolution from 20 nm to micrometres. The new video-rate Raman imaging capability, developed in objective 3, now allows the investigation of dynamic processes in complex mediums, such as living cells. These new National Measurement Institute (NMI) capabilities are the first of their kind in European NMIs, and can be accessed by industrial users to make quantitative RS measurements such as studying the absorption and metabolism of drugs in living cells.

Actual and potential impact

Dissemination of results

Awareness of metrology in RS fields was previously very limited, and significant efforts have been made to increase awareness of the project's results. 15 papers have been published in international journals (listed in the next section), and 43 conference presentations have been delivered. In addition, a Metrology Session was started in the largest international conference on Raman spectroscopy (ICORS); these sessions have been well attended and are vital for increasing awareness of metrology in RS. A dedicated website has also been set up to continue the activities of Raman metrology in collaboration with the international community.

Impact on standardization

Before this project there were no internationally accepted standards for Raman spectroscopy. 5 potential standards (reference standards and methods) have been identified in this project for pre-normative study, with the aim of developing these into ISO standards. A BIPM Consultative Committee (CCQM) pre-pilot study is in progress.

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 the National Physical Laboratory, 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. This has generated a 20% cost saving for product testing. 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 in objective three, 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.

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
3. Prospects of the emerging Raman scattering tools for surface and nanoanalysis. Debdulal Roy and Alasdair Rae **Journal Metrology Society of India** Dec 2013, Volume 28, Issue 4, pp285-297. DOI 10.1007/s12647-013-0092-7
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
6. Nanometrology. Dario Imbraguglio, Andrea Mario Giovannozzi and Andrea Mario Rossi Metrology and Physical Constants, book chapter in the section **MODERN METROLOGY** 09/2013, DOI: 10.3254/978-1-61499-326-1-193
7. Accurate measurement of enhancement factor in tip-enhanced Raman spectroscopy through elimination of far-field artefacts. Naresh Kumar, Alasdair Rae and Debdulal Roy **APPLIED PHYSICS LETTERS** 104, 123106 (2014) DOI: 10.1063/1.4869184"
8. Nanoscale mapping of catalytic activity using tip-enhanced Raman spectroscopy. Naresh Kumar, Bruno Stephanidis, Renato Zenobi, Andy Wain and Debdulal Roy **Nanoscale**, 7, 7133-7137 (2015) DOI: 10.1039/c4nr07441f
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
10. Tip-enhanced Raman Spectroscopy Principles and Applications:. Naresh Kumar, Sandro Mignuzzi, Weitao Su and Debdulal Roy **Techniques and Instrumentation** (2015) 2:9 DOI 10.1140/epjti/s40485-015-0019-5
11. State of the art Raman techniques for biological applications. Alasdair Rae, Rainer Stosch, Petr Klapetek, Angela R Hight Walker, Debdulal Roy **Methods**, Volume 68, Issue 2, pages 338-347, DOI: 10.1016/j.ymeth.2014.02.035
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

JRP start date and duration:	01 August 2012 (36 months)
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The EMRP is jointly funded by the EMRP participating countries within EURAMET and the European Union