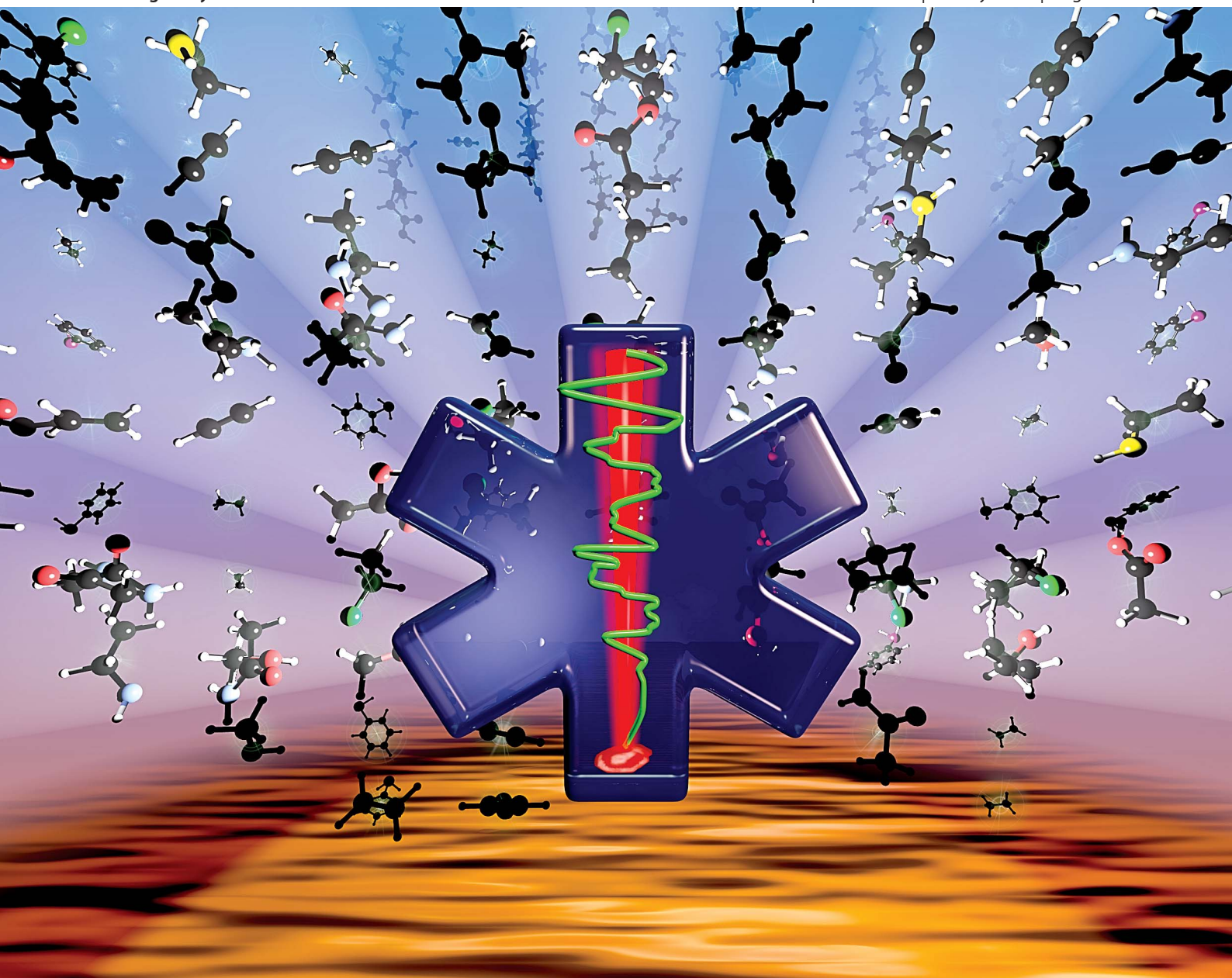


Analyst

www.rsc.org/analyst

Volume 138 | Number 14 | 21 July 2013 | Pages 3847–4204



Themed issue: Optical Diagnosis

ISSN 0003-2654

RSC Publishing

CRITICAL REVIEW

David I. Ellis *et al.*

Illuminating disease and enlightening biomedicine: Raman spectroscopy as a diagnostic tool



0003-2654(2013)138:14;1-X

Illuminating disease and enlightening biomedicine: Raman spectroscopy as a diagnostic tool

Cite this: *Analyst*, 2013, **138**, 3871

David I. Ellis,^{*a} David P. Cowcher,^a Lorna Ashton,^a Steve O'Hagan^a and Royston Goodacre^{ab}

The discovery of the Raman effect in 1928 not only aided fundamental understanding about the quantum nature of light and matter but also opened up a completely novel area of optics and spectroscopic research that is accelerating at a greater rate during the last decade than at any time since its inception. This introductory overview focuses on some of the most recent developments within this exciting field and how this has enabled and enhanced disease diagnosis and biomedical applications. We highlight a small number of stimulating high-impact studies in imaging, endoscopy, stem cell research, and other recent developments such as spatially offset Raman scattering amongst others. We hope this stimulates further interest in this already exciting field, by 'illuminating' some of the current research being undertaken by the latest in a very long line of dedicated experimentalists interested in the properties and potential beneficial applications of light.

Received 8th April 2013
Accepted 8th May 2013

DOI: 10.1039/c3an00698k

www.rsc.org/analyst

Introduction

The properties of light have been of interest to experimentalists for millennia. From the publication of Ibn al-Haytham's seven

volume treatise the 'Book of Optics' (Kitāb al-Manāẓir) in the early 11th century (leading to him being regarded as the father of modern optics¹), through to one of the very first publications in a scientific journal, Isaac Newton's paper on the theory of the properties of light² itself followed some years later by his famous book, *Opticks*, in 1704.³ Whilst separated by seven centuries, these two polymaths and their respective bodies of work shared similarities, perhaps the most important being that both were the result of systematic and methodical experimentation. In the century following the publication of Newton's

^aSchool of Chemistry, Manchester Institute of Biotechnology, The University of Manchester, 131 Princess Street, Manchester M1 7ND, UK. E-mail: D.Ellis@manchester.ac.uk

^bManchester Centre for Integrative Systems Biology, Manchester Institute of Biotechnology, University of Manchester, 131 Princess Street, Manchester M1 7DN, UK



David Ellis was educated on the Welsh coast at the University of Wales, Aberystwyth, obtaining a BSc in Environmental Science and a PhD in Analytical Biotechnology/Microbiology. His research involving the rapid and quantitative detection of food-borne bacteria using FT-IR and machine learning has been widely publicised, featuring on BBC TV and radio, the Science Museum in London, as well as

the national and international press (e.g. WIRED). He now works at the Manchester Institute of Biotechnology (MIB), as a Senior Experimental Officer undertaking research and managing the labs of Roy Goodacre (<http://biospec.net>) and Doug Kell (<http://dbkgroup.org>), in the School of Chemistry, University of Manchester, UK.



David Cowcher obtained an undergraduate Masters degree in Chemistry and Forensic Science in 2009 from the University of Manchester, which included a year working as an analytical chemist for GlaxoSmithKline. He is currently studying for a PhD with Roy Goodacre at the Manchester Institute of Biotechnology, University of Manchester, on the development of enhanced Raman scattering methods for biochemical analysis.



Opticks, as early as the mid-1800s, the interaction of light within tissue was being used by physicians to assist in disease recognition.⁴

It was during a sea voyage from India to England in 1921 that the brilliant Indian physicist C.V. Raman, another renowned experimentalist, undertook some on-board experiments which were later submitted to *Nature* in a letter called the 'The Colour of the Sea'.⁵ Unable to accept Lord Rayleigh's⁶ explanation that the colour of the sea was just a reflection of the colour of the sky,⁷ Raman's experiments showed that the colour of the sea was in fact a direct result of the molecular scattering of light and independent of absorption or the reflection of light from the sky.⁸ This was very closely followed by another letter to *Nature*

concerning the molecular scattering of light in liquids and solids.⁹ These experiments opened up a deep interest in C.V. Raman and a new field of research on his return to Kolkata (Calcutta) on the scattering of light,⁵ as well as the publication of another article on the molecular diffraction and quantum structure of light in the following year.¹⁰

Raman and collaborators such as K.S. Krishnan began a series of seminal experiments concerning the scattering of light in a large number of liquids, as well as theories about the potential applications of their experiments, which culminated in their discovery of the inelastic scattering effect named after Raman in 1928 on 28 February,¹¹ and his award of the Nobel Prize for Physics in 1930. This discovery was also independently observed by Landsberg and Mandelstam later in 1928 (see Fig. 1 for a timeline of events in Raman spectroscopy). There was a great deal of interest in the Raman effect and this not only aided the fundamental understanding about the quantum nature of light, and its interaction with matter at the molecular level, but also opened up a completely novel area of optics and spectroscopic research that is, particularly in terms of biological and biomedical applications,^{12,13} accelerating at a greater rate during the last decade than at any time since its inception.

Whereas infrared (IR) spectroscopies measure the absorption of energy, Raman spectroscopy measures the *exchange* of energy with electromagnetic (EM) radiation of a particular wavelength, usually provided by a monochromatic light source such as a laser in the visible to near-IR portion of the EM; although it is also possible to conduct experiments in the near- and deep-UV. From the exchange in EM energy a measurable Raman shift in the wavelength of incident laser light is observed, this is also referred to as the inelastic light scattering effect.^{14–16} It is usually the Stokes shift which is measured, as



Lorna Ashton studied for her BSc with the Open University before joining the University of Manchester where she obtained her PhD in Biomolecular Sciences and Raman Optical Activity. She has since worked as a post doctoral research associate specialising in Raman Spectroscopy and two-dimensional correlation analysis for the characterisation of conformational transitions in biomolecules. Lorna is currently

working as part of a BBSRC funded project for rapid evolution of enzymes and synthetic microorganisms developing Raman spectroscopy as a high-throughput analytical technique for industrial bioprocesses.



Steve O'Hagan gained BSc and MSc degrees in Chemistry at the University of Manchester. The MSc research was to characterise 'Molecular Beam Sources' using mass spectrometry and TOF kinetic energy measurements; computer simulations were also used. Steve gained a PhD in Chemistry at the University of Warwick; several mass spectrometry and chemometric techniques were

employed to analyse engine oils and additives. After several years working for commercial laboratories, he joined the University of Manchester as a Computer Officer, working with Doug Kell's and Roy Goodacre's research groups. Interests include: genetic programming; laboratory automation; analytical laboratory data analysis; chemometrics; as well as scientific visualization.



Roy Goodacre is a PhD graduate from the University of Bristol (UK) where he studied mass spectrometry of microbial systems. After a postdoc, Wellcome Trust fellowship and lectureship in the University of Wales, Aberystwyth, he is now Professor of Biological Chemistry at the University of Manchester (UK). His group's main areas of research (<http://www.biospec.net/>) are broadly

*within analytical biotechnology, metabolomics and systems biology. His expertise involves many forms of Raman spectroscopy (including deep UV resonance Raman and SERS), FT-IR spectroscopy, and mass spectrometry, as well as advanced chemometrics, machine learning and evolutionary computational methods. He is Editor-in-Chief of the journal *Metabolomics*, on the Editorial Advisory Boards of *Analyst* and *Journal of Analytical and Applied Pyrolysis*, a founding director of the *Metabolomics Society* and a director of the *Metabolic Profiling Forum*.*



Event/discovery



- 2018 ExoMars Rover, taking Raman spectroscopy to Mars
 2010 Video-rate *in vivo* SRS Raman imaging by Sunny Xie
 2010 SESORS reported by Graham, Faulds, Stone and Matousek
 2009 Boyle and Smith awarded Nobel Prize in Physics for CCD
 2005 SORS invented by Pavel Matousek
 2000 TERS developed by Volker Deckert
 1997 Single molecule SERS reported by Katrin Kneipp
 1990s Significant increase in commercial instrumentation
 1983 Enhancement of 10^{14} reported by Richard van Duyne
 1982 Use of Ag and Au colloids for SERS by Lee and Meisel
 1980s CCD detectors used for Raman
 1980s Fibre optics coupled with Raman
 1974 Fleischman and colleagues observe surface enhanced Raman scattering (SERS)
 1970s Raman coupled with microscopy
 1969 Invention of charge-coupled device (Willard Boyle and George Smith)
 1964 Townes, Basov and Prochorov awarded Nobel Prize in Physics for invention of the laser
 1960s Stimulated Raman and coherent anti-Stokes Raman (CARS) invented
 1960s Lasers used as light source for Raman spectrometers
 1957-59 Light amplification by stimulated emission of radiation constructed
 1953 First commercial Raman spectrometer
 1950 Observation of resonance Raman (RR) spectra
 1940s Invention of Raman grating spectrometer
 1930s Invention of the monochromator
 1930 Sir Chandrasekhara Venkata Raman awarded Nobel Prize in Physics
 1928 C.V. Raman and K.S. Krishnan: discovery of Raman effect experiments on 28 Feb
 1928 Landsberg and Mandelstam: independent observation of inelastic light scattering
 1927 Compton awarded Nobel Prize in Physics
 1923 Arthur Compton (effect): discovered inelastic X-rays & γ -rays scattering in matter
 1923 Adolf Smekal's prediction of the Raman effect
 1922 CV Raman's monograph published on the Molecular Diffraction of Light
 1921 CV Raman's experiments on the colour of the sea, showing that the colour was due to molecular diffraction
 1917 Albert Einstein first theorizes about stimulated emission (On the Quantum Theory of Radiation)
 1871-1899 Lord Rayleigh (John Strutt) further refines his theory of scattering in a series of papers
 1871 Rayleigh scattering first described

Year

Fig. 1 A timeline of events and discoveries in the history of Raman spectroscopy, Nobel Prizes marked in red (photo of Sir C.V. Raman in the sculpture park of Nehru Science Centre, Mumbai, India, by Prof. Paul O'Brien FRS, School of Chemistry, University of Manchester).

this has a higher probability of occurring than anti-Stokes.¹⁷ It is important to note that this shift is complementary to IR absorption and a spectroscopic 'fingerprint'^{13,17,18} of the same sample can be analysed and constructed by both vibrational spectroscopies. Whilst mid-IR spectroscopy is known to be intensely sensitive to and a high absorber of water, this is generally not the case with Raman spectroscopy as water is a weak scatterer.¹³ For biomedical and routine clinical applications (and with low laser output power at the point of contact), this allows for the direct collection *in vivo* of Raman spectra. The

utility of Raman spectroscopy has been demonstrated for a diverse and wide range of potential biological and biomedical applications, such as bacterial identification,^{19,20} chemical hazards and illicit substance detection,^{21,22} as well as food and product authentication,^{23,24} with a great deal of interest and research into its potential for disease diagnosis and use in biomedical applications seen during the last decade (Fig. 2).

Some of the previously documented limitations of Raman spectroscopy for biomedical applications have for example included issues such as weak scattering signals, subsequently

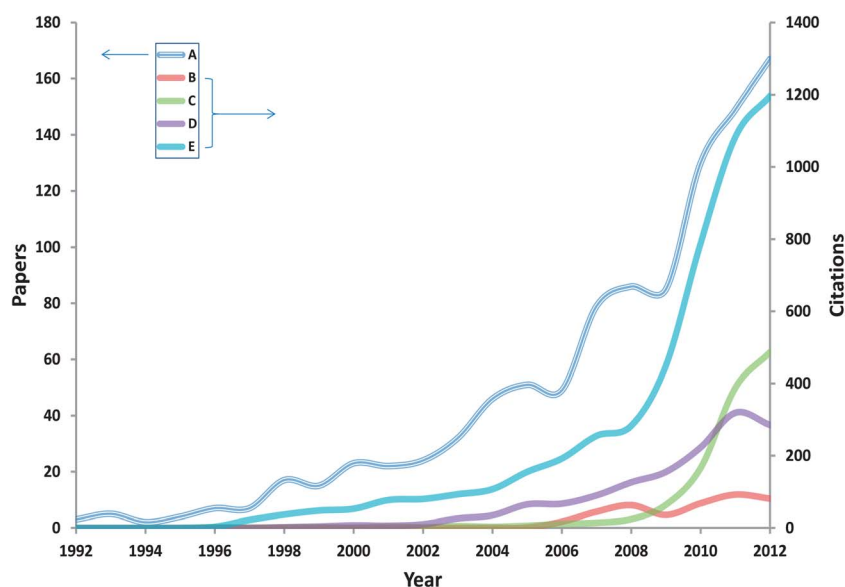


Fig. 2 Results of bibliometric analysis of the number of publications (A) and citations (B/C/D/E) per year listed on ISI Thomson Web of Science® (<http://wos.mimas.ac.uk/>) for the period 1992–2012, using the search terms (A) Raman AND disease, (B) spatially offset Raman AND disease, (C) SERS AND disease, (D) Raman endoscopy, (E) Raman imaging AND disease.



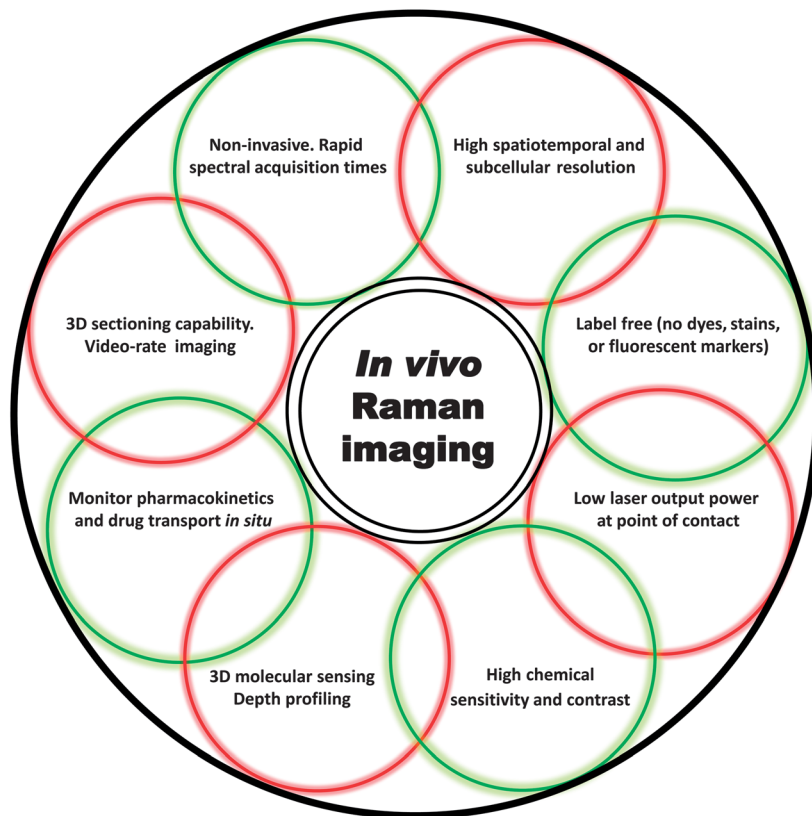


Fig. 3 A figurative (as opposed to fibre-optic) bundle of some of the key terms and demonstrated capabilities of *in vivo* Raman imaging mentioned throughout this article.

long spectral acquisition times, fluorescence from biological samples, and interference from silica within fibre-optics. However, within this exciting and highly research active era of biomedical Raman spectroscopy, and with the hard work and application of experimentalists (in the long tradition of those already mentioned above), these so-called limitations are being overcome by groups of scientists and engineers around the globe who are not content to remain within the bounds of current knowledge, or the confines of commercially available optics for that matter, and who are constantly pushing the field forward. This introductory overview focuses on some of the recent developments within this exciting field, highlighting a small number of high-impact studies in imaging, endoscopy, stem cell research, and other recent developments such as spatially offset Raman scattering (SORS), coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS), amongst others.

Imaging

Biomedical imaging is an extremely useful and important field which allows for the collection and processing of highly complex biochemical and physiological data, and the creation, manipulation, and in-depth analysis of three-dimensional coloured images to aid molecular sensing,²⁵ drug transport,²⁶ characterization of cells²⁷ and tissues,²⁸ and, perhaps the ultimate goal, the rapid diagnosis of disease (*vide infra*). Label-free optical imaging,

particularly *in vivo*, would be highly advantageous as dyes or fluorescent labels, which are needed as contrast agents, can be toxic or perturbative to the cell/tissue. Optical techniques have also been said to have the potential to be complementary to existing techniques such as magnetic resonance imaging (MRI), and they offer superior sensitivity and high spatial resolution (compared to MRI, see Fig. 3).²⁹ Whilst mass spectrometry (MS) has mass appeal for metabolomics for example³⁰ and can be used for chemical imaging of tissues and cells^{31,32} – including in 3D³³ – as well as being combined with vibrational spectroscopy,³⁴ imaging MS is a destructive analysis and the practical spatial resolution for MALDI-MS is 50–200 μm and for SIMS is 1 μm , thus Raman spectroscopy has considerable opportunities to shed light on disease.

In any review of biomedical imaging, and particularly one focussed on Raman spectroscopy, it would be a heedless omission not to include any one of a number of studies by the Sunney Xie group. A major contributor to biomedical imaging, based at Harvard University (<http://bernstein.harvard.edu>), this group's body of work includes both coherent Raman scattering techniques,³⁵ comprising of coherent anti-Stokes Raman scattering (CARS) microscopy^{36–38} and stimulated Raman scattering (SRS) microscopy.³⁹ Descriptions of these techniques can be found in Table 1 and it should also be noted that the signal from CARS is non-linearly proportional to species concentration whilst the signal from SRS is linearly proportional.



Table 1 Definition of some of the main approaches of Raman spectroscopy discussed in this review

Technique	Acronym	Short definition	References
Surface enhanced Raman spectroscopy	SERS	Requires close proximity/adsorption onto a roughened metal surface, a colloidal solution or a roughened electrode (usually Ag or Au). Enhancement explained by two processes; an <i>electromagnetic enhancement</i> effect (thought to dominate) and a charge transfer mechanism, known as <i>chemical enhancement</i> . Has a fluorescence quenching effect. Can tune to a specific chromophore for additional resonance enhancement (known as SERRS). Enhancements over normal Raman scattering of typically 10^3 to 10^6 .	19, 122, 136 and 137
Coherent anti-Stokes Raman spectroscopy	CARS	A multiphoton form of Raman spectroscopy based on a non-linear conversion of two lasers into a coherent high intensity beam in the anti-Stokes region. The emission is usually many orders of magnitude greater than spontaneous Raman scattering. Useful for obtaining spectra of fluorescing samples. Nonresonant background can complicate spectral assignment, limit sensitivity, and affect quantitative interpretation	27, 38, 41, 45 and 138
Stimulated Raman scattering	SRS	A multiphoton technique analogous to stimulated emission, where two lasers coincide on a sample. The sample is excited by colinear and tightly focused pump and Stokes beams. When the differences in known frequencies match a molecular vibration in the sample, the Stokes beam intensity increases and pump beam intensity decreases as a result of the coherent excitation of molecular vibration. Unlike CARS, it does not exhibit a nonresonant background and is a good technique for sensitive, high spatial resolution 3D imaging	25, 26, 39, 61, 63 and 139
Spatially offset Raman spectroscopy	SORS	Raman spectra are collected from locations spatially separated from the point of laser illumination on the sample surface. SORS allows for the isolation of chemically rich spectral information from distinct substructures or layers and through other barriers, not accessible via spontaneous Raman. Typical wavelengths used for biological tissue are 785 or 830 nm. Ideal for detecting disease in cells and tissue underlying other tissue types, such as bone through skin, cancer cells through muscle and lipid tissue for example	25, 26, 39, 61, 63 and 139

As briefly mentioned above, the signal from spontaneous Raman scattering is known to be weak, with potentially long integration times, which impacts by significantly reducing imaging speed (an important factor as *in vivo* samples are in constant motion at the micro scale). Although these nonlinear methods were first invented in the 1960s, coherent Raman scattering techniques can be said to have very recently surmounted any technical difficulties and imaging speed hurdles by enhancing the Raman signal level by up to five orders of magnitude,³⁹ and image collection speeds by three orders of magnitude. Indeed, on the subject of speed, one of the more memorable articles propelling CARS microscopy as a rapid method over a decade ago (for the identification of bacterial spores), had the unforgettable acronym FAST-CARS (femto-second adaptive spectroscopic techniques for coherent anti-Stokes Raman spectroscopy).⁴⁰

More recent reports have highlighted some of the potential limitations of CARS,²⁹ which has been said to make quantitative interpretation and applications other than lipid imaging⁴¹ unnecessarily challenging. These limitations can be particularly noticeable in the fingerprint region where spectral signals are said to be congested³⁹ and separating out weak from strong signals in this region is thus more problematic for CARS microscopic imaging. That being said, CARS microscopy has been

successfully demonstrated as a biomedical imaging method for a range of *ex vivo* biological samples such as brain structures,³⁸ colon tissue,⁴² and arterial tissue²⁷ for example, as well as *in vivo* studies including sciatic nerve tissue⁴³ and atherosclerotic plaque deposits⁴⁴ in animals, with a recent report also demonstrating the use of CARS microscopy in skin imaging of humans *in vivo*.⁴⁵

Recent articles within the last half decade have explored SRS as an alternative imaging technology, which, unlike CARS, does not exhibit a nonresonant background and so is inherently more quantitative, though like other nonlinear multiphoton techniques (including CARS), does allow for sensitive, high spatial resolution 3D imaging.⁴⁶ Whilst initial reports demonstrated stimulated Raman scattering's advantages over CARS (*e.g.* a lack of nonresonant background complications), it was said to not be suitable for bioimaging due to sample photodamage from excessive laser power⁴⁶ and had a limited spatial and spectral resolution as well as a slower image acquisition rate than CARS.⁴⁷ These challenges were rapidly overcome by the Xie group the following year using a multifaceted approach which lowered peak rates by three orders of magnitude! (resulting in no photodamage), optimised spectral resolution, increased sensitivity by four orders of magnitude than the previous year's report and surpassed the detection limit previously stated for CARS microscopy.⁴⁶



Not content with these and other leaps forward, three bio-imaging applications were then elegantly presented by Xie and co-workers. The first application monitored and imaged the uptake of polyunsaturated omega-3 fatty acids by living human cancer cells, specifically eicosapentaenoic acid (EPA). Using the Raman band at 3015 cm^{-1} (attributable to unsaturated fatty acids), and in contrast, the Raman band at 2920 cm^{-1} whose peak intensity is similar for saturated and unsaturated fatty acids, they were able to use SRS to follow the uptake of EPA by living cells, concluding that EPA is taken up by the cells and more strongly enriched in lipid droplets compared to other cellular organelles.⁴⁶ The second application presented the potential for tissue imaging without the requirement for staining, highlighting the 3D sectioning capability and subcellular resolution of SRS, using the CH_2 stretching vibration at 2845 cm^{-1} (see Table 2 for Raman band frequencies of biological interest).

This was demonstrated in a variety of mouse tissues; from neuron bundles in corpus callosum (highlighting myelin sheaths), thick brain tissue, and from depth profiles of ear tissue, in addition to comparative SRS and CARS images of stratum corneum (visually illustrating how the nonresonant background from CARS can complicate image interpretation). Finally, this seminal series of experiments showed the use of SRS to monitor drug delivery, namely deuterated dimethyl sulfoxide (DMSO), a skin penetration enhancer and retinoic acid (RA) used to treat a range of skin conditions and acute promyelocytic leukemia. Drug delivery into fresh mouse skin was monitored by tuning into the vibrations for DMSO at 670 cm^{-1} and RA at 1570 cm^{-1} (as well as lipids of subcutaneous fats at 2845 cm^{-1}).⁴⁶ This series of experiments elegantly demonstrated the potential for SRS as a new approach for studying pharmacokinetics *in situ*, sensitive label-free imaging, and molecular sensing in 3D in living cells and tissue.

Whilst the potential of SRS had been so ably demonstrated, the series of experiments above had been undertaken *ex vivo*. Well aware of the importance of *in vivo* optical imaging in biomedicine, and perhaps acutely mindful of the challenges required to achieve this goal, as well as the fact that SRS imaging had not yet been accomplished *in vivo* (in animals or humans), an article was published in 2010 demonstrating that *in vivo* SRS was not only possible (both in mice and humans), but that it could also be performed at video-rate speeds.²⁹ This high-speed imaging was achieved by modulating the intensity of the Stokes beam at 20 MHz and the group building their own all-analogue lock-in amplifier with a response time of ~ 100 ns (whereas this was previously limited by commercially available 100 μs lock-in amplifiers).²⁹ This new custom-built system raster scanned across a sample with a line rate of 8 kHz (100 ns per pixel at 512×512 pixels with up to 25 frames per s), increasing imaging speed by three orders of magnitude. In addition, to significantly increase the collection of back-scattered light from *in vivo* samples further in-house modifications were undertaken, such as changing the geometry of the photodetector and microscope objective. This involved exciting light from the objective through an aperture in the detector

Table 2 A selection of Raman band frequency assignments of biological interest^{140–144}

Band frequency (cm^{-1})	Vibration mode	Assignment
Protein secondary structure		
930–950	N–C α –C stretch	Skeletal stretch/ α -helix
1235–1259	N–H and C–H band	Amide III/ β -sheet
1260	N–H and C–H band	Amide III/disordered
1300–1340	N–H and C–H band	Amide III/ α -helix
1650–1655	H-bonded C=O stretch	Amide I/ α -helix
1670–1680	H-bonded C=O stretch	Amide I/ β -sheet and β -barrel
Amino acid residues		
508–545	S–S stretch	<i>Trans</i> and <i>gauche</i> conformers
655	C–S stretch	<i>Gauche</i> conformer
704	C–S stretch	<i>Trans</i> conformers
835/857	H-bonding of indole ring	Tyrosine Fermi doublet
875–880	H-bonding of indole ring	Tryptophan orientation
1008/1034	Phenyl ring	Phenylalanine
1551–1556	Indole ring	Tryptophan
1605	Phenyl ring	Phenylalanine
1615	Indole ring	Tyrosine
RNA and DNA		
813–816	O–P–O stretching	A-form helix
914–925	C–O and C–C stretching	Ribose–phosphate
1095	PO_2 symmetric stretch	B-DNA and Z-DNA marker
1135, 1235, 1395	Ring stretch	Uracil
1174, 1325, 1370	Ring stretch	Guanine
1245, 1275	Ring stretch	Cytosine
1256, 1514	Ring stretch	Adenine
1671	C=O stretch	Thymine
Sugars		
1000–1200	C–O and C–C stretch	β -D-Glucose, D-(+) dextrose
1025, 1047, 1155	C–O stretch from CH_2OH of carbohydrates	Glycogen
1267	NH_2 rocking	GlcNAc and GalNAc
1300–1500	CH_2 and CH_2OH deformations	β -D-Glucose, D-(+) dextrose
Lipids		
891, 908	CH_2 rocking	Fatty acid chain length
1080	PO_2 -symmetric stretch	Phospholipids
1259	PO_2 -asymmetric stretch	Phospholipids
1296	C–C stretch	Unbranched saturated fatty acids
1660	C=C stretch	Unsaturated lipid bonds
2873, 2931, 2961	C–H stretch	Acyl chains of lipids
2888, 2926	CH_2 asymmetric stretch	Saturated lipid bonds
3009	H–C=C stretch	Unsaturated lipid bonds

placed between the microscope objective and the sample, and specially designing a filter to block the modulated Stokes beam whilst transmitting the pump beam.²⁹



The effectiveness of this new video-rate SRS imaging technique was then demonstrated through a series of experiments such as skin imaging in living mice. The CH_3 stretching vibration at 2950 cm^{-1} was shown to mainly highlight proteins, as well as residual lipid structures and individual red blood cells in the viable epidermis, whilst the water signal at 3250 cm^{-1} was coincident with sebaceous glands in both positive and negative contrast. *In vivo* imaging of drug penetration of *trans*-retinol was demonstrated in living mouse skin, visualising with SRS imaging and 3D depth projection that penetration of the topically applied drug occurs along the hair shaft. This pathway was not observed in previous experiments with excised fresh tissue,⁴⁶ which was said to further highlight the importance of *in vivo* imaging, as it can lead to insights into the transport mechanisms of small molecules in living organisms.

Finally, a series of *in vivo* SRS imaging experiments were performed on human skin of a volunteer's forearm showing cell layers of the viable epidermis to a depth of $50\text{ }\mu\text{m}$, and the boundary (via observation of varying nuclear sizes) between the viable epidermis and stratum corneum when tuned into the CH_3 stretching vibration at 2950 cm^{-1} . The penetration-enhancing small molecule DMSO (which was deuterated) was applied to the skin, and its accumulation in the hair shaft and lack of complete penetration into the hair itself was imaged using the C–D stretching vibration at 2125 cm^{-1} . With image acquisition times of 150 ms and 37 ms respectively, these blur-free high-speed *in vivo* vibrational imaging experiments demonstrated the diagnostic potential of this technology in humans.²⁹

Stem cells

Stem cells,⁴⁸ stem cell therapy,^{49,50} and stem cell engineering⁵¹ are extremely important and highly topical areas of scientific research with huge potential benefits for the treatment of a wide range of diseases and biomedical applications. The ability to identify the phenotypic purity of live cells is absolutely crucial (and a noninvasive optical method would be highly beneficial), as excessive proliferation of unwanted phenotypes (*i.e.*, uncontrolled differentiation) following transplantation could result in undesirable consequences, such as tissue overgrowth and tumour formation for example.^{52,53} One of the recent studies by the Notingher group applied Raman spectroscopy to determine if it could be used as a label-free, noninvasive method to detect and image intrinsic chemical differences that could be used as molecular markers among highly heterogeneous stem cells populations; specifically, cardiomyocytes (CMs) derived from human embryonic stem cells (hESCs).⁵²

Using a custom-built instrument, Raman detection and imaging of molecular markers specific to hESC-derived CMs was carried out, along with retrospective phenotypic identification of all cells via immunofluorescence imaging integrated with the Raman microscope. Multivariate statistical analysis of Raman spectra and cross-validation methods were used to develop a model (from 50 CMs and 40 non-CMs within the same heterogeneous populations) to determine the true accuracy of phenotypic identification of CMs, the sensitivity and specificity parameters, and select discriminatory Raman bands. Raman

spectral images corresponding to the Raman bands identified by both the multivariate model and immunostaining of the same cells allowed for the accurate assignment of Raman molecular markers. The conclusions drawn from the results were that spectral differences were mainly attributable to glycogen and myofibrils, with glycogen being responsible for discrimination of CMs (with a band assignment at 860 cm^{-1}), and myofibril proteins providing a lesser contribution (with a band assignment at 938 cm^{-1}).⁵² This study demonstrated the potential of Raman spectroscopy for noninvasive phenotypic identification of stem cells, though the authors themselves stated that it was not yet practical for medical applications due to their long spectral acquisition times.

However, a later study by the same group on the same hESC-derived cardiomyocytes reduced spectral acquisition times by a hundredfold, from minutes per cell to 5 s per cell, without the need for raster scanning. When incorporating high-powered commercial lasers, this could be further reduced to cell sorting speeds of approximately 10 cells per s.⁵⁴ In addition, a recent study by the same group has successfully applied Raman to identify, image and quantify the differentiation status of live neural stem cells *in vitro*, where this time the spectral differences were said to be related to cytoplasmic RNA.⁵⁵ A number of other interesting studies involving Raman analysis of stem cell populations have also appeared in the literature very recently^{56–59} and this area of interest, not surprisingly, continues to grow and flourish.

As well as cellular differentiation, the location of specific drugs inside cells is also of interest as this may allow the elucidation of the pharmaceutical's site of action. A recent study addressing this enabled mapping the site of action of the HIV protease inhibitors indinavir and lopinavir in cervical carcinoma cells expressing the E6 oncogene from human papilloma virus (HPV).⁶⁰ This study demonstrated that indinavir undergoes enhanced nuclear accumulation in E6 expressing cells, indicating this as the site of action for this compound against the HPV. Further interesting studies showing the plethora of Raman imaging applications include a report on stimulated Raman photoacoustic imaging,⁶¹ surface enhanced Raman scattering (SERS) imaging using nanotags in live mice, as a potential multiplexed imaging detection method for multiple biomarkers in living subjects associated with a specific disease,⁶² quantitative multiplex SRS imaging,⁶³ noninvasive time-course imaging of apoptotic cells,⁶⁴ and multivariate image reconstruction methods for Raman hyperspectral datasets.⁶⁵ For those specifically interested in coherent nonlinear optical imaging, which includes stimulated Raman scattering, the reader is directed to a recent review by Min *et al.*,⁶⁶ others include a review of CARS microscopy,³⁷ as well as gold nanoparticles and imaging in medicine.⁶⁷

Endoscopy

The clinical potential for *in vivo* Raman endoscopy has been the subject of research for over a decade, since the first published report of *in vivo* Raman spectra of human gastrointestinal tissue measured during routine clinical endoscopy in 2000.⁶⁸ In the same year work by the Stone group incorporated Raman spectroscopy and clinical endoscopy to discriminate between



normal, dysplastic and cancerous laryngeal tissue.⁶⁹ Nick Stone's group, based in the UK (<http://www.exeter.ac.uk/>) have played a major role in pioneering the field of clinical optical diagnostics with (in addition to other vibrational spectroscopies) well received studies using Raman spectroscopy/ endoscopy to analyse a range of diseases/disorders such as Barrett's oesophagus⁷⁰ and bladder and prostate cancer.⁷¹ One of the recent studies by the Stone group involved the evaluation of the suitability of a custom-built fibre-optic Raman probe for the potential *in vivo* diagnosis of early onset oesophageal neoplasia. Whilst this involved *ex vivo* sampling, the results clearly demonstrated the potential for the rapid and accurate differentiation between benign tissue, Barrett's oesophagus and both dysplastic and malignant tissue.

The culmination of this study was a custom-built confocal Raman probe constructed by the group. This novel probe had been reported by the group previously,⁷² although by the time of the latest study, the probe had undergone some modifications which had improved its performance for spectral acquisition during oesophageal endoscopy. The 90 cm long, 2.7 mm diameter fibre-optic probe was designed to fit into the instrument channel of a standard clinical endoscope and to have direct contact with oesophageal epithelial tissue. The optics had been modified by incorporating a graded index lens at the tip (and the output power regulated to 60 mW), so that a sampling depth of 100 to 200 μm could be ensured. Collecting spectra from this depth meant that signals from deeper tissue structures would not obscure those collected from mucosal abnormalities such as early neoplastic changes,⁷³ and that these abnormalities could be quickly classified in timescales suitable to a clinical setting. The performance of the probe *ex vivo* had been evaluated for translational use for *in vivo* sample collection, with low laser power at the probe tip and short spectral acquisition times said to enable its routine use for oesophageal endoscopy and paving the way for *in vivo* clinical trials.⁷³ This group have recently published a review on *in vitro* and *in vivo* Raman spectroscopy as a potential routine tool for the rapid, noninvasive, early diagnosis of lesions and preventing development of cancer in the oesophagus.⁷⁴

Another group who have made a significant contribution to the field of Raman endoscopy in recent years is headed by Zhiwei Huang and based at the Optical Imaging Laboratory in Singapore (<http://www.bioeng.nus.edu.sg/optbioimaging/huang/>). Several of these studies utilised image-guided Raman endoscopy, which the group reported for the first time in 2009.⁷⁵ Whilst image-guided endoscopy is by no means novel,^{76,77} the integration of image-guided techniques with Raman endoscopy is relatively recent. The first report on this technique involved integrating Raman spectroscopy with trimodal imaging techniques (white-light reflectance, autofluorescence and narrow-band) and the development of a novel 1.8 mm Raman probe which filtered out interference from fluorescence as well as interference from silica from within optical fibres. This was demonstrated via the rapid collection of Raman spectra (<1 s) and the corresponding endoscopic images of different locations of the upper gastrointestinal tract of a healthy volunteer in real-time and *in vivo*.⁷⁵

Since then this group have published several articles using image-guided Raman endoscopy, demonstrating its potential as an *in vivo* real-time detection method for a variety of diagnostic applications. These studies have included, perhaps not surprisingly, the *in vivo* diagnosis of oesophageal cancer using this technique in conjunction with biomolecular modelling.⁷⁸ This involved collecting spectra from 75 oesophageal tissue sites from 27 patients of normal tissue (squamous mucosa) and malignant tumours. The cancerous tissue was said to show distinct Raman signals mainly associated with cell proliferation, lipid reduction, abnormal nuclear activity and neo-vascularisation. To estimate the biochemical composition of oesophageal tissue, biomolecular modelling was employed using six basis reference spectra from actin, collagen type I, DNA, histones, triolein and glycogen. This allowed for the construction of a linear discriminant analysis (LDA) model with a sensitivity of 97% and specificity of 95.2% for the *in vivo* diagnosis of oesophageal cancer.⁷⁸ These results have since been said to be extremely promising, but that image-guided Raman endoscopy is yet to be used to detect dysplasia or the early onset of cancer.⁷³

Nevertheless, the Huang group have previously demonstrated the potential of Raman spectroscopy for the detection of dysplasia, with a ball-lens fibre-optic probe, in the high wavenumber region (HW) ($2800\text{--}3700\text{ cm}^{-1}$) for the *in vivo* detection of cervical dysplasia (a HW Raman probe was first presented by Gerwin Puppels and co-workers for *in vitro* measurements of brain tissue in 2005⁷⁹). The perceived main advantages of HW Raman were said to be a significant reduction in fluorescence and background signal from optical fibres, more intense Raman signals (compared to the fingerprint region), and the possibility of an unfiltered single fibre Raman probe design for *in vivo* clinical use.⁸⁰ In addition to this study, several more recent reports have continued to investigate this area.^{81–83}

The *in vivo* detection of epithelial neoplasia in the stomach using image-guided Raman endoscopy has also been demonstrated, and significant differences between normal and cancerous gastric tissue were reported.⁸⁴ More recently, a study has been published showing the development of an online automated spectral diagnostics system integrated with image-guided Raman endoscopy for real-time *in vivo* diagnosis during endoscopic examination.⁸⁵ This system was built on a database of 2465 normal and 283 cancerous gastric tissue spectra acquired from 305 patients, with the system employing a variety of diagnostic algorithms. Other so-called *in vivo* real-time applications recently reported have involved transnasal image-guided Raman endoscopy of the larynx and nasopharynx, which the authors hoped would pave the way for realizing early diagnosis and detection of cancers and precancers of the head and neck.⁸⁶ All of these studies show promise and many have similarities in terms of rapid spectral acquisition times (absolutely crucial in *in vivo* studies), and novel developments in optical engineering, but there remains more to accomplish, such as the ability not only to discriminate cancer, but to classify and grade both cancerous and precancerous cells and tissue.^{87,88}



Recent developments

In terms of noninvasive biomedical Raman other very exciting developments are spatially offset Raman spectroscopy (SORS)⁸⁹ as well as transmission Raman, both techniques directly resulting from research involving depth profiling using Raman Kerr-gating methods.⁹⁰ SORS was invented and developed in the Central Laser Facility of the Rutherford Appleton Laboratory (<http://www.clf.rl.ac.uk>) in 2005 by Pavel Matousek and readers are directed to an excellent overview of this fast moving field by Matousek and Stone.⁹¹

The central difference in SORS is that Raman spectra are collected from different locations, spatially separated (offset) from the point of laser excitation on the sample (Fig. 4). As a consequence of photon diffusion processes within tissue the resultant Raman spectra contain different relative contributions from different depths within the sample and thus allows for the highly accurate chemical analysis of subsurface objects of interest.⁸⁹ These spectra acquired from different spatial offsets are processed to reveal pure Raman spectra of subcomponents from separate depth locations within tissue.⁹¹ SORS can be applied in a number of Raman collection and beam delivery geometries including single point collection, ring, and other pattern illumination.⁹¹ In respect to biomedical imaging this approach is said to be particularly useful when configured as inverse SORS, where the sample is illuminated by an adjustable ring-shaped laser beam (generated by a conical (axicon) lens) and the Raman light collected via fibres in the centre of the ring.⁹² The radius of this ring is said to define the spatial offset and as it is adjustable, this can be optimised to suit both the scattering properties and dimensions of each sample and covers a wider illumination zone on a sample surface than conventional SORS.⁹¹ SORS is said to be effective at tissue depths in excess of 500 μm , which is well beyond the accessible range of conventional confocal Raman spectroscopy. Applications can be as diverse and wide-ranging as noninvasive detection of pharmaceuticals through packaging,⁹³ detection of

hidden explosives and drug precursors behind opaque plastics and garments,^{21,94–96} as well as agricultural and food product analysis.⁹⁷

From the outset, Matousek and collaborators immediately realised the diagnostic potential of SORS with its ability for noninvasive subsurface probing of for example bone through skin⁸⁹ and dermatology studies.⁹² This foresight was quickly realised with the first SORS spectra of *ex vivo* bone collected in 2006^{98,99} from animal and human cadavers, and *in vivo* SORS demonstrated by Matousek during the same year.⁹² There have been several studies since including accurate *in vivo* assessment of bone composition using the carbonate (1070 cm^{-1}) -to-phosphate (958 cm^{-1}) ratio through the skin of live mice,¹⁰⁰ *in vivo* evaluation of bone grafts,^{101,102} transcutaneous *in vivo* monitoring of glucocorticoid induced osteoarthritis¹⁰³ and *in vivo* measurement and evaluation of subtle changes in bone composition.¹⁰⁴

A significant body of pioneering work forwarding Raman spectroscopy as a tool for breast cancer diagnosis was undertaken in the George R. Harrison Spectroscopy Lab at MIT (<http://web.mit.edu/spectroscopy/>) under the directorship of Michael S. Feld. This included identifying chemical differences in microcalcifications from benign and malignant breast lesions,^{105,106} demonstrating the real time capabilities of an *in vivo* Raman system during femoral bypass and breast lumpectomy surgeries,¹⁰⁷ and also involved testing of spectral diagnostic algorithms for breast cancer diagnosis.¹⁰⁸ The results from the Raman research on the chemical composition and identification of the different types of microcalcifications has led directly to others investigating the potential of SORS as a possible clinical adjunct to mammography, for the noninvasive diagnosis of breast cancer. As the changing concentration of carbonate substitution for phosphate ions in the calcium hydroxide lattice in microcalcifications may relate to the process of tumour cell metastasis, and the ability to measure the magnitude of this (as well as soft tissue signals) by Raman, could indicate the potential progression of this cancer.¹⁰⁹

Research by Stone, Matousek, and collaborators, demonstrated the proof-of-principle of SORS for potential *in vivo* breast cancer diagnosis in a model system using three calcification standards overlaid with various preparations of animal tissue (*i.e.* chicken breast tissue, with and without skin). For this work they utilised a continuous wave 827 nm laser with a spatial offset for collection of 3 mm, which enabled the probing and collection of Raman spectra from calcifications through up to 10 mm of tissue.¹⁰⁹ Previous results by this group using Kerr-gated Raman techniques had achieved penetration depths of 1 mm in comparison.⁹⁰ This SORS study demonstrated the collection of high quality Raman spectra and biochemical information measured through 8.7 mm of tissue, identifying the difference between three calcification standards.

Subsequent experiments by the same group applied transmission Raman spectroscopy in combination with chemometrics in similar model systems collecting Raman calcification signals from depths of 20 mm, this time through porcine tissue, as a breast tissue 'phantom'.¹¹⁰ This was said to reach the lower range for clinically relevant breast tissue thicknesses from

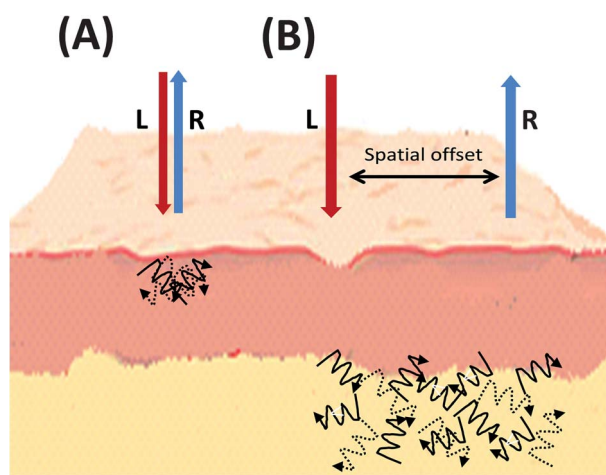


Fig. 4 Simplified graphical representation of (A) spontaneous Raman compared to (B) spatially offset Raman scattering, illustrating the spatial offset and deeper subsurface probing in skin tissue. L = laser light, R = Raman light.



mammographic screening (1.9 cm), with further work said to be required to reach the top of this range (5 cm).⁹¹ Whilst these studies utilised the carbonate-to-phosphate band ratio (similar to the SORS bone studies above¹⁰⁰), a more recent study showed that the intense Raman phosphate band at 960 cm^{-1} broadens and shifts as carbonate concentration (from calcium hydroxyapatites) increases in the calcifications.¹¹¹ This study was said to pave the way toward a new generation of noninvasive breast cancer screening methods based around SORS and transmission Raman spectroscopy.¹¹¹ Some very recent studies by the Mahadevan-Jansen group have investigated SORS for the real-time, intraoperative assessment of breast cancer tumour margins.¹¹² This same group then developed and tested a SORS probe with multiple source detector offset limits specific to this type of analysis, and published results acquired from 35 freeze-thaw breast cancer samples *in vitro*.¹¹³

Variants of SORS have also recently been used to augment analysis and include *in vivo* transcutaneous glucose sensing in rats¹¹⁴ and further demonstrated the accuracy and functionality of this in a later study.¹¹⁵ These studies employed what they term surface enhanced SORS (SESORS), first published in 2010,¹¹⁶ and presented at the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS) meeting in the USA during the same year. SESORS marry SERS techniques using nanoparticles and nanosurfaces with the subsurface probing of SORS, with a further study exploring this technique's potential for Raman imaging.¹¹⁷

On the subject of SERS, which has not been mentioned in any great detail here, many studies have appeared in the literature during the last few years related to disease detection and we would like to highlight just a modest selection of these. They of course include the multiplexed imaging of SERS nanotags *in vivo* already mentioned above,⁶² as well as other multiplexing studies, such as multiplex single nucleotide polymorphism (SNPs) genotyping coupled to SERS,¹¹⁸ multiplexed *in vivo* cancer detection using SERS NIR nanotags, demonstrating the excellent sensitivity, stability and tumour specificity of three bioconjugated nanotags,¹¹⁹ recent reviews on the area of SERS multiplexed detection for disease diagnostics,¹²⁰ as well as SERS cancer detection and imaging and the potential of SERS agents for targeted drug delivery and photothermal therapy.¹²¹

Several SERS studies involving immunoassays, including cancer detection,¹²² detection of a potential pancreatic cancer marker in serum,¹²³ and on-chip immunoassays using hollow gold nanospheres.¹²⁴ Fluorescent SERS gold co-functionalized nanorod probes for *in vivo* imaging of lymph node mapping and tumor targeting in mice,¹²⁵ high sensitivity *in vivo* detection of inflammation using gold nanoclusters conjugated to monoclonal antibodies¹²⁶ and the use of functionalised nanoparticles and SERS for the detection of DNA relating to disease.¹²⁷ With the recent interest concerning the potential resurgence in microbial disease, as well as bioterrorism, an article demonstrating a portable quantitative SERS system for detection of *Bacillus* spores at levels significantly lower than those previously reported for SERS has been published.¹²⁸ Fig. 5 shows SERS spectra of a biomarker from the spores of *Bacillus*, a species of which *Bacillus anthracis* is the cause of the acute, and mostly

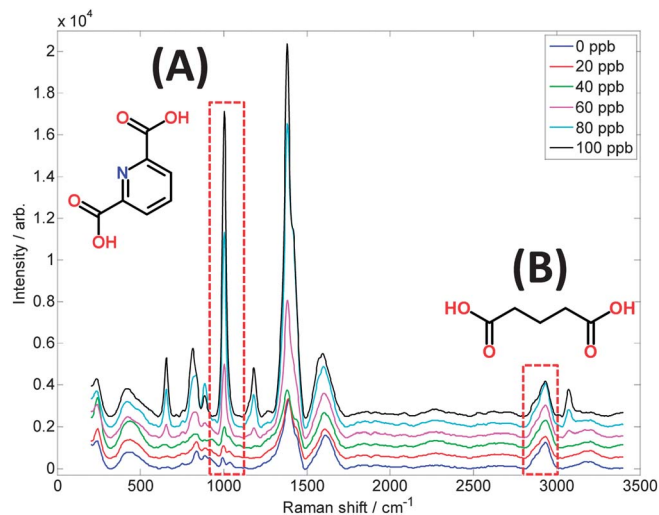


Fig. 5 SERS spectra of dipicolinic acid, a biomarker for *Bacillus* spores, measured using citrate-reduced silver colloid and a portable 633 nm Raman spectrometer. The concentration range 0–100 ppb in the sample volumes used is of equivalent magnitude to the infective dose of inhalation anthrax. Highlighted are: (A) the pyridine ring breathing vibration and structure of dipicolinate at 1006 cm^{-1} , and (B) the C–H stretching vibration and structure of glutaric acid at 2934 cm^{-1} , which is included as an internal standard to allow accurate quantification.

lethal, disease anthrax. The detection limit of this dipicolinic acid biomarker is estimated at 5 ppb (30 nM).¹²⁸

In addition to those primary articles and reviews already mentioned above, other recently published reviews and books on an array of Raman-based topics which may be of interest include those concerning *in vivo* and *in vitro* analysis,¹²⁹ Raman scattering in pathology,¹³⁰ optical tumour margin identification in the larynx,¹³¹ data-classification algorithms for spectral analysis,¹³² recent advances in gold nanoparticle based assays for detecting and identifying microbes,¹³³ and emerging Raman applications and techniques in biomedical and pharmaceutical fields.¹³⁴

Concluding remarks

We hope that whilst only an introductory overview to some of the more recent work in this field, the range and scope of the studies shown here elegantly demonstrate the development and exploitation of Raman spectroscopy as a medical diagnostic tool. We believe this truly is an exciting field, where those at the forefront are propelling it forwards and are simply not content to remain within the confines of current knowledge or commercially available optics. These studies highlight the necessary interdisciplinary nature of this field, with inspirational contributions from a range of analytical scientists and technologists including clinicians, biologists, chemists, optical engineers, as well as statisticians and chemometricians. These multidisciplinary inputs and fresh approaches are invigorating this field, unlocking the doors to new insights,¹³⁵ adding to knowledge, and opening up new dimensions and avenues of study, as well as potential clinical applications, for biomedical Raman spectroscopy.



Acknowledgements

DIE would like to thank *Analyst* for their kind invitation to write this article and the patience of all in the editorial team during its construction, as well as Prof. Paul O'Brien FRS, Dr Steve O'Hagan and Howbeer Muhamad Ali for assistance with graphics and software. RG, LA and DPC are very grateful to the UK BBSRC, EPSRC, Avacta group plc., and industrial members of the Bioprocessing Research Industry Club (BRIC) for funding this work.

References

- M. T. Masoud and R. Masoud, *Br. Med. J.*, 2006, **332**, 120.
- I. Newton, *Phil. Trans.*, 1671, **6**, 3075–3087.
- A. R. Hall, *All was light: An introduction to Newton's Opticks*, Clarendon Press, Oxford, 1993.
- R. RichardsKortum and E. SevickMuraca, *Annu. Rev. Phys. Chem.*, 1996, **47**, 555–606.
- R. Singh and F. Riess, *Curr. Sci.*, 1998, **75**, 965–971.
- J. Strutt, *Philos. Mag.*, 1899, **47**, 375–394.
- Anon, American Chemical Society International Historic Chemical Landmarks, The Raman Effect, http://portal.acs.org/portal/acs/corg/content?_nfpb=true&_pageLabel=PP_SUPERARTICLE&node_id=515&use_sec=false&sec_url_var=region1&__uuid=59c1b22b-50f3-4af9-b4b5-d5ad83faf161, accessed 27 March 2013.
- C. V. Raman, *Nature*, 1921, **108**, 367.
- C. V. Raman, *Nature*, 1921, **108**, 402–403.
- C. V. Raman, *Nature*, 1922, **109**, 444–445.
- C. V. Raman and K. S. Krishnan, *Nature*, 1928, **121**, 501–502.
- D. I. Ellis, *Bioanalysis*, 2011, **3**, 1189–1194.
- D. I. Ellis and R. Goodacre, *Analyst*, 2006, **131**, 875–885.
- N. B. Colthup, L. H. Daly and S. E. Wiberly, *Introduction to Infrared and Raman Spectroscopy*, Academic Press, New York, 1990.
- P. Hendra, C. Jones and G. Warnes, *Fourier Transform Raman Spectroscopy*, Ellis Horwood, Chichester, 1991.
- B. Schrader, *Infrared and Raman spectroscopy: methods and applications*, Verlag Chemie, Weinheim, 1995.
- D. I. Ellis, W. B. Dunn, J. L. Griffin, J. W. Allwood and R. Goodacre, *Pharmacogenomics*, 2007, **8**, 1243–1266.
- D. I. Ellis, G. G. Harrigan and R. Goodacre, in *Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis*, ed. R. Goodacre and G. G. Harrigan, Kluwer Academic, Boston, 2003, pp. 111–124.
- R. M. Jarvis, A. Brooker and R. Goodacre, *Anal. Chem.*, 2004, **76**, 5198–5202.
- R. M. Jarvis and R. Goodacre, *Anal. Chem.*, 2004, **76**, 40–47.
- B. Cletus, W. Olds, E. L. Izake, S. Sundarajoo, P. M. Fredericks and E. Jaatinen, *Anal. Bioanal. Chem.*, 2012, **403**, 255–263.
- M. E. Farrell, E. L. Holthoff and P. M. Pellegrino, in *Chemical, Biological, Radiological, Nuclear, and Explosives*, ed. A. W. Fountain, SPIE-Int. Soc. Optical Engineering, Bellingham, 2012, vol. 8358.
- D. I. Ellis, V. L. Brewster, W. B. Dunn, J. W. Allwood, A. P. Golovanov and R. Goodacre, *Chem. Soc. Rev.*, 2012, **41**, 5706–5727.
- D. I. Ellis, D. Broadhurst, S. J. Clarke and R. Goodacre, *Analyst*, 2005, **130**, 1648–1654.
- C. W. Freudiger, W. Min, G. R. Holtom, B. W. Xu, M. Dantus and X. S. Xie, *Nat. Photonics*, 2011, **5**, 103–109.
- B. G. Saar, L. R. Contreras-Rojas, X. S. Xie and R. H. Guy, *Mol. Pharmaceutics*, 2011, **8**, 969–975.
- H. W. Wang, T. T. Le and J. X. Cheng, *Opt. Commun.*, 2008, **281**, 1813–1822.
- R. Manoharan, Y. Wang and M. S. Feld, *Spectrochim. Acta, Part A*, 1996, **52**, 215–249.
- B. G. Saar, C. W. Freudiger, J. Reichman, C. M. Stanley, G. R. Holtom and X. S. Xie, *Science*, 2010, **330**, 1368–1370.
- W. B. Dunn, A. Erban, R. J. M. Weber, D. J. Creek, M. Brown, R. Brietling, T. Hankemeier, R. Goodacre, S. Neumann, J. Kopka and M. R. Viant, *Metabolomics*, 2013, **9**, S44–S66.
- E. G. Armitage, H. L. Kotze and N. P. Lockyer, *Metabolomics*, 2013, **9**, S102–S109.
- P. M. Angel and R. M. Caprioli, *Biochemistry*, 2012, DOI: 10.1021/bi301519p.
- J. S. Fletcher and J. C. Vickerman, *Anal. Chem.*, 2013, **85**, 610–639.
- R. Masyuko, E. J. Lanni, J. V. Sweedler and P. W. Bohn, *Analyst*, 2013, **138**, 1924–1939.
- Coherent Raman Scattering Microscopy*, ed. J. X. Cheng and X. S. Xie, CRC Press, Abingdon, Oxon, UK, 2012.
- W. M. Tolles, J. W. Nibler, J. R. McDonald and A. B. Harvey, *Appl. Spectrosc.*, 1977, **31**, 253–271.
- J. X. Cheng and X. S. Xie, *J. Phys. Chem. B*, 2004, **108**, 827–840.
- C. L. Evans, X. Y. Xu, S. Kesari, X. S. Xie, S. T. C. Wong and G. S. Young, *Opt. Express*, 2007, **15**, 12076–12087.
- X. Zhang, M. B. J. Roeffaers, S. Basu, J. R. Daniele, D. Fu, C. W. Freudiger, G. R. Holtom and X. S. Xie, *ChemPhysChem*, 2012, **13**, 1054–1059.
- M. O. Scully, G. W. Kattawar, R. P. Lucht, T. Opatrny, H. Pilloff, A. Rebane, A. V. Sokolov and M. S. Zubairy, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 10994–11001.
- T. Hellerer, C. Axang, C. Brackmann, P. Hillertz, M. Pilon and A. Enejder, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 14658–14663.
- C. Krafft, A. A. Ramoji, C. Bielecki, N. Vogler, T. Meyer, D. Akimov, P. Rosch, M. Schmitt, B. Dietzek, I. Petersen, A. Stallmach and J. Popp, *J. Biophotonics*, 2009, **2**, 303–312.
- T. B. Huff and J. X. Cheng, *J. Microsc.*, 2007, **225**, 175–182.
- C. Matthaus, S. Dochow, G. Bergner, A. Lattermann, B. F. M. Romeike, E. T. Marple, C. Krafft, B. Dietzek, B. R. Brehm and J. Popp, *Anal. Chem.*, 2012, **84**, 7845–7851.
- H. G. Breunig, R. Buckle, M. Kellner-Hofer, M. Weinigel, J. Lademann, W. Sterry and K. Konig, *Microsc. Res. Tech.*, 2012, **75**, 492–498.
- C. W. Freudiger, W. Min, B. G. Saar, S. Lu, G. R. Holtom, C. W. He, J. C. Tsai, J. X. Kang and X. S. Xie, *Science*, 2008, **322**, 1857–1861.



- 47 E. Ploetz, S. Laimgruber, S. Berner, W. Zinth and P. Gilch, *Appl. Phys. B: Lasers Opt.*, 2007, **87**, 389–393.
- 48 F. M. Watt and B. L. M. Hogan, *Science*, 2000, **287**, 1427–1430.
- 49 I. Klimanskaya, N. Rosenthal and R. Lanza, *Nat. Rev. Drug Discovery*, 2008, **7**, 131–142.
- 50 D. A. Robinton and G. Q. Daley, *Nature*, 2012, **481**, 295–305.
- 51 S. F. Badylak, D. J. Weiss, A. Caplan and P. Macchiarini, *Lancet*, 2012, **379**, 943–952.
- 52 F. C. Pascut, H. T. Goh, N. Welch, L. D. Buttery, C. Denning and I. Notingher, *Biophys. J.*, 2011, **100**, 251–259.
- 53 B. E. Reubinoff, M. F. Pera, C. Y. Fong, A. Trounson and A. Bongso, *Nat. Biotechnol.*, 2000, **18**, 399–404.
- 54 F. C. Pascut, H. T. Goh, V. George, C. Denning and I. Notingher, *J. Biomed. Opt.*, 2011, **16**, 045002.
- 55 A. Ghita, F. C. Pascut, M. Mather, V. Sottile and I. Notingher, *Anal. Chem.*, 2012, **84**, 3155–3162.
- 56 A. Downes, R. Mouras, P. Bagnaninchi and A. Elfick, *J. Raman Spectrosc.*, 2011, **42**, 1864–1870.
- 57 S. O. Konorov, H. G. Schulze, C. G. Atkins, J. M. Piret, S. A. Aparicio, R. F. B. Turner and M. W. Blades, *Anal. Chem.*, 2011, **83**, 6254–6258.
- 58 S. O. Konorov, H. G. Schulze, J. M. Piret, R. F. B. Turner and M. W. Blades, *J. Raman Spectrosc.*, 2011, **42**, 1135–1141.
- 59 H. G. Schulze, S. O. Konorov, N. J. Caron, J. M. Piret, M. W. Blades and R. F. B. Turner, *Anal. Chem.*, 2010, **82**, 5020–5027.
- 60 D. H. Kim, R. M. Jarvis, J. W. Allwood, G. Batman, R. E. Moore, E. Marsden-Edwards, L. Hampson, I. N. Hampson and R. Goodacre, *Anal. Bioanal. Chem.*, 2010, **398**, 3051–3061.
- 61 V. V. Yakovlev, H. F. Zhang, G. D. Noojin, M. L. Denton, R. J. Thomas and M. O. Scully, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 20335–20339.
- 62 C. L. Zavaleta, B. R. Smith, I. Walton, W. Doering, G. Davis, B. Shojaei, M. J. Natan and S. S. Gambhir, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 13511–13516.
- 63 D. Fu, F. K. Lu, X. Zhang, C. Freudiger, D. R. Pernik, G. Holtom and X. S. Xie, *J. Am. Chem. Soc.*, 2012, **134**, 3623–3626.
- 64 A. Zoladek, F. C. Pascut, P. Patel and I. Notingher, *J. Raman Spectrosc.*, 2011, **42**, 251–258.
- 65 M. Miljkovic, T. Chernenko, M. J. Romeo, B. Bird, C. Matthaus and M. Diem, *Analyst*, 2010, **135**, 2002–2013.
- 66 W. Min, C. W. Freudiger, S. J. Lu and X. S. Xie, in *Annual Review of Physical Chemistry*, ed. S. R. Leone, P. S. Cremer, J. T. Groves and M. A. Johnson, Annual Reviews, Palo Alto, 2011, vol. 62, pp. 507–530.
- 67 E. Boisselier and D. Astruc, *Chem. Soc. Rev.*, 2009, **38**, 1759–1782.
- 68 M. G. Shim, L. Song, N. E. Marcon and B. C. Wilson, *Photochem. Photobiol.*, 2000, **72**, 146–150.
- 69 N. Stone, P. Stavroulaki, C. A. Fulljames, M. Birchall and H. Barr, in *Biomedical Spectroscopy: Vibrational Spectroscopy and Other Novel Techniques*, ed. A. Mahadevanjansen and G. J. Puppels, SPIE-Int. Soc. Optical Engineering, Bellingham, 2000, vol. 1, pp. 120–128.
- 70 C. Kendall, N. Stone, N. Shepherd, K. Geboes, B. Warren, R. Bennett and H. Barr, *J. Pathol.*, 2003, **200**, 602–609.
- 71 P. Crow, A. Molckovsky, N. Stone, J. Uff, B. Wilson and L. M. Wongkeesong, *Urology*, 2005, **65**, 1126–1130.
- 72 J. C. C. Day, R. Bennett, B. Smith, C. Kendall, J. Hutchings, G. M. Meaden, C. Born, S. Yu and N. Stone, *Phys. Med. Biol.*, 2009, **54**, 7077–7087.
- 73 L. M. Almond, J. Hutchings, C. Kendall, J. C. C. Day, O. A. C. Stevens, G. R. Lloyd, N. A. Shepherd, H. Barr and N. Stone, *J. Biomed. Opt.*, 2012, **17**, 081421.
- 74 L. M. Almond, J. Hutchings, N. Shepherd, H. Barr, N. Stone and C. Kendall, *J. Biophotonics*, 2011, **4**, 685–695.
- 75 Z. W. Huang, S. K. Teh, W. Zhen, J. H. Mo, K. Lin, X. Z. Shao, K. Y. Ho, M. Teh and K. G. Yeoh, *Opt. Lett.*, 2009, **34**, 758–760.
- 76 X. D. Li, S. A. Boppart, J. Van Dam, H. Mashimo, M. Mutinga, W. Drexler, M. Klein, C. Pitris, M. L. Krinsky, M. E. Brezinski and J. G. Fujimoto, *Endoscopy*, 2000, **32**, 921–930.
- 77 R. L. P. Rhoten, M. G. Luciano and G. H. Barnett, *Neurosurgery*, 1997, **40**, 632–637.
- 78 M. S. Bergholt, W. Zheng, K. Lin, K. Y. Ho, M. Teh, K. G. Yeoh, J. B. Y. So and Z. Huang, *Technol. Cancer Res. Treat.*, 2011, **10**, 103–112.
- 79 L. F. Santos, R. Wolthuis, S. Koljenovic, R. M. Almeida and G. J. Puppels, *Anal. Chem.*, 2005, **77**, 6747–6752.
- 80 J. H. Mo, W. Zheng, J. J. H. Low, J. Ng, A. Ilancheran and Z. W. Huang, *Anal. Chem.*, 2009, **81**, 8908–8915.
- 81 S. Duraipandian, W. Zheng, J. Ng, J. J. H. Low, A. Ilancheran and Z. W. Huang, *Anal. Chem.*, 2012, **84**, 5913–5919.
- 82 A. F. Garcia-Flores, L. Raniero, R. A. Canevari, K. J. Jalkanen, R. A. Bitar, H. S. Martinho and A. A. Martin, *Theor. Chem. Acc.*, 2011, **130**, 1231–1238.
- 83 K. Lin, D. L. P. Cheng and Z. W. Huang, *Biosens. Bioelectron.*, 2012, **35**, 213–217.
- 84 Z. W. Huang, S. K. Teh, W. Zheng, K. Lin, K. Y. Ho, M. Teh and K. G. Yeoh, *Biosens. Bioelectron.*, 2010, **26**, 383–389.
- 85 S. Duraipandian, M. S. Bergholt, W. Zheng, K. Y. Ho, M. Teh, K. G. Yeoh, J. B. Y. So, A. Shabbir and Z. W. Huang, *J. Biomed. Opt.*, 2012, **17**, 081418.
- 86 M. S. Bergholt, K. Lin, W. Zheng, D. P. C. Lau and Z. W. Huang, *J. Biomed. Opt.*, 2012, **17**, 077002.
- 87 M. J. Baker, E. Gazi, M. D. Brown, J. H. Shanks, N. W. Clarke and P. Gardner, *J. Biophotonics*, 2009, **2**, 104–113.
- 88 M. J. Baker, E. Gazi, M. D. Brown, J. H. Shanks, P. Gardner and N. W. Clarke, *Br. J. Cancer*, 2008, **99**, 1859–1866.
- 89 P. Matousek, I. P. Clark, E. R. C. Draper, M. D. Morris, A. E. Goodship, N. Everall, M. Towrie, W. F. Finney and A. W. Parker, *Appl. Spectrosc.*, 2005, **59**, 393–400.
- 90 R. Baker, P. Matousek, K. L. Ronayne, A. W. Parker, K. Rogers and N. Stone, *Analyst*, 2007, **132**, 48–53.
- 91 P. Matousek and N. Stone, *J. Biophotonics*, 2013, **6**, 7–19.
- 92 P. Matousek, E. R. C. Draper, A. E. Goodship, I. P. Clark, K. L. Ronayne and A. W. Parker, *Appl. Spectrosc.*, 2006, **60**, 758–763.
- 93 C. Eliasson and P. Matousek, *Anal. Chem.*, 2007, **79**, 1696–1701.



- 94 C. Eliasson, N. A. Macleod and P. Matousek, *Anal. Chem.*, 2007, **79**, 8185–8189.
- 95 E. L. Izake, B. Cletus, W. Olds, S. Sundarajoo, P. M. Fredericks and E. Jaatinen, *Talanta*, 2012, **94**, 342–347.
- 96 L. Lee, A. Frisby, R. Mansson and R. J. Hopkins, in *Optics and Photonics for Counterterrorism and Crime Fighting VII Optical Materials in Defence Systems Technology VIII and Quantum-Physics-Based Information Security*, ed. C. Lewis, D. Burgess, R. Zamboni, F. Kajzar, A. A. Szep, M. T. Gruneisen, M. Dusek and J. G. Rarity, SPIE-Int. Soc. Optical Engineering, Bellingham, 2011, vol. 8189.
- 97 D. T. Yang and Y. B. Ying, *Appl. Spectrosc. Rev.*, 2011, **46**, 539–560.
- 98 M. V. Schulmerich, K. A. Dooley, M. D. Morris, T. M. Vanasse and S. A. Goldstein, *J. Biomed. Opt.*, 2006, **11**, 060502.
- 99 M. V. Schulmerich, W. F. Finney, V. Popescu, M. D. Morris, T. M. Vanasse and S. A. Goldstein, in *Biomedical Vibrational Spectroscopy III: Advances in Research and Industry*, ed. A. Mahadevanjansen and W. H. Petrich, SPIE-Int. Soc. Optical Engineering, Bellingham, 2006, vol. 6093, p. O930.
- 100 M. V. Schulmerich, J. H. Cole, J. M. Kreider, F. Esmonde-White, K. A. Dooley, S. A. Goldstein and M. D. Morris, *Appl. Spectrosc.*, 2009, **63**, 286–295.
- 101 P. I. Okagbare, F. W. L. Esmonde-White, S. A. Goldstein and M. D. Morris, *Analyst*, 2010, **135**, 3142–3146.
- 102 P. I. Okagbare, F. W. L. Esmonde-White, S. A. Goldstein and M. D. Morris, in *Photonic Therapeutics and Diagnostics VII*, ed. N. Kollias, B. Choi, H. Zeng, H. W. Kang, B. E. Knudsen, B. J. F. Wong, J. F. R. Ilgner, K. W. Gregory, G. J. Tearney, L. Marcu, H. Hirschberg, S. J. Madsen, A. Mandelis, A. Mahadevanjansen and E. D. Jansen, SPIE-Int. Soc. Optical Engineering, Bellingham, 2011, vol. 7883.
- 103 J. R. Maher, J. Inzana, M. Takahata, H. A. Awad and A. J. Berger, in *Biomedical Vibrational Spectroscopy V: Advances in Research and Industry*, ed. A. Mahadevanjansen and W. Petrich, SPIE-Int. Soc. Optical Engineering, Bellingham, 2012, vol. 8219.
- 104 P. I. Okagbare, D. Begun, M. Tecklenburg, A. Awonusi, S. A. Goldstein and M. D. Morris, *J. Biomed. Opt.*, 2012, **17**, 090502.
- 105 A. S. Haka, K. E. Shafer-Peltier, M. Fitzmaurice, J. Crowe, R. R. Dasari and M. S. Feld, *Cancer Res.*, 2002, **62**, 5375–5380.
- 106 A. S. Haka, K. E. Shafer-Peltier, M. Fitzmaurice, J. Crowe, R. R. Dasari and M. S. Feld, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 12371–12376.
- 107 J. T. Motz, S. J. Gandhi, O. R. Scepanovic, A. S. Haka, J. R. Kramer, R. R. Dasari and M. S. Feld, *J. Biomed. Opt.*, 2005, **10**, 031113.
- 108 A. S. Haka, Z. Volynskaya, J. A. Gardecki, J. Nazemi, R. Shenk, N. Wang, R. R. Dasari, M. Fitzmaurice and M. S. Feld, *J. Biomed. Opt.*, 2009, **14**, 054023.
- 109 N. Stone, R. Baker, K. Rogers, A. W. Parker and P. Matousek, *Analyst*, 2007, **132**, 899–905.
- 110 N. Stone and P. Matousek, *Cancer Res.*, 2008, **68**, 4424–4430.
- 111 M. M. Kerssens, P. Matousek, K. Rogers and N. Stone, *Analyst*, 2010, **135**, 3156–3161.
- 112 A. Mahadevan-Jansen, M. D. Keller, E. Vargis, B. Caldwell, T. Q. Nguyen, N. D. Granja, M. Sanders and M. C. Kelley, *Spectroscopy*, 2011, 48–58.
- 113 M. D. Keller, E. Vargis, N. D. Granja, R. H. Wilson, M. A. Mycek, M. C. Kelley and A. Mahadevan-Jansen, *J. Biomed. Opt.*, 2011, **16**, 077006.
- 114 J. M. Yuen, N. C. Shah, J. T. Walsh, M. R. Glucksberg and R. P. Van Duyne, *Anal. Chem.*, 2010, **82**, 8382–8385.
- 115 K. Ma, J. M. Yuen, N. C. Shah, J. T. Walsh, M. R. Glucksberg and R. P. Van Duyne, *Anal. Chem.*, 2011, **83**, 9146–9152.
- 116 N. Stone, K. Faulds, D. Graham and P. Matousek, *Anal. Chem.*, 2010, **82**, 3969–3973.
- 117 N. Stone, M. Kerssens, G. R. Lloyd, K. Faulds, D. Graham and P. Matousek, *Chem. Sci.*, 2011, **2**, 776–780.
- 118 A. J. Lowe, Y. S. Huh, A. D. Strickland, D. Erickson and C. A. Batt, *Anal. Chem.*, 2010, **82**, 5810–5814.
- 119 K. K. Maiti, U. S. Dinis, A. Samanta, M. Vendrell, K. S. Soh, S. J. Park, M. Olivo and Y. T. Chang, *Nano Today*, 2012, **7**, 85–93.
- 120 J. A. Dougan and K. Faulds, *Analyst*, 2012, **137**, 545–554.
- 121 M. Vendrell, K. K. Maiti, K. Dhaliwal and Y.-T. Chang, *Trends Biotechnol.*, 2013, **31**, 249–257.
- 122 J. H. Granger, M. C. Granger, M. A. Firpo, S. J. Mulvihill and M. D. Porter, *Analyst*, 2013, **138**, 410–416.
- 123 G. F. Wang, R. J. Lipert, M. Jain, S. Kaur, S. Chakraborty, M. P. Torres, S. K. Batra, R. E. Brand and M. D. Porter, *Anal. Chem.*, 2011, **83**, 2554–2561.
- 124 H. Chon, C. Lim, S. M. Ha, Y. Ahn, E. K. Lee, S. I. Chang, G. H. Seong and J. Choo, *Anal. Chem.*, 2010, **82**, 5290–5295.
- 125 J. Qian, L. Jiang, F. H. Cai, D. Wang and S. L. He, *Biomaterials*, 2011, **32**, 1601–1610.
- 126 R. McQueenie, R. Stevenson, R. Benson, N. MacRitchie, I. McInnes, P. Maffia, K. Faulds, D. Graham, J. Brewer and P. Garside, *Anal. Chem.*, 2012, **84**, 5968–5975.
- 127 D. Graham, R. Stevenson, D. G. Thompson, L. Barrett, C. Dalton and K. Faulds, *Faraday Discuss.*, 2011, **149**, 291–299.
- 128 D. P. Cowcher, X. Yun and R. Goodacre, *Anal. Chem.*, 2013, **85**, 3297–3302.
- 129 S. Wachsmann-Hogiu, T. Weeks and T. Huser, *Curr. Opin. Biotechnol.*, 2009, **20**, 63–73.
- 130 Z. J. Smith, T. R. Huser and S. Wachsmann-Hogiu, *Anal. Cell. Pathol.*, 2012, **35**, 145–163.
- 131 O. R. Hughes, N. Stone, M. Kraft, C. Arens and M. A. Birchall, *Head Neck-J. Sci. Spec. Head Neck*, 2010, **32**, 1544–1553.
- 132 C. Krafft, G. Steiner, C. Beleites and R. Salzer, *J. Biophotonics*, 2009, **2**, 13–28.
- 133 M. A. Syed and S. H. A. Bokhari, *J. Biomed. Nanotechnol.*, 2011, **7**, 229–237.
- 134 *Emerging Raman Applications and Techniques in Biomedical and Pharmaceutical Fields (Biological and Medical Physics*,



- Biomedical Engineering*), ed. P. Matousek and M. D. Morris, Springer, Heidelberg, 2010.
- 135 D. I. Ellis and R. Goodacre, *Curr. Opin. Biotechnol.*, 2012, **23**, 22–28.
- 136 R. A. Alvarez-Puebla and L. M. Liz-Marzan, *Small*, 2010, **6**, 604–610.
- 137 X. M. Qian and S. M. Nie, *Chem. Soc. Rev.*, 2008, **37**, 912–920.
- 138 H. F. Wang, Y. Fu, P. Zickmund, R. Y. Shi and J. X. Cheng, *Biophys. J.*, 2005, **89**, 581–591.
- 139 K. J. Blow and D. Wood, *IEEE J. Quantum Electron.*, 1989, **25**, 2665–2673.
- 140 H. Abramczyk, B. Brozek-Pluska, J. Surmacki, J. Jablonska and R. Kordek, *J. Mol. Liq.*, 2011, **164**, 123–131.
- 141 J. M. Benevides, S. A. Overman and G. J. Thomas, *J. Raman Spectrosc.*, 2005, **36**, 279–299.
- 142 J. De Gelder, K. De Gussem, P. Vandenabeele and L. Moens, *J. Raman Spectrosc.*, 2007, **38**, 1133–1147.
- 143 B. Singh, R. Gautam, S. Kumar, B. N. V. Kumar, U. Nongthomba, D. Nandi, G. Mukherjee, V. Santosh, K. Somasundaram and S. Umopathy, *Curr. Sci.*, 2012, **102**, 232–244.
- 144 Z.-Q. Wen, *J. Pharm. Sci.*, 2007, **96**, 2861–2878.

