Check for updates

*Focal Point Review*

Applied Spectroscopy
2018, Vol. 72(7) 987–1000
© The Author(s) 2018
Reprints and permissions:
sagepub.co.uk/journalsPermissions.nav
DOI: 10.1177/0003702818764672
journals.sagepub.com/home/asp
SAGE

# Surface-Enhanced Raman Scattering (SERS) in Microbiology: Illumination and Enhancement of the Microbial World

**Malama Chisanga, Howbeer Muhamadali, David I. Ellis, and Royston Goodacre**

## Abstract

The microbial world forms a huge family of organisms that exhibit the greatest phylogenetic diversity on Earth and thus colonize virtually our entire planet. Due to this diversity and subsequent complex interactions, the vast majority of microorganisms are involved in innumerable natural bioprocesses and contribute an absolutely vital role toward the maintenance of life on Earth, whilst a small minority cause various infectious diseases. The ever-increasing demand for environmental monitoring, sustainable ecosystems, food security, and improved healthcare systems drives the continuous search for inexpensive but reproducible, automated and portable techniques for detection of microbial isolates and understanding their interactions for clinical, environmental, and industrial applications and benefits. Surface-enhanced Raman scattering (SERS) is attracting significant attention for the accurate identification, discrimination and characterization and functional assessment of microbial cells at the single cell level. In this review, we briefly discuss the technological advances in Raman and Fourier transform infrared (FT-IR) instrumentation and their application for the analysis of clinically and industrially relevant microorganisms, biofilms, and biological warfare agents. In addition, we summarize the current trends and future prospects of integrating Raman/SERS-isotopic labeling and cell sorting technologies in parallel, to link genotype-to-phenotype in order to define community function of unculturable microbial cells in mixed microbial communities which possess admirable traits such as detoxification of pollutants and recycling of essential metals.

## Keywords

Raman spectroscopy, surface-enhanced Raman scattering, SERS, isotopic labeling, Fourier transform infrared, FT-IR, metabolic fingerprinting, microbes, biofilms, biowarfare

Date received: 12 January 2018; accepted: 12 February 2018

## Introduction

Microorganisms are ubiquitous in the environment and found in and on mammalian hosts in a complex systematic relationship;[1] in these environments these organisms occur predominantly as complex multi-organism biofilms. The microbial world forms a huge family of organisms that exhibit the greatest phylogenetic diversity on Earth and thus colonize virtually our entire planet.[2,3] Due to this diversity and subsequent complex interactions, the vast majority of microorganisms such as *Pseudomonas putida* and *Azotobacter chroococcum* are directly involved in a huge number of natural bioprocesses which contribute a central role toward the homeostasis of life on Earth,[4] whilst a small and important minority which includes *Vibrio cholerae, Campylobacter*, and *Mycobacterium tuberculosis* is responsible for various infectious diseases.[5,6] The ever-increasing demand for environmental monitoring, sustainable ecosystems, food security, and improved healthcare systems drives the continuous search for inexpensive but rapid, sensitive, reproducible, automated, and portable techniques for the detection of microbial isolates and understand their interactions for clinical, environmental, and industrial applications and benefits.[7–9]

During the early experimentation and exploration of the microbial world, simple light microscopes were largely used to identify and classify microbes based on their morphological dynamics which demanded extensive understanding

School of Chemistry, Manchester Institute of Biotechnology, University of Manchester, UK

**Corresponding author:**
Royston Goodacre, School of Chemistry, Manchester Institute of Biotechnology, University of Manchester, Manchester, M1 7DN, UK.
Email: roy.goodacre@manchester.ac.uk

of taxonomy.[10] Since then, the science of microbiology has rapidly evolved to such an extent that scientists have now gained deeper insights and understanding of the genetic make-up and corresponding phenotypes of these organisms, and how they interact within their own diverse communities.[11,12] This is mainly due to the advances in instrumentation and data analysis software which generate a great deal of information from DNA, RNA, proteins, metabolites, and lipid biomarkers giving rise to the genomics, transcriptomics, proteomics, metabolomics, and lipidomics scientific fields, respectively.[13–15] In the so-called post-genomics era, complementary investigations for high-throughput analyses at the level of gene expression, protein translation as well as post-translational modifications and metabolic networks analysis are desirable. These integrated analyses play a central role in understanding complex biochemical and biological mechanisms to facilitate comprehensive identification, discrimination and more interestingly, functional analysis of microbes.[16–18] To achieve this challenging task, many researchers have successfully demonstrated the application of various analytical platforms, including molecular techniques,[17] matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry,[19,20] and whole-organism metabolic fingerprinting techniques,[21] for extensive microbial analysis. Here, we discuss the technological advancements, limitations, and current and potential future applications of vibrational spectroscopy, with particular emphasis on surface-enhanced Raman scattering (SERS), as a global biochemical fingerprinting technique to complement existing knowledge in microbial characterization.

## Classical and Vibrational Spectroscopy Platforms for Bacterial Analysis

Until relatively recently culture-based methods have been routinely used to assess cellular phenotypes, despite being excessively time-consuming and mainly limited to studies involving microbial populations.[22,23] In times of emergency due to the breakout of highly contagious life-threatening bacterial diseases, such a delay would lead to huge increases in morbidity and mortality rates. Additionally, only a small fraction of microorganisms ($<1\%$) can be cultivated to suitable biomass level under normal laboratory conditions which is universally recognized as a key limitation in contemporary microbial analysis.[24–27] Previous research has demonstrated the development and applications of molecular bio-sensing techniques based on polymerase chain reaction (PCR) for investigating microbe-specific DNA sequences and enzyme-linked immunosorbent assays (ELISA).[8,28–30] These tools, which permit more rapid and simultaneous detection of several bacterial cells than culture-based methods, have greatly improved our understanding of bacterial diversity and physiology in complex communities. While obviously sensitive and specific for studying culturable and unculturable organisms of clinical and environmental importance, these techniques are relatively costly, for example PCR and ELISA require large libraries of specific primers and antibodies respectively, to detect a wide range of different bacterial species under investigations.[31,32] Additionally, they are labor-intensive, prone to contamination, difficult to employ for low-level microbial detection, cannot differentiate between live and dead cells, do not provide information on spatial localization of cells and if not carefully optimized the temperature profile in PCR can potentially alter microbial components of a mixed community.[33] Ideally, efficient and effective environmental monitoring, infection control and epidemiological assessments primarily require user-friendly, sensitive, reproducible and rapid with minimal sample preparation experimental procedures for routine microbial identification. Within the last decade, vibrational spectroscopy has gained huge popularity as a metabolic fingerprinting tool for rapid, label-free and high-throughput screening of a wide range of microbial cells and biological agents.[34–36] Vibrational spectroscopy based on Fourier transform infrared (FT-IR) and Raman provide specific "whole-organism molecular fingerprints" (spectra) comprising of distinct vibrational modes which are qualitatively (peak position) and quantitatively (peak height or area) measurable. Thus, FT-IR and Raman offer a wealth of exciting and practicable information for studying comprehensive intrinsic biochemical composition and variations which are representative of genetic changes modified by the environment (phenotype).[37] This information is exploited to identify and characterize bacterial species even of closely related cell biochemistry,[21,38] and to assess microbial cell functionality.[39] Due to the nondestructive nature of FT-IR and Raman measurements, samples can be recovered following an analysis and used for further downstream studies.[40] Compared to classical methods of typing bacteria, FT-IR and Raman have superior spatial resolution, sensitivity, and require little or no sample preparation as well as minimal background training.[41,42] Figure 1 illustrates the general sample preparation scheme for vibrational spectroscopic techniques in microbial analysis. Notably, sample preparation for FT-IR includes a drying step usually at 55 °C to dehydrate the sample completely to avoid interference from water.

Fourier transform infrared spectroscopy is based on the principle that when sample molecules interact with infrared radiation, the electric dipole moment of IR-active molecular bonds changes through atomic displacement, and begin to vibrate at higher frequencies (Figure 2).[7] The vibrational modes of a multitude of biologically relevant molecules such as carbohydrates, proteins, nucleic acids and lipids in bacterial cells contain one or more chemical bonds (viz. C=O, C–N, N–H, O–H etc.) and thus they are frequently detected in the mid-IR (Table 1) as distinct fundamental characteristic bands and used to characterize microbes.[43,44]

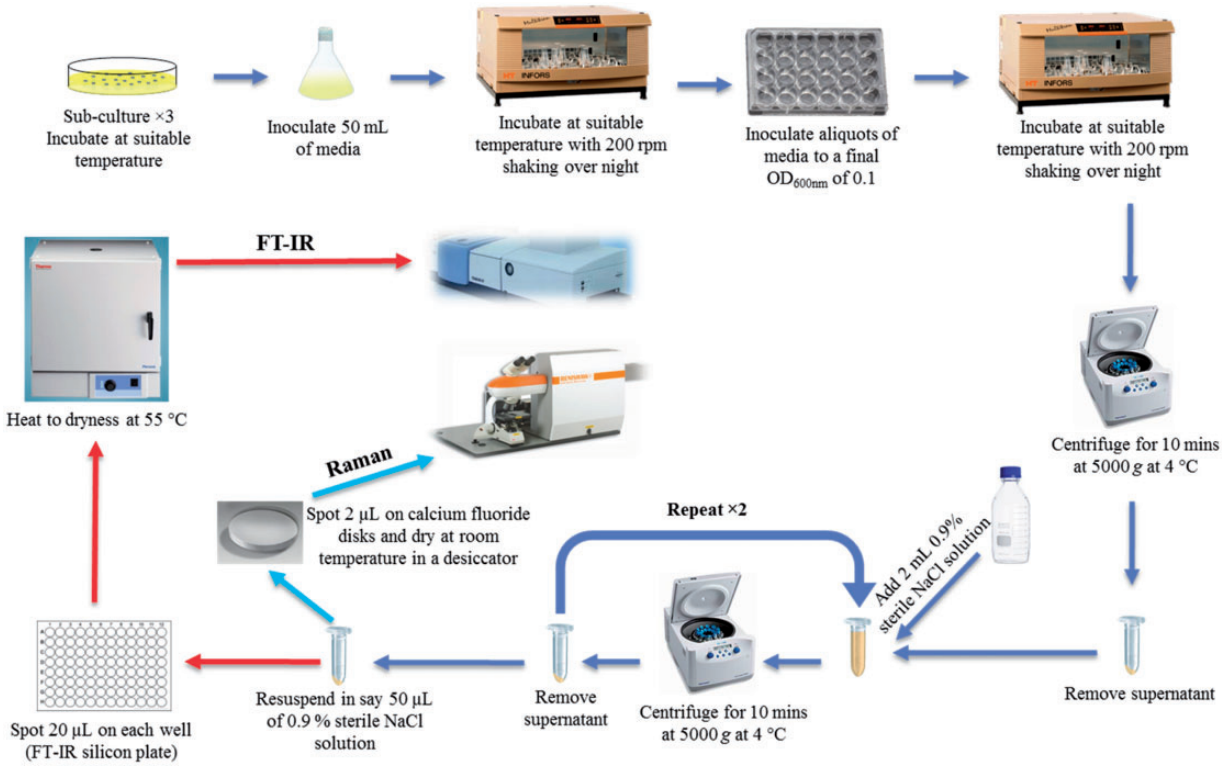


**Figure 1.** General flow diagram for sample preparation for FT-IR and Raman–SERS fingerprinting analysis of microbial cells. Prior to FT-IR measurements, microbial samples are dried at 55 °C to evaporate water molecules that may otherwise interfere with spectral interpretation.

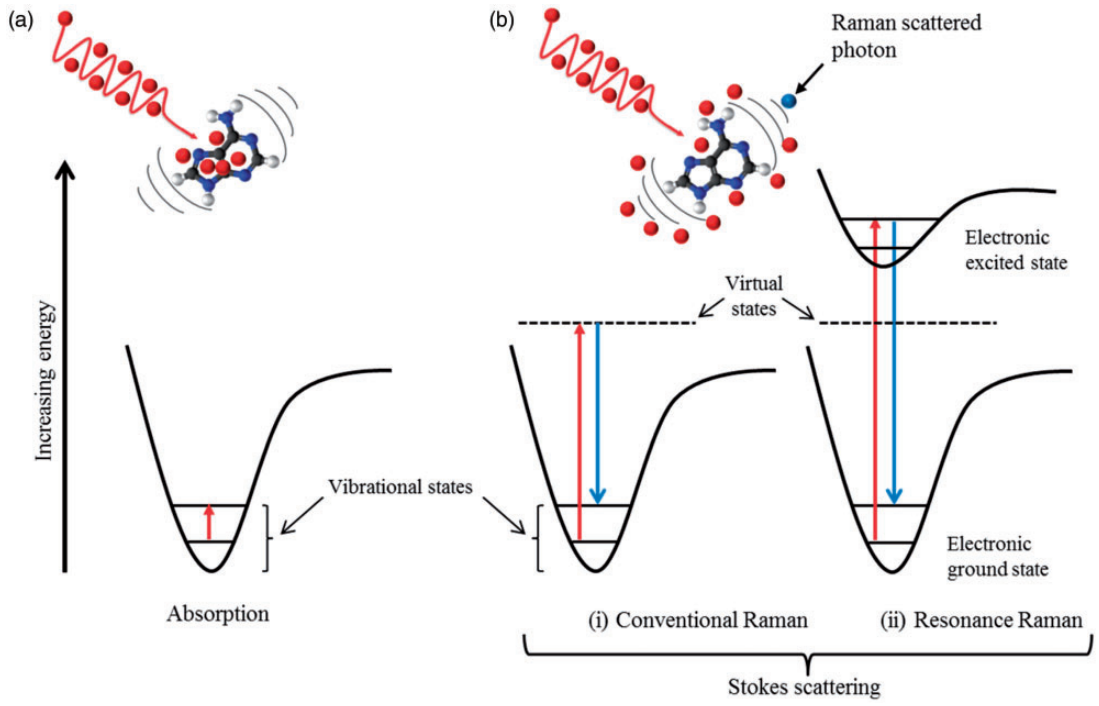


**Figure 2.** Schematic illustration of the three vibrational spectroscopic processes based on (a) IR absorption and (b) Stokes Raman scattering: (i) conventional Raman or SERS; (ii) resonance Raman. The Morse curves show the molecular energy transitions, which occur when an investigated sample interacts with radiation of appropriate wavelength.

**Table I.** Major spectral band assignments for FT-IR, Raman, and SERS (tentative) of bacteria.[39,64,151,155]

| Wavenumber ($cm^{-1}$) | Band assignment* | | |
|---|---|---|---|
| | FT-IR | Raman | SERS |
| 3200–3500 | O–H, N–H str proteins and carboxylic acids | – | – |
| 2800–3100 | $CH_3$ and $CH_2$ str carbohydrates, lipids, proteins | $CH_3$ and $CH_2$ str carbohydrates, lipids, proteins | $CH_3$, $CH_2$ and $=CH_2$ str carbohydrates, lipids, proteins, olefins |
| 1655 | C=O, N–H str amide I | C=O, N–H str. amide I | – |
| 1620 | – | – | C=C str olefinic |
| 1575–1590 | – | Guanine, adenine ring br | C–N str proteins, DNA, Amides |
| 1520–1550 | C–N, N–H str amide II | – | C–H bend or C=C str |
| 1440–1460 | $CH_2$ def lipids, proteins, carbohydrates | $CH_2$ def. lipids, proteins, carbohydrates | $CH_2$ def. lipids, proteins, carbohydrates |
| 1320–1340 | – | – | Adenine ring br |
| 1230–1295 | Amide III | Amide III | Amide III |
| 1155 | – | – | C–N str, amides, DNA, adenine |
| 1087 | P=O str FAD, phosphodiesters | C–O str amides, proteins | C–O str. amides, proteins |
| 1004 | – | Phenylalanine ring br | – |
| 785–840 | – | Cytosine, uracil, tyrosine | Cytosine, uracil, tyrosine |
| 720–740 | $CH_2$ def lipids, proteins, carbohydrates | Adenine | Adenine, FAD, NAG |
| 665 | – | Guanine | Nucleic acids |
| 640 | – | Tyrosine | Nucleic acids |

*str = stretching, def = deformation, br = breathing.

Fourier transform infrared spectroscopy is a very versatile tool with many applications in food quality control and environmental monitoring due to its powerful screening ability. As shown by Helm and colleagues, FT-IR combined with chemometrics provides accurate means to classify and identify various infectious bacteria including *Streptococcus, Staphylococcus*, and *Legionella* species.[45] Similarly, the results obtained by Maquelin et al. reveal that FT-IR exhibits similar accuracy and reliability level (~98%) as traditional "gold standard" tools in the identification of soil bacterial and fungal species such as *Escherichia coli, Enterococcus faecium, Candida albicans*, and *Candida glabrata* recovered from blood culture.[46] With the resurgence of surface-enhanced infrared absorption (SEIRA), which improves the sensitivity of absorption bands through plasmonic materials, and the development of nanoscale infrared microscopy which permits accurate analysis and high resolution (~2 μm) imaging of single microbial cells, FT-IR spectroscopy will potentially offer valuable information to allow extensive biochemical structure elucidation and highly accurate classification of novel uncultivated prokaryotic microbial systems.[47–50] However, one drawback of FT-IR is that it registers intense broad bands due to the strong absorption of infrared radiation by water molecules, which may mask other spectral features and interfere with spectral interpretation. Nonetheless, this problem is usually overcome by the dehydration of a sample, application of attenuated total reflectance (ATR), use of heavy water ($D_2O$) as the solvent, or subtraction of water peaks.[51–53]

By contrast, Raman spectroscopy provides chemical information through Stokes (as well as anti-Stokes but this is less efficient) scattered photons (Figure 2) from Raman-active symmetrical molecular vibrations (e.g., C=C, S–S, N=N, C–H, etc.), whose electron densities shift unsymmetrically (anisotropically) to induce polarizability when a sample is irradiated with light in the visible to mid-IR range.[54] Therefore, Raman offers complementary biochemical and structural information to FT-IR absorption measurements due to different selection rules, except in centrosymmetric biochemical molecules in which case the rule of mutual exclusion applies.[21,53,55] Interestingly, Raman exhibits exceptional advantages over FT-IR in microbial analysis, as water is a weak scatterer of light and thus does not cause any interference with spectral interpretation. This makes Raman more appropriate for in vivo analysis of microbial systems that occur naturally in aqueous environments.[26]

Within recent years Raman microspectroscopy has been increasingly attracting considerable attention for bacterial analysis, particularly due to advancements in technology which includes optical fibers for in situ online analysis, strong monochromatic lasers, whose radiation wavelength

is within the diffraction limit ($\bar{\upsilon}^2$ or $\lambda^{-2}$, where $\bar{\upsilon}$ and $\lambda$ are wavenumber and wavelength, respectively), and highly sensitive pixelated charge-coupled devices (CCD) connected to a confocal microscopy system.[55–57] With these improvements, the Raman spectral fingerprints of bacteria can be measured at much higher spatial resolution (lateral and depth resolution of $<1\,\mu m$ and $1–2\,\mu m$, respectively; about the size of a single bacterial cell) and contrast from very small sample weights ($\sim 1$ pg).[39] Additionally, the moveable motorized encoded sample stage with a step size of typically 100 nm permits stable, accurate and repeatable Raman measurements of samples in any physical state. Coupled with robust novel cell sorting technologies such as magnetic nanoparticles (MNPs),[58] laser-induced forward transfer (LIFT)-assisted Raman activated cell ejection (RACE), and Raman tweezers,[59] Raman spectroscopy is proving to be a very powerful tool for studying detailed and subtle information about the physiological dynamics, spatial localization and functional attributes of single microbial cells of desirable traits in complex natural environments as demonstrated by several authors.[26,60,61]

Although the non-resonance Raman instrumentation has been greatly improved both mechanically and optically, its scattering efficiency is still intrinsically poor. Typically only $\sim 1$ in $10^6$–$10^8$ photons undergo inelastic Raman scattering resulting in limited sensitivity and long spectral collection times.[62] Additionally, many biological samples tend to fluoresce in the visible to near-IR in which case Raman spectral features are obscured making it difficult for qualitative and quantitative analysis.[63] The quest for techniques which exhibit ultra-high sensitivity to low concentrations of biomass has been showing much promise in recent years. Resonance Raman spectroscopy (RRS), where laser excitation matches the electronic transition states of the investigated sample molecules (Figure 2), is frequently used to increase the Raman signal by $10^2$–$10^4$ orders of magnitude.[64] Resonance Raman spectroscopy selectively enhances Raman signals of chromophoric segments of metabolites and biopolymers which exhibit large Raman cross-sections, and in the deep ultraviolet (e.g., 244 nm), preferentially from conjugated aromatic molecular moieties such as nucleic acids and aromatic amino acids.[65–67] This means that an RRS spectrum has fewer highly intense and well resolved vibrational modes which are easier to interpret than conventional Raman.[66,68] Thus, in addition to simplifying spectral interpretation, RRS provide higher sensitivity, allowing for quicker bacterial analysis, and can reduce the fluorescence background if the laser wavelength is below the absorption range (usually $< 290$ nm).[69,70] Many researchers have reported the application of RRS to identify and discriminate a wide range of bacteria and to probe microbial functions, largely due to the variability in peak intensity ratios and absolute intensities within a cell.[70–73] However, poor selectivity makes it less suitable for whole-organism bacterial typing since it is biased towards a limited range of chromophoric groups which are essentially present in all bacterial species as illustrated by Lopez-Diez and Goodacre's work on Gram-positive *Bacillus* and *Brevibacillus* genera.[70] As a result, RRS spectra may not provide large discriminatory variations in biochemical composition and capabilities, and hence the general metabolic activity required for the accurate identification and clear-cut differentiation of bacteria at species and strain level. Furthermore, the photochemical effects involved as a consequence of the absorption of strongly energetic UV-Vis radiation focused to sample area of $\sim 1\,\mu m^2$ resulting in $10^4$–$10^6$ W $m^{-2}$ power density range may cause sample degradation.[37,41] However, as demonstrated by Gaus et al. sample degradation can be minimized by reducing the spectral acquisition time and spinning the sample on a microscope stand to avoid continuous and prolonged exposure of microbial cells to the high-power laser beam.[68]

Alternatively, the intensity of the Raman effect can be amplified when sample molecules adsorb onto, or are in immediate proximity to a roughened metal surface, colloidal suspension or thin metal film via SERS.[74,75] Ideally, stable plasmonic metals predominantly Ag- and Au-based nanoparticles (NPs) with negatively charged surfaces derived from the reducing agent used in NP production or small positive dielectric constants are commonly used, as they exhibit high absorptivity and large enhancement factors (EF) when they are exposed to the electric field of radiation.[76] The SERS approach provides unique favorable benefits including fluorescence quenching effects due to fluorescence resonance energy transfer (FRET) from biomolecules in close proximity to metal NPs (which does not depreciate the Raman emission process),[77,78] reduced band overlap, shorter spectral acquisition times and the ability to eliminate photodegradation.[54] Historically, the SERS effect was first observed and reported by Martin Fleischmann and co-workers for pyridine adsorbed onto the surface of roughened silver electrodes.[79] However, SERS as we know it today was to a large degree interpreted by Jeanmaire and Van Duyne in 1977,[80] and later confirmed by Albrecht and Creighton in the same year.[81] These two reports proposed two separate theories, the long range (nanometer scale) electromagnetic (EM) and the short range (atomic scale) charge transfer (CT) mechanisms, respectively, as the two potential factors contributing to the dramatic increase in Raman signal intensity. According to the EM theory, conventionally accepted to be the major contribution to the SERS effect, a spectral signal is significantly enhanced when incident radiation excites localized surface plasmon oscillations on the surface of NPs resulting in typical EF of $\sim 10^6$–$10^8$.[82,83] This interaction generates regions of amplified electric fields around NP surfaces generally called "hot spots" within which sample molecules with enhanced signals reside or are in close proximity to.[84,85] In contrast, the CT mechanism is

assumed to involve electronic coupling between the Raman-active components of the sample molecules and NPs surface which results in an increase in the Raman cross-section.[86] It is postulated that highly electronegative atoms and ionizable functional groups such as $–CO_2H$, $–NH_2$, and $–N\equiv N–$ in sample molecules form chemical bonds with NP surfaces.[87] Due to the migration of electron densities across molecule–NP chemical bonds, polarizability is enhanced which eventually amplifies the Raman signal by $\sim 10–10^2$ fold if the excitation laser wavelength is resonant with NP-molecule charge transfer electronic states.[88] Although EM and CT enhancements have improved our understanding of SERS substantially there is currently no fully elaborated and conclusive explanation for signal enhancement to date, that is to say, it is still very hard to generate computer simulations of SERS spectra and thus this topic is still the subject of intense debate.[89]

Surface-enhanced Raman scattering exhibits virtually all the advantages of conventional Raman but adds quantitative ultra-sensitivity up to single molecule detection,[90–92] to enhance its robust discriminatory ability. Moreover, since inexpensive and easily prepared NPs are used as the Raman signal enhancing media for sample molecules of low concentrations, stable, and information-rich SERS signals can be achieved with low cost spectrometers. When combined with molecular techniques such as immunoassays and robust chemometrics, SERS is a viable alternative candidate that can compete fairly well with traditional gold-standard techniques in terms of cost but with the additional advantages of portability, rapidity, multiplexing, high specificity, and selectivity. Thus, it is perhaps not surprising that SERS has proliferated into diverse scientific fields for various applications such as clinical,[93,94] discovery of biological and chemical warfare markers,[95] antimicrobial resistance,[96,97] environmental studies,[4,98] and microbial ecology,[99] as shown by the results of a bibliometric analysis of SERS related reports within the last decade (Figure 3).

## Applications of SERS in Microbial Analysis

### *Identification of Bacteria in Clinical Biochemistry*

As already stated above, whilst the vast majority of microbes play an absolutely vital role to the maintenance of life on Earth, opportunistic and pathogenic bacteria and fungi cause a plethora of well-known infections and diseases in humans, such as urinary tract infections (UTIs), pneumonia, waterborne diarrheal diseases, sepsis, and tuberculosis, as indicated by annual global health reports.[100] For example, invasive fungal infections especially those which are caused by *Aspergillus* and *Candida* species are common causes of morbidity and deaths in immunocompromised patients.[101] The most common way of tackling microbial infections is the use of antibiotics or antifungal agents which are prescribed to patients based on the clinical signs and symptoms they exhibit.[102] Although these therapeutic strategies that target surrogate indicators or symptoms of the presence and progression of microbial infections may succeed at times, there is still a high probability of missing the target etiological agents, in which case infections are then likely to persist or recur. Therefore, there is a requirement for the identification of pathogenic bacteria or fungi linked to disease-specific symptoms accurately and reliably, for the early intervention of improved, accelerated, and targeted treatments.[98,103,104] This approach would certainly prevent a mismatch between therapeutic strategies and target pathogen, facilitate desirable prognosis and reduce antibiotic/antifungal resistance.[105]

Within the last few years, SERS has been shown to be effective in detection and identification of specific pathogenic microorganisms associated with UTIs,[97,106] pneumonia,[107] and anthrax,[95,108] in order to guide personalized clinical intervention.[109] The research reported by Shanmukh et al. indicated the potential of using SERS in clinical medicine to study the molecular biochemistry of highly pathogenic viruses in blood.[110] This SERS method identified and differentiated between adenovirus type 6, rhinovirus type 4, and human immunodeficiency virus

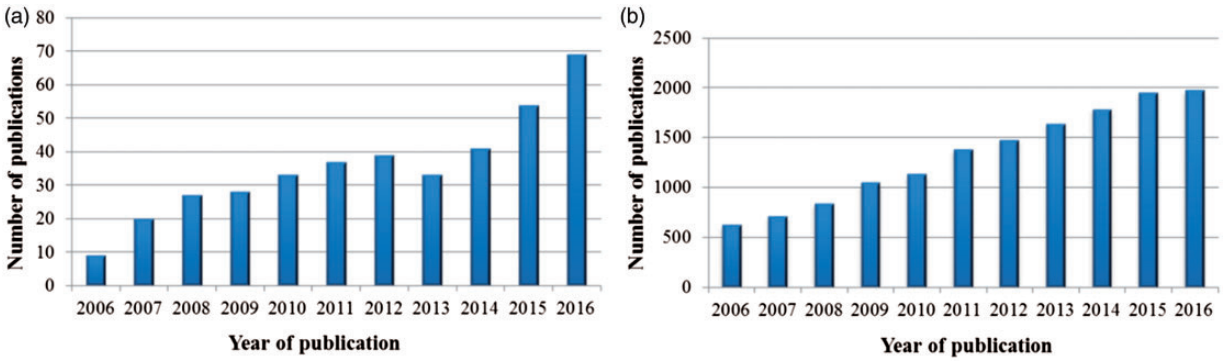


**Figure 3.** Bar charts illustrating increasing number of publications on SERS per year from 2006 to 2016 listed in Web of Science Core collection search engine using the search term (a) "surface enhanced Raman" or SERS bacteria and (b) surface enhanced Raman or SERS (accessed on 9 October, 2017).

(HIV) correctly based on variations in spectral features and intensities of SERS signals ascribed to the nucleic acids guanine and adenine. This interesting study highlights the ability of SERS to detect trace concentrations of deadly viruses making it a potentially suitable candidate to reveal subclinical and/or asymptomatic infections such as candidemia.[94] With the continuous development of portable Raman spectrometers, SERS can be employed for rapid point-of-care (POC) diagnosis in healthcare especially in rural and remote areas where such infections are highly prevalent. Figure 4 illustrates how SERS fingerprints and principal component analysis (PCA) can be used to differentiate among microbes of clinical and environmental importance. Using PC1 and PC2, all the three strains namely *Bacillus cereus* (Gram-positive), *Pseudomonas putida*, and *E. coli* cells (both Gram-negative) were clearly discriminated. The tentative assignment of the SERS spectral bands in Figure 4 is summarized in Table 1.

## *Characterization of Biofilms*

Biofilms represent an interfacial collection (community) of multispecies pathogenic and non-pathogenic microorganisms (e.g., bacteria, fungi, protozoa) embedded within complex matrix of extracellular polymeric substances (EPS) of, predominantly, microbial origin.[111] The growth of microbes and the maintenance of biofilm integrity are largely sustained by biopolymers such as carbohydrates, lipids, proteins, humic-like materials, and nucleic acids abundantly found in the EPS. Thus, EPS acts as a supply of energy sources and water reservoir, protection against environmental and physical stress and as a communication medium among microbial cells (quorum sensing).[12] For this reason, microbial cells in biofilms can live longer and subsequently predate mammals and plants. Due to this fact, pathogenic biofilms have the potential to be used as agents of bioterrorism. In clinical medicine, biofilms are associated with the majority (80%) of human microbial infections in healthcare centers. As noted by Donlan and colleagues, biofilms comprising predominantly pseudomonads, staphylococci and *Enterobacter* species are commonly formed on the surfaces of in-dwelling medical devices and instruments.[12,112] When used on especially hospitalized immunocompromised patients (e.g., during an operation and/or organ transplant) pathogen-infested medical devices can deposit biofilms into human body where they cause various infections which can lead to substantial healthcare costs and increased mortality rates.[113] In light of this, there is a need to develop a deeper understanding of microbial diversity and the chemical composition of biofilms in order to identify and develop appropriate biocides and workable antifouling strategies.[114] In industrial wastewater treatment, more in-depth knowledge of biofilms would allow for effective bioremediation strategies.[115]

Ideally, analytical platforms that combine rapidity, portability and simplicity are desirable to understand biofilms and combat biofilm infections.[114] Confocal laser scanning microscopy (CLSM) and staining strategies have been successfully used to investigate the molecular constituents and characterize the three-dimensional structures of biofilms at microscopic scale.[116] However, CLSM lacks the desired specificity and penetration depth demanded by the analysis of diverse molecular composition of biofilms. Neu et al. characterized glycoconjugates within EPS matrix in environmental biofilms in situ using fluor-conjugated lectins staining strategy.[117] Their results revealed heterogeneity in molecular composition of the biofilms with high spatial resolution. Despite being useful, the staining method is difficult to employ for comprehensive elucidation of diverse polymeric materials with multiple binding sites present in a complex EPS matrix. By contrast, Raman and FT-IR spectroscopy provide noninvasive, rapid, and holistic qualitative and quantitative means of measuring accurate and characteristic vibrational modes arising from carbohydrates, proteins,

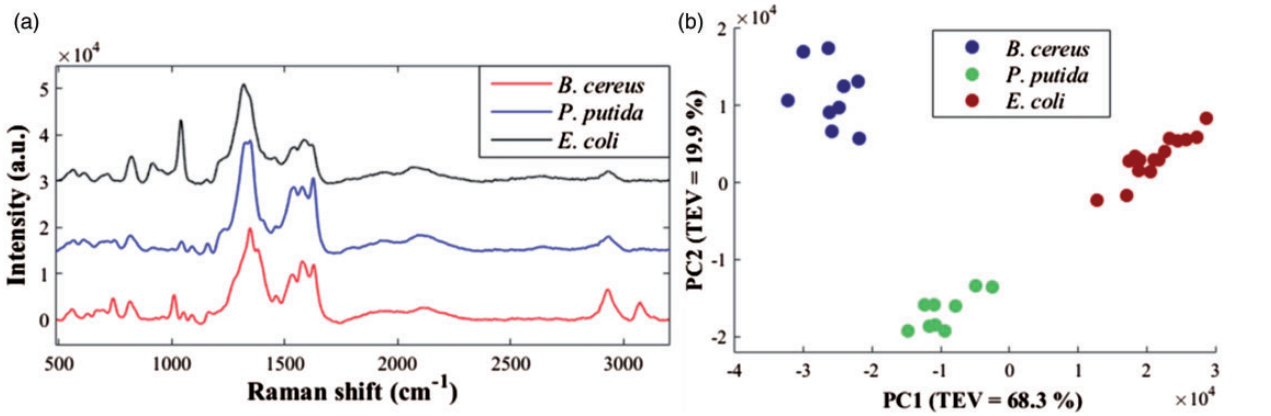


**Figure 4.** (a) SERS spectra employed to identify and differentiate among *E. coli* (black), *P. putida* (blue), and *B. cereus* (red). (b) PCA scores plot of the SERS spectra demonstrating the high reproducibility and discriminatory power of the SERS approach.

lipids, nucleic acids, etc., present in the EPS matrix at ~1 μm spatial resolution in real time.[118,119] Recently, Ivleva and colleagues demonstrated that when Raman is complemented by SERS, highly reproducible, characteristic and integrated spectral information, free of water signals and background fluorescence could be achieved. This method exhibited huge potential for on-site unequivocal interpretation of results which are attainable from various constituents of the aqueous multispecies biofilms in situ.[120] Most importantly, label-free SERS enhances the Raman signal to improve sensitivity to trace amounts of biopolymers in EPS at different stages of biofilm formation, and reduces spectral collection times from minutes to a few seconds.[121–123] However, for future investigations of biofilms, a combination of CLSM, Raman, SERS and stable isotopic labeling is envisaged for a comprehensive elucidation of the molecular composition and their spatial distribution in complex EPS matrices during developmental and maturation stages of biofilm formation.[119]

## *Detection of Dipicolinic Acid (DPA), a Biological Warfare Marker*

The development and applications of SERS as a highly sensitive and specific fingerprinting technique to detect and quantify biological warfare markers has focused on causative agents of anthrax. Anthrax is a well known and highly transmissible disease caused by *Bacillus anthracis*, gaining further notoriety when it was used for bioterrorism purposes in the United States of America in 2001.[124] *B. anthracis* spores were circulated in letters and other mail packages in Florida and New Jersey to their intended targets. Since *B. anthracis* forms a spore by developing a hard protective proteinaceous coat around the cellular genetic material,[125] it can survive harsh conditions like UV, heat, droughts, pH fluctuations, and harsh chemicals for significant periods.[126,127] If inhaled, the spores germinate into active vegetative states and produce toxic chemicals associated with swelling of the lungs and internal bleeding as the reactivated cells multiply rapidly.[128] To accelerate response strategies with respect to the bioterrorism attacks and threats using inhalation anthrax, it has become increasingly important to detect and identify specific anthrax spores rapidly for quick intervention of therapeutic strategies. Techniques such as traditional culturing and PCR have been commonly used as the gold standard to identify and characterize *Bacillus* spores including those of virulent subspecies.[129,130] Nonetheless, the time required to complete bacterial analysis using these techniques is typically in the range of several hours to days.[131] A viable alternative is to detect dipicolinic acid (DPA; pyridine-2,6-dicarboxylic acid), a unique characteristic chemical marker produced in late sporulation, and which comprises about 1–14% of the dry weight of a spore.[126] Dipicolinic acid has been detected by many research groups using ultraviolet RR,[132] mass spectrometry,[133,134] chromatography,[135] potentiometric sensors,[136] and FT-IR.[134,137]

Qualitative and quantitative analytical approaches utilizing SERS with lower limits of detection (LOD) have shown considerable promise in the last two decades. The direct quantitative detection of trace levels of DPA down to 29.9 nM (well below the infective dose of *B. anthracis* ~$10^4$ cells) and 10 μM using inexpensive silver colloids has been reported.[95,138] Jarvis et al. demonstrated a clear qualitative correlation between the SERS spectral profile of DPA extracted from *Bacillus* spores and that of their vegetative cells,[139] and subsequent studies exploiting superhydrophobic copper wires showed that the DPA signal allowed for the detection of just 18 spores.[108] There are other chemical compounds produced by *Bacillus* spores that have been detected as anthrax biomarkers. The study by Gao and colleagues provides a recent example of the potential to detect and quantify poly-γ-D-glutamic acid (PGA) in serum, another anthrax biomarker, using SERS employing PGA-conjugated gold NPs within an automated microfluidic environment.[140] It is clearly evident that this SERS-based analytical platform for measuring PGA exhibited higher detection sensitivity (LOD = 100 pg/mL) than ELISA (LOD = 0.1–1.0 μL).[140] This illustrates the capability of SERS to compete with established protocols based on immunosorbent assays to detect deadly biowarfare. Nevertheless SERS has poor selectivity,[141] and its ability to discriminate between DPA biomarkers produced by different non-pathogenic environmental species and subspecies of various organisms within the same genus is yet to be demonstrated.[95,138] However, within a battlefield scenario the few seconds to minutes within which SERS detects DPA warrants the use of other confirmatory techniques to ascertain whether the detected DPA is produced by infectious bacterial spores.

## *Functional Analysis of Microorganisms Using Stable Isotopic Labeling Technique*

Within the recent two decades, stable isotope probing (SIP) has emerged to be the state-of-the-art means of studying functional properties and substrate degradation pathways of individual unculturable members of a mixed microbial community directly.[61,142–144] Stable isotope probing employs the "you are what you eat" concept,[145] which involves the incubation of cells with stable isotopes. This is followed by detection of the isotopic content in cellular biomarkers such as polar-lipid-derived fatty acids (PLFA),[146] nucleic acids (DNA/RNA),[147–149] and simultaneous identification of distinct microbes actively metabolizing the labeled substrate. Substrates labeled with stable atoms of carbon ($^{13}$C) and nitrogen ($^{15}$N) are commonly employed as single or dual labels in microbial cells, which can use them to maintain their normal life. In terms of vibrational spectroscopy, spectral bands of the cells metabolizing

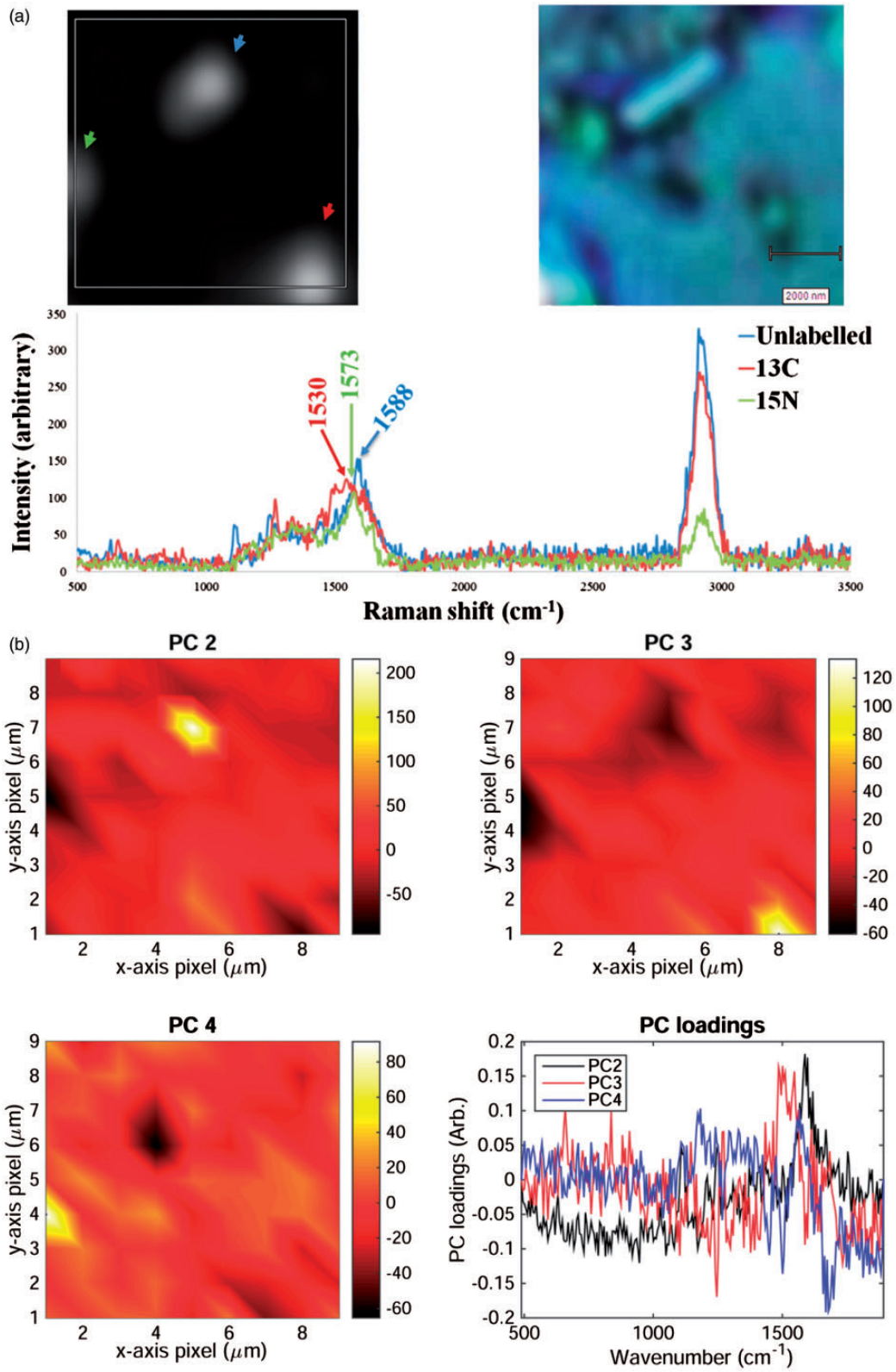


**Figure 5.** SERS image of isotopically labeled intact single *E. coli* cells. (a) SERS and corresponding white light image of three single *E. coli* cells, and representative spectra of each single bacterium (indicated by the arrows). The gray shading represents peak intensity at 1588 $cm^{-1}$. (b) PCA scores images reconstruction of the SERS spectral data collected between 500–1800 $cm^{-1}$, and their corresponding loadings plot; the first PC score is not shown as this only differentiates between bacterial biomass and $CaF_2$ background. (Reproduced from Chisanga et al.,[151] with permission from The Royal Society of Chemistry.)

isotopically enriched substrates shift to lower wavenumbers (red shift) and are clearly distinguished from those that do not incorporate the labeled isotopes, whose peaks remain on the same wavenumbers before and after the labeling process. Very recently, we demonstrated the feasibility of using FT-IR, Raman spectroscopy, and multivariate analysis to detect *E. coli* cells labeled with varying ratios of $^{12}C/^{13}C$-glucose and/or $^{14}N/^{15}N$-ammonium chloride at population and single cell levels.[39] The results showed that cells enriched with varying amounts of $^{13}C$ and/or $^{15}N$ labeled substrates could be differentiated from each other based on their Raman and FT-IR spectral band shifts. This illustrated the exciting prospects of using FT-IR and Raman-SIP as complementary tools to identify microbial cells at different levels of substrate incorporation in a complex microbial community.

Similarly, Huang et al. successfully identified citrate degrading microbes using *reverse* isotopic labeling with unlabeled citrate.[99] Initially, cells were grown on $^{13}C$-glucose and their Raman bands shifted to lower wavenumbers showing full incorporation of $^{13}C$ to cells followed by incubation of the same cells on $^{12}C$-citrate as a single carbon source. The red shifted Raman bands of *Acinetobacter baylyi* ADP1 due to $^{13}C$-glucose incorporation reverted back to their initial wavenumbers after growth on $^{12}C$-citrate. There were no spectral shifts observed for *E. coli* DH5α-GFP cells under the same conditions as they could not utilize citrate compound. Interestingly, $D_2O$ was also used to sort metabolically active cells grown on $^{12}C$-citrate as a carbon source. The Raman spectrum of *A. baylyi* ADP1 shows a broad peak in 2050–2300 $cm^{-1}$ region ascribed to C–D stretch which was absent in the spectrum of *E. coli* DH5α-GFP, confirming the general metabolic activity of *A. baylyi* ADP1 in the presence of $^{12}C$-citrate. This technique, termed reverse isotopic labeling, illustrates the viability of employing unlabeled substrates as sorting criteria of microbial cells that degrade organic pollutants whose labeled analogues are either too expensive or not commercially available.

The application of SERS to observe functional activities of bacterial communities is showing considerable promise, and SERS-SIP applied in this vein was first reported by Kubryk et al. in 2015,[73] and three other reports have emerged to date.[4,150,151] Recently SERS-SIP revealed the possibility of applying SERS imaging to detect and identify $^{13}C$ and $^{15}N$ labeled cells in a community from very low concentration of biomass using in situ synthesis of Ag NPs (Figure 5).[151] Figure 5 shows clear shifts of SERS bands to lower wavenumbers for $^{13}C$ and $^{15}N$ incorporated cells due to protein (C–N) vibrations and corresponding images highlighting differentiation of bacteria at the single-cell level. Although Raman and SERS-SIP is showing increasing potential in microbial ecology, spectral features measured from bacteria which are at different stages of cell growth show significant variation.[71] Furthermore, the variations in environmental conditions (e.g., pH) can alter the composition of bacterial cells resulting in changes in Raman or SERS spectral fingerprints. This may complicate spectral interpretation and the identification of microbes may be very difficult, though spectral shifts could be detected when heavy atoms are incorporated. In modern integrated analysis, these problems could be overcome by employing genomics-based tools, e.g., fluorescence in situ hybridization (FISH) and RACE,[152,153] to screen cells in order to confirm Raman or SERS fingerprints as demonstrated previously.[61,153,154]

## Conclusion

In this review article, we have discussed the applications of SERS spectroscopic methods and techniques for the identification, differentiation, and discrimination of a range of microbes in several areas of topical interest. Much emphasis has been placed on the current and future potential applications of SERS in diverse microbial studies, taking advantage of the continuous technological advances in Raman instrumentation which employs relatively inexpensive materials to increase their sensitivity further in order to detect extremely low concentrations of analytes. This is particularly important in addressing high rising food poisoning cases for example, as well as dealing with potential bioterrorism threats involving deadly microbial pathogens and biowarfare agents, where minute concentrations would cause serious harm to human communities be they military or civilian. Additionally, we believe that when FT-IR, Raman, and SERS are integrated with other tools such as CLSM, genomics-based analytics, and multivariate chemometrics, they will increasingly present an interesting and innovative platform for the comprehensive characterization of infectious microbes and biofilms, significantly expanding on current knowledge. Additionally, it is believed that the development of on/at/in-line Raman techniques would provide rapid, efficient and cost-effective analysis for monitoring microbial infections as well as environmental pollution in real time on-site without the need to transport the samples to the laboratory. In contemporary microbial ecology, these complementary tools, coupled with robust cell sorting techniques including robust MNPs and RACE, and metabolomics, may elucidate the link between uncultivated microbial cells (gene) and various observable microbially mediated environmental bioprocesses (functions) they are associated with. This knowledge will be vital in improving bioremediation processes, for instance in community wastewater treatment plants (as well as those located in the increasing number of nuclear power plants) where the identity of pollutant-degrading microbes is absolutely essential in helping to degrade and maintain the levels of a wide range of pollutants with less or non-intoxicating limits.

## Acknowledgments

We would like to thank The Royal Society of Chemistry for giving us permission to reproduce Figure 5 from Chisanga et al.[151] RG would like to sincerely thank *Applied Spectroscopy* for their kind invitation to write this Focal Point Review article.

## Conflict of Interest

None declared.

## Funding

This work was supported by the Commonwealth Scholarship Commission, UK, the European Commission's Seventh Framework Program (STREPSYNTH; project number 613877), and UK BBSRC (grant number BB/L014823/1).

## ORCID iD

Royston Goodacre http://orcid.org/0000-0003-2230-645X

## References

1. R. Goodacre. "Metabolomics of a Superorganism". J. Nutr. 2007. 137(Suppl. 1): 259–266.
2. C. Griebler, T. Lueders. "Microbial Biodiversity in Groundwater Ecosystems". Freshwater Biol. 2009. 54(4): 649–677.
3. A. Oren. "Archaea". In: Encyclopedia of Life Sciences. Chichester, UK: John Wiley and Sons, 2010, pp.1–10.
4. L. Cui, K. Yang, G.W. Zhou, W.E. Huang, et al. "Surface-Enhanced Raman Spectroscopy Combined with Stable Isotope Probing to Monitor Nitrogen Assimilation at Both Bulk and Single-Cell Level". Anal. Chem. 2017. 89(11): 5794–5801.
5. S. Elias, E. Banin. "Multi-Species Biofilms: Living with Friendly Neighbors". FEMS Microbiol. Rev. 2012. 36(5): 990–1004.
6. H. Chiyangi, J.B. Muma, S. Malama, J. Manyahi, et al. "Identification and Antimicrobial Resistance Patterns of Bacterial Enteropathogens from Children Aged 0–59 Months at the University Teaching Hospital, Lusaka, Zambia: A Prospective Cross Sectional Study". BMC Infect. Dis. 2017. 17: 117.
7. D.I. Ellis, W.B. Dunn, J.L. Griffin, J.W. Allwood, et al. "Metabolic Fingerprinting as a Diagnostic Tool". Pharmacogenomics. 2007. 8(9): 1243–1266.
8. V. Velusamy, K. Arshak, O. Korostynska, K. Oliwa, et al. "An Overview of Foodborne Pathogen Detection: In the Perspective of Biosensors". Biotechnol. Adv. 2010. 28(2): 232–254.
9. H. Muhamadali, A. Subaihi, M. Mohammadtaheri, Y. Xu, et al. "Rapid, Accurate, and Comparative Differentiation of Clinically and Industrially Relevant Microorganisms via Multiple Vibrational Spectroscopic Fingerprinting". Analyst. 2016. 141(17): 5127–5136.
10. A.J.M. Wollman, R. Nudd, E.G. Hedlund, M.C. Leake. "From Animaculum to Single Molecules: 300 Years of the Light Microscope". Open Biol. 2015. 5(4): 150019.
11. V. Torsvik, L. Ovreas. "Microbial Diversity and Function in Soil: From Genes to Ecosystems". Curr. Opin. Microbiol. 2002. 5(3): 240–245.
12. R.M. Donlan, J.W. Costerton. "Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms". Clin. Microbiol. Rev. 2002. 15(2): 167–193.
13. J. Handelsman. "Metagenomics: Application of Genomics to Uncultured Microorganisms". Microbiol. Mol. Biol. Rev. 2004. 68(4): 669–685.
14. C. Rinke, P. Schwientek, A. Sczyrba, N.N. Ivanova, et al. "Insights into the Phylogeny and Coding Potential of Microbial Dark Matter". Nature. 2013. 499(7459): 431–437.
15. D.I. Ellis, H. Muhamadali, D.P. Allen, C.T. Elliott, et al. "A Flavour of Omics Approaches for the Detection of Food Fraud". Curr. Opin. Food Sci. 2016. 10: 7–15.
16. O. Fiehn. "Combining Genomics, Metabolome Analysis, and Biochemical Modelling to Understand Metabolic Networks". Comp. Funct. Genomics. 2001. 2(3): 155–168.
17. M.A. Nadkarni, F.E. Martin, N.A. Jacques, N. Hunter. "Determination of Bacterial Load by Real-Time PCR Using a Broad-Range (Universal) Probe and Primers Set". Microbiology. 2002. 148(Pt. 1): 257–266.
18. R. Goodacre, S. Vaidyanathan, W.B. Dunn, G.G. Harrigan, et al. "Metabolomics by Numbers: Acquiring and Understanding Global Metabolite Data". Trends Biotechnol. 2004. 22(5): 245–252.
19. T. Krishnamurthy, P.L. Ross, U. Rajamani. "Detection of Pathogenic and Non-Pathogenic Bacteria by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry". Rapid Commun. Mass Spectrom. 1996. 10(8): 883–888.
20. M. Hariu, Y. Watanabe, N. Oikawa, M. Seki. "Usefulness of Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry to Identify Pathogens, Including Polymicrobial Samples, Directly from Blood Culture Broths". Infect. Drug Resistance. 2017. 10: 115–120.
21. H. Muhamadali, D. Weaver, A. Subaihi, N. AlMasoud, et al. "Chicken, Beams, and Campylobacter: Rapid Differentiation of Foodborne Bacteria via Vibrational Spectroscopy and MALDI-Mass Spectrometry". Analyst. 2016. 141(1): 111–122.
22. P. Houpikian, D. Raoult. "Traditional and Molecular Techniques for the Study of Emerging Bacterial Diseases: One Laboratory's Perspective". Emerg. Infect. Dis. 2002. 8(2): 122–131.
23. R.E. Fernandez, A. Rohani, V. Farmehini, N.S. Swami. "Review: Microbial Analysis in Dielectrophoretic Microfluidic Systems". Anal. Chim. Acta. 2017. 966: 11–33.
24. R.I. Amann, W. Ludwig, K.H. Schleifer. "Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation". Microbiol. Rev. 1995. 59(1): 143–169.
25. M.S. Rappe, S.J. Giovannoni. "The Uncultured Microbial Majority". Annu. Rev. Microbiol. 2003. 57: 369–394.
26. W.E. Huang, M.Q. Li, R.M. Jarvis, R. Goodacre, et al. "Shining Light on the Microbial World: The Application of Raman Microspectroscopy". In: A.I. Laskin, S. Sariaslani, G.M. Gadd, editors. Advances in Applied Microbiology. San Diego, CA: Elsevier Academic Press, Inc., 2010, pp.153–186.
27. P.K. Agrawal, S. Agrawal, R. Shrivastava. "Modern Molecular Approaches for Analyzing Microbial Diversity from Mushroom Compost Ecosystem". 3 Biotech. 2015. 5(6): 853–866.
28. W.T. Liu, T.L. Marsh, H. Cheng, L.J. Forney. "Characterization of Microbial Diversity by Determining Terminal Restriction Fragment Length Polymorphisms of Genes Encoding 16S rRNA". Appl. Environ. Microbiol. 1997. 63(11): 4516–4522.
29. E. Reiss, T. Obayashi, K. Orle, M. Yoshida, et al. "Non-Culture Based Diagnostic Tests for Mycotic Infections". Med. Mycol. 2000. 38(Suppl. 1): 147–159.
30. L. Armbrecht, P.S. Dittrich. "Recent Advances in the Analysis of Single Cells". Anal. Chem. 2017. 89(1): 2–21.
31. U. Nubel, F. Garcia Pichel, G. Muyzer. "PCR Primers to Amplify 16S rRNA Genes from Cyanobacteria". Appl. Environ. Microbiol. 1997. 63(8): 3327–3332.
32. O. Lazcka, F.J. Del Campo, F.X. Munoz. "Pathogen Detection: A Perspective of Traditional Methods and Biosensors". Biosens. Bioelectron. 2007. 22(7): 1205–1217.
33. J.L. Nielsen, C. Kragelund, P.H. Nielsen. "Combination of Fluorescence In Situ Hybridization with Staining Techniques for Cell Viability and Accumulation of PHA and polyP in Microorganisms in Complex Microbial Systems". In: S.P. Cummings, editor. Bioremediation: Methods, Protocols. Totowa, NJ: Humana Press Inc., 2010, pp.103–116.

34. X.N. Lu, H.M. Al-Qadiri, M.S. Lin, B.A. Rasco. "Application of Mid-Infrared and Raman Spectroscopy to the Study of Bacteria". Food Bioprocess Technol. 2011. 4(6): 919–935.
35. J.P. Harrison, D. Berry. "Vibrational Spectroscopy for Imaging Single Microbial Cells in Complex Biological Samples". Front. Microbiol. 2017. 8: 675.
36. L.B. Kong, P.F. Zhang, P. Setlow, Y.Q. Li. "Characterization of Bacterial Spore Germination Using Integrated Phase Contrast Microscopy, Raman Spectroscopy, and Optical Tweezers". Anal. Chem. 2010. 82(9): 3840–3847.
37. K. Maquelin, L.P. Choo-Smith, T. van Vreeswijk, H.P. Endtz, et al. "Raman spectroscopic Method for Identification of Clinically Relevant Microorganisms Growing on Solid Culture Medium". Anal. Chem. 2000. 72(1): 12–19.
38. K. Maquelin, L. Dijkshoorn, T.J.K. van der Reijden, G.J. Puppels. "Rapid Epidemiological Analysis of Acinetobacter Strains by Raman Spectroscopy". J. Microbiol. Methods. 2006. 64(1): 126–131.
39. H. Muhamadali, M. Chisanga, A. Subaihi, R. Goodacre. "Combining Raman and FT-IR Spectroscopy with Quantitative Isotopic Labeling for Differentiation of *E. coli* Cells at Community and Single Cell Levels". Anal. Chem. 2015. 87(8): 4578–4586.
40. J. Doherty, G. Cinque, P. Gardner. "Single-Cell Analysis Using Fourier Transform Infrared Microspectroscopy". Appl. Spectrosc. Rev. 2017. 52(6): 560–587.
41. R. Petry, M. Schmitt, J. Popp. "Raman Spectroscopy: A Prospective Tool in the Life Sciences". ChemPhysChem. 2003. 4(1): 14–30.
42. M. Pilling, P. Gardner. "Fundamental Developments in Infrared Spectroscopic Imaging for Biomedical Applications". Chem. Soc. Rev. 2016. 45(7): 1935–1957.
43. D. Naumann, D. Helm, H. Labischinski. "Microbiological Characterizations by FT-IR Spectroscopy". Nature. 1991. 351(6321): 81–82.
44. M.J. Baker, J. Trevisan, P. Bassan, R. Bhargava, et al. "Using Fourier Transform IR Spectroscopy to Analyze Biological Materials". Nat. Protoc. 2014. 9(8): 1771–1791.
45. D. Helm, H. Labischinski, G. Schallehn, D. Naumann. "Classification and Identification of Bacteria by Fourier-Transform Infrared Spectroscopy". J. Gen. Microbiol. 1991. 137(1): 69–79.
46. K. Maquelin, C. Kirschner, L.P. Choo-Smith, N.A. Ngo-Thi, et al. "Prospective Study of the Performance of Vibrational Spectroscopies for Rapid Identification of Bacterial and Fungal Pathogens Recovered from Blood Cultures". J. Clin. Microbiol. 2003. 41(1): 324–329.
47. M. Osawa. "Surface-Enhanced Infrared Absorption". In: S. Kawata, editor. Near-Field Optics, Surface Plasmon Polaritons. Berlin: Springer-Verlag, 2001, pp.163–187.
48. F. Le, D.W. Brandl, Y.A. Urzhumov, H. Wang, et al. "Metallic Nanoparticle Arrays: A Common Substrate for Both Surface-Enhanced Raman Scattering and Surface-Enhanced Infrared Absorption". ACS Nano. 2008. 2(4): 707–718.
49. S. Kriegel, T. Uchida, M. Osawa, T. Friedrich, et al. "Biomimetic Environment to Study *E. coli* Complex I Through Surface-Enhanced IR Absorption Spectroscopy". Biochemistry. 2014. 53(40): 6340–6347.
50. A. Dazzi, C.B. Prater. "AFM-IR: Technology and Applications in Nanoscale Infrared Spectroscopy and Chemical Imaging". Chem. Rev. 2017. 117(7): 5146–5173.
51. D.I. Ellis, D. Broadhurst, D.B. Kell, J.J. Rowland, et al. "Rapid and Quantitative Detection of the Microbial Spoilage of Meat by Fourier Transform Infrared Spectroscopy and Machine Learning". Appl. Environ. Microbiol. 2002. 68(6): 2822–2828.
52. C.L. Winder, R. Goodacre. "Comparison of Diffuse-Reflectance Absorbance and Attenuated Total Reflectance FT-IR for the Discrimination of Bacteria". Analyst. 2004. 129(11): 1118–1122.
53. W. Jiang, A. Saxena, B. Song, B.B. Ward, et al. "Elucidation of Functional Groups on Gram-Positive and Gram-Negative Bacterial Surfaces Using Infrared Spectroscopy". Langmuir. 2004. 20(26): 11433–11442.
54. E. Smith, G. Dent. Modern Raman Spectroscopy: A Practical Approach. Oxford: Blackwell Science, 2005.
55. N. AlMasoud, Y. Xu, D.I. Ellis, P. Rooney, et al. "Rapid Discrimination of *Enterococcus faecium* Strains Using Phenotypic Analytical Techniques". Anal. Methods. 2016. 8(42): 7603–7613.
56. C.G. Xie, Y.Q. Li. "Confocal Micro-Raman Spectroscopy of Single Biological Cells Using Optical Trapping and Shifted Excitation Difference Techniques". J. Appl. Phys. 2003. 93(5): 2982–2986.
57. J.W. Chan, A.P. Esposito, C.E. Talley, C.W. Hollars, et al. "Reagentless Identification of Single Bacterial Spores in Aqueous Solution by Confocal Laser Tweezers Raman Spectroscopy". Anal. Chem. 2004. 76(3): 599–603.
58. D.Y. Zhang, J.P. Berry, D. Zhu, Y. Wang, et al. "Magnetic Nanoparticle-Mediated Isolation of Functional Bacteria in a Complex Microbial Community". ISME J. 2015. 9(3): 603–614.
59. Y.Z. Song, H.B. Yin, W.E. Huang. "Raman Activated Cell Sorting". Curr. Opin. Chem. Biol. 2016. 33: 1–8.
60. C. Xie, J. Mace, M.A. Dinno, Y.Q. Li, et al. "Identification of Single Bacterial Cells In Aqueous Solution Using Confocal Laser Tweezers Raman Spectroscopy". Anal. Chem. 2005. 77(14): 4390–4397.
61. Y. Wang, Y.T. Ji, E.S. Wharfe, R.S. Meadows, et al. "Raman Activated Cell Ejection for Isolation of Single Cells". Anal. Chem. 2013. 85(22): 10697–10701.
62. L.E. Jamieson, S.M. Asiala, K. Gracie, K. Faulds, et al. "Bioanalytical Measurements Enabled by Surface-Enhanced Raman Scattering (SERS) Probes". Annu. Rev. Phys. Chem. 2017. 10: 415–437.
63. L. Zeiri, S. Efrima. "Surface-Enhanced Raman Spectroscopy of Bacteria: The Effect of Excitation Wavelength and Chemical Modification of the Colloidal Milieu". J. Raman Spectrosc. 2005. 36(6–7): 667–675.
64. K. Maquelin, C. Kirschner, L.P. Choo-Smith, N. van den Braak, et al. "Identification of Medically Relevant Microorganisms by Vibrational Spectroscopy". J. Microbiol. Methods. 2002. 51(3): 255–271.
65. S. Chadha, R. Manoharan, P. Moenneloccoz, W.H. Nelson, et al. "Comparison of the UV Resonance Raman Spectra of Bacteria, Bacterial Cell Walls, and Ribosomes Excited in the Deep UV". Appl. Spectrosc. 1993. 47(1): 38–43.
66. Q. Wu, T. Hamilton, W.H. Nelson, S. Elliott, et al. "UV Raman Spectral Intensities of *E. coli* and Other Bacteria Excited at 228.9, 244.0, and 248.2 nm". Anal. Chem. 2001. 73(14): 3432–3440.
67. R.M. Jarvis, R. Goodacre. "Ultra-Violet Resonance Raman Spectroscopy for the Rapid Discrimination of Urinary Tract Infection Bacteria". FEMS Microbiol. Lett. 2004. 232(2): 127–132.
68. K. Gaus, P. Rosch, R. Petry, K.D. Peschke, et al. "Classification of Lactic Acid Bacteria with UV-Resonance Raman Spectroscopy". Biopolymers. 2006. 82(4): 286–290.
69. W.H. Nelson, R. Manoharan, J.F. Sperry. "UV Resonance Raman Studies of Bacteria". Appl. Spectrosc. Rev. 1992. 27(1): 67–124.
70. E.C. Lopez-Diez, R. Goodacre. "Characterization of Microorganisms Using UV Resonance Raman Spectroscopy and Chemometrics". Anal. Chem. 2004. 76(3): 585–591.
71. U. Neugebauer, U. Schmid, K. Baumann, W. Ziebuhr, et al. "Towards a Detailed Understanding of Bacterial Metabolism—Spectroscopic Characterization of *Staphylococcus epidermidis*". ChemPhysChem. 2007. 8(1): 124–137.
72. N. Kunapareddy, J. Grun, R. Lunsford, S. Nikitin, et al. "Multiwavelength Resonance Raman Characterization of the Effect of Growth Phase and Culture Medium on Bacteria". Appl. Spectrosc. 2015. 69(8): 966–971.
73. P. Kubryk, J.S. Kolschbach, S. Marozava, T. Lueders, et al. "Exploring the Potential of Stable Isotope (Resonance) Raman Microspectroscopy and Surface-Enhanced Raman Scattering for the Analysis of

Microorganisms at Single Cell Level". Anal. Chem. 2015. 87(13): 6622–6630.
74. D. Cialla, A. Marz, R. Bohme, F. Theil, et al. "Surface-Enhanced Raman Spectroscopy (SERS): Progress and Trends". Anal. Bioanal. Chem. 2012. 403(1): 27–54.
75. A.B. Zrimsek, N.H. Chiang, M. Mattei, S. Zaleski, et al. "Single-Molecule Chemistry with Surface- and Tip-Enhanced Raman Spectroscopy". Chem. Rev. 2017. 117(11): 7583–7613.
76. A.J. Haes, C.L. Haynes, A.D. McFarland, G.C. Schatz, et al. "Plasmonic Materials for Surface-Enhanced Sensing and Spectroscopy". MRS Bull. 2005. 30(5): 368–375.
77. W.R. Premasiri, D.T. Moir, M.S. Klempner, N. Krieger, et al. "Characterization of the Surface Enhanced Raman Scattering (SERS) of Bacteria". J. Phys. Chem. B. 2005. 109(1): 312–320.
78. V.N. Pustovit, T.V. Shahbazyan. "Fluorescence Quenching Near Small Metal Nanoparticles". J. Chem. Phys. 2012. 136(20): 204701.
79. M. Fleischmann, P.J. Hendra, A.J. McQuillan. "Raman Spectra of Pyridine Adsorbed at a Silver Electrode". Chem. Phys. Lett. 1974. 26(2): 163–166.
80. D.L. Jeanmaire, R.P. Van Duyne. "Surface Raman Spectroelectrochemistry: Part I. Heterocyclic, Aromatic, and Aliphatic Amines Adsorbed on the Anodized Silver Electrode". J. Electroanal. Chem. Interfacial Electrochem. 1977. 84(1): 1–20.
81. M.G. Albrecht, J.A. Creighton. "Anomalously Intense Raman Spectra of Pyridine at a Silver Electrode". J. Am. Chem. Soc. 1977. 99(15): 5215–5217.
82. K.A. Willets, R.P. Van Duyne. "Localized Surface Plasmon Resonance Spectroscopy and Sensing". Annu. Rev. Phys. Chem. 2007. 58: 267–297.
83. B. Sharma, R.R. Frontiera, A.I. Henry, E. Ringe, et al. "SERS: Materials, Applications, and the Future". Mater. Today. 2012. 15(1–2): 16–25.
84. A. Campion, P. Kambhampati. "Surface-Enhanced Raman Scattering". Chem. Soc. Rev. 1998. 27(4): 241–250.
85. S. Schlucker. "Surface-Enhanced Raman Spectroscopy: Concepts and Chemical Applications". Angew. Chem.-Int. Edit. 2014. 53(19): 4756–4795.
86. Y. Liu, H.B. Zhou, Z.W. Hu, G.X. Yu, et al. "Label and Label-Free Based Surface-Enhanced Raman Scattering for Pathogen Bacteria Detection: A Review". Biosens. Bioelectron. 2017. 94: 131–140.
87. R.S. Das, Y.K. Agrawal. "Raman Spectroscopy: Recent Advancements, Techniques and Applications". Vib. Spectrosc. 2011. 57(2): 163–176.
88. R.A. Tripp, R.A. Dluhy, Y.P. Zhao. "Novel Nanostructures for SERS Biosensing". Nano Today. 2008. 3(3–4): 31–37.
89. D. Graham, R. Van Duyne, B. Ren. "Surface-Enhanced Raman Scattering". Analyst. 2016. 141(17): 4995–4995.
90. S.M. Nie, S.R. Emory. "Probing Single Molecules and Single Nanoparticles by Surface-Enhanced Raman Scattering". Science. 1997. 275(5303): 1102–1106.
91. K. Kneipp, Y. Wang, H. Kneipp, L.T. Perelman, et al. "Single Molecule Detection Using Surface-Enhanced Raman Scattering (SERS)". Phys. Rev. Lett. 1997. 78(9): 1667–1670.
92. X.M. Qian, S.M. Nie. "Single-Molecule and Single-Nanoparticle SERS: From Fundamental Mechanisms to Biomedical Applications". Chem. Soc. Rev. 2008. 37(5): 912–920.
93. R.M. Jarvis, R. Goodacre. "Discrimination of Bacteria Using Surface-Enhanced Raman Spectroscopy". Anal. Chem. 2004. 76(1): 40–47.
94. S. Mabbott, D. Thompson, N. Sirimuthu, G. McNay, et al. "From Synthetic DNA to PCR Product: Detection of Fungal Infections Using SERS". Faraday Discuss. 2016. 187: 461–472.
95. S.E.J. Bell, J.N. Mackle, N.M.S. Sirimuthu. "Quantitative Surface-Enhanced Raman Spectroscopy of Dipicolinic Acid—Towards Rapid Anthrax Endospore Detection". Analyst. 2005. 130(5): 545–549.
96. H.B. Zhou, D.T. Yang, N.P. Ivleva, N.E. Mircescu, et al. "Label-Free In Situ Discrimination of Live and Dead Bacteria by Surface-Enhanced Raman Scattering". Anal. Chem. 2015. 87(13): 6553–6561.
97. W.R. Premasiri, Y. Chen, P.M. Williamson, D.C. Bandarage, et al. "Rapid Urinary Tract Infection Diagnostics by Surface-Enhanced Raman Spectroscopy (SERS): Identification and Antibiotic Susceptibilities". Anal. Bioanal. Chem. 2017. 409(11): 3043–3054.
98. R.A. Halvorson, P.J. Vikesland. "Surface-Enhanced Raman Spectroscopy (SERS) for Environmental Analyses". Environ. Sci. Technol. 2010. 44(20): 7749–7755.
99. Y. Wang, Y.Z. Song, Y.F. Tao, H. Muhamadali, et al. "Reverse and Multiple Stable Isotope Probing to Study Bacterial Metabolism and Interactions at the Single Cell Level". Anal. Chem. 2016. 88(19): 9443–9450.
100. M.L. Cohen. "Changing Patterns of Infectious Disease". Nature. 2000. 406(6797): 762–767.
101. R. Saral. "Candida and Aspergillus Infections in Immunocompromised Patients: An Overview". Rev. Infect. Dis. 1991. 13(3): 487–492.
102. Y. Schmiedel, S. Zimmerli. "Common Invasive Fungal Diseases: An Overview of Invasive Candidiasis, Aspergillosis, Cryptococcosis, and Pneumocystis Pneumonia". Swiss Med. Wkly. 2016. 146: w14281.
103. O. Fiehn. "Metabolomics: The Link Between Genotypes and Phenotypes". Plant Mol. Biol. 2002. 48(1–2): 155–171.
104. D.B. Kell. "Systems Biology, Metabolic Modelling and Metabolomics in Drug Discovery and Development". Drug Discovery Today. 2006. 11(23–24): 1085–1092.
105. A.A. Cleveland, M.M. Farley, L.H. Harrison, B. Stein, et al. "Changes in Incidence and Antifungal Drug Resistance in Candidemia: Results From Population-Based Laboratory Surveillance in Atlanta and Baltimore, 2008–2011". Clin. Infect. Dis. 2012. 55(10): 1352–1361.
106. R.M. Jarvis, R. Goodacre. "Characterisation and Identification of Bacteria Using SERS". Chem. Soc. Rev. 2008. 37(5): 931–936.
107. Y. Cheong, Y.J. Kim, H. Kang, S. Choi, et al. "Rapid Label-Free Identification of *Klebsiella pneumoniae* Antibiotic Resistant Strains by the Drop-Coating Deposition Surface-Enhanced Raman Scattering Method". Spectrochim Acta, Part A. 2017. 183: 53–59.
108. M. Cheung, W.W.Y. Lee, D.P. Cowcher, R. Goodacre, et al. "SERS of Meso-Droplets Supported on Superhydrophobic Wires Allows Exquisitely Sensitive Detection of Dipicolinic Acid, an Anthrax Biomarker, Considerably Below the Infective Dose". Chem. Commun. 2016. 52: 9925–9928.
109. D.K. Trivedi, K.A. Hollywood, R. Goodacre. "Metabolomics for the Masses: The Future of Metabolomics in a Personalized World". New Horiz. Transl. Med. 2017. 3(6): 294–305.
110. S. Shanmukh, L. Jones, J. Driskell, Y.P. Zhao, et al. "Rapid and Sensitive Detection of Respiratory Virus Molecular Signatures Using a Silver Nanorod Array SERS Substrate". Nano Lett. 2006. 6(11): 2630–2636.
111. L. Hall-Stoodley, J.W. Costerton, P. Stoodley. "Bacterial Biofilms: From the Natural Environment to Infectious Diseases". Nat. Rev. Microbiol. 2004. 2(2): 95–108.
112. U. Romling, C. Balsalobre. "Biofilm Infections, Their Resilience to Therapy and Innovative Treatment Strategies". J. Intern. Med. 2012. 272(6): 541–561.
113. J.W. Costerton, P.S. Stewart, E.P. Greenberg. "Bacterial Biofilms: A Common Cause of Persistent Infections". Science. 1999. 284(5418): 1318–1322.
114. H. Wu, C. Moser, H.Z. Wang, N. Hoiby, et al. "Strategies for Combating Bacterial Biofilm Infections". Int. J. Oral Sci. 2015. 7(1): 1–7.
115. B.S. McSwain, R.L. Irvine, M. Hausner, P.A. Wilderer. "Composition and Distribution of Extracellular Polymeric Substances in Aerobic Flocs and Granular Sludge". Appl. Environ. Microbiol. 2005. 71(1): 1051–1057.
116. J.R. Lawrence, G.D.W. Swerhone, G.G. Leppard, T. Araki, et al. "Scanning Transmission X-ray, Laser Scanning, and Transmission Electron Microscopy Mapping of the Exopolymeric Matrix of

Microbial Biofilms". Appl. Environ. Microbiol. 2003. 69(9): 5543–5554.
117. T.R. Neu, G.D.W. Swerhone, J.R. Lawrence. "Assessment of Lectin-Binding Analysis for In Situ Detection of Glycoconjugates in Biofilm Systems". Microbiology. 2001. 147(Pt. 2): 299–313.
118. J. Schmitt, H.C. Flemming. "FTIR-Spectroscopy in Microbial and Material Analysis". Int. Biodeterior. Biodegrad. 1998. 41(1): 1–11.
119. N.P. Ivleva, P. Kubryk, R. Niessner. "Raman Microspectroscopy, Surface-Enhanced Raman Scattering Microspectroscopy, and Stable-Isotope Raman Microspectroscopy for Biofilm Characterization". Anal. Bioanal. Chem. 2017. 409(18): 4353–4375.
120. N.P. Ivleva, M. Wagner, H. Horn, R. Niessner, et al. "In Situ Surface-Enhanced Raman Scattering Analysis of Biofilm". Anal. Chem. 2008. 80(22): 8538–8544.
121. Y.Q. Chao, T. Zhang. "Surface-Enhanced Raman Scattering (SERS) Revealing Chemical Variation During Biofilm Formation: From Initial Attachment to Mature Biofilm". Anal. Bioanal. Chem. 2012. 404(5): 1465–1475.
122. E. Efeoglu, M. Culha. "In Situ-Monitoring of Biofilm Formation by Using Surface-Enhanced Raman Scattering". Appl. Spectrosc. 2013. 67(5): 498–505.
123. S. Polisetti, N.F. Baig, N. Morales-Soto, J.D. Shrout, et al. "Spatial Mapping of Pyocyanin in Pseudomonas Aeruginosa Bacterial Communities Using Surface Enhanced Raman Scattering". Appl. Spectrosc. 2017. 71(2): 215–223.
124. J.A. Jernigan, D.S. Stephens, D.A. Ashford, C. Omenaca, et al. "Bioterrorism-Related Inhalational Anthrax: The First 10 Cases Reported in the United States". Emerg. Infect. Dis. 2001. 7(6): 933–944.
125. A.O. Henriques, C.P. Moran. "Structure, Assembly, and Function of the Spore Surface Layers". Annu. Rev. Microbiol. 2007. 61: 555–588.
126. P. Setlow. "Spores of Bacillus subtilis: Their Resistance to and Killing by Radiation, Heat and Chemicals". J. Appl. Microbiol. 2006. 101(3): 514–525.
127. P.T. McKenney, A. Driks, P. Eichenberger. "The Bacillus subtilis Endospore: Assembly and Functions of the Multilayered Coat". Nat. Rev. Microbiol. 2013. 11(1): 33–44.
128. M.J. Leggett, G. McDonnell, S.P. Denyer, P. Setlow, et al. "Bacterial Spore Structures and Their Protective Role in Biocide Resistance". J. Appl. Microbiol. 2012. 113(3): 485–498.
129. Y.A. Qi, G. Patra, X.D. Liang, L.E. Williams, et al. "Utilization of the rpoB Gene as a Specific Chromosomal Marker for Real-Time PCR Detection of Bacillus anthracis". Appl. Environ. Microbiol. 2001. 67(9): 3720–3727.
130. J.S. Gulledge, V.A. Luna, A.J. Luna, R. Zartman, et al. "Detection of Low Numbers of Bacillus anthracis Spores in Three Soils Using Five Commercial DNA Extraction Methods With and Without an Enrichment Step". J. Appl. Microbiol. 2010. 109(5): 1509–1520.
131. C.A. Bell, J.R. Uhl, T.L. Hadfield, J.C. David, et al. "Detection of Bacillus anthracis DNA by LightCycler PCR". J. Clin. Microbiol. 2002. 40(8): 2897–2902.
132. R. Manoharan, E. Ghiamati, R.A. Dalterio, K.A. Britton, et al. "UV Resonance Raman Spectra of Bacteria, Bacterial Spores, Protoplasts and Calcium Dipicolinate". J. Microbiol. Methods. 1990. 11(1): 1–15.
133. M.B. Beverly, F. Basile, K.J. Voorhees, T.L. Hadfield. "A Rapid Approach for the Detection of Dipicolinic Acid in Bacterial Spores Using Pyrolysis/Mass Spectrometry". Rapid Commun. Mass Spectrom. 1996. 10(4): 455–458.
134. R. Goodacre, B. Shann, R.J. Gilbert, E.M. Timmins, et al. "Detection of the Dipicolinic Acid Biomarker in Bacillus Spores Using Curie-Point Pyrolysis Mass Spectrometry and Fourier Transform Infrared Spectroscopy". Anal. Chem. 2000. 72(1): 119–127.
135. P. Di Filippo, D. Pomata, C. Riccardi, F. Buiarelli, et al. "Muramic and Dipicolinic Acids in Atmospheric Particulate Matter as Biomarkers of Bacteria and Bacterial Spores". Anal. Bioanal. Chem. 2017. 409(6): 1657–1666.
136. Y.X. Zhou, B. Yu, K. Levon. "Potentiometric Sensor for Dipicolinic Acid". Biosens. Bioelectron. 2005. 20(9): 1851–1855.
137. J. Forrester, N.B. Valentine, Y.F. Su, T.J. Johnson. "Chemometric Analysis of Multiple Species of Bacillus Bacterial Endospores Using Infrared Spectroscopy: Discrimination to the Strain Level". Anal. Chim. Acta. 2009. 651(1): 24–30.
138. D.P. Cowcher, Y. Xu, R. Goodacre. "Portable, Quantitative Detection of Bacillus Bacterial Spores Using Surface-Enhanced Raman Scattering". Anal. Chem. 2013. 85(6): 3297–3302.
139. R.M. Jarvis, A. Brooker, R. Goodacre. "Surface-Enhanced Raman Scattering for the Rapid Discrimination of Bacteria". Faraday Discuss. 2006. 132: 281–292.
140. R. Gao, J. Ko, K. Cha, J.H. Jeon, et al. "Fast and Sensitive Detection of an Anthrax Biomarker Using SERS-Based Solenoid Microfluidic Sensor". Biosens. Bioelectron. 2015. 72: 230–236.
141. R.S. Golightly, W.E. Doering, M.J. Natan. "Surface-Enhanced Raman Spectroscopy and Homeland Security: A Perfect Match?". ACS Nano. 2009. 3(10): 2859–2869.
142. I.D. Bull, N.R. Parekh, G.H. Hall, P. Ineson, et al. "Detection and Classification of Atmospheric Methane Oxidizing Bacteria in Soil". Nature. 2000. 405(6783): 175–178.
143. H.T.S. Boschker, J.J. Middelburg. "Stable Isotopes and Biomarkers in Microbial Ecology". FEMS Microbiol. Ecol. 2002. 40(2): 85–95.
144. M.G. Dumont, J.C. Murrell. "Stable Isotope Probing–Linking Microbial Identity to Function". Nat. Rev. Microbiol. 2005. 3(2): 499–504.
145. J.D. Neufeld, M. Wagner, J.C. Murrell. "Who Eats What, Where and When? Isotope-Labelling Experiments are Coming of Age". ISME J. 2007. 1(2): 103–110.
146. H.T.S. Boschker, S.C. Nold, P. Wellsbury, D. Bos, et al. "Direct Linking of Microbial Populations to Specific Biogeochemical Processes by $^{13}$C-Labelling of Biomarkers". Nature. 1998. 392(6678): 801–805.
147. J.D. Neufeld, M.G. Dumont, J. Vohra, J.C. Murrell. "Methodological Considerations for the Use of Stable Isotope Probing in Microbial Ecology". Microb. Ecol. 2007. 53(3): 435–442.
148. S. Radajewski, P. Ineson, N.R. Parekh, J.C. Murrell. "Stable-Isotope Probing as a Tool in Microbial Ecology". Nature. 2000. 403(6770): 646–649.
149. M. Manefield, A.S. Whiteley, R.I. Griffiths, M.J. Bailey. "RNA Stable Isotope Probing, a Novel Means of Linking Microbial Community Function to Phylogeny". Appl. Environ. Microbiol. 2002. 68(11): 5367–5373.
150. P. Kubryk, R. Niessner, N.P. Ivleva. "The Origin of the Band at Around 730 $cm^{-1}$ in the SERS Spectra of Bacteria: A Stable Isotope Approach". Analyst. 2016. 141(10): 2874–2878.
151. M. Chisanga, H. Muhamadali, R. Kimber, R. Goodacre. "Quantitative Detection of Isotopically Enriched E. coli Cells by SERS". Faraday Discuss. 2017. 205: 331–343.
152. R. Amann, B.M. Fuchs. "Single-Cell Identification in Microbial Communities by Improved Fluorescence In Situ Hybridization Techniques". Nat. Rev. Microbiol. 2008. 6(5): 339–348.
153. Y.Z. Song, A.K. Kaster, J. Vollmers, Y.Q. Song, et al. "Single-Cell Genomics Based on Raman Sorting Reveals Novel Carotenoid-Containing Bacteria in the Red Sea". Microb. Biotechnol. 2017. 10(1): 125–137.
154. W.E. Huang, K. Stoecker, R. Griffiths, L. Newbold, et al. "Raman-FISH: Combining Stable-Isotope Raman Spectroscopy and Fluorescence In Situ Hybridization for the Single Cell Analysis of Identity and Function". Environ. Microbiol. 2007. 9(8): 1878–1889.
155. A. Walter, A. Marz, W. Schumacher, P. Rosch, et al. "Towards a Fast, High Specific and Reliable Discrimination of Bacteria on Strain Level by Means of SERS in a Microfluidic Device". Lab Chip. 2011. 11(6): 1013–1021.