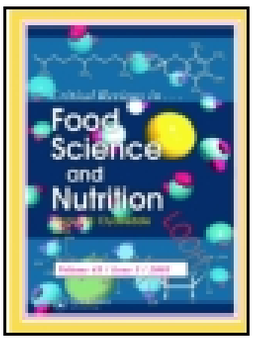

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REVIEW

Recent developments in Raman spectral analysis of microbial single cells: Techniques and applications

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ABSTRACT

The conventional microbial cell analyses are mostly population-averaged methods that conceal the characteristics of single-cell in the community. Single-cell analysis can provide information on the functional and structural variation of each cell, resulting in the elimination of long and tedious microbial cultivation techniques. Raman spectroscopy is a label-free, noninvasive, and *in-vivo* method ideal for single-cell measurement to obtain spatially resolved chemical information. In the current review, recent developments in Raman spectroscopic techniques for microbial characterization at the single-cell level are presented, focusing on Raman imaging of single cells to study the intracellular distribution of different components. The review also discusses the limitation and challenges of each technique and put forward some future outlook for improving Raman spectroscopy-based techniques for single-cell analysis. Raman spectroscopic methods at the single-cell level have potential in precision measurements, metabolic analysis, antibiotic susceptibility testing, resuscitation capability, and correlating phenotypic information to genomics for cells, the integration of Raman spectroscopy with other techniques such as microfluidics, stable isotope probing (SIP), and atomic force microscope can further improve the resolution and provide extensive information. Future focuses should be given to advance algorithms for data analysis, standardized reference libraries, and automated cell isolation techniques in future.

KEYWORDS

Single-cell analysis; microorganisms; Raman spectroscopy; microfluidics; stable isotope probing; atomic force microscope

Introduction

Abundant methods are available to study the microbial community, however, they often fail to differentiate the functional difference between single cells of the same community or cells from closely related communities and do not link genomic information to single cells, disregarding the fact that individual cells are the basic unit of life (Bowers, Doud, and Woyke 2017). In recent years there has been an increasing interest in studying communities on a single cell level to understand the function and role of individual cells, which has prominent implications on cell development and evolution . Deep knowledge about the genomics, transcriptomics, proteomics, and metabolomics of a single cell in a community can be used as a bottom-up approach to comprehend the ecological role and dynamic changes of the community as a whole. Moreover, single-cell assessment unfolds an opportunity to investigate uncultivable microorganisms, which can have a tremendous impact on food safety and security (Yuan et al. 2017).

The majority of the methods adopted for the analysis of the biological activity of cells are population averaged measurement, which reflects only the dominant characteristics of the population, thus undermining the distinct traits of the rare subpopulation of cells (C. Zhang et al. 2021). Single-cell measurement can act as a framework for understanding the population dynamics and functional fluctuations between each cell. Multi-omics approaches normally include a collection of different profiling strategies such as genomics, proteomics, transcriptomics, and metabolomics, and can be used as the building blocks for obtaining complete profiling of a single cell (Hu, Pu, and Sun et al. 2021). Phenotypical homogeneity in a vast population of cells often mask transcriptional diversity among cells and the link between transcriptome and biological characteristics are commonly neglected. The critical step in the single-cell analysis is to successfully isolate a single, viable, and intact cell from a cell suspension. Several methods including mouth pipetting, robotic micromanipulation, fluorescence-activated cell sorting (FACS), and microfluidic platforms have been employed for this purpose. Among them, mouth pipetting is a simple

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and rapid technique suitable for sensitive molecules such as RNA, while robotic manipulation, FACS, and microfluidics require cell labeling and sophisticated pieces of equipment (Geng and Huang 2018; Hwang, Lee, and Bang 2018). The isolation technique is selected based on the targeted molecule, the nature of the cell, and the proceeding genomic sequencing. The whole-cell genomic variations can be related to transcriptional variation, which is directly related to several phenotype characteristics of the cell (Imdahl et al. 2020). The development of a technique for the detection and profiling of single-cell microorganisms can lead to shorten or eliminate the long and tedious cell cultivation methods, thus leading to several interdisciplinary works between microbiology, food safety, and biotechnology (Germond, Ichimura, Chiu et al. 2018; ; D. W. Sun et al. 2021).

The conventional single-cell analysis includes labeling specific cells with a fluorescent probe and measuring the scattering and fluorescence intensity by passing through a laser light source (Zhou, Pu, and Sun 2020; D. Zhang et al. 2021). Flow cytometric techniques such as FACS have been employed for counting, sorting, and profiling of single-cell bacteria labeled with a fluorescent dye. Stoichiometric binding of the florescent label allows multi-parametric chemical characterization of cells when passing through a laser light source (Sibanda and Buys 2017). A wide variety of labeling agents from common fluorescent dyes to quantum dots have been used for their unique capabilities (Lee, Sun, et al. 2019). In addition, fluorescence in-situ hybridization (FISH) is an advanced and powerful technique, in which fluorescently labeled DNA probes are used to bind to rRNA of cells for targeting a specific group of microorganisms, and the procedure involves partial cell wall lysis to allow fluorescent probe hybridization that hinders *in-vivo* analysis. However, the development of a fluorescent probe for FISH analysis of living cells is greatly unexplored (Batani et al., 2019).

Metabolomic profiling of a single cell is extremely complex due to several factors including 1) extreme low concentration of metabolites production from a single cell and unlike genome, metabolite cannot be amplified, 2) rapid metabolomic turnover, and 3) diversity of metabolites produced (Fessenden 2016). In recent years, nanoelectrospray ionization mass spectroscopy has been used for single-cell metabolomics combined with a small pipette that can sample even separate subcellular materials and directly generate ion, but the reproducibility of the data produced is significantly too low to represent cellular heterogeneity (Mizuno et al. 2008). Several other mass spectroscopy (MS) based imaging techniques have gained popularity in single-cell metabolomics due to their high spatial resolution, and matrix-assisted laser desorption/ionization mass spectroscopic (MALDI-MS) imaging has proved to be suitable for tissue analysis as a single microbial cell is much smaller than its spatial resolution (~50 μm) (Neumann et al. 2019). In addition, the secondary ion mass spectrometry (SIMS) with a spatial resolution of ~1 μm also shows potential in single-cell analysis, but the application only extends to imaging of ions and ionized lipids on the cell surface and the capabilities of the technique to detect intracellular metabolites are yet to be explored (Singh et al. 2020).

Raman spectroscopy is an effective, label-free, *in-vivo* and nondestructive technique for extracting the chemical fingerprint of an individual cell to provide insight into biomaterials such as nucleic acids (DNA and RNA), proteins, carbohydrates, and lipids (Huang et al. 2021; Hussain et al. 2020). Single-cell Raman analysis overcomes the limitation of low cell count and matrix complexity of the biological sample and provides results within minutes without any tedious sample pretreatment. Technical advancements in Raman mapping have led to delivering spatially resolved chemical information, thereby offering an opportunity to monitor biochemical and biophysical changes throughout the whole cell (Wang, He, et al. 2020a). Raman spectroscopy can acquire the spectra from a very small sample volume, normally $<1\ \mu m^3$, which is applicable to study changes in physiological and phenotypic characteristics of living cells (Li, Zhu, and Sun 2020; Hussain, Sun, and Pu 2019). Several obstacles should be overcome when using Raman spectroscopy to study single cells, including weak Raman signal and low signal-to-noise ratio (Li et al. 2012), and therefore the integration of Raman spectroscopy with other techniques can open up more unique opportunities for screening, detection, characterization, and imaging of cells from biological systems (Wu, Pu, and Sun 2021). This high-resolution method has been employed in many biomedical applications such as cancer cell diagnosis (Bhamidipati et al. 2018), cell cytotoxicity analysis (Mao et al. 2018), stem cell differentiation, and nerve cell analysis (Chen et al. 2020). However, the capabilities of single-cell Raman spectroscopy in other areas have not yet been explored. Thus, there is an enormous untapped potential of single-cell Raman spectroscopy in the area of food safety and quality applications (W. Zhang, Ma, and Sun 2020).

There are a few relevant reviews available on the topic. He, Wang, et al. (2019a) discussed the recent development in the Ramanome technology platform including Raman activated cell ejection (RACE), Raman spectroscopy coupled with microfluidics, and optical tweezers for single-cell sorting of microorganisms, while Lee, Sun, et al. (2019) summarized the application of plasmonic nanostructures and coherent Raman scattering for single-cell bio-imaging. However, these reviews focus on the application of Raman spectroscopy in tissue engineering and the pharmaceutical industry, and an in-depth comparison of recently emerged Raman based techniques for single-cell analysis of microorganisms is not yet available. Therefore, this review focus on the potential of Raman based techniques including conventional Raman spectroscopy, tip-enhanced Raman spectroscopy (TERS), Raman spectroscopy combined with SIP and microfluidics, and stimulated Raman spectroscopy for single-cell analysis. The working principle, recent applications, and advantages and limitations for each technique are discussed (Table 1) and challenges and future outlook are also presented.

Table 1. Comparison of advantages and limitation of single-cell Raman spectroscopic methods.

Techniques	Resolution	Applications	Advantages	Limitations	References
Raman spectroscopy	0.5-1 μm	Biomolecule detection and cell sorting	Simple and easy handling and noninvasive; Raman activated cell sorting	Nonspecific measurement; high fluorescence background	Song et al. (2017)
Raman spectroscopy with microfluidics	0.5-1 μm	Single-cell trapping, biomolecule detection and imaging	Isolation, culturing and simultaneous measurement; analysis of multiple cell types against multiple stimuli at single-cell level	Unsuitable for high motile cells; complex design and fabrication process for microfluidic platforms	Sun et al. (2019)
SRS	0.5-1 μm	Label-free imaging with chemical specificity	No resonant background; vibrational excitation of otherwise dormant molecules; simultaneous detection on multiple metabolites	Narrowband excitation; use of long-wavelength reducing the spatial resolution	Hong et al. (2018)
TERS	<1 nm	Surface chemical imaging of cells at nanometer length scales	<1 nm spatial resolution; measurement of sub-cellular component distribution	Background signal from carbon contamination on a tip; cell damages during measurement	Richards et al. (2003), Berezin et al. (2017)
Raman - SIP	0.5-1 μm	Analysis of overall metabolic activity of cells	Semi-quantitative metabolic detection; high throughput sorting	Difficulty in differentiating VBNC cells from cultivable cells; lack of ^{13}C-labeled or ^{15}N-labeled derivatives limiting application	Tao et al. (2017)

Note: SRS = Stimulated Raman spectroscopy; TERS = Tip enhanced Raman spectroscopy; SIP = Stable isotope probing; VBNC = Viable but non-culturable.

Fundamentals of Raman single-cell analysis

Raman spectroscopy has gained the attention of researchers as a tool for *in-vivo* analysis of cells. The fingerprint spectra obtained from vibrational modes of cellular components such as nucleic acid, protein, carbohydrate, and lipids can reveal the metabolic activity, physiological state, and, morphological characterization of cells. Table 2 summarizes recent application developments in using different Raman spectroscopic techniques for the analysis of single cells.

Raman spectroscopy

High spatial resolution and compatibility with aqueous samples make Raman spectroscopy an ideal tool for single-cell analysis (Hussain, Sun, and Pu 2020; Hussain, Pu, and Sun 2021). Große et al. (2015) used Raman imaging to investigate the infection process of *Staphylococcus aureus* and revealed that intracellular and extracellular cells were biologically different, which provided new insight into intracellular adaptation strategies inside host cells. The noninvasive nature of Raman spectroscopy allows continuous monitoring of molecular constituents of a single cell to investigate the dynamic growth of cells within their natural environment (Hussain, Pu, and Sun 2020b). For example, Ren et al. (2017) were able to differentiate the growth phase of a single *Lactobacillus casei* cell with an accuracy of 91.2% using single-cell Raman spectroscopy and showed that the technique could be applied for heterogeneous cell populations since the technique is independent of cellular markers. However, the spectra produced by a complex biological system such as a yeast cell are a result of several overlapping contributions from cellular components such as polysaccharides, proteins, and nucleic acids. Thus, combining Raman spectroscopy with multivariate analysis helps to unravel hidden information in the spectra (K. Wang et al. 2020a; K. Wang et al. 2020b). The Raman spectroscopic analysis was able to identify two different myristyl myristate in Euglena cells and the chain length of different esters through multivariate curve resolution analysis (Iwasaki et al. 2019). Moreover, Nekvapil et al. (2021) studied the effect of Mn and Zn doped ferrite nanoparticles (20 mg/L) exposure on *Coelomoron pussilum* using single-cell micro-Raman spectroscopy and revealed that the change in Raman shift (C = C) generated due to carotenoids in the cyanobacteria could indicate the physiological response of cells to the presence of nanoparticles.

Confocal Raman spectroscopy has proved as an efficient method to analyze intercellular components in genetically engineered microbial cells (Kochan et al. 2018). For example, Kochan et al. (2019) used confocal Raman spectroscopy to detect and track cyclopropane fatty acids in genetically engineered *Saccharomyces cerevisiae* cell. The CRS imaging of single cells revealed that the abundance and composition of lipid bodies were different for engineered and control cells and the degree of unsaturation of lipid bodies was predicted using the ratio of bands corresponds to ν(C = C) (1660 cm^{-1}) and δ(CH2) (1448 cm^{-1}) in the engineered cell. Germond, Ichimura, Horinouchi et al. (2018b) proved that Raman spectroscopic signals could be used as an indicator for gene expression in *E. coli* for

Table 2. Summary of recent applications of Raman based techniques for single-cell analyses.

Technique	Cell type	Application	Imaging	Reference
Raman spectroscopy	*Calciodinellum operosum aff.*	Anhydrous β-guanine crystals distribution	Mapping at peak 650 cm^{-1}	Jantschke et al. (2019)
Hyperspectral stimulated Raman scattering microscopy	*Enterococcus faecalis*	Antibiotic susceptibility analysis by monitoring the glucose metabolic activity	Incorporation of glucose d_7 by mapping peak at 2178 cm^{-1}	Hong et al. (2018)
Hyperspectral stimulated Raman scattering microscopy	*Tetrahymena thermophila*	Nanoparticles uptake and subcellular distribution	Mapping at peak 2930 cm^{-1} for TiO_2 and 2875 cm^{-1} for Fe_2O_3 nanoparticles	Huang et al. (2018)
Raman spectroscopy	*Euglena gracilis*	Intracellular bio-molecular distribution during wax ester fermentation	Mapping at peak 425 cm^{-1} and 1731 cm^{-1} for paramylon and wax esters distribution	Iwasaki et al. (2019)
Stimulated Raman imaging	*Candida albicans*	Differentiation of fluconazole-susceptible, and resistant cells	Mapping at 2120 cm^{-1} (C-D) and 2850 cm^{-1} (C-H) for lipid accumulation	Karanja et al. (2017)
Raman spectroscopy	*Saccharomyces cerevisiae*	Metabolic product heterogeneity due to genetic modification	Spatial distribution of saturated (1444 cm^{-1}), unsaturated fatty acids (1656 cm^{-1}) and triglycerides (1740 cm^{-1})	Kochan et al. (2019)
SERS	Murine macrophage cell	Endogenous hypochlorous acid and peroxynitrite detection	Radiometric imaging upon I_{389}/I_{551} for peroxynitrite and I_{983}/I_{551} for hypochlorous acid	Li et al. (2018)
Laser-trapping Raman spectroscopy	*Biflagellate C. reinhardtii*	In vivo lipid profiling of oil-producing microalgae	–	Wu et al. (2011)
RACE combined with SIP labeling	Synechococcus spp. Pelagibacter spp	Cell sorting and characterization of carbon-fixing bacteria	–	Jing et al. (2018)
RACE	*Escherichia coli* Cyanobacteria	Isolation of carotenoid containing cells from the Red Sea	–	Song et al. (2017)
Raman integrated mid-infrared photo-thermal microscopy	*Staphylococcus aureus*	Cell chemical composition	–	Li, Zhang, et al. (2019)
TERS	*Halobacterium salinarum*	Localization of purple membrane patches on cell surface	–	Deckert-Gaudig et al. (2012)
TERS	*Saccharomyces cerevisiae*	Glucose dehydrogenase protein accumulation	–	Naumenko et al. (2013)
TERS	*Bacillus subtilis* spore	Nanoscale chemical imaging	PC2 and PC3 score maps from PCA	Rusciano et al. (2014)
TERS	*Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, and Staphylococcus aureus*	Gram-positive and negative bacterial cell differentiation	–	Berezin et al. (2017)
Raman spectroscopy	*Lactobacillus casei*	Bacterial cell growth phase differentiation	–	Ren et al. (2017)
SERS -Microfluidic droplet technique	Cancer cells	Extracellular metabolic product detection	–	Sun et al. (2019)
Raman spectroscopy with glass microfluidics	*Euglena gracilis*	Paramylon biogenesis monitoring	Spatial distribution of $_{12}C$-paramylon, $_{13}C$-paramylon, and chlorophyll	Ota et al. (2019)
Resonance Raman microfluidics system with SIP	*Synechocystis sp*	Isotope labeled cell sorting	–	McIlvenna et al. (2016)
Hyperspectral Stimulated Raman Scattering Microscopy	*Caenorhabditis elegans*	Fatty acid metabolic pathway	Mapping at 2110 cm^{-1} for deuterated fatty acids and 2850 cm^{-1} for total lipid level	Fu, Zhou, et al. (2014)

Note: SERS = Surface-enhanced Raman spectroscopy; RACE = Raman activated cell ejection; SIP = Stable isotope probing; TERS = Tip-enhanced Raman spectroscopy; PCA = Principle component analysis; PC = Principle component.

antibiotic resistant genes and the two-dimensional principal component analysis followed by discrimination analysis revealed the spectral regions that contributed to the discrimination of different antibiotic resistance modes of action.

Therefore, Raman spectroscopy is considered the most promising vibrational spectroscopic technique for real-time analysis of complex biological systems, subcellular structures (Kochan et al. 2018), and changes in chemical composition in single cells (Rzeczycki et al. 2019).

Raman spectroscopy with microfluidics

Microfluidics is considered a powerful tool for manipulating single cells with high precision and in a short time. Several specific microfluidic structures such as flow shortcut structure, U-shape capture, micro-well/micro-cavity arrays (Deng et al. 2019), shallow channels with dam and/or parallel semi-closed micro-channels structures (Ota et al. 2019) and optical tweezers (Dochow et al. 2011) have been reported

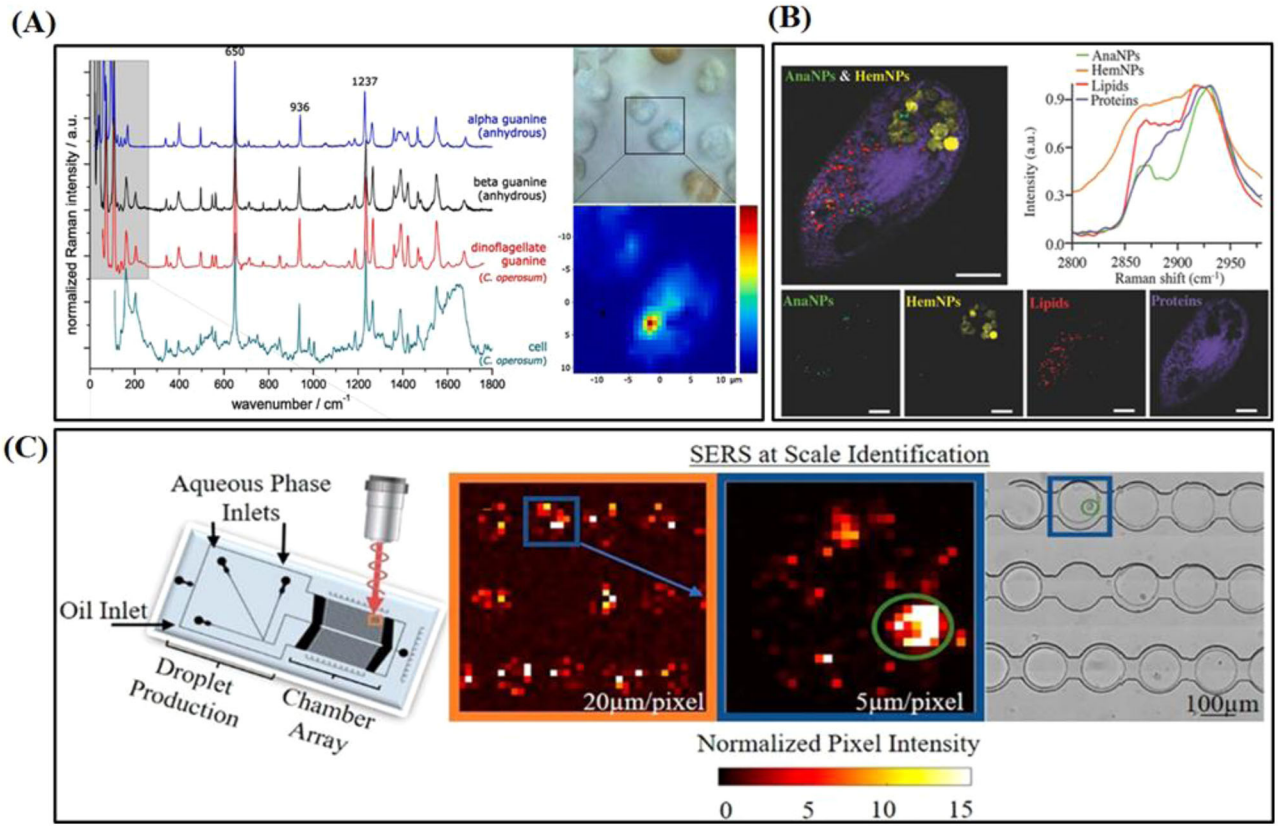

Figure 1. (A) Raman maps showing the intracellular distribution of guanine in a single marine dinoflagellate by plotting the peak intensity at 650 cm^{-1} (Jantschke et al. 2019), (B) hyperspectral stimulated Raman scattering image of the distribution of nanoparticles in *Tetrahymena thermophila* (Huang et al. 2018), and (C) schematic of the optofluidic platform for culturing and identification of cells (Willner et al. 2018).

previously. The most common choices of material for the development of a microfluidic platform for *in-situ* single-cell analysis are glass and poly-dimethylsiloxane (PDMS), which have high transmittance in the visible region and produce no/fewer peaks in the Raman spectra. Even though glass shows more advantages including low background noise interference and physical rigidity over conventional polymer, glass is rarely employed due to the difficulty in bonding different parts together (Ota et al. 2019). Integrating Raman spectroscopy with techniques such as microfluidics allows isolation of a single cell, culturing in microenvironments, and simultaneous monitoring of cells in situ (K. Wang, Sun, Pu, Wei, et al. 2019). In this combined technique, microfluidic channels facilitate the separation of a single cell from a colony and the pumps and valves provide the necessary growth medium and reagents at nano-litre volume. Single-cell isolation of microorganisms is more difficult as compared with other cells due to its high motility, microscopic size, few interspecies variability and matrix complexity.

Recently, droplet microfluidics has gained attention because it allows single-cell monitoring of a cell encapsulated in a single droplet. Generally, a single cell in a droplet of 1 pL–10 nL of the culture medium is encapsulated in an immiscible oil carrier through T-junction channels. So far, microfluidic Raman spectroscopy has proved to be successful in exploring antibiotic susceptibility (Dietvorst et al. 2020), cell sorting (McIlvenna et al. 2016), metabolic response to the environment (Sun et al. 2019), and intra/extracellular variability (Willner et al. 2018) (Figure 1C). The performance of this technique can be further improved by the incorporation of nanoparticles into micro-droplet or by introducing a new surface-enhanced Raman scattering (SERS) - microfluidic droplet technique. The nanoparticles facilitate absorption and separation of metabolite and highly sensitive label-free detection of multiplexed metabolites at the single-cell level (Hu et al. 2020a; Hussain et al. 2021). The encapsulation of cell in isolated single droplet concentrates the metabolite and accelerate the process of diffusion (Sun et al. 2019). The introduction of microfluidics plays a significant role in enabling single-cell genomic sequencing as it can isolate and process a small sample volume without contamination or sample loss (Uzunoglu et al. 2019). It can also accelerate the study of other single-cell omics such as proteomics and transcriptomics. Further, the microfluidic platform enables analysis of the response of multiple cell types against multiple stimuli at a single-cell level (Cao et al. 2016).

Stimulated Raman spectroscopy (SRS)

SRS is equipped with two laser pulses, one at stokes frequency and the other at pump frequency. Both lasers are focused on the sample in such a way that the stokes photons that are previously generated by normal Raman scattering are added to the excitation beam, which results in stimulated Raman scattering. This SRS offers 5 order of magnitude enhancement for strong modes and diminishes weak modes

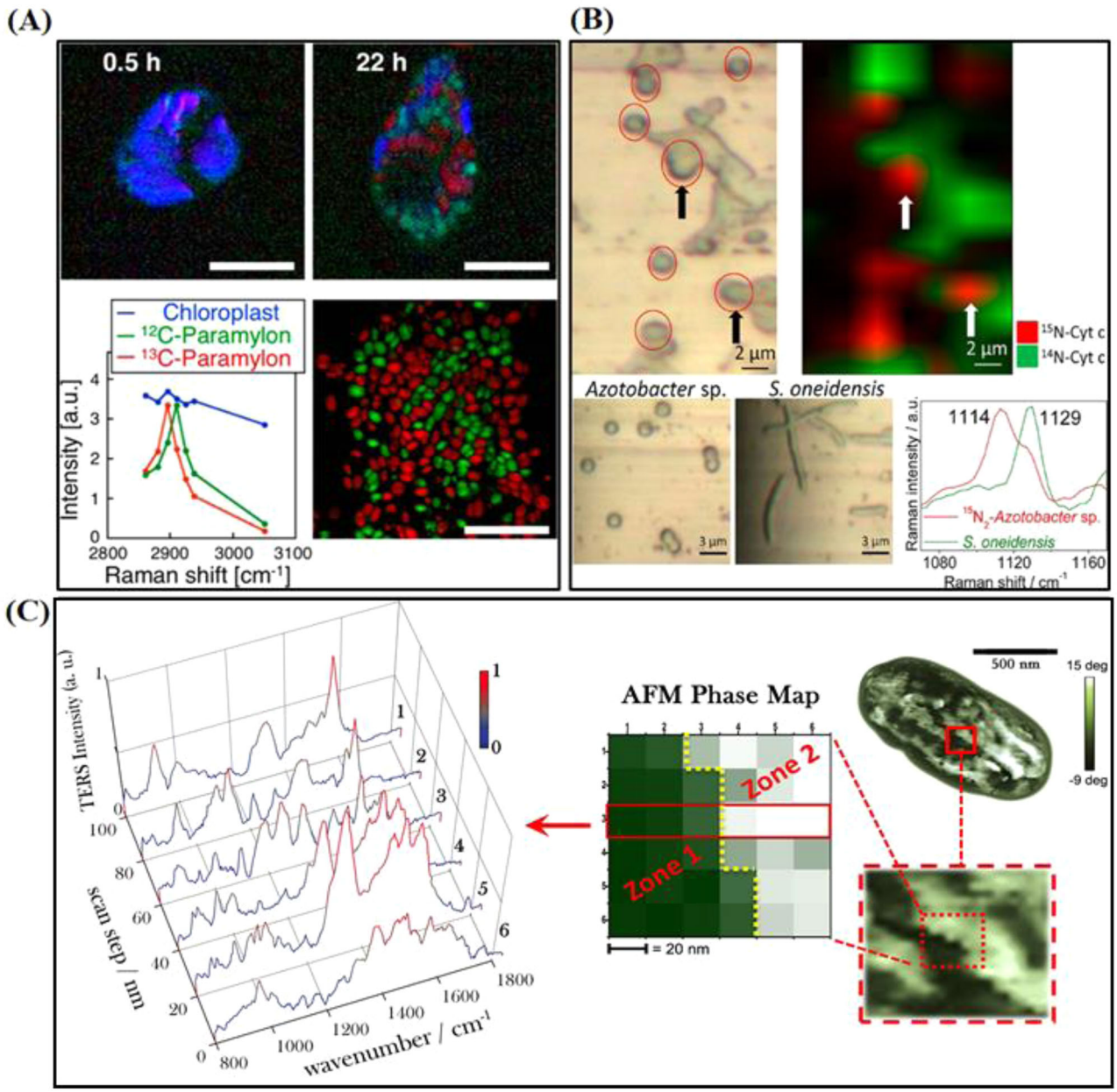

Figure 2. (A) Resonance Raman spectroscopic imaging of single *E. gracilis* cell after incubation with paramylon-induction medium (Ota et al. 2019), (B) SIP - Raman image showing N_2 fixing (red) and non-N_2-fixing (green) bacterial cells (Cui et al. 2018), and (C) TERS maps showing clear distinction of ridges on the surface of spores (Rusciano et al. 2014).

that are shown in the regular Raman spectra (Liao et al. 2015). Speed and chemical specificity are the two most important attributes of SRS imaging and the chemical specificity is achieved by either stacking a series of SRS images at different Raman shifts successively or collecting a complete spectrum at each spatial location (Lin et al. 2018).

SRS has been found to be effective in analyzing a broad spectrum of biomolecules including DNA, RNA, proteomes, phospholipids, and triglycerides in living cells with alkaline as tag molecules (Wei et al. 2014). Compared with spontaneous Raman scattering, SRS is free of non-resonant background and encourages vibrational excitation of otherwise dormant molecules by a magnitude of 10^7. Moreover, SRS mapping enables the simultaneous detection of multiple metabolic products (Fu, Zhou, et al. 2014). Further, the hyperspectral stimulated Raman scattering imaging was able to study the spatiotemporal distribution of two classes of neutral lipids, triacylglycerols, and cholesteryl esters, in single *Saccharomyces* sp. yeast cell (Fu, Yu, et al. 2014). This unique imaging technique can be extended to study other subcellular components with no sample preparation and the sensitivity of the imaging can be improved by tagging an alkaline group to analyte compound (Wei et al. 2014). For example, Huang et al. (2018) investigated the accumulation and distribution of α-Fe_2O_3 and TiO_2 nanoparticles in *Tetrahymena thermophila* with SRS mapping. Their study showed different uptake routes and competition between dissimilar nanoparticles, which resulted in a difference in

the subcellular distribution of α-Fe_2O_3 and TiO_2 nanoparticles (Figure 1B), and when the pump laser was kept at 840 nm and the stokes laser was at ≈ 2300 cm^{-1}, only the pump-probe signal was detected and a vibrational silent region was generated, where the signals from lipid and proteins in the cells could be eliminated.

Tip-enhanced Raman spectroscopy (TERS)

The combination of Raman spectroscopy with atomic force microscopy or scanning tunneling microscopy creates a powerful analytical tool referred to as tip-enhanced Raman spectroscopy (TERS), which provides improved spatial resolution as compared with SERS. The special feature of TERS is a single plasmonically active scanning probe tip that provides enhancement at nanometer length scales and it also serves as a topography scanner and surface plasmon resonance generator simultaneously.

TERS has been shown to be able to provide chemical information with <1 nm spatial resolution and allow measurements of the sub-cellular distribution of molecules within living cells. Studies have shown that the gold and silver tips were preferred for excitations from 633 to 785 and 488 to 568 nm, respectively (Deckert-Gaudig et al. 2017). The nanometer level penetration depth of the TERS probe allows the sub-surface analysis of single cells, which are inaccessible by other methods. In recent years, TERS has been employed for the characterization of DNA and RNA (He, Wang, et al. 2019b), protein interaction (Agressott et al. 2020), and intracellular component mapping (Shibata et al. 2020).

Naumenko et al. (2013) analyzed glucose dehydrogenase protein accumulation in the periplasmic space of the cell envelop of genetically modified *Saccharomyces cerevisiae* using μ-TERS. The spectrum was able to identify protein cluster formation at the subcellular level in a single cell *in-vivo*. In addition, the use of various statistical tools can help to deduce more information from the spectra to shed light on complex biological systems. Rusciano et al. (2014) investigated the surface heterogeneity of *Bacillus subtilis* spore and generated statistically significant chemical maps of spore surface chemistry.

Generally, the spatial variability is difficult to interpret from TERS spectra, but it is possible to distinguish the characteristics of the spore surface on nanoscale level using basis analysis and principal component analysis (PCA). PCA can provide an unbiased TERS map to show a clear difference in the chemical composition of ridges on the surface of spores formed to adjust changes in the volume of cells during different environmental conditions (Figure 2C).

Hence, TERS is a nondestructive and label-free method for surface chemical imaging of single cells at the nanoscale level. However, despite the advantages of TERS, only a very few studies are available because TERS demands a wide range of skill sets including nanoparticles synthesis, Raman spectroscopy, atomic force microscopy probe preparation, laser optical alignment, and cellular biology (Shibata et al. 2020).

Stable isotope probing (SIP) – Raman spectroscopy

Theoretically, the Raman shift represented by a bond vibration is inversely proportional to the square root of atomic mass, which indicates that when an atom is replaced with its heavier isotope, the Raman signal shifts to the lower region. In the single-cell analysis the shift in Raman spectra as a result of isotope incorporation during incubation can be used as an indicator for overall metabolic activity, nitrogen/carbon fixation (Cui et al. 2018; Taylor et al. 2017), resuscitation of viable but non-culturable (VBNC) cells (Guo et al. 2019), and antimicrobial/antibiotic susceptibility (Yang et al. 2019). The isotopes used for stable isotope probing (SIP) include ^{13}C, ^{15}N, and ^{2}H, in which ^{13}C is the most suitable choice for single-cell microbial studies. However, D_2O labeling provides accurate information about the metabolic changes in microbial cells as it does not change the substrate molecular structure. During the cultivation of microbial cells in the presence of deuterated substrate, the viable or metabolically active cells assimilate deuterium into their cell biomass through H/D exchange. The intensity of distinctive C–D Raman band (2000–2300 cm^{-1}) and/or intensity ratio of $(C-D)/((C-H)+(C-D))$ can be used as a factor for qualitative and semi-quantitative analysis of the metabolic activity of cells (Wang, He, et al. 2020b). Table 3 lists the recent applications of SIP-Raman spectroscopy in the single-cell analysis.

The C-D band formation over time in cells can be ascribed to biological metabolic pathways including carbon catabolite repression (Kumar et al. 2016), nitrogen assimilation (Cui et al. 2018), and cellulose degradation (Olaniyi et al. 2019). Therefore, SIP-Raman analysis can differentiate metabolically active cells from inactive/dead cells and the degradation pathway of deuterated substrates and deduce the function of microbial cells in the complex biological environment (Wang, He, et al. 2020b). Taylor et al. (2017) used the SIP-Raman spectroscopy as a noninvasive approach to investigate the growth rate of microorganisms extensively at the single-cell level. They revealed that the redshift in the carotenoid peak was proportional to the ^{13}C-bicarbonate enrichment in the media based on time course Raman spectra of cyanobacterium (*Synechococcus* sp.) incubated in ^{13}C-bicarbonate, and ascribed this shift in peak to the semi-quantitative detection of extracellular and intracellular metabolic product accumulation.

The single-cell Raman spectroscopy combined with SIP is an effective tool to decipher the functional and ecological roles of different microorganisms in the environment (Li, Bi, et al. 2019). However, the lack of commercial availability of ^{13}C-labeled or ^{15}N-labeled derivatives of substrates limits the use of SIP-Raman for single-cell analysis.

Characterization of single-cell microorganisms by Raman techniques

Cell sorting and differentiation

Isolation of cells based on specific characteristics from a bulk heterogeneous population allows us to further study a

Table 3. Summary of study of single microbial cell characterization using SIP-Raman spectroscopy.

Isotope	Cell	Application	C-D assignment	Reference
D_2O	*Euglena gracilis*	Photosynthetic activity	Peak at 2168 cm^{-1} for glucose	Yonamine et al. (2017)
$^{15}N_2$	*Azotobacter chroococcum, Rhizobium radiobacter, Bradyrhizobium japonicum*	Nitrogen fixation	Peak at1664 cm^{-1} for cytochrome c amide I bond	Cui et al. (2018)
D_2O	*Escherichia coli Staphylococcus aureus Pseudomonas sp. Aeromonas sp.*	Resuscitation of VBNC cells metabolic activity	2040 – 2300 cm^{-1} as a universal biomarker for metabolic activity	Guo et al. (2019)
D_2O	*Escherichia coli Bacillus subtilis Salmonella typhimurium Marinobacter adhaerens*	Isotope labeled microbial cells sorting	Peak generation at 2040-2300 cm^{-1} region	Lee, Palatinszky, et al. (2019)
$_{13}C$	*Synechococcus sp.,*	Carbon fixation	Peak at 1521 cm^{-1} (C = C) for carbon metabolism	Taylor et al. (2017)
D_2O	*Enterococcus sp., Clostridium sp. Lactobacillus sp Escherichia coli*	Total chemical profiling	Peaks at 2220 cm^{-1}, 2160 cm^{-1} and 2109 cm^{-1} representing DNA proteins and lipids	Wang, He, et al. (2020)
D-glucose-d_{12} naphthalene-d_8	*Escherichia coli Pseudomonas putida*	Anabolic pathways of carbon sources; biosynthesis of fatty acids and phenylalanine	2070 – 2300 cm^{-1} as a universal biomarker for metabolic activity	Xu et al. (2017)
D_2O	*Escherichia coli*	Resuscitation of VBNC cells metabolic activity	Peak at 2200 cm^{-1} for total metabolic activity	Zhang et al. (2018)
D_2O	*Pseudarthrobacter oxydans, Massilia phosphatilytica, Burkholderia cepacia, Bacillus megaterium,* and *Chryseobacterium gleum*	Phosphate release	Peaks at 2040 – 2300 cm^{-1} representing phosphate release	Li, Bi, et al. (2019)
D_2O	*Providencia vermicola, Bacillus safensis, Bacillus cereus, Bacillus tequilensis,* and *Bacillus velezensis*	Carboxy-methyl cellulose degradation	Peak generation at 2040 – 2300 cm^{-1}	Olaniyi et al. (2019)
D_2O	*Streptococcus mutans, Streptococcus gordonii, Streptococcus sanguinis,* and *Lactobacillus fermentum*	Antimicrobial effects	2040 to 2300 cm^{-1} as a universal biomarker for metabolic activity	Tao et al. (2017)
D_2O	*Salmonella enterica, Shigella flexneri, Proteus vulgaris, Klebsiella variicola, Escherichia fergusonii, Providencia rettgeri, Klebsiella singaporensis, Klebsiella pneumoniae*	Rapid antibiotic susceptibility testing	2040 to 2300 cm^{-1} as a universal biomarker for metabolic activity	Yang et al. (2019)
$_{13}C$-citrate and D_2O	*Escherichia coli Acinetobacter baylyi*	Carbon assimilation and general metabolic activity	Peak at 2187 cm^{-1} representing carbon metabolism	Wang et al. (2016)
$^5N_2$	*Escherichia coli, Pseudomonas putida, Bacillus megaterium,* and *Azotobacter chroococcum*	Nitrogen assimilation	Peak at 730 cm^{-1} representing adenine production	Cui et al. (2017)

Note: VBNC = Viable but non-culturable.

subpopulation, find the link between phenotype and genotype, and assist in genome sequencing. Most of the existing technology such as FACS primarily relies on fluorescent labeling, which does not apply to intracellular molecules and may result in nonspecific binding (Nitta et al., 2020). The lack of external labeling makes Raman activated cell sorting (RACS) a more suitable technique for cell sorting and discrimination as compared with other cell sorting techniques. RACS consists of a high-resolution Raman spectroscopic system and a cell isolation system such as microfluidics, tweezer, or cell ejection system. RACS facilitates the separation of cells based on the fingerprint spectra contributed by intracellular components rather than cell morphology, thus making the separated cells viable for single-cell genomics. Song et al. (2017) used RACE to separate carotenoid-containing bacterial cells from the red seawater sample and the information was used to connect phenotype and genotype of cells. Moreover, McIlvenna et al. (2016) used a Raman-microfluidic system for sorting of cyanobacteria based on differences in the culture medium (^{12}C or ^{13}C carbon source) and achieved 96.3% accuracy at a flow rate of 100 $\mu m\ s^{-1}$.

SIP-Raman enables the sorting of isotope-labeled microbial cells from complex biological samples and can be used for single-cell culturing or genomic analysis. Lee, Palatinszky, et al. (2019) employed a complex platform

combining microfluidics, optical tweezing, isotope labeling, and Raman spectroscopy for sorting of bacteria and exhibited an accuracy of 98.3 ± 1.7% with 3.3–8.3 cells min^{-1} throughput. From the Raman spectrum obtained, cell index was calculated to indicate the presence of a cell in the optical tweezers and labeling index to indicate deuterium in a cell. This RACS method can also be tuned for the sorting of other microbial structures that show characteristic Raman spectra (Lee, Palatinszky, et al. 2019).

Deckert-Gaudig et al. (2012) employed TERS for the characterization and localization of specific membrane on *Halobacterium salinarum* surface and showed that the spectrum was able to distinguish purple membrane patches on the red membrane of the bacterial cell surface. The spectra also showed many features of the cell membrane that were not found in the normal Raman spectra, paving the way for analyzing structural and functional characteristics of different cell structures. Further, Berezin et al. (2017) showed the ability of TERS to differentiate gram-positive and gram-negative bacterial species. Since the change in gram-positive and negative bacteria mainly depends on lipid bilayer, the study focused on the 2800-3000 cm^{-1} region and found that for gram-negative bacteria an enhancement of 60% was observed for tip-in than tip-out, whereas a 5% reduction in intensity was observed for gram-positive bacteria.

Statistical tools such as multivariate chemometric analysis can acquire detailed information from spectral data for differentiating microbial cells even to strain level. Dina et al. (2021) used a semi-supervised clustering method of fuzzy c-means in combination with PCA, i.e., fuzzy linear discriminant analysis (FLDA) for the classification of spectra obtained from 3 bacterial species (*Staphylococcus aureus, Enterococcus faecalis,* and *Pseudomonas aeruginosa*) and showed that the application of FLDA resulted in a correct classification rate of 100% but the cross-validation showed a correct classification rate of only 50%. Similarly, Yan et al. (2021) combined Kernel principal component analysis (KPCA) with the decision tree algorithm to differentiate spectral data from 23 common bacterial strains at the single-cell level and reported that the intricate nature and similarities of the spectral data obtained from a single bacterial cell demanded a four-level classification model that showed 95.8% correct rate of prediction. Thus, single-cell Raman spectroscopy combined with machine learning can be used as an analytical tool for rapid diagnostics of food-borne pathogens (Hu et al.2020b; Hussai, Pu, and Sun 2020a).

Metabolic activities

The profiling of metabolomic transformations is the most effective way to understand the response of a living cell to the changes in environmental conditions (He et al., 2020; K. Wang, Sun, Pu, and Wei 2019b). The metabolome acts as the power source and medium for communication and can be directly correlated with the phenotype of the cell. A deep insight into the metabolic and transcriptional regulations of single cells can shed light on the fundamental aspect of the nitrogen cycle, the differentiation of microorganisms based on their carbon metabolisms in a mixed culture, and the metabolic pathway of metals, sulfur, and halogens (Gao, Huang, and Tao 2016). SRS has been used for lipid profiling of live cells as the CH_2 bond in lipids generates a strong signal when the pump and stroke frequencies match with the C-H bond frequency (D'Arco et al. 2017). The addition of microfluidics to SRS offers an opportunity for *in-situ* analysis of lipid metabolic pathways at single-cell resolution (Cao et al. 2016).

Xu et al. (2017) used deuterated glucose and naphthalene to analyze the metabolic pathway of carbon and fatty acid in *Escherichia coli* and *Pseudomonas putida* at the single-cell level. The spectra revealed that the intensity of the C-D vibration (2070–2300 cm^{-1}) was directly proportional to the extent of deuteration. Due to the difference in the metabolic pathway, the conversion rate of lipid from deuterated naphthalene was observed to be lower than that of glucose. Yonamine et al. (2017) investigated the photosynthetic activity of *Euglena gracilis* by analyzing the C-D band variation in D_2O derived metabolites at the single-cell level. The C-D vibrations in extracted paramylon from the cells indicated the incorporation of deuterium into cells through photoautotrophic carbohydrate metabolism. Further, Olaniyi et al. (2019) used D_2O labeled Raman spectroscopy for semi-quantitative analysis of carboxy-methyl-cellulose (CMC) degradation by 7 bacterial species isolated from cassava wastewater and the degradation ability was expressed in terms of the $C-D$ ratio, showing that *Bacillus velezensis* had the highest CMC degrading ability as validated by the endoglucanase activity. Besides, the C-D ratio for the bacteria cultivated in glucose was 60% higher than in CMC, indicating the preference of the bacteria to simple sugars. Moreover, Wang et al. (2016) showed a simultaneous analysis of carbon assimilation and metabolic interaction of two bacterial species *Acinetobacter baylyi* and *Escherichia coli* through co-labeling. They introduced a reverse labeling process, in which the ^{13}C labeled cells were cultured in a ^{12}C environment and the reversion of ^{13}C cells (970 cm^{-1}) to ^{12}C cells (1006 cm^{-1}) was found to be time-dependent, which also provided detailed information about the carbon flow in single cells. The ^{13}C peaks (970 cm^{-1}) of *Acinetobacter baylyi* reversely shifted back to ^{12}C peak (1006 cm^{-1}) within 7 h but no significant change was shown in *E. coli*. However, when co-cultured, *E. coli* showed significant metabolic activity by utilizing the metabolic byproduct of *Acinetobacter baylyi* as confirmed by mass spectroscopic analysis, which suggested that the associated byproducts could be putrescine and phenylalanine. This reverse Raman-SIP can be used as a reliable method to analyze microbial interactions between two different species at the single-cell level (Wang et al. 2016).

Antimicrobial effects

Fast and accurate antimicrobial and antibiotic susceptibility testing is important for choosing a suitable agent to treat infectious diseases and identify resistant strains. Raman spectroscopy with and without isotope labeling has immense

potential in antimicrobial susceptibility testing on the single-cell level (L. Wu et al. 2020). The high sensitive metabolite mapping ability of SRS imaging was applied in antimicrobial susceptibility testing of *Candida albicans* (Karanja et al. 2017). The imaging revealed that lipogenesis profiling could predict the susceptibility of bacteria to fluconazole and SRS imaging offers rapid susceptibility testing as only a few hours are necessary to analyze a full metabolic cycle. In addition, Hong et al. (2018) developed a universal method based on stimulated Raman imaging for antibiotic susceptibility testing applicable for a wide spectrum of bacteria irrespective of the mechanism of inhibition.

Single-cell SIP - Raman spectroscopy can expedite antimicrobial susceptibility testing in identifying pathogenic bacteria in clinical samples and defining minimum inhibitory concentration (MIC) to assess the efficacy of drugs. MIC of antimicrobial agents was calculated as the minimum dose and the median value of CD/(CD + CH) at 8 h of drug exposure was ≤ 0 and the standard deviation among the individual cells was ≤ 0.005 (Tao et al. 2017). The measurement of antimicrobial efficiency through analyzing metabolism inhibition is rapid, quantitative, and low-cost and the efficiency can be used as a reference for recommending the dose and duration of antibiotics. Moreover, Yang et al. (2019) were able to reduce the total assay time to 2.5 h. Zhang et al. (2018) employed D_2O-labeled single-cell Raman spectroscopy to examine the time-dependent recovery of metabolic activity in *E. coli* bacteria at VBNC state induced by ultraviolet (UV- C) irradiation. The metabolic activity of VBNC cells was proportional to the level of incorporation of D from D_2O into cells, and the higher the UV-C dosage was, the more the damage on bacterial cell and the less the ability of the cell to recover during post-incubation.

However, although the D_2O-labeled single-cell Raman spectroscopy technique provides information about resuscitation and metabolic heterogeneity, it cannot differentiate VBNC cells from cultivable cells. Further, Guo et al. (2019) extended the study to other bacteria including *Aeromonas* sp., *Pseudomonas* sp., *E. coli,* and *Staphylococcus aureus* in drinking water and showed that the CD/(CD + CH) ratio from the Raman spectrum declined from 95.7% to 47.9% with the increase of dosage from 10 to 200 mJ/cm^2. Therefore, D_2O-labeled single-cell Raman spectroscopy can provide fundamental data to optimize doses for the water disinfection process, but the procedure and obtained spectrum are expected to get complicated for application in biological media.

Biomolecule accumulation

The growth media of microorganism contain specific biomolecules, which act as a biomarker for certain stains and the intracellular production leads to the accumulation of biomolecules inside the cell (Gaimster et al. 2014). Jantschke et al. (2019) used Raman spectroscopic imaging to analyze the distribution of a specific component of β-guanine crystals in a single *Calciodinellum operosum* cell (Jantschke et al. 2019) (Figure 1A). The time-resolved Raman imaging acquired from a single *Euglena gracilis* cell provided information about the dynamic intracellular distribution of myristyl myristate (wax ester) (Iwasaki et al. 2019). However, the spectra produced by a complex biological system such as a yeast cell are the result of several overlapping contributions from cellular components such as polysaccharides, proteins nucleic acids, etc, and combining Raman spectroscopy with multivariate analysis can thus unravel hidden information in the spectra, as indicated by Iwasaki et al. (2019), who identified two different myristyl myristate in *Euglena* cells and the chain length of different esters through multivariate curve resolution analysis. Moreover, the principal component analysis of the single-cell spectral data from four microalgae (*Arthrospira platensis*, *Chlorella vulgaris*, *Phaeodactylum tricornutum,* and *Scenedesmus obliquus*) revealed that the intensity at the peak of 1514 cm^{-1} was negatively correlated with total phenolic and flavonoid content in the cell structure (Gouda et al. 2021).

Ota et al. (2019) conducted the time-resolved Raman analysis of a single *Euglena gracilis* cell cultured in glass microfluidic device paramylon biogenesis and demonstrated that Raman mapping could provide information about the accumulation of paramylon in a single cell (Figure 2A). The H_2O and CO_2 in *E. gracilis* were replaced with stable isotope-labeled ^2H_2O and $^{13}CO_2$ to analyze the metabolic products. This replacement with a heavier isotope promoted the Raman signal to move to a lower wavenumber position. The technique was used to analyze 30 cells simultaneously and can be further applied for single-cell isolation and analysis of other microorganisms.

The specific metabolic capabilities of different microorganisms have been studied in detail using single-cell SIP-Raman spectroscopy. Cui et al. (2018) employed strong resonance Raman spectroscopy (RRS) signals of $^{15}N_2$-labeled cytochrome c in *Azotobacter* sp. *Rhizobium radiobacter*, and *Bradyrhizobium japonicum* to analyze their nitrogen-fixing capabilities. The characteristic peak of cytochrome c at 1129 cm^{-1} shifted to 1114 cm^{-1} due to the incorporation of $^{15}N_2$ into cell structure and the peak area ratio ($Area_{1114}$/($Area_{1114}$ + $Area_{1129}$)) showed a linear relationship with $^{15}N_2$ concentrations in the culture medium. Furthermore, Raman imaging was employed to differentiate $^{15}N_2$-labeled *Azotobacter* sp. from *Shewanella oneidensis* in a co-culture, although both species contained cytochrome c (Figure 2B). In another attempt to understand nitrogen assimilation in various microorganisms, Cui et al. (2017) employed SERS−^{15}N SIP approach and showed that the band at 730 cm^{-1} best represented the characteristic peak for nitrogen assimilation in a single cell.

Challenges and future work

Single-cell Raman spectroscopy is an effective label-free method to understand the biological activity of single cells in complex matrixes. Understanding the functional fluctuations of a single cell in a community is a difficult task due to the lack of high-resolution analytical methods and limitations of the existing techniques. Therefore, despite

significant developments in single-cell Raman spectroscopic techniques, many challenging issues still remain.

- The cell undergoes several different chains of events during the cell growth cycle, resulting in several distinguishable growth phases and metabolic states during its life. The noninvasive nature of Raman spectroscopy supports the characterization of metabolic states of the microbial cell. However, it is challenging to select a cell for analysis without any bias due to the growth stage, as the metabolic activity varies according to the age of the cell. Therefore it is incredibly important to consider the implications of the cell cycle itself on Raman spectroscopic measurements because the subtle change in the metabolism of single cells can result in false-positive results.
- RACE offers an effective method to isolate a single cell based on a specific trait, but the sensitivity is indirectly proportional to throughput. This low throughput is the major limiting factor that prevents the application of RACE in the sorting of microbial cells. Although Raman labeling can improve the output, it demotes downstream sequencing analysis, thus effort must be made to improve the throughput without compromising the sensitivity. For example, laser capture microdissection (LCM) can be employed to isolate cells with specific traits from their environment. The application of this microdissection technique has been extensively studied in tissue engineering, but the potential of LCM in microbial analysis is yet to be discovered, which can be a topic for future studies.
- Apart from the use of microfluidics for isolation for Raman measurements, optical tweezers are a promising alternative for micromanipulation and sorting of single cells. Optical tweezers have been proven to have the ability of 3D optical manipulation and can thus be combined with Raman spectroscopy for rapid screening and isolation of a single cell. Optical tweezers can control the movement of a single cell in x, y, and z directions and can trap the cell under a Raman microscope. Since both Raman spectroscopy and optical tweezers rely on laser beam for measurement and trapping of the cell respectively, the development of a system combining these two technologies should be investigated in future.
- Effective interpretation and visualization of the abundant data produced by Raman spectra is still a major challenge, especially in biological samples. However, along with the development of various sorting platforms, a standardized algorithm for data analysis should also be developed. The abundant data produced during imaging should be followed by machine learning-based cell classifiers for processing and interpreting information in the future. This could result in creating a steady and credible database for differentiating bacterial strains based on the massive data collected from various microbial species and strains. Additionally, the lack of a reference library for comparison slows down the extraction of chemical information and interpretation, thus the development of a library should be considered in future studies, which should boost the application of this fingerprinting technique.
- Another major concern in Raman spectroscopic techniques at the single-cell level is the oversight of colony-level interactions such as extracellular signaling between cells and the environment. Thus, care should be given to incorporate such properties into experimental design and data interpretation. The solemn purpose of the single-cell analysis is to eliminate the population-averaged assumptions about the microbial cells, but cell-cell interactions during growth is an important phenotypical characteristic possessed by the cell. Thus the integration of single-cell analysis with population-averaged techniques is expected to be a robust approach for a better understanding of microorganism behavior, and future efforts should be devoted to this area.
- Overall, the evolution of automated systems, the advancement of machine learning, accurate isolation techniques, and the assortment of reference datasets would put Raman spectroscopy milestones ahead in single-cell analysis. Therefore, it is expected that the application of single-cell Raman spectroscopy can be extended to several disciples of research including pharmaceutical, medical, food safety, and genetic engineering in future.

Conclusions

Analysis of microorganisms at the single-cell level can unearth previously unknown characteristics and behavior of cells, which are impossible to detect in culture-based bulk measurements. This review provides a summary of recent developments in Raman spectroscopic methods for the analysis of single cells.

The natural weak signal of Raman spectroscopy during the analysis of biological samples can be overcome by employing enhancement by nanoparticles (SERS), choosing Raman active molecule with natural enhancement capabilities as biomarkers (RRS) and/or by introducing additional stokes photon. Furthermore, introducing traceable Raman tag such as deuterium isotope into cells (Raman-SIP) helps in understanding the metabolic pathway of fundamental molecules. Integration of microfluidics can facilitate simultaneous culturing and monitoring of time-resolved single-cell analysis and atomic force microscopy (TERS) can provide the surface and sub-surface chemical mappings of single cells.

SCRS is an evolutionary technique for analyzing the structural and functional characteristics of a single cell. SCRS is a nondestructive and label-free method, which provides information inaccessible by culture-dependent method about single cells at the nanoscale level. The effect of environmental stress on single-cell microorganisms is measured mainly based on the change in the metabolic activity of the cell. The combination of Raman spectroscopy with single-cell isolation technique and statistical data analysis methods put forth a powerful technique for single-cell analysis. These techniques have tremendous application potentials in environmental and biological analyses including antimicrobial

and antimicrobial susceptibility, metabolic pathway analysis, intracellular biological event, lipid droplet in yeast, metabolic profiling, and nanoparticles uptake. The development of laboratory-scale Raman spectroscopy is relatively straightforward and simple in term of instrumentation and skill set, but tremendous efforts should be made to improve the throughput of single-cell analysis.

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