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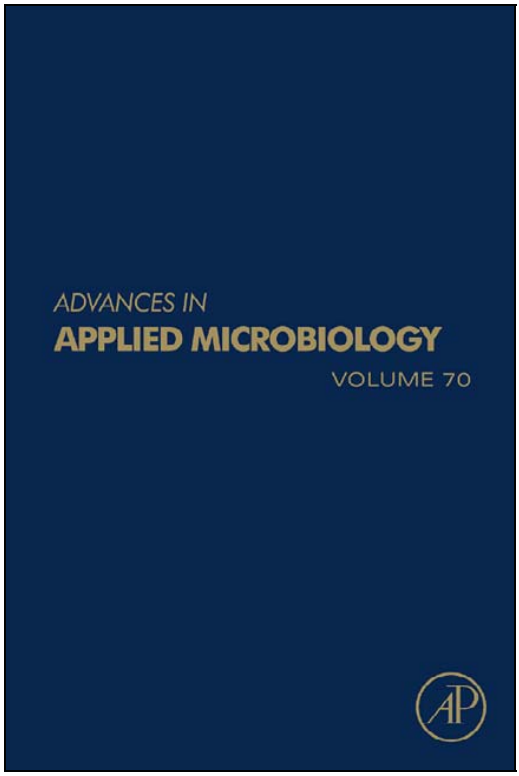

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CHAPTER 5

Shining Light on the Microbial World: The Application of Raman Microspectroscopy

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Contents

Abstract

Raman microspectroscopy is a noninvasive, label-free, and single-cell technology for biochemical analysis of individual mammalian cells, organelles, bacteria, viruses, and nanoparticles. Chemical information derived from a Raman spectrum provides

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comprehensive and intrinsic information (e.g., nucleic acids, protein, carbohydrates, and lipids) of single cells without the need of any external labeling. A Raman spectrum functions as a molecular "fingerprint" of single cells, which enables the differentiation of cell types, physiological states, nutrient condition, and variable phenotypes. Raman microspectroscopy combined with stable isotope probing, fluorescent *in situ* hybridization, and optical tweezers offers a culture-independent approach to study the functions and physiology of unculturable microorganisms in the ecosystem. Here, we review the application of Raman microspectroscopy to microbiology research with particular emphasis on single bacterial cells.

I. INTRODUCTION

Sydney Brenner is often quoted as saying ''Progress in science depends on new techniques, new discoveries and new ideas, probably in that order'' (*Nature*, June 5, 1980), and science can be significantly pushed forward by introducing new technologies and research tools, notably X-ray for the DNA helix structure, DNA sequencing technology and microarrays for molecular biology. Single-cell microbiology has emerged as an important concept due to the discovery of microbial cellular heterogeneity in terms of gene expression, phenotypes, pathogenesis, and physiological states (Amann and Fuchs, 2008; Avery, 2006; Brehm-Stecher and Johnson, 2004; Czechowska *et al*., 2008; Davey and Kell, 1996; Dethlefsen and Relman, 2007; Elowitz *et al*., 2002; Kuypers and Jorgensen, 2007; Marcy *et al*., 2007; Musat *et al*., 2008; Muzzi *et al*., 2007; Raes and Bork, 2008; Sabelnikov and Kempf, 2008; Stepanauskas and Sieracki, 2007). In addition, over the last two decades, microbiologists realized that the vast majority of microbes (>99%) in the natural environment are currently unculturable using traditional culture-dependent approaches (Amann *et al*., 1995; Daniel, 2005; Huang *et al*., 2009b; Venter, 2003; Venter *et al*., 2004; Whitman *et al*., 1998). To explore the behavior and functions of unculturable microorganisms, it has become necessary to develop culture-independent tools such as metagenomics (Brehm-Stecher and Johnson, 2004; Handelsman, 2004; Schloss and Handelsman, 2003) and single-cell-based technologies (Brehm-Stecher and Johnson, 2004; Huang *et al*., 2004, 2007c, 2009b). To date, many single-cell technologies have been successfully applied to microbiology (for a review, see Brehm-Stecher and Johnson, 2004). Among them, Raman microspectroscopy offers a unique opportunity which enables one to interrogate at the biochemical level and manipulate microbes at single cell level in their natural habitat.

The Raman effect was first discovered by C. V. Raman in 1928, and it is the inelastic scattering of photons from a sample. Normal (Rayleigh)

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scattering is elastic, whereby the energy (or wavelength) of the scattered photons is unchanged from the incident light. Raman scattering is inelastic, so that the scattered photons have an energy (or wavelength) which is either greater or smaller than that of the incident light. The loss (or gain) in energy during the scattering process is caused by interaction of the photon with vibrational modes of the chemical bonds within the sample. Thus, a typical Raman spectrum will comprise a number of Raman peaks which are indicative of particular chemical bonds and their vibrations. Particular bond types (e.g., C–H, N–H, C=O) always appear in a particular spectral region, but their precise spectral position will depend on their immediate environment. Raman scattering provides detailed information about chemical composition—however, the effect is usually weak since only 1 in 10^6–10^8 photons incident on the sample will undergo Raman scattering (the rest will take part in competing processes, such as Rayleigh elastic scattering, fluorescence, heating, etc.). Historically, several constraints, such as low sensitivity, interference from fluorescence background and data interpretation, have hampered Raman spectroscopy to a broader application. However, in recent years, those problems have been largely overcome by introducing advances in laser technology, photon measurements using charged coupled devices (CCDs), and computation. Lasers produce extremely high intensity monochrome photons thereby resulting in much stronger Raman signals. Sample fluorescent background can be minimized by using different lasers, such as deep-red or near-infrared lasers in the range of 630–1060 nm (Carey, 1999), or moving to the deep UV range (200–260 nm) (Nelson *et al.*, 1992). Since the 1970s the discovery of surface-enhanced Raman scattering (SERS) makes Raman spectroscopy even more attractive because SERS can enhance Raman signals by a factor of 10^{6-14} (Lombardi and Birke, 2009), enabling the detection of single molecules (Kneipp *et al.*, 1997; Nie and Emery, 1997), and in general Raman spectroscopy has shown its great potential to become ultrasensitive chemical analysis (Kneipp *et al.*, 1999b).

Raman microspectroscopy that combines Raman spectroscopy with an optical microscopy was first introduced by Puppels *et al.* (1990) and has engendered thousands of articles thereafter. Raman microspectroscopy is a noninvasive technique to acquire chemical signals from a small volume of samples ($<1\ \mu m^3$) such as bacteria (Huang *et al.*, 2004). Raman microspectroscopy employs a monochrome laser as a light source to acquire Raman signal from a single bacterial cell. The same laser can also generate optical trapping which can be used to manipulate bacterial cells in aqueous solutions (Huang *et al.*, 2009a; Xie and Li, 2003). If there are no resonance effects different lasers should generate theoretically same Raman spectra. We have employed 532, 633, and 785 nm lasers to interrogate single bacterial cells and found that the 532 nm laser produce best single cell Raman spectrum (SCRS) in terms of signal-to-noise ratio with

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negligible fluorescence background. A typical SCRS contains more than thousand bands, providing intrinsic profile of cells without any external labeling (Fig. 5.1). Raman spectra at the range of 500–2000 cm^{-1} contain rich biological information (Fig. 5.1), from nucleic acids, proteins, polysaccharides, carbohydrate, and lipids (Table 5.1). Such spectral information offers a signature of the molecular structures, cellular compositions, and physiological states. Over the past few years, Raman microspectroscopy has been combined with optical tweezers and epifluorescent microscope to develop Raman tweezers (Xie and Li, 2002, 2003; Xie *et al.*, 2002, 2005a,b) and Raman microspectroscopy combined fluorescent *in situ* hybridization techniques (Raman-FISH) (Huang *et al.*, 2007c, 2009a,b).

Raman microspectroscopy has been shown to differentiate cell phenotypes, metabolism states, and physiological changes. Raman microspectroscopy, armed with SERS, optical tweezers, and FISH, could provide a huge opportunity to characterize and sort single bacterial cells, and it may open a new frontier to studying natural microbial communities, without the need for prior cultivation. There are many excellent reviews on Raman spectroscopy (Carey, 1999; Chan *et al.*, 2008; Harz *et al.*, 2009; Kneipp, 2007; Kneipp *et al.*, 1999b, 2006c; Lombardi and Birke, 2009; Maquelin *et al.*, 2002b; Petry *et al.*, 2003; Wachsmann-Hogiu *et al.*, 2009; Wagner, 2009). Here, we review the application of Raman microspectroscopy to microbiology research with particular emphasis the study of single bacterial cells.

II. IDENTIFICATION OF MICROBIAL SPECIES USING SINGLE CELL RAMAN SPECTRA

There is an increasing requirement for the rapid identification of microbes in samples for environmental, public health, and medical studies. For the situation that requires a very fast identification and localization of pathogens or specific microbes in their microniches, one option is to use an imaging approach such as Raman microspectroscopy (Chan *et al.*, 2004; Huang *et al.*, 2004, 2007b; Rosch *et al.*, 2005; Xie *et al.*, 2005a). Raman microspectroscopy is a label-free and noninvasive physicochemical technology, able to reveal intrinsic chemical information about individual cells, and this may be specific elicitation of gene expression, biosynthesis of compounds, cell components, characteristic structures, physiological states, or metabolic profiles. Raman spectroscopy has been used to classify different bacterial species at colony (Choo-Smith *et al.*, 2001; Jarvis and Goodacre, 2004; Maquelin *et al.*, 2000, 2002a) and single cell levels (Chan *et al.*, 2004; Huang *et al.*, 2004, 2007b; Rosch *et al.*, 2005; Xie *et al.*, 2005a), as well as from slurries (Goodacre *et al.*, 1998). Since closely related bacteria could have different Raman profiles, Raman microspectroscopy

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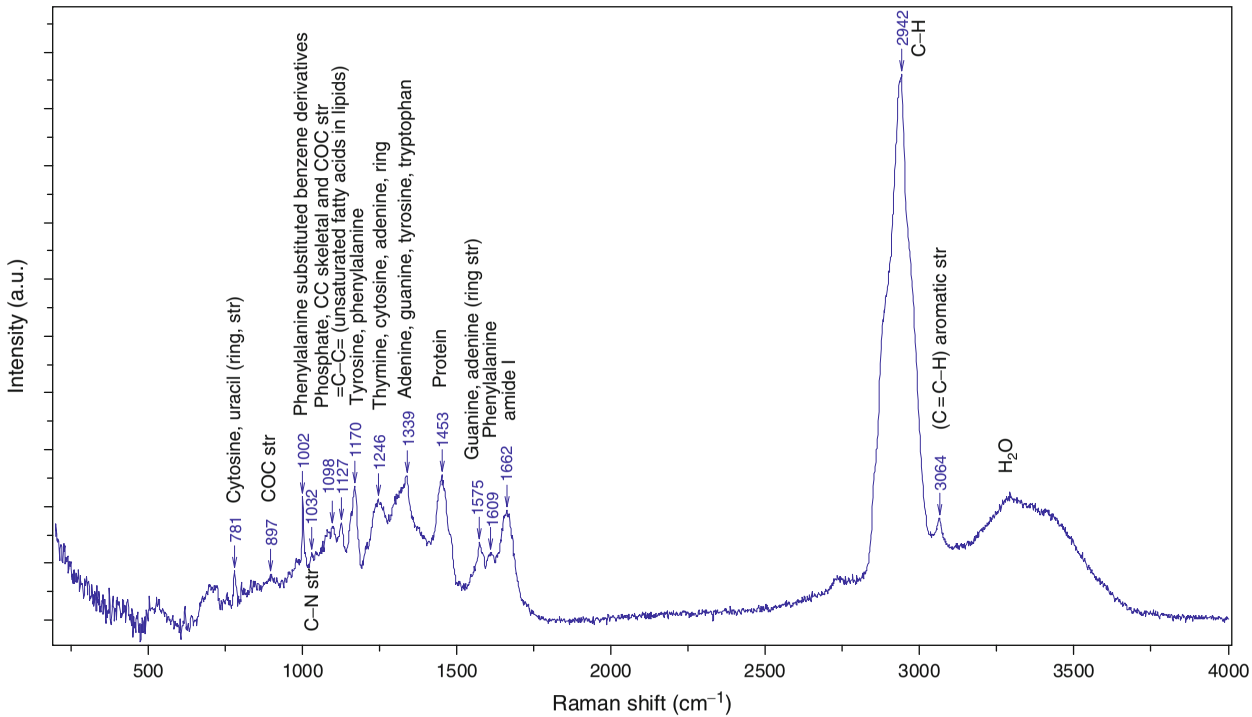

FIGURE 5.1 A typical Raman spectrum of a single cell of *Acinetobacter baylyi* ADP1 using 532 nm laser; the laser on the single cell was 35 mW and acquisition time was 10 s. Most Raman signals are at range of 600–2000 cm^{-1}.

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TABLE 5.1 Assignment of some bands frequently in Raman spectra respective of biological query

Frequency (cm^{-1})	Assignment	References
3240	Water	Harz *et al.* (2009)
3059	(C=C–H) aromatic str	Maquelin *et al.* (2002b)
2975	CH_3 str	Maquelin *et al.* (2002b)
2935	C–H str	Maquelin *et al.* (2002b), Harz *et al.* (2009)
2870–2890	CH_2 str	Maquelin *et al.* (2002b)
1735	>C=O ester str	Maquelin *et al.* (2002b)
1650–1680	Amide I	Maquelin *et al.* (2002b)
1663	Amide I	
1658	Unsaturated lipids	van Manen *et al.* (2005)
1614	Tyrosine	Maquelin *et al.* (2002b)
1605–1606	Phenylalanine	Maquelin *et al.* (2002b)
1582, 1593	Protein	Maquelin *et al.* (2002b), Kneipp *et al.* (2006b)
1575–1578	Guanine, adenine (ring str)	Maquelin *et al.* (2002b)
1573	C=C, N–H def, and C–N str (amide II)	Schuster *et al.* (2000b)
1516	C=C str, of sarcinaxanthin	Rosch *et al.* (2005)
1510	Adenine, or C=C str, carotenoids	Uzunbajakava *et al.* (2003a)
1505, 1518, 1532, 1578	Adenine, cytosine, guanine	Uzunbajakava *et al.* (2003a)
1482–1487	Nucleic acids	Schuster *et al.* (2000a)
1441	Lipids	van Manen *et al.* (2005)

(*continued*)

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TABLE 5.1 (*continued*)

Frequency (cm^{-1})	Assignment	References
1440–1460	C–H_2 def	Maquelin *et al.* (2002b)
1431–1481	Protein marker band 1451	Uzunbajakava *et al.* (2003b)
1421–1427	Adenine, guanine	Uzunbajakava *et al.* (2003a), Kneipp *et al.* (2006b)
1375	Thymine, adenine, guanine	Uzunbajakava *et al.* (2003a)
1336–1339	Adenine, guanine, tyrosine, tryptophan	Uzunbajakava *et al.* (2003a), Harz *et al.* (2009)
~1320	Amide III, C–H def	Schuster *et al.* (2000b)
1304	Adenine, amide III	Uzunbajakava *et al.* (2003a)
1295	CH_2 def	Maquelin *et al.* (2002b)
1214, 1240, 1254	Thymine, cytosine, adenine, ring ν	Uzunbajakava *et al.* (2003a)
1254	Adenine, amide III	Uzunbajakava *et al.* (2003a)
1220–1290	Amide III random, lipids	Schuster *et al.* (2000b)
1267	Lipids	van Manen *et al.* (2005)
1209	Tyrosine, phenylalanine, protein, amide III	Uzunbajakava *et al.* (2003a)
1175	Tyrosine, phenylalanine	Uzunbajakava *et al.* (2003a)
1155–1157	C–C str, of sarcinaxanthin, carotenoids	Rosch *et al.* (2005)
1154	ν(CC, CN), ρ(CH_3)	Maquelin *et al.* (2002b)
1145–1160	C–C, C–O ring breath, assym	Schenzel and Fischer (2001), Rosch *et al.* (2004)

(*continued*)

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TABLE 5.1 (*continued*)

Frequency (cm^{-1})	Assignment	References
~1130	=C–C= (unsaturated fatty acids in lipids)	Schuster *et al.* (2000b)
1102	> PO_2^- str (sym)	Maquelin *et al.* (2002b)
1100	Glass background	Schuster *et al.* (2000b)
1098–1099	Phosphate, CC skeletal, and COC str from glycosidic link	Maquelin *et al.* (2002b)
1085	C–O str	Maquelin *et al.* (2002b)
1061	C–N and C–C str	Maquelin *et al.* (2002b)
1054	Nucleic acids, CO str; protein, C–N str	Uzunbajakava *et al.* (2003a)
1032	Phenylalanine; C–N str	Uzunbajakava *et al.* (2003a)
1030–1130	Carbohydrates, mainly –C–C– (skeletal), C–O, def (C–O–H)	Schuster *et al.* (2000b)
~1004	Phenylalanine, substituted benzene derivatives	Maquelin *et al.* (2002b)
897	COC str	Maquelin *et al.* (2002b)
858	CC str, COC 1,4 glycosidic link	Maquelin *et al.* (2002b)
~850	Buried tyrosine	Maquelin *et al.* (2002b)
~830	Exposed tyrosine	Maquelin *et al.* (2002b)
838	DNA	Deng *et al.* (1999)
813	A-type helices in RNA	Uzunbajakava *et al.* (2003b)
810–820	Nucleic acids (C–O–P–O–C in RNA backbone)	Schuster *et al.* (2000b)
778–785, 792	Cytosine, uracil (ring, str)	Maquelin *et al.* (2002b), Uzunbajakava *et al.* (2003a)

(*continued*)

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TABLE 5.1 (*continued*)

Frequency (cm^{-1})	Assignment	References
748–751	O–P–O sym str	Takai *et al.* (1997)
752	T ring str	Uzunbajakava *et al.* (2003a)
730	A ring str	Uzunbajakava *et al.* (2003a)
720	Adenine	Maquelin *et al.* (2002b)
665	Guanine	Maquelin *et al.* (2002b)
640	Tyrosine (skeletal)	Maquelin *et al.* (2002b)
620	Phenylalanine (skeletal)	Maquelin *et al.* (2002b)
550 range	Glass background	Schuster *et al.* (2000b)
540	COC glycosidic ring def	Maquelin *et al.* (2002b)
520–540	S–S str	Maquelin *et al.* (2002b)
481	Skeletal modes of carbohydrates (starch)	Schuster *et al.* (2000b)
407	Skeletal modes of carbohydrates (glucose)	Schuster *et al.* (2000b)

Note: str = stretching; def = deformation; sym = symmetric; asym = antisymmetric

can be a very sensitive tool, and it has been used to discriminate between different *Acinetobacter* strains (Maquelin *et al.*, 2006) and different strains of *E. coli* (Jarvis *et al.*, 2004). However, bacterial Raman spectra may reflect more bacterial phenotypes than genotypes. Many environmental events (e.g., temperature, pH, micronutrient condition) and variable gene expression could affect the composition of individual cells and cause variations in SCRS, which may blur the boundaries of species groups. In practice, these drawbacks should be considered carefully. In order to reduce the biological variations of SCRS, one possible solution is to ensure that the bacterial species growing at the same condition and more replicate measurements are taken. Of course, for scenarios where microcosms are analyzed directly, the need for cultivation is obviated.

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III. LINKING MICROBIAL SPECIES, SPATIAL DISTRIBUTION, AND THEIR FUNCTIONS

One important goal of environmental microbiology is to link particular functional attributes to particular microorganisms *in situ*, since most natural bacteria have yet to be cultured in the laboratory (Amann *et al.*, 1995; Daniel, 2005; Huang *et al.*, 2009b; Venter, 2003; Venter *et al.*, 2004; Whitman *et al.*, 1998). The combined use of stable isotope tracers (stable isotope probing or ''SIP''), coupled with identification methods (e.g., 16S-rRNA sequencing) (Manefield *et al.*, 2002a,b; Radajewski *et al.*, 2000, 2003), currently holds the greatest promise for this purpose. However, these methodologies are reliant on the extraction and homogenization of biomarker molecules from their native environment, and therefore are destructive and do not permit examination of spatial localization of the live organisms at the microscale. FISH has proved a powerful tool for quantifying the presence and localization of bacteria based upon 16S rRNA sequences (Amann and Fuchs, 2008; Amann *et al.*, 2001), but these methods are dependent upon the availability of suitably discriminate probes. In addition, both SIP—DNA/RNA extraction approach and FISH are destructive to the microbial cells under interrogation.

It has recently been found that some carbon-associated bands of bacterial SCRS shifted to lower wavenumber or higher wavelength (red-shift) if the bacteria were fed with ^{13}C-carbon substrates (Huang *et al.*, 2004, 2007c, 2009b). Figure 5.2A shows a comparison of the SCRS of a naphthalene degrader after growing in ^{12}C- and ^{13}C-naphthalene. Some Raman bands of ^{13}C-labeled cells are strong and sharp (e.g., 1002 cm^{-1} from phenylalanine), and the shifts can be so great that the change of the bands can be used to indicate the quantitative ^{13}C-integration at the single cell level (Huang *et al.*, 2007c, 2009b; Wagner, 2009). We showed that the phenylalanine band shifted 36 cm^{-1} from ^{12}C-SCRS at 1002 cm^{-1} to ^{13}C-SCRS at 966 cm^{-1} (Fig. 5.2A). We have also recently found that the stable isotope nitrogen also caused SCRS red-shift. Figure 5.2B shows that some bands of ^{15}N-SCRS of *E. coli* red-shifted in comparison of ^{14}N-SCRS after the cells were grown in M9 medium in which ^{15}N- and ^{14}N-NH_4Cl were the sole nitrogen sources. ^{15}N-NH_4Cl did not affect the phenylalanine band at 1002 cm^{-1}, and most red-shifts of the SCRS were related to nucleic acids (Fig. 5.2B). The adenine and guanine bands shifted 9 cm^{-1} from ^{14}N-SCRS at 1575 cm^{-1} to ^{15}N-SCRS at 1566 cm^{-1} (Fig. 5.2B). Therefore, coupled with SIP, the SCRS could be used to link single bacteria with their carbon and nitrogen metabolic activities, and also enables us to quantitatively estimate the factions of ^{13}C-incoporation in single cells by calculating the intensity ratio of the red-shift bands (Huang *et al.*, 2007c, 2009b).

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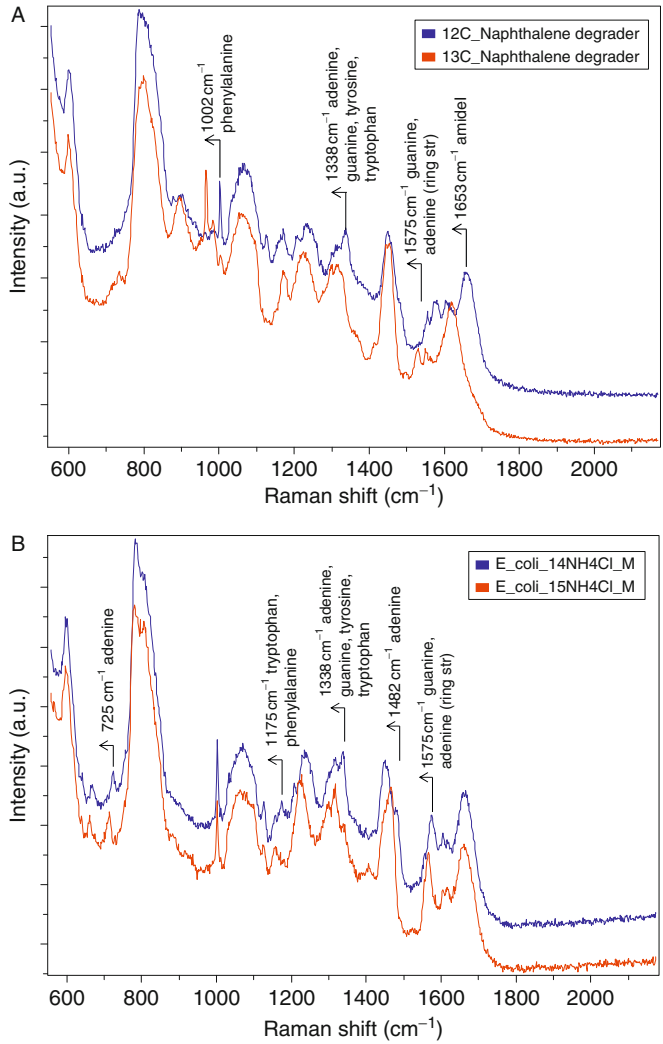

FIGURE 5.2 The red-shift of SCRS caused by stable isotope labeling. The comparison of ^{13}C- (red or light line) and ^{12}C- (blue or dark line) SCRS for a naphthalene degrader growing on naphthalene as the sole carbon source (A); and ^{15}N- (red or light line) and ^{14}N- (blue or dark line) SCRS *E. coli* grown in M9 in which N-NH_4Cl was the sole nitrogen source (B).

Raman-FISH has been developed as a new tool for single cell structure function analyses in complex microbial communities (Huang *et al.*, 2007c, 2009b). The Raman-FISH approach has enabled the direct identification and quantification of the metabolism of the labeled compound *in situ* to a

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key microbial group which was known to possess the capability from independent measures (selective isolation and molecular characterization of the strains). Coupled with SIP, Raman-FISH has helped to identify and measure *in situ* ^{13}C-naphthalene degraders within a microbial community from a complex groundwater system and proved that an uncultured species—*Acidovorax* sp.—played the key role in naphthalene biodegradation, rather than the three culturable naphthalene biodegrading *Pseudomonas* sp. obtained from the same groundwater (Huang *et al.*, 2009b). This was the first report to demonstrate that the unculturable bacteria in a natural environment should not be ignored because they could play a critical functional role in the ecosystem. The key advantage of the Raman-FISH is that it links bacterial identity, spatial localization, and their metabolic functions down to the single cell level (resolution of the technique is typically 1 μm). It has the benefits of both SIP and FISH approaches under a single analysis umbrella, which offers a great tool for the investigation of the ecological functions of many uncultured bacteria in the natural environment.

IV. RAMAN TWEEZERS TO MEASURE AND MANIPULATE SINGLE MICROBIAL CELLS

A laser beam can produce an attractive or repulsive force, depending on a sample's refractive index. Optical tweezers was first developed by Ashkin and his colleagues back in 1970, and was soon applied to manipulate and study single cells (Ashkin, 1970, 1980; Ashkin and Dziedzic, 1987; Ashkin *et al.*, 1986, 1987). Optical tweezers has now become an important tool in biological studies because it enables one to use the laser beam to manipulate single cells or organelles and revealing the interactions of proteins, small molecules, and DNA (Ashkin, 1992; Ashkin *et al.*, 1990; Block *et al.*, 1989; Chiou *et al.*, 2005; Greenleaf and Block, 2006; Greenleaf *et al.*, 2007; Herbert *et al.*, 2006; Neuman *et al.*, 1999; Svoboda and Block, 1994; Wang *et al.*, 1997). Optical tweezers can trap particles from 5 nm to tens of μm with typical forces of 100 aN to more than 100 pN (Grier, 2003), thereby allowing the trapping and manipulation of single bacteria cells in aqueous environments.

Raman tweezers, which couples optical tweezers with Raman microspectroscopy, has been applied to trap and measure single blood cells (Xie *et al.*, 2002) and *E. coli* (Xie and Li, 2003). Over the past few years, Raman tweezers has been explored to identify single bacterial cells and spores (Alexander *et al.*, 2003; Chan *et al.*, 2004; De Gelder *et al.*, 2007b; Hamden *et al.*, 2005; Mannie *et al.*, 2005; Ramser *et al.*, 2007; Xie *et al.*, 2004, 2005a,b, 2007), and measure dynamic changes in cell composition (Chen *et al.*, 2009; Peng *et al.*, 2009). In clinical microbiology, Raman tweezers

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through the use of laser controlling allows the analysis of lethal pathogens without direct contact (Chen *et al.*, 2006; De Gelder *et al.*, 2007b; Petrov, 2007). In environmental microbiology, Raman tweezers is especially useful, because it enables the noninvasive measurement of cells in their natural habitat thus not perturbing the cells. The Raman spectra collected from such an environment reflects the ecophysiological and metabolic states of single cells in real time with high spatial resolution, and to elucidate the spatial distribution of bacteria in their native habitat (Patzold *et al.*, 2008). Since Raman tweezers is able to measure live cells, it has also been used to directly monitor the process of bacterial physiology or gene expression such as bacterial lysis (Chen *et al.*, 2009), *Bacillus* spore germination (Chen *et al.*, 2006), and *E. coli* protein expression (Chan *et al.*, 2007; Ramser *et al.*, 2007; Xie *et al.*, 2007).

Since Raman tweezers can undertake both measurement and manipulation at the single cell level, it has the potential to achieve Raman-activated cell sorting (RACS) (Huang *et al.*, 2009a; Lau *et al.*, 2008). A RACS system combined with microfluidic device is suitable to recover living cells with high yield. More importantly, microfluidic-device-based Raman sorting can be readily integrated with following-on process such as cell incubation, microreactors, chemical analysis, and PCR (El-Ali *et al.*, 2006; Wang *et al.*, 2005). Unlike fluorescence-activated cell sorting (FACS), which mainly differentiates cells using fluorescent signal which are introduced by either externally dye-labeling or genes encoded to fluorescent proteins, Raman sorting can have more criteria to identify and sort cells without any labeling or pretreatments. However, a key challenge to achieve a high throughput Raman sorting is that cellular Raman signals are usually very weak, which requires longer acquisition time (>1 s) for one SCRS, and advanced chemometrics may be needed for spectral deconvolution.

A few efforts to address this constraint have been made recently. To prove the concept, Huang and colleagues have introduced bacterial cells into capillary tubes, characterized single bacteria according to the ^{13}C-Raman shift with a 30-s acquisition time, and then isolated the single cells by breaking the capillary tubes (Huang *et al.*, 2009a). After isolation, the cells trapped in capillary tubes were recovered by centrifugation, and the genome of the isolated single cells has been amplified by multiple displacement amplification (Dean *et al.*, 2001; Huang *et al.*, 2009a). To reduce the photodamage that may be caused by high frequency laser, the authors used a 532 nm laser to measure cellular Raman spectra and an infrared laser (1014 nm) to manipulate cells (Huang *et al.*, 2009a). Microfluidic devices and Raman tweezers have also been combined to an integrated optofluidic platform, and this approach has been used to identify and to sort two different leukemia cells lines as a proof-of-principle of RACS (Lau *et al.*, 2008). However, in both studies (Huang

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et al., 2009a; Lau *et al.*, 2008) the Raman acquisition times for single cells were too long (30–120 s) to allow for high throughput cell sorting. It is possible to enhance the Raman signal using the SERS effect that has the potential to reduce SCRS down to subseconds because it is able to enhance Raman spectra by 6–14 orders of magnitude (Lombardi and Birke, 2009). A Raman spectral flow cytometry has been constructed to sort polymer microspheres (polystyrene/divinylbenzene) labeled with the SERS tags (Watson *et al.*, 2008, 2009); these authors have shown that the SERS effect has enabled Raman signals from an individual particle with acquisition time as short as 10 μs (Watson *et al.*, 2009). Although the SERS-tagged Raman spectral flow cytometry is only limited to sort particles, it is likely that a high throughput RACS can be achieved by labeling individual cells with the SERS tags because the SERS tags may provide more ''colorful'' criteria for sorting.

We believe that RACS would provide the opportunity to measure single bacteria in their natural habitat and potentially be applied to sort those bacteria cells according to their characteristic SRCS. Reducing Raman acquisition time would be a critical for RACS, and cells labeled with specific SERS tags could offer a possible solution.

V. SURFACE-ENHANCED RAMAN SCATTERING (SERS)

There is a range of scientific fields where the detection and characterization of small amounts of microbial biomass would be extremely advantageous. These include medical, food, and environmental microbiology, where the objective would be to obtain a phenotypic measurement from small numbers of bacteria or single cells, thereby avoiding the necessity of time-consuming cell culture. Also of concern is that the cell culture step, required for traditional microbiological analysis and most analytical approaches, can have a deleterious effect on organisms due to the selective conditions for growth imposed by the choice of culture medium. Furthermore, the biochemical or phenotypic fingerprint expressed by cells grown in culture as opposed to their environment of origin can differ dramatically, and therefore any results from *in vitro* analysis may not be translatable to the actual *in vivo* system being studied.

Much effort has been invested in the development of traditional analytical science techniques for microbiological analysis, including the mass spectrometry-based methods and vibrational spectroscopy. Mass spectrometry (MS) methods such as pyrolysis MS (PyMS), and the hyphenated MS approaches of gas chromatography and liquid chromatography MS (GC-, LC-MS), offer superb sensitivity at the ppb level, and with increasingly accurate MS detectors can be used for profiling of chemical components in complex bacterial samples (Cheung *et al.*, 2009;

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Fletcher *et al.*, 2006; Frank *et al.*, 2008; Hewavitharana *et al.*, 2007; Timmins *et al.*, 1998). However, the instrumentation is often very expensive to purchase and maintain; sample preparation and throughput is time-consuming; the instruments require highly specialized skills to operate, are generally not portable; and very difficult challenges in the deconvolution, analysis, and interpretation of results still remain.

By contrast, vibrational spectroscopies are less expensive in terms of equipment and operation, with greater sample throughput and fewer sample preparation requirements, and can be readily automated for routine analysis, and readily miniaturized. However, they are unable to compete with MS approaches in terms of limit of detection or specificity of chemical identification for the analysis of chemically complex samples. Although techniques such as FT-IR and Raman spectroscopy have been used to great effect in bacterial identification studies (Harz *et al.*, 2005; Naumann, 1985), including at the single cell level (Huang *et al.*, 2004, 2007b), there is a need to improve the sensitivity of these methods for more rapid analysis of trace amounts of biomass.

The SERS effect was first discovered in 1974 (Fleischmann *et al.*, 1974) and confirmed in 1977 by Jeanmaire and Van Duyne, and independently by Albrecht and Creighton in the same year (Albrecht and Creighton, 1977; Jeanmaire and Vanduyne, 1977). In these pioneering SERS experiments, enhancement factors of 10^5–10^6 were achieved from pyridine on the surface of a silver electrode. The general method to achieve a good SERS response is the ''direct'' or very close contact of samples and rough metal surfaces. There exist variations in preparation of rough metal surfaces and the method to put samples on them (for general review of SERS, see Moskovits, 1985; Otto *et al.*, 1992).

Ever since SERS has been investigated for the chemical fingerprinting of bacterial cells (Culha *et al.*, 2008; Jarvis and Goodacre, 2004a, 2008; Jarvis *et al.*, 2008; Naja *et al.*, 2007; Wilson *et al.*, 2007; Zeiri and Efrima, 2006; Zeiri *et al.*, 2002), an excellent review article on this subject has been written (Efrima and Zeiri, 2009). SERS can dramatically enhance the number of incident photons converted to inelastically scattered light in a Raman experiment, allowing for single-molecule detection under certain conditions (Nie and Emory, 1997). SERS utilizes a roughened coinage metal substrate (silver and gold are the most common metals used), often a colloidal suspension or patterned surface (Zhang *et al.*, 2005). It has been generally agreed that there are two mechanisms contributing SERS. The major one is electromagnetic enhancement due to the resonance of metal surface plasmon and incident radiation, and the other one is chemical enhancement caused by charge transfer between sample molecules and the metal substrate (GarciaVidal and Pendry, 1996; Moskovits, 1985; Otto *et al.*, 1992). Based on the hypothesis of the population pumping effect of SERS, SERS can enormously increase the Raman cross section to

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10^{-16} cm^2 per molecule which means an enhancement factor up to 10^{14} and the potential to detect a single molecule (Kneipp *et al.*, 1996). Several SERS experiments have confirmed its capability to probe a single molecule (Kneipp *et al.*, 1997; Nie and Emery, 1997), and a recent article provided more details about single-molecule detection by SERS (Kneipp *et al.*, 2006d).

In practice, SERS can be implemented together with other modifications of normal Raman scattering to suit specific purposes, for example, surface-enhanced resonance Raman scattering (SERRS) (Graham and Faulds, 2008; Johannessen *et al.*, 2007; Jordan *et al.*, 2005; Vosgrone and Meixner, 2005), surface-enhanced hyper Raman scattering (SEHRS) (Kneipp *et al.*, 1999a, 2006a), and tip-enhanced Raman scattering (TERS; a combination of SERS and atomic force microscopy) (Anderson, 2000; Neugebauer *et al.*, 2006; Pettinger *et al.*, 2004; Stockle *et al.*, 2000). Due to its high sensitivity, SERS can be used for the rapid detection of the intrinsic chemical signatures of microorganisms. Compared with fluorescence, which also gives a strong spectroscopic signal, SERS has many advantages because it provides molecular structural information as well as high signal-to-noise ratio.

Zeiri and coworkers reported that by using aqueous redox reactions of $AgNO_3$ and $NaBH_4$, silver particles can be deposited on bacteria surfaces or synthesized inside bacteria; the consequent SERS spectra show high similarity between different bacteria species; these spectra are mainly from cell surface and dominated by riboflavin's SERS signature (Efrima and Zeiri, 2009; Zeiri *et al.*, 2002). Jarvis and Goodacre (2004), for the first time, generated robust SERS signals from bacterial cells to allow for the differentiation of microorganisms at species and strain levels. Jarvis *et al.* (2008) recently reported that by using the ability of the bacterium *Geobacter sulfurreducens* to provide electron chain to extracellular metal ions reduction, silver particles can be deposited on cell surfaces, and by an unclear mechanism gold particles can be generated inside cells, SERS spectra were recorded from both silver and gold labeled cells. Premasiri *et al.* (2005) recorded SERS spectra of bacterial cells of different species by depositing cells on a gold nanoparticle-coated SiO_2 surface; Patel *et al.* (2008) followed the same method to obtain SERS spectra of bacterial cells and demonstrated its potential capability to rapidly identify pathogens. Kao *et al.* (2008) obtained reproducible SERS spectra from two bacterial strains on a gold/silver rough surface and demonstrated that differences in SERS spectra can be observed between Gram-positive and Gram-negative bacteria. Sujith *et al.* (2008) observed SERS spectra from silver nanoparticles-labeled living yeast cells and reported fluctuations in SERS spectra both spatially and temporally, while Shanmukh *et al.* (2006) obtained SERS spectra from different viruses deposited on a rough silver surface and achieved differentiation among species.

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SERS has brought many new techniques and insights into microbiology, and it is under rapid development (Jarvis and Goodacre, 2008). At the whole microorganism level, greatly enhanced Raman signals can provide a better basis for characterization of microorganisms than normal Raman spectra. SERS can be also used to dramatically enhance the signal from selected molecules in microorganisms and exclude interferences of other molecules, for example, one can design a selective bonding between metal nanoparticles or external Raman chromophores and biological molecules of interest; in other words, SERS provides an alternative labeling method to conventional procedures such as fluorescent labeling, isotope labeling, and so on. SERS has also shown its potential as an imaging technique (Keren *et al*., 2008; Kneipp *et al*., 2009; Vo-Dinh *et al*., 2005), and at the molecular biology level, as a single-molecule detection-capable method, SERS can act as the source of signals in DNA/protein microarray. Besides its ultrahigh sensitivity, SERS makes microarrays more ''colorful'': unlike fluorescent probes which often emit broad overlapping spectra, one has much more choices in SERS probes because vibrational spectra contain way more information than broad fluorescence spectra. This leads to more powerful multiplex assays, such as SERS-based DNA/protein microarray. SERS can also be used to investigate DNA/protein microarray and DNA sequencing (Chen *et al*., 2008; Faulds *et al*., 2008; Isola *et al*., 1998; Kneipp *et al*., 1998; Sheng *et al*., 1991; Vodinh *et al*., 1994).

The SERS technique has been applied to a range of microbiological detection and identification problems, and there is a great deal of interest in developing tailored SERS substrates for bacterial analysis (Alexander and Le, 2007; Chu *et al*., 2008). The main drawback to the approach is the difficulty in obtaining consistently reproducible spectra from samples, particularly those with great biochemical complexity such as microorganisms. It is well understood that SERS spectral profiles are greatly influenced by the absorbance geometry of analytes, and since this leads to both qualitative and quantitative variance in measurements (even from the same sample), there are still challenges to overcome in the analysis of these data.

VI. SINGLE CELL RAMAN SPECTROSCOPY AND MEASURING MICROBIAL METABOLIC POTENTIAL

In Section II, we have discussed that SCRS has hitherto been used to differentiate between bacteria at the species level and even between different strains. Raman microspectroscopy technique also has the potential to differentiate phenotypes among isogenic (genetic identical) populations. For example, SCRS of *Clostridium acetobutylicum* ATTC 824

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in early, middle, and late phases were different, indicating their metabolic and physiological states (Schuster *et al.*, 2000a). It has also been shown that the SCRS of *Acinetobacter baylyi* ADP1, *E. coli* DH5α and *Pseudomonas fluorescens* SBW25 are different in 8- and 22-h growth phases (Huang *et al.*, 2004). The analysis of SCRS of an opportunistic pathogen—*Staphylococcus epidermidis*—indicated the change of protein and nucleic acids components at different growth times (Neugebauer *et al.*, 2007). The authors demonstrated that SCRS analysis reflected changes in bacterial metabolism, revealed the molecular basis of biofilm formation, and therefore allowed for the understanding of the mechanism of bacterial pathogenesis (Neugebauer *et al.*, 2007). Bacteria in different metabolic and physiological states or organisms that have experienced stresses would be expected to contribute to significant changes in whole-cell chemical composition, and this could be detected using SCRS. It has also been shown that the SCRS of *Burkholderia xenovorans* LB400 changed after it was exposed to four different environmental pollutants: phenanthrene, dodecane, 3-chlorobiphenyl, and pentachlorophenol (Singer *et al.*, 2005), illustrating that bacteria have different stress response models to different chemicals. Raman microspectroscopy has been used to detect the variation of chemical profiles of *P. fluorescens* SBW25 when grown on different carbon sources and when transitioning into starvation (Huang *et al.*, 2007b). Single cell Raman spectra analysis shows that the lipids bands (750 and 1130 cm^{-1}) of *P. fluorescens* SBW25 after 9-day starvation have been significantly reduced in comparison to fresh cells (Fig. 5.3).

Due to the stochastic fluctuation of gene expression, individual bacterial cells within isogenic microbial population have shown phenotypic heterogeneity (Avery, 2006). Phenotypic heterogeneity is a survival strategy of microbes, enabling a rapid adaptation to a shifted environment without the need for genetic mutation (Fraser and Kaern, 2009; Kaern *et al.*, 2005). Because gene expression involves a series of binding and interaction of DNA, RNA, protein, and small molecules which is resulted from molecular random encounters, stochastic fluctuation is an intrinsic property of bacterial gene expression (Kaern *et al.*, 2005), and it has been shown that even a single-molecule event could switch a cell's phenotype (Choi *et al.*, 2008). The heterogeneity of phenotypes and metabolic and physiological states leads to different chemical profiles for individual bacteria within an isogenic population, and such difference can be detected by Raman microspectroscopy. By way of example, at the same growth phase, SCRS of *C. acetobutylicum* ATTC 824 were different although the cells were visibly identical, indicating the variations of gene expression of individual cells (Schuster *et al.*, 2000a,b).

At present, most studies on phenotypic heterogeneity and stochastic gene expression rely on cloning genes encoded with fluorescent proteins (e.g., green fluorescent protein or GFP) into cells and examined variation

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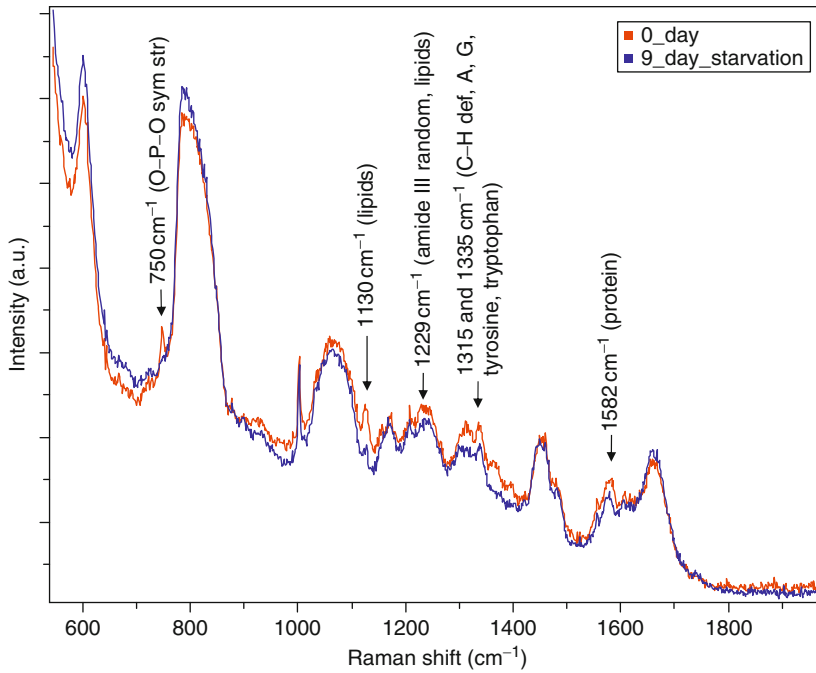

FIGURE 5.3 A comparison of single cell Raman spectra of *Pseudomonas fluorescens* SBW25 from fresh cultures (red or light spectrum) and after 9-day starvation (blue or dark spectrum).

of gene expression using fluorescent proteins as signals. SCRS could provide better signals than fluorescent signals to reveal bacterial spatial distribution because it indicates bacterial internal traits and does not need any external labeling. In addition, SCRS reflects bacterial metabolic functions; especially, the red-shifted SCRS arising from stable-isotope-labeling is able to indicate unambiguously bacterial incorporation of target substrates. Like fluorescent imaging, Raman microscopy can also produce chemical (pseudo-) images based on Raman spectra (Fig. 5.4). Figure 5.4 shows a Raman image of the distribution of ^{13}C- bacterial cells (green) among other ^{12}C- (red) cells on a glass surface (blue) with a high resolution of 0.1 μm, and this false color map clearly indicates the distribution of two types of cells (Fig. 5.4). Since this image contains 24,048 pixels in which each pixel contains 1022 Raman reading from 550 to 2160 cm^{-1}, the Raman image contains much greater information than FISH images. Using the same dataset, many different Raman images can be obtained according to the definition of Raman bands. A Raman image can also be produced using a number of characteristic Raman bands (Hermelink *et al.*, 2008; Rosch *et al.*, 2005). Using *Legionella bozemanii* L2165, *Bacillus cereus* ATCC 10987, and *Bacillus thuringiensis* DSM 5815 as model systems,

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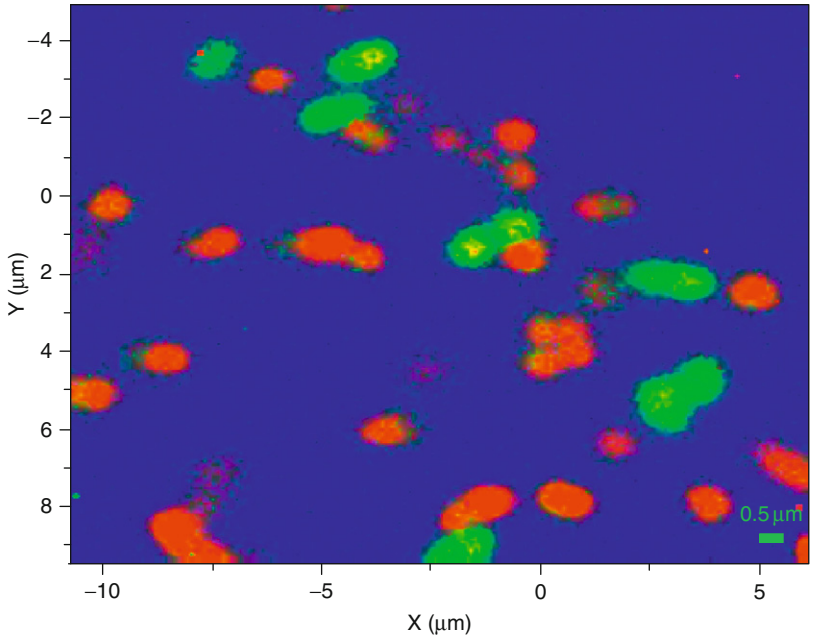

FIGURE 5.4 A Raman image of bacterial community on a glass surface; ^{13}C-cells: green or bright and ^{12}C-cells: red or grey background: blue (1s $\times$1 per pixel, each pixel contains 1022 Raman pixels from 550 to 2160 cm^{-1}, 0.1 μm step size, 24,048 pixels). A LabRAM HR LabSpec5 (Horiba Scientific, UK) was employed to generate these Raman data.

Hermelink *et al*. used Raman microspectroscopy to interrogate and visualize Poly-*b*-hydroxybutyric acid (PHB) (at 1726 cm^{-1}) and Ca-DPA (at 1018 cm^{-1}) within single cells and their distribution in the isogenic populations (Hermelink *et al*., 2008). This work provides direct evidence of the spatial heterogeneity and variation of gene expression of single cells within the isogenic populations (Hermelink *et al*., 2008). A key advantage of Raman image over FISH image is that it uses bacterial internal chemical information to generate an image without the need of prior knowledge of DNA sequence and external labeling. However, the challenge is that a Raman image currently takes longer time, usually a few hours while a FISH image only a few seconds. Recent hardware/software developments do now allow ultrafast confocal Raman mapping—with acquisition times down to sub-μs per pixel it is possible to acquire detailed Raman images in the matter of seconds or minutes. However, such techniques are still dependent on samples which offer relatively strong signals—in this respect single cell bacteria remain less than ideal.

Unlike fluorescent labeling techniques that usually employs fluorescent proteins (e.g., GFP, RFP, and YFP) or dyes as marks to report the gene

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expression, the Raman images can be generated from characteristic bands of SCRS without the need of any marker genes or dyes. Raman images generated from entire spectral SCRS profiles enable a multicriteria, non-invasive, real-time, and quantitative monitoring of molecular structures, intermolecular interactions, and metabolic dynamics of single cells. SRCS provides label-free global chemical profiles of single cells which potentially bridges genetic–protein–metabolism and provides fundamental new insights in individual-level analyses of composition, competition, function (e.g., drug resistance), biochemistry, and gene-expression within isogenic populations.

VII. RAMAN SPECTRA DATA ANALYSIS

SCRS is complex, containing Raman signals from all molecules within a single cell. Since Raman measures vibrations of molecular bonds and biological molecules share some bonds (e.g., C–H, C=O, etc.), the Raman spectra of different biological molecules overlap thereby presenting difficulties in data interpretation. As mentioned in Section I, data interpretation is critical to the application of Raman microspectroscopy. Table 5.1 gives a summary of the main Raman bands assigned to specific biological molecules. A reference database of Raman spectra of pure biological molecules is available online to download http://www.analchem.ugent.be/Raman/Html/refdata.php (De Gelder *et al.*, 2007a).

With Raman spectroscopy of microorganisms, the analyst is typically interested in quantifying biochemical changes in a dynamic system, or classifying different samples based upon their phenotype. A single Raman spectrum usually consists of many hundreds of data points representing the intensity of inelastic scattering across the spectrum. Therefore, the data are multivariate and the main data analysis themes, reported widely in the literature, are different flavors of multivariate linear regression or discriminant analysis.

There are a wide range of multivariate and machine learning classification algorithms, and it is not possible to cover all the permutations here. Different flavors of algorithm may satisfy the particular needs of the researcher for certain data analyses. In exploratory analysis or outlier detection unsupervised methods can be used, that rely on natural variance in the data to reduce the dataset from what may be many thousands of measurements to a handful of latent variables. This means that the derived model scores can be visualized on biplots or 3D plots which allow for simple outlier detection, or in some cases observation of separation between the different classes studied. Examples of this type of algorithms are principal components analysis (PCA; Jolliffe, 1986), independent component analysis (ICA; Lee, 1998), and factor analysis (Chatfield and

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Collins, 1980). Another approach is unsupervised hierarchical cluster analysis (HCA; Manly, 1994): this uses distance-based metrics to offer valuable insights into the relationships between dependent variables (samples) and independent variables (measurements) simultaneously, as has been shown to great effect in gene expression and transcriptomic studies (Eisen *et al.*, 1998). The additional benefit with this approach is that the model output can be visualized with a single two-dimensional plot, called a dendrogram, which depicts the distance between clusters.

It is often the case that these unsupervised approaches are unable to provide an adequate solution to a regression or classification problem from Raman data, when measurements are acquired from a complex microbiological system. This is due to the fact that there may only be very subtle spectral changes occurring that describe the effect being studied, and these are most often masked by natural variance in the data arising from general biological variability. In addition, the execution of an experiment may introduce nonbiological variance, which means that the variation between samples across the dataset will not necessarily be correlated with the biological differences that one intends to model, and this can lead to false discoveries (Ioannidis, 2005; Ioannidis *et al.*, 2001; Todd, 2006; Wacholder *et al.*, 2004). Therefore, powerful supervised multivariate methods can be used that require a model training process. In classification problems using Raman data, the following are used often: discriminant function analysis (DFA; Manly, 1994), canonical variates analysis (CVA; Krzanowski, 1988), partial least squares discriminant analysis (PLS-DA; Wold *et al.*, 2001), and classification and regression trees (CART; Hwang and Weng, 2000). With DFA and CVA a data reduction step is usually required because these algorithms are unable to handle collinearities (multiple variables accounting for the same variance within the data; Dixon and Massey, 1983; MacFie *et al.*, 1978). Therefore, PCA can be applied to derive a series of uncorrelated latent variables (principal components) as inputs to these supervised algorithms (Goodacre and Berkeley, 1990). In calibration problems where a quantitative output is needed (e.g., level of toxin exposure to a bacterium), partial least squares regression (PLSR) and support vector regression (SVR) are used frequently (Martens and Næs, 1989).

One important aspect of supervised learning is the need to apply rigorous crossvalidation procedures to the model (Brereton, 1992; Broadhurst and Kell, 2006; Goodacre *et al.*, 2007). This can be achieved through a number of approaches, but all methods essentially use a process of projecting samples that are independent from the training data in to the model, following which an objective statistical assessment of the model accuracy can be made. Crossvalidation is critical to the avoidance of model overtraining, where too much information specific to the training data is used to build the model, and it is therefore impossible to

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generalize (i.e., used for predictions on blind samples; Seasholtz and Kowalski, 1993).

Multivariate analysis (MVA) methods often allow for the interpretation or ''mining'' of data through spectral weights or loadings to derive a shortlist of spectral features that contribute most to the derived model, and therefore can be used to obtain knowledge from the data that is pertinent to the problem being addressed (Duda *et al.*, 2001; Hastie *et al.*, 2001). However, these figures can often be difficult to interpret due to their complexity; and alternative data mining/inductive reasoning algorithms are often used to mine biochemical information from the data. For feature selection from Raman data, evolutionary algorithm (EA) computation methods, such as genetic algorithms (GAs; Bäck *et al.*, 1997; Goldberg, 1989; Mitchell, 1995; Fig. 5.5) and genetic programming (GP; Holland, 1992, 1998), are very powerful tools, that can be used in conjunction with one or other of the supervised learning analysis methods mentioned earlier, to select variable subsets that are most important for classification. These evolutionary search algorithms can be used to find combinations of only a handful of variables that provide a good solution, rather than the best solution, to a classification problem. They are described as heuristic algorithms, which means they are good at finding a range of suboptimal solutions, rather than the global optimum (Holland, 1992). Apart from the obvious reward of gaining useful knowledge from Raman data, evolutionary approaches are very efficient search algorithms for feature selection and have advantages in multivariate modeling. Many of the measured variables will not be relevant, and it is known from the statistical literature that better (i.e., more robust) predictions can often be obtained when only the most relevant input

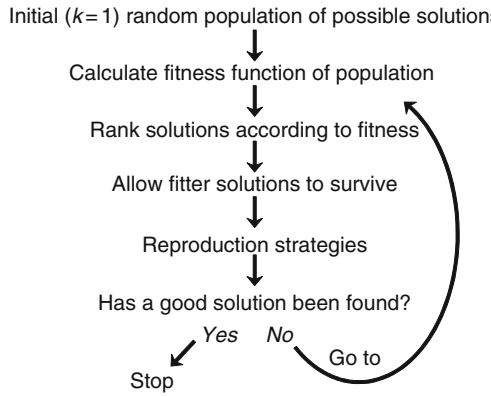

FIGURE 5.5 The evolution procedure employed by a GA. The criterion for a good solution (fitness) is usually based on setting a threshold error between the known target and the models response.

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variables are considered (Seasholtz and Kowalski, 1993), that is, that ''parsimonious'' models tend to generalize better (Goodacre, 2005). We have used GAs for the analysis of spectroscopic data (Jarvis and Goodacre, 2005) and have generated a freely available computation package for chemometrics (Jarvis *et al.*, 2006) which can be downloaded via (http://pychem.sourceforge.net/).

VIII. CONCLUSION AND FUTURE PROSPECTS

Raman microspectroscopy is a very useful physicochemical tool to the study of microorganisms. The advantages of this approach are listed as follows:

1. It is a noninvasive technique. It measures samples without damaging them (Huang *et al.*, 2004; Schuster *et al.*, 2000a,b; Xie and Li, 2003);
2. It requires small samples and is able to focus on a volume as small as 1 μm^3, which enables the measurement of microbes at single cell level without the need of cultivation (Huang *et al.*, 2004, 2007c, 2009b; Rosch *et al.*, 2005; Schuster *et al.*, 2000a);
3. Raman tweezers can be used to manipulate and measure bacterial cells in an aqueous environment as water contributes minimal bands for a Raman spectrum (Carey, 1982; Huang *et al.*, 2004, 2007c; Schuster *et al.*, 2000a);
4. It measures intrinsic molecular information of cells *in vivo* without the need of external labeling or extraction (Huang *et al.*, 2004, 2007c; Petry *et al.*, 2003). A Raman image could provide the information of cellular phenotypes and gene expression without introducing gene cloning or external labeling;
5. It is able to acquire more than 1000 Raman bands from a single cell in 1–30 s over a Raman shift range of 500–2000 cm^{-1}, which provides comprehensive information of cellular molecular compositions, structures, and physiological states (Huang *et al.*, 2004, 2007a,b,c);
6. It can be extremely sensitive, able to discriminate closely related bacteria, or to detect even single molecule and single nanoparticle with the use of the SERS effect (Jarvis and Goodacre, 2004; Kneipp *et al.*, 2006a).

In conclusion, the Raman spectra of bacterial cells (and indeed fungal and those from higher organisms) can be regarded as their chemical fingerprints, and such ''whole organism fingerprints'' (Goodacre *et al.*, 1998) can be used to classify bacteria in terms of species and physiological states by multivariate statistical analyses. Raman spectroscopy is a powerful evolving technique and offers great analysis potential to microbiologists in environmental, food-processing, and clinical laboratories.

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