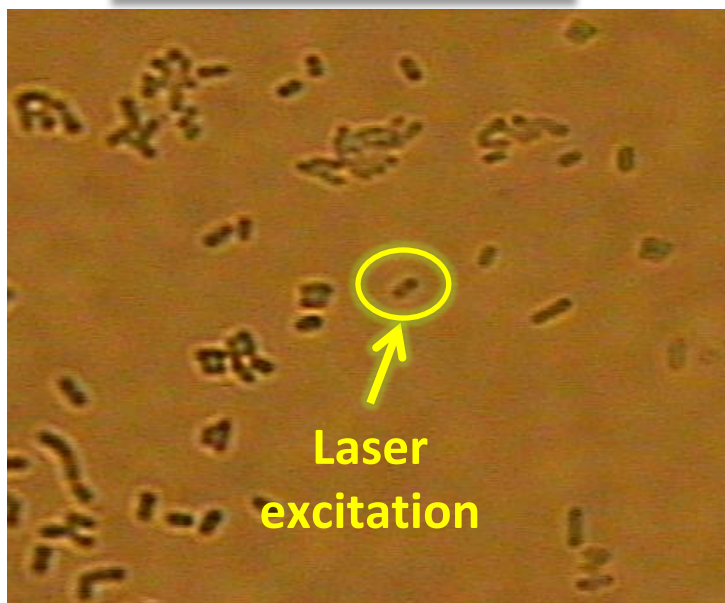


Microbial community

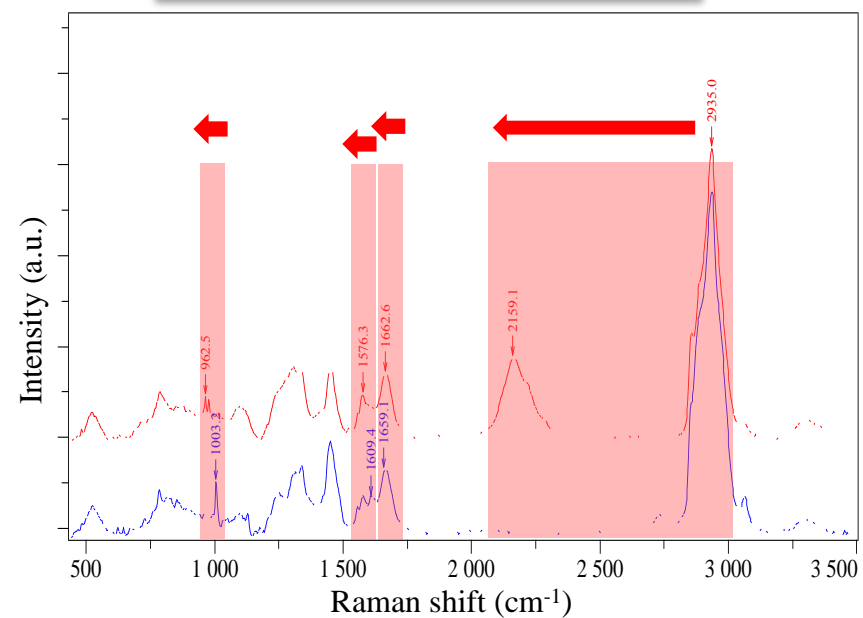


Laser
excitation

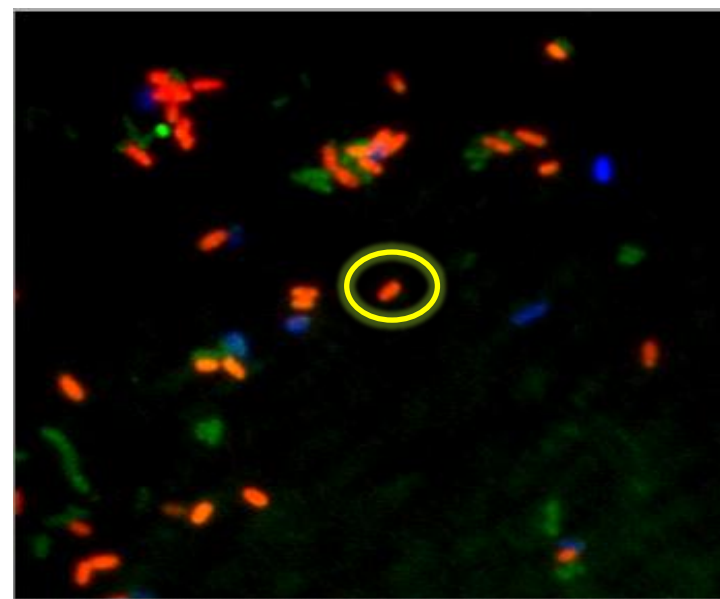
Single cell
metabolic activity

Single cell identity
and spatial position

Raman-SIP revealing
phenotypic profile



FISH probing
phylogenetic identity



Highlights:

1. Single cell Raman spectra provide fast, non-destructive, label-free, and intrinsic biochemical profiles of single microbial cells
2. Some bands of single cell Raman spectra shift if cells are labeled with stable isotopes such as ^{13}C , ^{15}N and ^2H .
3. Combined with fluorescence *in-situ* hybridization (FISH), isotope-induced Raman band shifts link microbial metabolic activity and cell identity at the single cell level.

Single Cell Stable Isotope Probing in Microbiology using Raman Microspectroscopy

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Key Words: single cell, Raman spectroscopy, stable isotope probing, deuterium, metabolism

Abstract

Microbial communities are essential for most ecosystem processes and interact in highly complex ways with virtually all eukaryotes. Thus, a detailed understanding of the function of such communities is a fundamental prerequisite for microbial ecologists, applied microbiologists and microbiome researchers. Using single cell Raman microspectroscopy, biochemical fingerprints of individual microbial cells can be obtained in a fast and nondestructive manner. If combined with stable isotope probing (SIP), Raman spectroscopy can directly reveal functions of single microorganisms in their natural habitat. This review provides an update on various SIP-approaches suitable for combination with different Raman scattering techniques and illustrates how single cell Raman SIP can be directly combined with the omics-centric analysis pipelines generally applied to investigate microbial communities.

Introduction

Understanding the function of microbial communities is a major challenge due to their often enormous diversity and the complexity of interactions occurring between community members and between them and the abiotic and biotic environment. Today, such communities can be directly studied using meta-omics approaches [1] and if combined with DNA-, RNA-, protein- or lipid-SIP, functional properties of community members are directly revealed [2-5]. While many fascinating discoveries were obtained with this toolbox, it (i) often fails to decipher functional differences between closely related community members, (ii) provides no information on functional differences between single cells of the same population, (iii) does not link genomic or functional information to individual cells as the basic unit of life and (iv) masks all spatial information on the occurrence of community members. The recent advent of single cell genomics [6,7] overcomes some of these limitations, although (i) obtaining closed genomes from single microbial cells is generally still not possible, and (ii) only a fraction of single-cell genomes can be recovered from a specific sample [8*]. Furthermore, transcriptomic and proteomic studies based on single microbial cells are still in their infancy [9] or (yet) not feasible. Consequently, SIP-techniques on single cells fill a major void in the toolbox of microbiologists as they enable direct observations of functional properties of individual microbial community members.

Isotope probing and simultaneous identification of single microbial cells within complex communities is possible since 1999 when the combination of fluorescence *in situ* hybridization (FISH) and microautoradiography was invented [10]. This approach is widely used in microbial ecology, but its dependency on radioisotopes limits substrate labelling options and applications. Furthermore, this technique is destructive and does not enable follow-up studies on cells with a certain function. During the last years single cell probing

with stable isotopes became possible by analyzing microbial communities after incubation with labelled substrates with either secondary ion mass spectrometry (SIMS) or Raman microspectroscopy [11].

Different Raman techniques exist. For microbiological applications most frequently spontaneous Raman spectroscopy is applied. This technique measures the frequency change of incident photons due to energy exchange between molecular bonds and incident photons. A spontaneous Raman spectrum is independent from the wavelength of the incident photons, and typically presented in wavenumbers, which are the differences of inverse frequencies of the energy-changed photons and the inverse frequency of the incident photons. As a Raman spectrum reports vibrational, rotational, and other low-frequency modes in a system which is defined by intrinsic properties of molecules, it represents a chemical fingerprint of a molecule or a system. Resonance Raman (RR) occurs when the frequency of the incident photons nearly coincides with the electronic excitation frequency of a molecule. Under RR conditions, the intensities of Raman bands from the molecule are selectively enhanced by a factor of 10^3 to 10^5 . Surface-enhanced Raman scattering (SERS) also offers strongly enhanced Raman signals for those molecules being in very close spatial contact with rough metal surfaces or plasmonic nanostructures. For a more encompassing overview on the basic principles of these and other Raman techniques we refer the reader to reference [12]. Raman microspectroscopy offers a label-free and non-destructive way to obtain a chemical fingerprint of single microbial cells in their natural habitat [12] (Fig. 1). The spectroscopic analysis can be performed in water and requires virtually no sample preparation. The obtained single cell fingerprints enable direct identification of many storage compounds, pigments and other interesting compounds [13-17] (Fig. 1). However, identification of microbes living in their natural habitat based on these fingerprints is in most cases not possible, as the Raman fingerprint of a cell, indicating its chemical composition, changes with changing

environmental conditions and is dependent on the growth stage of the analyzed cell. In this context it is important to keep in mind that Raman microspectroscopy can be directly combined with FISH and thus obtaining Raman spectra of microbes identified by rRNA-targeted nucleic acid probes within their habitat is possible [18,19] (Fig. 1). While the chemical composition of a cell can already contain hints to its function, more direct insights into functional properties can be obtained by directly combining stable isotope probing with ^{15}N -, ^{13}C - and ^2H -isotopes and Raman microspectroscopy [17-22] (Fig. 1).

SIP-Raman theory

Raman spectra are generated due to inelastic light scattering when incident light and molecular bonds experience energy exchange. Taking one vibrational model as an example, diatomic molecules are thought as two masses (m_1 and m_2) on a spring, and they have a reduced mass μ calculated by the equation $\mu = \frac{m_1 m_2}{m_1 + m_2}$ [23]. The wavenumber of the bond vibration ($\bar{\nu}$, cm^{-1}) is described by the equation $\bar{\nu} = \frac{1}{2\pi c} \sqrt{\frac{k}{\mu}}$, where c is the speed of light (ms^{-1}), k is the force constant of a diatomic bond (Nm^{-1}). Therefore, the wavenumber is inversely proportional to the square root of the reduced mass. When an atom is replaced by its heavier isotope, μ increases, leading to a smaller wavenumber. Taking deuterium substitution of hydrogen in C-H bond as an example, μ is nearly doubled, leading to a new to original wavenumber ratio being 0.73 (for C-D replacement of C-H). In Raman-SIP, isotope-dependent shifts of bands in single cell Raman spectra (SCRS) can be used as indicators for isotope incorporation after incubation experiments with an isotope-labeled substrate, whilst such shifts cannot be observed in cells from control experiments with an unlabeled substrate. The control experiments are essential when working with microbial communities in order to exclude that an unknown compound in an unlabeled cell has a Raman band that is used as an indicator for isotope incorporation. Interestingly, band shifts caused by isotope incorporation

in microbial cells can also be observed with Fourier transform infrared (FT-IR) spectroscopy [24], but this method cannot be performed in the presence of water, has less distinguishably sharp spectral bands, lower spatial resolution and cannot be directly combined with FISH.

To date, most of the studies using SIP-Raman focused on ^{13}C , ^{15}N and ^2H to replace their primordial isotopes (^{12}C , ^{14}N and ^1H) respectively, and these applications will be discussed in this review in detail. Oxygen (O) and sulfur (S) isotopes can be potentially applied to SIP-Raman, too, but to the best of our knowledge this has not been examined yet.

^{13}C -labeling for Raman-based single cell isotope probing

^{13}C -labeled compounds are most widely applied in SIP experiments with microbes and ^{13}C -incorporation into the biomass of a microbial cell can be readily observed via Raman microspectroscopy. Huang *et al.* [20] showed that Raman spectra from individual *Pseudomonas fluorescens* cells grown with ^{13}C -glucose as the sole carbon source contained bands at lower wavenumbers if compared to the spectra from unlabeled cells. The most prominent band shift appeared for the band at 1001 cm^{-1} in unlabeled cells, which is assigned to the symmetric benzene ring breathing mode (e.g. from phenylalanine), that shifted to 967 cm^{-1} in cells cultured with 100% ^{13}C -glucose. A more complete list of Raman band shifts after ^{13}C incorporation into cellular biomass is provided in Table 1 and a typical Raman shift of SCRS caused by ^{13}C incorporation is shown in Fig. 2A. The ratio of the 967 cm^{-1} to the 1001 cm^{-1} band increases with increasing amounts of incorporated carbon allowing to quantify the degree of labeling of the analyzed cells. Importantly, these SIP analyses are also possible with paraformaldehyde-fixed cells for FISH and can thus directly be combined with the identification of microbes by fluorescently labeled probes [18]. For example, the SIP-Raman-FISH technology was applied to a naphthalene contaminated groundwater sample and

an uncultivated *Acidovorax* sp. was identified to play a key role in naphthalene degradation *in situ* [18,19]. Furthermore, using the above described band shift, it was demonstrated using incubation experiments with ^{13}C -phenylalanine that elementary bodies of chlamydia are metabolically active outside of their host cells [25]. This finding obtained by Raman-based single cell SIP broke a long-lasting hypothesis in chlamydial biology that stated that the extracellular life stages of these intracellular bacteria are spore-like and metabolically inactive. This example also illustrates another important point for researchers interested in using single cell Raman-SIP in environmental samples. Many microbes can take up amino acids including phenylalanine from the environment. If phenylalanine is not synthesized *de novo* (and not applied as labeled derivative as in the above mentioned chlamydia experiment) by a bacterial cell then its 1001 cm^{-1} band will not shift in the presence of a ^{13}C -labeled substrate like glucose or cellulose despite the fact that it is actively metabolizing the substrate [26*]. In such cases other ^{13}C -induced band shifts have to be used for Raman-based SIP. More generally, this example illustrates that like all other SIP techniques, Raman-SIP is dependent on incorporation of the stable-isotope from the labeled substrate into the biomass.

^{13}C -incorporation into cells can not only be observed via monitoring the symmetric benzene ring breathing mode. For example, in yeasts SIP experiments have been performed using shifts of a band at 1602 cm^{-1} (position in unlabeled cells) originating predominantly from the $\text{C}=\text{C}-\text{C}=\text{C}$ symmetric stretch of the 5,7-diene structure in ergosterol [27], but other band shifts have also been exploited [28]. In *C. elegans* the 1001 cm^{-1} phenylalanine band is masked by another band of unknown origin and could thus not be used for quantitative SIP predator-prey experiments with ^{13}C -labeled *E. coli* offered as food to the nematode [29*]. However, this problem was solved by exploiting the Raman band at 747 cm^{-1} of cytochrome C (suggested to originate from thymine in the original paper) in the eukaryotic cells for quantifying ^{13}C -incorporation [29*]. Cytochrome c is abundant in the mitochondria of eukaryotic cells and

possesses a few strong and unique Raman bands at 747, 1128, 1311 and 1583 cm^{-1} if excited by a 532-nm laser due to Raman resonance scattering [30,31]. Furthermore, carotenoids of photosynthetic bacteria also show due to RR at a 532 nm excitation wavelength three very strong and characteristic bands that shift proportional to the ^{13}C -content of the cells. This effect can be exploited for identifying and quantifying the activity of phototrophs in samples incubated with $^{13}\text{C}\text{-NaHCO}_3$ via Raman microspectroscopy [17]. ^{13}C -single-cell SIP using Raman microspectroscopy has a detection limit of about 10% $^{13}\text{C}/^{12}\text{C}+^{13}\text{C}$ of a cell of interest if based on shifts of the symmetric benzene ring breathing mode [18] or carotenoid RR bands [17]. Due to the strong signals of the carotenoid RR bands SIP of phototrophs requires only very short measurement times enabling high-throughput counting of active cells in microfluidic chambers [32]. Recently, RR-SIP has also been exploited by targeting shifts of the cytochrome c bands in *Geobacter metallireducens* in order to observe the ^{13}C -uptake of single bacterial cells [33**]. In the same study, ^{13}C -incorporation into *E. coli* biomass could be observed by exploiting the very sensitive SERS technique. In the SERS spectra a band at 733 cm^{-1} that was assigned to adenine or adenine-containing molecules was shifted to 720 cm^{-1} in ^{13}C -labeled *E. coli* cells [33**]. More generally, SERS has a few interesting characteristics that renders it attractive for microbial ecology studies [34,35]. It selectively enhances some Raman signals dependent on the properties of the applied nanoparticle and incident laser wavelength and this effect only happens at short distances within 1-4 nm range around nanoparticles. Furthermore, SERS might also be able to reduce the often problematic fluorescence interference to Raman signals (unpublished data). On the other hand, obtaining reproducible SERS signals with bacterial cells is very challenging and spectral assignment for spontaneous Raman bands may be inapplicable to SERS.

¹⁵N-labeling for Raman-based single cell isotope probing

Nitrogen consists of two stable isotopes, ¹⁴N and ¹⁵N, and its fourteen radioactive isotopes are all short-lived, with the longest half-life being 9.965 min. Therefore, radioisotope-based techniques are not suitable for N metabolism analysis. In Raman analysis, the red-shift caused by ¹⁵N substitution is not as large as that caused by ¹³C-replacement (Table 1) [21], and most red-shifts of the Raman bands are related to nucleic acids. [21,24,36] (Fig. 2B). Since Raman shifts caused by ¹⁵N replacement are buried in other Raman bands, it is not trivial to unequivocally identify ¹⁵N-labeled cells in complex communities. It is thus not surprising that till today no reports on this have been published. However, we recently obtained promising results by combining the SERS method [37] with ¹⁵N labeling of microbial cells causing shifts of band at 728 cm⁻¹ that was assigned to adenine ring breathing (pure adenine-containing compounds were used as controls). Interestingly, a linear correlation was observed between the extent of the band shift and the degree of ¹⁵N-labeling of the cell (Fig. 3).

Recently, Muhamadali et al. [24] compared the Raman spectra of *Escherichia coli* biomass obtained under different ratios and combinations of ¹³C/¹²C-glucose and ¹⁵N/¹⁴N-ammonium chloride, as the sole carbon and nitrogen sources, respectively. Multivariate analyses of the spectra revealed clustering patterns directly correlated with the ratio of the isotopically labeled content of the medium. However, the Raman spectra of cells grown with combinations of both ¹³C and ¹⁵N were dominated by the ratio of ¹³C rather than both isotopes. Furthermore, it should be noted that most analyses reported in this publication were not performed on the single cell level but obtained from bulk measurements of concentrated biomass and that all data were obtained from cell harvested at the same growth stage, minimizing Raman spectra variations caused by other factors than incorporation of stable isotopes. Thus, double-labeling Raman-SIP studies using ¹⁵N and ¹³C-labeled substrates for

analyzing function properties of microbial cells in complex communities is probably not feasible without further improvements.

²H-labeling for Raman-based single cell isotope probing

²H (D)-labeling is often used to trace the metabolism of lipids and their storage patterns in eukaryotic cells via Raman microspectroscopy and Raman imaging as the signals of CD stretching vibrations between 2000 and 2300 cm⁻¹ can be easily detected by spontaneous Raman spectroscopy due to their location in an otherwise silent region [38] of the spectrum of cells [39,40]. Using Coherent anti-Stokes Raman Scattering (CARS) the sensitivity of imaging –CD₂ stretching vibrations can be enhanced by several orders of magnitude compared to conventional Raman microscopy enabling monitoring of lipid metabolism in living eukaryotic cells with high temporal and spatial resolution [41]. In CARS spectral focusing of the laser energy into a single Raman band of interest occurs, but the resulting lack of spectral information can be overcome by combining CARS imaging with confocal Raman spectrometry using the same picosecond laser [42]. Different deuterated substrates have been used to monitor metabolic features of eukaryotic cells. For example, recently D-glucose labeling and Stimulated Raman Scattering (SRS) was applied to monitor lipogenesis in single living cells with high spatial and temporal resolution [43**]. Similarly, proteins can be metabolically labeled and detected using Raman spectroscopy by incubating cells with deuterated phenylalanine causing a shift of the 1001 cm⁻¹ phenylalanine-band to 959 cm⁻¹ [44]. Combination of labeling with deuterated amino acids and SRS imaging even allows generating spatial maps illustrating the ratio between newly synthesized and total proteins in mammalian cells [45*]. Deuterium-labeled substrates were also successfully used for Raman-based single cell SIP of microbes [22**,33], but it should be noted that depending on the

labeled substrate offered and the metabolic pathways present in the target organisms, not all compounds in the cell will become deuterated. For example, growth of *Geobacter metallireducens* GS-15 with ^2H -acetate did not cause any shift of *cytochrome c* Raman bands [33**].

Studies exploiting isotope-labeled substrates for functional analyses of microbial communities have two important limitations. Firstly, isotopically labeled derivatives of many substrates of interest are very expensive or not even commercially available. Secondly, addition of labeled substrates changes the composition of the natural substrate pool, and thus might change the activities of community members. SIP studies using labeled water [5,46] are helpful to circumvent these problems, but were until recently not explored for single cell isotope probing. In 2015 Berry *et al.* [22**] demonstrated using deuterated water (D_2O) that the incorporation of deuterium into the biomass (mainly during the reductive steps of lipid biosynthesis [47]) of active bacterial and archaeal community members can be unambiguously detected via the newly-appearing C-D signature Raman band (2040 - 2300 cm^{-1}). A typical Raman shift of SCRS caused by ^2H (D) incorporation is shown in Fig. 2C. This Raman signature band is already detectable before cell division has been completed and occurs in fast growing cells after 20 minutes. This method, which can also be combined with FISH [22**], thus allows microbiologists to recognize and identify all metabolically active members of a microbial community on the single cell level. Ideally, samples are concentrated before addition of heavy water in order to finally reconstitute the natural substrate concentrations. Furthermore, heavy water labeling can also be exploited for hunting microbes metabolizing any substrate of interest. For this purpose, D_2O is added in combination with the unlabeled substrate and those microbes (identified by FISH) showing increased deuterium incorporation compared to a control experiment without added unlabeled substrate represent cells stimulated by the added substrate. The suitability of this approach for investigating

complex microbial communities was demonstrated by identifying degraders of several complex carbohydrates in mice cecum microbiota [22**] and quantifying active microbial cells in native as well as glucose-amended soils [26*].

Given the clear Raman band shifts caused by ^{13}C or deuterium incorporation into the biomass of microbial cells, it seems likely that double-labeling studies with these isotopes to investigate microbial community members will be possible in the future.

Combining SIP-Raman with Single Cell Genomics

Due to its non-destructive nature, single cell Raman-SIP can also be used to sort microbial cells with a certain function from a complex microbial community for subsequent genomic analyses. After an initial proof of principle experiment with ^{13}C -labeled cells from pure cultures [48], Berry *et al.* sorted individual deuterated cells from a mouse cecal microbial community after stimulation with unlabeled glucosamine or mucin in the presence of heavy water using an optical tweezers and subsequently successfully amplified their genomes using multiple displacement amplification [22**]. This approach is widely applicable, but still has a relatively low throughput. To overcome this limitation, we are currently developing an automated microfluidic-based Raman-SIP cell sorter for targeted high-throughput genomic analyses of microbial cells labeled with stable isotopes.

Conclusions and Outlook

Raman-SIP using ^{13}C -labeled or deuterated substrates including heavy water can now routinely be applied for functional analyses of microbial community members. Its nondestructive manner offers ample opportunities for sorting of cells with a desired function

and enhancing standard single-cell genomic or metagenomic pipelines. And if combined with FISH, it is often an attractive alternative for more tedious FISH-microautoradiography [10] or FISH-NanoSIMS [11] analyses.

Nevertheless, there is still room for improvement. Undoubtedly, the biggest challenge is that spontaneous Raman signals are naturally weak, especially on the single cell level, causing a need for relatively long measurement times per cell (but still generally no more than some seconds). Raman signal enhancement can be achieved by advanced Raman techniques like RR, CARS, SRS and SERS, but while progress has been made in applying these techniques for measuring labeled cells, these techniques are either not yet widely available (CARS, SRS) or generally applicable (RR, SERS) especially in complex samples containing numerous impurities generating high background Raman signals.

Raman signal enhancement is also required to increase the throughput of automated Raman-SIP cell sorters or for Raman imaging approaches in microbial consortia. Furthermore, the detection limit for stable isotopes in a single microbial cell as measured by single cell Raman-SIP is comparable with the one of bulk DNA or RNA stable isotope probing, but this method is significantly less sensitive than (destructive) NanoSIMS measurements. Another challenge is that Raman bands of microbial cells are often assigned to several compounds simultaneously (an overview of Raman band assignment for biological molecules is provided in supplementary Table S1). While this does not hamper the detection of labeled cells, it complicates interpretation of metabolic pathways contributing to isotope incorporation into cellular macromolecules. Further experimental and database comparison work is needed to improve band assignments. But even without these refinements, single cell Raman-SIP is already a valuable amendment of the toolbox of microbiologists interested in revealing the function of microbial cells directly in their natural environment.

Acknowledgement

We acknowledge support from EPSRC (EP/M002403/1), NERC (NE/M002934/1) and BBSRC sLoLa, (BB/M000265/1) in the UK and NSFC (31400436), CAS (XDB15040100) in China. MW was supported by an ERC Advanced Grant (NITRICARE, 294343) as well as by the Emerging Technologies Opportunity Program, which is supported by the Office of Science of the US Department of Energy under Contract DE-AC02-05CH11231.

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Table 1 Major Raman bands shifted in microbial cells due to isotope substitution. It should be noted that in isotope labeling experiments depending on the type of substrate and the metabolic pathways expressed in the analyzed cell not all shifts reported here must always be detectable. Band shifts will only occur if the isotope is actually incorporated into the respective molecule within the cell.

Raman bands of unlabeled cells (cm ⁻¹)	Isotope	The extent of Raman shift (cm ⁻¹)	Assignment	References
787	¹³ C	-17	O- P- O breathing, Cytosine, uracil	[20]
1001-1003	¹³ C	-37	Symmetric ring breathing mode of phenylalanine	[20]
1247	¹³ C	-14	Amide III	[20]
1342	¹³ C	-15	CH stretching of adenine	[20]
1578	¹³ C	-47	Ring stretching of guanine and adenine	[20]
1663	¹³ C	-35	C=C of unsaturated lipid, amide I	[20]
729	¹⁵ N	-14	Adenine ring breathing	[21]
787	¹⁵ N	-3	O- P- O breathing, Cytosine, uracil	[21]
1174	¹⁵ N	-10	C-H in-plane bending of tyrosine or phenylalanine	[21]
1247	¹⁵ N	-14	Amide III	[21]
1342	¹⁵ N	-19	CH stretching of adenine	[21]
1480	¹⁵ N	-10	Purine base of guanine and adenine	[21]
1578	¹⁵ N	-7	Ring stretching of guanine and adenine	[21]
1001-1003	² H	-42	Symmetric ring breathing mode of phenylalanine	[44]*
~2850	² H	-745	CH stretching of lipid	[41]**
2800-3100	² H	-800	CH stretching of lipid and protein	[22]

* D-phenylalanine rather than D₂O was used;

**measured by CARS

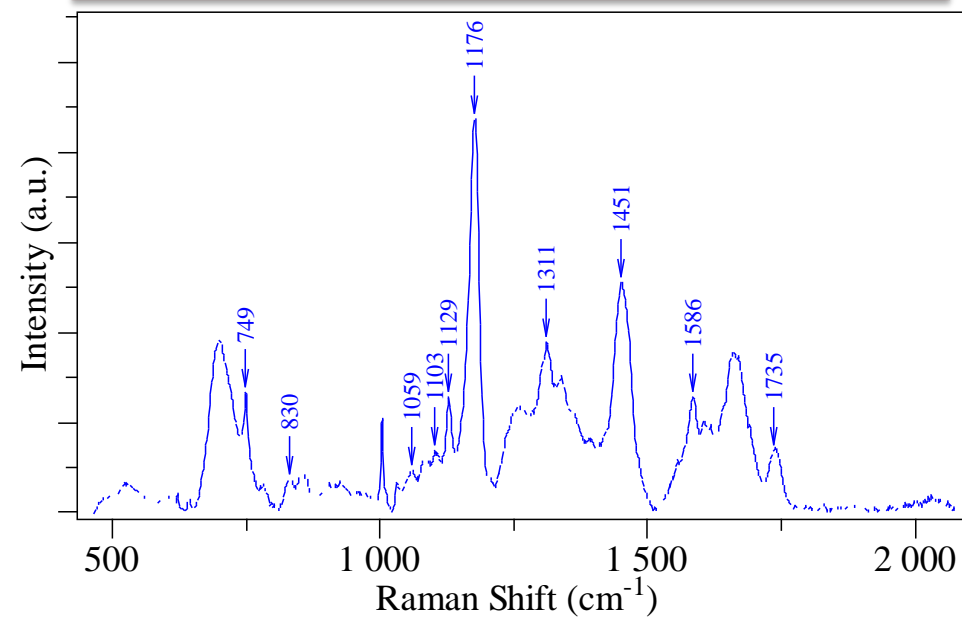
Figure legends

Figure 1. Overview on SIP-Raman technology. Upper panel: Typical single cell Raman spectrum showing signature peaks of poly-3-hydroxybutyrate (830, 1059, 1103, 1451, 1735 cm^{-1}) and polyphosphate (1176 cm^{-1}) as storage products. Furthermore, the typical cytochrome peaks from RR are visible (749, 1129, 1311 and 1586 cm^{-1}). See also supplementary Table S1 for assignment of Raman bands to biological molecules. Lower panel: Combination of FISH (left) with Raman-SIP (right) using ^{13}C -labeled naphthalene in a groundwater microbial community. Depicted Raman spectra are from FISH identified *Pseudomonas* sp. (green) and *Acidovorax* sp. (red) cells.

Figure 2. Shifts of Bands in SCRS caused by incorporation of different stable isotopes. (A) fully ^{13}C -labeled *E. coli* (red) and unlabeled *E. coli* (blue). (B) Fully ^{15}N -labeled *E. coli* (red) and unlabeled *E. coli* (blue). A few bands, such as 729 cm^{-1} (adenine ring breathing), 787 (cytosine, uracil ring breathing), 1176 (guanine ring stretching), 1247 (Amide III), 1341 (nucleic acid vibration mode), and 1577 cm^{-1} (guanine, adenine ring stretching) are significantly shifted due to ^{15}N incorporation. (C) Deuterated *E. coli* (red) due to growth in 40% D_2O -containing medium and unlabeled *E. coli* (blue). Depending on the metabolism of the microbial cell (e.g. autotrophic vs. heterotrophic) additional deuterium-induced peak shifts might occur. It should be noted that the exact position of bands shifting due to isotope incorporation sometimes varies in different publications. This is often caused by the use of different gratings causing different spectral resolution.

Figure 3. SERS spectra of single *E. coli* DH5a cells with different levels of ^{15}N incorporation when growing in various concentrations of $^{15}\text{N}\text{-NH}_4\text{Cl}$. The detection limit is 10% ^{15}N incorporation ($P < 0.05$). The power of the 532-nm laser on single cells was 0.05 mW. (A) A sharp and clear Raman band at 728 cm^{-1} (adenine like compound) shifted due to ^{15}N incorporation into cellular biomass. (B) Relationship between the ^{15}N ratio in single cells and Raman shift.

Raman spectrum as phenotypic fingerprint of a single cell



Single cell stable isotope probing

Metabolism

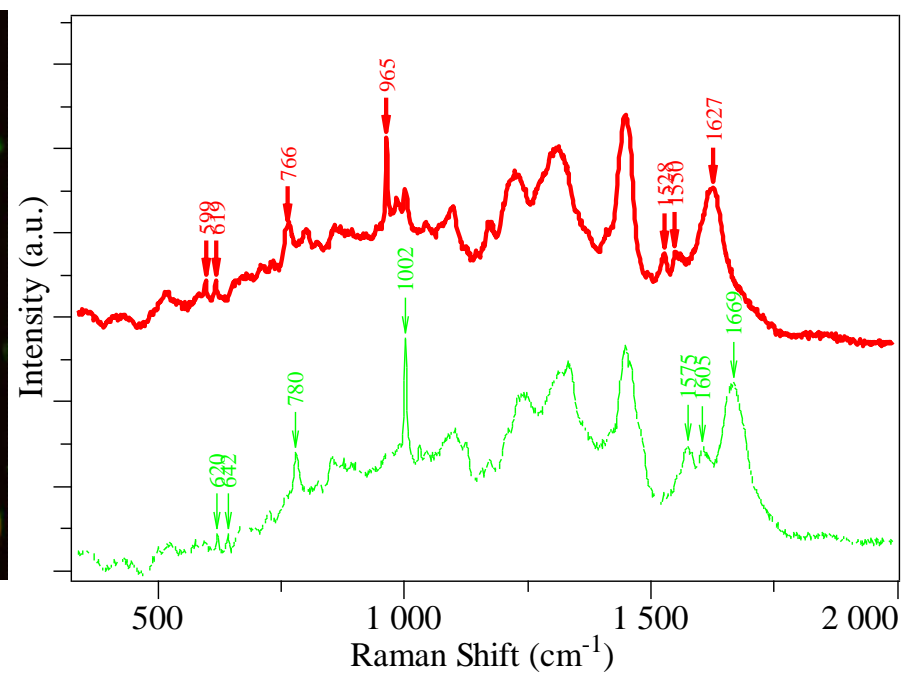
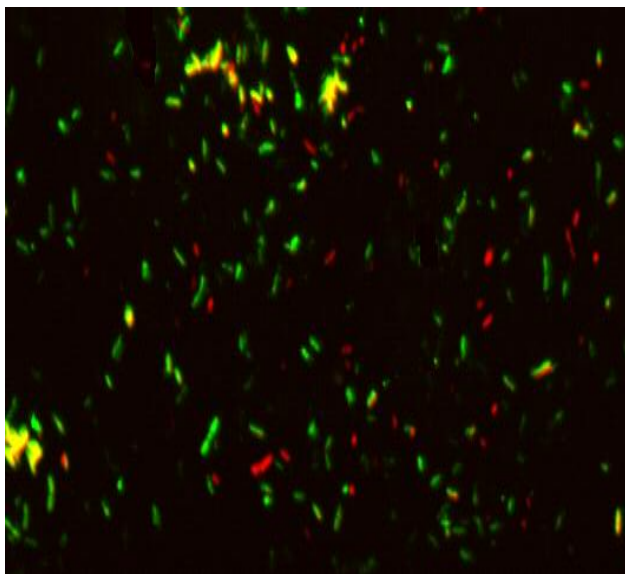
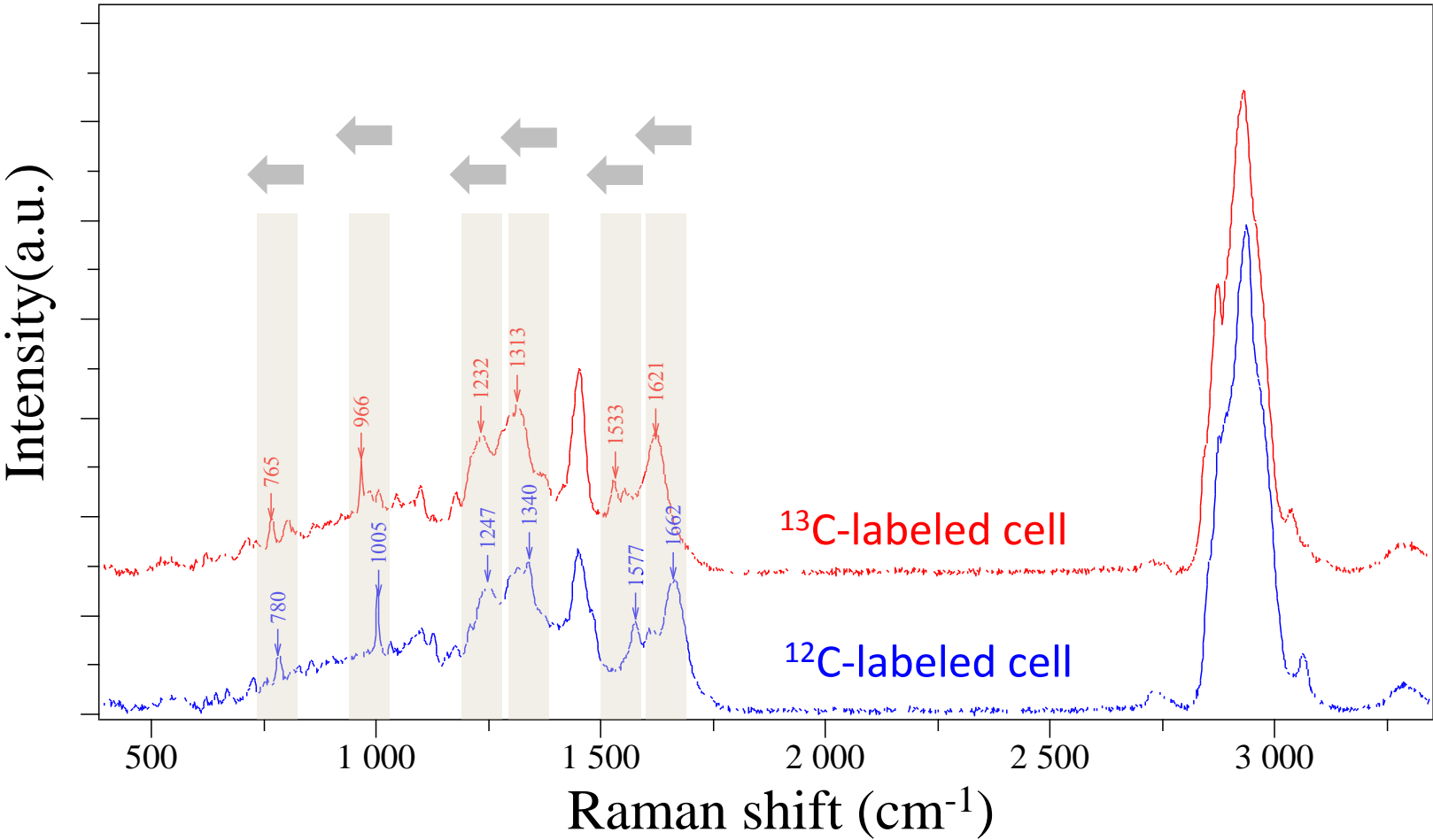
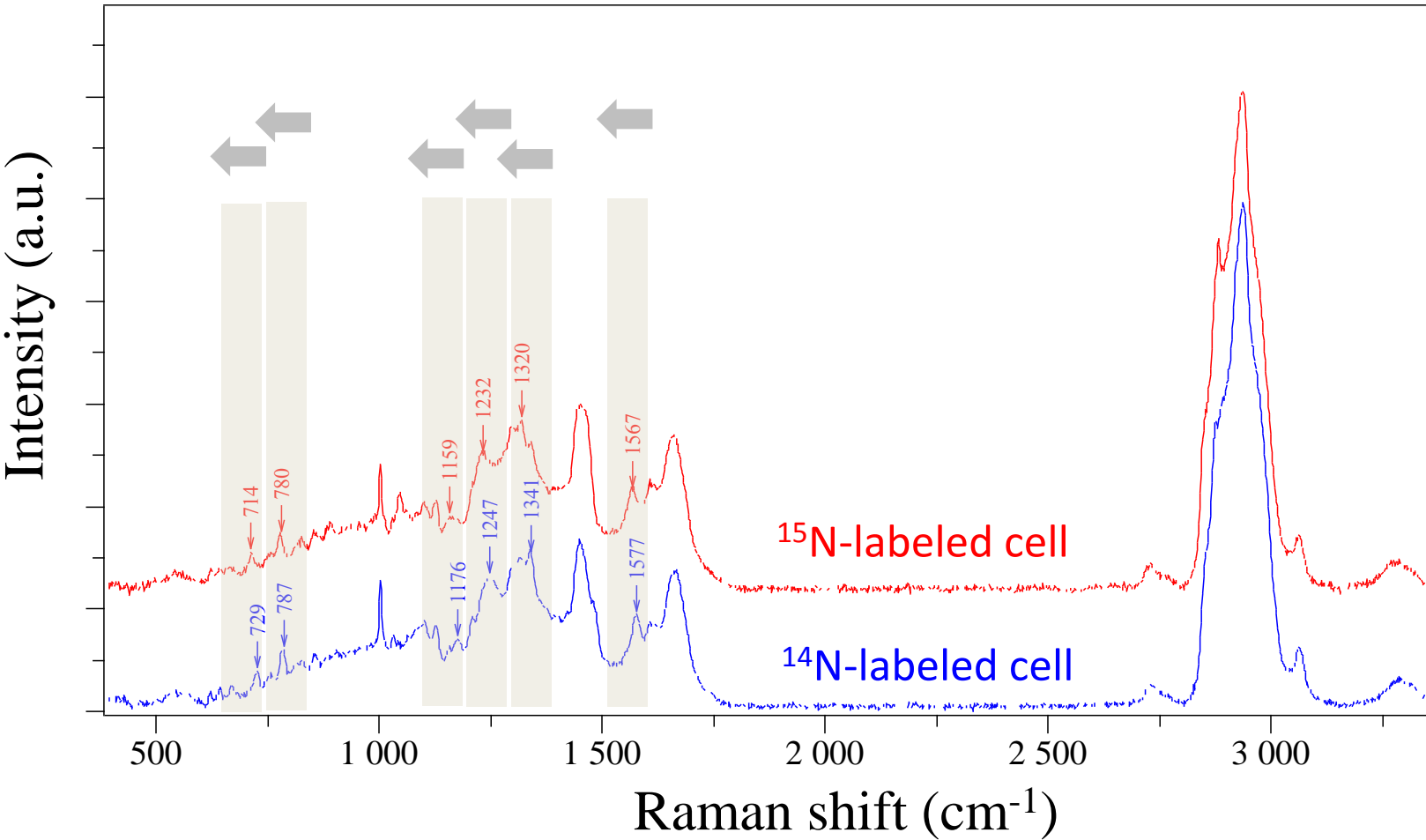


Figure2

A



B



C

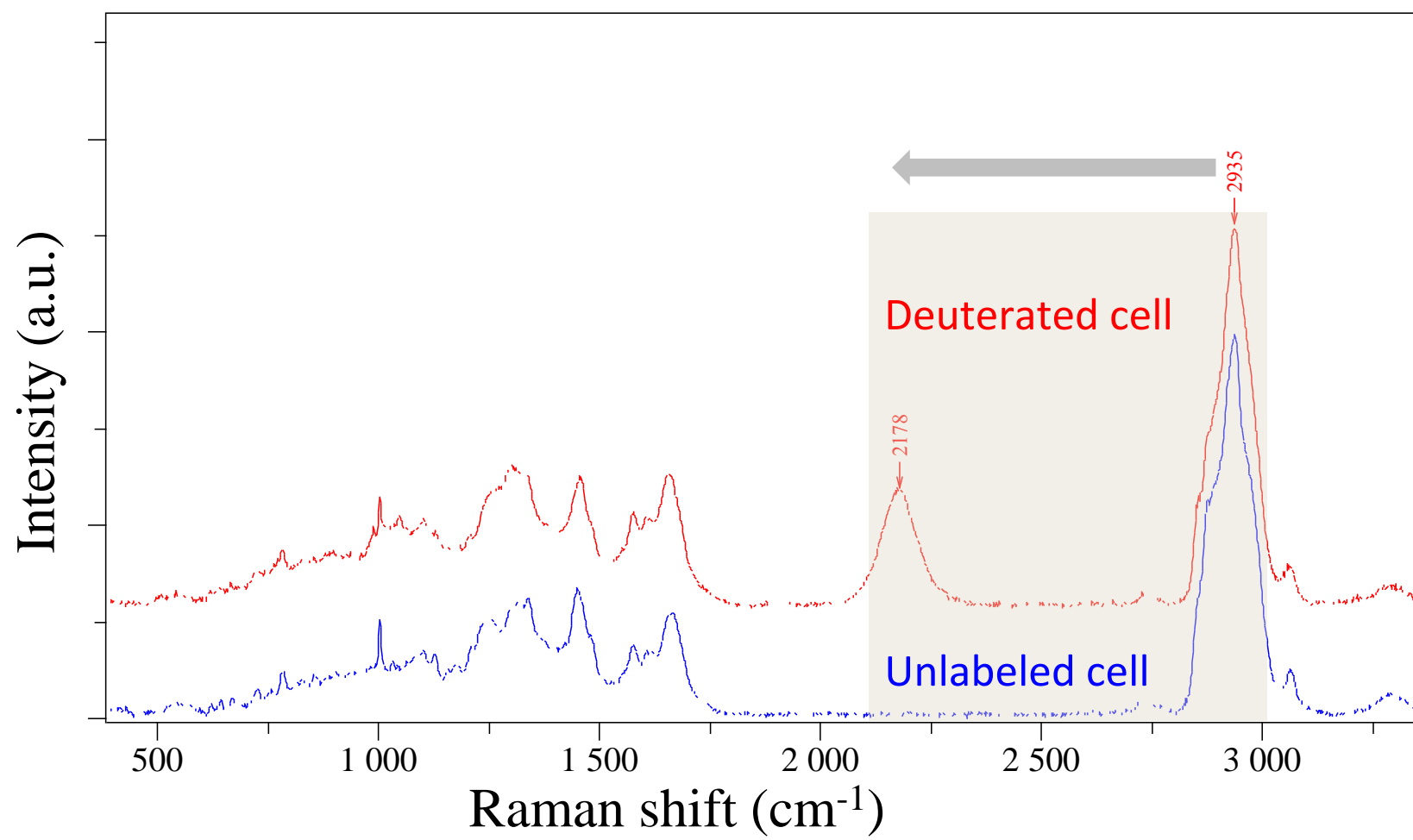
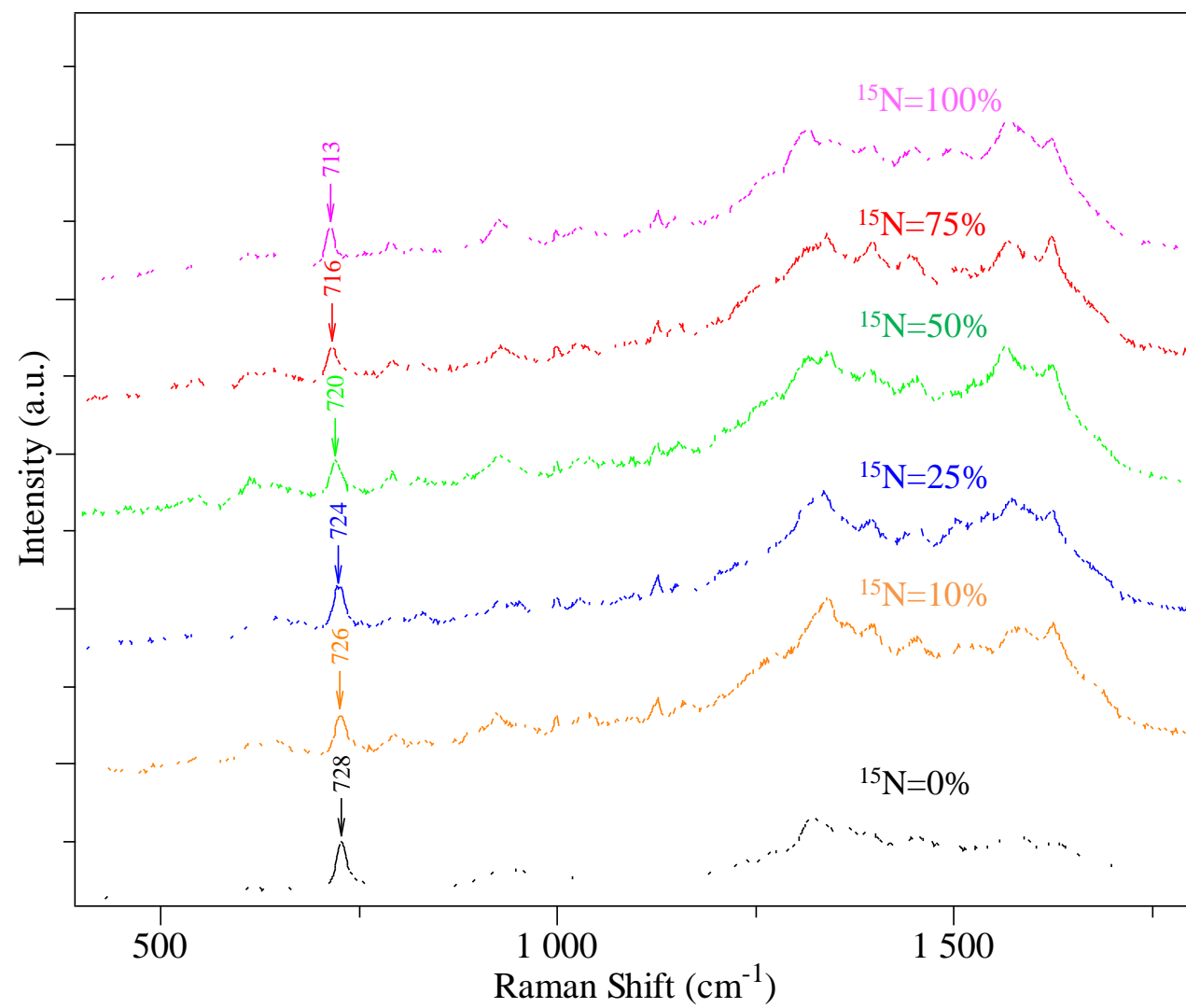
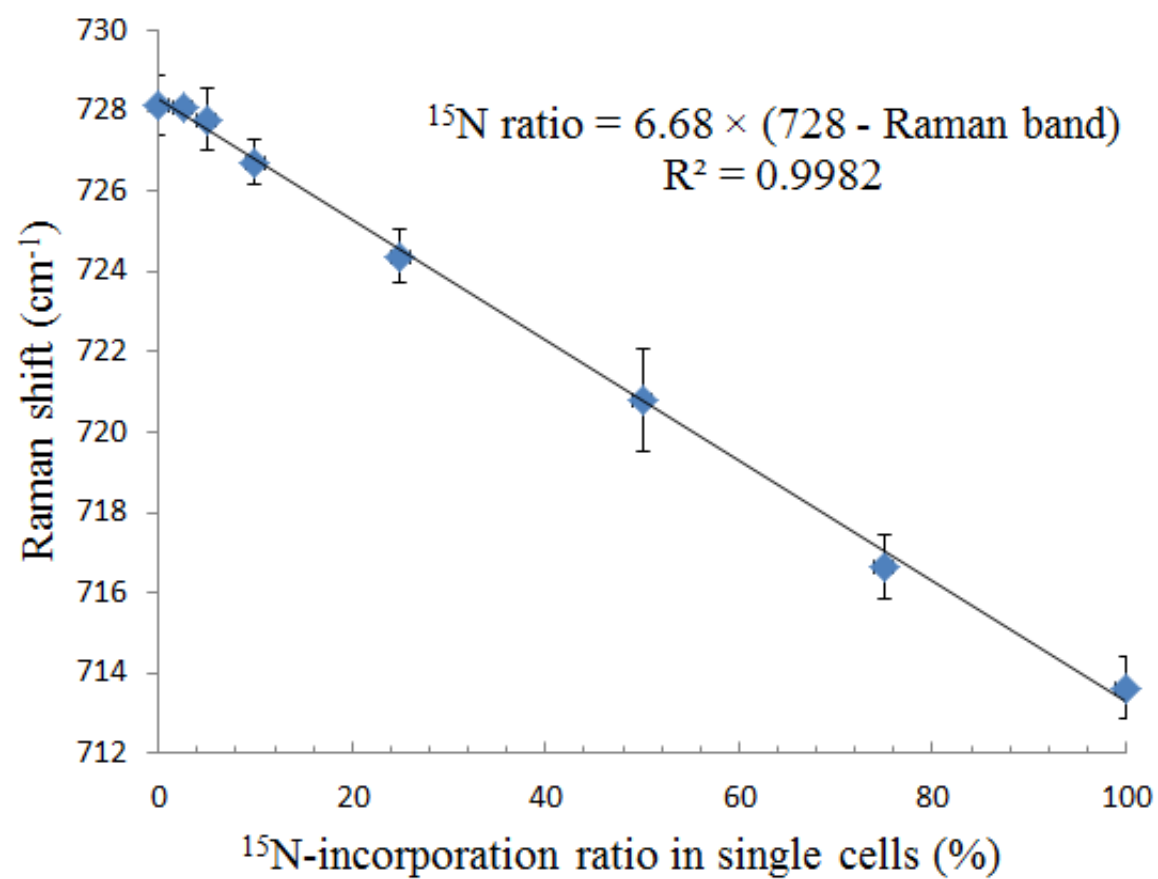


Figure3

A**B**

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