Raman $^{18}$O-labeling of bacteria in visible and deep UV-ranges

Georgette Azemtsop Matanfack$^{1,2,3}$ | Aikaterini Pistiki$^{1,2,3}$ | Petra Rösch$^{1,3*}$ | Jürgen Popp$^{1,2,3}$

$^{1}$Institute of Physical Chemistry and Abbe Center of Photonics (IPC), Friedrich-Schiller-University Jena, Jena, Germany
$^{2}$Leibniz Institute of Photonic Technology a member of the Leibniz Research Alliance Leibniz Health Technology (Leibniz-IPHT), Jena, Germany
$^{3}$Research Campus Infectognostics e.v. Jena, Jena, Germany

*Correspondence
Petra Rösch, Institute of Physical Chemistry and Abbe Center of Photonics (IPC), Friedrich-Schiller-University Jena, Helmholtzweg 4, 07743 Jena, Germany. Email: petra.roesch@uni-jena.de

Funding information
Bundesministerium für Bildung und Forschung, Grant/Award Number: 01E11701; Deutsche Forschungsgemeinschaft, Grant/Award Number: CRC 1076

Abstract
Raman stable isotope labeling with $^2$H, $^{13}$C or $^{15}$N has been reported as an elegant approach to investigate cellular metabolic activity, which is of great importance to reveal the functions of microorganisms in native environments. A new strategy termed Raman $^{18}$O-labeling was developed to probe the metabolic activity of bacteria. Raman $^{18}$O-labeling refers to the combination of Raman microspectroscopy with $^{18}$O-labeling using $H_2^{18}$O. At an excitation wavelength of 532 nm, the incorporation of $^{18}$O into the amide I group of proteins and DNA/RNA bases was observed in Escherichia coli cells, while for an excitation wavelength electronically resonant with DNA or aromatic amino acid absorption at 244 nm $^{18}$O assimilation was detected using chemometric tools rather than visual inspection. Raman $^{18}$O-labeling at 532 nm combined with 2D correlation analysis confirmed the assimilation of $^{18}$O in proteins and nucleic acids and revealed the growth strategy of E. coli cells; they underwent protein synthesis followed by nucleic acid synthesis. Independent cultural replicates at different incubation times corroborated the reproducibility of these results. The variations in spectral features of $^{18}$O-labeled cells revealed changes in physiological information of cells. Hence, Raman $^{18}$O-labeling could provide a powerful tool to identify metabolically active bacterial cells.

KEYWORDS
$^{18}$O-labeling, 2D-correlation spectroscopy, bacterial cells, Raman microspectroscopy, stable isotope labeling

1 | INTRODUCTION

The trend toward Raman stable isotope labeling has gained increasing attention in clinical diagnostics [1–7] and environmental microbiology over the past decade [8–17]. Raman spectroscopy is a vibrational technique, which provides intrinsic information on the overall biochemical composition of microorganisms [18–20]. The concept of stable
isotope labeling is based on the replacement of single atoms (e.g., $^{12}\text{C}$, $^{14}\text{N}$, $^1\text{H}$ and $^{16}\text{O}$) by their stable heavier isotopes ($^{13}\text{C}$, $^{15}\text{N}$, $^2\text{H}$ and $^{18}\text{O}$) in biomolecules, such as proteins, lipids and nucleic acids [7, 21]. This isotopic substitution leads to a red-shift of the vibrational frequency of the functional groups involved, that is, a shift toward lower wavenumbers, due to increased atomic mass [7, 21]. Owing to the benefit of frequency shift, the heavier isotopes might serve as tracers to track the metabolic activity of microorganisms [7, 22–27].

The characterization of isotopically labeled bacteria using Raman spectroscopy with visible excitation wavelengths has been reported. For instance, Yang et al. amended clinical samples with heavy water (D$_2$O) for a rapid antibiotic susceptibility testing of bacteria causing urinary tract infections [6]. By evaluating the amount of incorporated deuterium in bacteria, the authors could differentiate between antibiotics susceptible and resistant bacteria. The metabolic activity of resistant bacteria was not inhibited by the tested antibiotics thus, resistant bacteria exhibited more deuterium accumulation than sensitive bacteria [6]. Tao et al. used the same deuterium labeling approach to test the efficacy of drugs to inhibit the metabolic activity of oral bacteria [4]. The study reported on the detection of viable but not culturable (VBNC) cells, since the antimicrobial agents inhibited the bacterial growth of VBNC cells but not their metabolic activity [4]. Hence, the VBNC cells assimilated a considerable amount of deuterium from D$_2$O. The study pointed the VBNC cells as the cause of many latent and recurring infections [4]. Olaniyi et al. identified cellulose degrading bacteria in a soil microbial community using deuterium labeling [12]. They found a strong correlation between ATP production and deuterium assimilation. From this they concluded that the larger the amount of accumulated deuterium in the bacterial cells, the higher the metabolic activity of cells [12]. Taubert et al. tracked active bacteria in groundwater using Raman deuterium labeling [14]. After a subsequent genomic analysis, the authors were able to link active bacteria to their ecological function in situ [14]. Kumar et al. demonstrated the mechanism of carbon catabolic repression in naphthalene degrading bacteria by combining two distinct labeled substrates, namely $^{13}\text{C}$-glucose and fully deuterated glucose [10]. Cui et al. identified N$_2$-fixing soil bacteria owing to $^{15}\text{N}$ induced Cyt c band shifts [28]. Based on the positive correlation found between the Cyt c band shift and the $^{15}\text{N}$ percentage, the authors could quantify the extent of N$_2$ fixation of various soil bacteria [28]. Angel et al. optimized the $^{15}\text{N}$ tracer method by tracing $^{15}\text{N}$ incorporation into microbial RNA [29]. The optimized $^{15}\text{N}$-RNA method improved the sensitivity to identify soil bacteria involved in N$_2$-fixation [29].

Up to now, only the isotopes $^{13}\text{C}$, $^2\text{H}$ and $^{15}\text{N}$ have been employed in Raman stable isotope labeling of microorganisms. To the best of our knowledge studies with stable isotopes and bacteria using deep UV-resonance Raman spectroscopy at 244 nm have never been reported.

The present study combines for the first time $^{18}\text{O}$ labeling and Raman microspectroscopy for the characterization of metabolically active bacterial cells. We hypothesized that the accumulation of $^{18}\text{O}$ could be detected in proteins and nucleic acids. In order to do so, we combined the advantages of non-resonant Raman microspectroscopy at 532 nm and UV-resonance Raman spectroscopy at 244 nm excitation wavelength, to analyze the phenotypic features of bacterial cells exposed to H$_2$$^{18}\text{O}$. The Raman excitation wavelength of 532 nm is chosen to obtain information about proteins and the excitation wavelength of 244 nm to obtain information about both nucleic acids and the aromatic amino acid composition in bacterial cells.

2 | EXPERIMENTAL SECTION

2.1 | Bacterial growth and sample preparation

The bacterial strain *Escherichia coli* DSM 501 was pregrown on a M9 minimal medium (Sigma Aldrich) agar plate at 37°C. For each sample, a colony was taken from the M9 plate with an inoculation loop and suspended in 18 mL M9 minimal medium (1 g/L ammonium chloride, 6 g/L disodium hydrogen phosphate, 3 g/L potassium dihydrogen phosphate, 0.5 g/L sodium chloride, 2 mL/L 1 M magnesium sulfate solution, 20 mL/L 20% glucose solution, pH 7.4), prepared with either water (H$_2$O) or 97% $^{18}\text{O}$-water (H$_2$$^{18}\text{O}$) (Sigma Aldrich). The bacterial cultures were grown with 120 rpm rotational shaking at 37°C, for 24, 48 and 72 h. Three biological replicates were prepared.

For single cells Raman measurements in the visible range (532 nm) 1 mL of the bacterial culture was centrifuged for 5 min at 5000 g and 4°C. The supernatant was removed, and the pellet was resuspended in 1 mL of distilled water to discard the medium. Three washing steps with distilled water were performed. The resulting pellet was resuspended in 1 mL distilled water. Then 10 μL of the resulting suspension was spotted onto nickel foil and allowed to air dry.

For bulk Raman measurements in the deep UV range (244 nm) 5 mL of the bacterial culture was centrifuged for 5 min at 5000 g and 4°C. The washing steps were analog to those of the measurements at 532 nm. The pellet resulting from the last washing step was resuspended in 10 μL distilled water and the cell suspension was spread on a quartz slide and dried at room temperature.
2.2 | Raman measurements

The Raman measurements in the VIS region were performed at a single cell level using a Raman microscope (BioParticleExplorer, Rap.ID Particle Systems GmbH, Berlin, Germany). The setup was equipped with a 532 nm solid-state frequency-doubled Nd:YAG laser (LCM-S-111-NNP25; Laser-export Co.Ltd.) and a 100x air objective (MPLFLN-BD, Olympus, NA = 0.90) with a spot size below 1 μm focusing the laser light of approximately 9 mW onto single bacterial cells. A single-stage monochromator (HE 532, Horiba Jobin Yvon) equipped with a 920 lines/mm grating allowed the 180° backscattered Raman light to be diffracted and then detected by a thermoelectrically cooled charge-coupled device (CCD) camera (DV401-BV; Andor Technology). The spectral resolution was approximately 8 cm$^{-1}$ and the integration time of 15 s per single bacterial cell was chosen.

For the bulk biomass measurements in the deep UV region, the Raman spectra were recorded using a Raman microscope (HR800, Horiba/Jobin-Yvon, Bensheim, Germany) with a 244 nm frequency-doubled Argon-ion laser (Innova 300, FReD, Coherent, Dieburg, Germany) and a focal length of 800 mm. The setup was equipped with a 2400 lines/mm grating and a ×20 magnification antireflection coated objective (LMU UVB) with a numerical aperture of 0.4. The width of the entrance slit was set to 400 μm and the exposure time was 15 s with 10 accumulations. The maximal laser power of about 18 mW was chosen, leading to about 0.5 mW on a sample. To minimize possible photodegradation, the sample was rotated at a speed of 30 rpm and moved in the x, y direction after each rotation to cover a large sample area. The Raman scattered light was detected by a nitrogen-cooled CCD camera. The spectral resolution was approximately 2 cm$^{-1}$.

2.3 | Data preprocessing

The data preprocessing of spectra acquired in the VIS and UV regions were carried out with the software GNU R [30] using in-house written scripts. First the cosmic spikes were removed from the spectra [31]. Then the wavenumber axis of VIS-spectra was calibrated using acetylaminophenol as reference [32]. Subsequently, the sensitive nonlinear iterative peak (SNIP) clipping algorithm [33] with 30 and 40 iterations was applied to remove the fluorescence background in the spectra recorded in the VIS and UV regions, respectively. The spectra were then vector normalized and averaged across groups. Principal component analysis (PCA) was subsequently applied to all spectra to reduce the data dimensionality. Linear discriminant analysis (LDA) was then performed to classify $^{18}$O-labeled and non-labeled cells. The classification performance was evaluated by the
leave-one-batch-out cross-validation with the optimal number of principal components. A 2D correlation analysis was applied to the time-dependent Raman spectra of E. coli cells using the R package corr2D [34]. The 2D synchronous and asynchronous maps were interpreted according to the Noda’s rules [35].

3 | RESULTS AND DISCUSSION

To compare the wavelength dependent Raman spectra of E. coli cells, samples from the same batch culture were used.

In Figure 1A the characteristic features of bacterial cells are observed at 2935 cm\(^{-1}\) (\(\nu(\text{C–H})\)), 1666 cm\(^{-1}\) (\(\nu(\text{C=O})\) of amide I), 1576 cm\(^{-1}\) (DNA), 1450 cm\(^{-1}\) (\(\delta(\text{CH}_2/\text{CH}_3)\)), 1240 cm\(^{-1}\) (amide III), 1003 cm\(^{-1}\) (phenylalanine) and 781 cm\(^{-1}\) (DNA/RNA). The band positions conform to previously reported Raman studies (Table S1) on bacteria [19, 36–41]. The most prominent difference between the spectra of E. coli cells grown in H\(_2\)O (non-labeled) and those grown in \(^{18}\)O-water (\(^{18}\)O-labeled) is observed in the amide I band. For more detailed examination, the amide I band has been enlarged and depicted in Figure 1B. A comparison of the mean Raman spectra shows that at the time points 24, 48 and 72 h the amide I band of \(^{18}\)O-labeled cells is shifted to lower wavenumber positions and has decreased in intensity. In addition to the amide I band and the nucleic acid peak at 781 cm\(^{-1}\) attributed to DNA/RNA contribution was red-shifted to about 775 cm\(^{-1}\) in the spectra of cells incubated with H\(_2\)\(^{18}\)O (Figure 1C). The red-shift was already visible at 24 h of incubation and became more pronounced over time. The variations observed in the position and intensity of the amide I band and the nucleic acid peak at 781 cm\(^{-1}\) indicated the incorporation of \(^{18}\)O from H\(_2\)\(^{18}\)O into E. coli cells. The band superposition in the fingerprint region of Raman spectra makes it more challenging to detect small spectral changes related to the assimilation of \(^{18}\)O.

The application of Raman spectroscopy with electronically resonant excitation wavelengths in the deep UV region reduces the fluorescence background and provides an optimal signal-to-noise ratio due to the resonance Raman enhancement effect of biomolecules [40, 42–44]. For an excitation wavelength at 244 nm in particular vibrations due to aromatic amino acids and nucleic acids are resonantly enhanced. Figure 2 shows the Raman spectra of cells excited at 244 nm. The spectral assignment is in accordance with previously published UV-Raman spectroscopic studies of bacteria (Table S2) [40, 43, 45].

The band at 1640 cm\(^{-1}\) is attributed to the \(\nu(\text{C=O})\) of thymine and cytosine, whereas the signal at 1617 cm\(^{-1}\) is assigned to the \(\nu(\text{C=C})\) of the aromatic amino acids tyrosine, tryptophan and phenylalanine. Additional contributions arising from the ring breathing modes of aromatic amino acids were observed at 1365 cm\(^{-1}\) (tyrosine) and 1011 cm\(^{-1}\) (tryptophan and phenylalanine). The bands at 1176 and 762 cm\(^{-1}\) can also be attributed to tyrosine and tryptophan, respectively. The signal at 1575 cm\(^{-1}\) is assignable to the \(\nu(\text{C=C})\) and \(\nu(\text{C=N})\) modes of guanine and cytosine, while the signal at 1485 cm\(^{-1}\) is due to the ring vibrations of all nucleobases. The combination of adenine and tyrosine gives rise to the broad band at about 1335 cm\(^{-1}\), whereas the combination of adenine and thymine contributes to the band at about 1241 cm\(^{-1}\). The signal at 1530 cm\(^{-1}\) can be assigned to cytosine. The differences between labeled and non-labeled spectra for the same excitation wavelength are difficult to detect by simple visual inspection (Figures 1 and 2). For better visualization, multivariate data analysis was performed on the 532 and 244 nm-spectra of each time point (24, 48, and 72 h). PCA was first applied for

**FIGURE 2** Mean Raman spectra of Escherichia coli cells excited at 244 nm after 24, 48 and 72 h of incubation in water (blue spectra) and H\(_2\)\(^{18}\)O (red spectra). The spectra were shifted vertically for clarity. Corresponding peak assignments are provided in Table S2.
dimensionality reduction. Then LDA was performed with leave-one-batch-out cross-validation to classify 18O-labeled and non-labeled cells. At each time point both classes were very well separated with more than 92% accuracy. The classification results are summarized in Table S3. The loadings plots resulting from the PCA-LDA classification are depicted in Figure 3. Negative loadings are characteristics of non-labeled cells, whereas positive difference signals represent 18O-labeled cells. For all three time points the loadings plot of 532 nm spectra (Figure 3A) showed a negative difference signal at about 1666 cm\(^{-1}\) and a positive difference signal at about 1648 cm\(^{-1}\). The signal at 1666 cm\(^{-1}\) could be assigned to the unlabeled amide I band, while the signal at 1648 cm\(^{-1}\) might correspond to the amide I band labeled with 18O. The difference between the peak position of both bands indicates a red-shift caused by the incorporation of 18O in the C=O stretching group of amide I. The negative difference peak at about 781 cm\(^{-1}\) was characteristic of non-labeled cells, while the positive difference peak at about 775 cm\(^{-1}\) originated from the nucleic acid contribution of 18O-labeled cells, as shown in Figure 1C.

The peak shifts observed and the differences in the loadings plotted hint changes in the biochemical composition of bacterial cells incubated with the labeled substrate. Hence, these observations from the non-resonant Raman difference spectra excited at 532 nm indicate that E. coli cells used 18O-labeled water (H\(_2\)\(^{18}\)O) during metabolic processes. The increase of intensity of the peaks at 2983 and 2890 cm\(^{-1}\) at 72 h incubation could be due to nutrient depletion in the medium and the adaptation of the cell metabolism to the present conditions.

At 244 nm, the loadings plot showed that the differentiation between 18O-labeled and non-labeled cells was mainly based on DNA signatures due to a resonant excitation (Figure 3B). The feature differences in the guanine and adenine ring breathing vibrations at 1479 and 1491 cm\(^{-1}\) and the tryptophan symmetric stretching vibration at 780 and 762 cm\(^{-1}\) could represent red-shifts that were not visible in the mean spectra. In general, the contributions of ring breathing modes of guanine, cytosine, adenine and thymine appear at about 1485 cm\(^{-1}\) (Table S2). The peak at about 1491 cm\(^{-1}\) was assigned to the guanine, cytosine, and thymine contribution of non-

---

**FIGURE 3** Loadings vectors resulting from the principal component analysis (PCA)-linear discriminant analysis (LDA) classification of 18O-labeled and non-labeled *Escherichia coli* cells after 24, 48 and 72 h of incubation. A, 532 nm excitation and B, 244 nm excitation.
labeled cells. In contrast to adenine, both guanine and cytosine have a carbonyl group \((\text{C}=\text{O})\) which is bound to the purine and pyrimidine ring, respectively. Thymine, however, comprises two carbonyl groups (Figure S1). Hence, the signal at about 1479 cm\(^{-1}\) can be attributed to the substitution of \(^{16}\text{O}\) by \(^{18}\text{O}\) in guanine, cytosine and thymine bases. In UV-resonance Raman spectroscopy, only the Raman intensities of vibrational modes associated with the electronic transition (Franck-Condon active modes) are enhanced [18]. Similarly, to the 532 nm excitation, a possible red-shift is present in the nucleic acid band at 780 cm\(^{-1}\) indicating that the same vibrational modes were detected by both excitation wavelengths. Another red-shift is present in the loadings of the 48 h incubation, at 828 cm\(^{-1}\), which is not present in the other studied time points and is assigned to tryptophan. The absence of this shift in the other time points could indicate adaptations of cellular metabolism to the consumption of available nutrients in the medium. Unlike DNA and aromatic amino acids, the amide I group of proteins absorbs light at even shorter wavelengths, that is, in the deeper UV-range at 197–206 nm [46]; hence, the signal of amide I is not resonantly enhanced for 244 nm excitation. Consequently, the incorporation of \(^{18}\text{O}\) into the amide I group could in contrast to non-resonant excitation at 532 nm not easily be detected in the UV resonance Raman spectra of \(E. \text{coli}\) cells. However, a peak of weak intensity \((1659 \text{ cm}^{-1})\) assignable to the amide I signal was present in the loadings plot and characteristic for \(^{18}\text{O}\)-labeled cells (Figure 3B). Although no peak shift was detected by the visual inspection of the UV resonance Raman spectra of \(^{18}\text{O}\)-labeled, it is noteworthy that chemometric methods such as PCA-LDA classification allowed to visualize the differences between \(^{18}\text{O}\)-labeled cells and non-labeled cells. Hence, Raman \(^{18}\text{O}\) labeling in deep-UV range can be combined with chemometrics to study the changes in the genotype of bacteria.

Since a peak shift was already detected in the 532 nm spectra at 24 h, we checked whether \(^{18}\text{O}\)-assimilation was time-dependent. For this purpose, we recorded the Raman spectra of earlier incubation times (4, 8, 12 and 18 h) using 532 nm excitation wavelength, to check at which time point \(^{18}\text{O}\) was assimilated in \(E. \text{coli}\) cells. The mean spectra of earlier incubations are depicted in Figure 4A. It is observed that a red-shift occurred in the amide I band (Figure 4B) and the nucleic acid signal (Figure 4C) appeared already after 4 h of incubation with \(\text{H}_2^{18}\text{O}\). The peak shifts were also present at intermediate time points. And later, after 18 h of incubation the red-shifts were more pronounced in both amide I and nucleic acid signals.

The amide I signal shifted from 1666 to about 1648 cm\(^{-1}\), while the DNA/RNA signal shifted from 781 to about 775 cm\(^{-1}\) after 18 h of incubation with \(\text{H}_2^{18}\text{O}\). These observations indicated that \(E. \text{coli}\) cells performed protein synthesis and DNA replication and thus were metabolically active during the monitored period. The \(^{18}\text{O}\)-induced peak shifts seem to be much smaller.
than the shifts induced by other stable isotopes such as $^2$H (D), $^{13}$C and $^{15}$N. It could be that only a small fraction of amide I has been converted and that we see a mixed signal in the amide I region. A possible reason for this could be the different metabolic pathways used by bacteria to assimilate each substrate. It is likely that the chemical pathway of $^{18}$O from H$_2^{18}$O into biomolecules is more restricted than that of $^{13}$C from glucose (commonly used in labeling of bacteria), resulting in a lower labeling efficiency. This would explain both the low Raman intensity and the small shift observed in the amide I band.

To investigate the incorporation of $^{18}$O into proteins and nucleic acids in more details and to resolve overlapping bands, 2D-correlation analysis was applied using all the spectra recorded at 532 nm excitation wavelength. The fundamentals of 2D-correlation spectroscopy were described by Noda and others [35, 47]. Figure 5 depicts synchronous and asynchronous 2D spectra in the range 1750 to 1520 cm$^{-1}$ generated from the time-dependent (4-72 h) spectral variations of E. coli cells. The red and blue areas in the 2D maps represent positive and negative correlation, respectively, and the cross-peaks are read in the upper part of the diagonal. The synchronous map of $^{18}$O-labeled cells (Figure 5A) is dominated by a strong autopeak at about 1650 cm$^{-1}$, due to the C–O stretching mode of proteins. This autopeak means that the amide I signal changed significantly over time. The signal at 1650 cm$^{-1}$ has a positive correlation with the bands at about 1620 cm$^{-1}$ (tyrosine) and

![Figure 5](image-url)

**Figure 5** 2D synchronous and asynchronous (*) maps of the 1750 to 1520 cm$^{-1}$ spectral region of Escherichia coli cells incubated with H$_2^{18}$O, A and water, B. Red color: positive correlation, blue color: negative correlation. Autopeaks are developed along the diagonal in the synchronous maps. Cross-peaks are located at off-diagonal positions in the synchronous and asynchronous maps.
1560 cm$^{-1}$ (DNA), indicating that the amide I signal changed in the same direction with tyrosine and DNA signals. The corresponding asynchronous map developed a “four-leave-clover cluster” pattern in the amide I band with a positive (at about 1666 cm$^{-1}$) and a negative (at about 1648 cm$^{-1}$) cluster highlighted by the dashed circles near the diagonal. The appearance of well-resolved lines in the clusters indicates that peaks were present at different positions, suggesting that the composition of amide I signal was heterogeneous, due to the substitution of $^{16}$O by $^{18}$O atoms. The sign of cross-peaks in the asynchronous map suggested that the spectral changes at 1648 cm$^{-1}$ occurred earlier than those at 1666 cm$^{-1}$. This is to say the assimilation of $^{18}$O into the C=O group first causes the red-shift of the peak before affecting the signal intensity. The same information resulted from the 2D maps of the nucleic acid peak at about 781 cm$^{-1}$, which showed that the DNA/RNA peak was heterogeneous with a positive and a negative cross-peak (Figure S2A); the corresponding asynchronous map (Figure S2B); corroborated that the peak shift preceded the change in signal intensity.

The amide I signal of $^{18}$O-labeled cells showed a positive cross-peak with the nucleic acid peak at 781 cm$^{-1}$ (Figure S3). The sign of the cross-peak in the asynchronous map revealed that the amide I signal changed prior to the nucleic acid peak. This means that $E. coli$ cells incorporated $^{18}$O into proteins first and then into nucleic acids. This indicates that $E. coli$ cells started with protein synthesis during the adaptation phase (within 4 h of incubation) and later performed the DNA replication or RNA synthesis, allowing protein synthesis from 24 h onward, where $E. coli$ cells should have reached the steady state. This growth strategy may explain the variations observed in the amide I signal (Figures 1 and 4A), and thus indicate that the observed decrease in the intensity of the amide I band of $^{18}$O-labeled cells (Figure 4A) was not only due to $^{18}$O-uptake.

Unlike the synchronous map of $^{18}$O-labeled cells, which developed cross-peaks only with the amide I signal, the synchronous map of water treatment shows a block of autopeaks and positive correlated cross-peaks in the region of 1750 to 1520 cm$^{-1}$ (Figure 5B). The corresponding asynchronous map (Figure 5B*) developed no cross-peak near the diagonal, indicating that the amide I signal of non-labeled cells was homogenous. The patterns of both synchronous and asynchronous maps of $^{18}$O-labeled cells (Figure 5A, A*) were significantly different from those of non-labeled cells (Figure 5B, B*), indicating that the variations caused by $^{18}$O assimilation in bacterial cells can be distinguished from those resulting from normal bacterial growth with water. Therefore, 2D correlation analysis can be combined with Raman $^{18}$O-labeling to study the metabolic dynamics of bacteria. The results of Raman $^{18}$O-labeling at 532 and 244 nm demonstrated that the variations in spectral features were related to changes in the biochemical composition of bacterial cells. Thus, this labeling approach might allow the access to physiological and genetical information of bacterial cells.

4 | CONCLUSION

The present study demonstrates for the first time the use of $^{18}$O stable isotope in the labeling strategies of bacteria. The results showed that 4 h of incubation are sufficient to label $E. coli$ cells with $^{18}$O. The Raman spectra of $^{18}$O-labeled bacterial cells excited at 532 nm showed the accumulation of $^{18}$O in the amide I group and DNA/RNA bases (cytosine/thymine/uracil), suggesting that protein and nucleic acid metabolisms were active in $E. coli$ cells. Hence, the presence of a peak-shift is a reliable indicator for $^{18}$O-uptake. The combination of Raman $^{18}$O-labeling at 532 nm with 2D correlation analysis revealed the growth strategy of $E. coli$ cells, which performed protein synthesis in the latent and steady phases, and DNA replication in the exponential phase. Hence, Raman $^{18}$O-labeling combined with 2D correlation analysis can serve to monitor the metabolic activity of single bacterial cells.

However, for electronic resonant excitation at 244 nm $^{18}$O uptake was not detected by visual inspection. The non-detection of $^{18}$O uptake in the UV resonance Raman spectra leads to the following question: Are Raman modes sensitive to $^{18}$O uptake not resonantly enhanced at 244 nm, or in other words was the selected UV excitation wavelength at 244 nm not suitable to detect $^{18}$O incorporation in nucleic acids and aromatic amino acids? Future work also applying different UV excitation wavelengths is needed to answer this question. The results of this study showed that Raman $^{18}$O-labeling approach at 532 nm is sensitive to the metabolic activity of both proteins and nucleic acids. Hence, this labeling approach could detect fluctuations in physiological information of bacteria and thus reveal the presence of metabolically active cells. The presented approach might offer opportunities for future research and the development of applications in different fields. For instance, Raman $^{18}$O-labeling will be of great advantage to investigate phosphate solubilizing bacteria, since phosphate lacks stable isotopes. Furthermore, Raman $^{18}$O-labeling could be applied to identify antimicrobial resistant bacteria by monitoring the metabolic dynamics of proteins and nucleic acids in response to antimicrobial agents. We believe that Raman $^{18}$O-labeling might represent a promising tool to probe the general metabolic activity of bacterial cells in a very effective and simple way.
ACKNOWLEDGMENTS
This study is part of the Collaborative Research Center AquaDiva (CRC 1076) of the Friedrich Schiller-University Jena funded by the Deutsche Forschungsgemeinschaft (DFG). The financial support of the project CarbaTech (FKZ 01EI1701) by the BMBF is gratefully acknowledged. Open Access funding enabled and organized by Projekt DEAL.

CONFLICTS OF INTEREST
The authors declare no potential conflicts of interest.

DATA AVAILABILITY STATEMENT
Data available on request from the authors.

ORCID
Petra Rösch https://orcid.org/0000-0001-6179-3719

REFERENCES
[1] G. Azemtsop Matanfack, J. Rüger, C. Stiebing, M. Schmitt, J. Popp, J. Biophotonics 2020, 13, e202000129.
[2] G. Azemtsop Matanfack, M. Taubert, S. Guo, R. Houhou, T. Bocklitz, K. Küsel, P. Rösch, J. Popp, Anal. Chem. 2020, 92, 11429.
[3] Y. Song, L. Cui, J. Á. S. López, J. Xu, Y.-G. Zhu, I. P. Thompson, W. E. Huang, Sci. Rep. 2017, 7, 16648.
[4] Y. Tao, Y. Wang, S. Huang, P. Zhu, W. E. Huang, J. Ling, J. Xu, Anal. Chem. 2017, 89, 4108.
[5] Y. Wang, J. Xu, L. Kong, T. Liu, L. Yi, H. Wang, W. E. Huang, C. Zheng, J. Microbiol. Biotechnol. 2020, 13, 572.
[6] K. Yang, H.-Z. Li, X. Zhu, J.-Q. Su, B. Ren, Y.-G. Zhu, L. Cui, Anal. Chem. 2019, 91, 6296.
[7] B. Lorenz, C. Wichmann, S. Stockel, P. Rosch, J. Popp, Trends Microbiol. 2017, 25, 413.
[8] D. Berry, E. Mader, T. Bocklitz, A. Ramoji, U. Neugebauer, M. Foerster, C. Kroegel, M. Bauer, J. Popp, Chemom. Int. Ed. 2016, 155, 1.
[9] T. Dörfer, T. Bocklitz, N. Tarcea, M. Schmitt, J. Popp, Z. Phys. Chem. 2011, 225, 753.
[10] K. Liland, B.-H. Mevik, R. Canteri, 2020. https://cran.r-project.org/web/packages/baseline/baseline.pdf
[11] R. Geitner, R. Fritsch, T. Bocklitz, J. Popp, J. Stat. Softw. 2019, 90, 1. https://doi.org/10.18637/jss.v090.i03
[12] I. Noda, A. E. Downrey, C. Marcott, J. L. Story, Y. Ozaki, Appl. Spectrosc. 2000, 54, 236A.
[13] S. Meisel, S. Stöckel, M. Elschnner, F. Melzer, P. Rösch, J. Popp, Environ. Microbiol. 2012, 78, 5575.
[14] D. Kusić, B. Kampe, P. Rösch, J. Popp, Water Res. 2014, 48, 179.
[15] M. Harz, P. Rösch, J. Popp, Cytometry A 2009, 75A, 104.
[16] A. Rygula, K. Majzner, K. M. Marzec, A. Kaczor, M. Pilarczyk, M. Baranska, J. Raman, Spectrosc. 2013, 44, 1061.
[17] U. Neugebauer, U. Schmid, K. Baumann, W. Ziebuhr, J. Popp, ChemPhysChem 2007, 8, 124.
[18] J. De Gelder, K. De Gussem, P. Vandenaeele, L. Moens, J. Raman, Spectrosc. 2007, 38, 1133.
[19] M. Harz, R. A. Claus, C. L. Bockmeyer, M. Baum, P. Rösch, K. Kentouche, H.-P. Deigner, J. Popp, Biopolymers 2006, 82, 317.
[20] A. Silge, R. Heinke, T. Bocklitz, C. Wiegang, U.-C. Hipler, P. Rösch, J. Popp, Anal. Bioanal. Chem. 2018, 410, 5839.
[21] O. Žukovskaja, S. Kloß, M. G. Blango, O. Ryabchikov, O. Kniemeyer, A. A. Brakhage, T. W. Bocklitz, D. Cialla-May, K. Weber, J. Popp, Analyst 2018, 90, 8912.
[22] N. Tarcea, M. Harz, P. Rösch, T. Frosch, M. Schmitt, H. Thiele, R. Hochleitner, J. Popp, Spectrochim. Acta A Mol. Biomol. Spectrosc. 2007, 68, 1029.
SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.