Imaging isotopically labeled bacteria at the single cell level using high-resolution optical infrared photothermal spectroscopy

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ABSTRACT: We report that the cellular uptake of stable-isotope-labeled compounds by bacteria can be probed at the single cell level using infrared spectroscopy and this monitors the chemical vibrations affected by the incorporation of “heavy” atoms by cells, and thus can be used to understand microbial systems. This presents a significant advance as most studies have focused on evaluating communities of cells due to the poor spatial resolution achieved by classical infrared microspectrometers and, to date, there is no study evaluating the incorporation of labeled compounds by bacteria at single cell levels using infrared spectroscopy. The development of new technologies and instrumentations that provide information on the metabolic activity of a single bacterium is critical, as this will allow for a better understanding of the interactions between microorganisms as well as the function of individual members and their interactions in different microbial communities. Thus, the present study demonstrates the ability of a novel far-field infrared imaging technique, optical photothermal infrared (O-PTIR) spectroscopy, as a tool to monitor the uptake of 13C-glucose and 15N-ammonium chloride by *Escherichia coli* bacteria at single cell levels using spectral signatures recorded via single-point and imaging modes. An additional novelty is that imaging was achieved using six vibrational bands in the Amide I and II regions which were analysed with chemometrics employing partial least squares-discriminant analysis to predict 13C/12C and 15N/14N simultaneously.

INTRODUCTION

Fourier transform infrared (FTIR) spectroscopy has been widely used as a tool to interrogate the overall biochemistry of biological samples based on the vibrations of molecules that are infrared active such as proteins, nucleic acids, carbohydrates, and lipids1. FTIR spectroscopy is by far the most used method to acquire infrared spectra of bacterial systems among the technologies currently available and has been well succeeded as a whole-organism fingerprinting tool to investigate the development of antibiotic resistance by bacteria2,3, classification and identification of microbial species and strains4-8, as well as for studying the changing phenotype during growth of bacterial populations9,10. Infrared chemical imaging enables simultaneous evaluation of the morphology and abundance of molecules in biological systems, but the long excitation wavelength of mid-infrared (mid-IR) radiation (3−30 μm) and the lack of high numerical aperture (NA) objectives provide spatial resolution of approximately 3-14 μm, with the information rich fingerprint region (1800-800 cm-1) having an approximate spatial resolution of 7-14 μm, which makes the technique unsuitable for imaging subcellular structures or single microorganisms (with dimensions typically in the range 1-2 µm). Consequently, most bacterial studies using FTIR spectroscopy have mainly focused on the analysis of bulk samples instead of single bacterium. To overcome the spatial resolution challenge, the technology of infrared spectroscopy was combined with near-field optical imaging, resulting in the development of techniques capable of mapping infrared-active modes with nanoscale (< 100 nm) spatial resolution such as with atomic force microscopy-infrared spectroscopy (AFM-IR) and infrared scattering-scanning near-field microscopy (IR-SNOM)11. Both methods operate by combining the sub-micron spatial resolution provided by an AFM tip with the high chemical specificity of infrared spectroscopy12. These two techniques are fundamentally different regarding their basic principles of operation. Briefly, AFM-IR measures the light absorbed by the sample, whereas IR-SNOM is based on the scattered light13. The high spatial resolution achieved by nanoscale infrared spectroscopy allows the investigation of single cell bacterium, which is not possible with a state-of-the-art infrared microscope. So far, AFM-IR has been applied to investigate the cell wall of Gram-negative and Gram-positive bacteria14, detection of antibiotic resistance15, evaluation of bacteria infected by viruses16, as well as for monitoring the production of polyhydroxybutyrate17 and the distribution of biomolecules such as lipids18 and proteins19 in bacterial cells. Despite the high-resolution provided by scanning-probe techniques, these methods are complex in terms of instrumentation, laborious, have limited field of view, require contact and long time for acquiring data20-22.

Recently, a novel far-field infrared imaging technique based on the photothermal effect of infrared radiation has been proposed to provide chemical images in a fast manner with sub-micron spatial resolution. This emerging method, so-called optical photothermal infrared (O-PTIR) spectroscopy, measures the photothermal response of a sample illuminated by a tunable mid-IR laser beam. The spatial resolution is determined by a visible probe beam that is used to detect the photothermal response, therefore the spatial resolution is limited now by the short visible wavelengths and is wavelength-independent in the infrared range23. Previous studies have demonstrated the potential of infrared photothermal imaging to analyze single bacterial cells using the fingerprint21 and high wavenumber region20 of mid-IR. Therefore, the present study aims to demonstrate, for the first time, the ability of O-PTIR spectroscopy to evaluate the incorporation of 13C and 15N labeled compounds by *E. coli* at single cell level using spectral signatures recorded via both single-point and imaging modes. Incorporating “heavy” atoms into bacterial cells has been widely used to understand their ecophysiology as well as their role in biogeochemical cycles24, biotechnological processes25, and human-disease interaction26,27. The cellular uptake of stable-isotope-labeled atoms by bacterial cells can be probed by vibrational spectroscopic methods such as Raman and infrared spectroscopies due to their ability to monitor the chemical vibrations affected by the incorporation of isotopically labeled molecules by cells28. Whilst the diffraction limit of Raman allows sufficient spatial resolution to probe single cells, despite the widespread use of infrared spectroscopy to investigate the incorporation of heavy atoms by bacteria, most of these stable isotope probing (SIP) studies have focused on evaluating bulk samples and, to date, there is no study analyzing isotopically labeled bacteria at single cell level. Development and application of novel methodologies such as O-PTIR spectroscopy is critical for better understanding of bacterial metabolism at the single cell level and bypassing the prolonged traditional culturing techniques, which may allow for understanding the interactions between microorganisms, as well as the functions of individual members and their interactions in their natural microbial communities.

EXPERIMENTAL SECTION

**Growth conditions:** Unless otherwise stated, all reagents used in the experiments were purchased from Fisher (Fischer Scientific, U.K.). *Escherichia coli* K-12 MG1655 cells were grown in minimal medium supplemented with 12C- or 13C-glucose (99 atom % homogeneously labelled; from Sigma-Aldrich, U.K.) as the sole carbon source (5 g/L), and 14N- or 15N-NH4Cl (98 atom % homogeneously labelled; Sigma-Aldrich, U.K.) as the only nitrogen source (1 g/L), resulting in four growth conditions (12C14N, 13C14N, 12C15N, and 13C15N). Bacterial cells were transferred to sterile 24-well plates (Greiner Bio-one, U.K.) as 1.5 mL aliquot replicates (*n* = 3) and incubated in a Multitron standard shaker incubator (INFORS-HT, Bottmingen, Switzerland) for 15 h at 37°C with 170 rpm shaking. In order to reduce sample loss by potential evaporation, the plates were sealed with sterile Breathe-Easy membranes (Sigma-Aldrich, U.K.).

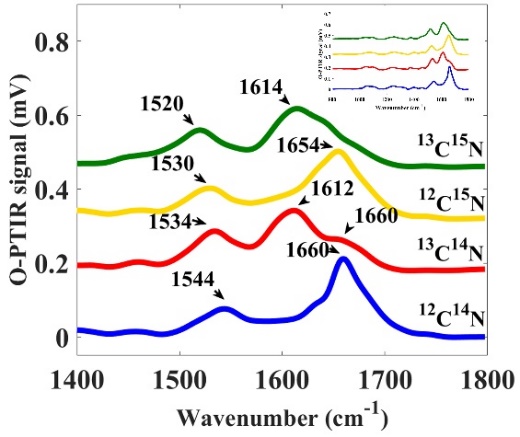
**Sample preparation:** After the incubation period, 1.3 mL aliquots from each of the samples were transferred to a 2 mL microcentrifuge tube and centrifuged for 10 min at 5000 x*g* using a benchtop Eppendorf Microcentrifuge 5424R (Eppendorf Ltd., Cambridge, U.K.). The supernatant was discarded and the biomass was resuspended in 2 mL of deionized water. A total of 5 µL of the cell suspension from each condition (12C14N, 13C14N, 12C15N, and 13C15N) was spotted onto four different CaF2 substrates and left to dry in a desiccator at room temperature. Additional 2 µL of each sample was placed on another substrate and mixed in order to generate O-PTIR chemical maps and thus to evaluate the signatures from labeled and unlabeled cells.

**O-PTIR spectroscopy:** The working principle of O-PTIR spectroscopy is based on measuring the photothermal response of a sample illuminated by infrared radiation. The infrared beam causes specific IR vibrations and thus induces a temperature increase that locally changes the sample surface and refractive index; therefore, the photothermal effect can manifest itself both mechanically and optically29. The magnitude of the photothermal effect is proportional to the infrared radiation that is being absorbed by the sample during vibration and is probed by measuring the intensity and propagation of a visible laser focused on the same spot illuminated by the infrared beam. Thus, O-PTIR spectroscopy can be described as a “pump–probe” technique. O-PTIR measurements were acquired on single-point and imaging mode using mIRage Infrared Microscope (Photothermal Spectroscopy Corp., Santa Barbara, USA), with the pump consisting of a tunable four-stage QCL device, while the probe beam is a continuous wave (CW) 532 nm laser. Spectral data were collected in reflection mode through a 40×, 0.78 NA, 8 mm working distance Schwarzschild objective. Single-point spectral data were acquired over the spectral region 800 to 1800 cm−1, with 2 cm−1 spectral resolution, and 50 scans per spectrum. Single frequency images were collected at 500 nm step size by tuning the QCL device to the frequencies corresponding to amide I and II vibrations (1660, 1614, 1544, 1534, 1530, and 1520 cm-1) of labeled and unlabeled cells. Instrument control and data collection were performed using PTIR Studio software supplied by the manufacturers.

**Data analysis:** Spectral data collected on single-point mode were subjected to principal component analysis (PCA). Spectra were submitted to baseline correction, smoothed via Savitzky-Golay filter using a polynomial of second order in an eleven-point window (of approximately 22 cm-1 size) and vector normalized and mean centered prior PCA. All collected data were processed using MATLAB software version 2011a (Mathworks Inc., Natwick, USA). Chemical maps showing isotope incorporation were reconstructed using multivariate analysis of partial least squares-discriminant analysis (PLS-DA). In this process PLS2 was used to predict 13C/12C and 15N/14N simultaneous. The input data (*X*-data) were 6 selected bands from the Amide I and II regions at 1520, 1530, 1535, 1544, 1614, and 1660 cm-1 from data extracted from the full spectral data from single cells (data from Fig. 1). The output data (*Y*-data) comprised two *Y* columns (one for C and the other for N) that encoded the isotope patterns where -1 -1 (was from cells comprising 12C 14N), +1 -1 (13C 14N), -1 +1 (12C 15N), +1 +1 (13C 15N). The PLS algorithm was calibrate with these data and 4 latent variables (PLS factors) were used for modelling. This model was then challenged with the combination of the 6 single frequency images (individually shown in Fig. 4) and the two *Y* outputs plotted as a gray colour maps where black was negative and white was positive.

RESULTS AND DISCUSSION

Figure 1 illustrates typical O-PTIR spectra acquired from *E. coli* bacteria at single cell levels grown on combinations of labeled (13C, 15N) and unlabeled (12C, 14N) carbon and nitrogen sources. Several shifts are observed on the spectral range associated with the amide I and II regions (1400-1800 cm-1), which have been previously reported as the main bands affected by the uptake of 13C-glucose and 15N-ammonium chloride30.



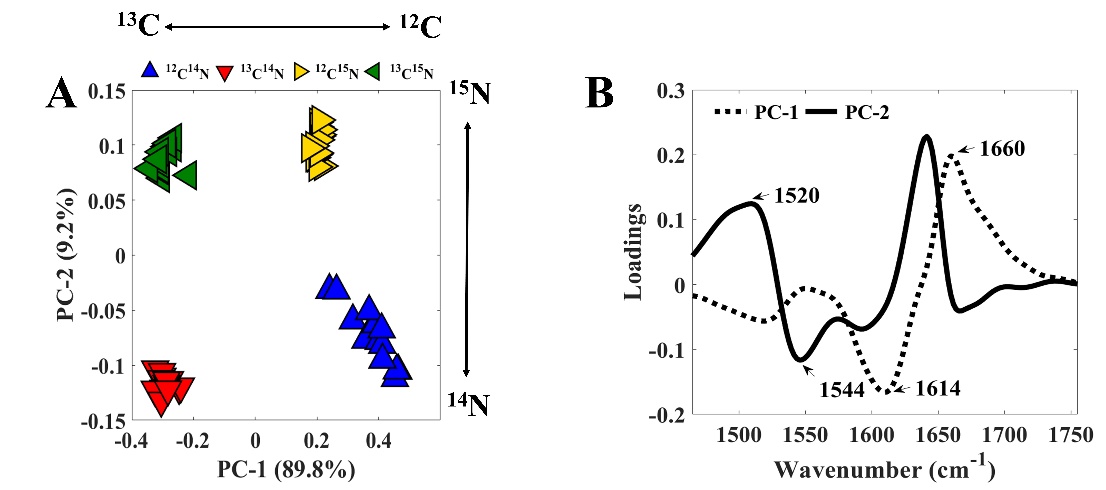
**Figure 1.** Amide I and II region (1400-1800 cm-1) of O-PTIR spectra acquired from single *E. coli* cells incubated with 12C14N (blue line), 13C14N (red line), 12C15N (yellow line), and 13C15N (green line). The fingerprint region (800-1800 cm-1) is illustrated on the top-right. The plots are offset for clarity.

The amide I vibration, peaking at 1660 cm-1 in unlabeled cells, arises mainly from the C=O stretching vibration coupled with smaller contributions from the out-of-phase C−N stretching, the CCN deformation and the NH in-plane bend from the backbone of proteins31. Two peaks (1612 and 1660 cm-1) are detected in the amide I region from spectra acquired from cells grown on 13C-glucose and unlabeled ammonium chloride. The vibration peaking at 1612 cm-1 arises from proteins of cells that incorporated 13C atoms and presented higher intensity compared to the signal at 1660 cm-1. Our findings indicate a shift of 6 cm-1 toward lower wavenumber region (1654 cm-1) from cells cultivated on 15N-ammonium chloride, which is probably due to the contribution of chemical bonds with nitrogen atoms that are coupled to the C=O stretching vibration from amide I. The shift induced by the incorporation of heavy atoms is more apparent in the 13C15N-labeled cells, which illustrated the highest shift (∆46 cm-1). Amide II mode is assigned to the out-of-phase combination of the N−H in plane bend (~60% contribution) and the C−N stretching vibration (~40% contribution) with smaller contributions from the C−O in plane bend and the C−C and N−C stretching vibrations31. Amide II from cells grown with unlabeled ammonium chloride and glucose is identified at 1544 cm-1. *E. coli* cells cultivated on 13C-glucose and 14N-ammonium chloride illustrated a shift of 10 cm-1 (1534 cm-1) towards lower wavenumbers, while the incorporation of 15N resulted in a shift to 1530 cm-1. Cells grown with both compounds illustrated amide II band peaking around 1520 cm-1. Regardless of the compound used to label isotopically the bacterial cells, all the altered peaks exhibited shifts toward the low wavenumber region (so called redshift), which is associated with an increase in the reduced mass (µrm) resulting from the incorporation of heavier isotopically labeled molecules by the cells30. Table 1 compares the spectral signatures obtained for amide I and II in the present study to our previous work30 using FTIR spectroscopy on bulk bacterial samples, to compare the alterations in the chemical vibrations of *E. coli* under similar growth conditions.

Table 1. Spectral signatures (peak maxima) obtained through FTIR and O-PTIR spectroscopies from labeled and unlabeled *E. coli* cells grown in similar conditions.

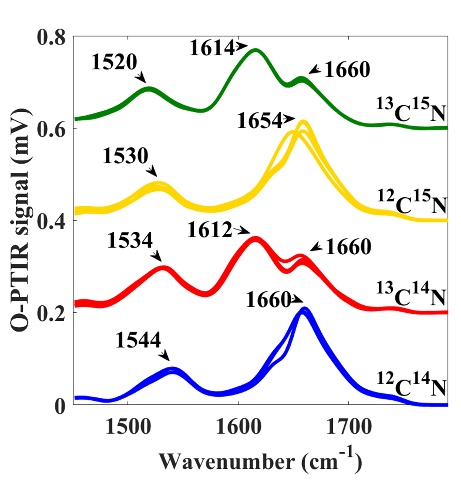
|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Amide I (cm-1)** | | **Amide II (cm-1)** | |
| Grown | Bulk  FTIR | Single cell  O-PTIR | Bulk  FTIR | Single cell  O-PTIR |
| **12C14N** | 1655 | 1660 ± 0.9 | 1545 | 1544 ± 0.5 |
| **13C14N** | 1616 | 1612 ± 0.5 / 1660 ± 1.72 | 1535 | 1534 ± 0.8 |
| **12C15N** | 1655 | 1654 ± 1 | 1530 | 1530 ± 1.4 |
| **13C15N** | 1616 | 1614 ± 1.28 | 1520 | 1520 ± 0.8 |

The effects of reduced mass on the detected vibrational frequencies were investigated in our previous work using FTIR spectroscopy, in which the percentage errors of the detected wavenumbers were less than 4% compared to their theoretically calculated reduced mass30. According to Table 1, chemical shifts obtained in the present study are in agreement with our previous findings30, therefore indicating that the magnitude of the chemical shifts are in-line with the values predicted by theory. Table 1 shows low standard deviations for peak positions obtained through O-PTIR, whereas data collected via FTIR spectroscopy presented zero standard deviation. These findings reflect the high reproducibility of chemical shifts from cells within same preparation as well as the superior ability of O-PTIR to probe biochemical changes at single cell level. Peak position of amide I obtained by O-PTIR and FTIR spectroscopies are slightly different. It is important to notice that data acquired by both technologies may vary due to the ability of each method in probing bacterial cells at community (bulk) and single cell levels. The signal obtained through FTIR is the combination of signatures from many individual cells in a community that interact with the relatively large area of the



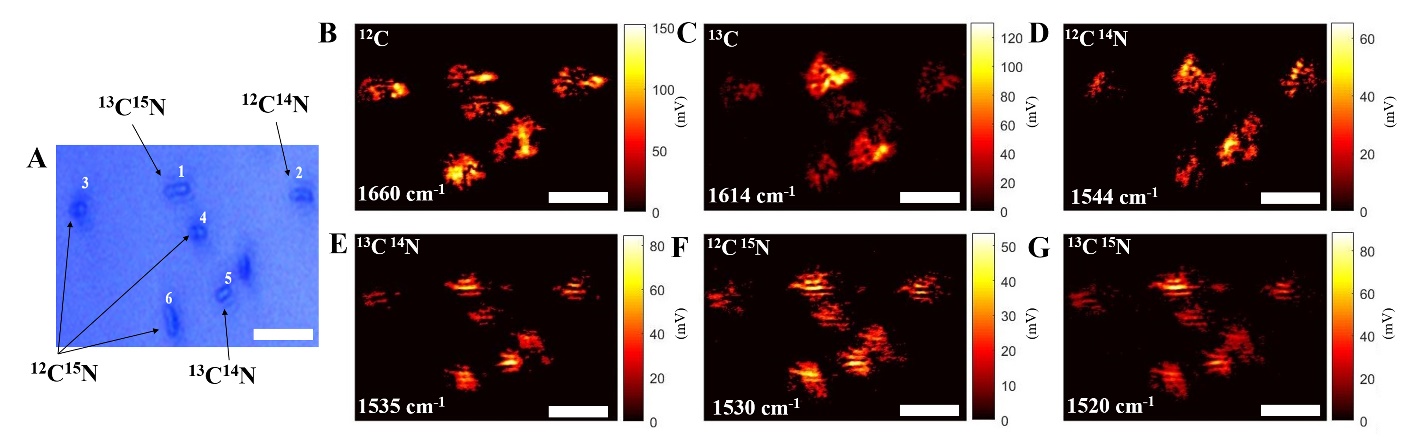
**Figure 2.** (a) PCA scores plot of preprocessed O-PTIR spectra of single *E. coli* cells grown with 12C/13C-glucose and/or 14N/15N-ammonium chloride where each point is the score related to the spectrum from a single bacterium; values in parentheses are the percentage total explained variance (TEV). (b) PCA loadings plot of the O-PTIR spectral data with PC1 (dashed line) and PC2 (full line) showing the major peaks affected by the labelling process and contributing to the separation.

polychromatic IR beam (for FTIR this was ~1 mm supplied by heated SiC30). On the other hand, the higher spatial resolution achieved by O-PTIR provides accurate information from bacteria at single cell levels and reveals the variation in the metabolic activity of the cells in the community. Thus, O-PTIR allows one to monitor the uptake of heavy atoms by individual cells, whereas FTIR spectroscopy provide the average signal from a community of cells illuminated by the IR beam. Therefore, spectral differences may be expected when comparing results obtained by both techniques. To examine the ability of O-PTIR to differentiate bacteria cultivated on 12C/13C-glucose and/or 14N/15N-ammonium chloride, single-point spectra were recorded on CaF2 substrates containing dispersed single bacteria grown in the four conditions and the resultant spectra were subjected to principal component analysis (PCA) in order to evaluate any specific separation resulting from the isotopic incorporation. The multivariate analysis investigation of the data was carried out using the amide I and II region (1450-1800 cm-1) of spectra as input data.



**Figure 3.** O-PTIR spectra acquired from a substrate containing a mixture of *E. coli* cells (*n* = 14) grown on 12C/13C-glucose and/or 14N/15N-ammonium chloride. The spectral signatures indicate three unlabeled cells (blue line); five 13C-labeled bacteria (red line); four 15N-labeled cells (yellow line), and two bacteria labeled with both compounds (green line). The plots are offset for clarity

The resultant PCA scores plot (Figure 2a) displayed a clear separation between spectra of cells grown with 13C/12C and 15N/14N. The first principal component (PC-1), which accounts for 89.8% of the total explained variance (TEV), retains information related to the effects of 13C/12C-glucose. 13C-labeled cells (13C14N and 13C15N) were grouped on the negative side of PC-1 axis, while bacteria grown on 12C-glucose (12C14N and 12C15N) were on the positive side. Consequently, PC-1 illustrated positive loadings on 1660 cm-1 and negative loadings for 1614 cm-1 (Figure 2b), which correspond to the vibrations of amide I from unlabeled and 13C-labeled cells. By contrast, the effects of 14N/15N-ammonium chloride on cells can be observed along PC-2 axis, which accounts for only 9.2% of TEV. 15N-labeled cells (12C15N and 13C15N) were grouped on the positive side of PC-2, whereas bacteria cultivated on 14N-ammonium chloride (12C14N and 13C14N) were clustered on the negative side. The loadings indicated that discrimination obtained along the second principal component is mainly related to amide II vibrations (1548 cm-1 from unlabeled bacteria and 1520 cm-1 from 15N-labeled cells). These findings indicate that amide I vibrations largely accounts in classifying cells cultivated on 13C/12C-glucose, while amide II retains information associated to the incorporation of 15N atoms into the cells. The lower contribution of amide II in the separation of bacteria grown in different conditions is perhaps not surprising considering that the amide I signature is mainly dominated by the C=O vibrations and the contribution of nitrogen atoms are minimal. Amide I band is also more intense than amide II peak due to the change in dipole moment with respect to the distance (∂µ/∂x) of C=O stretching vibrations is higher than the variation associated to the vibrations of amide II (mainly N−H in plane bend and the C−N stretching vibrations). Furthermore, the band peaking at 1614 cm-1 (amide I) due to the incorporation of 13C atoms by bacteria arises in a spectral region with relatively less signal interference from other vibrations, compared to the shift associated to the amide II (1520 cm-1) for the labeled cells, indicating the major contribution of 13C rather than 15N isotopes on the data discrimination achieved by PCA. These findings are in entire agreement with our previous results, where we analyzed bulk cells with FTIR, and both bulk and single cell



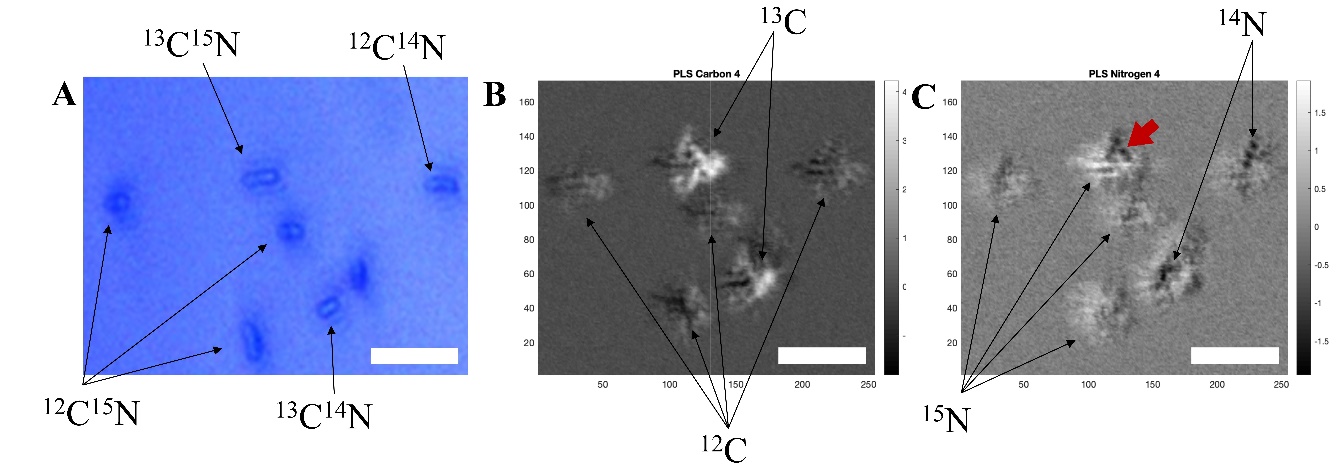
**Figure 4.** (A) Optical image showing one unlabeled bacterium (cell 2); one 13C-labeled bacterium (cell 5), three cells grown on 15N-ammonium chloride (cells 3, 4, 6), and one cell cultivated on both isotopically labelled compounds (cell 1). (B-G) Single wavenumber images collected through O-PTIR imaging by tuning the QCL device to the frequencies corresponding to amide I and II vibrations on unlabeled and labelled cells (1660, 1614, 1544, 1534, 1530, and 1520 cm-1. Scale bar is 2 µm

infrared measurements showed a higher contribution 13C labelling in the PCA model which could be due to higher carbon content in comparison to nitrogen (C/N ratio 5.9±1.1)30,32.

Spectral data were also recorded from a substrate containing a mixture of unlabeled and labeled cells (*n* = 14 cells) in order to evaluate the ability of O-PTIR in identifying the condition that the cells were grown within an artificial community. The signatures obtained for all 14 cells are shown in Figure 3, in which it is possible to identify three unlabeled cells; five 13C-labeled bacteria; four 15N-labeled cells, and two bacteria labeled with both elements. Spectral data of all 14 cells were subjected to PCA and the results were similar to those obtained from bacterial cells grown in different conditions and analyzed individually, which was shown in Figure 2: 12C/13C-labeled cells are discriminated along PC-1 (93.1%), whereas bacteria cultivated on 14N/15N-ammonium chloride are grouped along PC-2 (3.4%) (Figure S1a). The loadings related to PC-1 indicate major contributions of amide I, while PC-2 is mostly dominated by vibrations peaking at amide II region (Figure S1b).

The comparison of loadings related to PC-1 and PC-2 obtained from spectra recorded from labeled and unlabeled cells on the same substrate, as well as from different substrates, containing bacteria cultivated on each condition (Figure S1c) illustrated very similar findings, indicating the ability of O-PTIR in identifying the uptake of the different isotopically labelled substrates.

Although infrared spectroscopy has been extraordinarily successful as a tool for investigating biochemical features of bacterial cells, the low spatial resolution achieved by infrared microspectrometers where the IR light is supplied by a globar, or indeed Synchrotron source, have limited the study of bacterial organisms to the analysis of bulk samples and only a few studies using AFM-IR or IR-SNOM have evaluated a bacterium at the single cell level. To date, there is no reported study of imaging isotopically labeled bacteria using infrared spectroscopy. Besides the higher spatial resolution achieved by O-PTIR in comparison to FTIR spectroscopy, O-PTIR imaging enables the acquisition of discrete frequency images due to the tunable infrared laser (Quantum Cascade Laser, QCL) device used as infrared source, which provides information about the distribution of chemical species while improving the speed of data collection compared to the time-consuming hyperspectral images obtained by classical infrared microspectroscopy. Thus, single wavenumber images were collected by tuning the QCL device to the frequencies corresponding to amide I and II vibrations (1660, 1614, 1544, 1534, 1530, and 1520 cm-1 (identified from Figure 1 and Table 1) on labeled and unlabeled cells from a region of interest containing cells grown in different conditions (*n* = 6 cells) in order to evaluate the ability of O-PTIR in identifying the incorporation of 13C/15N atoms by cells on imaging mode. The optical image shown in Figure 4a illustrates the region of interest containing seven unlabeled and labeled bacterial cells. Single-point O-PTIR spectral data acquired from cells numbered 1-6 are illustrated in Figure S2, in which we identified one unlabeled bacterium (cell 2); one 13C-labeled bacterium (cell 5), three cells grown on 15N-ammonium chloride (cells 3, 4, 6), and one cell cultivated on both isotopically labelled compounds (cell 1). Single frequency images obtained for wavenumbers associated to amide I and II are illustrated on Figure 4b-g. Bacterial cells grown on 13C-glucose can be easily identified in the single frequency image obtained for 1614 cm-1 (Figure 5c) due to the high O-PTIR signal recorded for the cells that were numbered as 1 (13C15N) and 5 (13C14N) in Figure 5a. On the other hand, the localization of bacteria cultivated on 15N-ammonium chloride is not so clear. As previously described, 15N-labeled cells are mainly discriminated by information retained in the amide II vibrations. However, O-PTIR signal recorded from wavenumbers corresponding to the peak positions of amide II in labeled and unlabeled cells (1520, 1530, 1535, 1540 cm-1) provided poor contrast for the identification of 14N/15N-labeled bacteria due to the relatively similar intensities registered in the surrounding wavenumbers. By contrast, the main effects of the incorporation of 13C atoms are observed in the vibrations associated to amide I, in which 13C-labeled bacteria present a peak arising at 1614 cm-1, which has relatively less signal interference from other vibrations, therefore enhancing the contrast between the cells that were grown on 13C/12C-glucose on the images.



**Figure 5.** (A) Optical image displaying labelled and unlabeled bacterial cells. (B, C) Images reconstructed using the outputs obtained through PLS2 applied to the O-PTIR signal extracted from bands peaking at 1520, 1530, 1535, 1544, 1614, and 1660 cm-1 from single cell data shown in Figure 1. (B) Predictions of 13C/12C where dark pixels indicate 12C-labelled cells and light pixels 13C incorporation into bacteria. (C) Predictions of 15N/14N where dark pixels show 14N-labelled cells and light pixels 15N incorporation. The cell highlighted by the red arrow presents both dark and light pixels, which may be associated to the incorporation of both 13C and 15N atoms. Scale bar is 2 µm.

Single-frequency images were subjected to PLS in order to enhance the contrast between labelled and unlabeled cells. Figure 5b shows the distribution of cells grown on 13C/12C-glucose, in which the highlighted cells represent 13C-labeled bacteria. These findings agree with the results shown on Figure 4c. Figure 5c depicts the image reconstructed by using the PLS component related to the incorporation of 15N atoms by the cells, in which the dark pixels indicate cells cultivated on 14N-ammonium chloride, and light pixels those that have incorporated 15N into their biomass. The cell highlighted by the red arrow presents both dark and light pixels, which may be associated to the incorporation of both 13C and 15N atoms. All chemical images provided information about the localization and number of cells that were probed by the laser. However, the morphological details obtained in the images such as shape and size were not clear representation of *E. coli* cells, as the rod-shaped morphology typically observed for *E. coli* was not clearly evident in these single wavenumber chemical images.

CONCLUSIONS

In this study, O-PTIR spectroscopy was used as a tool to monitor the incorporation of 13C-glucose and 15N-ammonium chloride by *E. coli* bacteria at single cell level using spectral signatures recorded via single-point and single wavelength imaging modes. This new method holds a number of key advantages over classical infrared spectroscopy such as higher spatial resolution, absence of spectral distortions due to scatter artifacts (e.g., Mie scattering), simple sample preparation, the measurement is contactless, as well as the acquisition of discrete frequency images in a fast manner, which enables to evaluate the distribution of chemical species on the sample. We have shown that O-PTIR spectra acquired on single-point mode can be used to identify the incorporation of stable-isotope-labeled compounds such as 13C-glucose and 15N-ammonium chloride by *E. coli* cells.

We have previously used Raman spectroscopy to measure metabolism in single bacteria by distinguishing isotopically labeled cells30; however, this study did not include imaging (chemical mapping) due to the long collection times needed to collect Raman spectra of sufficient quality. The current study builds on this previous work and shows that O-PTIR can also be used to characterise single bacterial cells that have incorporated stable-isotope-labeled compounds. We found significant advantages of O-PTIR are both the speed of analysis and its non-destructive nature.

Regarding imaging with O-PTIR, we were able readily to identify cells grown on different carbon sources (12C or 13C) and despite the lack of accuracy in identifying the incorporation of 15N atoms by bacteria as well as the morphological details such as shape and size of the cells, all chemical images provided information about the localization and number of cells that were probed by the laser. Hence, this emerging new technique presents further opportunities as a whole-organism fingerprinting tool to investigate the metabolism of a single bacterium in a range of biological applications, shedding light on the interaction between microorganisms as well as the role of each individual in microbial communities

ASSOCIATED CONTENT

**Supporting Information**

This material is available free of charge at <http://pubs.acs.org>:

Figure S1, PCA scores and loadings from O-PTIR spectra; Figure S2, O-PTIR spectra from cells in Figure 4a (PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes  
MK is employed by Photothermal Spectroscopy Corporation who manufacture the O-PTIR instrument used in this study. All other authors declare no competing financial interest.

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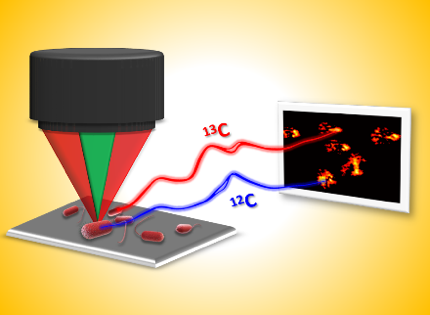
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