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 nitrogen-fixing bacteria in soil communities by
 resonance Raman spectroscopy with <sup>15</sup>N<sub>2</sub> labelling

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### TOC/Abstract art



23 ABSTRACT

Nitrogen (N) fixation is the conversion of inert nitrogen gas (N<sub>2</sub>) to bioavailable N 24 25 essential for all forms of life. N2-fixing microorganisms (diazotrophs), which play a key role in global N cycling, remain largely obscure because a large majority are 26 27 uncultured. Direct probing of active diazotrophs in the environment is still a major challenge. Herein, a novel culture-independent single-cell approach combining 28 resonance Raman (RR) spectroscopy with <sup>15</sup>N<sub>2</sub> stable isotope probing (SIP) was 29 developed to discern N<sub>2</sub>-fixing bacteria in a complex soil community. Strong RR 30 signals of cytochrome c (Cyt c, frequently present in diverse N<sub>2</sub>-fixing bacteria), 31 along with a marked <sup>15</sup>N<sub>2</sub>-induced Cyt c band shift, generated a highly 32 distinguishable biomarker for  $N_2$  fixation. <sup>15</sup> $N_2$ -induced shift was consistent well with 33 <sup>15</sup>N abundance in cell determined by isotope ratio mass spectroscopy. By applying 34 this biomarker and Raman imaging, N<sub>2</sub>-fixing bacteria in both artificial and complex 35 soil communities were discerned and imaged at the single-cell level. The linear band 36 shift of Cyt c versus  ${}^{15}N_2$  percentage allowed quantification of  $N_2$  fixation extent of 37 diverse soil bacteria. This single-cell approach will advance the exploration of 38 hitherto uncultured diazotrophs in diverse ecosystems. 39

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41 Keywords: single-cell resonance Raman,  $N_2$ -fixing bacteria,  ${}^{15}N_2$  stable isotope 42 probe, cytochrome c, soil community

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### 45 **INTRODUCTION**

Nitrogen (N) is an essential element sustaining all forms of life on Earth. Biological 46 fixation is a critical process converting inert nitrogen gas (N<sub>2</sub>) to bioavailable N 47 48 (ammonia or nitrate) required by all living organisms for biosynthesis. It has been estimated that over half of the fixed N sustaining the world's population is supplied 49 by biological fixation.<sup>1,2</sup> This process is exclusively performed by bacteria and 50 archaea (diazotrophs) in free-living form<sup>3</sup> or in symbiosis with coral,<sup>4</sup> higher plants,<sup>5</sup> 51 or animals in different ecosystems.<sup>6-9</sup> Despite the importance of biological N<sub>2</sub> 52 53 fixation, active diazotrophs along with their distribution in ecosystems, remain largely unknown.<sup>10,11</sup> One important reason is that a large proportion of diazotrophs 54 55 remain uncultured to date.

56 Molecular methods exploiting nitrogenase *nifH* genes have been widely used to evaluate the potential of N<sub>2</sub> fixation in diverse ecosystems in a culture-independent 57 fashion.<sup>9,10,12,13</sup> Microorganisms expressing *nifH* genes, including a large portion of 58 uncultured bacteria, were reported to have the genetic potential for  $N_2$  fixation.<sup>7,10,14</sup> 59 However, discordance between presence or transcription of nifH and N2-fixation 60 activity has been revealed,<sup>12,13</sup> suggesting that species with genetic potential do not 61 necessarily fix N<sub>2</sub>. Hence, a functional or phenotypic means of identification of 62  $N_2$ -fixing microorganisms is urgently needed. Stable isotope probing (SIP) with  ${}^{15}N_2$ 63 is reported to be the only direct and unambiguous means for identifying N2-fixing 64 bacteria and quantifying biological N<sub>2</sub> fixation.<sup>15,16</sup> Combining <sup>15</sup>N<sub>2</sub> SIP with 65 single-cell level characterization provides additional advantages of allowing one to 66 bypass the need for culture, whilst imaging of distribution of N<sub>2</sub>-fixation species in 67 complex communities such as bacteria-protist symbionts.7 15N2-SIP combined with 68 high spatial resolution secondary ion mass spectroscopy (SIMS) - especially 69

70 NanoSIMS - is one of the very few available techniques that can probe active N<sub>2</sub>-fixing bacteria at the single-cell level.<sup>6,7,10,12,17,18</sup> By applying single-cell 71 NanoSIMS to analyze <sup>15</sup>N<sub>2</sub>-incubated sample, a novel cyanobacterial group actively 72 fixing N<sub>2</sub> was discovered in a microbial mat; in contrast, despite expressing genetic 73 potential, a  $N_2$ -fixing deltaproteobacteria was excluded due to a lack of  ${}^{15}N$ 74 enrichment.<sup>10,12</sup> NanoSIMS imaging of an in vivo <sup>15</sup>N-labeled protist inhabiting 75 wood-eating roach revealed N<sub>2</sub> fixation in bacterial ectosymbionts of protist.<sup>7</sup> These 76 applications advance the exploration of uncultured diazotrophs. Nevertheless, SIMS 77 78 is a destructive approach and thus does not allow important downstream genomic sequencing or even cultivation of active N<sub>2</sub>-fixing cells. 79

80 Raman spectroscopy is an attractive non-destructive method capable of providing 81 information about the chemical bonds of various biomolecules of bacteria and discerning functional bacteria at the single-cell level.<sup>19,20</sup> For some cells with 82 specific pigments such as cytochrome c (Cyt c), carotenoids or chlorophyll, strong 83 resonance Raman (RR) signals can be excited if the laser wavelength is within - or 84 close to - the electronic transition of molecules.<sup>21-27</sup> Selective enhancement of RR 85 generates a highly characteristic Raman feature of pigment-containing bacteria. 86 Measurement time can also be greatly reduced, facilitating a relatively rapid spectral 87 acquisition such as Raman imaging. Raman imaging is similar to NanoSIMS 88 89 imaging by providing information regarding the spatial distribution of bacteria in microbial communities.<sup>21,23</sup> More importantly, in combination with <sup>13</sup>C or D<sub>2</sub>O-SIP, 90 single-cell Raman or RR can detect functional or active cells in their natural habitat, 91 based on the Raman shift induced by substitution of 'light' atom in a chemical bond 92 by its 'heavier' isotope.<sup>19,28,29</sup> Despite these advantages, <sup>15</sup>N-induced shifts are far 93 less distinguishable than <sup>13</sup>C or <sup>2</sup>D-induced, and the few reports of single-cell Raman 94

with <sup>15</sup>N-SIP were all limited to pure-cultured bacteria.<sup>19,30</sup> Recently, owing to the 95 finding of clear <sup>15</sup>N-induced shifts in surface-enhanced Raman spectra (SERS) of 96 bacteria,<sup>19,31</sup> SERS was successfully applied for bulk analysis of N assimilation by 97 environmental bacterial community in a wetland.<sup>31</sup> However, because SERS 98 substrates can easily be contaminated by environmental medium and damaged by 99 laser, single-cell SERS has not been achieved in complex environmental microbial 100 communities. In this regard, normal Raman or RR spectroscopy is more feasible 101 towards investigating environmental bacteria. To date, there is no report using 102 103 single-cell Raman to study N<sub>2</sub>-fixing bacteria in complex ecosystems. Identifying an indicative Raman band associated with N2 fixation is the priority for the potential 104 applicability of this approach. 105

Herein, we showed for the first time that  ${}^{15}N_2$ -induced shifts in the resonance Raman band of Cyt c are a sensitive and robust indicator of N<sub>2</sub>-fixation. This biomarker was then used to discern and image the location of N<sub>2</sub>-fixing bacteria in both artificial and complex soil communities at the single–cell level. The linear band shift *versus*  ${}^{15}N_2$  percentages provided a good means to compare N<sub>2</sub> fixation extent of diverse soil bacteria. This work provides a novel single-cell resonance Raman approach to discern, image and compare fixation extent of N<sub>2</sub>-fixing soil bacteria.

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### 114 MATERIALS AND METHODS

115 **Strains, media and growth conditions.** Four model  $N_2$ -fixing bacteria, including 116 two free-living *Azotobacter* sp. (AS1.222) and *Azotobacter chroococcum* 117 (ACCC10096), two symbiotic *Rhizobium radiobacter* (ATCC15955) and 118 *Bradyrhizobium japonicum* (ACCC15067), were purchased from Guangdong 119 Culture Collection Center of Microbiology, China. *Azotobacter* sp. and *A*.

chroococcum were cultured in N-free azotobacter media containing 20 g  $L^{-1}$ 120 mannitol, 0.2 g  $L^{-1}$  KH<sub>2</sub>PO<sub>4</sub>, 0.8 g  $L^{-1}$  K<sub>2</sub>HPO<sub>4</sub>, 0.2 g  $L^{-1}$  MgSO<sub>4</sub> 7H<sub>2</sub>O, 0.1 g  $L^{-1}$ 121 CaSO<sub>4</sub> 2H<sub>2</sub>O, and trace amount of Na<sub>2</sub>MoO<sub>4</sub> 2H<sub>2</sub>O and FeCl<sub>3</sub> at 28 °C and 180 rpm. 122 *R. radiobacter* and non-N<sub>2</sub>-fixing strain *Shewanella oneidensis* MR-1 were grown in 123 Luria Bertani (LB) broth containing 10 g L<sup>-1</sup> tryptone (Oxoid Ltd., England), 5 g L<sup>-1</sup> 124 yeast extract (Oxoid Ltd., England), and 10 g  $L^{-1}$  NaCl at 28 °C and 180 rpm. B. 125 *japonicum* were grown in media containing 1 g  $L^{-1}$  yeast exact, 200 mL of soil 126 extract and 10 g L<sup>-1</sup> mannitol at 28 °C and 180 rpm. Unless otherwise stated, 127 chemicals were purchased from Sinopharm Chemical Reagent Co., China. 128

Soil sample collection. Park soil samples were collected from grassland at a depth < 5 cm in the campus of Institute of Urban Environment, Xiamen, China (24°36′39.90″N 118°03′33.48″E). The soil samples were homogenized and sieved through a 0.6-mm sieve to remove small stones, grass roots and other debris, then stored at 4 °C prior to use.

Incubation of model N<sub>2</sub>-fixing bacteria and soil microcosm with <sup>15</sup>N<sub>2</sub> gas. An 134 aliquot of 20 mL N-free azotobacter media was filled into a 40-mL crimp-top vial 135 136 and then inoculated with 20 µL of Azotobacter sp. or A. chroococcum after 24-hour culture. Vials were sealed, and air in the headspace was replaced with mixture gas 137 consisting of  ${}^{15}N_2$  (99 atom%, purity >98.5%, Aladdin, China) and oxygen (volume 138 ratio of N<sub>2</sub> to O<sub>2</sub> is 4:1) of different volumes to achieve  ${}^{15}N_2$  of different percentages 139  $({}^{15}N_2/({}^{15}N_2+{}^{14}N_2))$ . Considering that  ${}^{15}N$  content is 99% in commercial  ${}^{15}N_2$  and 0.36% 140 in natural abundance,<sup>32</sup> the final  $^{15}$ N abundance relative to N<sub>2</sub> in air in the headspace 141 was 99.36%, 49.68%, 25.02%, 10.22%, 0.36%, respectively. Bacteria incubated with 142 different percentages of <sup>15</sup>N<sub>2</sub> were harvested after culturing for 48 h. Soil microcosm 143 contained 2 g of park soil in a 12 mL crimp-top vial. Vials were sealed, purged with 144

145  $O_2$  for 10 min to remove air and then replaced with appropriate volume of  ${}^{15}N_2$  to 146 achieve 80%  ${}^{15}N_2$  and 20% oxygen in the headspace. The control soil microcosm 147 was supplied with lab air.  ${}^{15}N_2$ -incubated soil microcosm and control were amended 148 with 500 uL of 0.5 M glucose solution and incubated at room temperature (ca. 25 °C) 149 under low light conditions for 12 days. All of these incubations were performed in 150 triplicate.

Extracting bacteria from soil microcosms by Nycodenz density gradient 151 separation. Nycodenz density gradient separation was used to extract bacteria from 152 soil. A modified protocol from a previous report was used.<sup>17</sup> Briefly, soils 153 post-incubation were homogenized in 10 mL PBS (NaCl 8 g L<sup>-1</sup>, KCl 0.2 g L<sup>-1</sup>, 154 Na<sub>2</sub>HPO<sub>4</sub> 1.44 g L<sup>-1</sup>, KH<sub>2</sub>PO<sub>4</sub> 0.24 g L<sup>-1</sup>) supplemented with 0.5% (v/v) Tween 20 155 (Aladdin) to detach soil particle-associated cells by vigorously vortexing for 30 min 156 at room temperature. To separate bacteria from soil particles, 1 vol of the 157 as-prepared soil slurries were introduced into an Eppendorf tube containing 1 vol of 158 Nycodenz ( $\geq$ 98%, Aladdin) stock solution carefully. Nycodenz stock was prepared 159 by dissolving 8 g of Nycodenz in 10 mL sterile water, producing a final density of 160 1.42 g·mL<sup>-1</sup>. This density is proper for efficient capture of soil bacteria and 161 separation from soil particles.<sup>17,33</sup> The tube was then centrifuged at 14000 g for 90 162 163 min at 4 °C. The upper and middle aqueous layers containing bacteria were collected and mixed with 10 vol PBS in a fresh centrifuge tube. The bacteria inside were then 164 collected by centrifuging at 5000 rpm for 10 min and washed with ultrapure water 165 twice for further Raman analysis. 166

Single-cell Raman measurement and Raman mapping acquisition. To prepare
bacteria for single-cell Raman measurements, bacterial solution from model strains
or soil samples were washed twice by DI water by centrifuging at 5000 rpm for 3

170 min and then adjusting to a proper concentration in order to obtain single-cell dispersion on aluminum (Al) foil substrate.<sup>34</sup> An aliquot of 2 µL of the as-prepared 171 bacteria were loaded on Al foil and air-dried at room temperature. To construct an 172 artificial community consisting of Azotobacter sp. and S. oneidensis, one bacterial 173 solution was applied to the Al foil and allowed to air-dry whereupon the second was 174 applied to the same spot and also allowed to dry. Single-cell Raman spectra and 175 Raman mapping were obtained using a LabRAM Aramis (HORIBA Jobin-Yvon) 176 177 confocal micro-Raman system equipped with a 600 g/mm grating. Excitation was provided by a 532-nm Nd: YAG laser. A 100× dry objective with a numerical 178 aperture of 0.9 (Olympus) was employed. For single-cell Raman measurements, 179 180 acquisition time was 5 s and 25 spectra were acquired from each bacterial sample. Raman mapping was employed to generate Raman images of artificial communities 181 and also soil bacterial community containing N<sub>2</sub>-fixing bacteria. The step-size was 182 set at 1.5 µm in a rectangular mapping area with acquisition time of 2 s on each 183 184 point.

Raman spectral and mapping data analysis. LabSpec5 software (HORIBA 185 Jobin-Yvon) was used to process single-cell Raman spectra. After spectral extraction 186 187 and baseline subtraction (polynomial, degree 6), mean spectra from each sample were calculated. The overlapped 1114 (C-<sup>15</sup>N) and 1129 cm<sup>-1</sup> (C-<sup>14</sup>N) bands were 188 deconvoluted using the GaussLorenz peak fitting function and the resulting peak 189 area was used to calculate the intensity ratio of these two bands. Principal 190 component analysis (PCA) and the required spectral pre-processing were performed 191 using IRootLab toolbox (https://code.google.com/p/irootlab/) running on MATLAB 192 2012a.<sup>35,36</sup> Spectral pre-processing included spectral extraction (from 1095 to 1145 193 cm<sup>-1</sup>), rubberband subtraction and vector normalization. PCA was then performed by 194

195 reducing spectral variables to 10 factors accounting for more than 99% of the total variance. The resulting PCA 1-D (PC1) scores were plotted against  ${}^{15}N_2$  percentages. 196 OriginPro8.5 was used to perform statistical calculation of average value  $\pm$  standard 197 deviation and linear regression fitting. One-way ANOVA with Tukey's Multiple 198 comparison test was conducted in GraphPad Prism 5 for significance analysis of 199 <sup>15</sup>N-induced Raman band change; P < 0.05 was considered significant. Direct 200 classical least square (DCLS) modelling in Labspec 5 was employed to construct a 201 202 Raman image of N<sub>2</sub>-fixing and non-N<sub>2</sub>-fixing bacteria based on multidimensional Raman mapping data matrix. C-<sup>15</sup>N band at 1114 cm<sup>-1</sup> and C-<sup>14</sup>N at 1129 cm<sup>-1</sup> were 203 selected as the model, and then DCLS operation finding the linear combination of 204 205 model spectra that matched most closely the original data was applied to all traces of the original data sets to construct Raman image of each component. 206

Quantification of <sup>15</sup>N incorporation in N<sub>2</sub>-fixing bacteria by isotope ratio mass 207 spectroscopy (IRMS). To measure the bulk isotope composition (<sup>15</sup>N %) of 208  $N_2$ -fixing bacteria incubated with different percentages of  ${}^{15}N_2$  to natural  $N_2$  in air, 209 0.01-0.05 mg (dry weight) of <sup>15</sup>N-labeled lyophilized bacteria and 0.15-0.18 mg of 210 urea was placed in a tin capsule. For non labeled N2-fixing bacteria, 1 mg of 211 lyophilized cell powder was put in a tin capsule. Urea of 0.30-0.34 mg was used as 212 213 standard. Samples were analyzed with an elemental analyzer (Flash HT 2000 Thermo Fisher) coupled via a ConFlo IV device to the IRMS (Delta V advantage). 214 Isotope composition was calculated using the equation below: 215

At<sup>15</sup>N%=( $M_{urea}$ ×46.67% ×0.367%+ $M_{bacteria}$ ×9.77% ×<sup>15</sup>N% )/( $M_{urea}$ ×46.67%+ $M_{bacteria}$ × 9.77%), where At<sup>15</sup>N% is the percentage of <sup>15</sup>N in total N and can be measured by IRMS,  $M_{urea}$  and  $M_{bacteria}$  are the weight of urea and bacteria in tin capsule, 0.367% is the natural abundance of <sup>15</sup>N in urea, 46.67% and 9.77% are the total nitrogen content in urea and bacteria respectively,  ${}^{15}N\%$  is the abundance of  ${}^{15}N$  in bacteria that will be calculated.

### 222 RESULTS AND DISCUSSION

# Resonance Raman spectra of cytochrome c is a common signal in diverse N<sub>2</sub>-fixing bacteria.

225 To identify a common Raman signal in diverse N<sub>2</sub>-fixing bacteria, we examined four model N<sub>2</sub>-fixing bacteria including two rhizobium-originated (B. Japonicum 226 and R. radiobacter) and two free-living (Azotobacter sp. and A. chroococcum), and 227 five environmental N<sub>2</sub> fixers isolated from soil by plating soil slurry onto N-free agar 228 plates. Only N<sub>2</sub>-fixing bacteria can form colonies on these agar plates since 229 atmospheric N<sub>2</sub> provided the sole nitrogen source required for their growth. These 230 231 soil N<sub>2</sub>-fixing isolates were affiliated to the genera of Azotobacter, Rhizobium and Raoultella based on 16S rRNA sequencing and phylogenetic analysis (Figure S1a). 232 These genera were reported to be from N<sub>2</sub>-fixer group.<sup>9,37</sup> To further confirm N<sub>2</sub> 233 fixation potential of these strains, Dinitrogenase reductase genes (nifH), which are 234 the most widely used marker gene to identity N<sub>2</sub>-fixing bacteria, were amplified and 235 236 visualized on agarose gel. All the model and soil N2-fixer isolates displayed a band specific to nifH genes, which, however, are absent in non N2-fixing S. oneidensis, 237 238 E.coli and non-templated control (Figure 1a). Phylogenetic analysis of nifH amplicons further supported that these five soil isolates were N<sub>2</sub> fixers (Figure S1b). 239

Single-cell Raman spectra were acquired from four model N<sub>2</sub>-fixing bacteria and all colonies (approximately 32) formed by N<sub>2</sub>-fixing soil bacteria (Figure 1b). It is interesting to find that all of them display characteristic Raman bands of Cyt c at 749 cm<sup>-1</sup> (pyrrole breathing mode), 1129 [v(C-N)], 1312 [ $\delta$ (C-H)], and 1589 cm<sup>-1</sup> [v(C-C)], which are almost the same as that of pure Cyt c. <sup>21,38,39</sup> Strong Cyt c signal

was also recently reported in symbiotic rhizobia isolated from legumes nodules.<sup>40</sup> 245 These observations demonstrate that Cyt c is commonly present in both free-living 246 and symbiotic N<sub>2</sub>-fixing bacteria of either model or environmental strains. The 247 existence of Cyt c in diazotrophs is associated with the crucial role of Cyt c in 248 protecting respiration as a terminal oxidase by rapid consumption of O<sub>2</sub> affecting the 249 activity of O<sub>2</sub>-labile nitrogenase.<sup>41</sup> In addition, intensities of Cyt c relative to Amide 250 I at 1664 cm<sup>-1</sup> were found to fluctuate in these  $N_2$ -fixer. Both the physiological state 251 of bacteria and the redox state of Cyt c were reported to affect Cyt c intensity. For 252 instance, significantly higher Cyt c signal was observed in Rhizobium 253 *leguminosarum* by. *viciae* isolated from nodules than those grown in culture;<sup>40</sup> 254 255 reduced state of Cyt c generated more intense signal than the oxidized state due to shift in electronic transition.<sup>23</sup> In addition, Raman signal of Cyt c is much stronger 256 than that of other intracellular bacterial constituents, such as bands at 1002 cm<sup>-1</sup> 257 (phenylalanine), 1240 cm<sup>-1</sup> (protein), 1450 cm<sup>-1</sup> (lipid) and 1664 cm<sup>-1</sup> (protein), 258 despite their higher abundance. Cyt c is a hemeprotein showing maximum electronic 259 absorption at around 550 nm,<sup>42</sup> matching well with the 532 nm laser and thus 260 generating a selective resonance Raman enhancement. 261

Not only N<sub>2</sub>-fixing bacteria show Cyt c RR peaks, RR signals of Cyt c have also been reported in nitrifier bacteria, annamonx bacteria, and electron-generating bacteria such as *S. oneidensis* (Figure 1b) and *Geobacter*.<sup>21,23</sup> Therefore, despite the fact that the strong and characteristic Raman signal of Cyt c makes it highly distinguishable, it is insufficient to identify N<sub>2</sub>-fixing bacteria because Cyt c is also present in other bacteria that are unable to fix nitrogen. Among the four RR bands of Cyt c, the band at 1129 cm<sup>-1</sup> was assigned to C-N stretch, providing a good potential target to incorporate  ${}^{15}N_2$  stable isotope into C-N. The resulting Raman shift would then provide additional evidence for N<sub>2</sub> fixation.

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# <sup>15</sup>N<sub>2</sub>-induced Raman shifts in cytochrome c are a biomarker of N<sub>2</sub>-fixing bacteria

274 To test this hypothesis, we incubated Azotobacter sp. and A. chroococcum in N-free medium with different percentages of  ${}^{15}N_2$  relative to N<sub>2</sub> in air, *i.e.*, 99.36%, 275 49.68%, 25.02%, 10.22% and 0.36% (<sup>15</sup>N natural abundance). Mean single-cell 276 Raman spectra from approximately 25 individual cells under each <sup>15</sup>N<sub>2</sub> incubation 277 conditions are shown in Figure 2a and 2b. It is notable to observe that the 1129  $\text{cm}^{-1}$ 278 band (C-N stretch) at 0.36%  $^{15}N_2$  shifted markedly to 1114 cm<sup>-1</sup> at 99.36%  $^{15}N_2$ . The 279 around 15 cm<sup>-1</sup> shift is very close to that the 13 cm<sup>-1</sup> shift observed in  ${}^{15}N$ 280 isotopically labeled pure Cyt c,<sup>43</sup> confirming that it is the substitution of light <sup>14</sup>N 281 with heavier <sup>15</sup>N in the C-N bond resulting in a decrease of vibrational frequency of 282 C-N stretch. By comparison, no obvious shifts were observed in other Cyt c bands 283 wherein N was absent: 749 cm<sup>-1</sup> (ring breathing), 1312 cm<sup>-1</sup> (C-H), 1589 cm<sup>-1</sup> (C-C), 284 or bacterial composition-related bands. Figure 2b shows the enlarged spectra 285 including exclusively 1114 and 1129 cm<sup>-1</sup> bands. With increasing  ${}^{15}N_2$ , the 1129 286 cm<sup>-1</sup> band (C-<sup>14</sup>N) decreased whilst the 1114 cm<sup>-1</sup> band (C-<sup>15</sup>N) increased. The 287 co-existence of 1129 cm<sup>-1</sup> and 1114 cm<sup>-1</sup> in individual bacteria indicates that only a 288 proportion of N in intracellular Cyt c is replaced by the heavier <sup>15</sup>N. 289

By deconvoluting these two bands using peak fitting in Labspec software (Figure S2), peak area ratio of 1114 cm<sup>-1</sup> band against the sum of peak area of 1114 cm<sup>-1</sup> and 1129 cm<sup>-1</sup> bands (Area<sub>1114</sub>/(Area<sub>1114</sub>+Area<sub>1129</sub>)) from individual spectrum were plotted against <sup>15</sup>N<sub>2</sub> percentages. A linear relationship with  $R^2$  of 0.968 was obtained.

To achieve a rapid analysis of large numbers of spectra from single cells and avoid possible errors in deconvolution of overlapped spectra, PCA was also performed on spectral profile between 1095 and 1145 cm<sup>-1</sup> (Figure 2c). Scores along PC1, accounting for 87.5% of variance, generated an even better linear relationship with <sup>15</sup>N<sub>2</sub> percentages ( $R^2 = 0.998$ ) than the peak area ratio ( $R^2 = 0.968$ ).

To validate Raman results of N<sub>2</sub> fixation, bulk isotope analysis by isotope ratio 299 mass spectroscopy was used to quantify how much N was fixed in bacteria. The 300 abundance of <sup>15</sup>N in bacteria was determined to be 0.36% (close to natural 301 abundance of <sup>15</sup>N), 5.89%, 15.01%, 29.97%, and 66.16%, and linearly increased 302 with percentage of atmospheric incubation  ${}^{15}N_2$  (Figure 2d), whereas non-N<sub>2</sub> fixer S. 303 oneidenis containing Cyt c and E. coli only displayed <sup>15</sup>N of natural abundance. This 304 measurement fully demonstrated that bacteria had incorporated  ${}^{15}N$  and a higher  ${}^{15}N_2$ 305 percentage resulted in a higher incorporation extent and Raman shift. A further 306 correlation analysis indicated that <sup>15</sup>N content of bacteria corresponded linearly to 307 PC1 scores (Figure 2e), providing a way to measure the extent of N<sub>2</sub> fixation by RR 308 spectral profile of Cyt c at single-cell level, protist.<sup>16,44,45</sup> <sup>15</sup>N abundance in bacteria 309 was around three-fifth of the  ${}^{15}N_2$  percentage, indicating that N<sub>2</sub> fixers were not fully 310 labeled by <sup>15</sup>N, consistent with Raman result that both 1114 and 1129 cm<sup>-1</sup> bands 311 were observed. Detection limit of Raman-<sup>15</sup>N<sub>2</sub> SIP was defined as the minimum <sup>15</sup>N<sub>2</sub> 312 that can induce significant spectral change. Spectral change was analyzed by PCA 313 and the resulting PC1 scores were used for significance analysis of <sup>15</sup>N-induced 314 spectral change via one-way ANOVA. Detection limit was determined to be 10.22% 315  $^{15}\mathrm{N}_2,$  corresponding to 5.89%  $^{15}\mathrm{N}$  abundance in bacteria, at which spectra were 316 significantly different from that in air (one-way ANOVA, P < 0.05). The linear band 317 shifts were also obtained in A. chroococcum (Figure S3) with similar slope and 318

intercept (y = 0.098x - 0.5059) to that of *Azotobacter* sp. (y = 0.091x - 0.5411) based on PC1 scores.

Bacteria without Cyt c like E. coli display peaks at around 1123 cm<sup>-1</sup> 321 (carbohydrate), which is close to  $C^{-14}N$  and  $C^{-15}N$  at 1129 and 1114 cm<sup>-1</sup> (Figure S4), 322 but much lower, thus will not interfere <sup>15</sup>N-induced shift. Bacteria containing 323 carotenoid show strong RR signal at 1155 and 1511 cm<sup>-1</sup>. Although the band at 1155 324 cm<sup>-1</sup> has some overlap with C-<sup>14</sup>N band of Cyt c at 1129 cm<sup>-1</sup>, it separates well with 325  $C^{-15}N$  band at 1114 cm<sup>-1</sup>, thus exerting no effect on identification of  ${}^{15}N_2$  fixation. 326 Cyanobacteria can also fix  $N_2$ ,<sup>12</sup> however, its fluorescence was too strong to observe 327 Raman signal (Figure S5), so RR combined with  ${}^{15}N_2$  is not enough to detect 328 cyanobacteria as N<sub>2</sub> fixers. The further application of SERS with <sup>15</sup>N<sub>2</sub> excited with a 329 laser out of fluorescence can provide a solution. 330

The above observations indicate that the marked  ${}^{15}N_2$ -induced shift in C-N bond of Cyt c is a sensitive and highly distinguishable biomarker for N<sub>2</sub>-fixing bacteria. The linear shift also provides a good means towards evaluating N<sub>2</sub>-fixing extent in a quantitative manner.

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### **Probing and Raman imaging of N<sub>2</sub>-fixing bacteria in artificial communities**

Important advantages of single-cell measurements lie in the ability to discern and image N<sub>2</sub>-fixing bacteria in a complex community. Herein, an artificial community comprising *S. oneidensis* and  $^{15}N_2$ -incubated *Azotobacter* sp. was constructed. Both species contain Cyt c, while only *Azotobacter* sp. can fix nitrogen. Raman imaging was used to discern and locate  $^{15}N_2$ -fixing *Azotobacter* sp. in this artificial community. 343 Figure 3a shows a photomicrograph of such an artificial community with Azotobacter sp. appearing as spherical and S. oneidensis as rod-shaped. Because of 344 their highly distinguishable shapes, the distribution of the two species is visually 345 relatively clear except when clumped on top of each other. By using the 1114 cm<sup>-1</sup> 346 and 1129 cm<sup>-1</sup> bands characteristic of  ${}^{15}$ N-labeled N<sub>2</sub>-fixing Azotobacter sp. and S. 347 oneidensis (<sup>14</sup>N) respectively (Figure 3c), Raman imaging was acquired and 348 pseudo-color Raman image was generated (Figure 3b). Red regions were identified 349 as Azotobacter sp. whilst green regions as S. oneidensis. The distribution of both 350 bacterial species in Raman images is consistent with the conventional 351 photomicrographs. For example, the encircled spherical Azotobacter sp. in the 352 photomicrograph can be found in the same place as that shown in red in the 353 354 corresponding Raman image, as is the case for S. oneidensis. This result demonstrates the accuracy of Raman imaging in indicating the site of N<sub>2</sub>-fixing 355 bacteria. In addition, Azotobacter sp. that were clumped with S. oneidensis (labeled 356 with arrows) were hard to confirm their presence based on the photomicrographic 357 image (Figure 3a), but can be conclusively identified to be Azotobacter sp. based on 358 the red spot in the Raman image, demonstrating the potential of Raman imaging in 359 locating N<sub>2</sub>-fixing cells in microbial communities. 360

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## 362 Probing and Raman imaging N<sub>2</sub>-fixing bacteria of different activities in soil 363 communities

We further applied  ${}^{15}N_2$  incubation and Raman imaging to reveal N<sub>2</sub>-fixing bacteria in soil microbial communities. Soils harbor a multitude of our Earth's biodiversity and also a high diversity of N<sub>2</sub>-fixing bacteria in either free form or in symbiosis with plants,<sup>46</sup> representing the main site wherein biological N<sub>2</sub> fixation is

368 naturally carried out. Herein, park soil was collected from a grassland that had been left fallow for a long period of time without application of fertilizer, providing a high 369 probability of detecting N<sub>2</sub>-fixing bacteria. Soil was placed in 12-ml vials filled with 370 a mixture gas of  ${}^{15}N_2$  ( ${}^{15}N$  99.36%) and O<sub>2</sub> at volume ratio of 4:1. After a 12-day 371 incubation, the soil samples were collected. To date, application of Raman 372 spectroscopy for single-cell investigations in soil systems has been very limited,<sup>17</sup> 373 because soil microorganisms are dispersed in a high background of soil particles. 374 Separation of bacteria from soil particles is a necessity for either Raman or SIMS 375 measurement.<sup>17</sup> Soil bacteria were detached from soil particles via gradient density 376 centrifugation reported previously.<sup>17</sup> High-background soil particles can be largely 377 reduced for both Raman and SIMS measurement. Figure 4 shows representative 378 379 Raman spectra of individual soil bacteria with different phenotypes, including N<sub>2</sub>-fixing bacteria identified by 1114 cm<sup>-1</sup> (C-<sup>15</sup>N) band (i), bacteria containing Cyt c 380 but unable to fix N<sub>2</sub> (ii), bacteria containing Cyt c and carotenoid of different 381 382 contents but unable to fix  $N_2$  (iii, iv), as well as bacteria without any pigments (v). The above finding indicates a high diversity of bacteria with different phenotypes in 383 soil. 384

Raman imaging was then applied to soil bacteria to discern and image N<sub>2</sub>-fixing 385 bacteria. Seven areas ranging from  $20 \times 20$  to  $30 \times 30 \ \mu\text{m}^2$  were mapped. Because of 386 the high diversity of soil bacteria, not every area contained both N2-fixer and non-N2 387 fixer. Two areas containing both were shown in Figure 5. The photomicrograph 388 (Figure 5a, 5b left) shows soil bacteria of different shapes and sizes, but provides no 389 information on function. In contrast, we can use spectral profile covering 1114 cm<sup>-1</sup> 390 and 1129 cm<sup>-1</sup> band (Figure 5c red and green) to discriminate N<sub>2</sub>-fixing bacteria 391 from non-N<sub>2</sub>-fixing bacteria. The resulting Raman images clearly reveal that the red 392

dots are N<sub>2</sub>-fixing bacteria (labelled as <sup>15</sup>N), and the green dots are non-N<sub>2</sub>-fixing 393 bacteria containing Cyt c (Figure 5a, 5b right). Encircled bacteria (labeled with 'no 394 Cyt c' in Figure 5a) do not exhibit any pigment signal (Figure 5c, bottom curve 395 labeled with 'no Cyt c') and thus appear as a dark point in the Raman image. Figure 396 5b shows photomicrographic and Raman images taken from another independent 397 location. A total of six N<sub>2</sub>-fixing bacteria were discerned in the two areas, including 398 two (labelled as <sup>15</sup>N-2 and <sup>15</sup>N-3) in close contact with non-N<sub>2</sub>-fixing bacteria. The 399 green irregular shape connected with the <sup>15</sup>N-2 red dot shows good consistency with 400 401 the photomicrographic image, indicating a sufficient spatial resolution in Raman images. These observations demonstrate that Raman images can discern N<sub>2</sub>-fixing 402 403 bacteria from soil communities based on their isotopic composition.

 $^{15}N_2$ -fixation extent by soil bacteria was also revealed based on spectral profile of 404 C-15N and C-14N bands. Single-cell Raman spectra from a total of 29 individual 405 N<sub>2</sub>-fixing soil bacteria including the six identified above were incorporated together 406 with the spectra of Azotobacter sp. incubated with different percentages of  ${}^{15}N_2$ 407 (Figure 2c). As a comparison, three Raman spectra from soil bacteria similar to 408 Azotobacter sp. incubated with 0.36% <sup>15</sup>N<sub>2</sub> were also incorporated; this clearly 409 indicates the presence of non- or weak N2-fixing bacteria in soil. PCA was then 410 performed on spectra of N2-fixer and non or weak N2-fixer from soil to generate 1-D 411 412 PCA scores (on the right of Figure 2c labeled as 'soil bacteria'). A larger variation in PC1 scores was observed in soil bacteria than Azotobacter sp. incubated with the 413 same 99.36% <sup>15</sup>N<sub>2</sub>, indicating heterogeneous N<sub>2</sub>-fixing extent from diverse soil 414 bacteria. Based on the linear correlation between <sup>15</sup>N content measured by IRMS and 415 PC1 scores of Azotobacter sp. incubated with different atmospheric <sup>15</sup>N<sub>2</sub>% (Figure 416 2e), <sup>15</sup>N content of soil bacteria were determined to be from 0 to 84.40 (on the right 417

of Figure 2e) by inputting PC1 scores of soil bacteria to the linear fitting equation of <sup>15</sup>N% in cells =  $38.79 + 75.18 \times PC1$ . The even larger <sup>15</sup>N incorporation extent in most N<sub>2</sub>-fixing soil bacteria than *Azotobacter* sp. should be related to the much longer incubation time of soil with <sup>15</sup>N<sub>2</sub> (12 days) than *Azotobacter* sp. (2 days).

### 422 CONCLUSIONS

This is the first demonstration that single-cell resonance Raman spectroscopy with 423  $^{15}N_2$ -SIP can discern, image and compare the extent of N<sub>2</sub>-fixation of diverse 424 N<sub>2</sub>-fixing bacteria in complex soil communities in a culture-independent fashion. 425 Cyt c was demonstrated as a universal N<sub>2</sub>-fixation biomarker by investigating both 426 model and environmental strains screened from soils. Its strong resonance Raman 427 signal, together with a remarkable <sup>15</sup>N-induced C-N band shifts of Cyt c, provided a 428 robust biomarker to distinguish N2-fixing bacteria from non-N2-fixing bacteria with 429 430 or without Cyt c. Raman imaging at micrometer resolution facilitated the location of N<sub>2</sub>-fixing bacteria in both artificial and soil communities. The linear correlation of 431 C-N band profile with  ${}^{15}N_2$  percentages allowed a quantitative evaluation of  $N_2$ 432 433 fixation extent of diverse soil bacteria.

For future work, this single-cell resonance Raman- $^{15}N_2$  SIP approach can be applied to important N<sub>2</sub>-fixing symbiont systems. The further combination with single-cell isolation and genome sequencing suitable for soil microorganisms will also be developed, in order to reveal the precise ecological role of largely unexplored uncultured diazotrophs (microbial dark matter) in diverse ecosystems.

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513 **Supporting Information Available:** Experimental description of 16s rRNA and *nifH* 514 gene sequencing. Phylogenic trees. Deconvolution of Raman band and correlation of 515 band area ratio with  ${}^{15}N_2$  percentage. Raman spectra of other types of N<sub>2</sub>-fixing 516 bacteria and related bacteria.

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Figure 1. Gel image of PCR product of *nifH* genes specific for N<sub>2</sub> fixation (a) and
single-cell resonance Raman spectra (b) from N<sub>2</sub>-fixing bacteria isolated from soil
(red), model N<sub>2</sub>-fixing bacteria (blue), non N<sub>2</sub>-fixing *S. oneidensis* and *E. coli* (black).
'No template control' in gel image is the negative control without DNA template for
PCR.

Figure 2. (a) Single-cell resonance Raman spectra of Azotobacter sp. incubated with 552  $^{15}N_2$  of 99.36%, 49.68%, 25.02%, 10.22%, 0.36% relative to N<sub>2</sub> in air. (b) Spectral 553 region including exclusively 1114 cm<sup>-1</sup> (C-<sup>15</sup>N) and 1129 cm<sup>-1</sup> (C-<sup>14</sup>N) bands 554 extracted from a. (c) Correlation between incubation  ${}^{15}N_2$  percentage versus 555 <sup>15</sup>N-induced spectral changes in single cell based on PCA 1-D scores. Each point is a 556 557 measurement of a single cell, and around 25 cells were measured. (d) Quantification of bulk <sup>15</sup>N content in Azobacter sp. from the same incubation as in Raman as 558 detected by isotope ratio mass spectroscopy. <sup>15</sup>N abundance in E. coli and S. 559 oneidensis incubated with 99.36%  $N_2\,was$  also shown. (e) Correlation between  $^{15}N$ 560 content of Azobacter sp (from d) and PC1 scores (from c). Data points on the right of 561 c and d labeled as soil bacteria were from active N<sub>2</sub>-fixing and non or weak N<sub>2</sub>-fixing 562 soil bacteria. 563

**Figure 3.** (a) Photomicrograph of a mixed artificial community containing N<sub>2</sub>-fixing *Azotobacter* sp. incubated with  ${}^{15}N_2$  in N-free medium and non-N<sub>2</sub>-fixing *S. oneidensis* grown in LB medium - two images showing the different shapes (isospherical vs. rod-shaped) of these two bacteria are shown below. (b)

Corresponding Raman image from the same area as in a. (c) Typical Raman spectra acquired from *Azotobacter* sp. and *S. oneidensis* in a and b. Bands at 1114 cm<sup>-1</sup> (C-<sup>15</sup>N) and 1129 cm<sup>-1</sup> (C-<sup>14</sup>N), representing <sup>15</sup>N<sub>2</sub>-fixing (red) and non-N<sub>2</sub>-fixing bacteria (green), were employed to construct the Raman image.

**Figure 4.** Single-cell Raman spectra of diverse soil bacteria extracted from soil after 12-day incubation with  ${}^{15}N_2$ . Each spectrum represents a characteristic phenotype. i, N<sub>2</sub>-fixing bacteria; ii, bacteria containing Cyt c but unable to fix N<sub>2</sub>; iii and iv, bacteria containing both Cyt c and carotenoid but unable to fix N<sub>2</sub>; v, bacteria without any pigment.

**Figure 5.** (a, b) Photomicrograph (left) and Raman images (right) of bacteria extracted from soil microcosms incubated with  ${}^{15}N_2$  for 12 days. (c) Resonance Raman spectra of single cells from six N<sub>2</sub>-fixing bacteria ( ${}^{15}N$ -1, 2, 3, 4, 5, 6), non-N<sub>2</sub>-fixing bacteria ( ${}^{14}N$ ), and bacteria without Cyt c (no Cyt c) in a and b. Bands at 1114 cm<sup>-1</sup> (C- ${}^{15}N$ ) and 1129 cm<sup>-1</sup> (C- ${}^{14}N$ ) were used to construct the Raman images exhibiting N<sub>2</sub>-fixing bacteria as red, non-N<sub>2</sub>-fixing bacteria containing Cyt c as green, and bacteria without Cyt c as black.

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Figure 1











Figure 5