Visualizing and isolating iron-reducing microorganisms at single cell level

Cuifen Gan[1], Rongrong Wu[1] Yeshen Luo[1], Jianhua Song[1], Dizhou Luo[1], Bei Li[2,3], Yonggang Yang[1]*, Meiying Xu[1]

1 Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, State Key Laboratory of Applied Microbiology Southern China, Guangdong Institute of Microbiology, Guangdong Academy of Sciences, Guangzhou 510070, China

2 The State Key Lab of Applied Optics, Changchun Institute of Optics, Fine Mechanics and Physics, CAS, 130033 Changchun, China

3 HOOKE Instruments Ltd., 130033 Changchun, China

Corresponding author: Y. Yang. Guangdong Institute of Microbiology, Guangzhou 510070, China. Tel.: +86 20 87684471; fax: +86 20 87684587. E-mail: yyg117@163.com.

Abstract: Iron-reducing microorganisms (FeRM) play key roles in many natural and engineering processes. Visualizing and isolating FeRM from multispecies samples are essential to understand the in-situ location and geochemical role of FeRM. Here, we visualized FeRM by a "turn-on" Fe^{2+}-specific fluorescent chemodosimeter (FSFC) with high sensitivity, selectivity and stability. This FSFC could selectively identify and locate active FeRM from either pure culture, co-culture of different bacteria or sediment-containing samples. Fluorescent intensity of the FSFC could be used as an indicator of Fe^{2+} concentration in bacterial cultures. By integrating FSFC with a single cell sorter, we obtained three FSFC-labeled cells from an enriched consortia and all of them were subsequently evidenced to be capable of iron-reduction and two unlabeled cells were evidenced to have no iron-reducing capability, further confirming the feasibility of the FSFC.

Importance: Visualization and isolation of FeRM from samples containing multispecies are commonly needed by researchers from different disciplines, such as environmental microbiology, environmental sciences and geochemistry. However, no available method has been reported. In this study, we provide a solution to visualize FeRM and evaluate their activity even at single cell level. Integrating with single cell sorter, FeRM can also be isolated from samples containing multispecies. This method can be used as a powerful tool to uncover the in-situ or ex-situ role of FeRM and their interactions with ambient microbes or chemicals.

Keywords: iron reducing bacteria, extracellular electron transfer, fluorescent chemodosimeter, sediment

1. Introduction

Iron minerals are widespread in anoxic subsurface environments and can be used as electron acceptors by many microorganisms.[1] In natural environments, those iron-reducing microorganisms (FeRM) not only play a key role in the reduction of minerals and humic substances but also participate in the oxidation of sulfur compounds and organic matters.[2-4] Moreover, FeRM are important in many engineered processes such as the wastewater treatment, bioremediation and bioelectrochemical systems.[5] Microbial iron-reducing process is an ancient respiration on the earth.[1] However, many novel electron transfer strategies (e.g. bacterial nanowire, direct inter-cellular electron transfer) possessed by FeRM were just recognized in recent years.[6,7]

To explore the role of FeRM in various environments, visualizing FeRM is a common need by researches on environmental, microbiological and earth sciences as it can provide essential information such as the location, amount or even activity of FeRM. However, FeRM are phylogenetically rather ubiquitous and thus there is no 16S rRNA or functional gene based assay to detect them so far. Phenanthroline-based spectrophotometric method has been used mostly to evaluate the capability of FeRM.[8] However, this method is unavailable to identify, locate or quantify FeRM from a multispecies consortia. Recently, some methods targeting cytochromes or extracellular electron transfer processes similar to iron reduction (including azo-dye reduction, tungsten trioxide reduction) have been reported.[9-11] However, these methods are unsuitable for visualizing FeRMs in a multispecies consortia, because (i)

cytochrome proteins are commonly shared by both FeRM and other bacteria; (ii) some FeRM do not reduce other extracellular electron acceptors[12]; (iii) it is hard to use such methods to locate FeRM in complex samples at single cell level.

A common characteristic of FeRM is that, the ferrous phosphate or ferrous carbonate generated from iron reduction can be adsorbed by extracellular polymeric substances, and thus creating a Fe^{2+}-accumulated layer on the cell surface of FeRM.[13-15] This Fe^{2+}-layer can be maintained by the reducing forces from outer membrane redox proteins such as *c*-type cytochromes. Therefore, Fe^{2+}-selective fluorescent chemosensor may provide a convenient and sensitive tool to visualize FeRM in different environments. Several Fe^{2+}-specific fluorescent probes have been developed for mammalian cells but none of them has been tested in microorganisms.[16-19] In contrast to the intracellular Fe^{2+} detection in mammalian cells, several challenges must be considered for a fluorescent probe for FeRM. For examples, the FeRM-probe should be nonreactive to other microorganisms as Fe^{2+} can also be adsorbed to the surfaces of them, especially in environments containing high concentration of Fe^{2+}.

Once FeRM cells can be visualized with fluorescence, single cell sorting techniques (e.g. microfluidic devices, laser tweezer or laser ejection) can be used to isolate them and their partner microbes from where they are observed. Thus, both in situ and ex-situ roles and mechanisms of each targeted FeRM cell can be understood. Guided by this aim, we synthesized an oxygen-depleting Fe^{2+}-specific fluorescent chemosensor (FSFC) which showed high sensitivity and selectivity to Fe^{2+}. This

89 FSFC also showed high feasibility in visualizing FeRM in pure-cultured and
90 multispecies systems. Integrating with single cell sorting technique, this probe could
91 facilitate identification and isolation of FeRM from an enriched sediment consortia.

92 **2. Materials and methods**

93 **2.1 Synthesis of N-butyl-4-phenyltellanyl-1, 8-naphthalimide (FSFC)**

94 This probe was selected due to its repeatable and simple synthesis method (Fig.
95 S1).[16] Firstly, 4-bromo-N-butyl-1, 8-naphthalimide was synthesized.[16,20] In brief, 5.0 g
96 4-bromo-1,8-naphthalic anhydride and 3 mL n-butylamine were dissolved in 90 mL
97 ethanol and refluxed in 82 °C for 6 h. Then the mixture was filtered to obtain a
98 wine-red solution. After evaporation with a rotary evaporator (90 °C, 80 rpm, until all
99 ethanol was evaporated), the crude product was purified by column chromatography
100 (silica gel, ethyl acetate: petroleum ether = 1: 50) to get a pale yellow solid product
101 (4.8 g). Secondly, N-butyl-4-phenyltellanyl-1, 8-naphthalimide (FSFC) was
102 synthesized by a modified method based on a previous report.[16] 1.02 g diphenyl
103 ditelluride and 60 mL ethanol were added to a 150 mL three-neck flask flushed with
104 nitrogen. The suspension was cooled to 0 °C, then 0.24 g sodium borohydride was
105 dissolved in 12 mL ethanol and slowly dropt into the three-neck flask. After the red
106 color faded, the reaction mixture was heated to reflux in 83 °C. Then, a mixture of
107 cuprous iodide (0.41 g, 2.2 mmol) and 4-bromo-N-butyl-1, 8-naphthalimide (0.59 g,
108 1.8 mmol) were added. The mixture was stirred and refluxed for 30 min in a nitrogen
109 atmosphere. After cooling to room temperature, the black mixture was filtered to
110 remove insoluble materials. Then, the black filtrate was evaporized on a rotary

111 evaporator. The residue was washed by ethanol and filtered again. After evaporation
112 (90 °C, 80 rpm, until all ethanol was evaporated), the residue was purified by column
113 chromatography (silica gel, ethyl acetate: petroleum ether = 1:125) to obtain a yellow
114 solid product of FSFC (0.72 g). The final yellow product was dissolved in acetonitrile
115 to get a 5 mM stock solution. It was further diluted by phosphate buffer saline (PBS,
116 containing 3.6 g/L $Na_2HPO_4 \cdot 7H_2O$, 0.27 g/L KH_2PO_4, 8 g/L NaCl, and 0.2 g/L KCl,
117 pH 7.2) and stored in dark before use.

118 **2.2 Sensitivity and selectivity test of FSFC.**

119 Aqueous solutions of ferric citrate ($FeC_6H_5O_7 \cdot 5H_2O$), ammonium iron (II)
120 sulfate hexahydrate ($H_8FeN_2O_8S_2 \cdot 6H_2O$), $MnCl_2$, $ZnCl_2$, $CaCl_2$, $MgCl_2$, $NiCl_2 \cdot 6H_2O$,
121 $CuCl_2$, $Co(NO_3)_2 \cdot 6H_2O$, $CdCl_2 \cdot 2.5H_2O$, NaCl and KCl, were used for the selectivity
122 and sensitivity tests of Fe^{3+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, Na^{+},
123 K^{+} respectively. Millipore water was used to prepare all kinds of aqueous solution.
124 For each experiment, fresh Fe^{2+} solution was prepared before using.

125 Sensitivity of FSFC towards Fe^{2+} was tested by a PerkinElmer LS 45
126 fluorescence spectrometer. Typically, the sensitivity of FSFC was carried by
127 incubating the FSFC (50 μM) with 0, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM, 200 μM,
128 500 μM, 1000 μM and 2000 μM of Fe^{2+} (ammonium iron sulfate hexahydrate,
129 $H_8FeN_2O_8S_2 \cdot 6H_2O$) for 30 min. The reaction solution (3 mL final volume for each
130 solution) was added into a quartz cell for fluorescence measurements with an
131 excitation wavelength (λ_{ex}) at 445 nm and the emission wavelength (λ_{em}) from 480 nm
132 to 600 nm.

Selectivity of FSFC towards Fe^{2+} was investigated by incubating 50 μM FSFC with 100 μM of various specified cations (Fe^{3+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, Na^{+}, K^{+} in ferric citrate ($FeC_6H_5O_7 \cdot 5H_2O$), ammonium iron (II) sulfate hexahydrate ($H_8FeN_2O_8S_2 \cdot 6H_2O$), $MnCl_2$, $ZnCl_2$, $CaCl_2$, $MgCl_2$, $NiCl_2 \cdot 6H_2O$, $CuCl_2$, $Co(NO_3)_2 \cdot 6H_2O$, $CdCl_2 \cdot 2.5H_2O$, NaCl and KCl), respectively for 30 min. The reaction solution (3 mL final volume for each solution) was added into a quartz cell for fluorescence measurements with an excitation wavelength (λ_{ex}) at 445 nm and the emission wavelength (λ_{em}) at 530 nm.

2.3 Pure culture strains and growth conditions.

To test the selectivity of FSFC to FeRM, pure bacteria cultures including three known FeRM (*Shewanella decolorationis* strain S12, *S. oneidensis* MR-1 and *Geobacter sulfurreducens* PCA),[5,6,21,22] three non-FeRM (a *ccmA*-mutant *S. decolorationis* S22, *Massilia rivuli* FT92W, *Duganella lacteal* FT50W) incapable of iron-reduction,[21,23] and five pure cultured bacteria newly isolated from sediments with unknown iron-reduction capacity (*Paenibacillus motobuensis* Iβ12, *Ciceribacter* sp. F217, *Sphingobium hydrophobicum* C1, *Bacillus* Iβ8, *Lysinibacillus varians* GY32) were used. Further information of those bacteria were listed in Table 1. All bacteria (except for *G. sulfurreducens* PCA) were firstly grown aerobically in Luria-Bertani (LB) medium. The bacteria cells were washed with sterilized PBS for two times and then inoculated to N_2-flushed anaerobic lactate medium (LM, containing 2.0 g/L lactate, 0.2 g/L yeast extract, 12.8 g/L $Na_2HPO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, and 1.0 g/L NH_4Cl) with an initial OD_{600} of 0.1. 3 mM of ferric citrate or poorly

crystalline Fe(III) oxides was used as the electron acceptor in the LM, unless otherwise stated. The cultures were grown at 33 °C. *G. sulfurreducens* PCA (initial OD_{600} = 0.08) was anaerobically cultivated using freshwater medium containing acetate (10 mM) as electron donor and ferric citrate (4 mM) or poorly crystalline Fe(III) oxides (4 mM) as electron acceptor.[6] The poorly crystalline Fe(III) oxides were prepared as previous report.[6] Meanwhile, the iron reducing capability of those bacteria was tested with traditional Phenanthroline-based method.[8]

2.4 Co-culture grown in liquid medium and sediment

Different co-culture systems was used to test whether FSFC can distinguish the FeRM from non-FeRM in the same bacteria culture. The co-culture systems includes: (i) co-culture system of *S. decolorationis* S12 and non-FeRM *L. varians* GY32 in N_2-flushed anaerobic LM solution with 3 mM of ferric citrate for 8 h; (ii) 10 mL of the above co-culture system was inoculated with 1 g of sterilized river sediment (obtained from Shijing River, Guangzhou, China), cultivated for 8 h.

2.5 Fluorescence Imaging.

The fluorescence imaging of the pure culture or co-culture samples were obtained with a confocal laser scanning microscopy (CLSM, LSM 700, Zeiss) after being stained by 50 μM FSFC for 15 min. FSFC concentration higher than 0.5 mM may cause toxicity to bacteria (Fig. S2). 10 μL of the stained cultures was dripped on a glass slide with small piece of cover slide and then observed under the CLSM with an excitation wavelength (λ_{ex}) at 445 nm for FSFC. Propidium iodide (PI, λ_{ex}= 490 nm, Thermo Fisher) was used as a fluorescent indicator for evaluating the activity of

177 the bacterial cells, only cells with low activities and impaired cell membrane can be
178 stained by PI.

179 **2.6 FSFC-based single cell isolation**

180 An enriched iron-reducing biofilm consortia was used to test that whether FSFC can
181 selectively label FeRM in a complex microbial community. This consortia was made
182 by inoculating 1 g of sediment into 100 mL LM containing 5 mM of ferric citrate in
183 an anaerobic serum bottle. Six graphite plates (1 × 1 × 0.1 cm) were added in the
184 culture for biofilm growth. 80% of the enriched culture was replaced with fresh LM
185 containing 5 mM of ferric citrate for every two weeks. After being enriched for
186 eight-weeks, three of the graphite plates were fetched and stained by FSFC and PI
187 after a gentle wash in sterilized PBS. The stained biofilms were observed under
188 CLSM. Biofilms on the other three graphite plates were scraped by a sterilized cotton
189 swab. The resulting biofilm cells were suspended in 5 ml PBS and stained by 50 μM
190 FSFC for 15 min. 50 μL of the FSFC-stained sample was transfer to a glass slide
191 designed for single cell ejection and observed under fluorescence mode of the single
192 cell precision sorter (PRECI SCS, HOOKE Instruments). The selected cells (with or
193 without fluorescence) on the slides were ejected by a laser beam controlled by PRECI
194 SCS software. 7 bacterial cell with fluorescence and 6 bacterial cells without
195 fluorescence were ejected from the slide into a collector containing sterilized PBS by
196 low power laser (0.5 to 1 μJ, varied according to the cell shape and adsorption force
197 on the slide surface). The collected single bacteria was then anaerobically cultivated
198 in LB medium containing 2 mM of ferric citrate. The grown bacteria were then

199 cultivated in the same freshwater medium used for *G. sulfurreducens* PCA with ferric
200 citrate as sole electron acceptor and acetate as electron donor.

201 **3. Results and discussions**

202 **3.1 Sensitivity, selectivity and stability of FSFC**

203 FSFC was non-fluorescent ("off" state) in the absence of Fe^{2+} due to the heavy-atom
204 effect of the tellurium atom on the naphthalimide fluorophore. Fe^{2+} can trigger the
205 detelluration reaction of FSFC and cause a strong fluorescence ("on" state).[16] As
206 evidenced by GC-MS (Fig. S3), the purity of the FSFC product was 94.2%. Fig. 1A
207 showed that FSFC exhibit very weak background fluorescence in the absence of Fe^{2+}.
208 Upon the addition of Fe^{2+} from 0 to 2000 μM, the fluorescence emission increased
209 accordingly. Fig. 1B showed a linear relation between the fluorescence intensity (FI)
210 and the logarithm of Fe^{2+} concentration. The theoretical limit of detection (LOD) was
211 calculated to be 6.3 μM (based on the formula LOD = 3 × σ/m, σ is the standard
212 deviation of the response at the lowest tested concentration and m is the slope of the
213 concentration-FI response).[24] Generally, the concentration of Fe^{2+} in practical
214 environments varied from several to hundreds of μM and could be up to several mM
215 in FeRM cultures.[25,26] Therefore, FSFC could be used as an alternative Fe^{2+} sensor or
216 FeRM-label for most environmental and experimental samples.

217 Other metal ions in practical environments may affect the fluorescence response
218 of FSFC to Fe^{2+}. Fig. 1C showed that all tested metal ions (except for Fe^{2+}) had no
219 significant fluorescence response to FSFC individually. When co-existing with Fe^{2+},
220 metal ions K^+, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+} had little effects on the Fe^{2+}-FSFC fluorescence

while Cu^{2+}, Mn^{2+} and Co^{2+} could affect the fluorescence to some extents. In typical natural environments, the concentrations of Co^{2+}, Cu^{2+} and Mn^{2+} are generally several orders of magnitude lower than that of Fe^{2+},[25] e.g. the concentration of manganese was two orders of magnitude lower than that of iron (8 vs 800 μM) in the sediments of Yaquina Bay Estuary,[25] indicating that the effects of other metal ions will be small for tests with naturally environmental samples. It should be noted the effects of other metal ions on FSFC fluorescence may increase with their concentrations (Fig. S4). For some industrial wastewaters containing high concentration of metal ions, the samples should be diluted or Fe^{2+} should be artificially elevated before using FSFC to visualize FeRM.

Fig. 1D showes a stability comparison between FSFC fluorescence and the traditional o-phenanthroline method. The FI of FSFC remained stable within 5 h (deviation < 5%) while the signal of traditional phenanthroline-method increased by over 10% within 2 h. Therefore, FSFC had better stability (within 5 h) compared to the phenanthroline-method, which also means that FSFC has particularly advantage in the studies needing long-time operations or including large number of samples.

3.2 Fluorescence imaging of viable FeRM reducing soluble and solid Fe^{3+}.

Iron reducing capability of *Shewanella* and *Geobacter* species has been extensively demonstrated in previous studies[2,6,7,15,27,28,]. Moreover, it has been reported that ferrous phosphate and ferrous carbonate aggregate on cellular surfaces during the iron-reduction by FeRM.[15] Our results also showed that compared to the non-FeRM (0.1-0.3 μM/μg bacteria protein), much higher concentration of Fe^{2+}

accumulated on the cell surface of *S. decolorationis* S12 (1.1 μM/μg bacteria protein) and *S. oneidensis* MR-1 (1.2 μM/μg bacterial protein) when exposing to the same Fe^{2+}-containing culture (Fig. S5), which further supported the reasonability of using Fe^{2+}-probe to identify FeRM. Fig. 2 showed that the *S. decolorationis* S12 in iron-reducing medium displayed significant fluorescence while the cells grown aerobically (without Fe^{3+}) have no fluorescence, indicating that cell surface-adsorbed Fe^{2+} can selectively turn-on the fluorescence of FSFC. Moreover, the FI on S12 cell surface increased correspondingly with the Fe^{2+} concentration in the culture supernatant (Fig. 2BD). By integrating with PI, a fluorescent dye targeting inactive bacteria (with impaired cellular membrane), it can be seen that FSFC only label the active iron-reducing S12 cells (Fig. 3). This result demonstrated that FSFC was selectively targeted to the active iron-reducing strain S12 cells rather than the inactive or non-FeRM strain S12 cells, probably because the Fe^{3+}-reducing activity of inactive cells was low and thus the Fe^{2+} accumulation layer cannot be maintained on the surfaces of such cells.

Considering that iron exist mainly as solids in natural environments, the reduction process of poorly crystalline Fe(III) oxides by strain S12 was also investigated. Due to the low reducing capability of strain S12 on poorly crystalline Fe(III) oxides, almost no fluorescence was observed in the first 2 days (Fig. 2E, F). The results showed that FSFC had no fluorescence response to poorly crystalline Fe(III) oxides. Over the next 5 days, fluorescence on S12-cells gradually increased with the increase in Fe^{2+} concentration (Fig. 2G-H). The FI was much lower of S12

grown with poorly crystalline Fe(III) oxides compared to that with ferric citrate, which was corresponding to the different reduction rates of strain S12 with the two forms of Fe^{3+}. In the system with either soluble Fe^{3+} or poorly crystalline Fe(III) oxides, FI on the cells showed linear relationship to the ambient Fe^{2+} concentration (Fig. 2I, J). We also tested the performance of FSFC with *S. oneidensis* MR-1 reducing ferric citrate or poorly crystalline Fe(III) oxides which showed consistent results with that with *S. decolorationis* S12 (Fig. S6).

Geobacter has different extracellular electron transfer pathways compared to *Shewanella*.[5,7] When using ferric citrate as electron acceptor, the FI on *G. sulfurreducens* PCA cell increased with the Fe^{2+} concentration which was similar to the two *Shewanella* species. However, when reducing poorly crystalline Fe(III) oxides, only *G. sulfurreducens* PCA cells attached to the poorly crystalline Fe(III) oxides showed fluorescence while planktonic cells showed weak or no fluorescence (Fig. S7). The different fluorescent performances between *Shewanella* and *Geobacter* when reducing poorly crystalline Fe(III) oxides may be explained by their extracellular electron transfer pathways: *Shewanella* can secret soluble electron mediators to dissolve and reduce poorly crystalline Fe(III) oxides without attaching to the particles while *G. sulfurreducens* PCA can only reduce poorly crystalline Fe(III) oxides via outer membrane cytochrome *c* or e-pili after attaching to the particles.[5,6,7] These results demonstrated that FSFC can visualize FeRM reducing either dissolved ferric citrate or solid poorly crystalline Fe(III) oxides. Moreover, the FI on the bacteria surface can be considered as an indicator of the Fe^{2+} concentration in the pure cultures

reducing ferric citrate. However, it should be noted that the FI of different *G. sulfurreducens* PCA cells on the aggregates of poorly crystalline Fe(III) oxides varied largely (Fig. S7), indicating different physiological status of them at single cell level.

3.3 Evaluating the iron-reducing capability of different bacteria

In addition to iron-reducing capability, bacteria from different genera usually have different shapes, surface properties and metabolites that may affect the fluorescence of FSFC. To further analyze the selectivity of FSFC, we used FSFC to test five blind bacterial samples (five bacteria newly isolated from sediment with unknown iron-reducing performance, Table 1), with *S. decolorationis* S12, *S. oneidensis* MR-1 as positive controls (capable of iron-reduction) and *ccmA*-mutant S22 (deficiency in producing mature c-type cytochromes),[21] *S. oneidensis* MR-1 without electron donor, *Massilia rivuli* FT92W, *Duganella lacteal* FT50W as negative controls (incapable of iron-reduction).[23] As expected, *S. decolorationis* S12, *S. oneidensis* MR-1 showed fluorescence while the negative controls showed no fluorescence (Fig. 4, Fig. S8). Among the five blind bacterial samples, only *Paenibacillus motobuensis* Iβ12 had fluorescence but the FI was lower than that of *S. decolorationis* S12. The other bacteria have no fluorescence (Fig. 4A-G). Traditional o-phenanthroline method showed consistent results that only *P. motobuensis* Iβ12 had iron-reducing capability and its iron-reducing rate is much lower than that of *S. decolorationis* S12 (0.14 vs 0.58 mM/h). The results indicated that FSFC could be used as a simple and visualizing method to identify and evaluate the iron-reducing capability of different bacteria.

3.4 Visualizing FeRM from bacterial co-cultures

Co-culture of FeRM and bacteria with other functions is an important way to understand the interaction between FeRM and other bacteria. In such co-culture systems, one possible problem challenging FSFC is that the Fe(II) generated by FeRM may adsorbed to non-FeRM and render the later fluorescence. To test whether FSFC can identify FeRM in co-culture systems, we co-cultured a filamentous non-FeRM *L. varians* GY32 and *S. decolorationis* S12 using lactate as electron donor. As shown in Fig. 5A, the rod-shape strain S12 showed strong fluorescence while the filamentous bacteria *L. varians* GY32 have no fluorescence in the same iron-reducing culture. It can be seen that FSFC can selectively visualize the FeRM in microbial samples containing FeRM and non-FeRM. The result was consistent with that Fe^{2+} accumulated on the surface of non-FeRM is much less even in the same Fe^{2+}-containing environment (Fig. S5). By integrating with a flow cytometer, we could separate the iron reducing bacterium *S. decolorationis* S12 from a co-culture of two rod-shape bacteria (*S. decolorationis* S12 and *S. hydrophobicum* C1, Fig. S9) by the fluorescence, suggesting potential application of FSFC for FeRM with properly controlled flow cytometer or other microfluidic techniques. However, the bacteria samples for microfluid- or microdroplet-based techniques must be simple and well-separated. The pretreatment of most environmental samples which contain aggregates or filamentous bacteria will be challenging for such microfluidic techniques.

To evaluate the feasibility of FSFC in more complex environments, FSFC was

used to the co-culture of *L. varians* GY32 and *S. decolorationis* S12 in sterilized sediment containing ferric citrate. Fig. 5C showed that in the sediments without co-culture, only a minority of particles showed fluorescence probably due to the inherent Fe^{2+} on those sediment particles and no bacteria-like particles showed fluorescence. The results showed that FSFC had little background fluorescence in sediments and the unviable (sterilized) microorganisms in sediment could not trigger the fluorescence of FSFC. In the co-culture system, short-rod strain S12 showed significant fluorescence while the filamentous bacteria *L. varians* GY32 had no fluorescent, indicating the feasibility of FSFC for visualizing FeRM in sediment-containing environments. However, it should be noted that a minor portion of particles in sediments also had fluorescent response to FSFC probably because some particles can absorb the Fe^{2+} generated by FeRM. A proper dilution or filter could be used to remove the particles from the sediment samples.

3.5 Visualizing and isolating single cell FeRM from multispecies consortia

In addition to visualizing FeRM, isolating FeRM from multispecies samples is a general and important need for understanding the iron-associated biogeochemical processes.[29] The selective fluorescent of FSFC to FeRM provide the possibility of isolating single FeRM cell from multispecies with a single cell isolating platform. Fig. S10 shows that *S. decolorationis* S12 can be distinguished and isolated from the co-culture containing wild strain S12 and mutant strain S22 by integrating FSFC with a laser-based single cell sorter. The laser power used to eject the single microbial cell (< 1 μJ) with this platform was three-order of magnitude lower than the power that

(several mJ) may hurt cell viability.[30]

We combined FSFC and PI to label the biofilms in an enriched iron-reducing reactor. CLSM showed that the active FeRM cells were mainly located at the outer layer of the biofilms while the inner (bottom) layer biofilms cell showed low activity and little FSFC fluorescence (Fig. 6A). This activity profile was similar with that of the biofilms respiring with nitrate or azo dyes as electron acceptors[31], indicating the Fe^{3+} was inaccessible to the inner biofilm layers and thus only the outer layer biofilm cells can reduce Fe^{3+} and maintain high activity. Microbial community analysis showed that the diversity of the enriched biofilm consortia was significantly decreased compared to the initial community (Fig. S11). Gram-positive bacteria were dominant in the enriched consortia. After addition of FSFC to the suspended biofilm consortia, both fluorescent bacteria and non-fluorescent bacteria were observed (Fig. S11). Seven single cells with fluorescence and six single cells without fluorescence were isolated from the enriched consortia using the single cell sorter (Fig. 6). Three of the isolated fluorescent single cells (named S1, S2, S3) were successfully cultivated and all of them could use acetate as electron donor to reduce ferric citrate (Fig. 6F). The 16S rRNA genes of the isolated FeRM S1 (accession number MT947627), S2 (accession number MT947628) were close to *L. fusiformis* NBRC15717 (similarity 99.84%) and *L. pakistanensis* NCCP-54 (similarity 100%), respectively. S3 (accession number MT947629) was close to *Paenibacillus glucanolyticus* NBRC 15330 (similarity 99.25%), respectively. *Lysinibacillus* commonly exists in various environments such as sediment or wastewater.[32-34] Although the capability of several

375 *Lysinibacillus* strains using electrodes as electron acceptors have been reported,[33,34]
376 our results present evidence that the genus *Lysinibacillus* can reduce iron.
377 *Paenibacillus* is also a common gram-positive bacterial genus and several species in
378 this genus have been demonstrated to reduce iron.[35,36] On the other hand, two of the
379 non-fluorescent single cells with 16S rRNA genes similar to *Bacillus terrae* RA99
380 (similarity 99.28%, accession number MT947630) and *P. barengoltzii* NBRC 101215
381 (similarity 99.58%, accession number MT947631) were successfully cultivated and
382 had no iron-reducing capacity (Fig. 6F). *B. terrae* was identified as a new aerobic
383 species from rhizosphere soils while *P. barengoltzii* NBRC 101215 was an aerobic
384 bacterium that can degrade chitin.[37, 38] The results also suggested that FSFC could be
385 used as a novel and efficient method to isolate FeRM from different environments by
386 integrating with single cell isolation techniques.
387 This study reports a method that can visualize and isolate FeRM from bacterial
388 cultures containing multispecies or even sediments. The FSFC has high sensitivity,
389 selectivity and stability to Fe^{2+} and low background fluorescence in both liquid and
390 sediment environments. In pure cultures or co-cultures containing FeRM, FSFC could
391 selectively visualize the active FeRM. By integrating with single cell sorting
392 technique, targeted FeRM could be efficiently obtained from samples at single cell
393 level. This novel method could be a powerful tool serving for obtaining novel FeRM
394 and for a deeper understanding of the biogeochemical role of FeRM in different
395 environments. It should be noted that the isolation and cultivation conditions should
396 be modified according the samples and aims. For example, although the method

reported here can be applied to visualize and isolate to obligate anaerobic FeRM (e.g. *Geobacter*) at single cell level, exposure of the samples in air will decrease their culturability and the experiments should be done under an anaerobic condition. For unknown microbial samples, the sorting laser power and cultivating medium should also be optimized.

ASSOCIATED CONTENT

Supporting Information

Figure S1. Synthesis route and function mechanism of N-butyl-4-phenyltellanyl-1, 8-naphthalimide (FSFC).

Figure S2. Toxicity of FSFC on *S. decoloratIonis* S12 cultivated aerobically in LB medium.

Figure S3. GC-MS analysis of the synthesized products.

Figure S4. FI of FSFC in solutions containing 100 μM Fe^{2+} and different concentrations of Mn^{2+}.

Figure S5. Fe^{2+} collected from the cell surfaces of different bacteria.

Figure S6. FSFC-stained *S. oneidensis* MR-1 reducing soluble and solid Fe^{3+}.

Figure S7. FSFC-stained *G. sulfurreducens* PCA reducing soluble and solid Fe^{3+}.

Figure S8. Fluorescent images of control bacteria.

Figure S9. Flow cytometry scatter plots of strain S12 and *S. hydrophobicum* C1.

Figure S10. Single cell isolation and determination of strain S12 and *ccmA*-mutant S22 from their co-culture.

Figure S11. Microbial composition and the FSFC-staining of the enriched iron-reducing consortia.

AUTHOR INFORMATION

Corresponding Author

*Email: yyg117@163.com

Notes

The authors declare no competing financial interest.

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562 **Figure legends**

563 **Fig. 1** The sensitivity, selectivity and stability of FSFC in Fe^{2+}-containing solution. (A)
564 Response of FSFC fluorescence spectra to different concentrations of Fe^{2+}. (B)
565 Relationship between the concentration of Fe^{2+} and the FI. Insert shows the linear
566 relationship between FI and the logarithm of Fe^{2+} concentrations. (C) Selectivity tests
567 of FSFC to Fe^{2+}. Black bars indicate fluorescence response of FSFC to deionized
568 water (blank) and deionized water containing different metal cations (M^{+}), red bars
569 indicate fluorescence response of FSFC to different cations combined with Fe^{2+}. (D)
570 Relative stability of FSFC and traditional o-phenanthroline-based method.

571

572 **Fig. 2** Fluorescence response of FSFC to strain S12 using oxygen, soluble Fe^{3+} or

573 solid Fe^{3+} as electron acceptor. (A) S12 respiring with oxygen or at 0 h in
574 Fe^{3+}-reducing medium; (B-D) S12 respiring with soluble Fe^{3+} for 1, 3, 5 h,
575 respectively; (E-H) S12 respiring with solid Fe^{3+} for 0, 3, 5, 7 days, respectively; (I, J)
576 Fe^{2+} concentration and the corresponding FI of strain S12 with soluble Fe^{3+} or solid
577 Fe^{3+}, respectively.

578

579 **Fig. 3** PI-FSFG co-staining on strain S12. Fluorescence imaging of the iron reducing
580 S12 stained with PI for low viability cells (A, λex= 490 nm) and FSFC for
581 iron-reducing cells (B, λex= 445 nm).

582

583 **Fig. 4** Fluorescence images of FSFC to different bacterial cultures containing ferric
584 citrate. (A) Ciceribacter sp. F217, (B) S. hydrophobicum C1, (C) Bacillus Iβ8, (D) L.
585 varians GY32, (E) P. motobuensis Iβ12, (F) S. decolorationis S12, (G) the
586 iron-reduction of different strains. (Scale bar: 5 μm).

587

588 **Fig. 5** Fluorescence images of S. decolorationis S12 and L. varians GY32 co-culture.
589 (A) Fluorescence mode image of the co-culture (Fe^{2+}concentration: 2.3 mM),
590 magnified from the red rectangle area in the insert; (B) Light-fluorescence merged
591 image of the co-culture in liquid medium (Fe^{2+}concentration: 2.3 mM), magnified
592 from the red rectangle area in the insert; (C, D) Light-fluorescence merged image of
593 the sediments with and without co-culture, respectively (Fe^{2+}concentration: 1.9 mM).

594

595 **Fig. 6** FSFC-based single cell isolation and iron-reducing capability test. (A) Vertical

596 section view of enriched iron-reducing biofilm, red indicates PI-stained cells and
597 green indicates FSFC-labeled cells. (B)Light-fluorescence merged image area of the
598 suspended iron-reducing biofilms. Cell 1 (non-fluorescent) and 2 (fluorescent) are two
599 typically targeted cells to be isolated. The dark cross is a land-mark designed on the
600 glass slide. (C, D) Images before and after the laser-ejection of cell 1 from the slide to
601 a collecting pore containing PBS. (E, F) Images before and after the laser-ejection of
602 cell 2, respectively. (G) Iron-reduction capability of the isolated bacteria.
603

604 **Table 1.** Information of bacterial strains

Bacteria strain	Gram-staining	Cell shape	Iron reduction
S. decolorationis S12	Negative	Rod	Yes
S. decolorationis S22	Negative	Rod	No
S. oneidensis MR-1	Negative	Rod	Yes
G. sulfurreducens PCA	Negative	Rod	Yes
M. rivuli FT92W,	Negative	Rod	No
D. lacteal FT50W	Negative	Rod	No
Ciceribacter sp. F217	Negative	Rod	Unknown
S. hydrophobicum C1	Negative	Rod	Unknown
Bacillus sp. Iβ8	Positive	Rod	Unknown
L. varians GY32	Positive	Filament	Unknown
P. motobuensis Iβ12	Positive	Rod	Unknown

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